ALMA MATER STUDIORUM - UNIVERSITY OF BOLOGNA

FACULTY OF AGRICULTURE

PhD IN FOOD SCIENCE

Food safety in wine: optimization of analytical controls and evaluation of production technologies

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Index

Preface

In the last ten years, the safe use of food products has been big acceleration in the field of scientific studies in order to be able to protect human health.

Security, in terms of the absence of chemical contaminants, physical and toxic agents of biological origin, it must be the most important and indispensable requirement of quality.

The quality and safety of food can be affected by many factors involved in the different stages of production, such as raw materials, techniques of production and conservation. Quality and food safety, therefore, should be guaranteed not only by respecting the laws, even for the control systems implemented by the manufacturer.

In this regard, the Community EC Regulation 178/2002, establishing the general rules for the traceability of food, allowed further protect consumers. However, to do the appropriate monitoring, it is indispensable to have analytical methodologies more reliable and speedy, that allow to carry out specific checks with limited use of resources.

The sector of beverages (e.g. wine, beer, fruit juices, soy milk, etc.) needs special attention, because the biotechnological process of transformation is quite complex.

The search for chemical contaminants of natural origin such as biogenic amines and mycotoxins has been particularly important in recent years, because these substances are dangerous to human health.

Biogenic amines are organic bases which are often in wines and other fermented foods and can cause a number of problems to humans. Some are already present in the must, others are produced and accumulated during the winemaking, by yeasts (during alcoholic fermentation), or bacteria (during malolactic fermentation) or other microorganism responsible for alterations of wine.

Mycotoxins, highly toxic molecules produced by several fungal species, have a stable configuration and a mutagenic and carcinogenic action. Concerning beverages, especially wine, is present ochratoxin A, produced by various species in the *Penicillium* and *Aspergillus* genera which widely occur in nature. The European Community established, with regulation 123/2005 dated 26th January 2005, the legal limit of ochratoxin A content in the Italian and European wines that is 2 micrograms/kg (ppb).

The following research has been proposed to validate and then apply innovative analytical methodologies for the determination of ochratoxin A and biogenic amines in white and red wines. The study was therefore conducted on different separate strands:

A) Simultaneous determination analysis of amino acids and biogenic amines in wines;

B) Validation of a HPLC method for simultaneous determination of amino acids and amines with precolumn-derivatization with 9-Fluorenyl-methoxycarbonyl chloride (FMOC-Cl) and UV detection using a monolithic column;
C) A survey of amino acids and biogenic amines in wines produced in the Emilia Romagna region;
D) Determination of biogenic amines in red wines: influence of enological practices and composition of wine;
E) Evaluation of different clean-up and analytical methods for the determination of OTA.

1 Biogenic amines

1.1 Definition

Biogenic amines are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones (Askar A. and Treptow H., 1986; Maijala R.L. *et al*., 1993; Silla Santos M.H., 1996). They are organic bases with low molecular weight and are synthesized by microbial, vegetable and animal metabolisms (Brink B. *et al.*, 1990). Biogenic amines in food and beverages are formed by enzymes of raw material or are generated by microbial decarboxylation of amino acids (Brink B. *et al.*,1990; Halasz A. *et al*., 1994) but it has been found that some of the aliphatic amines can be formed "in vivo" by amination from corresponding aldehydes (Maijala R.L., 1993).

The chemical structure of biogenic amines can either be:

- aliphatic (putrescine, cadaverine, spermine, spermidine);
- aromatic (tyramine, phenylethylamine);
- heterocyclic (histamine, tryptamine) (Silla Santos M.H., 1996).

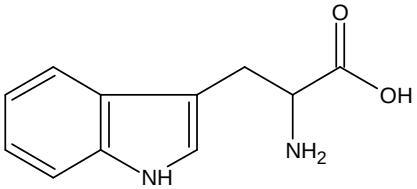
Amines such as polyamines, putrescine, spermidine, spermine and also cadaverine are indispensable components of living cells and they are important in the regulation of nucleic acid fraction and protein synthesis and probably also in the stabilization of membranes (Bardocz S. *et al.*, 1993; Maijala R.L. *et al*., 1993; Halasz A. *et al*., 1994; Silla Santos M.H., 1996).

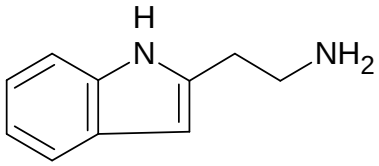
1.2 Mechanism of amines formation

Amine build-up usually results from decarboxylation of free amino acid by enzymes of bacterial origin. Amino acid decarboxylation takes place by removal of the α-carboxyl group to give the corresponding amine. Arginine is easily converted to agmatine, or as result of bacterial activity can be degraded to ornithine from which putrescine is formed by decarboxylation. Lysine can be converted by bacterial action into cadaverine. Histidine can, under certain conditions, be decarboxylated to histamine. Tyramine, tryptamine and β-phenylethylamine come by the same manner from tyrosine, tryptophan and phenylalanine, respectively.

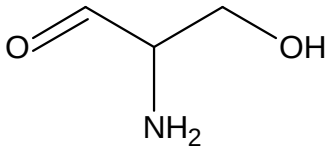
Proteolysis, either autolytic or bacterial, may play a significant role in the release of free amino acids from tissue proteins which offer a substrate for decarboxylases reactions (Shalaby A.R., 1996).

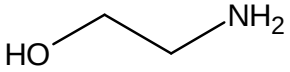
The precursors of the main biogenic amines are:



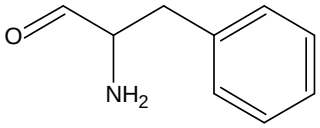


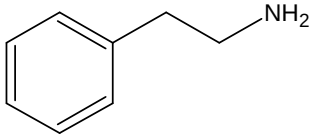
tryptophan → tryptamine



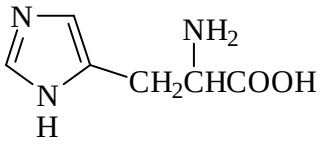


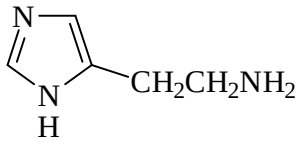
serine → ethanolamine



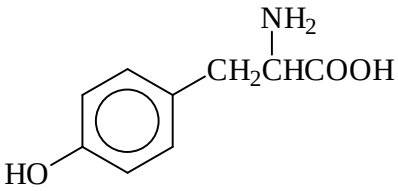


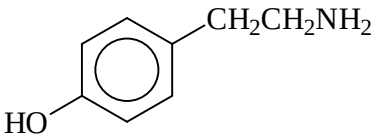
phenylalanine → phenyl ethylamine



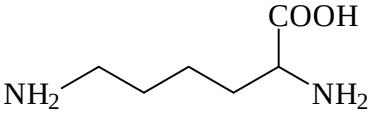


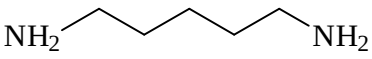
histidine → histamine



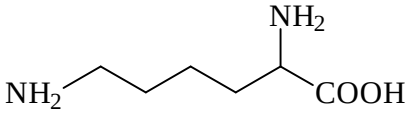


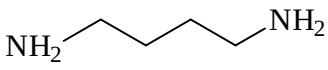
tyrosine → tyramine



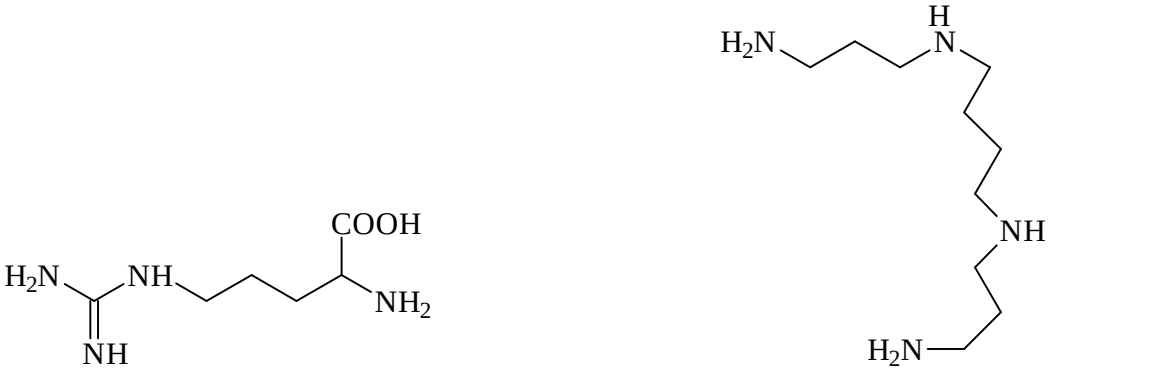


lysine → cadaverine

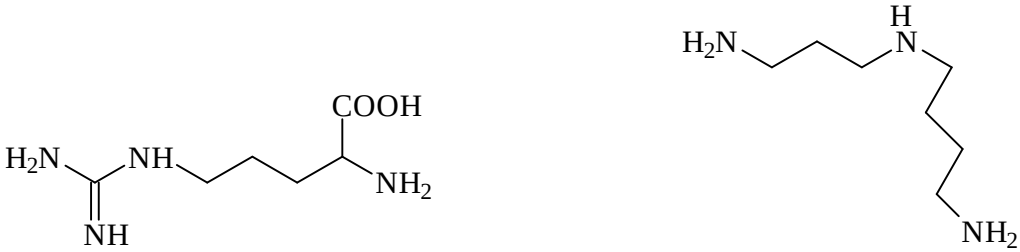




ornithine → putrescine



arginine → spermine



arginine → spermidine

arginine → agmatine

Prerequisites for biogenic amine formation by microorganisms are:

1) availability of free amino acids (Joosten H.M.L.J., 1988; Marklinder I. and Lonner C., 1992; Soufleros E. *et al*., 1998);

2) presence of decarboxylase-positive microorganisms (Tiecco T. *et al*., 1986; Brink B. *et al.*, 1990; Huis in't Veld J.H.J. *et al*., 1990);

3) conditions that allow bacterial growth, decarboxylase synthesis and decarboxylase activity (Brink B. *et al.*, 1990; Silla Santos M.H., 1996; Coton E. *et al.*, 1998; Gardini F. *et al.*, 2005).

