CHAPTER 6

GRAPES, WINE AND OTHER DERIVATIVES. OTA CONTENT

6.1. WORLD-WIDE PRODUCTION OF GRAPES, WINE AND OTHER GRAPE-DERIVATIVES

Grape is the fruit of a vine in the family *Vitaceae* (Table 7). It is commonly used for making grape juice, wine, jelly, wine, grape seed oil and raisins (dried grapes), or can be eaten raw (table grapes).

Table 7. Scientific classification of grapes.

Kingdom:	*Plantae*
Division:	*Magnoliophyta*
Class:	*Magnoliopsida*
Order:	*Vitales*
Family:	*Vitaceae*
Genus:	*Vitis*

Many species of grape exist including:

- *Vitis vinifera*, the European winemaking grapes.
- *Vitis labrusca*, the North American table and grape juice grapes, sometimes used for wine.
- *Vitis riparia*, a wild grape of North America, sometimes used for winemaking.
- *Vitis rotundifolia*, the muscadines, used for jelly and sometimes wine.
- *Vitis aestivalis*, the variety Norton is used for winemaking.
- *Vitis lincecumii* (also called *Vitis aestivalis* or *Vitis lincecumii*), *Vitis berlandieri* (also called *Vitis cinerea* var. helleri), *Vitis cinerea*, *Vitis rupestris* are used for making hybrid wine grapes and for pest-resistant rootstocks.

The main reason for the use of most *V. vinifera* varieties in wine production is their high sugar content, which after fermentation, produce a wine with an alcohol content of 10 % or slightly higher. Grape varieties of *V. vinifera* have a great variation of composition. Skin pigment colours vary from greenish yellow to russet, pink, red, reddish violet or blue-black. The colour of red wines comes from the skin, not the juice. The juice is normally colourless, though some varieties have a pink to red colour. Juice flavours vary from bland to strong. Although many people incorrectly assume that red grapes have the most health benefits, the fact is that grapes of all colours have comparable benefits. Some of the health benefits of red wine that are not found in white wine are because of some compounds of the skin, as only red wine is fermented with the skins.

Other grape species used for wine include *V. labrusca* and *V. rotundifolia*. Neither of them usually contains sufficient sugar at maturity to make wine with an alcohol content of 10 %. Sugar must be added to produce a stable wine from these grapes and they will also present more acidity.

Vineyards need very precise climatologic characteristics to prosper. Ripening period should be long enough to ensure a good maturation of the berries and winter should be cold enough to let vines repose. Certain daily amount of light, temperature and water, are other requirements. In general, vines grow better in temperate climates situated in latitudes comprised between 30 and 50º north and south (Figure 6).

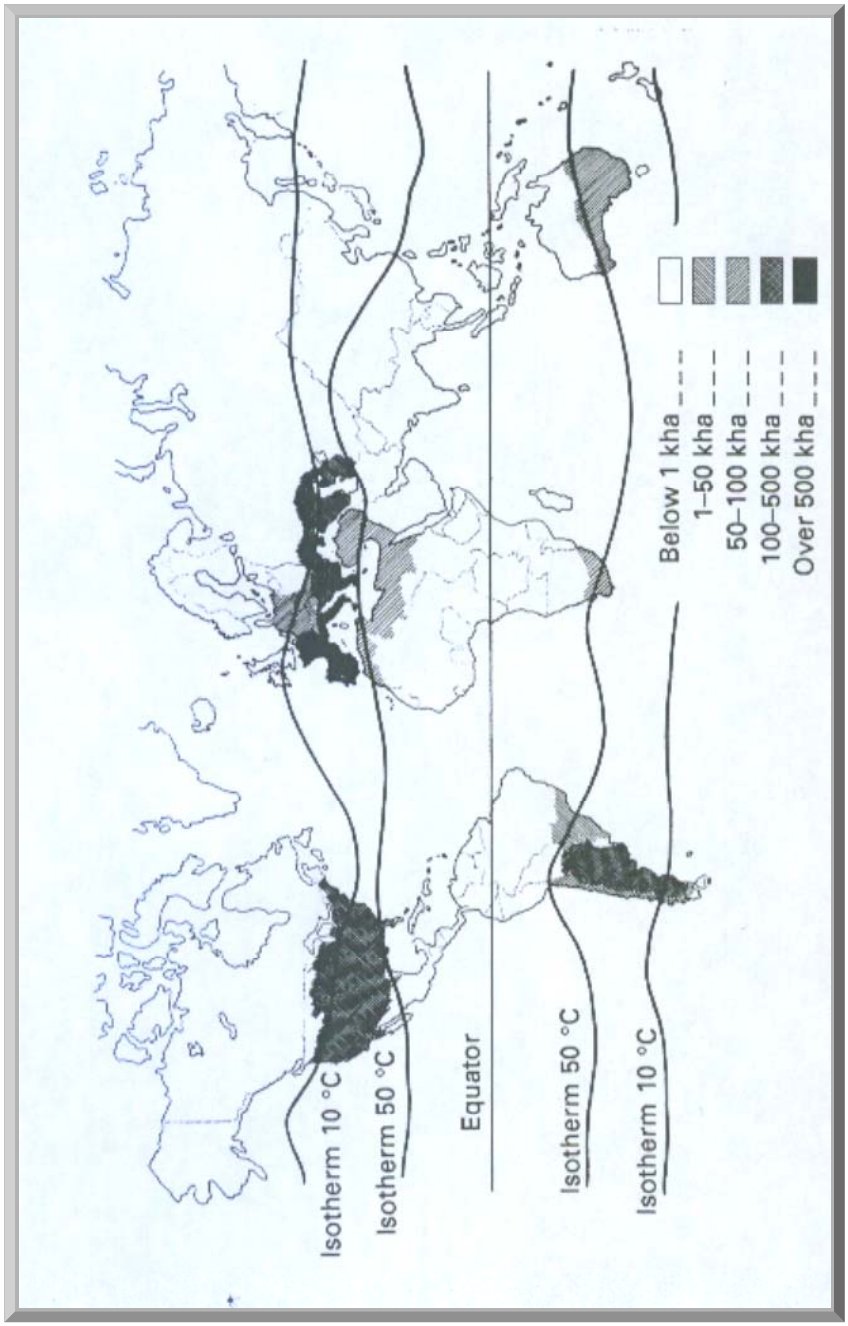

Figure 6. Distribution of wine grape plantings in the world (Mullins et al., 1992).

6.1.1. World-wide surface of vineyard

The maximum world-wide surface of vineyards was achieved at the end of the 70's with 10.2 million hectares, decreasing latter since 1998. So far, it slightly escalated to finally stabilize around 8 million hectares (Figure 7). The reason of this latest increase is the recent expansion of the Asiatic viticulture, especially in China, whereas some years ago, the increase was attributed to the development of viticulture in South America and USA.

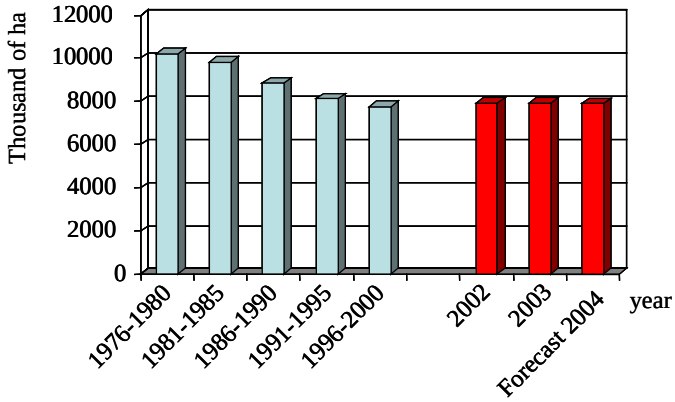

Figure 7. Evolution of world-wide area of vineyards since 1976 (OIV, 2005).

European wine producing areas have gradually decreased since 1975/76, following the introduction of a ban on new plantings and abandonment premia. This reduction has accelerated annually since the 90's. From 1976 to 1996, the areas under vines in the EU decreased from 4.5 to 3.4 million ha, which represents an annual decrease rate of 1.4 %, almost 56 000 ha/year. In the last few years however, the rate of reduction has clearly slowed down. In addition, the vineyards within the EU have, in general, aged, as they have not been replanted at a sufficient rate, although there are exceptions in certain regions.

Although in 2004 EU wine harvest fell below initial forecasts, for the first time since 2001 European production increased. Under the EU's wine regime, current planting of vines is strictly regulated and controlled in terms of acreage and allowed varieties. Controls remain in place to encourage the production of quality wines while discouraging the production of poor quality. New plantings of wine grapes are forbidden until July of 2010, except under certain circumstances.

In 2004, nearly 60 % of the world-wide area planted with vines was located in the EU. European wine-growing varies from one Member State to another, and even from one region to another, not only as regards the degree of specialisation of the wine holdings, but

also as regards the size of the vineyard and the type of wine produced. Asia is the second continent with more vineyards (21.5 %), most of them destined to grapes for direct consumption. The rest, is distributed between the three remaining continents, where nearly 12 % correspond to North and South America (Figure 8).

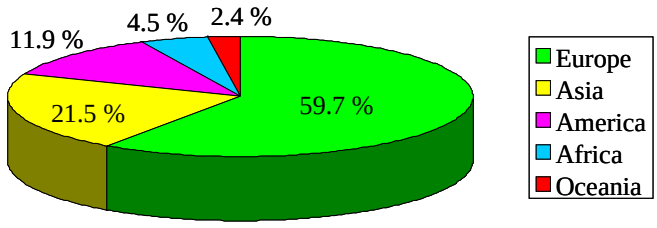

Figure 8. Areas planted with vines distributed by continent in 2004 (OIV, 2005).

Spain is the leading country in vineyard extension, with 1.19 million hectares. It is important to highlight the position of Turkey, China and Iran, included among the first seven principal countries in surface of vineyard (Figure 9).

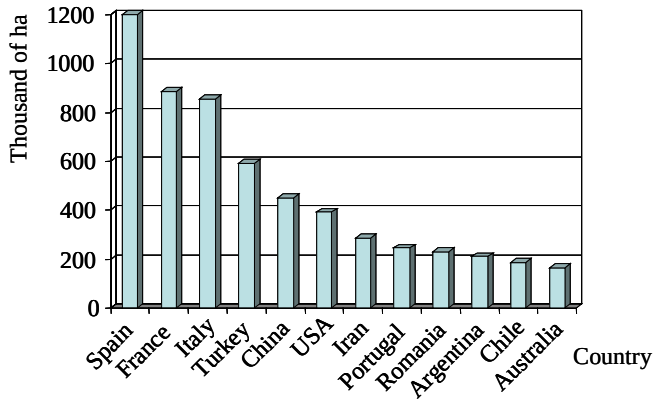

Figure 9. Areas planted with vines of the 12 leading countries in 2004 (OIV, 2005).

6.1.2. World-wide grape production

6.1.2.1. Total grape production

The global grape production is the result of combining the evolution of the surface planted with wines, the climatic differences and the innovations in the production techniques along the years. The production of grapes in 2004 is estimated in 66 million metric tonnes (Figure 10).

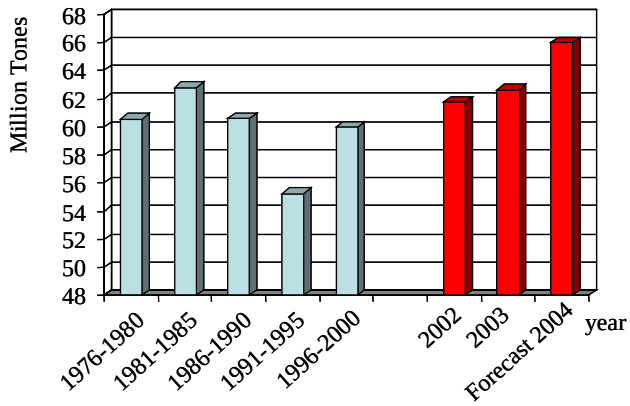

Figure 10. Evolution of the world-wide grape production since 1976 (OIV, 2005).

Approximately half of the global grape production comes from Europe, followed by Asia (22.7 %) and America (18.5 %) (Figure 11).

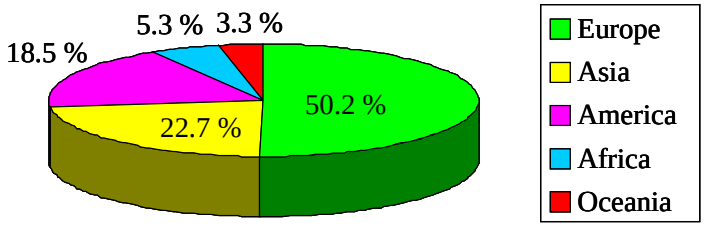

Figure 11. Production of grapes distributed by continent in 2004 (OIV, 2005).

There are little differences in the classification of the main grape-producing countries and the leaders in the surface planted with vines. Exactly, Portugal and Romania are emplaced by South Africa and Germany, taking the 11th and 12th position, respectively (Figure 12).

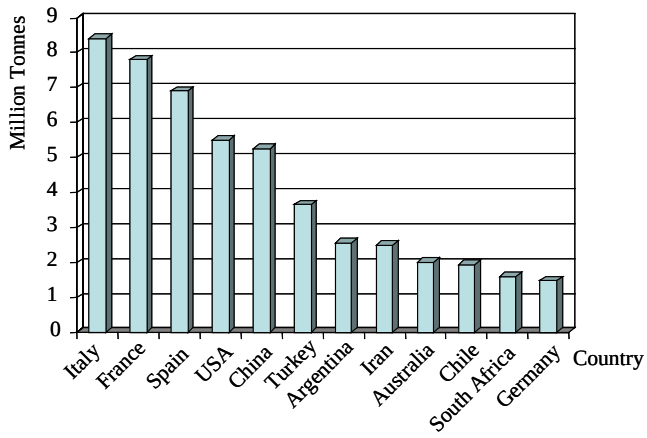

Figure 12. Total grape production of the 12 leading countries in 2004 (OIV, 2005).

6.1.2.2. Table grape production

In the last three years, the production of table grapes (grapes for direct consumption) has remained stable, with approximately 16.3 million tonnes, much higher than the productions registered since the half 80's (Figure 13).

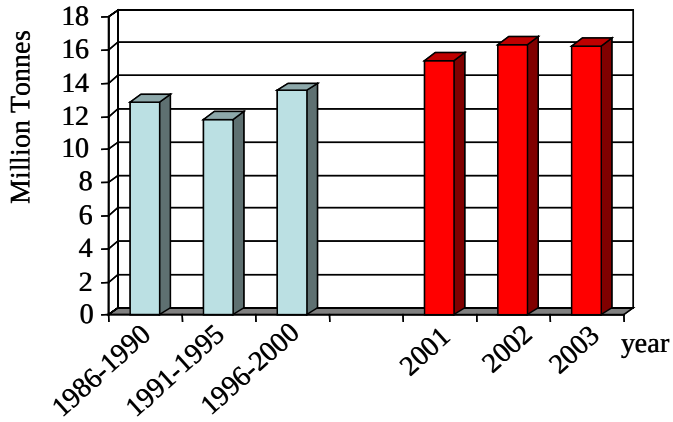

Figure 13. Evolution of the world-wide table grape production since 1986 (OIV, 2005).

Table grape production distribution among continents differs from the total grape production distribution, as Asia overtakes EU in the production of table grapes, with more than 50 % and 21 % of the total, respectively. Approximately, the remaining one quarter of the production comes from America and Asia, whereas Oceania production of table grapes is almost negligible (Figure 14).

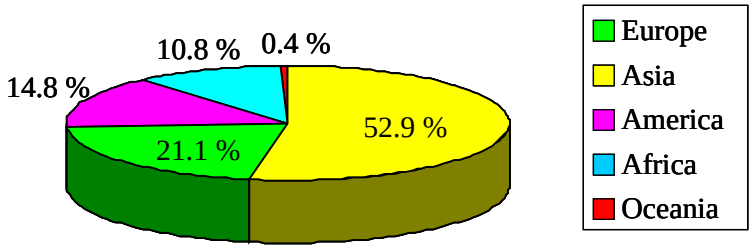

Figure 14. Production of table grapes distributed by continent in 2004 (OIV, 2005).

China, Turkey, Iran and India leader the production of table grapes in Asia, meanwhile Italy is the main producer in Europe, USA in North America, Chile and Brazil in South America and Egypt in Africa (Figure 15).

