Survey of Ochratoxin A in South African Wines

M.A. Stander[1]* and P.S. Steyn[2]**

1) Department of Chemistry, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa
2) Division of Research Development, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa

Submitted for publication: May 2001
Accepted for publication: March 2002
Key words: Ochratoxin A, mycotoxin, nephrotoxin, wine, HPLC, immunoaffinity clean up, quantification, South Africa

The mycotoxin, ochratoxin A (OTA), an important nephrotoxin, teratogen and carcinogen, is mainly produced as a secondary metabolite of *Aspergillus* and *Penicillium* species. The mycotoxin is a common contaminant of various feedstuffs and of foodstuffs such as grains, coffee and wine. The levels of OTA have been established in 122 South African wines, representative of a variety of wine cultivars from various wine-producing regions of the country. The method of analysis was based on the method of Visconti *et al.* (1999): it consisted of immunoaffinity clean up, quantification on reversed phase HPLC with fluorescence detection and confirmation of the presence of OTA by synthesising its methyl ester. The levels of OTA of most of the red and white wines were substantially lower than the maximum permissible levels of OTA in wines, as suggested by the European Union. However, high levels of OTA were found in a few noble late harvest wines. This first comprehensive survey unambiguously shows that OTA presents no cause for concern to the South African wine industry.

Ochratoxin A (OTA) (Fig. 1), an important mycotoxin, is produced by various *Penicillium* and *Aspergillus* species. It causes kidney diseases such as Danish porcine nephropathy in pigs and nephropathy in poultry (Van der Merwe *et al.*, 1965; Krogh *et al.*, 1988). OTA is also associated with similar diseases in humans including Balkan Endemic Nephropathy in the Balkan countries and urinary tract tumors in countries in North Africa (Creppy *et al.*, 1993; Creppy, 1999). The mycotoxin has been detected in various foodstuffs such as dried fruits, coffee, maize, sorghum, wheat, pulses and wine (Marquardt & Frohlich, 1992; Steyn & Stander, 1999). OTA has been detected in the blood of more than 70% of the people tested in certain countries, indicating widespread consumption of contaminated foods and that the toxin has a very long half-life in animals and possibly also in humans (Petkova-Becharova *et al.*, 1988; Breitholtz *et al.*, 1991; Creppy *et al.*, 1991; Bacha *et al.*, 1993; Uedo, 1998; Thuvander, *et al.*, 1999). This supposition was confirmed by the recent observation of Stander *et al.* (2001a) that the elimination half-life of OTA in monkeys was 19-21 days.

The suggested maximum tolerable level for OTA intake for an adult human is 5 ng/kg bodyweight per day (JECFA, 1995). The European Union suggested a maximum permissible level of OTA in wine as 200 pg/mL. Surveys done in various European countries over the past five years on the occurrence of OTA in various foodstuffs reported low levels of OTA in wine on their retail markets (Burdaspal & Legarda, 1999). The data indicated that an average person (60 kg) who consumes 156.8 mL red wine per day would derive approximately 1-2.8% of the daily allowable OTA intake from that source. In such circumstances wine contributes only to a small extent to the daily allowable OTA intake. However, Wolff *et al.* (2000) found in a comprehensive study of German foods and liquors that wine and champagne may be the

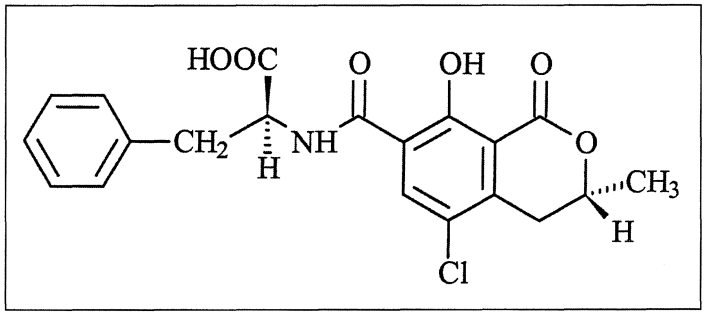

FIGURE 1
Chemical structure of Ochratoxin A.

source of 12.5% of the daily OTA intake, compared to a variety of breads as 35.6% of the source of OTA in Germany.