1.3 Biogenic amines in food

In virtually, all food that contain proteins or free amino acids are subject to conditions enabling microbial or biochemical activity; biogenic amines can be expected. The total amount of the

different amines formed strongly depends on the nature of the food and the microorganisms present (Brink B. *et al*., 1990).

Biogenic amines are present in a wide range of food products including fish products, meat products, dairy products, wine, beer, vegetables, fruits, nuts and chocolate (Askar A. and Treptow H., 1986; Brink B. *et al.*, 1990; Halasz A. *et al.*, 1994; Silla Santos M.H., 1996; Shalaby A.R., 1996; Soufleros E. *et al*., 1998).

In non-fermented foods, the presence of biogenic amines above a certain level is considered as indicative of undesired microbial activity, therefore, the amine level could be used as an indicator of microbial spoilage. However, the presence of biogenic amines in food does not necessary correlate with the growth of spoilage organisms, because they are not all decarboxylase-positive (Santos C. *et al.*, 1985; Vidal-Carou M.C. *et al.*, 1990a; Silla Santos M.H., 1996).

Levels of histamine, putrescine and cadaverine usually increase during spoilage of fish and meat whereas levels of spermine and spermidine decrease during this process (Brink B. *et al*., 1990).

During the preparation of fermented food, the presence of many kinds of microorganisms can be expected, some of them are capable of producing biogenic amines. Most products, in which lactic bacteria grow, contain considerable amounts of putrescine, cadaverine, histamine, and tyramine (Brink B. *et al.*, 1990).

FISH. Scombroid fish have most commonly been associated with incidents of histamine intoxication (scombrotoxicosis). The formation of histamine in scombroid and other marine fish containing abundant endogenous histidine has been attributed to microbial action rather than to endogenous histidine decardoxylase activity (Baranowski J.D. *et al.*, 1985; Halasz A. *et al*., 1994). The histidine can be catabolized in two ways in fish muscle. The amino acid deamination to obtain urocanic acid or the histidine decarboxylation to form histamine (Mackie M. and Fernandez J., 1977). The deamination activity is the principal way in normal physiological conditions; decarboxylation activity can be most important in other circumstances, e.g. bacterial contamination (Vidal M.C. and Mariné A., 1984).

Different biogenic amines (histamine, putrescine, cadaverine, tyramine, spermine, spermidine) have been detected in fish such as mackerel, herring, tuna, sardines (Yoshida A. and Nakamura A., 1982; Vidal M.C. and Mariné A., 1984; Baranowski J.D., 1985; Silla Santos M.H., 1996; Merialdi G. *et al*., 2001).

Other amines, such as trimethylamine and dimethylamine are present in fish and fish products at levels determined by the fish freshness (Hotchkiss J.H., 1989; Pfundstein B. *et al*., 1991; Silla Santos M.H., 1996).

FRESH FRUIT, JUICES AND VEGETABLES. Amines were found to be widespread in fruits and vegetables (Lovenberg W., 1973; Shalaby A.R., 1996).

Several juices, nectars and lemonades made from oranges, raspberries, lemons, grapefruits, mandarins, strawberries, currant and grapes contain different biogenic amines in variable concentrations: putrescine is the most important (Maxa E. and Brandes W., 1993).

Halasz A. *et al*. (1994) have reported high amine levels in orange juice (noradrenaline, tryptamine), tomato (tyramine, tryptamine, histamine), banana (tyramine, noradrenaline, tryptamine, serotonin) and plum (tyramine, noradrenaline) and spinach leaves (histamine).

Phenylethylamine is also a natural constituent of cocoa beans and thus occurs in chocolate, chocolate products and confectionery containing chocolate. Some species of mushrooms also contain high levels of phenylethylamine and in white and black pepper and soy sauce high levels of pyrrolidine have been detected (Pfundstein B. *et al.,* 1991).

Fermented vegetables represent another class of food from which biogenic amines have been isolated. The main biogenic amines in sauerkraut are histamine, tyramine, putrescine and cadaverine, while β-phenylethylamine is found only in minor quantities (Taylor S. *et al.,* 1978; Brink B. *et al.*, 1990; Silla Santos M.H., 1996).

MEAT AND FERMENTED MEAT PRODUCT. Meat and meat products have been reported to contain tyramine, cadaverine, putrescine, spermine, and spermidine (Koehler P.E. and Eitenmiller R.R., 1978; Santos Buelga C. *et al*., 1986; Stratton J.E. *et al.*, 1991; Shalaby A.R., 1996).

Maijala R.L. *et al.* (1993) detected increased concentrations of histamine and tyramine during sausage fermentation. Fermentation may be important in the formation of histamine in certain types of sausage. Semi-dry sausages are fermented for short periods often with lactic acid cultures, while dry sausages are allowed to ferment from the action of natural microflora for a long period. During the sausage ripening process, the histamine concentration increases at least 10-fold during the first 3 days of ripening (Silla Santos M.H.,1996; Shalaby A.R., 1996). In general, quite variable quantities of biogenic amines were reported for sausages. The variable concentration could be due to the variation of the ripening process time (Cantoni C. *et al.,* 1974), the variation and difference of decarboxylase activity of the natural microflora responsible for fermentation and the biosynthesis and metabolism of such amines in addition to variations in the manufacturing process, the great variation in the type and quality of the meat used, the proportion of meat content included and the length of maturation (Shalaby A.R., 1996).

CHEESE AND DAIRY PRODUCTS. After fish, cheese is the most commonly implicated food item associated with histamine poisoning and the first reported case occurred in 1967 in Netherlands and involved Gouda cheese (Stratton J.E. *et al.,* 1991). The most important biogenic

amines occurring in cheeses are tyramine, histamine, putrescine, cadaverine, tryptamine and β-phenylethylamine (Sumner S.S. *et al*., 1985; Stratton J.E. *et al*., 1991; Besançon X. *et al*., 1992; Celano G.V. *et al*., 1992; Diaz-Cinco M.E. *et al*., 1992; Moret S. *et al*., 1992; Halasz A. *et al*.,1994*;* Silla Santos M.H., 1996). During cheese ripening, casein is slowly degraded by proteolytic enzymes, leading to an increase of free amino acids content (Joosten H.M.L.G. and Olieman C., 1986), which can be subjected to subsequent breakdown reactions and catalyzed by specific bacterial decarboxylases to give rise to the formation of CO_2 and amines. Therefore, biogenic amines are gradually increased with different levels especially histamine, putrescine and cadaverine by extending cheese ripening time. Processed cheeses which are subjected to high temperature during manufacturing contained appreciable levels of biogenic amines (Shalaby A.R., 1996).

WINE AND BEER. Wine, like other fermented foods, is an ideal substrate for amine production, as its manufacturing process involves not only available free amino acids, but also the possible presence of decarboxylase-positive microorganisms and the environmental conditions that allow the growth of microorganisms, as well as the activity of decarboxylase enzymes.

The formation of biogenic amines has been associated to the alcoholic fermentation (Vidal-Carou M.C. *et al*., 1990b) and/or the malolactic fermentation (Lonvaud-Funel A. and Joyeux A., 1994; Coton E. *et al.*,1998; Lonvaud-Funel A., 2001). Bad sanitary conditions, during the winemaking process, also contributed to an increased formation of biogenic amines (Vidal-Carou M.C. *et al.*, 1991; Zee J.A. *et al.*,1983).

The formation of biogenic amines in wine is dependent upon the presence of certain microorganisms as well as on the content of the precursor amino acids, duration of the initial fermentation phase, period of contact of must with grape skin, levels of sulfure dioxide, pH and duration of wine contact with yeast lees (Vidal-Carou M.C. *et al.*, 1990b; Martelli A. *et al*., 1997). Biogenic amines can also be present in the must itself (Daeschel M.A., 1996).

Many types of biogenic amine have been detected in both white and red wine: tyramine, histamine, tryptamine, monomethylamine, 2-phenethylamine, putrescine, cadaverine, spermidine (Zee J.A. *et al.*, 1983; Lehtonen P. *et al*., 1992; Bauza T. *et al*., 1995a; Silla Santos M.H., 1996).

In beer the presence of biogenic amines is related to the yeast activity (*Saccharomyces cerevisiae, Saccharomyces carlsbergensis)* or to microbiological contamination (*Lactobacillus, Pediococcus).* Formation of some amines in beer has been related to unsanitary conditions during production and to raw material quality, because of hop (*Humulus lupulus*) is a natural source of histamine (Martelli A. *et al.*,1997).