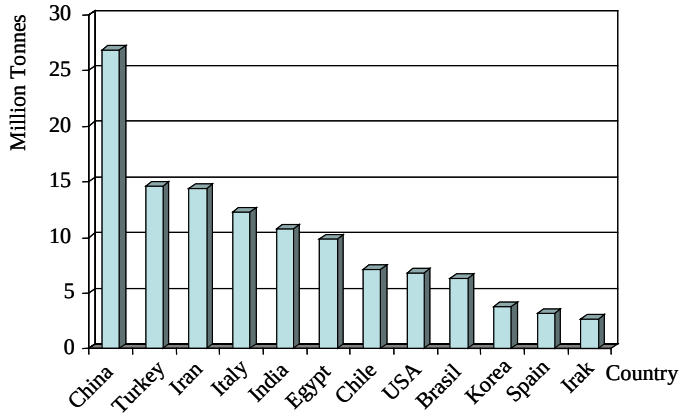

Figure 15. Table grape production of the 12 leading countries in 2004 (OIV, 2005).

6.1.3. World-wide grape consumption

The consumption of fresh grapes is increasing since 1995, being 15.7 million tonnes in 2003 (Figure 16).

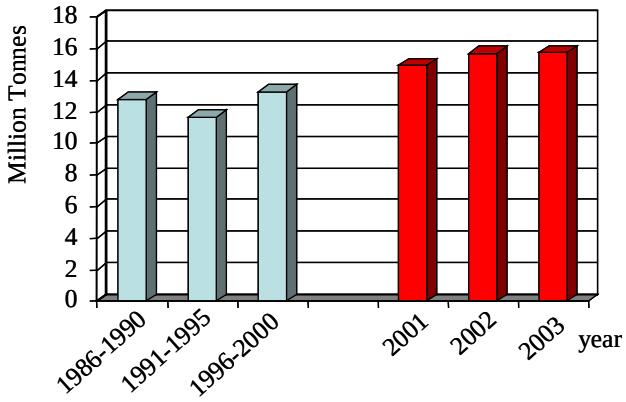

Figure 16. Evolution of the world-wide consumption of fresh grapes since 1986 (OIV, 2005).

The highest consumption of fresh grapes is detected in Asia (approx. 55 %), followed by Europe (22.6 %), America (12.6 %) and Africa (9.7 %) (Figure 17).

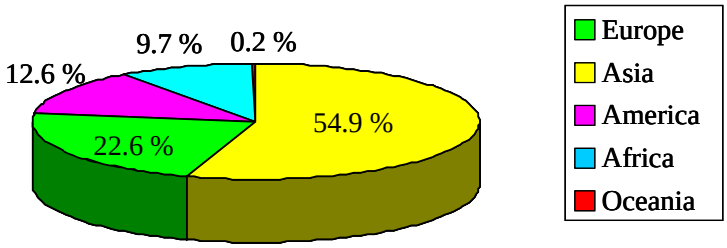

Figure 17. Consumption of fresh grapes distributed by continent in 2004 (OIV, 2005).

China is the principal country in the consumption of fresh grapes, with more than 2.7 million tonnes consumed per year. It is followed by Iran, Turkey and India, with more than 1.0 million tonnes consumed in each country, stating the importance of Asia in grape

consumption. Only two European countries, Italy and Germany, appear among the 12 leading countries in grape consumption (Figure 18).

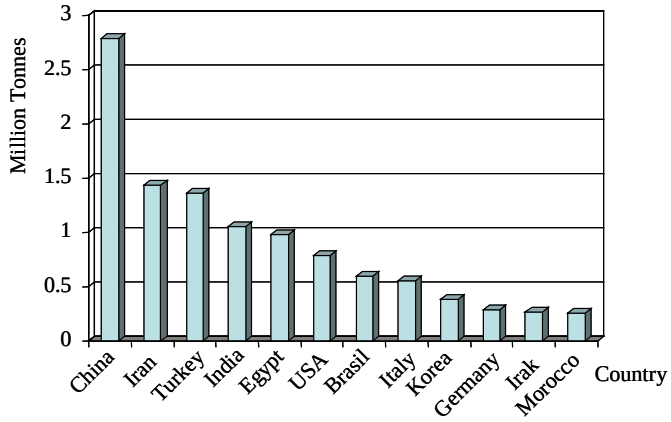

Figure 18. Consumption of grapes of the 12 leading countries in 2004 (OIV, 2005).

6.1.4. World-wide wine production

Wine production is characterised by very marked annual fluctuations, due, on the one hand, to climatic effects and, on the other hand, to cultivation methods. In spite of yearly fluctuations, a significant negative trend in wine production over the last twenty years has been observed: from a level of 333.6 million hl in 1980, average production has fallen 21 % at the beginning of nineties and showing a little increase to 272.6 million hl in the period 1996-2000. Nowadays, wine production has increased again, reaching approximately 295 million hl in 2004 (Figure 19).

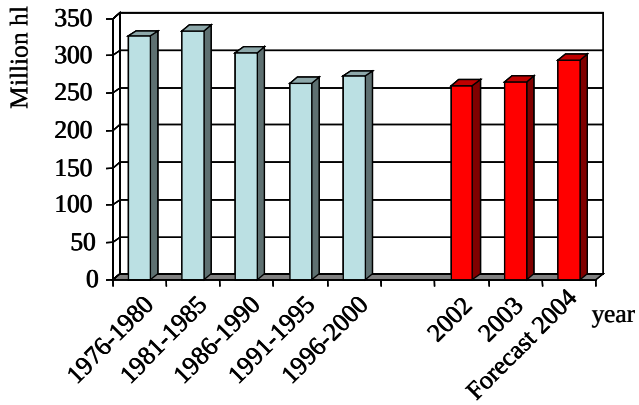

Figure 19. Evolution of the world-wide wine production since 1976 (OIV, 2005).

With production fluctuating between 152 and 165 million hl (70 % of the world wine production) during the last five years, the EU is, by far, the world's leading wine producer, followed by America, with 16 % of the global production. The other continents represent between 4 and 5 % of wine production (Figure 20).

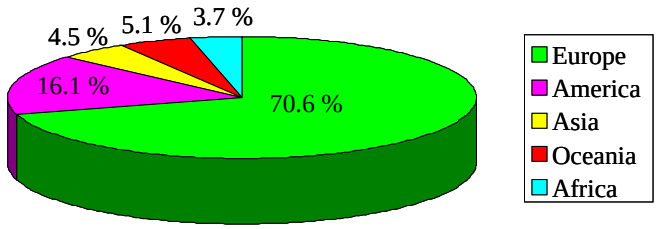

Figure 20. Production of wine distributed by continent in 2004 (OIV, 2005).

The main wine producing countries over the world are indicated in figure 21, being France, Italy and Spain the three major producers.

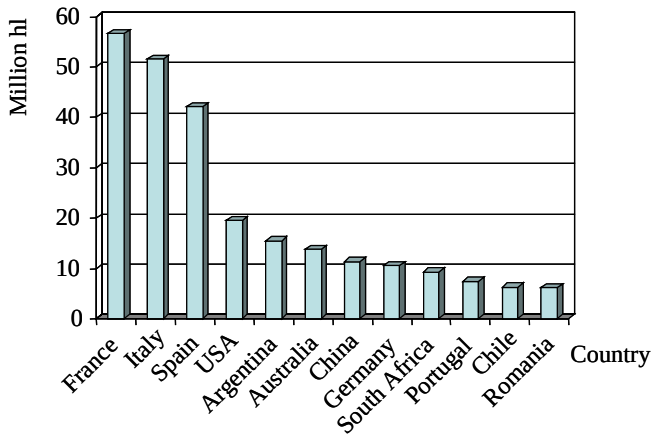

Figure 21. Wine production of the 12 leading countries in 2004 (OIV, 2005).

6.1.5. World-wide wine consumption

The amount of wine consumed in 2004 over the world was estimated in 235.7 million hl. The main consumers are in Europe, which account for almost of 68 % of world consumption, followed by the Americans (20 %), leaving the third position to Asians (7 %). However, from 1986 to 1996, total wine consumption in the EU decreased by 10 million hl. This fall reflects a significant downward trend connected in particular to changes in life style, in consumer behaviour, in the role that wine plays in food, etc.

The 2004 classification of wine consumption according to the country, shows that the eight leader countries consumed approximately 154 million hl, which means the two thirds of the world-wide demand (Figure 22). Logically, the highest wine consumption corresponds with the traditional wine growing countries; in particular, in wine producing countries of southern Europe, the consumption is more or less double the EU average (Figure 23). However, the Community average masks important disparities between Member States: production in Spain has fallen significantly during the last twenty years where levels of consumption per capita had been the highest; contrary, the consumption in Denmark, which is a non wine producer, is considerably high (approx. 30 litres per capita per annum).

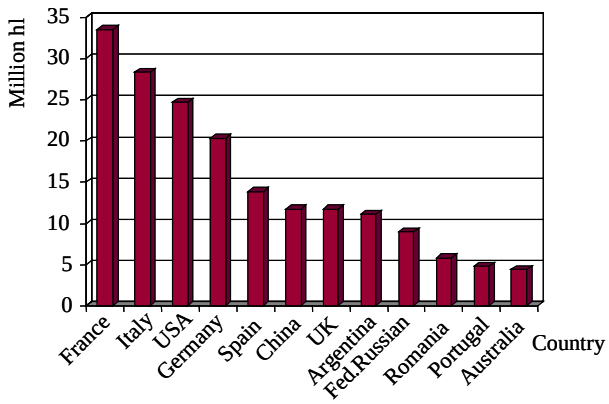

Figure 22. Wine consumption of the 12 leader countries in 2004 (OIV, 2005).

The EU is both the leading world exporter and importer. While France, Italy and Spain are the main exporting countries of wine; Germany, followed by UK, are the main importers (OIV, 2005). Wine producing and exporting countries aim to obtain good quality safe products and maintain this throughout the productive chain, therefore, a special attention to OTA contamination should be paid.

To sum up, the EU occupies a leading position on the world wine market, accounting for 60 % of wine-growing areas, 70 % of production, 68 % of global consumption and approximately 70 % of exports in global terms.

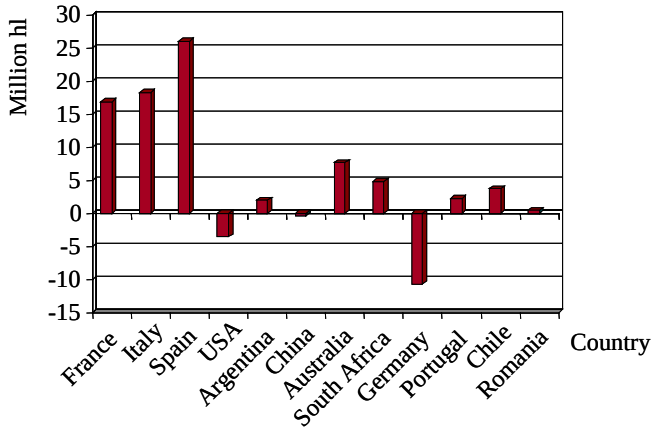

Figure 23. Difference between wine production and consumption for the main producing countries in 2004 (OIV, 2005).

6.1.6. World-wide dried grape production

There is an increasing trend in the production of dried grapes in the last years, reaching the 1220 thousand of tonnes in 2003 (Figure 24).

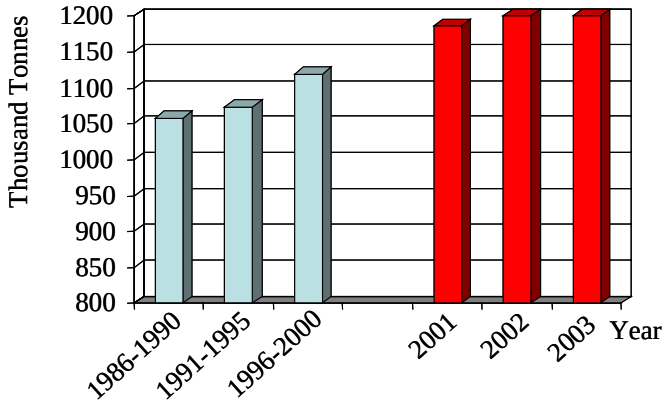

Figure 24. Evolution of the world-wide dried grapes production since 1986 (OIV, 2005).

The statistics of 2003 show that two continents control more than three quarters of the world-wide production of dried grapes: Asia (51 %) and America (36 %) (Figure 25).

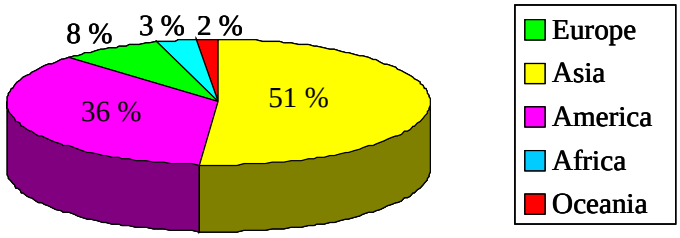

Figure 25. Production of dried grapes distributed by continent in 2003 (OIV, 2005).

USA and Turkey are the two main producer countries of dried grapes, with more than 350 thousand tonnes each. After them, only Iran produced more than 200 thousand tonnes, and the rest of countries in the classification produce around or below 50 thousand tonnes (Figure 26).

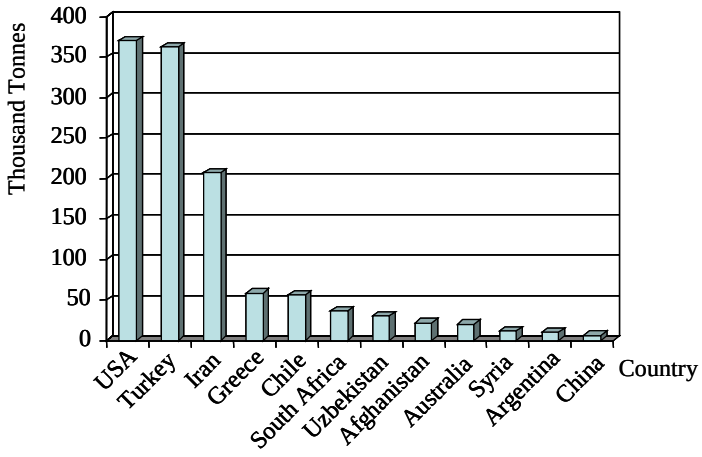

Figure 26. Dried grapes production of the 12 leading countries in 2003 (OIV, 2005).

6.1.7. World-wide dried grape consumption

Parallel of the present world-wide increase in dried grape production, there is a global increase in the consumption of these fruits (Figure 27).

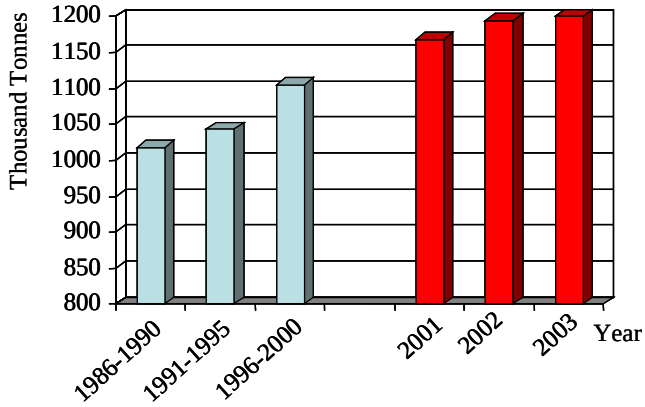

Figure 27. Evolution of the world-wide dried grapes consumption since 1986 (OIV, 2005).

Although Europe is a minor producing country of dried fruits, the 28 % of the total consumption belongs to this continent. Consequently to its low production and high consumption, EU is the main importer region of dried fruits. Equal amounts of dried fruits are consumed in America, meanwhile the highest consume is reported in Asia, with the 38 % of the world-wide consumption (Figure 28).

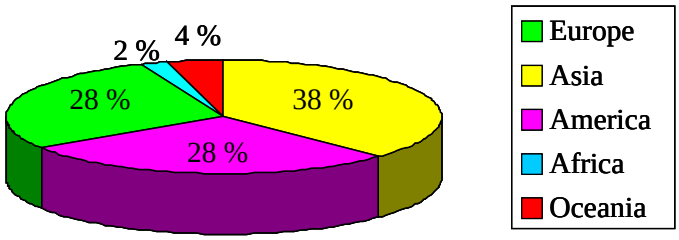

Figure 28. Consumption of dried grapes distributed by continent in 2003 (OIV, 2005).