The highest levels of OTA were found in wines from North Africa and some of the Southern European countries. The highest levels of OTA detected by Zimmerli & Dick (1996) were found in wine produced in North Africa (three wines contained OTA at levels of 194, 292 and 388 pg/mL). These higher levels may be ascribed to either the hotter, more humid climates of these countries or to different wine-producing practices (Zimmerli & Dick, 1996). This observation was confirmed by Majerus & Otteneder (1996) and again recently by Otteneder & Majerus (2000), who found higher levels of OTA in wine from Southern European regions than in the wine from Northern European regions. Two unspecified South African wines were also analyzed in the study by Zimmerli & Dick (1996) and found to contain OTA at levels of 24 and 81 pg/mL). The question remains whether these differences in OTA levels are caused by the different soils, climates and cultivars or by different wine-producing techniques. In a recent study of Portuguese wines no OTA was observed in any of the

*Present address: Microsep, PO Box 391647, 2018 Bramley, South Africa.
**Corresponding author.

Acknowledgements: The authors thank Winetech and the MRC, Tygerberg, for financial support.

port wine or Vinho Verde samples (Festas *et al.*, 2000). In a comprehensive study of European wines Otteneder & Majerus (2000) found that the red wine samples from the northern cultivating area showed a contamination of 12% in contrast to those from the southern area that showed a contamination of about 95%. In a related study Larcher & Nicolini (2001) investigated the levels of OTA in wines of Trentino, a cool climate region of Northern Italy; they reported only very low levels of OTA in wines and musts. The investigations confirm the importance of production region, ambient temperatures and humidity on the development of OTA in grapes and the wine-making process.

The results reported (Zimmerli & Dick, 1996; Ospital *et al.*, 1998; Burdaspal & Legarda, 1999; Visconti *et al.*, 1999) found higher levels of OTA in red than in white wines. The higher levels of contamination may be ascribed to the fact that at the beginning of infection the fungi grow at the outside of the grapes, and in the production of red wines the longer contact of the must with the skins of the grapes will extract more OTA. Zimmerli & Dick (1996) found that OTA is formed prior to alcoholic fermentation; therefore, fungal contamination and OTA formation can occur in the vineyards or while the grapes are being transported or during storage. When the grapes are in a more advanced stage of fungal infection, OTA occurs throughout the fruit, leading to similar levels in white and red wine (see results on noble late harvest). *Penicillium* species produce OTA over a temperature range of 4-31°C, whereas *Aspergillus* species prefer higher temperatures (12-39°C). Storing the grapes at low temperatures may thus reduce the production of OTA during storage. Vines under physiological stress (due to drought, excessive water and cold weather) are also more susceptible to fungal infections. Another possible cause of OTA formation is the storage of wines in old wooden barrels, in which case the fungal contamination may have occurred owing to improper barrel sanitation.

OTA is a remarkably stable compound that can only be completely hydrolysed by heating under reflux for 48 h in 6 M hydrochloric acid (Van der Merwe *et al.*, 1965). The adsorption of OTA to activated carbon (or silica gel followed by gelatin) causes a decrease of OTA of more than 50%. "Toxical" (a mixture of activated carbon and silica gel) followed by gelatin could be used to remove >90% of the OTA (Dumeau & Trione, 2000). However, activated charcoal also removes beneficial substances like anthocyanins and flavonoids from the wine. OTA can also be hydrolysed to the less toxic ochratoxin α and phenylalanine by enzymes like carboxypeptidase A (Doster & Sinnhuber, 1972; Stander *et al.*, 2001b), lipases (Stander *et al.*, 2000a) and certain yeasts, such as *Rhodotorula*, *Cryptococcus* and *Pichia* species (Steyn *et al.*, 2000).

The objective of this analytical study was to determine the levels of OTA in several South African wines, as well as to explore any regional and/or cultivar influences.

MATERIALS AND METHODS

Analytical method

The method of Visconti *et al.* (1999) was used for the analysis.

