

## ABSTRACTS

### *International Workshop*

## **OCHRATOXIN A IN GRAPES AND WINE: PREVENTION AND CONTROL**

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FACULTY OF AGRICULTURE PIACENZA – CAVIRO - ISPA

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### SECTION 1

# **Ochratoxin A and toxigenic fungi in grapes**

## LECTURES

### TOXICITY AND CARCINOGENICITY OF OCHRATOXIN A

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Ochratoxin A (OTA) is a mycotoxin which may contaminate a variety of agricultural products, resulting in chronic human exposures. OTA is nephrotoxic and induces renal tumors in rodents, whereby it exhibits significant sex- and species differences. Male rats are most susceptible to OTA carcinogenicity and repeated administration of low doses of OTA (up to 210 µg/kg b.w.) for two years results in high incidences of renal adenomas and carcinomas arising from the straight segment (S3) of the proximal tubule epithelium. Kidney tumors in OTA exposed rats develop with a relative rapid onset and are characterized by their malignant and aggressive behavior. Interestingly, no increase in tumor incidence was observed following treatment with 21 µg/kg b.w, suggesting a non-linear dose-response for renal tumor formation by OTA. However, the mechanism of tumor formation by OTA in the kidney is not well defined and controversial results regarding mode of action have been published.

OTA is not mutagenic in *Salmonella typhimurium* but weak genotoxic effects have been observed in some mammalian cell systems. Regarding the potential of OTA to covalently bind to DNA, conflicting results have been obtained. While experiments using radiolabeled (<sup>3</sup>H or <sup>14</sup>C) OTA and liquid scintillation counting or accelerator mass spectrometry indicate lack of formation of covalent DNA-adducts, spots detected by <sup>32</sup>P-postlabeling have been attributed to treatment with OTA. However, these putative DNA-adducts have not been shown to contain OTA or part of the OTA molecule and so far no structural information has been provided. Consistent with the absence of DNA-binding of radiolabeled OTA, studies on biotransformation *in vivo* and *in vitro* indicate that OTA is poorly metabolized and does not form reactive intermediates capable of interacting with DNA. Recently, the structures of a carbon- and an oxygen-bonded OTA-deoxyguanosine adduct which are formed by photoirradiation of OTA in the presence of deoxyguanosine have been reported and suggested to be involved in OTA carcinogenicity. However, formation of these potential adducts in relevant activation systems *in vitro* or in rats treated with OTA *in vivo* could not be confirmed by LC-MS/MS or <sup>32</sup>P-postlabeling, consistent with results of the DNA binding studies.

Using the comet assay, OTA has been shown to induce DNA strand breaks in target and non-target tissues of male rats treated with OTA. Interestingly, a similar degree of DNA damage was observed in rats treated with OTB, despite the lower toxicity of OTB. Moreover, the presence of DNA damage did not correlate with histopathological alterations which were evident in the kidney, but not in the liver, suggesting that the observed DNA damage by itself may not be sufficient to trigger tumor formation. In liver and kidney, the extent of DNA damage was further enhanced in the presence of Fpg-glycosylase, which is known to convert oxidative DNA damage into strand breaks, indicating the presence of oxidative DNA lesions. In addition, a small but not significant increase in the incidence of chromosomal aberrations (essentially chromatid and chromosome type deletions) was observed in splenocytes from rats treated with OTA *in vivo* and subsequently cultured *in vitro* to express chromosomal damage. These aberrations are also compatible with oxidative DNA-lesions since they are not typically caused by chemical carcinogens which form covalent DNA-adducts. Taken together, these data suggest that OTA may cause genetic damage in both target and non-target tissues independent of direct interaction with DNA. However, the lack of target-organ specific induction of DNA-damage strongly suggests that additional events are required for renal tumor formation by OTA.

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### BLACK ASPERGILLI AND OTA PRODUCTION

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Members of the Section *Nigri* are world-wide in distribution as soil saprophytes. Many are food spoilage organisms. They are commonly present in vineyards and have the ability to cause berry rot, known as *Aspergillus* rot or black mould, both on white and red grape varieties. Furthermore, some species within the

Section *Nigri* are ochratoxin A (OTA) producers, and so correct identification at species level is essential. The taxonomic problems within this group of black aspergilli will be discussed. Whilst some species such as *Aspergillus carbonarius* and the uniseriate members of the group are distinct species, many form an aggregate centred on *Aspergillus niger*. For these reasons black aspergilli isolated during the four year study on the mycoflora of European vineyards were classified into three groups: *A.carbonarius*, *A.niger* aggregate, and the "uniseriates".