The main consumer countries of dried-vine fruits are shown in figure 29.

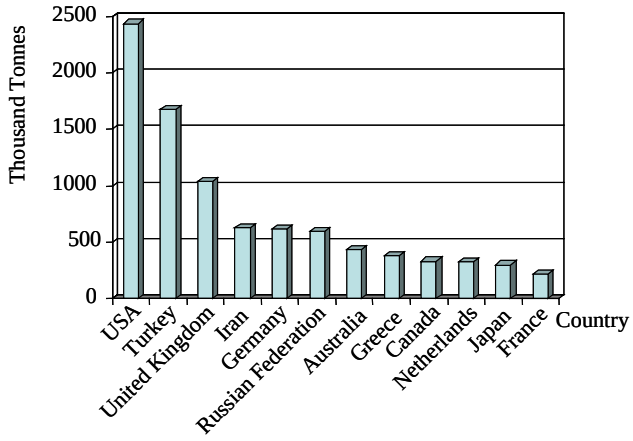

Figure 29. Dried grapes consumption of the 12 leading countries in 2003 (OIV, 2005).

6.2. SPANISH PRODUCTION OF GRAPES, WINE AND OTHER GRAPE-DERIVATIVES

The wine-producing sector is a highly important one in Spain, due not only to the economic value it generates, but also to the population it employs and the role it plays in environmental conservation.

6.2.1. Extension of Spanish vineyard

Spain dedicates 1.2 million hectares to grape cultivation, of which 97 % is destined to wine grapes, and continues to encompass the largest vineyard area in the EU and the world (more than one third of the total EU surface area, followed by France and Italy with 25 % each, which accounts for more than 15 % world-wide). In a country where the winemaking tradition dates back to the times of the Romans, the grape vine occupies third place in cultivated surface area, after cereals and olive groves.

Table 8 shows the surface dedicated to winegrowing and the production of wine in each region within the Spanish country. Nearly half of the total vineyards are located in Castilla-La Mancha (583.000 ha in 2003), the area with the greatest vineyard surface area in the world, followed by Extremadura, Comunidad Valenciana, Castilla y León and Cataluña. La Rioja, on the other hand, has the largest vineyard area in proportion to total cultivated surface area.

The major surface area planted with table grape vineyards is found in Eastern (C. Valenciana) and Southern (Andalucía) regions of Spain, whereas nearly 100 % of the total vineyards surface in the northern regions, are destined to wine-making grapes cultivars.

Table 8. Vineyard surface (ha) in different regions of Spain (2002-03) (MAPA, 2003).

Spanish regions	Table grapes vineyards	Wine grapes vineyards	Dry grape vineyards	Others	Total vineyards surface
Galicia	–	33326	–	–	33326
P. de Asturias	–	110	–	–	110
Cantabria	–	42	–	–	42
País vasco	–	13214	–	–	13214
Navarra	–	24416	–	–	24416
La Rioja	–	42855	–	–	42855
Aragón	285	42290	–	–	42575
Cataluña	48	64876	–	116	65040
Baleares	66	1890	–	–	1956
Castilla y León	128	70516	–	–	70644
Madrid	15	18455	–	–	18470
Castilla-La Mancha	231	583585	13	35	583864
C. Valenciana	11627	74471	–	–	86098
R. de Murcia	6211	45535	–	–	51746
Extremadura	732	86713	40	–	87485
Andalucía	4298	38439	–	–	45289
Canarias	132	18826	–	19	18977
Spain	23773	1159559	2605	170	1186107

6.2.2. Spanish table grape production

The evolution of the production of table grapes in Spain for nearly 20 years is shown in figure 30.

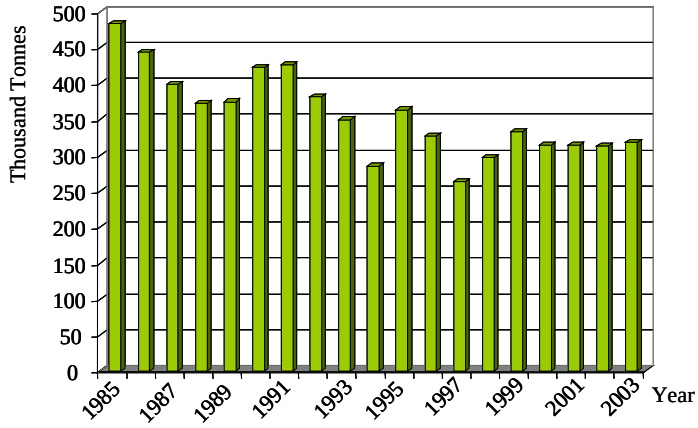

Figure 30. Evolution of the Spanish table grape production since 1985 (MAPA, 2003).

6.2.3. Spanish wine production

The geographical location of Spain, its different climates and variety of soil makes the peninsula ideal for the production of wines with widely varied characteristics. According to the latest figures from the Spanish Ministry of Agriculture (MAPA), Spanish wine production for the 2003/2004 harvest amounted to 46.4 million hl (including must), an increase of 12 % over the previous year.

The production of wine in Spain is different according to each region (Table 9). Castilla-La Mancha has the highest wine production (approx. 16 million hl), producing mostly table wines (87 %). Cataluña occupies the second place in the ranking, with 3.1 million hl produced, most of it destined to the production of qualified wine (90.4 %). The third position is for C. Valenciana, with half production of Quality Wines Produced in a Specific Region (VCPRD) and half of table wines. Galicia, La Rioja, Castilla y León and Andalucía are also high wine producers in this country, with a production around 1.4 million hl in 2002-03 each.

Table 9. Wine production (hl) in different regions of Spain (2002-03) (MAPA, 2003).

Spanish regions	VCPRD wine	Table wine	Other wines	Total
Galicia	405354	323137	694460	1422951
P. de Asturias	–	–	3600	3600
Cantabria	–	–	894	894
País vasco	404476	450	4694	409620
Navarra	652006	29157	8988	690151
La Rioja	1353479	65795	–	1419274
Aragón	630415	200616	16833	847864
Cataluña	2807291	223445	74587	3105323
Baleares	22577	–	8277	30854
Castilla y León	775996	616738	12488	1405222
Madrid	117178	415449	–	532627
Castilla-La Mancha	1517462,9	14084061	479409	16080933
C. Valenciana	1286117	1268250	380	2554747
R. de Murcia	290385	467151	–	757536
Extremadura	683789	2652357	–	3336146
Andalucía	948126,17	585030	254908	1788064,2
Canarias	39289	114503	–	153792
Spain	11933941	21046139	1559518	34539598

In general, a little more than half of the Spanish wine production is destined to table wines, whereas 30 % ends in VCPRD wines (D.O. wines) (Figure 31). However, an upward trend in the production of D.O. wines has been registered nowadays, while the surface area and the production of table wines has diminished in the last years (Figure 32).

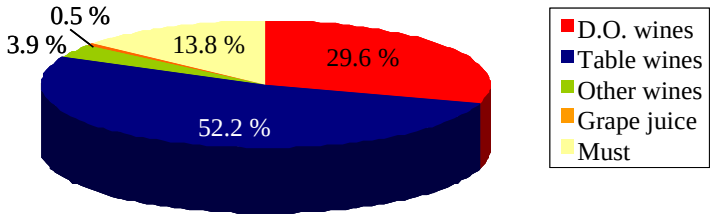

Figure 31. Production of the different types of grape-derivatives in Spain (2002-2003) (MAPA, 2003).

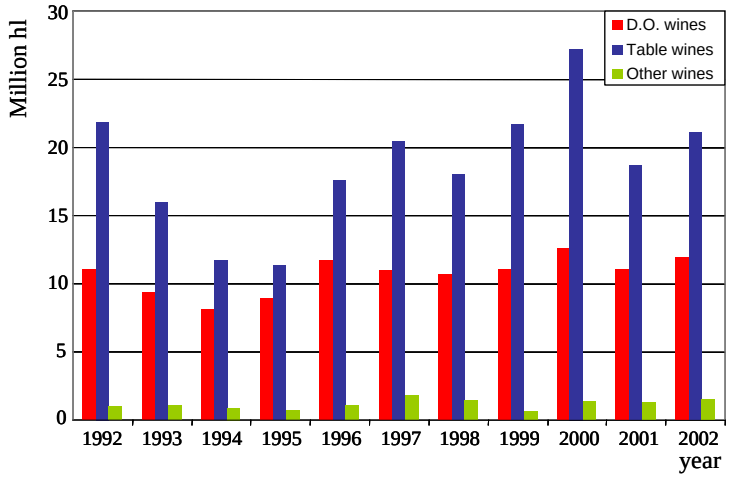

Figure 32. Evolution of the Spanish production of the main wine types since 1992 (MAPA, 2003).

Approximately one third of the total Spanish wine production is exported. From an average of 7 million hl exported in the three-year period from 1994 to 1996, the figure rose to an average of nearly 10.5 million hl in the successive three-year periods, with certain significant annual fluctuations due to changes in Spanish or EU production. Of the 14.5 million hl exported in 2004, 87.1 % represented still wines, 7.4 % sparkling wines and 2.2 % sweet wines.

6.2.4. Spanish wine Designations of Origin

Vineyards are cultivated in all the 17 Autonomous regions into which the country is divided. The Spanish Department of Agriculture controls the quality of Spanish wines through a labelling process which establishes 63 different wine regions or *Denominaciones de Origen* (Designations of Origin) (Table 10; Figure 33). Only 25 % of Spanish wines have been granted this prestigious label.

Apart of being an indicator of the geographical region, Designations of Origin (D.O.) point the production processes, quality, and personality of each wine variety. The official definition includes two basic components:

1) The quality, personality, and uniqueness of products derived from their geographical origin, which implies both certain conditions of soil and climate and certain growing and manufacturing practices.

2) The recognition and assignation of value of these differentiating qualities by consumers.

Quality depends not only on the grape varieties used, but also on the soil and climatic conditions where they are grown. That is why D.O. is so important when determining a wine's quality and authenticity. Its regulations do not merely guarantee that a wine has been produced using a particular grape variety, they look into all of the geographical factors that influence the final product, maintain strict control over the amounts produced, enological practices, and the quality of the wines made in each area.

Table 10. Wine Designation of Origin in Spain distributed by Autonomous regions.

Region	Designation of Origin		
Andalucía	- Condado de Huelva - Jerez-Xérèz-Sherry	- Málaga - Manzanilla San Lúcar de Barrameda	- Montilla-Moriles - Sierra de Málaga
Aragón	- Calatayud - Campo de Borja	- Cariñena - Cava[a]	- Somontano
Baleares	- Binissalem	- Mallorca	- Pla i Llevant
Canarias	- Abona - El Hierro - Lanzarote	- La Palma - Tacoronte-Acentejo - Valle de Güímar	- Valle de la Orotava - Ycoden-Daute-Isora
Castilla y León	- Bierzo - Cigales	- Ribera de Duero - Rueda	- Toro
Castilla-La Mancha	- Almansa - Dominio de Valdepusa - Jumilla[a]	- La Mancha - Manchuela - Méntrida	- Mondéjar - Ribera del Júcar - Valdepeñas
Cataluña	- Alella - Ampurdán - Cataluña - Cava[a] - Conca de Barberà	- Costa Brava - Costers del Segre - Montsant - Penedés - Pla de Bages	- Priorato - Tarragona - Terra Alta
Extremadura	- Cava[a]	- Ribera del Guadiana	
Galicia	- Monterrey - Rías Baixas	- Ribeiro - Ribeira Sacra	- Valdeorras
Madrid	- Vinos de Madrid		
Murcia	- Bullas	- Jumilla[a]	- Yecla
Navarra	- Cava[a]	- Navarra	- Rioja[a]
País Vasco	- Cava[a] - Chacolí de Bizkaia	- Chacolí de Alava - Chacolí de Getaria	- Rioja[a]
La Rioja	- Cava[a]	- Rioja[a]	
Comunidad Valenciana	- Alicante - Cava[a]	- Valencia	- Utiel-Requena

[a]Designations of Origin including more than one region.

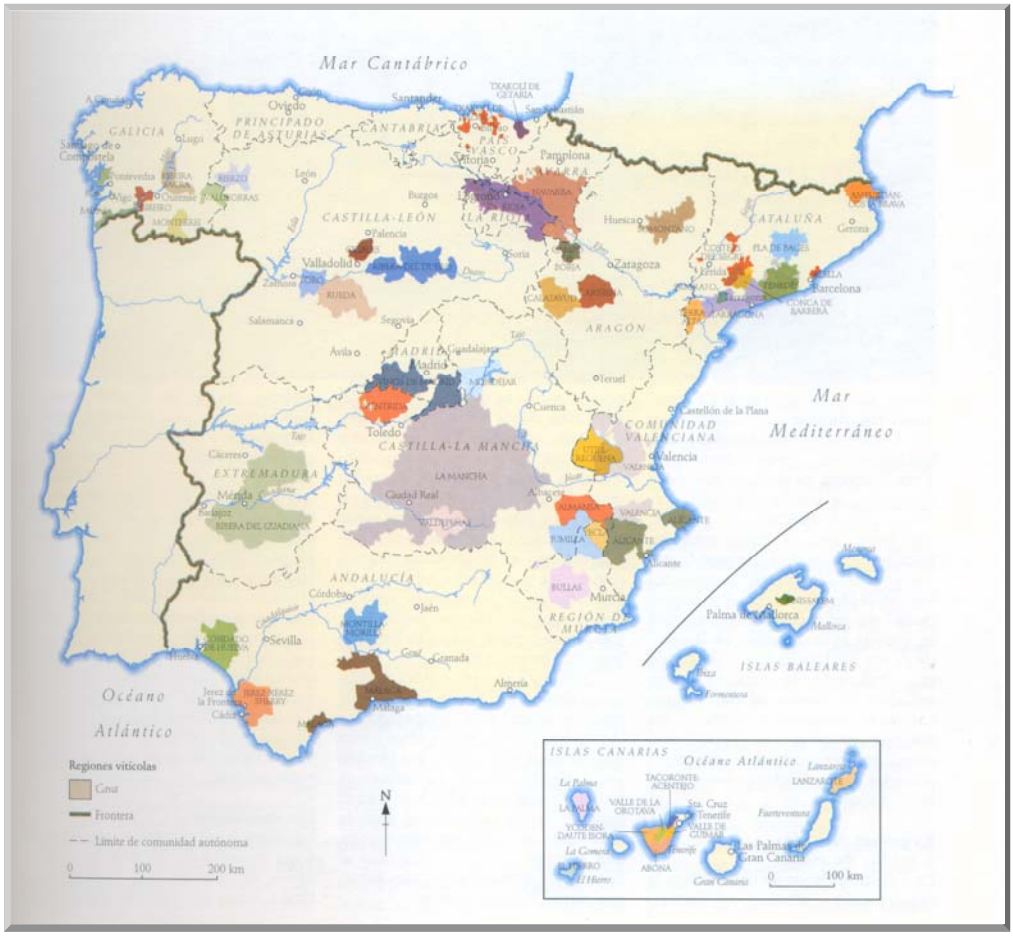

Figure 33. Main wine producing regions in Spain (Larouse, 2000).

6.3. OTA CONTENT IN DIFFERENT GRAPE-DERIVATIVES

6.3.1. OTA in grapes, grape juices and musts

Must is the juice of freshly pressed grapes, prior to fermentation into wine. Must contains various quantities of pulp, skins, stems, and seeds, called pomace or grape solids, which typically comprise between 7-23 % of the total weight of the must. These components, and the time they are allowed to be in contact with the juice, are critical to the final character of the wine.

To know the concentration of OTA in grapes, several bunches must be randomly sampled, berries crushed and the extracted must analysed. Only a couple of studies reporting OTA analysis of 'natural' musts could be found. On the one hand, Battilani et al. (2002) found significant amounts of OTA in musts from Italian grapes –concentrations not provided-, observing differences between years and vineyards. On the other, Sage et al. (2002) found eight contaminated musts, out of 11 samples made from French grapes with concentrations of OTA ranging from 10 to 461 ng l^{-1}.