Clean up

Wine samples (25 mL) were diluted with a solution (25 mL) containing polyethylene glycol 8000 (1% m/v) and sodium bicarbonate (5% m/v). The diluted extract was filtered through micro-fibre filter paper and transferred to a glass syringe barrel (10 mL) fitted to an immunoaffinity column (Vicam). The remaining diluted extract was kept at -20°C in a sealed container. The filtered diluted extract was passed through the immunoaffinity column at one drop per second until air came through the column. A saline solution (5 mL, 2.5% sodium chloride and 0.5% sodium bicarbonate) followed by distilled water (5 mL) was passed through the column at 1-2 drops per second until air came through. The fraction containing OTA was eluted from the column with methanol (2 mL, HPLC grade) at a flow rate of 1-2 drops per second, after which the eluant was evaporated to dryness under a stream of nitrogen at 60°C. The extract was reconstituted in the HPLC mobile phase (250 µL) and injected onto the HPLC (Fig. 2). After HPLC analysis the remaining extracts of the samples, which tested positive for OTA, were immediately evaporated to dryness in a stream of nitrogen at 60°C, and the methyl esters of OTA prepared as confirmation.

High-Performance Liquid Chromatography

A Waters 2690 system fitted with a fluorescence detector, thermostatic column compartment, auto sampler and Millennium software was used. The excitation wavelength was set at 331 nm and the emission wavelength at 460 nm. Separation was achieved on a C_{18} reversed phase column (4.6 mm x 150 mm, 5 µm, Discovery, Supelco, Bellefonte, PA, USA) at 30°C, employing an isocratic mobile phase of methanol/water/acetic acid (60:40:2), an injection volume of 50 µL and a flow rate of 1 mL/min.

Confirmation of OTA in wine samples

The dried wine extract (after HPLC analysis) was reacted with boron trifluoride methanol complex (0.5 mL, 20% solution in methanol, supplied by Merck) for 30 minutes at 60°C. The sample was evaporated to dryness under a stream of nitrogen at 60°C, reconstituted in the HPLC mobile phase (250 µL) and injected as an aliquot (50 µL) onto the HPLC. The appearance of a peak (retention time 16 minutes) corresponding to the retention time of the methyl ester of OTA and the disappearance of the OTA peak (retention time 11 minutes) were regarded as confirmation of the presence of OTA in the sample (Fig. 3).

Reference Standards

OTA was extracted from Durum wheat inoculated with *Aspergillus ochraceus* (Stander *et al.*, 2000b). OTA was recrystallised from chloroform and its purity and identity were confirmed by the melting point, 600 MHz NMR spectroscopy and HPLC analysis. A stock solution was prepared from OTA (5.5 mg/mL methanol), the concentration of which was confirmed by UV spectroscopy. The stock solution was kept at -20°C. An external calibration curve for OTA was prepared daily. The limit of detection for OTA was determined to be 0.003 ng (at S/N 1:3) and the limit of quantification (at S/N 1:10) as 0.01 ng per injection. The recovery of the OTA varied between wines; the percentage recovery was 98 ± 5% for red wine (Pinotage cultivar) at a spiked level of 48 pg/mL (n=6). The reproducibility of the method was better at higher levels with a %RSD of 2.2 (n=6) at a level of 104 pg/mL spiked red wine.

RESULTS AND DISCUSSION

Two separate studies were undertaken to determine the levels of OTA in South African wines: a preliminary study of the levels of the OTA in wines from the medium to low price ranges obtained from local retail stores (Table 1) and a second more comprehensive