Halasz A. *et al.* (1994) investigated the influence of technological conditions of beer production on biogenic amine formation. Barley variety, malting technology, wort processing and fermentation conditions seem to affect the total biogenic amines content of beer.
The amount of biogenic amines in beer and wine is considerably lower than in other fermented foods such as cheese (Martelli A. *et al.*,1997; Soufleros E. *et al.,* 1998).

1.4 Biogenic amines in wine and factors that influence their presence

The presence of amines in musts and wines is well documented in the literature (Ough C.S. *et al.*, 1981; Zee J.A. *et al.*, 1983; Radler F. and Fath K.P., 1991; Mafra I. *et al.*, 1999). However, the processes that generate these amines, together with the factors that influence their quantitative and qualitative presence are in some cases not well defined yet (Radler F. and Fath K.P., 1991; Herbert P. *et al.,* 2005; Guerrini S. *et al*., 2005).
They may be three possible origins of biogenic amines in wines (Lafon-Lafourcade S. and Joyeux A., 1976):
a) some amines are already present in the must, namely histamine and tyramine (Mayer K. and Pause G., 1973; Buteau C. *et al.*, 1984; Vidal-Carou M.C. *et al.*, 1990b);
b) they are formed by yeast during alcoholic fermentation (Buteau C. *et al.*,1984; Vidal-Carou M.C. and Marinè-Font A., 1985) and/or they are formed by the action of bacteria involved in the malolactic fermentation (Aerny J., 1985; Bertrand A. *et al.,* 1989; Vidal-Carou M.C. *et al.*, 1990c; Bauza T. *et al*., 1995b).
Furthermore, Buteau C. *et al.* (1984); Vidal-Carou M.C. *et al.* (1991); Lehtonen P. (1996); Bravo-Abad F. (1996) indicated the possibility that biogenic amines are formed in wine by the action of contaminant microorganism such as the enteric bacteria *Klebsiella* and *Proteus.* Cerutti G. and Remondi L. (1972) indicated out that "a wine produced in optimal conditions, from a hygienic point of view, should be nearly free of amines". Likewise Zappavigna R. *et al.* (1974) pointed out that technological conditions of the winemaking process and the quality of raw materials employed have a definite influence on the intensity of amine biogenesis. On the basis, histamine has been proposed as an indicator of defective manufacturing (Battaglia R. and Frolich P., 1978) or as a quality parameter of wines (Coppini D. *et al.,* 1973; Inigo B. and Bravo F., 1980).
The amines more commonly found in wine are histamine, tyramine, putrescine, cadaverine (Vidal-Carou M.C. *et al.,* 1990b; Lehtonen P., 1996; Soufleros E. *et al.,* 1998; Vazquez-Lasa M.B. *et al.,* 1998; Coton E. *et al.,*1999; Lonvaud-Funel A., 2001).

It is generally accepted that red wine contain higher concentrations of biogenic amines than white wines (Lafon-Lafourcade S. and Joyeux A., 1976; Zee J.A. *et al.*, 1983; Cilliers J.D. and Van Wyk C.L., 1985; Cerutti G. *et al.*, 1986; Vidal-Carou M.C. *et al.*, 1990c; Bauza T. *et al.*, 1995b; Kallay M. and Body-Szalkai M., 1996; Vazquez-Lasa M.B. *et al.*, 1998; Gerbaux V. and Monamy C., 2000; Arena M.E. and Manca de Nadra M.C., 2001; Galgano F. *et al.*, 2003; Leitao M.C. *et al.*, 2005; Bover-Cid S. *et al.*, 2006). It can be explained by the difference in the vinification techniques.

The variability in biogenic amines contents of wine could be explained on the basis of differences in:

- type of soil (Baucom T.L. *et al.*, 1986);
- variety (Zee J. *et al.*, 1983; Beatriz M.A.G. *et al.*, 1998; Nicolini G. *et al.*, 2003; Bertoldi D. *et al.*, 2004; Landete J.M. *et al.*, 2005a) and degree of maturation of the grape (Ough C.S., 1971; Herbert P. *et al.*, 2005);
- raw material quality (Karmas E., 1981; Onal A., 2007);
- precursor free amino acids (Cerutti G. *et al.*, 1978; Soufleros E. *et al.*, 1998);
- contact time of must and grape skin (Ough C.S., 1971; Inigo B. and Bravo F., 1980; Guitart A. *et al.*, 1997; Martin-Alvarez P.J. *et al.*, 2006);
- action of yeast in alcoholic fermentation (Vidal-Carou M.C. *et al.*, 1990b; Torrea Goni D. and Ancin Azpilicueta C., 2001; Caruso M. *et al.*, 2002; Torrea Goni D. and Ancin Azpilicueta C., 2002; Valero E. *et al.*, 2003);
- alcohol content (Landete J.M. *et al.*, 2004; Vidal-Carou M.C. *et al.*, 1990b);
- sulfur dioxide concentration (Rivas-Gonzalo J.C. *et al.*, 1983; Vidal-Carou M.C. *et al.*, 1990b; Ferrer S. and Pardo I., 2005);
- added nutrients (Gloria M.B.A. *et al.*, 1998);
- pH (Cerutti G. and Remondi L., 1972; Aerny J., 1990; Vidal-Carou M.C. *et al.*, 1990b; Lonvaud-Funel A., 2001; Landete J.M. *et al.*, 2004; Landete J.M. *et al.*, 2005a; Ferrer S. and Pardo I., 2005);
- quantity and type of fining agents (Jakob L., 1968; Spettoli P., 1971; Kallay M. and Body-Szalkai B., 1996; Eder R. *et al.*, 2002);
- duration of wine contact with yeast lees and marcs (Bauza T. *et al.*, 1995a; Coton E. *et al.*, 1999; Lonvaud-Funel A., 2001);

- action of lactic acid bacteria in the malolactic fermentation (Delfini C., 1989; Vidal-Carou M.C. *et al.,* 1990b; Lonvaud-Funel A. and Joyeux A., 1994; Coton E. *et al.,*1998; Lonvaud-Funel A., 2001; Guerrini S. *et al.,* 2002; Landete J.M. *et al.,* 2005a; Palacios A. *et al.,* 2005);
- time and storage conditions (Gonzalez J.M. *et al.*, 1977; Ough C.S. *et al.,* 1981; Gonzalez A. and Ancin Azpilicueta C., 2006; Landete J.M. *et al.,* 2005a);
- possible microbial contamination during winemaking (Zee J.A. *et al.,* 1983; Vidal-Carou M.C. *et al.,* 1991).

Some of these factors increase the concentration of precursor amino acids of biogenic amines in the medium, while other favor the development of microorganisms with the ability to form amines (Torrea Goni D. and Ancin Azpilicueta C., 2001).

1.5 Influence of winemaking process on the production of biogenic amines

Some amines, such putrescine, may already be present in grapes (Brodequis M. *et al.,* 1989) whereas other can be formed and accumulated during winemaking (Rivas-Gonzalo J.C. *et al.,*1983; Vidal-Carou M.C. *et al.,* 1990b; Bauza T. *et al.,* 1995a; Martin-Alvarez P.J. *et al.,* 2006).

The main factor affecting its formation during vinification are free amino acid concentrations and the presence of microorganisms able to decarboxylate these amino acids.

1.5.1 TYPE OF SOIL AND GRAPES HEALTH

The composition and type of soil can influence the content of biogenic amines. Di- and polyamine metabolism is dependent on external conditions and a major shift in nitrogen and amine metabolism can occur when plants are starved of nutrients, or exposed to osmotic shock or atmospheric pollution (Bouchereau A. *et al.,* 1999).

Some Authors (Broquedis M. *et al.*, 1989; Adams D.O. *et al.,* 1990; Vaz de Arruda Silveira R.L. *et al.,* 2001) observed that potassium deficiency increase the levels of putrescine in grapevine leaves. This amine could be accumulated in the grapes, and, as a result, remain in the wine. Viticulture practices that do not prevent potassium deficiency may contribute to increase the content of putrescine in wine (Leitao M.C. *et al.,* 2005).

In the grapevine, biotic stress, such as B*otrytis cinerea*, can also alter the composition of grape berries, increasing amines content (Hajos G. *et al*., 2000). *Botrytis cinerea* metabolism induces a decrease in the water content of the grapes, while the content of sugar, amino acids, biogenic amines (polyamine) increases and the taste improves (Sass-Kiss A. *et al.,* 2000).

1.5.2 VARIETY AND DEGREE OF GRAPES RIPENING

It was noticed that grape variety (Soleas G.J. *et al.*, 1999; Hernandez-Orte P. *et al*., 2006; Soufleros E.H. *et al.,* 2007), region of production and vintage can influence free amino acids and amines content of musts and wines (Zee J. *et al.,* 1983; Beatriz M.A.G. *et al.,* 1998; Nicolini G. *et al.,* 2003; Bertoldi D. *et al*., 2004; Landete J.M. *et al.*, 2005b; Herbert P. *et al*., 2005).

The different degree of grape maturity and the duration of maceration may also influence the nitrogenous components in the raw materials used for fermentation (Ough C.S., 1971; Herbert P. *et al*., 2005).

During maturation, amino acids content increases from 2 to 5 times and it becomes the main type of nitrogen (50-90 % of total nitrogen). It decreases, during over ripening and in rotted berries, in dry conditions (Ribéreau-Gayon P. *et al.,* 1998b).