During the last few years, papers regarding the presence of OTA in grape and its processed products have been increased. Several authors have analysed the OTA content in 'commercial' grape juices and musts (Table 11). These products differ from natural musts because they undergo one or several processes such as filtration, homogenisation, aromatisation, etc., before commercialisation (Noguera, 1974). OTA occurrence in these products was widely reviewed in 6.3.4.2. Furthermore, a study including some samples of commercial Spanish musts (n=20) and grape juices (n=10) has been carried out to contribute to these data (see 6.4.3.).

Table 11. Studies of OTA occurrence in grape juices and musts. Concentrations are not given as they have been published in a review (see 6.3.4.2.).

Sample	n	Country	Reference
Red commercial grape juices White commercial grape juices Grape juices extracts	8 3 17	Switzerland	Zimmerli and Dick (1996)
Red grape juice White grape juice	14 6	various	Majerus and Otteneder (1996)
Grape must Grape juice alone or mixed with other fruit juices	8 10	Spain	Burdaspal and Legarda (1999)

(…/…)

Red grape juice White grape juices	64 27	Germany	Majerus et al. (2000)
White musts Red musts Red concentrated musts White concentrated must Concentrated rectified musts	20 20 16 1 6	Italy	Larcher and Nicolini (2001)

6.3.2. OTA in dried vine fruits

Raisins are dried grapes that can be eaten raw, used in cooking and baking or as ingredients in muesli, biscuits, cakes, etc. During summer, when grapes have attained their optimum sweetness, farmworkers carefully hand-pick the grape bunches and let them dry on rows of clean paper trays next to the vines. The grapes dry naturally in the sun for two or three weeks. The process can be done also indoor in industrial controlled driers. Raisins have high concentration of sugars, and if stored for a long period the sugar crystallises inside the fruit. This makes the fruit gritty, but does not affect the usability.

In USA, the term 'raisin' refers to any form of dried grape. California raisins, both sun-dried dark naturals and goldens, are made by drying Thompson seedless grapes. Dark naturals are sun dried and ferment in the process, while goldens are flame dried. Another variety of seedless grape, the Black Corinth, is also sun dried to produce Zante currants, mini raisins that are much darker in colour and have a tart, tangy flavour. In other countries, especially in Australia, specific varieties are given separate names, such as raisins, sultanas and currants. In particular, raisins are largest, sultanas are intermediate, while currants are the smallest. Raisins are also produced in Greece especially in the areas of Peloponessus, Crete and smaller islands. The main variety used in this country is the sultana. The grapes are mostly sun-dried thus producing seedless raisins of average size and golden colour. A notable exception to this rule is the grape variety cultivated especially for the purpose of raisin production in Corinth that give darker and bigger type of raisin, not seedless, named Corinthian (www.en.wikipedia.org/wiki/Raisin).

Some Mediterranean countries make special sweet wines using dried grapes by direct exposition to sun, sometimes classified as *dessert wines*. This process is feasible only in some warm or semi-arid regions of these countries, such the South of Spain.

Data on natural occurrence of OTA in dried vine fruits has increased in the last few years. The incidence of OTA in these products is generally high, as well as the OTA concentration (Table 12). The possibility of mechanical damage during harvesting and the prolonged time available for fungal growth during drying increase the probability of OTA to be formed. However, an important problem detected is the heterogeneity of

samples. Thus, validated sampling methods are required to ensure that the results of analysis are truly representative of the larger initial samples.

The unexpectedly high levels of OTA in dried vine fruits were of immediate concern. Slayne (2001) concluded that, based upon dietary consumption patterns, the levels of OTA in dried vine fruits are safe, but recommended that industry should reduce levels to the lowest technologically achievable.

Dried grape fruits, which have low water activity (a_w), are generally resistant to microbial attack. However, different surveys of mycoflora in dried fruits have been carried out (Abarca et al., 2003; Leong et al., 2004; Magnoli et al., 2004; Valero et al., 2005a), all pointing out black *Aspergillus* and mainly *A. carbonarius*, as the responsible for the OTA levels detected in these fruits. Adaptation to environmental conditions of sun-drying, and a strong dominance of black aspergilli among the common mycobiota of grapes at these conditions, are suggested as the two main reasons supporting the prevalence of black aspergilli in sun dried grapes (Valero et al., 2005b).

Table 12. Percentage and concentration of dried fruit samples naturally contaminated with OTA.

Purchased in:	Currant (% +)	Sultana (% +)	Raisin (% +)	Dried fruits[a] (% +)	Reference
UK and Norway	n=20; 95 % 0.2-53.6 µg kg^{-1}	n=20; 85 % 0.2-18.1 µg kg^{-1}	n=20; 85 % 0.2-20.0 µgkg^{-1}	-	McDonald et al. (1999)
UK	-	-	-	n=301; 95 % 0.1-41 µg kg^{-1}	MAFF (1999)
Germany	-	-	-	95 %	Engel (2000)
Finland France	-	-	71 % 46 %	-	Miraglia and Brera (2002)
Czech Republic	-	-	n=48; 25-58 % 0.5-63.6 µg kg^{-1}	-	Ostry et al. (2002)
Sweden	n=118 (currant and sultana); 84 % 0.1-34.6 µg kg^{-1}		-	-	Möller and Nyberg (2003)
Greece	n=54; 80 % mean:1.3 µg kg^{-1}	n=27; 63 % mean:0.6 µgkg^{-1}	-	-	Stefanaki et al. (2003)
Canada	n=2; 100 % 0.1-4.85 µg kg^{-1}	n=66; 59 % 0.1-26.0 µg kg^{-1}	n=85; 79 % 0.1-26.6 µg kg^{-1}	-	Lombaert et al. (2004)
Argentina	-	-	-	n=50; 74 % mean: 6.3 µg kg^{-1}	Magnoli et al. (2004)

[a] Specific fruit not identified.

6.3.3. OTA in vinegar

Vinegar, from the French word 'vinaigre' meaning sour wine, is a sour liquid made from the oxidation of ethanol in wine, cider, beer, or similar alcoholic products. Vinegar is typically 3-5 % by volume acetic acid, and natural vinegars also contain smaller amounts of tartaric acid, citric acid, and others. Vinegar may be started by the addition of what is called 'mother of vinegar'. The oxidation is carried out by acetic acid bacteria, as was shown in 1864 by Louis Pasteur.

Vinegar may also contain OTA. Few studies analysed its content and their results are summarized in table 13.

Table 13. Percentage and concentration of OTA-contaminated vinegar samples.

Sample	n	OTA+	Range	Country	Reference
Apple and fruit vinegar Wine vinegar Balsamic vinegar	18 38 29	6 % 50 % 83 %	0.01-0.02 μg l^{-1} 0.01-1.9 μg l^{-1} 0.01-4.35 μg l^{-1}	Germany	Majerus et al. (2000)
Balsamic vinegar	3	100 %	0.10-0.50 μg l^{-1}	France	Markaki et al. (2001)

6.3.4. OTA in wine

The presence of OTA in wine has been reported by several authors from the entire world since its first description in 1996 (Zimmerli and Dick, 1996). Until now, OTA and its related compounds are the only mycotoxins detected in wine.

Most of the studies carried out before 2001 were compiled in order to obtain a general overview of OTA occurrence in wines from different countries over the world, and the results are shown in the following paper (see 6.3.4.2.):

Bellí, N., Marín, S., Sanchis, V. and Ramos, A.J. 2002. Review: Ochratoxin A in wines, musts and grape juices: occurrence, methods of analysis and regulations. *Food Science and Technology International* 8, 325-335.

This revision included more than two thousand samples, with OTA being more commonly detected in red wine samples (mean: 71 %; median: 90 %), with mean content of 0.04-1.80 μg l^{-1} (max. 7.6 μg l^{-1}), followed by rosé wines (mean and median: 66 %), 0.02-1.35 μg l^{-1} (max. 2.4 μg l^{-1}), and white wines (mean 45 %; median: 34 %) with 0.01-0.53 μg l^{-1} (max.

1.2 μg l^{-1}). The number of dessert wines reported to contain OTA was in general high (60 % -100 %), with mean contents of 0.01-3.8 μg l^{-1} (max. 15.3 μg l^{-1}). Some authors also found OTA in grape juices, what stated a significant contribution of these products to the OTA exposure of children. Their studies were also reviewed. Other aspects presented in this review were the influence of the geographical origin of the samples, concluding that there is not a clear evidence of a North-South gradient in wine OTA contamination. Agricultural and manufacture practices in wine making were also mentioned, as they have an effect on the OTA content of wines. All the information collected suggests that the intake of OTA related to grape and its derivatives is a real risk, especially for consumers of red wine, dessert wine, and although not included in the review, dried vine fruits.

6.3.4.1. Analytical determination of OTA in wines

The analytical methodology to determine OTA in wines usually includes the following steps: extraction, purification of the extract (clean-up), separation, detection, quantification and confirmation of identity. There are a number of choices for each stage, and the combination chosen depends on operator preferences or on the equipment availability. Numerous analytical methods developed before 2001 for the detection and measurement of OTA in wine were pointed out in the final part of the review (see 6.3.4.2.).

Various extraction protocols for OTA in wines are described in the literature, most of them using toluene, chloroform or a hydrogen carbonate and polyethylene glycol (PEG) solution in the extraction step.

The most important development in the field of clean-up methods until now is the use of immunoaffinity columns (IAC). These columns are composed of monoclonal antibodies specific for OTA, which are immobilized into small plastic cartridges. The principle is that the extract is forced through the column and ochratoxins are left bound to the recognition site of the immunoglobulin. Extraneous material is washed off the column with water or aqueous buffer, and the ochratoxin is eluted with an appropriate solvent (e.g. acetonitrile). Then, the eluate is analysed for final separation and determination of the toxin. Direct application of the wine onto the IAC is possible.

Separation of the components of the extract and detection procedure are often achieved using high performance liquid chromatography (HPLC) with fluorescence detection (FD), but thin layer chromatography (TLC) and enzyme-linked immunosorbent assay (ELISA) are also applied. Detection by TLC is based on its blue fluorescence under UV radiation but the sensitivity of this technique is not always high. ELISA technique may be qualitative or semi-quantitative and is particularly attractive for rapid screening purposes. In addition, some methods have used mass spectrometry (MS) to identify OTA.

The identity of OTA is confirmed, most of the times, by the formation of a methyl ester of OTA, after derivatizate the extracts with a boron trifluoride methanol complex. Another confirmatory method is the degradation of OTA by carboxypeptidase with formation of ochratoxin α.

The Association of Official Analytical Chemists (AOAC International) and the European Standardization Committee (CEN), the European equivalent of the International Organization for Standardization (ISO), have a number of standardized methods of analysis for mycotoxins that have been validated in formal interlaboratory method validation studies, and this number is gradually growing. The latest edition of Official Methods of Analysis of AOAC International (Horwitz, 2000) contains approximately 40 validated methods for mycotoxin determination, and a recent review has been published about the validation of methods of analysis for mycotoxins (Gilbert and Anklam, 2002).
Finally, some kits for the detection of OTA in several foodstuffs have been recently developed. In particular, there is a rapid and easy ELISA kit (Biokit OTA assay kit, Tepnel Biosystem), that enables the detection of this mycotoxin in dried vine fruits and in white wines, and also in cereal products and green coffee, at levels below those proposed in the legislation. World-wide changes in legislation ever increase the need for more precise and sensitive mycotoxin analytical methods.

6.3.4.2. Review: OTA in wines, musts and grape juices: occurrence, regulations and methods of analysis

Ochratoxin A (OTA) in wines, musts and grape juices: occurrence, regulations and methods of analysis

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ABSTRACT

This work gives a general overview of ochratoxin A (OTA) occurrence in wines and the methodology for OTA analysis. The results of more than two thousand samples have been taken into account to quite extensively describe the present situation of OTA contamination of wine. According to these data, OTA is much more commonly detected in red wines than in rosé and white wines, and OTA concentration is remarkably higher than in the latter ones. Thus OTA could be detected in 45 % (median = 34 %) of white wine samples, whereas it was detected in 66 % (median = 66 %) of rosé and 71 % (median = 90 %) of red wine samples. When comparing the wines from Northern and Southern regions, the latter showed a higher contamination than those from the Northern area. It has been suggested that OTA accumulation could be due to fungi belonging to the genus *Aspergillus* in wines from Southern European countries because the crops are exposed to elevated temperatures, which favour growth of OTA-producing *Aspergillus* species over *Penicillium*. High performance liquid chromatography (HPLC) associated with fluorescence detection preceded by extraction of OTA using commercially available immunoaffinity columns (IAC) is currently the most applied method for OTA determination in wines.

KEY WORDS: ochratoxin A, mycotoxins, wine, must, grape juice

***Food Science and Technology International* 8, 325-335 (2002)**

INTRODUCTION

Mycotoxins are fungal secondary metabolites that could be present in foods as a consequence of fungal growth, and are harmful to animals and humans. Ochratoxins (cyclic pentaketids, dihydroisocoumarin derivatives linked to an L-phenylalanine moiety) are mycotoxins produced by some *Aspergillus* and *Penicillium* species. They were originally isolated in South Africa in 1965 as metabolites from a strain of *Aspergillus ochraceus* (Van der Merwe et al., 1965). Although a wide range of ochratoxin derivatives can be isolated from laboratory cultures it is usually only ochratoxin A (OTA) and occasionally ochratoxin B which occur in mouldy products. OTA (7-(L-β-phenylalanyl-carbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocumarin) is the most toxic compound of this group, and therefore, it is receiving increasing attention.

Natural occurrence of OTA in food has been broadly documented since 1969 (Shotwel et al., 1969). OTA has been widely detected in food of vegetal origin mainly in cereals (barley, wheat, maize, oat, etc.) and their by-products (Speijers and Van Egmond, 1993; Trucksess et al., 1999), in green coffee (Jørgensen, 1998; Trucksess et al., 1999), and also in spices (Hubner et al., 1998). OTA has also been detected in some drinks as coffee (Ueno et al., 1991; Nakajima et al., 1997; Téren et al., 1997; Bucheli et al., 1998; Burdaspal and Legarda, 1998; Jørgensen, 1998; Ueno, 1998), beer (Jørgensen, 1998; Ueno et al., 1998), and grape juice (Zimmerly and Dick, 1996; Ueno, 1998; Burdaspal and Legarda, 1999; Visconti et al., 1999). Moreover, recent investigations show that wines commonly contain ochratoxin (Majerus and Otteneder, 1996; Zimmerli and Dick, 1996; Burdaspal and Legarda, 1999; Visconti et al., 1999; Cerutti et al., 2000; Majerus et al., 2000; Tateo et al., 2000; Filali et al., 2001; Larcher and Nicolini, 2001; Markaki et al., 2001; Pietri et al., 2001; Soleas et al., 2001).

The main contributor to the dietary intake of OTA seems to be cereals and cereal products, but there is also a risk to human health not only through the intake of contaminated foods of vegetal origin, but also through foods of animal origin. OTA has been detected in pork and poultry meat (Kuiper Goodman and Scott, 1989), pig blood and kidneys, milk (Skaug, 1999), as well as in human blood and mother's milk (Höhler, 1998).

OTA-producing species

Apart from *Aspergillus ochraceus*, other species of *Aspergillus* section *Circumdati* (*A. ochraceus* group) have been identified as OTA producers: *Aspergillus auricomus*, *A. melleus*, *A. muricatus*, *A. ostianus*, *A. petrakii*, *A. sclerotiorum* and *A. sulphureus*. These members are the main ochratoxin producers in the Aspergilli (Varga et al., 2001). Recently, *A. albertensis* and *A. alliaceus* has been transferred from section *Circumdati* to *Aspergillus* section *Flavi* (Varga et al., 2000), and they have been identified as OTA-

producers, as well as some members of *Aspergillus* section *Aspergillus* (*A. glaucus* group) as *A. glaucus* (or *Eurotium herbariorum*), *A. sydowii* and *A. repens* (Varga et al., 2001). Furthermore, OTA has also been established as a metabolite of different species of the section *Nigri*, such as *A. niger*, *A. carbonarius*, *A. awamori* and *A. foetidus* (Abarca et al., 1994; Téren et al., 1996). There are other OTA-producing *Aspergillus* species that produce occasionally small amounts of OTA and their ability to produce OTA was not confirmed by other authors.