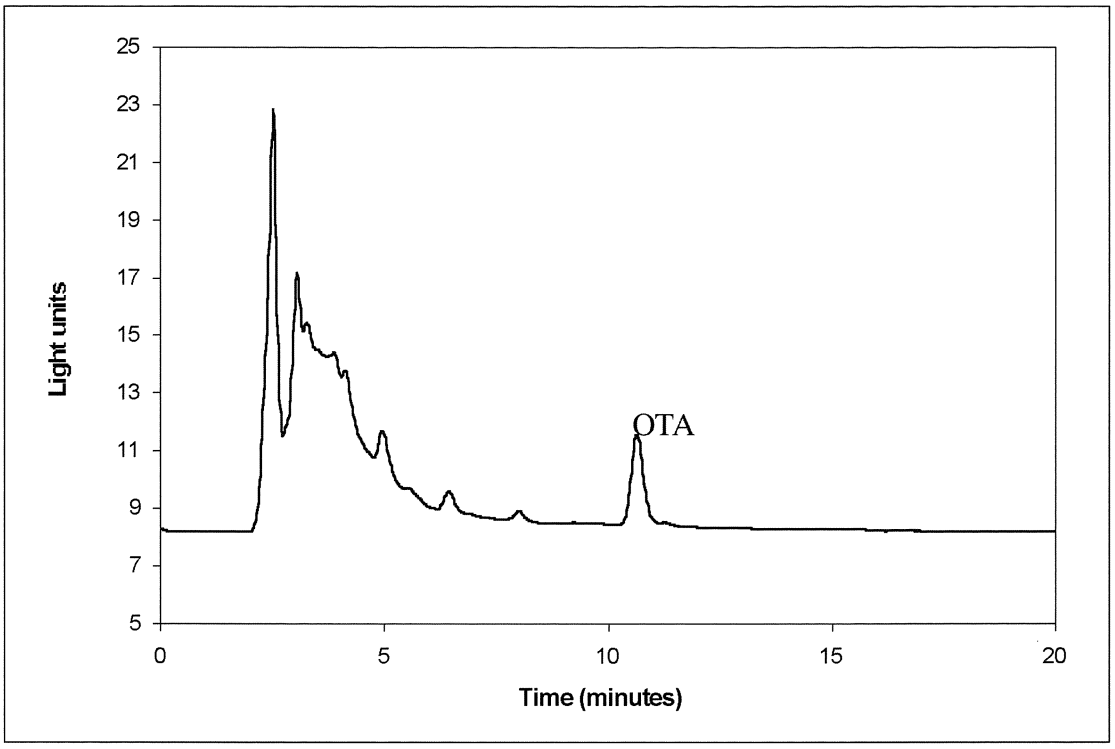

FIGURE 2

HPLC chromatogram of a red wine sample, after immunoaffinity cleanup, containing 104 pg/mL ochratoxin A (Retention time 10.2 minutes).