1.5.3 DURATION OF SKIN MACERATION

During alcoholic fermentation, the duration of skin maceration is the first factor that affect extraction of some compounds present in the skin, such as phenolic compounds, which are responsible for wine color. However, maceration affects the extraction not only of phenolics but also of other grape components, such as proteins, polysaccharides and also amino acids, which are the precursors of biogenic amines (Ribéreau-Gayon P., 1998a). For this reason, it is important to have a thorough knowledge of influence of the duration of maceration on the accumulation of biogenic amines in wines (Martin-Alvarez P.J. *et al.,* 2006). These results obtained are in agreement with previous studies that showed that longest maceration times could favor a greater production of biogenic amines (Bauza T. *et al*., 1995b; Guitart A. *et al.,* 1997).

1.5.4 USE OF PECTOLYTIC ENZYMES

Commercial pectolytic enzymes are used in winemaking to increase juice yields, facilitate pressing and filtering, to provide a great clarity to must and wines, to have a good extraction of phenols and aromas. However, mainly depending on the composition of these commercial preparations, they may also produce concomitant effects, such as an important proteolytic activity, which can lead to hydrolysis of proteins and peptides and release of amino acids (Ferrer S. and Pardo I., 2005). Martin-Alvarez P.J. *et al.* (2006) reported that there was little change in biogenic amine composition as a consequence of the use of commercial pectolytic enzymes. Only the mean concentration of phenylethylamine and cadaverine were affected by the use of these enzymes. However, it is interesting to note that the mean values of these amines were lower in the wines with supplements of pectinases compared with the wines manufactured without enzymes, showing that, apparently, use of these enzymes does not favor accumulation of amines in wines.

1.5.5 CONTACT WITH YEAST LEES

An extended contact with lees of yeast leads to higher amine content, than a short contact (Cerutti G. *et al*., 1987). If wines are maintained in contact with yeast lees, lactic acid bacteria find more peptides and free amino acids to hydrolyze and decarboxylate. This explains the higher level of amines in some wines which are produced with an extended lees contact (Coton E. *et al.,* 1999; Lonvaud-Funel A., 2001).

Martin-Alvarez P.J. *et al.* (2006) compared wines aged or not with lees and they found that the mean concentration of methylamine and putrescine were higher in wines aged on yeast lees. This was probably because through contact of wine with lees, the proteins are initially hydrolyzed to peptides of different molecular weight and these peptides are later degraded further to amino acids and amines as consequence of yeast and bacteria lysis. These results agree in part with those of Bauza T. *et al.* (1995a), who also found a higher production of tyramine and putrescine in wines added with lees and inoculated with lactic bacteria.

1.5.6 MALOLACTIC FERMENTATION

Malolactic fermentation (MLF) is an important biological process in winemaking because it reduces wine acidity and, if carried out by proper strains of lactic acid bacteria (LAB), it improves the flavor and the microbial stability during the wine aging (Davis C.R. *et al*., 1985). MLF is therefore considered essential for most red and some white wines. *Oenococcus oeni*, due to its acid tolerance, is the most frequent bacterial species occurring in wine having spontaneous MLF and thus it is also the preferred bacterium used as starter culture in induced MLF. However, *O. oeni* has recently been found capable of producing a wide range of biogenic amines (Lonvaud-Funel A., 2001; Guerrini S. *et al.,* 2002).

1.5.7 LACK OF HYGIENE

Formation of some amines in wines has been related to unsanitary conditions during the winemaking process (Vidal-Carou M.C. *et al.,* 1991).

Bacterial contamination in wineries is an important factor (Marquardt P. *et al.*,1963; Coton E. *et al.,* 1999), especially during the malolactic fermentation, where efficient control is necessary to avoid amine formations; all cleaning and disinfection methods are important. Addition of sulfur dioxide in wines inhibits microorganism and directly influences amine contents (Zee J.A. *et al.*, 1983).

In wines, high levels of histamine have been related to spoilage bacteria mainly *Pediococcus spp.* (Aerny J., 1985; Delfini C., 1989). *Pediococcus* can be present in wine, but usually in a few number of cells.

1.5.8 WOOD AGING

Wood aging can effect biogenic amines contents of wines because it could increase the microbial contamination risk (Bauza T. *et al*., 1995a; Bauza T. *et al.*, 1995b; Gerbaux V. and Monamy C., 2000; Woller R., 2005).

An investigation on the evolution of amines in red wines during aging in American oak barrels (Q*uercus alba)* and French oak barrels (*Quercus sessilis)* from the Allier and Nevers regions was carried out by Jimenez Moreno N. *et al*. (2003). The results showed that the evolution of amines were similar in all three types of oak woods. Histamine and tyramine were produced at the beginning of the aging process, although they were not accumulated in the wines, probably due to their degradation. The production of histamine during the first months of aging was probably due to the proliferation of microorganism with decarboxylase activity and could be favored by the release of amino acids at the end of fermentation because of yeast autolysis and alteration of yeast plasma membrane. Putrescine was the most abundant amine in the wines; its concentration increased to an important extent during aging as it did not undergo degradation. The concentration of cadaverine increased slightly at the first stage of aging and, like putrescine, did not degrade at all.

1.6 Reduction of biogenic amines content in wine

Clarification is the best oenological treatment to reduce the biogenic amines content in wine. Clarification can be carried out by physical methods (sedimentation, flotation, centrifugation and filtration) or by fining agents addition (gelatine, albumin, casein) or by pectolytic enzymes addition (Ribéreau-Gayon P. *et al*., 1998b).

Research carried out by Enartis (www.enartis.it) on bentonite, pvpp, silica sol, active charcoal, tannin and albumin showed that only bentonite was effective in reducing the amines content. A decrease in the amines content was directly related to the amount of bentonite (www.enartis.it; Mannino M. *et al*., 2006).

Kally M. e Body-Szalkai M. (1996) observed that, in red wines, 80 g/hL of bentonite reduced histamine content by 60 % and more with a higher amount of bentonite. The wine color must be considered, because bentonite reduce it.

Other Authors put in evidence the efficiency of bentonite in amines content decreasing (Jakob L., 1968; Spettoli P., 1971; Mannino M. *et al.*, 2006).

Mannino M. *et al*. (2006) carried out trials on different fining agents (bentonite, tannin and gelatin) to verify the possibility to reduce the amount of bentonite in amines removing. They found that the addition of tannin before bentonite addition is useful to reduce amines content of

2-6 time. So, it is possible to decrease the amount of bentonite added to wine and to preserve wine sensory characteristics.

The clarification induces a loss of nitrogen compounds. The use of silica sol induces a good decrease of nitrogen compounds, while pectolytic enzymes improves the amino acids content n relation to their activity on proteins and peptides (Guitart A. *et al.*, 1998).

The clarification carried out with physical treatments not always induces a decrease in amino acids amount.

1.7 Microorganism producing biogenic amines in wine

Usually amines production results from the presence of bacteria that are capable of decarboxylating amino acids (Gale E.F., 1946).

Musts and wines are very selective media, which can support growth of only few species of lactic acid bacteria (LAB). Four genera are represented: *Lactobacillus, Pediococcus, Leuconostoc* and *Oenococcus*. During alcoholic fermentation, the LAB population is mainly composed of *Pediococcus* along with *Oenococcus oeni*. The homofermentative lactobacilli, the major type present on grapes, disappear quickly after the start of alcoholic fermentation in favor of *Leuconostoc mesenteroides* which, at the end of the fermentation, is replaced by *O. oeni* (Moreno-Arribas M.V. *et al*., 2003).

Among lactic acid bacteria, *O. oeni* is the main species present in wine and the best adapted to carry out the malolactic fermentation at the low pH of wine (Wibowo D. *et al*., 1985). If biogenic amines formation is associated to MLF, it would be expected that *O. oeni* has the enzymes for breakdown of peptides and decarboxylation of amino acids present in wine in this stage (Leitao M.C. *et al.*, 2000).

1.8 Factors affecting activity of lactic acid bacteria in wine

Sulphur dioxide. The antimicrobial activity of SO_2 is based on their ability to pass across cell membrane.

The few data in literature regarding the relationship between this antiseptic and biogenic amines accumulation in wine generally report that SO_2 added at high levels decreases biogenic amines formation (Rivas-Gonzalo J.C. *et al.*, 1983; Vidal-Carou M.C. *et al*., 1990c) since it inhibits the development of microorganisms with amino acid decarboxylase activity (Palacios A. *et al.*, 2005). In fact, Bauza T. *et al.* (1995a) demonstrated that, in the presence of 100 mg/L and more of this antiseptic, the accumulation of biogenic amines in wine was greatly reduced. Red wines with about 40 mg/L of SO_2 contained more tyramine (8 mg/L) than histamine (2 mg/L), while the

ratio changed (4 and 7 mg/L respectively) if the sulfitation reached 85 mg/L (Vidal-Carou M.C. *et al.*, 1990b).

The SO_2 effect on biogenic amines accumulation depend also on other variables. In fact, at the higher pH values an increase of SO_2 caused a diminution of tyramine concentration. An opposite effect was observed when pH decreased (Gardini F. *et al*., 2005).

A sulfitation after racking must be avoid, if the malolactic fermentation is requested (Aerny J., 1985; Bauza T. *et al.*, 1995b). Sulphur dioxide delays the start of MLF because *Leuconostoc oenos* is negatively influenced. In these conditions P*ediococcus* strains, producer of biogenic amines, is favored (Aerny J., 1990; Ingargiola M.C. and Bertrand A., 1992). So, the use of sulphur dioxide must be delayed after MLF.