As part of the study on ochratoxin producing mycoflora of grapes, several black *Aspergillus* strains were isolated and tested for their OTA producing abilities. Some strains which were morphologically similar to *A.carbonarius*, a strong OTA producer, did not produce the toxin in detectable amounts. This has been validated and described as a new species.

## RAPID DETECTION METHODS FOR OCHRATOXIN A-PRODUCING FUNGI: STATE OF THE ART

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Nowadays, combinations of traditional mycological and molecular methods are used for the rapid detection of ochratoxin A (OTA) producing fungi. These species are included in the genera *Aspergillus* and *Penicillium*. There is a long list of species cited as OTA-producing fungi in the genus *Aspergillus*, but few of them are known to contaminate foods with this mycotoxin. OTA contamination of food was until recently believed to be produced only by *Aspergillus ochraceus* and by *Penicillium verrucosum*, which affect mainly dried stored foods and cereals respectively, in different regions of the world. However, recent surveys have clearly shown that some species belonging to the black aspergilli, including the *Aspergillus niger* aggregate and *Aspergillus carbonarius*, are sources of OTA in food commodities such as wine, grapes, dried vine fruits among others. Isolation or detection of an ochratoxigenic species from a food may suggest the involvement of OTA in it. However, the presence of this species in a food does not invariably indicate that this mycotoxin will be present. This food may not have provided suitable compounds for its production, or other environmental factors such as water activity, temperature or pH may prevent the biosynthesis of this toxin. On the other hand, not all the strains belonging to an ochratoxigenic species are mycotoxin producers.

Different methods have been developed to detect OTA producing fungi. Traditional mycological methods are time consuming and require taxonomical and chromatography expertise. Direct microscopic examination of the food, isolation and culture techniques followed by chemical characterization of the secondary metabolites produced by the isolates are included in these group of methods. Some differential culture media such as DYSG agar or coconut cream agar have been used for facilitating the detection of OTA producing species. Complementing these conventional methods, various immunological techniques have been also adapted to detect and quantify OTA, providing user friendly formats (e.g. lateral flow devices) making easy the detection of the mycotoxin produced by the isolates. Different molecular diagnostic methods for an early detection of ochratoxigenic fungi, using mainly PCR techniques, have been also proposed. Two main approaches have been used to detect the presence of these fungi. They are based on the detection of species-specific DNA fragments of OTA producing species (e.g. rDNA genes, anonymous specific sequences, ...) and the detection of DNA sequences of genes involved in OTA biosynthesis (e.g. polyketide synthase genes). One of the goals of these techniques is to differentiate between toxigenic and non-toxigenic strains belonging to species known to produce OTA. To date, one of the problems is that little is known about the genes involved in the OTA biosynthesis.

## MOLECULAR METHODS TO DETECT AND CHARACTERIZE BLACK ASPERGILLI FROM GRAPE IN EUROPE

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Members of *Aspergillus* belonging to section *Nigri* are distributed worldwide and are mainly responsible for the ochratoxin A accumulation in grapes and wine, particularly in Southern Europe.. Taxonomy of *Aspergillus* Section *Nigri* has been largely studied by means of morphological and cultural criteria, but it is still controversial and needs further elucidation, resulting problematic even for expert mycologists. Limited information is available on the species composition and genetic variability of black Aspergilli strains occurring on grapes and to this

respect, there is not unanimous evaluation among *Aspergillus* taxonomists, moreover, the accurate identification of *Aspergillus* species in the Section *Nigri* is of great importance because of the toxic profile of the single species could be different and could expose the contaminated food commodities to different toxicological risks. The increasing use of molecular methods in fungal diagnosis has provided more tools for answering taxonomic questions that common morphological identification procedures leave unsolved. Various PCR primers pairs were recently developed for species and gene-specific detection and identification of atypical producers of ochratoxin A. Primers were applied in pure cultures and in contaminated sample materials in conventional and in real-time PCR applications.

Different strategies will be presented in order to obtain nucleotide sequence information useful in designing diagnostic PCR assays.

## **FTIR FINGERPRINTING OF MUST AND WINE REVEALS *ASPERGILLUS CARBONARIUS* AND OCHRATOXIN A (OTA) CONTAMINATION.**

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1 - Ochratoxin A (OTA) is a mycotoxin found in many human food products. Low levels can be found in wines made from grapes contaminated with the fungus *Aspergillus carbonarius*.