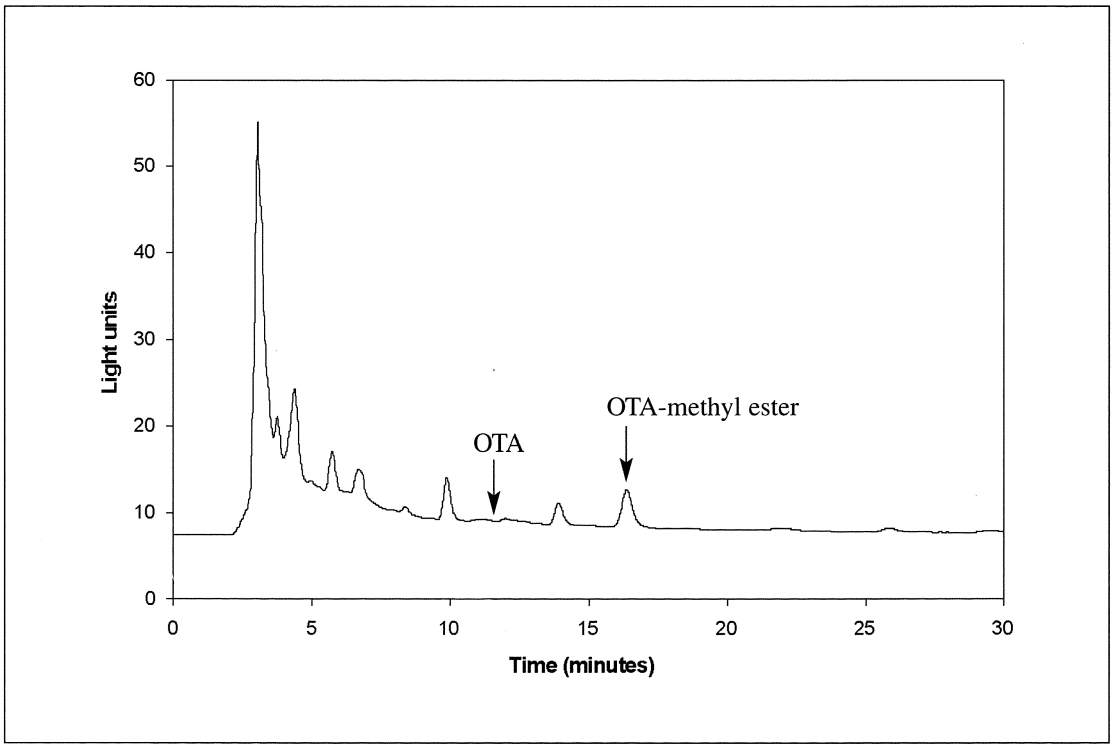

FIGURE 3

HPLC chromatogram of a red wine sample containing Ochratoxin A after esterification.

TABLE 1

Levels and distribution of OTA in South African wines (preliminary study).

Wine	Number of wines	Median	Levels	<10 pg/mL	10-50 pg/mL	50-150 pg/mL	150-200 pg/mL	>200 pg/mL
Red	16	14 pg/mL	<10 – 217 pg/mL	6	3	6	0	1
White	15	< 10 pg/mL	<10 – 195 pg/mL	9	1	4	1	0
Dessert	5	130 pg/mL	<10 – 270 pg/mL	1	1	2	0	1

TABLE 2

The levels of OTA in wines from the different wine-producing regions in South Africa.

Region	Number of wines analysed	Number of wines with an OTA level of <10 pg/mL	Median (pg/mL)	Highest level (pg/mL)
Swartland	5	5	<10	<10
Stellenbosch	20	15	<10	92
Durbanville	5	3	<10	48
Worcester	4	2	<10; 12*	57
Robertson	11	7	<10	126
Klein Karoo	2	2	<10	<10
Somerset West (Helderberg)	10	6	<10	100
Overberg	2	0	42; 110*	110
Franschhoek	2	2		
Elephants River	6	5	<10	55
Orange River	2	1	<10; 17*	17
Paarl	9	6	<10	30

*Two values are reported for the median owing to the small number of samples analysed.

TABLE 3

Levels of OTA in the different South African wine cultivars.

Cultivars	Number of wines analysed	Number of wines with an OTA level of <10 pg/mL	Median (pg/mL)	Highest level (pg/mL)
Red				
Cabernet Sauvignon	14	13	<10	21
Merlot	5	4	<10	31
Shiraz	11	7	<10	100
Pinotage	11	9	<10	16
Pinot Noir	1	1		
Tinta Barocca	2	2		
Rouge	2	2		
Blend (red)	3	2	<10	30
White				
Chardonnay	10	6	<10	126
Chenin Blanc	6	3	<10	57
Sauvignon Blanc	6	4	<10	110
Gewürztraminer	3	0	17	26
Rhine Riesling	1			17
Weisser Riesling	1			27
Others				
Noble late harvest wines	3	0	1732	2672
Fortified wines	7	3	21	168

study of South African wines representative of the higher price range, export-quality wines. The latter wines were entered for the South African Veritas Competition in 2000 (Tables 2 and 3).

Wines were selected to be representative of the different wine regions and the different cultivars that are produced in South Africa. A perusal of Tables 1 to 3 shows that the majority of wines had levels of OTA well below the guideline of the European Union for the maximum permissible level of OTA in wine (200 pg/mL). None of the different cultivars or wine-producing regions had markedly higher OTA levels, with the exception of noble late harvest wines. The high levels of OTA in noble late harvest wines may be attributed to the natural exposure of the older grapes, used in the production of these wines, to noble rot (*Botrytis*) and possibly some toxinogenic *Penicillium* and *Aspergillus* fungi.

It is of interest to note that the values of OTA in wines reported in the preliminary study were slightly higher than those in the second study; the observation may be associated with the differences in the quality of the grapes and the wine-producing practices.