After malolactic fermentation, wine is sulfited in order to eliminate yeast and bacteria which are no more desirable. This would normally prevent any changes in composition due to microorganisms. However, several compounds change in level and this is the case of biogenic amines. In Burgundy wines, histamine, tyramine and putrescine showed an increase in Chardonnay and Pinot noir during malolactic fermentation, and also during aging (Gerbaux V. and Monamy C., 2000). Histamine and tyramine content continuously increased. In their study, Gerbaux V. and Monamy C. showed that a more active phase was between the fourth and eighth month after malolactic fermentation. It was obvious that sulphur dioxide did not completely stop all the biochemical reactions triggered by bacteria. Due to high pH, a situation which is becoming more and more frequent, SO_2 is less active and it is accentuated in red wines due to its combinations to polyphenols (Lonvaud-Funel A., 2001).

The use of lysozyme is suggested to replace sulphur dioxide in lactic bacteria control. Lysozyme is more specific than SO_2 in lactic bacteria control and its activity improves when the pH rises (Gerbaux V. and Monamy C., 2000). An addition of 125 a 250 mg/L of lysozyme before MLF stops the increase of biogenic amines.

pH. It is the most important factor determining not only the biological activity of bacteria in wine but also their variety (Lonvaud-Funel A. and Joyeux A., 1994; Gerbaux V. and Monamy C., 2000; Landete J.M. *et al.*, 2005b). The higher the pH, the more complex the bacterial microflora, because pH acts as a selective factor of microorganisms in wine (Lonvaud-Funel A., 2001). At high pH, biogenic amines are always produced in high amounts (Lonvaud-Funel A. and Joyeux A., 1994). This is a consequence of an easier total growth and of the greater bacterial diversity. White wines, which are generally rich in acidic compounds, contain lower biogenic amine concentrations than red wines (Gerbaux V. and Monany C., 2000).

Ethanol. Lactic bacteria are sensitive to ethanol by 8-10 %. Cocci are more sensitive than bacilli.

The influence of ethanol is related to pH and SO_2. In wines with high pH and low amounts of SO_2, MLF can occur also if the ethanol content is high (Schieri G., 1991).

Temperature. Lactic bacteria can normally growth in a range of 10-30 °C, out of this range their metabolism is reduced or stopped. The optimum temperature is 20-25 °C for *Leuconostoc* and 25-30 °C for *Lactobacillus*. At 35 °C the growth of lactic bacteria can be stopped (Schieri G., 1991).

Temperature is influenced by ethanol; if the alcohol is 13-14 %, the optimum temperature decreases (Ribéreau-Gayon P. *et al*., 1998a).

If MLF starts, lactic bacteria can complete it also if temperature falls down (Ribéreau-Gayon P. *et al*., 1998a).

The influence of temperature on lactic bacteria growth is also relate to pH and SO_2. A good level of one of this parameters can positively influence the bacteria metabolism, also if the other parameters are bad (Palacios A. *et al.,* 2005). For example, if the pH is good, lactic bacteria can growth better than in a wine with a low pH, also with high content of ethanol and SO_2 (Ribéreau-Gayon P. *et al*., 1998b).

Nutrition. The amount of biogenic amines is strictly related to the presence of nutrients in wine (Lonvaud-Funel A. and Joyeux A., 1994; Palacios A. *et al.,* 2005).

The lactic bacteria needs of organic compounds: sugars, amino acids and organic acids.

Sugars are the best nutrient for lactic bacteria because they provide energy stored in ATP molecules. Also citric acid and arginine provide energy to lactic bacteria. Malolactic fermentation and histidine decarboxylation are useful to conserve energy (Ribéreau-Gayon P. *et al.,* 1998a).

Lactic bacteria are not able to synthesize amino acids on the contrary of yeasts (Schieri G., 1991). Amino acids must be present in wine to have lactic bacteria growth (Coton E. *et al.,* 1999). The different strains have different needs: cocci are more exigent than bacilli. Normally, alanine, arginine, cystine, glutamine, histidine, leucine, phenylalanine, serine, tryptamine, tyramine and valine are necessary together or in part. Amino acids are usually used to synthesize new proteins or to provide energy (arginine and histidine) (Ribéreau-Gayon P. *et al.,* 1998a).

After alcoholic fermentation yeast lees undergo proteolysis and release amino acids and peptides in the medium. This release is useful for the following activity of lactic bacteria.

Other factors can influence lactic bacteria growth:

Osmotic pressure. Most bacteria cannot survive at high osmotic pressure or at low water activity. With sugar concentration of 40 % or more lactic bacteria cannot growth, even if they can survive.

O**xygen**. Lactic acid bacteria derive benefit from the increase of the oxidoreduction potential of wine in order to multiply or at least to improve their existence temporarily (Millet V. *et al.*, 1995).

1.9 Toxicological effect

Biogenic amines, such as tyramine and β– phenylethylamine, have been proposed as the starters of hypertensive crisis in certain patients and dietary-induced migraine. Another amine, histamine, has been implicated as the causive agent in several outbreaks of food poisoning. Histamine intake ranged within 8-40 mg, 40-100 mg and higher than 100 mg may cause slight, intermediate and intensive poisoning, respectively (Parente E. *et al.*, 2001). Nout M.J.R (1994) pointed out that the maximum daily intake of histamine and tyramine should be in the range of 50-100 mg/kg and 100-800 mg/kg, respectively; over 1080 mg/kg tyramine becomes toxic. Putrescine, spermine, spermidine and cadaverine have not adverse health effect, but they may react with nitrite to form carcinogenic nitrosoamines and also can be proposed as indicators of spoilage (Hernandez-Jover T. *et al.*, 1997). Tryptamine can induce blood pressure increase, therefore causes hypertension, however there is no regulation on the maximum amount of tryptamine consummation in sausage in some countries (Shalaby A.R., 1996).

Food poisoning may occur especially in conjunction with potentiating factors such as monoamine oxidase inhibiting (MAOI) drugs, alcohol, gastrointestinal diseases and other food containing amines. Histaminic intoxication, hypertensive crisis due to interaction between food and MAOI anti-depressants and food-induced migraines are the most common reactions associated with the consumption of food containing large amounts of biogenic amines (Marine-Font A. *et al*., 1995). The diamines (putrescine and cadaverine) and the polyamines (spermine and spermidine) favor the intestinal absorption and decrease the catabolism of the above amines, thus, potentiating their toxicity (Bardocz S., 1995). Formation of nitrosoamines, which are potential carcinogens, constitutes an additional toxicological risk associated to biogenic amines, especially in meat products that contain nitrite and nitrate salts as curing agents (Scanlan R.A., 1983).

Determination of the exact toxicity threshold of biogenic amines in individuals is extremely difficult, since the toxic dose is strongly dependent on the efficiency of the detoxification mechanisms of each individual (Halasz A. *et al*., 1994). Normally, during the food intake process in the human gut, low amounts of biogenic amines are metabolized to physiologically less active degradation products. This detoxification system includes specific enzymes such as diamine oxidase (DAO). However, upon intake of high loads of biogenic amines in foods, the detoxification system is unable to eliminate these biogenic amines sufficiently. Moreover, in the

case of insufficient DAO activity, caused for example by generic predisposition, gastrointestinal disease or inhibition of DAO activity due to secondary effects of medicines or alcohol, even low amounts of biogenic amines cannot be metabolized efficiently (Bodmer S. *et al.*, 1999). Some biogenic amines, e.g., histamine and tyramine, are considered as antinutritional compounds. For sensitive individuals they represent a health risk, especially when their effects is potentiated by other substances. Poisoning by histamine with its allergy-like symptoms is usually related to the consumption of scombroid fish such as tuna or mackerel (Veciana Nogue M.T. *et al.,* 1997) and is considered to be one of the commonest forms of food intoxication reported.

1.10 Analytical methods

There are two reasons for determination of amines in food: the first is their potential toxicity; the second is the possibility of using them as food quality markers.

Analytical determination of biogenic amines is not simple because of their structure and because they are usually present at low levels in a complex matrix.

Biogenic amines in food have been determined in different ways, including thin-layer chromatography (TLC), gas chromatography, capillary electrophoretic method (CE) and high performance liquid chromatography (HPLC) (Busto O. *et al*., 1996). All the analytical techniques mentioned have an associated pre- or post- chromatographic treatment to improve the selectivity and sensitivity of different methods. However, it is not always necessary to resort to these treatments because the sample can be directly injected as long as there is a suitable detection system, both in GC and HPLC (Seiler N., 1977).

Many analytical methods have been proposed for the analysis of amino acids and biogenic amines. The most useful RP-HPLC method include precolumn derivatization. Since the aliphatic biogenic amines do not have chromophore groups which give pronounced absorption in the UV-Vis region, derivatization techniques have to be resorted in order to increase the sensitivity of the detection (Seiler S. and Demisch L., 1978).

Obtaining fluorescent derivates is the most widely used method since it can determine low concentration of amines with great sensitivity and selectivity.