2 - The European Community regulation (EC) 123/2005 sets a 2 µg.kg<sup>-1</sup> limit on OTA concentrations in wine.

3 - Standard OTA analyses in wine (e.g. NF EN 14133) are based on immunoassay processes and HPLC. Immunoenzymatic quantitative measurements (ELISA test) being a good alternative to HPLC. However, both analytical methods are difficult and expensive to run. There is strong interest in looking for alternative methods enabling larger and faster analytical capacities.

4 – We show that *A. carbonarius* grown on the grape berries, besides OTA secretion, results on the production of other metabolites like gluconic acid, citric acid, and glycerol. The concentration of these compounds is correlated both to the *A. carbonarius* development and the OTA concentration itself.

5 – Thus, *A. carbonarius* alters the must and wine composition. We show here that the Infra-Red spectrum using Fourier Transformation (FTIR) fingerprint of must and wine, is useful both to assess the alteration, and to quantify the damage of grape before harvest. The OTA production is strongly correlated with the IR signature of *A. carbonarius* induced metabolites. These results show that it is possible to develop a predictive measure of OTA in wine made from spoiled grapes.

6 – The FTIR fingerprint for OTA in wine reported here provides a user friendly application for routine measurements at low cost and with immediate results. Although it cannot replace standard methods, it could play a complementary role early in the wine's life, providing assistance for preventives actions.

## **PHYLOGENETIC ANALYSIS OF *ASPERGILLUS CARBONARIUS* STRAINS PRODUCING OTA BY AFLP FINGERPRINTING**

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Morphological and molecular methods fail to discriminate *Black Aspergilli* at the intraspecific level. This prompted us to evaluate the use of the genome-spanning method AFLP to this group. As a part of this survey we have analyzed several *A. carbonarius* isolates. We have found that the AFLP profile differs among these isolates. In order to obtain statistically significant data, a study was conducted on 30 isolates, including collection strains and field isolates. The field isolates are mainly from Narbonne (France). We have measured the Ochratoxin A production and obtained the corresponding AFLP profiles.

Based on OTA production, the isolates were statistically (CHA) classified in several groups. Interestingly, one group comprises six isolates that did not produce any OTA. In order to establish their taxonomic position, we have sequenced the ITS1-5.8S-ITS2 DNA from all the isolates. All of them were shown to be *A. carbonarius*.

We have performed AFLP fingerprinting in all the isolates, using 2 couples of discriminating primers. The analysis of the AFLP profiles resulted in the identification of 140 polymorphic markers. Two clades were identified by phylogenetic analysis, these clades correlate with OTA production. However the bootstraps values are not concluding. We are currently analysing data obtained from two additional discriminating primers. These results will be discussed at the meeting.

## DEVELOPMENT OF A QUANTITATIVE REAL-TIME PCR ASSAY FOR THE DETECTION OF *ASPERGILLUS CARBONARIUS* IN GRAPES

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*Aspergillus carbonarius* is the main species responsible for the production of ochratoxin A (OTA) in wine grapes. To monitor and quantify *A. carbonarius* in grapes, a quantitative real-time PCR assay was developed as a possible tool for predicting the potential ochratoxigenic risk. DNA extraction from grape berries was performed by using conventional extraction and clean up through EZNA Hi-bond® spin columns. A TaqMan probe was used to quantify *A. carbonarius* genomic DNA in grape berries samples. An exogenous internal positive control was used to overcome DNA recovery losses due to matrix inhibition. The quantification of fungal genomic DNA in naturally contaminated grape was performed using the TaqMan signal versus spectrophotometrically-measured DNA quantities (Log10) calibration curve with a linearity range from 50 to 5x10<sup>-4</sup> ng of DNA. A positive correlation ( $R^2 = 0.92$ ) was found between *A. carbonarius* DNA content and OTA concentration in naturally contaminated grape samples. This is the first application of TaqMan real-time PCR for identifying and quantifying *A. carbonarius* genomic DNA occurring in grapes. The rapid DNA extraction method for grapes, together with the commercial availability of reagents and instrumentation, allows to perform a remarkable number of reproducible assays (96-well format) in less than 4 hrs.

## EVALUATION OF OCHRATOXIN A CONTAMINATION IN ITALIAN WINES

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Among most recently food matrices discovered to be susceptible to ochratoxin A (OTA) contamination, wine represents one of the most relevant. Since this discovery (1996), many researches have been performed on the origin and diffusion of the contamination as well as the identification of risky areas. In Italy, due to the relevance of the issue both from the health and from the economic point of view, the Ministry of Agriculture launched a project with the aim of thoroughly investigate the problem of OTA occurrence in Italian wines. In this paper the results of the investigation are shown.