Ochratoxin A has also become established as a metabolite of some *Penicillium* species. Few years after its first description, OTA was detected as a metabolite from strains of *Penicillium viridicatum* and some other *Penicillium* species. However, because of misidentified fungal strains and changes in *Penicillium* taxonomy, Pitt (1987) suggested that *Penicillium verrucosum* is the only known and confirmed *Penicillium* species that is able to produce OTA, although latter, other authors have found other *Penicillium* isolates producing OTA (Bridge et al., 1989; Ueno et al., 1991). *Penicillium verrucosum* is the most dominant ochratoxin A-producing contaminant of foods in colder regions, such as Northern Europe and Canada (Frisvad and Samson, 2000). Major habitats of *P. verrucosum* are cereals and other plant sources. However, *P. verrucosum* is also found as a contaminant on food products with large amounts of proteins and fats, such as meat and cheese (Gareis and Scheuer, 2000).

Adverse effects of OTA

OTA is receiving increasing attention world-wide because of the hazard it poses to human and animal health. OTA has many toxic effects, with renal damage being the most common and serious. Cytotoxicity has been clearly demonstrated in cultured kidney cell lines (Bondy and Armstrong, 1998). Besides being a potent nephrotoxic agent (Plestina, 1996; Stoev, 1998), OTA displays hepatotoxic, teratogenic, carcinogenic and immunosuppressive properties.

Population studies have shown the presence of measurable concentrations of OTA in the blood plasma of many apparently healthy people (Scott et al., 1998; Ueno et al., 1998).

In 1993, the International Agency for Research on Cancer (IARC) classified OTA as a possible human carcinogenic substance (Group 2B), based on research with laboratory animals; however, evidence concerning humans is still inconclusive (IARC, 1993). Moreover, OTA is suspected to be involved in Balkan endemic nephropathy (a fatal kidney disease occurring in some areas of south-eastern Europe) and in the high frequency of urinary tract tumours observed in some Balkan areas (Castegnaro et al., 1991; Petkova-Bocharova and Castegnaro, 1991). Human exposure occurs mainly through the consumption of contaminated products and the toxin is frequently found in

human blood and milk, because of the long elimination half-life, of the order of 35 days in human serum (Studer-Rohr et al., 1995).

OCCURRENCE IN WINES AND GRAPE JUICES

General Aspects

The occurrence of OTA in wine is linked to the presence of moulds on grapes. The most important species producing OTA in grapes are *A. ochraceus*, *Aspergillus* section *Nigri* and *P. verrucosum*. The high number of *Aspergillus* section *Nigri* found in the latest studies in grapes (Serra et al., 2001; Sage et al., 2002) suggested that they could be the main responsible for the frequent OTA contents in grape juices and wines. There is limited information on the conditions that favour the development of infection in grapes, although hot and humid conditions are associated with the growth of any mould (Stockley, 2000).

Since the first study on the occurrence of OTA in wines (Zimmerli and Dick, 1996), several studies have been carried out by different authors, using a broad variety of wines (red, white, rosé, dessert, etc.), musts and grape juices, as it is shown in Table 1. Their results are not directly comparable because of the differences in the methods of analysis. It is also evident that some of these results can be misleading, since they were obtained in studies involving a small number of samples. Furthermore, the percentage of samples containing OTA also depends on the detection limit (DL) value. The lower the DL, the higher the percentage of positive samples is, and therefore, higher DL values imply that probably many samples containing low values of OTA were not detected.

As the general suspect is that red wines are the most OTA contaminated, the number of red wine samples analysed in all the studies reviewed was much higher than the number of the white, rosé or dessert wines.

Percentage of Wine Samples Containing OTA

The global results show that the median of the percentage of positive samples in red wines is around 90% (mean=71%), followed by rosé wines (66%) (mean=66%) and white ones (34%) (mean=45%). All authors detected higher number of red wines containing OTA, followed by rosé and then by white wines (Majerus and Otteneder, 1996; Visconti et al., 1999; Cerutti et al., 2000; Majerus et al., 2000; Soleas et al., 2001), except for Zimmerli and Dick (1996), who found more rosé wine samples containing OTA than red wines, probably due to the difference in the number of samples analysed for each one (n=79 red wines and n=15 rosé wines).

Table 1. Results of several surveys about OTA occurrence in wines, musts, and grape juices.

Samples	n	Positives (%)	Mean (µg/L)	Median (µg/L)	Range (µg/L)	DL (µg/L)	Reference
White wine	69	65.2	0.020	n.a.	< DL- 0.267	0.003	Burdaspal and Legarda, (1999)
Rosé wine	32	90.6	0.031	n.a.	< DL - 0.161		
Red table wine	91	92.3	0.054	n.a.	< DL - 0.603		
Aperitif wines (Sherry type)	47	74.5	0.040	n.a.	< DL -0.254		
Sparkling wines	12	83.3	0.012	n.a.	< DL -0.037		
Dessert wines	16	93.7	1.048	n.a.	< DL –2.540		
Grape must and grape juice	18	100	0.045	n.a.	0.015-0.102		
Red wine from bottle	23	86.9	0.385	0.195	< DL-1.340	0.080	Cerutti et al., (2000)
Red wine from tetrapak	6	100	1.802	2.125	0.143-2.933		
Rosé wine from bottle	1	0	< DL	< DL	<DL		
Rosé wine from tetrapak	2	50	1.348	1.348	<DL-1.348		
White wine from bottle	18	50	0.264	0.195	<DL-0.456		
White wine from tetrapak	10	50	0.144	0.108	<DL-0.289		
White wine	7	100	0.072	0.048	0.028-0.540	0.01	Filali et al., (2001)
Rosé wine	3	100	0.223	0.09	0.04-0.54		
Red wine	20	100	0.912	0.785	0.04-3.24		
White wines (aged in steel)	27	7.4	0.015	0.015	0.01-0.02	0.01	Larcher and Nicolini, (2001)
Red wines (aged in steel)	36	22.2	0.041	0.04	0.01-0.1		
Red wines (aged in barrique)	8	0	0	0	-		
Organic wines	7	0	0	0	-		
Base sparkling	9	0	0	0	-		
Late-harvest and Vino Santo	7	0	0	0	-		
White and red musts	40	0	0	0	-		
Concentrated musts	17	100	1.55	0.85	0.06-6.18		
Concentrated Rectified musts	6	16.6	0.03	0.03	0.03		
White wine	41	34	n.a.	0.07	<DL-1.2	0.01	Majerus and Otteneder, (1996)
Rosé	14	43	n.a.	0.1	<DL –2.4		
Red wine	89	45	n.a.	0.19	<DL –7.0		
White grape juice	6	17	n.a.	n.a.	<DL -0.73		
Red grape juice	14	86	n.a.	1.8	<DL –4.7		
White wine	58	24	n.a.	<0.01	< DL- 1.4	0.01	Majerus et al., (2000)
Rosé wine	51	35	n.a.	<0.01	< DL- 2.4		
Red wine	172	46	n.a.	<0.01	< DL- 7.0		
White grape juice	27	78	n.a.	0.09	< DL- 1.3		
Red grape juice	64	88	n.a.	0.27	< DL- 5.3		
Red wine	31	100	n.a.	n.a.	0.010-3.4	0.002	Markaki et al., (2001)
White wine	6	16.6	0.16	0.16	<0.01 – 0.16	0.01	Ospital et al., (1998)
Rosé wine	2	50	0.11	0.11	<0.01 – 0.11		
Red wine	21	57	0.08	0.075	<0.01 – 0.27		
Red wine	96	85	0.419	0.090	<0.001 –3.177	0.001	Pietri et al., (2001)
White dessert wine	15	60	0.736	0.008	<0.001 – 3.856		
White wines	362	3.9	n.a.	n.a.	0.051-0.100	0.05	Soleas et al., (2001)
Red wines	580	16.6			0.051-0.200		
Red table wines packed in brick	31	96.7	0.94	1.045	<DL-3.80	0.013	Tateo et al., (2000)
Commercial Red wine	27	96.3	1.269	0.895	<DL -7.63	0.010	Visconti et al., (1999)
Home made Red wine	11	100	1.185	0.660	0.46-4.72		
Commercial Rosé wine	6	83.3	0.804	1.010	< DL- 1.15		
Home made Rosé wine	2	100	0.525	0.525	0.41-0.64		
Commercial White wine	7	28.6	0.045	0.045	< DL -0.06		
Home made White wine	2	100	0.535	0.535	0.10-0.97		
Dessert wine (Marsala)	1	100	0.29	-	0.29		
White wine	24	33.3	0.011	< DL	< DL-0.178	0.005	Zimmerli and

Rosé	15	93.3	0.025	0.019	< DL-0.123		Dick, (1996)
Red wine	79	78.5	0.039	0.013	< DL- 0.388		
Port	6	n.a.	0.011	< DL	< DL-0.017		
Sherry	2	100	0.042	n.a.	0.029-0.054		
Marsala	2	100	0.191	n.a.	0.044-0.337		
Malaga	3	100	0.290	n.a.	0.049-0.451		
Vermouth	2	n.a.	< DL	n.a.	n.a.		
Red commercial grape juices	8	n.a.	0.188	0.235	<DL-0.311		
White commercial grape juices	3	0	0.116	n.a.	<DL		
Grape-juice extracts	17	0	<DL	n.a.	<DL		

n.a. - not available; DL - detection limit.

The number of samples reported containing OTA is, in general, extremely high for dessert wines (100% Zimmerli and Dick, 1996; 93.7%, Burdaspal and Legarda, 1999, and 60% Pietri et al., 2001). The reason could be that grapes used to produce this kind of wines are generally in an advanced ripeness and sometimes they are already rotten. However, geographical area and climate may be also important factors involved, thus, the incidence in dessert wines from Northern Italy has been shown to be low (Larcher and Nicolini, 2001).

OTA Content in Wines

Amounts of OTA found in red wines are higher than in rosé wines followed by white wines. In general, OTA mean content in red wines ranged from 0.039 μg/L (Zimmerli and Dick, 1996) to 1.802 μg/L (Cerutti et al., 2000) very similar to that for rosé wines, which ranged from 0.025 μg/L (Zimmerli and Dick, 1996) to 1.348 μg/L (Cerutti et al., 2000). These average values are slightly higher than the ones for white wine samples, which ranged from 0.011 μg/L (Zimmerli and Dick, 1996) to 0.535 μg/L (Visconti et al., 1999). Majerus et al. (2000) suggested that the reason for the different levels of OTA concentration of white, rosé and red wines is the different wine-making technique, since longer mash standings in red wine could lead to higher OTA content in wine.

The maximum levels of OTA detected were 7.63 μg/L (Visconti et al., 1999), 2.4 μg/L (Majerus and Otteneder, 1996; Majerus et al., 2000) and 1.2μg/L (Majerus et al., 2000) for red, rosé and white wine, respectively.

OTA Occurrence in Grape Juices

In view of the suspected occurrence of OTA also in fruit-juices and their potential consumption by children, some researchers analysed grape and other fruit-juices samples too. The number of grape juices containing OTA was also very high, especially for red grape juice samples (86% Majerus and Otteneder, 1996; 88% Majerus et al., 2000), but

lower for white grape juice samples (17% Majerus and Otteneder, 1996; 78% Majerus et al., 2000; 0% Zimmerli and Dick, 1996). Furthermore, 100% positives were found by Larcher and Nicolini (2001) in concentrated musts and by Burdaspal and Legarda (1999) in both white and red grape musts and grape juices, but due to the limited number of samples analysed (n=17 and n=18, respectively) more data on the occurrence of OTA in grape juices are required. Again, products made from red grapes were more contaminated with OTA, probably due to the long time of enzymatic treatment at high temperature of the crude juice and the berries to improve the deep colour yield (Majerus et al., 2000).

Grape juices also seem to contain more OTA than some table wines, this suggested that the toxin in the wines was likely present prior the wines were made (Majerus et al., 2000). Furthermore, the comparison between grape juice and wine demonstrates that OTA has a relatively high stability against the alcoholic fermentation and the subsequent technological steps. Grape juice, apple (n=33) and orange juice (n=30) as well as black currant (n=19) and vegetable juices (n=78) were analysed by Majerus et al. (2000) and found apple and orange juice free from OTA; levels slightly above the limit of detection were detected in black currant, tomato and carrot juices. Similar results obtained Filali et al. (2001) who did not found OTA in 13 fruit juices (cocktail, orange, mango, peach, pineapple, clementine) and the only sample analysed containing OTA was a grape fruit juice (1.16 μg OTA/L). More data on the occurrence of OTA in grape juices are necessary because children are one of the main consumers and because juice consumption is more than that of wine.

Sample Origin Influencing on OTA Content

Most of the surveys show that the geographic region of origin of the wine has a strong influence on OTA contamination. The further Southern the provenance, the more common the occurrence and the greater the concentration of OTA found. Zimmerli and Dick (1996) were the first to point out a higher incidence of contamination in red wines from Southern Europe and Northern Africa, in comparison with the rest of wines they analysed. Majerus and Otteneder (1996) results, also indicate a North-South gradient of OTA level in wines, whereby samples from the Mediterranean area were contaminated more frequently. Recently, Pietri et al. (2001) found that wines produced in Southern Italy were markedly more contaminated than those from the North. A study of red wines originated from Mediterranean sea countries (Markaki et al., 2001) revealed that the more contaminated samples were one from Greece (2.35 μg/L), four from France (2.62 μg/L, 1.59 μg/L, 3.21 μg/L, and 3.40 μg/L), and one sample labelled European Union(1.54 μg/L). Furthermore, one sample from Italy and two samples from Morocco were found to be contaminated with OTA at levels of 0.892μg/L, 0.551μg/L and 0.554μg/L, respectively. Majerus et al. (2000) also analysed few samples from Tunisia and Algeria obtaining a range from 0.37 to 1.85 μg/L. Taking into account earlier results from

Morocco (10.5-15.6 μg OTA/L by Ospital et al., 1998; 100% OTA contaminated wine samples by Filali et al., 2001), there seems to be a tendency that samples from Northern Africa have even higher OTA concentrations than those from regions in Europe. This could be because of the high temperature and humidity that characterise the South climate conditions. By contrast, Majerus et al. (2000) compared the OTA contamination of some European samples and observed higher OTA contamination in the North (Germany, Northern France, Northern Italy) than in the South (Southern France, Spain and Greece) regions in Europe. In addition, Soleas et al. (2001) analysed the incidence of OTA in 942 commercial wines and found that European red wines was quite variable: Spain (33%), Portugal (22%), and Central Europe (22%) had higher percentages, while Greece (17%) and Italy (16%) had lower ones. Therefore, evidence for a North-South gradient in wine OTA concentrations is not clear from these data.

Agricultural and Manufacture Practices Influencing on OTA Content

Different practices used in grape cultivation, post-harvest and winemaking techniques (e.g. time period and condition of storage of the harvested grapes, type of maceration, time and temperature of fermentation) could also influence on OTA levels in wines and grape products (Otteneder and Majerus, 2000). Cerutti et al. (2000) found in 60 Italian wines (red, rosé and white), less amount of OTA in glass bottled wines than in those packed in Tetrapak. Similar results were obtained by Tateo et al. (2000). The reports of other authors also gave a guiding prices references of the wines analysed (Zimmerli and Dick, 1996; Markaki et al., 2001). Apart from them, there are not many studies comparing the OTA content with the quality of the wine. Therefore, it would be interesting to classify wines according to their ages (young wines, ageing or reserve wines) and investigate the relationship with their OTA content.

REGULATIONS

It cannot be excluded that certain mycotoxins, as OTA, ingested in minor quantities with our daily diet for an extended period may pose a risk to human health. The quantity of OTA ingested per day can be established on the basis of the analytically determined OTA concentrations of foods available on the market and the quantities consumed, or from the analytically established OTA concentrations on the blood sera.

In 1991, the FAO/WHO Joint Expert Committee on Food Additives (JECFA) after the evaluation of the nephrotoxicity of OTA, established a provisional tolerable weekly intake value for OTA of 112 ng OTA /kg body weight (16 ng/kg body weight/day). In the same year, the Nordic Working Group on Food Toxicology and Risk Evaluation, based on carcinogenicity data, considered that it would be prudent to reduce the tolerable daily OTA intakes to 5 ng/kg body weight/day. In 1998, the European Union Scientific

Committee for Food, and due to the toxicological properties of OTA, came to the same conclusion and estimated an acceptable safe level for daily OTA intakes below 5 ng/kg body weight/day. Previously, Zimmerli and Dick (1996) had suggested a lower provisional virtually safe dose to the magnitude of 0.5 ng/kg body weight per day on the basis on the results of Studer-Rohr et al. (1995), who demonstrated that the terminal half-life of OTA in humans is 10 times longer than in rats.