Typical reagents for precolumn derivatization are phenylisothiocyanate (PITC) (Bidlingmeyer B.A. *et al*., 1987); o-phthalaldehyde (OPA) (Jones B.N. and Gilligan J.P., 1983); 9-fluorenyl-methyl-chloroformate (FMOC-Cl) (Einarsson S. *et al.,* 1983); 1-fluoro-2,4-dinitrobenzene (FDNB) (Morton R.C. and Gerber G.E., 1988); 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (DNPAA) (Kochhar S. and Christen P., 1989); and dansyl-chloride (Thio A.P. and Tompkins D.H., 1989; Sarwar G. and Botting H.G.,1993; Romero R. *et al.,* 2000).

Each of these reagents have particular advantages and limitations.

Phenylisothiocyanate (PITC) reacts with both primary and secondary amino acids to yield stable phenylthiocarbamoyl derivatives, which can be detected via using UV absorption. The main drawback of this ligand is its low sensitivity (Bidlingmeyer B.A. *et al.*, 1984)

o-Phthalaldehyde (OPA) is itself nonfluorescent, but it reacts rapidly with primary amino acids at room temperature to form highly fluorescent isoindoles. The disadvantages of this method are the poor reactivity of OPA with secondary amino acids and the low stability of the reaction product (Lindroth P. and Mopper K., 1970; Roth M., 1971; Chen R.F. *et al.*, 1979; Furst P. *et al.*,1990)

1-Dimethylaminonaphthalene-5-sulfonyl (dansyl) chloride forms fluorescent adducts with amino acids band primary and secondary amines but lacks selectivity (it reacts with both -OH and -NH_2 groups) and requires rather long reaction times and high reaction temperatures (Tapuki Y. *et al.*, 1981; Marquez F.J. *et al*., 1986)

In contrast 9-fluorenyl-methoxycarbonyl chloride (FMOC-Cl) reacts rapidly with amino acids to form highly fluorescent and stable adducts (Haynes P.A. *et al.*, 1991; Ou K. *et al.*, 1996). The advantages of using FMOC-Cl are the reaction is straightforward, rapid and can be performed at ambient temperature; the reaction products are stable at room temperature and can react with both primary and secondary amines. The major disadvantage of FMOC-Cl is its reactivity towards water; after hydrolysis and decarboxylation, the fluorescent alcohol, FMOC-OH, elutes in the middle of the chromatogram. At high concentrations, FMOC-OH overlaps with other amino acids in the chromatogram, complicating the quantification of these amino acids. In as much as FMOC-Cl is also fluorescent, excess reagent should be removed before chromatography, by extraction or by addition of a second reagent (1-aminoadamantane, ADAM) ADAM is a hydrophobic amine that reacts with FMOC-Cl in excess only to form a complex (FMOC-ADAM), thus allowing for the reduction of the chromatographic interference of FMOC-OH formed in alkaline medium (Einarsson S. *et al*., 1983; Gustavsson B. and Betnér I., 1990; Chan E.C.Y. *et al.*, 2000).

1.11 Simultaneous determination analysis of amino acids and biogenic amines in wines

1.11.1 Aim of work

The aim of this work was to validate a new HPLC method for the simultaneous determination of amino acids and amines in musts and wines.

The validation of the method was in term of accuracy, precision, limit of detection and quantification.

1.11.2 Experimental

Samples

Four commercial wines from different Italian regions were analyzed. The samples were analyzed as the standard solution. The identification of the chromatographic peaks was confirmed using spiked samples.

Apparatus

An LC-1500 HPLC system (Jasco, Tokyo, Japan) was equipped with an MD-1510 diode-array detector set at 263 nm (λmax). Data were acquired and processed using Borwin-PDA Version 1,50 software (JMBS Developments, Grenoble, France). Samples were injected with a 20 µL loop using a 7125 valve (Rheodyne, Cotati, CA) onto a Luna RP-18 column (150 × 4 mm, 3 µm i.d.) protected with a guard column of the same material (Phenomenex, Torrance, USA). The column operated at 25 °C (Jones Chromatography, Mid Glamorgan, U.K.) with a flow rate of 0,5 mL/min.

Eluents

The separation was optimized using a mobile phase consisting two eluents.

Eluent A: sodium acetate ($NaCH_3COO$) 50 mM (Carlo Erba, Milan, Italy) in H_2O (Merck, Darmstad, Germany) adjusted to pH = 4,2 with glacial acetic acid (Carlo Erba, Milan, Italy).

Eluent B: CH_3CN (Merck, Darmstad, Germany).

Eluents were filtered through a 0,22 µm nylon membrane filter.

The binary gradient was constructed as described in table 1.

Luna column	
Time (min)	**A (%)**
0	68
3	68
14	59
40	42
45	35
90	0
100	0
105	68
110	68

Table 1 - HPLC elution program for amino acids and biogenic amines analysis with Luna column.

Reagents

- borate buffer: 1,24 g boric acid (H_3BO_3) (Merk, Germany) in 100 mL of water adjusted to pH = 8,25 with concentrated sodium hydroxide (NaOH);
- 9-fluorenyl-methoxycarbonyl chloride (FMOC-Cl) (Sigma-Aldrich, Milan, Italy) 0,015 M in 10 mL of acetonitrile (CH_3CN)(Merck, Darmstad, Germany);
- 1-ammino amantadine (ADAM) (Sigma-Aldrich, Milan, Italy) 0,03 M in 10 mL of water/ acetonitrile (H_2O/CH_3CN)(1:1, v/v);
- chloridric acid (HCl)(Carlo Erba, Milan, Italy).

Standard solution

Standards of 24 amino acids and 7 amines were dissolved in HCl 0,01M, then derivatized and filtered through a 0,45 µm polytetrafluorethylene (PTFE) membrane (Gyrodisc, Orange Scientific, Waterloo, Belgium) prior to HPLC analysis.

Arginine (Arg), Hydroxiproline (HydPro), Asparagine (Asn), Glutamine (Glu), Citrulline (Cit), Serine (Ser), Aspartic acid (Asp), Glutammic acid (Glu), Threonine (Thr), Glycine (Gly), Alanine (Ala), Tyrosine (Tyr), Proline (Pro), Methionine (Met), γ-aminobutyric acid (Gaba), Valine (Val), Phenilalanine (Phe), Triptophan (Trp), Cysteine (Cys), Isoleucine (Ile), Leucine (Leu), Methylamine (Meta), Tyramine (Tyrn), Histidine (His), Lysine (Lys), histamine (Hist) Cystine (Cys-Cys), Putrescine (Put), Cadaverine (Cad), Spermidine (Spermd), Spermine (Sperm) were taken as salts from Sigma-Aldrich, Milan, Italy.

Derivatization procedure

Amino acids and amines were derivatized (FMOC–AA) at room temperature using a precolumn procedure. An aliquot of 300 µL of sample (wine or a standard solution of amino acids and amines) was added to 600 µL of a 200 mM borate buffer (pH 10,0). Then, 600 µL of 15 mM FMOC-Cl (in CH_3CN) was added to the wine and derivatization occurred. After 2 min, the reaction was stopped by the addition of 600 µL of 300 mM ADAM (H_2O/CH_3CN)(1:1, v/v) to form the FMOC–ADAM complex. Then, after 2 minutes, the sample was filtered and analyzed by HPLC.

The derivatization of amino acids with FMOC-Cl requires an alkaline pH (≥ 8,0). Considering wine acidic pH and its natural buffering capacity, a preliminary sample alkalinization with sodium hydroxide (32 %) was studied and used before the derivatization procedure. Best results were achieved by using a 0,2 M borate buffer at pH 10 with a 2:1 buffer–wine ratio. The FMOC-amino derivatives were tested to be stable up to 100 min.

In order to verify the influence of pH and time on derivatization several times (2, 3, 5, 8, 10 and 15 minutes) for the first derivatization and several buffer at different pH (8,00; 8,25 and 8,40) were tested. Other Authors (Melucci D. *et al.*, 1999) verified that pH 8,25 and 2 minutes are the best solution for amino acids derivatization.

The simultaneous derivatization of amino acids and amines moved us to study also amines kinetics and pH of derivatization.

Validation

In order to assess the accuracy of the method, recoveries performances were carried out by spiking each sample with amino acid and amines at three concentration levels (0,04; 0,06; and 0,08 mM). Each of the resulting spiked samples were analyzed in triplicate.

The limits of detection (L.O.D.) were calculated from the amount of amino acids and amines required to give a signal-to-noise ratio of 3 (S/N =3) and the limits of quantification (L.O.Q.) were determined with a signal-to-noise ratio of 10 (S/N = 10).

Linearity was determined with 3 derivatizations of 5 different concentrations of amino acids standard, ranging from 2 to 50 mg/L for each amino acids, ranging from 26 to 655 mg/L for proline and from 0,5 to 11 mg/L for amines.

Precision was determined in terms of peak chromatographic areas and retention times, repeatability on the base of 7 injection of the same standard solution, derivatized on the same condition (pH = 8,25 and derivatization time = 2 min).

1.11.3 Results and discussion

Optimal conditions of derivatization are evaluated. In particular, pH and time needed for a full and repeatable derivatization of each compounds was evaluated.

In table 2 can be observed that amino acids have a more stable derivatization at pH 8,25, as reported by other Authors (Melucci D. *et al.,* 1999) than in the other 2 pH conditions. Also amines show a good stability at pH 8,25.

The time of 2 minutes resulted to be a good solution for both, amino acids and amines as shown in table 2.

Several eluition gradients were tested in order to have a good peaks separation. The best one is showed in table 1, but using this gradient, there are still 3 pairs of substances coeluited (asparagine and glutamine, lysine and cystine, putrescine and histamine).