The research has been carried out in the period 1998-2003. The collection of samples was statistically performed on the basis of the ratio of production volume/winery and of the diffusion of the main grape varieties. A total of 814 wine samples were analysed.

In the first part of the study a total of 463 wine samples were collected taking into account the distribution of production across the country (North N= 148, Middle N= 168 and South N=147). Due to the recognition of the severity of the contamination in red wine with respect to the white one, an higher percentage (63%) of red wine samples were taken with respect to the white ones (37%). Most of the samples derived from the 1998-1999 production. The analysis on the samples showed an higher percentage of contamination in the South deriving samples, especially for red wines. The percentage of contaminated red wine samples below 0.1 ng/ml was approximately 70% mainly in North and Central Italy. Vice versa, in South Italy red wine samples were found to be contaminated at approximately 25% in the range 0.1 – 1,00 ng/ml, at approximately 5% in the range 1,00 – 2,00 ng/ml and at approximately 3% at level above the new legal limit (2 ng/ml) put in force with EC Regulation 123/2005.

During the second part of the research, mainly dealing with the years 2000-2003, on the basis of the previous results only red and rosé wine samples (N= 351) were collected only in those regions (Sicily, Apulia, Calabria and Sardinia) where the contamination has previously been found more relevant. The percentage of contaminated samples below 0.1 ng/ml ranged from approximately 35% to 80% for the four interested regions, from 20% - 60% in the range 0.1 – 1,00 ng/ml, from 3% to 5% in the range 1,00 – 2,00 ng/ml and with a approximately 3% only in Apulia of samples contaminated at levels above the legal limit.

The mean levels of contamination were 0.33 µg/L, 0.21 µg/L, 0.11 µg/L and 0.08 µg/L for Apulia, Calabria, Sardinia and Sicily respectively.

The main conclusions of the research, mainly obtained with the  $\rho$  of Spearman correlation index, are the followings: i) OTA contamination is primarily related to red wine from the Southern part of Italy ii) By combining the found levels of contamination with the consumption data the exposure of the Italian wine consumers is not alarming iii) no correlation was found between level of OTA contamination and price/production year.

Further investigation is ongoing on wine samples related to the years 2004-2005.

## OCCURRENCE OF OCHRATOXIN A IN GREEK GRAPES, MUSTS AND WINES

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The objective of the present work was to obtain information on the occurrence of OTA in Greek wine grapes, musts and wines. Preliminary results are presented here, as the survey is in progress. The wine samples were commercial wines representative of different types, grape varieties, geographical area of cultivation and different vintages (2000 -2004). Sixty two wine samples (38 red, 18 white, 6 rose) were studied and the OTA concentration found to range between 0.01– 1.5 µg kg<sup>-1</sup> (40 positive samples) with the red wines containing higher levels compared to the white and rose ones. OTA was also quantified in wine grapes (10 samples) and musts (8 samples) of the vintage 2004 from the region of Peloponnese (South Greece). OTA was not detected in any sample of grapes (limit of detection 0.005 µg kg<sup>-1</sup>). Must samples were also investigated for OTA content during fermentation. Three samples at the first stage of fermentation contained OTA at concentrations from 0.1 to 7.2 µg kg<sup>-1</sup>. The high levels of OTA detected during the fermentation in certain variety did not remained after the end of fermentation.

## ANALYTICAL OPTIONS FOR OCHRATOXIN A IN WINE

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Ochratoxin A (OTA) is a mycotoxin which possesses carcinogenic, nephrotoxic, teratogenic, immunotoxic and possibly neurotoxic properties (Scientific Committee on Food, September 1998).

Human dietary exposure is greatest from cereals (based on an "average" European diet). Wine, especially red wine is the second most significant contributor (Professor R. Walker, Mycotoxins of growing interest, 3rd joint FAO/WHO/UNEP Conference on mycotoxins, Tunis 1999.)

Consequently, the European Commission has, in recent years, introduced maximum residue limits for OTA in cereals and dried fruit, while at the same time considering action with regard to a number of other food commodities. In January 2005, Commission regulation 123/2005 was introduced setting maximum residue limits for OTA in, among others, wine and wine based beverages. A maximum residue limit for liqueur wines is the possible outcome of a review of legislation expected before 30<sup>th</sup> June 2006.