The above-suggested limits are below the real OTA intakes, as revealed by a study of the dietary OTA intake by the European population (Jørgensen and Bilde, 1996). Eight countries from thirteen, participating in that project, gave an estimate of mean dietary intake for an average adult person, based on food occurrence and consumption data, from 0.7 to 4.6 ng/kg bw/day; the other five results, based on human blood plasma data, ranged from 0.2 to 2.4 ng/kg bw/day.

Up to now, OTA has been regulated only in a few food products in the European Union, such as cereals and their based products (5 μg OTA/kg) and dry grapes (3μg OTA/kg; EU Commission Regulation, 2002). There is a regulation in Italy also for beer (200 ng OTA/L) (Ministerio de la Sanità, 1999). There is no maximum level for ochratoxin in wines, although it is known that commercial wines with a high level of contamination may increase the total daily intake of OTA, especially in high wine-consuming countries. On the basis of limited data, the Codex Alimentarius Commission estimated that 15% of the total intake of this toxin is due to wine consumption (Codex Alimentarius Commission, 1998).

The European Union has proposed to regulate all foods and beverages for OTA. It has been proposed a maximum permitted concentration of 0.5 μg/L for wine and grape juice from 31 December 2002 (Stockley, 2000).

METHODS OF ANALYSIS OF OTA IN WINES

The establishment of legal limits for OTA in wines implies the need of developing simple, reliable and sensitive methods for monitoring the level of OTA occurrence in wine products.

Mycotoxins analysis generally consist of a first step involving the extraction of the toxin from the matrix, a second step of purification of the extract (clean-up) intended to eliminate any possible interference of the matrix and, finally, the detection by means of suitable analytical instruments (Pascale et al., 2000). Table 2 shows the comparison of analytical characteristics of chromatographic methods for wines, musts and grape juices.

Clean-up and concentration procedures appear generally necessary when low detection limit was required (Festas et al., 2000). Classic methods were liquid-liquid partition and

solid-phase extraction (SPE), but sometimes the cleaning effect was inadequate for the complexity of the matrices. The use of immunoaffinity columns (IACs) in the clean-up step, specifically studied for OTA, has simplified the clean-up methodology. The main advantage of these columns is that OTA is bound specifically to the antibody and the matrix interface can be removed nearly completely. Furthermore, IACs give an optimal performance in terms of precision and accuracy within a wide range of concentration and they also reduce noticeable the use of dangerous solvents. A combination of these methods is also possible. Zimmerli and Dick (1995, 1996) proposed to extract OTA from acidified wines by chloroform and then to apply the residue to an IAC for a further purification. Other researchers have commonly used the method proposed by those authors with slight modifications as Pietri et al. (2001), Burdaspal and Legarda (1999) or Markaki et al. (2001). It should be underlined that chloroform extraction after acidification was considered in many cases fundamental, because OTA binds to protein and some food samples cannot be directly applied to an IAC (Valenta, 1998). Nevertheless, there is a trend towards minimising the amounts of halogenated and toxic solvents for environmental and sanitary reasons. Moreover, a suitable clean-up procedure allowing the application of the sample directly onto the IACs could reduce the time of analysis improving the possibility of automation. Some authors proposed the analysis of OTA passing directly diluted samples of beer and wine through the IAC, with apparently good results in terms of recovery and reproducibility (Scott and Kanhere, 1995; Visconti et al., 1999).

Ospital et al. (1998) obtained satisfactory results in terms of recovery and sensitivity operating a sample clean-up with silica gel SPE cartridges. The method of analysis included toluene extraction and high-performance liquid chromatography (HPLC) with fluorescence detection. This method allowed OTA quantification in wine samples in 10 min. However, the extraction procedure is rather laborious, time-consuming and serious control of data should be done when preparing a set of assays should be done.

At present, three types of immunoaffinity columns are commercially available for the analysis of OTA: OchraTest (Vicam, USA), Ochraprep (Rhône-Diagnostic Technologies, UK) and RIDA Ochratoxin (R-Biopharm, Germany).

Castellari et al. (2000) compared three immunoaffinity clean-up procedures to analyse OTA in 11 red and white wines. The methods used were direct wine clean-up with Ochraprep and Ochratest columns, and clean-up with chloroform extraction and Ochraprep column (procedure derived from that proposed by Zimmerli and Dick (1995)). All of them gave comparable results in terms of recovery and precision. The direct clean-up showed satisfactory limit of quantification for OTA in wine. Also, repeatability was improved and the time of analysis was reduced if compared with the reference procedure involving a preliminary extraction of OTA with chloroform.

High performance liquid chromatography associated with fluorescence detection (FLD) has become the most popular method for OTA detection and quantification in wine and grape beverages. Chromatographic separations have been normally performed using RP-C_{18} columns and isochratic elution with diluted acidified acetonitrile (Valenta, 1998). Nevertheless, although this method has the required sensitivity and accuracy, it is both expensive and requires technical expertise. Hence, ion-pair chromatography, HPLC-tandem mass spectroscopy (MS) or time resolved luminescence have been also proposed for some food and beverages (Terada et al., 1986; Breitholtz et al., 1991; Vazquez et al., 1996; Becker et al., 1998; Corneli and Maragos, 1998). Soleas et al. (2001), developed and compared two methods for the quantification of OTA in wines and beers. The first used HPLC interfaced to a photodiode array detector (HPLC-PDA), and they concluded that it would be suitable for routine detection and assays of OTA in wines due to the simplicity of sample preparation, low cost and acceptable recovery combined with the unique specificity of the PDA detector. The second method employed was gas chromatography (GC) interfaced to a mass selective detector (MSD), and because of its excellent specificity combined with non ideal sensitivity and recovery it would be best used as a confirmation tool.

Immunochemical enzyme-linked immunosorbent assay (ELISA) methods were also attractive (Barna Vetro et al., 1996; Solti et al., 1997), but sometimes caused systematic overestimates if compared to chromatographic methods (Wilkes and Sutherland, 1998). Cerutti et al. (2000) used an enzyme immunoassay method (RIDASCREEN) following the indications available for beer. The basis of this test is the antigen-antibody reaction, the wells in the microtiter strips are coated with specific antibodies to OTA. By adding ochratoxin A standards or the sample solution and enzyme labelled OTA (enzyme conjugate), a competition for the antibody binding site starts. Any unbound enzyme is then removed in a washing step. Enzyme substrate and chromogen are added to the wells and incubated. Bound enzyme conjugate converts the colourless chromogen into a blue product. The addition of the stop reagent leads to a colour change from blue to yellow, which is measured at 450 nm. The absorption is inversely proportional to the OTA concentration in the sample.

Table 2. Comparison of analytical methods for OTA with HPLC detection for wines and grape juices.

Matrix (initial volume)	Extraction method	Clean-up	D/Q (injection volume)	Mobile phase (flow rate)	Column	DL (µg/L)	RA (µg/L)	Rt (min)	R (%)	CV (%)	Reference
Wine (5 ml)	SE (chloroform)	IAC (Ohratest, Vicam) + postcolumn addition of ammonia	HPLC-fluor (20 µl)	Gradient conditions n.a.	Reversed-phase n.a.	0.003	0.009-0.190	8	96.6-103.6	<10	Burdaspal and Legarda (1999)
Wine (100 ml)	-	IAC (OchraPrep, Rhone Diagnostic Technologies)	HPLC-fluor (25 µl)	52% Sodium acetate 4 mM-Acetic acid (19:1) 48% Acetonitrile Isocratic conditions (0.3 ml min^{-1})	HP Hypersil ODS (250 x 2.1 mm, 5µm), c.t.: 40ºC	0.005	5-200	7	94.5	n.a.	Bezzo et al. (2000)
Wine (100 ml)	SE (toluene)	SPE-PC (silica cartridges)	HPLC-fluor (20 µl)	52% Sodium acetate 4 mM-Acetic acid (19:1) 48% Acetonitrile Isocratic conditions (2 ml min^{-1})	100 RP-18 (250x4 mm, 5µm)	0.02	3.3-33	10	87-107	3.96-8.86	Festas et al. (2000)
Wine (100 ml)	-	SPE-PC (C_{18} cartridges)	HPLC-fluor (n.a.)	52% Sodium acetate 4 mM-Acetic acid (19:1) 48% Acetonitrile Isocratic conditions (1 ml min^{-1})	c.t.: 30ºC n.a.	5	n.a.	10	>80	<10	Jornet et al. (2000)
Wine, grape juice (100 ml)	SE (toluene)	SPE (silica cartridges)	HPLC-fluor (20 µl)	Acetonitrile-water-acetic acid (45:54:1) (0.3 ml min^{-1})	Superspher-100 RP18 (125x4 mm, 5µm)	0.01	n.a.	n.a.	n.a.	n.a.	Majerus et al. (2000)
Wine and vinegar (10 ml)	SE (chloroform)	IAC (OchraPrep, Rhone Diagnostic Technologies)	HPLC-fluor (50 µl)	Water-acetonitrile-acetic acid (500+500+20) (0.9 ml min^{-1})	C_{18} Lichrosphere 100 RP (250x4 mm, 5µm)	0.002	0.001-0.020	9.5	91.3 - 96.6	6-8	Markaki et al. (2001)
Wine (100 ml)	SE (toluene)	SPE (silica cartridges)	HPLC-fluor (20 µl)	52% Sodium acetate 4 mM-Acetic acid (19:1) 48% Acetonitrile (1 ml min^{-1})	Altech C_{18} n.a.	0.01	n.a.	n.a.	73-90.7	n.a.	Ospital et al. (1996)
Wine and	-	SPE	HPLC-PDA	Gradient conditions	Reversed-phase	0.05	0.5-10	7.6	83-94	4.0-	Soleas et al.

beer (100 ml)			(100 µl)	n.a.	Nucleosil C_{18} (250x4 mm, 5µm)					8.9	(2001)
Wine (50 ml)	SE (chloroform)	-	HPLC-fluor	Water-acetonitrile-acetic acid (66.0:33.2:0.8) (1.5 ml min^{-1})	Supelco RP-Amide C_{16} (250x4.6 mm, 5µm)	0.013	0.32-32	28	94-109	1.3-8.5	Tateo et al. (1999)
Wine (10 ml)	-	IAC (Ochratest, Vicam)	HPLC-fluor (100 µl)	Acetonitrile-water-acetic acid (99:99:2) (1.0ml min^{-1})	Reversed-phase Discovery C_{18} (150 x4,6 mm, 5µm)	0.01	0.01-30.0	6	88-103	0.2-9.2	Visconti et al. (1999)
Wine (5 ml)	SE (chloroform)	IAC + postcolumn addition of ammonia	HPLC-fluor (20 µl)	Isocratic conditions n.a.	Reversed-phase ODS1 c.t.: 50ºC n.a.	0.005	n.a.	5	74-91	6	Zimmerli and Dick (1996)

Abbreviations: c.t., column temperature; CV, coefficient of variation; DER, derivatization; DL, detection limit; D/Q, Detection/Quantification; fluor, fluorescence detector; GC, gas chromatography; IAC, immunoaffinity column; n.a., not available; PC, preconcentration; PDA, photodiode array detector; R, recovery; RA, Range of applicability; RP, reversed phase; Rt, retention time; SE, solvent extraction; SPE, solid-phase extraction

FINAL REMARKS

The OTA concentration detected in wine is significantly less than that generally detected in other dietary sources. However, human exposure to OTA comes more likely from low level contamination of a wide range of different foods than from a high level ingestion of a single food source (Bauer, 1987; Codex Alimentarius Commission, 1997; Pittet, 1998).

The results of several studies indicated that some absorbents are able to remove substantial amount of OTA from wine (Castellari et al., 2001; Dumeau and Trioné, 2000), however, good practices in winemaking and prevention could play an important role in reducing human risk of exposure to this toxin.

The potential concentration of OTA in wine depends on the presence of OTA producing moulds on grapes, so any measures that prevent fungal growth also hinder the formation of fungal toxins. It is accepted that post-harvest conditions, moisture, high temperature, aeration, substrate, grain-fungi interactions, and time of infection are favourable factors for its development (Codex Alimentarius Commission, 1997). So the fact that OTA contents in white wines are lower than in rosé or red ones can be related to some of these factors, being the winemaking process differences important. The higher amounts of OTA in red wines coming from Tetrapak could be originated by bad grape conditions at harvest. As regards the dessert wines, their consumption is occasional and the ingested quantities are generally limited, so, although sometimes having high levels of OTA, their contribution to daily OTA intake could be considered to be rather small. However, the results show the need for the analysis of higher numbers of samples for OTA, in order to explain the mechanism of its formation and the role of strong hygiene in winemaking.

Finally, rapid and accurate methods are necessary to ensure that the distributed wine products are safe and to allow public laboratories with national or regional responsibility for food quality control, wine producers, importers and exporters to analyse the highest number of samples in the shortest time.

ACKOWLEDGMENTS

The authors are grateful to the Catalonian Government (Direcció General de Recerca, Generalitat de Catalunya), the Spanish Government (CICYT, Comisión Interministerial de Ciencia y Tecnología, project AGL 2001 2974-C05-02) and the EC, Quality of Life Programme (QoL), Key Action 1 (KA1) on Food, Nutrition and Health (QLRT-2000-01761) for their financial support.

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6.4. SURVEY OF OTA IN SPANISH WINES

A total of 240 Spanish grape-based beverages [red and white wines from four different Spanish Designations of Origin (n=160), musts (n=20), grape juices (n=10), ordinary wines (n=20), special wines (n=20) and sparkling wines (n=10)] were analysed in order to obtain quantitative data on the occurrence of OTA in Spain. Information regarding the sample was taken from the labels of the bottles and collected according to Table 14. The work is shown in the following paper (see 6.4.3.):

Bellí, N., Marín, S., Duaigües, A., Ramos, A.J. and Sanchis, V. 2004. Ochratoxin A in wines, musts and grape juices from Spain. *Journal of the Science of Food and Agriculture* 84, 591-594.

Table 14. Information regarding wine and grape-derivative samples analysed for OTA.

Sample number:_____	
Sample:	□ D.O. Wine □ Ordinary wine □ Sparkling wine □Special wine □ Must □ Grape juice
Bottle opened:	
Designation of Origin:	□ La Rioja □ Costers del Segre □ Penedès □ Utiel-Requena
Type of wine:	□ Young wine □ *Crianza* □ *Reserva* □ *Gran Reserva*
Colour of wine:	□ Red □ Rosé □ White
Year:	
Brand:	
Variety/ies:	
Others:	

An aliquot of each sample was analysed following an official method for the determination of OTA in wine (Bezzo et al., 2000), using immunoaffinity columns and HPLC+FD (Figure 34). Results stated a broad contamination level as OTA was found in the different samples, but not significant differences among the geographical regions, the colour, the age or the price of the wine could be found.

Eighteen % of the samples tested contained detectable levels of OTA. The overall mean OTA concentration in red and white wines of the D.O. was 0.30 and 0.18 μg l^{-1}, respectively (ranges: 0.05-3.19 μg l^{-1} for red wines and 0.05-1.13 μg l^{-1} for white ones).

The percentage of wine samples with detectable amounts of OTA was higher in red wines (18.3%) than in white ones (10 %). OTA was also found in two of ten red ordinary wines (0.68 and 4.24 μg l^{-1}), whereas none of the white ordinary ones contained OTA. The mean OTA amount detected in sparkling wines was 0.44 μg l^{-1} (range 0.14-0.71 μg l^{-1}). Two of the twenty samples of musts contained OTA in lower levels (0.08 and 0.18 μg l^{-1}), whereas none of the grape juices samples contained it. Highest amounts of OTA were found in special wines (40 %) with a maximum of 15.25 μg l^{-1} in a muscatel sample.

Figure 34. Several steps –centrifugation, filtration and immunoaffinity column clean-up- in the OTA analysis of wine.

6.4.1. Characteristics of the Designations of Origin surveyed

Four Designation of Origin have been chosen in the present work to carry out the survey of OTA occurrence in Spanish wines. A description of each is presented afterwards (Tables 15-19), together with information regarding Conca de Barberà Designation of Origin, as this D.O. was included in the field work (see 7.5.1.). Geographical origin of the five D.O. is shown in Figure 35. Information was provided by the Spanish Institute for Foreign Trade (Instituto Español de Comercio Exterior, ICEX), which is the authorised governmental platform for the promotion of Spanish exports and Spanish companies' international growth (www.winesfromspain.com).