CONCLUSIONS

This paper reports on one of the most comprehensive studies ever undertaken into the occurrence of OTA in wine in one country. Wines derived from the main cultivars and wine-producing regions in Africa were included in the study. No regional influence was observed, although slightly higher levels of OTA occurred in the less expensive wines and in the few samples of noble late harvest wines. The South African red and white wines contained similar levels of OTA; however, researchers from the wine-producing areas of Western Europe reported that their red wines contained slightly higher OTA levels than their white wines.

This first study clearly shows that there is no apparent problem associated with the level of OTA in South African wines. However, the results obtained warrant further investigations into the factors influencing the occurrence of OTA in South African wines, e.g. the microbiological origin of OTA in local wines; the physiology of the toxinogenic fungus/fungi involved; the effect of seasonal and agricultural conditions; the stage of the specific microbiological infection of grapes and/or must; the rate of OTA production on the grapes and/or on the must; the rate of the decomposition of OTA during barrel or bottle maturation as well as during the storage of wines.

LITERATURE CITED

Bacha, H., Maroufi, K., Achour, A., Hammami, M. & Creppy, E.E., 1993. Ochratoxines et ochratoxicoses humaines en Tunisie. In: Creppy, E.E., Castegnaro, M. & Dirheimer G., (eds). Human ochratoxicosis and its pathologies. College INSERM/John Libbey Eurotext Ltd., 231. pp. 111-121.

Breitholtz, A., Olsen, M., Dahlback, A. & Hult, K., 1991. Plasma ochratoxin A levels in three Swedish populations surveyed using an ion-pair HPLC technique. Food Additives and Contaminants 5, 183-192.

Burdaspal, P.A. & Legarda, T.M., 1999. Ochratoxina A en vinos, mostos y zumos de uva elaborados en Espana y en ostros paises europeos (Ochratoxin A in wines and grape musts and juices produced in Spain and other European countries), Alimentaria (Madrid) 299, 107-113.

Creppy, E.E., 1999. Human ochratoxicosis. J Toxicol. Toxin Rev. 18, 277-293.

Creppy, E.E., Betbeder, A.M., Gharbi, A., Counord, J., Castegnaro, M., Bartsch, H., Moncharmont, P., Fouillet, B., Chambon, P. & Dirheimer, G., 1991. Human ochratoxicosis in France. Mycotoxins, endemic nephropathy and urinary tract tumours. In: Castegnaro, M., Plestina, R., Dirheimer, G., Chernozemsky, I.N. & Bartsch, H. IARC. Sc. Publications no. 115. Lyon. pp. 145-151.

Creppy, E.E., Castegnaro, M. & Dirheimer, G., 1993. Human ochratoxicosis and its pathologies. In: Proc. Int. Symp: Human ochratoxicosis and associated pathologies in Africa and developing countries, Bordeaux, France. Colloque INSERM, vol. 231, John Libbey Eurotext.

Doster, R.C. & Sinnhuber, R.O., 1972. Comparative rates of hydrolysis of ochratoxins A and *B in vitro*. Fd. Cosmet. Toxicol. 10, 389-394.

Dumeau, F. & Trione, D., 2000. Wine processing in relation to ochratoxin A. Vignevini 27, 79-81.

Festas, I., Herbert, P., Santos, L., Cabral, M., Barros, P. & Alves, A., 2000. Ochratoxin A in some Portuguese wines: Method validation and screening in port wine and Vinho Verde. Am. J. Enol. Vitic. 51, 150-154.

JECFA,1995. Joint FAO/WHO Expert Committee on Food Additives, 44th Meeting, Technical Report Series Nr. 589, Geneva, WHO.

Krogh, P., Gyrd-Hansen, H.B., Larsen, S., Nielsen, J.P., Smith, M., Ivanoff, C. & Meisner, H., 1988. Renal enzyme activities in experimental ochratoxin A-induced porcine nephropathy: Diagnostic potential of phospho-enolpyruvate carboxykinase and gamma-glutamyl transpeptidase activity. J. Toxicol. and Environ. Health 23, 1.