Consequently the wine industry will be expected to conform to the new maximum residue limit of 2ppb for OTA. This will lead to more analysis within the industry; by manufacturers, contract laboratories and, of course, official control by government laboratories.

R-Biopharm Rhone Limited are, as part of the R-Biopharm group, one of the worlds' largest manufacturers of monoclonal antibody based diagnostic test kits for analysis of mycotoxins; and as such offer several solutions to the problem of analysis of OTA in wine, irrespective of the amount of equipment available or the number of samples to be processed.

Three different techniques will be briefly described; quantitative analysis using immunoaffinity column clean up of the wine and concentration of the toxin followed by detection of OTA by HPLC; quantitative analysis of samples using ELISA for screening of large numbers of samples and qualitative analysis using a membrane based card system where no equipment is required.

In addition the CEN standard method of analysis and a FAPAS ring trial on OTA in wine will be reviewed.

## POSTERS

### **ASPERGILLUS IBERICUS: A NEW SPECIES OF THE SECTION NIGRI ISOLATED FROM GRAPES**

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As part of a study on the ochratoxin producing mycoflora of grapes, several *Aspergillus* strains were isolated and tested for their ochratoxin A (OTA) producing abilities. *Aspergillus* strains of the section Nigri which did not produce detectable amounts of OTA but which had a similar morphology to *A. carbonarius* were isolated from wine grapes and/or dried vine fruit in Portugal and Spain.

These strains, however, have characters that allow morphological distinction from the other species in the section, particularly the spore size (5 – 7 µm), which allows separation of the species from the two most common biserial species in section Nigri: *A. carbonarius* (7 – 9 µm) and *A. niger* and its aggregate species (3 – 5 µm). The strains are described here as belonging to a new species, named *A. ibericus*.

The validation of this new taxon is further supported by analysis of the ITS-5.8S rDNA and calmodulin gene sequences and by analysis of the amplified fragment length polymorphism (AFLP) patterns, which were consistent in separating these strains from other species in the section. As the *A. ibericus* sp. nov. strains do not produce OTA, they are interesting for biotechnological exploration as many metabolites with commercial value are produced by other species in the section.

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### **REAL-TIME PCR FOR QUANTITATIVE DETECTION OF ASPERGILLUS CARBONARIUS IN GRAPES AND MUSTS**

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Several species of *Aspergillus* and *Penicillium* have been reported among fungi involved in ochratoxin A (OTA) contamination in wine. The researches carried out in several southern and warmer regions of Europe and northern Africa showed that the main if not exclusive responsible of OTA contamination in wine is *Aspergillus carbonarius* (Bainier) Thom, a fungus developing on grape bunches in vineyards.

So far, identification and quantification of *A. carbonarius* were based on laborious and time-consuming techniques requiring deep knowledge on fungal taxonomy. *A. carbonarius* is indeed very similar to other species belonging to the group *Nigri* of the genus *Aspergillus*, as *Aspergillus niger* Van Tieghem that is more abundant than *A. carbonarius* on grape bunches. Risk assessment is nowadays crucial already in vineyards for planning appropriate crop protection strategies aimed at producing wine and/or other grape-derived commodities according to the maximal tolerable level of OTA established in 2.0 µg kg<sup>-1</sup> by the Reg. (CE) N. 123/2005 of 26.1.2005. Reliable methods for detection and quantification of *A. carbonarius* in grape, must and wine are therefore highly desirable in order to fulfil the need to protect consumer's health from the risks deriving from exposure to OTA.

This report deals with the development and validation of a quantitative detection assay for *A. carbonarius* based on real-time Scorpion polymerase chain reaction (PCR).

Sequence Characterized Amplified Region (SCAR) primers pairs were designed on three selected species-specific Random Amplified Polymorphic DNA (RAPD) markers. Two of them (OPA2<sub>800</sub>B and OPA3<sub>519</sub>C) proved selective yielding the expected amplification products with DNA from *A. carbonarius*, but no with DNA from other grapevine-associated micro-organisms (i.e. species of *Aureobasidium*, *Botryosphaeria*, *Botryotinia*, *Cladosporium*, *Eutypa*, *Fomitiporia*, *Penicillium*, *Phaeoacremonium*, *Phaeomoniella*, *Phomopsis*, *Rhizopus*, *Trichoderma*, *Erysiphe*). The OPA3<sub>519</sub>C primer pair was selected on the ground of the PCR sensitivity and

modified to obtain a Scorpion probe suitable for detecting a specific 100-bp amplicon by fluorescence emitted from FAM-fluorophore in real time PCR assay.