6.4.1.1. D.O. Conca de Barberà

D.O. established: 19 November 1985; Website: www.do-conca.org

Table 15. Main characteristics of Conca de Barberà Designation of Origin.

Vineyards	
Subzones	-
Area under vine	6000 ha
Altitude of vineyards	350-600 m
Soil types	Light-brown chalky topsoil over alluvial, limestone bedrock
Principal grapes (w)	Macabeo, Parellada
Principal grapes (r)	Garnacha, Trepat, Tempranillo
Climate	
Max. summer temperature	35 °C
Min. winter temperature	-6 °C
Average rainfall	500 mm annually
Average sunshine	2.500 hours annually
Production	
Maximum crop	10.000 kg ha^{-1}
Yield	70 %
Equivalent hl/ha	70 hl ha^{-1}
Production 2001	27000000 kg
Production 2002	30625000 kg
Production 2003	33171000 kg
Wineries	
Number of Wineries	16
Total exports - 2001	18547 hl (01/08/01-31/07/02)
Total exports - 2002	6792 hl (01/08/02-31/07/03)
Total exports - 2003	-

6.4.1.2. D.O. Costers del Segre

D.O. established: 17 May 1988.

Table 16. Main characteristics of Costers del Segre Designation of Origin.

Vineyards	
Subzones	Artesa, Garrigues, Pallars Jussà, Raïmat, Segrià, Valls del Riucorb
Area under vine	4156 ha
Altitude of vineyards	250-700 m
Soil types	Light sandy topsoil over limestone
Principal grapes (w)	Macabeo, Parellada, Xarel.lo, Chardonnay, Garnacha blanca, Riesling, Sauvignon Blanc, Albariño
Principal grapes (r)	Garnacha Tinta, Ull de Llebre (Tempranillo), Cabernet Sauvignon, Merlot, Monastrell, Trepat, Samsó, Pinot Noir, Syrah.
Climate	
Max. summer temperature	35 °C
Min. winter temperature	-5 °C
Average rainfall	450 mm annually
Average sunshine	3000 hours annually
Production	
Maximum crop	-
Yield	-
Equivalent hl/ha	70 hl ha^{-1} (*en vaso*); 120 hl ha^{-1} (*espaldera*)
Production – 2001	-
Production – 2002	-
Production – 2003	-
Wineries	
Number of Wineries	25
Total exports – 2001	17513 hl
Total exports – 2002	15204 hl
Total exports – 2003	17395 hl

(Last updated: April 2004)

6.4.1.3. D.O. Penedés

D.O. established: 28 June 1970; Website: www.dopenedes.es

Table 17. Main characteristics of Penedés Designation of Origin.

Vineyards	
Subzones	-
Area under vine	27542 ha
Altitude of vineyards	Sea level to 750 m
Soil types	Sandy rising to clay, up to 20 % carbonates, deep
Principal grapes (w)	Macabeo, Xarel.lo, Parellada, Subirat-Parent, Chardonnay
Principal grapes (r)	Tempranillo, Cabernet-Sauvignon, Garnacha Tinta, Cariñena, Monastrell, Samsó, Merlot
Climate	
Max. summer temperature	33 °C
Min. winter temperature	-3.5 °C
Average rainfall	525 mm
Average sunshine	2350 hours/year
Production	
Maximum crop	Red: 9324 kg ha^{-1}; white: 11700 kg ha^{-1}
Yield	-
Equivalent hl/ha	Red: 69 hl ha^{-1}; white: 86.5 hl ha^{-1}
Production 2001	-
Production 2002	-
Production 2003	-
Wineries	
Number of Wineries	153
Total exports - 2001	-
Total exports - 2002	-
Total exports - 2003	-

(Last updated: November 1992)

6.4.1.4. D.O. Rioja

D.O. established: 6 June 1925; Website: www.riojawine.com

Rioja was the first Spanish wine region to obtain D.O. status in 1925. In 1991, it was promoted to DOCa (Qualified Designation of Origin), a higher category reserved for wines maintaining a proven consistency and quality over a long period of time.

Table 18. Main characteristics of Rioja Designation of Origin.

Vineyards	
Subzones	Rioja Alavesa, Rioja Alta, Rioja Baja
Area under vine	62500 ha
Altitude of vineyards	350-650 m
Soil types	Rioja Alavesa: calcareous clay; Rioja Alta: calcareous clay, some ferruginous clay and alluvial soils; Rioja Baja: ferruginous clay and alluvial soils.
Principal grapes (w)	Viura, Malvasía Riojana, Garnacha Blanca
Principal grapes (r)	Tempranillo, Graciano, Garnacha, Mazuela
Climate	
Max. summer temperature	40°C
Min. winter temperature	-4°C
Average rainfall	450 mm annually
Average sunshine	2800 hours annually
Production	
Maximum crop	9000 kg ha^{-1} white; 6500 kg ha^{-1} red
Yield	72 %
Equivalent hl/ha	62 hl ha^{-1} white; 47 hl ha^{-1} red
Production 2001 (hl)	242347992 hl
Production 2002 (hl)	196823899 hl
Production 2003 (hl)	298418768 hl
Wineries	
Total	1391
Total exports - 2001	60405880 l
Total exports - 2002	72097169 l
Total exports - 2003	66138317 l

(Last updated: April 2004)

6.4.1.5. D.O. Utiel-Requena

D.O. established: 10 April 1957; Website: www.utielrequena.org.

Table 19. Main characteristics of Utiel-Requena Designation of Origin.

Vineyards	
Subzones	-
Area under vine	41000 ha
Altitude of vineyards	600-900 m
Soil types	Mostly brown soil over limestone
Principal grapes (w)	Macabeo, Merseguera, Planta Nova, Chardonnay
Principal grapes (r)	Bobal, Tempranillo, Garnacha, Cabernet Sauvignon and Merlot
Climate	
Max. summer temperature	40 °C
Min. winter temperature	-10 °C
Average rainfall	430 mm annually
Average sunshine	2800 hours annually
Production	
Maximum crop	Reds: 7500 kg ha^{-1} (traditional formations) and 9100 kg ha^{-1} (for wall bars formations); whites: 8000 kg ha^{-1} (traditional formations) and 9700 kg ha^{-1} (for wall bars formations)
Yield	74 %
Equivalent hl/ha	Reds 55.5 hl ha^{-1} (traditional formations) and 67.34 hl ha^{-1} (for wall bars formations); whites 59 hl ha^{-1} (traditional formations) and 71.53 hl ha^{-1} (for wall bars formations)
Production 2001	435907 hl D.O. wines
Production 2002	572411 hl D.O. wines
Production 2003	478251 hl D.O. wines
Wineries	
Number of Wineries	108
Total exports - 2001	167713 hl
Total exports - 2002	161112 hl
Total exports - 2003	278135 hl

(Last updated: April 2001)

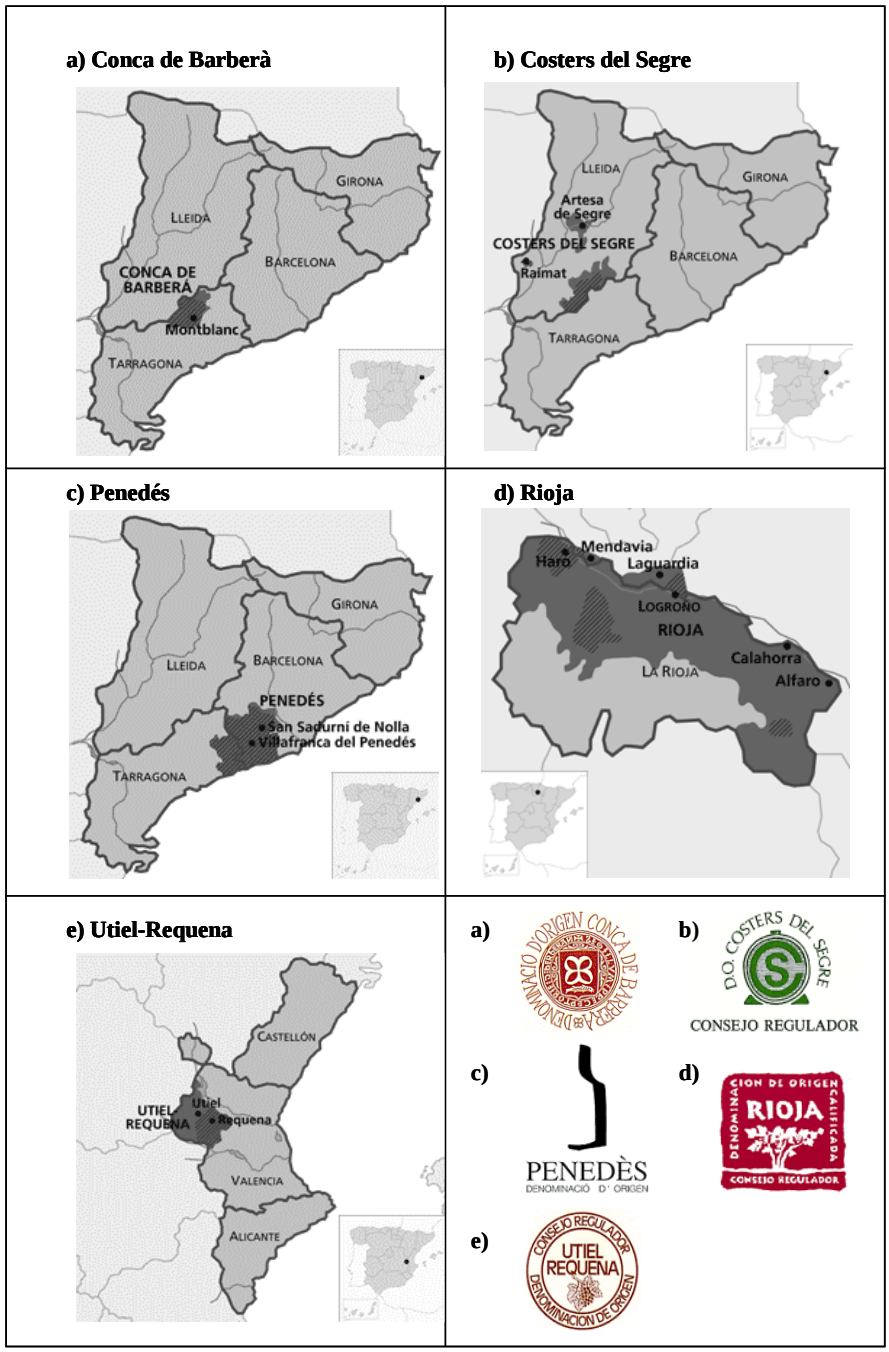

Figure 35. Location and logotypes of the Designations of Origin studied in this thesis.

6.4.2. Types of wine

Wine is the fermented juice of grapes. Only one species of grape, *Vitis vinifera*, is used for nearly all the wine made in the world (see 6.1.).

The primary categories of wine are **table wines** -also called still or natural wines-, **fortified wines** -wines with an extra dosage of alcohol-, and **sparkling wines** – champagne, cava, asti spumante, etc.-. This classification depends on the techniques of production, called vinification. Wine making is called Enology (or oenology), from the Greek words for ‘wine’ and ‘study’. The term ‘vintage’ signifies a single season's wine production, usually referring to the specific location in which a particular wine is produced.

Other classification of wines have been proposed, according to different criteria, as the quality of the vintage, the colour of the wine (red, white, rosé), their primary impression on the drinker's palate (dry, off-dry, fruity, sweet), etc.

All wines are made in a similar way, with variations depending on the type to be produced. The general steps are: harvesting, crushing, juice separation, treatment of the mass of crushed grapes and juice (called the must), fermentation, post fermentation treatment, clarification, aging, and bottling.

6.4.3. OTA in wines, musts and grape juices from Spain

Ochratoxin A in wines, musts and grape juices from Spain

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ABSTRACT

A survey about the occurrence of ochratoxin A (OTA) in 240 grape-based beverages was carried out. Red and white wines from four different Spanish Designations of Origin (n=160), musts (n=20), grape juices (n=10), ordinary wines (n=20), special wines (Malaga, Muscatel, Sherry, Vermouth, etc.) (n=20) and sparkling wines (n=10) were assayed for OTA content using immunoaffinity column clean-up and high-performance liquid chromatography with fluorimetric detection (detection limit: 0.05 μg l^{-1}). Forty-three (17.9 %) of the samples tested contained detectable levels of OTA. The overall mean OTA concentration in red and white wines of the Designations of Origin was 0.30 and 0.18 μg l^{-1} respectively (ranges: 0.05-3.19 μg l^{-1} and 0.05-1.13 μg l^{-1} respectively). The percentage of wine samples with detectable amounts of OTA was higher for red (18.3%) than for white (10 %) wines. OTA was also found in two of 10 red ordinary wines (0.68 and 4.24 μg l^{-1}), whereas none of 10 white ordinary wines contained OTA. The mean OTA amount detected in sparkling wines was 0.44 μg l^{-1} (range 0.14-0.71 μg l^{-1}). Two of the 20 must samples contained OTA at low levels (0.08 and 0.18 μg l^{-1}), while none of 10 grape juice samples contained OTA. Highest amounts of OTA were found in special wines (45 %), with a maximum of 15.25 μg l^{-1} in a muscatel sample.

KEY WORDS: Ochratoxin A, wine, grape beverages, immunoaffinity columns, HPLC, Spain

***Journal of the Science of Food and Agriculture* 84, 591-594 (2004)**

INTRODUCTION

Mycotoxins are a subgroup of secondary metabolites and their production is a common characteristic of the filamentous fungi. They may be formed in foods, raw materials for food production or animal feeds as a result of mould growth and are toxic to human and animals.[1]

Ochratoxin A is a mycotoxin produced by *Penicillium verrucosum* mainly in temperate climates and by a number of species of *Aspergillus* in warmer and tropical parts of the world.[2] OTA poses a risk to human health because it is nephrotoxic, teratogenic, immunotoxic and, possibly, neurotoxic. The International Agency for Research on Cancer (IARC) has classified OTA as a possible carcinogen to humans (group 2B).[3] Recently, the number of surveys on the occurrence of OTA in wine has increased and they show various levels of OTA.[4-22] Wine is a product widely consumed and, owing to its high OTA incidence, may represent an important source of OTA intake. OTA also occurs in other agricultural products such as grain, coffee, beans and dried fruit.[23,24]

Two new analytical methods, based on the use of immunoaffinity columns clean-up and high performance liquid chromatography, for the determination of OTA in wine and beer have been approved by the *European Committee for Standardization* (CEN) as European Standards and by the *Association of Official Analytical Chemists* (AOAC) *International* as first action Official Methods for the determination of OTA in wine and beer.[25] The latter has also been adopted as an official method by the *Office International de la Vigne et du Vin* (OIV)[26], and this was the method used in the present survey.

The aim of this work was to obtain quantitative data on the occurrence of OTA in Spanish grape-based beverages. The possible influence of some variables, such as the beverage categories, the aging wine receives, etc. was also studied. As OTA is a natural contaminant and its complete elimination is impossible, the major goal of wine producers should be to prevent and control OTA production in the field for human health safety.

EXPERIMENTAL

Samples

A total of 240 samples were analysed, all obtained from local supermarkets and small shops in Spain during 2002. All information about the samples was removed from the labels of the bottles. Wine samples ranged from low- to high-priced. Four Designations of Origin (La Rioja, Penedés, Utiel-Requena and Costers del Segre) were studied. Forty wines differentiated according to their maturation process (10 white young wine, 10 red young wine, 10 *crianza* and 10 *reserva*) from each Designation of Origin were analysed. The terms *crianza* and *reserva* refer to the aging a wine receives. Precise rules for the

aging process are defined for both qualifications by the governing body of each Designation of Origin. Twenty ordinary wines (low-priced, everyday wines; 10 red and 10 white), 20 musts (10 red and 10 white), 20 special wines (Malaga, mistelle, muscatel, sherry, vermouth, etc.), 10 sparkling wines (*cava*) and 10 grape juices were also analysed.