The addition of NaOH 0,1N after the use of borate buffer and before derivatization was founded as the best solution to obtain the desired pH.

Figure 1 shows a typical chromatogram obtained using Luna column.

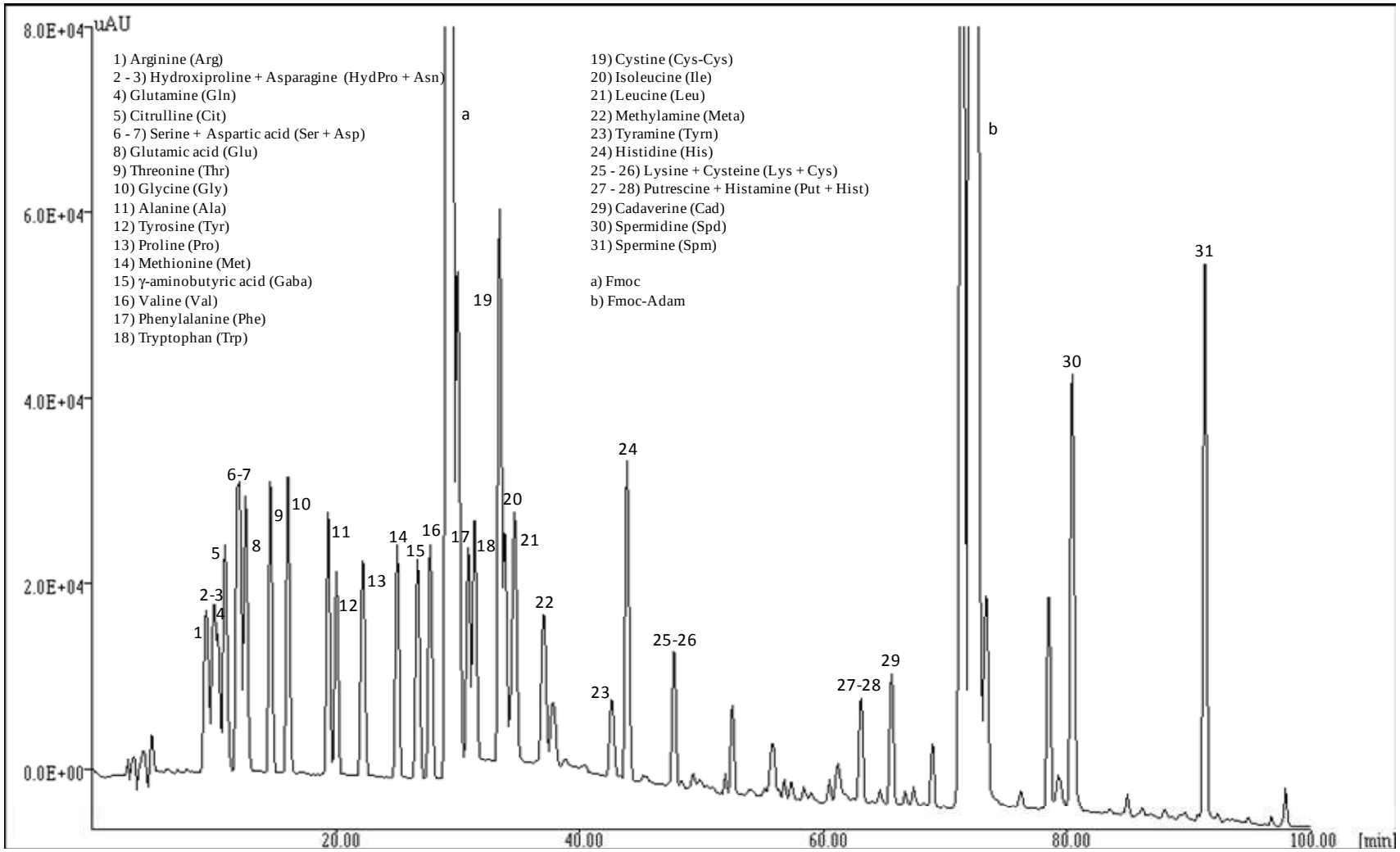


Figure 1 - Chromatographic separation of a biogenic amines and amino acids standard solution with Luna column

	pH = 8,00		pH = 8,25		pH = 8,40	
Arginine (Arg)	y = -871,92x + 358435	r^2 = 0,1833	y = 1915x + 352901	r^2 = 0,5706	y = -1456,3x + 371308	r^2 = 0,3416
Hydroxyproline (HydPro)	y = -200,16x + 320194	r^2 = 0,0133	y = 3275,1x + 337570	r^2 = 0,9519	y = 427,77x + 345669	r^2 = 0,0475
Asparagine (Asn)	y = 2988,2x + 316938	r^2 = 0,7066	y = -1364,5x + 375853	r^2 = 0,7766	y = 3320,4x + 323553	r^2 = 0,7553
Glutamine (Gln)	y = -905,9x + 185493	r^2 = 0,4574	y = -3655,1x + 202109	r^2 = 0,7429	y = 381,77x + 172274	r^2 = 0,0213
Citrulline (Cit)	y = -392,09x + 355577	r^2 = 0,0274	y = 424,2x + 372470	r^2 = 0,5647	y = 1224,5x + 368230	r^2 = 0,1749
Serine (Ser)	y = 167,33x + 317344	r^2 = 0,0061	y = -1659,1x + 428730	r^2 = 0,2478	y = -1766,7x + 430221	r^2 = 0,4425
Aspartic acid (Asp)	y = 3089,6x + 294079	r^2 = 0,8454	y = 7951x + 298808	r^2 = 0,8171	y = 7415,8x + 314408	r^2 = 0,6847
Glutamic acid (Glu)	y = 12973x + 253107	r^2 = 0,646	y = -561,99x + 393877	r^2 = 0,1462	y = 4201x + 384078	r^2 = 0,7311
Threonine (Thr)	y = -462,57x + 398269	r^2 = 0,042	y = -137,1x + 399619	r^2 = 0,0904	y = 414,61x + 401843	r^2 = 0,1111
Glycine (Gly)	y = -1266,2x + 40398	r^2 = 0,1302	y = -1558,6x + 404949	r^2 = 0,8133	y = 402,74x + 391365	r^2 = 0,1846
Alanine (Ala)	y = -359,29x + 350648	r^2 = 0,013	y = -2237,1x + 357928	r^2 = 0,8994	y = -355,19x + 361752	r^2 = 0,1495
Tyrosine (Tyr)	y = -13449x + 386599	r^2 = 0,9893	y = -24946x + 373262	r^2 = 0,9032	y = -17485x + 337322	r^2 = 0,9267
Proline (Pro)	y = -1619,3x + 385865	r^2 = 0,3579	y = -1187,1x + 358437	r^2 = 0,6696	y = -1132,7x + 384278	r^2 = 0,5805
Methionine (Met)	y = -2693,4x + 370370	r^2 = 0,7995	y = -5701,9x + 360286	r^2 = 0,9856	y = -2686,1x + 380636	r^2 = 0,9534
γ-Aminobutyric acid (Gaba)	y = -394,62x + 367432	r^2 = 0,0249	y = -8992,4x + 396480	r^2 = 0,8734	y = -6507,7x + 407128	r^2 = 0,6594
Valine (Val)	y = -2827,2x + 400908	r^2 = 0,7135	y = 2407,6x + 361593	r^2 = 0,791	y = -1895,8x + 400781	r^2 = 0,8505
Phenylalanine (Phe)	y = -5576,7x + 393032	r^2 = 0,9416	y = -3655,5x + 463428	r^2 = 0,5188	y = -3448,8x + 374928	r^2 = 0,8144
Tryptophan (Trp)	y = -6647,7x + 454995	r^2 = 0,9178	y = -1870,7x + 475892	r^2 = 0,5349	y = -6634,1x + 453576	r^2 = 0,9836
Cystine (Cys-Cys)	y = 1197,3x + 731848	r^2 = 0,2355	y = -2863,3x + 800413	r^2 = 0,1865	y = -2616,6x + 759356	r^2 = 0,6397
Isoleucine (Ile)	y = 2406,8x + 389034	r^2 = 0,7272	y = -138,78x + 404825	r^2 = 0,0135	y = -816,79x + 410610	r^2 = 0,6244
Leucine (Leu)	y = -10932x + 533757	r^2 = 0,3863	y = 1063,2x + 422123	r^2 = 0,2562	y = 829,23x + 548850	r^2 = 0,0629
Methylamine (Meta)	y = -1422,5x + 323708	r^2 = 0,3066	y = -1384,6x + 318231	r^2 = 0,3197	y = 719,71x + 315556	r^2 = 0,0639
Tyramine (Tyrn)	y = -14965x + 297874	r^2 = 0,9259	y = -16880x + 388394	r^2 = 0,9862	y = -14743x + 207566	r^2 = 0,8624
Histidine (His)	y = -16352x + 658099	r^2 = 0,9597	y = 3550,3x + 646016	r^2 = 0,9177	y = -17253x + 691967	r^2 = 0,9447
Cysteine (Cys)	y = -22034x + 422592	r^2 = 0,9795	y = -1865x + 323238	r^2 = 0,3232	y = -27378x + 461547	r^2 = 0,9478
Lysine (Lys)	y = -171,86x + 723762	r^2 = 0,0053	y = -104,82x + 754958	r^2 = 0,0029	y = -1098,1x + 748663	r^2 = 0,245
Histamine (Hist)	y = -21157x + 417678	r^2 = 0,9788	y = -12009x + 524746	r^2 = 0,8322	y = -22543x + 424314	r^2 = 0,9866
Putrescine (Put)	y = 1149,7x + 657532	r^2 = 0,1244	y = -14596x + 667307	r^2 = 0,7587	y = 1190,6x + 783444	r^2 = 0,0256
Cadaverine (Cad)	y = -115,85x + 236192	r^2 = 0,0157	y = 639,89x + 232725	r^2 = 0,0738	y = -458,54x + 241191	r^2 = 0,0252
Spermidine (Spermd)	y = -2755,6x + 753522	r^2 = 0,6576	y = -1197,5x + 680725	r^2 = 0,0071	y = 326,1x + 713768	r^2 = 0,002
Spermine (Sperm)	y = 209,76x + 809506	r^2 = 0,0012	y = -4327,6x + 813621	r^2 = 0,2114	y = 16608x + 682615	r^2 = 0,6516

Table 2 – Correlation between different derivatization times (2-15 min) and peak area (μAu x s)

(r^2= coefficient of determination)

Linearity of the method for each amino acids and amines assayed was tested.