Larcher, R. & Nicolini, G., 2001. Survey of ochratoxin A in musts, concentrated musts and wines produced or marketed in Trentino (Italy). J. Comm. Sci. 40, 69-78.

Majerus, P.B. & Otteneder, H., 1996. Nachweiss und Vorkommen von Ochratoxin A in Wein und Traubensaft. Deutsche Lebensmittel-Rundschau 92, 388-390.

Marquardt, R.R. & Frohlich, A.A., 1992. A review of recent advances in understanding ochratoxicosis. J. Ani. Sci. 70, 3968-3988.

Ospital, M., Cazabeil, J-M., Betbeder, A-M., Tricard, C., Creppy, E.E. & Medina, B., 1998. Ochratoxin A in wines. Union des Oenologues de France 169, 16-18.

Otteneder, H. & Majerus, P., 2000. Occurrence of ochratoxin A in wines: Influence of the type of wine and its geographical region. Food Additives and Contaminants 17, 793-798.

Petkova-Bocharova, T., Chernozemsky, I.N.B. & Castegnaro, M., 1988. Ochratoxin A in human blood in relation to Balkan Endemic Nephropathy and urinary system tumours in Bulgaria. Food Additives and Contaminants 5, 299-301.

Stander, M.A., Steyn, P.S., van der Westhuizen, F.H. & Payne, B.E., 2001b. A kinetic study into the hydrolysis of the ochratoxins and analogues by carboxypeptidase A. Chemical Research in Toxicology 14, 302-304.

Stander, M.A., Bornscheuer, U., Henke, E. & Steyn, P.S., 2000a. Screening of commercial lipases for the degradation of ochratoxin A. J. Agric. and Food Chem. 48, 5736-5739.

Stander, M.A., Steyn, P.S., Lübben, A., Miljkovic, A., Mantle, P.G. & Marais, G.J., 2000b. Influence of halogen salts on the production of the ochratoxins by *Aspergillus ochraceus* Wilh. J. Agric. and Food Chem. 48, 1865-1871.

Stander, M.A., Steyn, P.S., Nieuwoudt, T.W., Shephard, G.S., Creppy, E.E. & Sewram, V., 2001a. Toxicokinetics of ochratoxin A in vervet monkeys (*Cercopitheous aethiops*). Archives of Toxicology 75, 262-269.

Steyn, P.S. & Stander, M.A., 1999. Mycotoxins with special reference to the carcinogenic mycotoxins: Aflatoxins, ochratoxins and fumonisins. In: Ballantyne, B., Marrs, T.C. & Syversen, T. (eds). General and Applied Toxicology. MacMillan Reference Ltd., London. pp. 2145-2176.

Steyn, P.S., Stander, M.A. & Smit, M.S., 2000. Metabolic degradation of ochratoxin A by certain yeasts. Preliminary SA Patent Application No 2000214.

Thuvander, A., Paulsen, J. E., Axberg, K., Vidnes, A., Enghardt Barbieri, H., Trygg, K., Lund-Larsen, K., Bosnes, V., Hult, K. & Olsen, M., 1999. Levels of ochratoxin A in blood from Norwegian and Swedish blood donors. Estimated intake and correlation between blood levels and food consumption habits. Colloq.Sci.Int.Cafe 18, 276-280.

Uedo, Y,. 1998. Residue and risk of ochratoxin A in human plasma and beverages in Japan. Maikotokishin (Tokyo) 47, 25-32.

Van der Merwe, K.J., Steyn, P.S. & Fourie, L,. 1965. Mycotoxins. Part II. The constitution of ochratoxins A, B and C, metabolites of *Aspergillus ochraceus* Wilh. J. Chem. Soc 7083-7088.

Visconti, A., Pascale, M. & Centonze, G., 1999. Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. J. Chromat. A. 864, 89-101.

Wolff, J., Breach, H., Cholmakow-Bodechtel, C. & Engel, G., 2000. Belastung des Verbrauchers und der Lebensmittel mit Ochratoxin A. Abschlussbericht: Bundesministerium Für Gesundheit.

Zimmerli, B. & Dick, R., 1996. Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. Food Additives and Contaminants 13, 655-668.