Extraction and clean-up

The OIV method for the determination of specific mycotic contaminants randomly occurring in wines was used.[26] A 100 ml aliquot of each sample was brought to a pH value of 7.4 by adding 4M NaOH. Samples were centrifuged (3830 g, 15 min) and filtered (Whatman n. 1), then passed through immunoaffinity columns (Ochraprep, Rhône Diagnostics Technologies, Glasgow, UK) at a flow rate of 2-3 ml min^{-1}. Afterwards, the columns were washed with 20 ml of distilled water at a flow rate of 5 ml min^{-1} and finally dried in an air stream. Desorption was carried out by slowly passing 1.5 ml of methanol/acetic acid (98:2 v/v) solution through the column. The eluate was then evaporated to dryness under a stream of nitrogen at 40ºC and redissolved in 2 ml of mobile phase (acetonitril (Merck, Darmstadt, Germany) 48% - sodium acetate 4 mM/acetic acid (Prolabo, Briare, France) (19:1) 52%). All solvents were of HPLC grade. The solution was ready for the HPLC analysis.

Chromatographic conditions

A 25 µl aliquot of each final sample was injected into the HPLC system equipped with a fluorescence detector (Waters 474) (λ_{exc} 230 nm; λ_{em} 458 nm) and a C_{18} column (Waters Spherisorb 5 µm, ODS2, 4.6x250 mm, Milford, Massachusetts, U.S.A.). The analysis was performed under isocratic conditions at a flow rate of 1 ml min^{-1}. The detection limit of the analysis was 0.05 µg l^{-1}, based on a signal/noise ratio of 3:1. OTA was quantified by the external standard method. The ochratoxin standard was from *Aspergillus ochraceus* (Sigma-Aldrich, Steinheim, Germany). The standard solution was prepared in methanol (Merck) and confirmed by using an UV spectrophotometer. The required amounts were evaporated to dryness and dissolved in the mobile phase in order to prepare five solutions with OTA concentrations ranging from 2.5 to 50 µg l^{-1}. The calibration curve obtained was linear. The retention time of OTA under the conditions described was approximately 12 minutes.

Statistical analysis of the data

Analysis of variance was carried out on the amount of OTA detected in samples in order to identify any significant differences between beverages categories or Designation of Origins, using SAS version 8.02 (SAS Institute, Inc., Cary, N.C., U.S.A.).

RESULTS AND DISCUSSION

The results from the 240 samples are summarised in table 1. Forty-three (17.9%) of the samples contained detectable amounts of OTA. The overall mean OTA concentration in red and white wines of the Designations of Origin was 0.30 and 0.18 μg l^{-1} respectively (ranges: 0.05-3.19 μg l^{-1} and 0.05-1.13 μg l^{-1} respectively). The percentage of OTA-positive samples was higher for red (18.3%) than for white (10%) wines. Soleas *et al.*[15] obtained similar percentages (16.6% for red wines and 3.9% for white wines) (detection limit: 0.05 μg l^{-1}) in the analysis of 580 and 362 samples of red and white wines respectively. The percentages obtained by other authors were higher (90% -mean 71%- for red wine and 34% -mean 45%- for white wines), but their limits of detection were lower, as global results from a review on OTA in wines and grape beverages show.[27] OTA was also found in two of 10 red ordinary wines (0.68 and 4.24 μg l^{-1}), whereas no OTA was detected in any of the white ordinary wines. In previous surveys, higher numbers of red wines containing OTA and in higher amounts, followed by rosé and then by white wines, have been found.[4,5,7,9,10,13,21] Majerus *et al.*[10] suggested that the reason could be the different wine-making techniques employed, as red wines have longer mash standings.

Four of 10 sparkling wines contained detectable amounts of OTA (mean: 0.44 μg l^{-1}; range: 0.14-0.71 μg l^{-1}). A higher percentage of OTA-positive samples was found by Burdaspal and Legarda[4] (83.3%), but lower amounts (mean: 0.012 μg l^{-1}) (detection limit: 0.003 μg l^{-1}).

Two of 20 must samples contained low levels of OTA (0.08 and 0.18 μg l^{-1}), while none of the grape juice samples contained OTA. In view of the suspected occurrence of OTA in fruit juices and their potential consumption by children, some researchers analysed grape and other fruit juice samples too. They found higher percentages of grape juices and musts containing OTA and higher OTA levels, especially for red grape juice samples (86% (range: 0.01-4.7 μg l^{-1})[9], and 88% (range: 0.01-5.3 μg l^{-1})[10]), but lower for white grape juice samples (17% (range: 0.01-0.73 μg l^{-1})[9], and 78% (range 0.01-1.3 μg l^{-1})[10]). Furthermore, 100% positives were found by Larcher and Nicolini[7] in concentrated musts (range: 0.06-6.18 μg l^{-1}) and by Burdaspal and Legarda[4] in both white and red grape musts and grape juices (range: 0.015-0.102 μg l^{-1}). The dilution could be the reason for the low incidence of OTA in the samples of the present study, as most of the grape juices analysed were mixtures with high percentages of other fruit juices.

The highest OTA-containing samples were special wines (45%) (four muscatel, one mistelle, three Sherry and one vermouth), with a mean content of 4.47 μg l^{-1} and a maximum of 15.25 μg l^{-1} found in the mistelle sample. Dessert wines were the ones that contained the highest amounts of OTA. Burdaspal and Legarda[4] detected OTA in 15 of 16 samples of dessert wines (muscatel, Malaga, Marsala), the mean content being 1.048

μg l^{-1}. The amount of OTA obtained by Pietri *et al*[14] from the analysis of 15 Italian white dessert wines ranged from 0.001 to 3.856 μg l^{-1}; the authors concluded that, as the consumption of dessert wines is occasional and the ingested quantities are generally limited, their contribution to daily OTA intake could be considered to be rather small.

Table 1 . Occurrence of ochratoxin A (OTA) in Spanish grape-beverages.

Type	Colour	Positives/ Total	OTA mean concentration of positive samples (μg l^{-1})	Range (μg l^{-1})
Young wine La Rioja	White	0/10	-	ND
Young wine La Rioja	Red	4/10	1.22	ND-3.19
Wine La Rioja crianza	Red	1/10	0.07	0.07
Wine La Rioja reserva	Red	2/10	0.08	0.06-0.10
Young wine Penedés	White	3/10	0.43	ND-1.13
Young wine Penedés	Red	3/10	0.30	ND-0.69
Wine Penedés crianza	Red	6/10	0.11	ND-0.18
Wine Penedés reserva	Red	2/10	1.01	0.17-1.85
Young wine Costers Segre	White	0/10	-	ND
Young wine Costers Segre	Red	0/10	-	ND
Wine Costers Segre crianza	Red	1/10	0.06	0.06
Wine Costers Segre reserva	Red	0/10	-	ND
Young wine Utiel-Requena	White	1/10	0.31	0.31
Young wine Utiel-Requena	Red	1/10	0.15	0.15
Wine Utiel-Requena crianza	Red	1/10	0.47	0.47
Wine Utiel-Requena reserva	Red	1/10	0.11	0.11
Ordinary wines	White	0/10	-	ND
	Red	2/10	2.46	0.68-4.24
Sparkling wine	-	4/10	0.44	ND-0.71
Musts	White	2/10	0.13	0.06-0.18
	Red	0/10	-	ND
Grape juices	-	0/10	-	ND
Special wines	-	9/20	4.47	ND-15.25

Detection limit 0.05 μg l^{-1}; ND, not detected.

No statistical evidence for a relationship between the region of origin or the colour of the wine and the OTA content was found. Moreover, no difference in OTA content was found among young, *crianza*, and *reserva* wines, or between ordinary and Designation of Origin wines. Therefore, it is interesting to note that the data do not provide any evidence for a statistical correlation between the price level of the wines and their OTA content. Tateo *et al*[19], contrary to us, found higher OTA concentrations when analysing 31 samples of Italian red tetrabrik wines than those they found in good quality bottle wines.

There is no official maximum level for ochratoxin A in wines within the European Union although a maximum permitted concentration of 0.5 μg l^{-1} has been proposed for wine and grape.[28] Furthermore, the OIV suggested a maximum theoretical concentration of 2 μg l^{-1} for wines.[29] In our survey, this level was exceeded in six samples (two red wines and four special wines, three of the latter being dessert wines).

The most efficient way to protect consumers against OTA health hazards is to implement good agricultural practices to lower the presence of fungal strains on the grape and therefore reduce the possibility of OTA production during the winemaking process. Thus it is crucial to determine at which moment OTA is produced in field and then to establish suitable control strategies. Rates of OTA presence in wines also vary from one year to another depending on the meteorological conditions. Any measures that can prevent fungal growth, such as appropriate antifungal treatment of vines and a tight control of the winemaking process, will also hinder the formation of fungal toxins.

ACKNOWLEDGEMENTS

The authors are grateful to the Catalonian Government (Direcció General de Recerca, Generalitat de Catalunya), the Spanish Government (CICYT, Comisión Interministerial de Ciencia y Tecnología, project AGL 2001 2974-C05-02), and the EC, Quality of Life Programme (QoL), Key Action 1 (KA1) on Food, Nutrition and Health (QLRT-2000-01761) for their financial support. They also thank Tecnova S.A. (Spain) for technical asistance in the use of the immunoaffinity columns.

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6.5. OTA DECONTAMINATION OF WINES

OTA is stable in wine for at least one year (López de Cerain et al., 2002). Several physical, chemical and biological procedures have been proposed to decrease the OTA level in wines. Knowing that intensive pressing of pomace, prolonged drying of grapes, and storage in partially empty tanks increased OTA contamination of wines, good practices in winemaking and prevention would play an important role in reducing human risk of exposure to this toxin.

Alternatively to OTA decontamination, it has been suggested that some of the many different toxic effects of the mycotoxin OTA can be partially controlled by supplementing the diet with antioxidants, particularly vitamin E and possibly other agents such as carotenoids and flavonoids (Höhler, 1998).

6.5.1. Physical methods

Physical methods of separation are being used successfully in other food industries, as in the peanut and nutmeat industries throughout the world (Sinha, 1998). However, few studies regarding physical methods to detoxify wines of OTA have been found. Removing mouldy grapes or bunches before entering in the wine-making process to decrease the levels of OTA in wines, may not be a economically feasible practice for the wine industry. Wine filtration through a 0.45 μm membrane showed an 80 % decrease of OTA and could be used as another technique to diminish the levels of this toxin in wines (Gambuti et al., 2005).

6.5.2. Chemical methods

Results of several studies indicated that some adsorbents are able to remove substantial amount of OTA from wine. Castellari et al. (2001) found several enological fining agents able to remove the toxin in wines, potassium caseinate and activated carbon the best ones. Potassium caseinate was able to remove up to 82 % of OTA when used at 150 g hl^{-1}, while activated carbon showed the highest specific adsorption capacity owing to its high surface area per mass and the low adsorption of total polyphenols. Dumeau and Trioné (2000) achieved reductions of the OTA content of red wines up to 90 %, although sometimes, maximum reductions led to negative effects on wine quality, as reduction of the colour intensity and the polyphenols index of wines. Products like gelatine preparations, silica gel powder, even cellulose, gave good results. Enological decolorizing carbon was also able to remove up to 72 % of OTA in red wines in a recent study carried out by Gambuti et al. (2005), and it did not affect either polyphenol content and colour of wine, although this time, a decrease of several sensory odorants was observed.

6.5.3. Biological methods

Microbiological methods may be a good alternative to OTA detoxification in wines. The use of enzymes is far more convenient than the chemical detoxification methods, since it is substrate specific. Several reports of OTA biodegradation have been published. OTA can be hydrolysed to the less toxic ochratoxin α and phenylalanine by enzymes like carboxypeptidase A (Doster and Sinnhuber, 1972; Stander et al., 2001), lipases (Stander et al., 2000) and certain yeasts, such as *Rodotorula*, *Cryptococcus* and *Pichia* species (Steyn et al., 2000). Several *Aspergillus* species such as strains of *A. fumigatus, A. niger* (Varga et al., 2000), *A. clavatus, A. ochraceus, A. versicolor, A. wentii*, some other black aspergilli (Abrunhosa et al., 2002), were examined for their ability to degrade OTA, obtaining satisfactory results when tested in laboratory media. Abrunhosa et al. (2002) added to the list of the species able to degrade OTA, other genera frequently isolated from grapes: *Alternaria, Botrytis, Cladosporium* and *Penicillium*. In another study, OTA was successfully degraded by several species in the genera *Rhizopus*, degrading more than 95 % of the OTA added to YES medium within 16 days (Varga et al., 2004). Oenological *Saccharomyces* strains were also able to remove OTA from synthetic and natural grape juices (Bejaoui et al., 2004). This removal was rapid and improved by dead yeasts (heat-treated), lower pH and higher yeast concentration. In red wines, reductions were also achieved by using active dry yeast and yeast lees obtained from alcoholic fermentation (Moruno et al., 2005).

6.6. REGULATIONS

Mycotoxin regulations are generally implemented using a defined maximum limit and a sampling plan to detect and divert mycotoxin-contaminated products from food and feed markets. Several factors may influence the decisions taken by authorities to establish mycotoxins limits and regulations (van Egmond and Jonker, 2004). These include:

- Availability of toxicological data.
- Availability of data on the occurrence of mycotoxins in various commodities.
- Knowledge of the distribution of mycotoxin concentrations over commodities.
- Availability of reliable analytical methods.
- Legislation in other countries with which trade contacts exist.
- Need for sufficient food supply.

International legislation of food and feeds is established by Codex Alimentarius (CAC). The body responsible for the risk management in the CAC is the Codex Committee on Food Additives (CCFAC), which involves a scientifically based Risk Assessment of the toxicity of food, role belonging to the FAO/WHO Joint Expert Committee on Food Additives (JEFCA).

In the EU, the legal basis is the Council Regulation (EEC) 93/318/EEC of 8 February 1993 laying down Community procedures for contaminants in food. Specific EU maximum limits for mycotoxins and other contaminants in food are based on proposals from the European Commission (EC), which, in a Standing Committee procedure, shall be agreed by the Commission and the Member States, after the Scientific Committee for Food (SCF) has been heard. The SCF plays a similar expert advisory role in EU as JECFA does for Codex Alimentarius (Berg, 2003).

On a world-wide basis, at least 99 countries had mycotoxin regulations for food and/or feed in 2003, an increase of approximately 30 % compared to 1995. The total population in these countries represents approximately 87 % of the world's inhabitants (FAO, 2004). Many countries have set maximum levels for OTA in cereals, cereal-based products, and various have set different limits for both, such as the EU (5.0 and 3.0 $\mu g\ kg^{-1}$, respectively). Furthermore, limits for cereal-based foods for infants and young children have been established at 0.50 $\mu g\ kg^{-1}$ in the EU. OTA limits in coffee, beer, cocoa and spices and probably other foodstuff are nowadays under revision.

Given the significant contribution of wine to the OTA human exposure, regulatory limits for OTA levels in wines, have been recently established in the EU, to protect public health by preventing the distribution of unacceptably highly contaminated wines (Table 20). The maximum levels fixed will be applied to products produced from the 2005 harvest onwards.

The specific EU legislation for OTA, setting maximum limits for grape-derivatives appeared at the beginning of 2005:

- **Commission Regulation 123/2005/EC of 26 January 2005, amending Regulation 466/2001/EC as regards ochratoxin A.**

The distribution of the concentration of mycotoxins in products is an important factor to be considered in establishing regulatory sampling criteria. The distribution can be very heterogeneous, thus official sampling plans and reference analysis method for OTA have been established in many cases, as for cereals and dried fruit vines:

- **Commission Directive 2002/26/EC of 13 March 2002, laying down the sampling methods and the methods of analysis for the official control of the levels of ochratoxin A in foodstuffs.**

Table 20. Maximum levels of OTA in grape-derivatives products in the EU.

Products	Maximum levels OTA µg kg^{-1} (ppb)
- Dried vine fruit (currants, raisins and sultanas).	10.0
- Wine (red, white and rosé), including sparkling wines but excluding liqueur wines and wines with an alcoholic strength of not less than 15 % vol. and fruit wines.	2.0
- Other wine and/or grape must based beverages. Aromatised wines, aromatised wine-based drinks and aromatised wine-product cocktails. The maximum level for OTA applicable to these beverages is function of the proportion of wine and/or grape must present in the finished product.	2.0
- Grape juice, grape juice ingredients in other beverages, including grape nectar and concentrated grape juice as reconstituted. Fruit juice, including fruit juices from concentrates, concentrated fruit juice and fruit nectar.	2.0
- Grape must and concentrated grape must as reconstituted, intended for direct human consumption.	2.0

Legislators in Codex Alimentarius and in the EU and other international bodies will have to discuss how to use these data effectively as risk management tools in order to protect public health and promote international trade. The establishment of detailed and specific Codes of Practice and sampling plans are going to be important aspects.

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