DNA suitable for the PCR assay was extracted from fungal propagules collected from washing water of bunches or musts with a protocol based on CTAB buffer and Sepharose spin column purification.

Real-time PCR was carried out in a iCycler Thermal Cycler (Bio Rad Laboratories). Preliminarily a calibration curve was constructed. The linearity range was from  $10^3$  to  $10^6$  fungal propagules. The protocol permitted selective detection and quantification of about 4 ng of *A. carbonarius* DNA in the mixture of reaction (corresponding to  $0,8 \cdot 10^3$  conidia). Both artificially and naturally *A. carbonarius* contaminated samples of washing water of bunch or must were used to validate the procedure. The molecular protocol proved to be more rapid and reliable than the traditional quantification method based on plating of decimal dilutions of samples on semi-selective media. Thus it will be a useful tool for large-scale determination of *A. carbonarius* contamination aimed at assessing the risk of OTA contamination in wine starting from samples of bunches collected in vineyards.

## CHARACTERIZATION OF SOME PKS GENES IN *ASPERGILLUS CARBONARIUS* AND THEIR USE IN FUNGI DETECTION AND QUANTIFICATION IN GRAPES

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*Aspergillus carbonarius* is an ochratoxin producing fungus that has been considered to be responsible of the ochratoxin A (OTA) contamination in grapes and wines. The central enzyme responsible for the biosynthesis of OTA is a polyketide synthase (PKS) required for the synthesis of the polyketide dihydro-isocoumarin. In this study the diversity of polyketide synthase genes in *A. carbonarius* 2MU134 isolated from French vineyard has been investigated. Five different ketosynthase (KS) sequences (*AcLC35-4*, *AcLC35-6*, *AcKS9*, *AcKS10* and *Ac12RL-3*) have been identified using different pairs of primers previously designed to amplify the KS domains of PKSs by PCR on genomic DNA. The identified KS sequences were distributed in the reduced and the partially reduced clades on the phylogenetic tree. In order to monitor and quantify *A. carbonarius*, a specific primer pair *Ac12RL\_OTAF/R* has been designed from the AT domain of the sequence *Ac12RL-3* to amplify 141 bp PCR product. Among the mycotoxinogenic fungi tested only *A. carbonarius* gave positive result. This specific primer pair was also successfully employed in real time PCR conjugated with SYBR Green I dye in order to be used for the direct quantification of this fungus in grapes and must samples.

## IGS PCR AS AN ALTERNATIVE METHOD TO DIFFERENTIATE BETWEEN OCHRATOXIGENIC AND NON-ochratoxigenic STRAINS OF *ASPERGILLUS NIGER* AGGREGATE

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Ochratoxin A (OTA) has been reported as one of the most important toxic metabolites in various food commodities. On grapes, the main OTA producers are aspergilli belonging to the section *Nigri*. Only a small amount of the isolates of this taxon are able to produce OTA.

*Aspergillus* strains belonging to the *A. niger* aggregate, isolated from grapes sampled in various Italian regions, were analysed together with strains received from Italian and foreign collections, in order to ascertain the possibility to discriminate between the producing and the non-producing strains by means of molecular tools. Species identification was carried out observing the macro- and microscopic features of the colonies, using optical microscopes. The strains ochratoxigenicity was evaluated through Thin Layer Chromatography.

The species were classified as *Aspergillus niger* or *Aspergillus awamori* while *Aspergillus carbonarius*, a well known OTA-producer, was used as reference species.

After DNA extraction from the colonies, the PCR amplification of the Intergenic spacers (IGS) produced 440 bp amplicons in all the analysed strains. The dimension of the amplicon is not therefore sufficient to discriminate among the strains. The subsequent digestion of the IGS amplicon with the restriction endonuclease *Hinf* I unveiled a partial correlation with the OTA production. In particular, a profile type could be associated with the *A. niger* aggregate strains which lack the ability to produce OTA.

## OCCURRENCE OF OCHRATOXIN A IN WINE PRODUCED IN ITALY OVER SIX YEARS

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After the first detection of Ochratoxin A (OTA) in wine in 1996, several surveys were managed, mainly in Europe. In a previous investigation (2001), we confirmed that the geographic region of origin had a strong influence on OTA contamination: in fact, wines produced in southern Italy were markedly more contaminated. The overall median (mean) OTA concentration in the red wines produced in the four Italian areas (northwest, northeast, centre and south) was 0.002 (0.011), 0.090 (0.081), 0.134 (0.295) and 1.264 (1.233) µg/l. In 2005, the Commission of the European Communities (EC) fixed a limit of 2.0 µg/l for OTA in wine. The present paper presents the results of a survey, conducted from 1999 to 2004. A total of 537 red and 102 white Italian wines were analyzed. Analyses were carried out by an immunoaffinity clean-up step followed by HPLC separation and fluorescence detection. The limits of detection and quantitation for the method used were 0.005 and 0.015 µg/l, respectively; recovery values obtained from spiked red wine samples were in the range 92-96%. The geographic region of origin showed again a strong influence on OTA level: in fact, a gradual increase of contamination from northern to southern Italy was noticed. Red wines (table 1) produced in 1999 were the most contaminated: 32 (57%) samples from the south, 3 (6%) from the centre and 1 (4%) from the north contained OTA above the EC limit. In 2002, 4 samples (33%) from southern Italy showed an OTA level higher than 2 µg/kg. In 2003, 3 samples (12%) from southern and 2 (15%) from central Italy resulted above the EC limit. In samples of red wine collected in 2000, 2001 and 2004 the OTA content was always lower than 2 µg/l; moreover, in samples from vintage 2004 the OTA concentration never exceeded 1 µg/l.

White wines showed a trend similar to red wines, but the OTA level was generally lower. Only 1 sample of white wine, produced in southern Italy in 1999, exceeded 2 µg/l (8.86 µg/l). In samples from vintages 2000-2004, the OTA concentration was always lower than 1 µg/l.

Table 1 - Ochratoxin A concentration (µg/l) in red wines produced in Italy in the period 1999-2004.

Vintage 1999				
	No.	Mean	Median	Maximum
North	28	0.15	0.02	2.73
Centre	50	0.57	0.25	5.10
South	56	3.93	2.19	15.61
Total	134	1.89	0.35	
Vintage 2000				
	No.	Mean	Median	Maximum
North	21	0.19	0.07	0.79
Centre	3	0.02	0.03	0.03
South	0	//	//	//
Total	26	0.16	0.04	
Vintage 2001				
	No.	Mean	Median	Maximum
North	13	0.03	0.04	0.06
Centre	10	0.10	0.08	0.37
South	12	0.54	0.32	1.43
Total	35	0.22	0.07	

Vintage 2002				
	No.	Mean	Median	Maximum
North	23	0.01	<0.005	0.04
Centre	14	0.26	0.13	1.29
South	12	1.06	0.73	2.57
Total	49	0.34	0.03	
Vintage 2003				
	No.	Mean	Median	Maximum
North	137	0.04	0.02	0.59
Centre	13	0.94	0.83	2.13
South	24	0.94	0.60	3.07
Total	174	0.23	0.03	
Vintage 2004				
	No.	Mean	Median	Maximum
North	61	0.01	<0.005	0.20
Centre	22	0.11	0.10	0.36
South	36	0.25	0.16	0.70
Total	119	0.10	0.05	

## ELECTROCHEMICAL IMMUNOSENSOR FOR OCHRATOXIN A IN WINE

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A direct, competitive electrochemical enzyme-linked immunosorbent assay (ELISA) has been developed for the quantitative determination of ochratoxin A using polyclonal antibodies. The assay is carried out on carbon based screen printed electrodes. Optimisation of the ELISA competitive conditions, allowed us to realise an assay with improved analytical behaviour compared to the classical spectrophotometric ELISA based assay. The performance was comparable to a published monoclonal based assay. The assay gave a detection limit of 180 pg.ml<sup>-1</sup> and sensitivity of 6.1 ± 0.1 ng.ml<sup>-1</sup>. The immunosensor was challenged with wine to assess a matrix effect. Recoveries obtained were in the 70 - 118% range.

The proposed system was compared with the official method for determination of OTA in wine, accepted by OIV (Visconti, J AOAC Int. 2001, 84(6):1818-27). The comparison was carried out on white and red wines, fortified with known amount of OTA. During fermentation, at different steps, an aliquot of the sample was taken and analysed with the two different methods in order to check the trends of OTA level. The results obtained from both methods shown the same trend of the concentration values during 8 days for the two wines, also an overvaluation of the results obtained by immunosensor method.

## DETERMINATION OF OCHRATOXIN A IN WINERY BYPRODUCTS BY IMMUNOAFFINITY CLEAN-UP AND LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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A liquid chromatographic method was developed for the determination of ochratoxin A (OTA) in winery byproducts. Dried lees and grape pomace were extracted with a mixture of acetonitrile-water by shaking. An aliquot of the filtered extract was appropriately diluted with a solution of polyethylene glycol and sodium hydrogencarbonate, filtered and purified by an Ochratest immunoaffinity column. OTA was eluted from the column with methanol. The purified extract was dried, reconstituted with LC mobile phase and analysed by reversed-phase liquid chromatography with fluorometric detector. Mean recoveries of OTA from grape pomace spiked at levels between 1 ng/g and 100 ng/g ranged from 74% to 103%, with mean within-laboratory repeatability ranging from 4% to 11%. The limit of detection was 0.1 ng/g based on a signal-to-noise ratio of 3:1. Several samples of lees and grape pomace deriving from wine-making of Negroamaro and Primitivo were analysed for their OTA content. Grape pomace and lees of Negroamaro contained OTA at levels of 71.6 – 93.5 ng/g and 113.8 – 162.2 ng/g, respectively. Grape pomace and lees of Primitivo contained OTA at levels of 34.2 – 180.3 ng/g and 83.4 – 290.0 ng/g, respectively. The use of other extraction solvent mixtures (polyethylene glycol/sodium hydrogencarbonate or methanol/phosphoric acid/water) commonly used to extract OTA from grape berries, currants, raisins, sultanas gave lower OTA recoveries (up to -83%) from these naturally contaminated matrices.

## VALIDATION OF SCREENING TEST KIT OCHRACARD FOR THE DETERMINATION OF OCHRATOXINS IN WINE

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Ochratoxin A is produced by various moulds of the genera *Aspergillus* and *Penicillium* and is a known carcinogen and nephrotoxin of animals and humans. Ochratoxin A can be found in a variety of foodstuffs including cereals, dried vine fruits, coffee and wine.

In January 2005, the European Commission fixed a limit of 2.0 µg/l (ppb) for OTA in wine, therefore it is important to validate qualitative screening methods, at detection levels equal to and around the European legislative limits. In response to the new legislation, R-Biopharm Rhône, introduced OCHRACARD, a qualitative screening test for the determination of Ochratoxin A in cereal, dried fruit, coffee and wine. The following study aims to assess the performance of OCHRACARD on 30 naturally contaminated Italian red wine samples.

Ochratoxin A is extracted from the sample, filtered and passed through a immunoaffinity clean up column before being diluted and applied to OCHRACARD. Ochratoxin conjugate is then applied to the membrane and any unbound conjugate is removed by washing. A colourless substrate is added and the card is left to develop for 5 minutes. Finally a stop solution is applied to the membrane. A purple spot must appear at the control site to indicate that the test is valid (i.e. that all of the reagents have been added to the card in the correct order as advised). A purple spot at the sample site shows that the contamination level is less than 2.0 µg/l (ppb). No colour at the sample site indicates contamination at a higher level than the legal limit of 2.0 µg/l (ppb).

The 30 red wines analysed with Ochracard, were also purified using OCHRAPREP immunoaffinity columns and the results confirmed using HPLC with fluorescence detection.

The following table summarises the results obtained.

Sample	Ochracard result (ppb)	HPLC result (ppb)
1	less than 2	0,52
2	more than 2	3,79
3	less than 2	0,17
4	less than 2	0,98
5	less than 2	0,09
6	more than 2	4,18
7	less than 2	0,18
8	less than 2	0,89
9	more than 2	2,89
10	less than 2	1,13
11	more than 2	3,80
12	more than 2	4,50
13	more than 2	2,96
14	more than 2	1,26
15	less than 2	0,20
16	more than 2	2,28

Sample	Ochracard result (ppb)	HPLC result (ppb)
17	more than 2	2,20
18	more than 2	2,50
19	more than 2	2,59
20	more than 2	2,04
21	more than 2	6,20
22	more than 2	8,80
23	less than 2	1,98
24	more than 2	2,09
25	less than 2	1,65
26	less than 2	0,04
27	less than 2	1,22
28	less than 2	1,35
29	more than 2	2,02
30	less than 2	1,42
31	less than 2	1,13 ( 1,5 certified value)
32	more than 2	2,89 ( 3,0 certified value)

With the exception of one wine sample (N° 14) the results obtained using OCHRACARD were in agreement with HPLC. As part of the validation study, two wine reference materials (N° 31-32) were also analysed using OCHRACARD and using OCHRAPREP with HPLC and both methods confirmed the certified values. In conclusion OCHRACARD was found to be a suitable screening test for analysis of ochratoxin A in wine at the legal limit of 2.0 µg/l (ppb).