Calibration curve obtained for each compound have a good linearity and detemination coefficients, except for spermine which has r^2 = 0,6049 (Table 3).

y = ax + b	**a**	**b**	**r^2**
Arginine (Arg)	36608	-61076	0,9865
Hydroxyproline (HydPro)	62048	-2172	0,9980
Asparagine (Asn)	59675	-18544	0,9983
Glutamine (Gln)	41669	-82299	0,9933
Citrulline (Cit)	38638	-61676	0,9965
Serine (Ser)	67218	-102317	0,9931
Aspartic acid (Asp)	20085	8834	0,9915
Glutamic acid (Glu)	61867	-149782	0,9865
Threonine (Thr)	60977	-99795	0,9913
Glycine (Gly)	105144	-35424	0,9834
Alanine (Ala)	57352	-280	0,9980
Tyrosine (Tyr)	41387	-26498	0,9795
Proline (Pro)	56707	941066	0,9921
Methionine (Met)	50400	-54027	0,9876
γ-Aminobutyric acid (Gaba)	57960	-19333	0,9983
Valine (Val)	61774	-54407	0,9916
Phenylalanine (Phe)	55231	46271	0,9951
Tryptophan (Trp)	43922	-12684	0,9850
Cystine (Cys-Cys)	56771	-150618	0,9864
Isoleucine (Ile)	50116	41281	0,9682
Leucine (Leu)	59703	-70207	0,9914
Methylamine (Meta)	118544	-23437	0,9840
Tyramine (Tyrn)	48959	-5352	0,9817
Histidine (His)	59593	-205913	0,9900
Cysteine (Cys)	74248	-38037	0,9903
Lysine (Lys)	19170	42765	0,9913
Histamine (Hist)	117690	-14736	0,9821
Putrescine (Put)	78319	-3935	0,9943
Cadaverine (Cad)	71837	11696	0,9949
Spermidine (Spermd)	64046	-7294	0,9684
Spermine (Sperm)	24225	41037	0,6049

Table 3 - Linearity of calibration curve and

coefficients of determination $r^2 = \left[\frac{n\sum xy - (\sum x)(\sum y)}{\sqrt{n(\sum x^2) - (\sum x)^2}\sqrt{n(\sum y^2) - (\sum y)^2}} \right]^2$

The recovery of the method was determined. The results obtained are shown in table 4.

	Mean recovery (%)	**c.v. (%)**
Arginine (Arg)	99,6 ± 6,20	6,22
Hydroxyproline (HydPro)	102,4 ± 1,60	1,56
Asparagine (Asn)	101,7 ± 6,05	5,95
Glutamine (Gln)	95,8 ± 4,27	4,45
Citrulline (Cit)	103,9 ± 24,30	23,39
Serine (Ser)	96,7 ± 2,44	2,53
Aspartic acid (Asp)	100,8 ± 9,89	9,50
Glutamic acid (Glu)	103,9 ± 8,27	7,96
Threonine (Thr)	110,6 ± 7,95	7,18
Glycine (Gly)	99,2 ± 2,15	2,17
Alanine (Ala)	91,6 ± 7,62	8,17
Tyrosine (Tyr)	98,8 ± 5,37	5,26
Proline (Pro)	100,9 ± 1,26	1,25
Methionine (Met)	95,8 ± 4,43	4,62
γ-Aminobutyric acid (Gaba)	97,4 ± 12,75	12,26
Valine (Val)	96,2 ± 9,50	9,88
Phenylalanine (Phe)	100,6 ± 8,18	8,13
Tryptophan (Trp)	107,9 ± 7,23	6,49
Cystine (Cys-Cys)	99,6 ± 6,19	6,43
Isoleucine (Ile)	89,1 ± 7,58	8,51
Leucine (Leu)	88,8 ± 8,42	9,13
Methylamine (Meta)	92,7 ± 7,35	7,92
Tyramine (Tyrn)	100,8 ± 2,36	2,34
Histidine (His)	96,0 ± 6,42	6,69
Lysine (Lys)	91,2 ± 2,40	2,73
Cysteine (Cys)	102,6 ± 11,53	10,25
Putrescine (Put)	97,3 ± 13,00	12,51
Histamine (Hist)	96,5 ± 15,00	19,00
Cadaverine (Cad)	86,9 ± 8,26	9,16
Spermine (Sperm)	90,1 ± 7,48	8,31
Spermidine (Spermd)	91,9 ± 6,76	7,36

Table 4 – Accuracy for amino acids and amines determination
(c.v. = coefficient of variation = standard deviation/mean)

For most compound high recovery values were obtained (> 86,9 %),. Some variation coefficients are higher than 10 %, 23,39 for citrulline, 12,36 for gaba, 10,25 for cysteine, and 12,51 for putrescine. This result can be explained probably by lower stability of their derivatized products.

Concerning retention times, precision was calculated the same day (intraday) and in different days (interday) and expressed as c.v. (%). Value ranged from 0,01 % to 0,72 % (Table 5).

	mean time (min)	**intraday c.v. (%)**	**mean time (min)**	**interday c.v. (%)**
Arginine (Arg)	9,28	0,34	9,32	0,40
Hydroxyproline (HydPro) + Asparagine (Asn)	9,73	0,24	9,74	0,26
Glutamine (Gln)	10,12	0,24	10,07	0,34
Citrulline (Cit)	10,69	0,21	10,63	0,37
Serine (Ser)	11,76	0,25	11,76	0,25
Aspartic acid (Asp)	12,06	0,30	12,09	0,33
Glutamic acid (Glu)	12,23	0,32	12,17	0,44
Threonine (Thr)	14,34	0,14	14,14	0,72
Glycine (Gly)	15,75	0,11	15,58	0,56
Alanine (Ala)	18,96	0,09	18,78	0,50
Tyrosine (Tyr)	19,69	0,09	19,51	0,48
Proline (Pro)	21,67	0,08	21,45	0,52
Methionine (Met)	24,58	0,11	24,35	0,49
γ-Aminobutyric acid (Gaba)	26,51	0,08	26,27	0,45
Valine (Val)	27,18	0,07	26,92	0,48
Phenylalanine (Phe)	30,30	0,12	30,04	0,45
Tryptophan (Trp)	30,92	0,07	30,65	0,45
Cystine (Cys-Cys)	32,73	0,29	32,31	0,72
Isoleucine (Ile)	33,26	0,07	32,97	0,44
Leucine (Leu)	34,10	0,06	33,81	0,45
Methylamine (Meta)	37,17	0,06	36,88	0,39
Tyramine (Tyrn)	42,75	0,05	42,43	0,38
Histidine (His)	43,41	0,11	42,99	0,50
Cysteine + Lysine (Cys + Lys)	47,44	0,06	47,09	0,38
Putrescine + Histamine (Put + Hist)	63,19	0,03	62,77	0,33
Cadaverine (Cad)	65,74	0,02	65,34	0,31
Spermidine (Spermd)	80,76	0,01	80,38	0,24
Spermine (Sperm)	91,82	0,01	91,46	0,19

Table 5 - Precision of the method in relation to retention times of each compound (c.v. = coefficient of variation)

Regarding precision on the quantification (peak areas), results are interesting except those acquired interday for spermine and gaba (36,74 % and 10,20 % respectively) (Table 6).

	mean area (µAU x s)	intraday c.v. (%)	mean area (µAU x s)	interday c.v. (%)
Arginine (Arg)	3511191	1,55	3587905	1,83
Hydroxyproline (HydPro) + Asparagine (Asn)	2956227	0,67	2952980	0,89
Glutamine (Gln)	531105	1,15	542250	1,56
Citrulline (Cit)	1483559	1,69	1412228	2,92
Serine (Ser)	4288701	1,09	3917197	4,56
Aspartic acid (Asp)	650030	2,74	686648	3,71
Glutamic acid (Glu)	1990330	2,58	1832930	4,92
Threonine (Thr)	3052503	1,40	2991026	1,70
Glycine (Gly)	2967748	1,44	2973717	1,52
Alanine (Ala)	1321674	1,88	1197714	5,10
Tyrosine (Tyr)	1304165	2,47	1341159	2,74
Proline (Pro)	1497685	3,62	1464598	4,26
Methionine (Met)	1372189	1,63	1338091	2,14
γ-Aminobutyric acid (Gaba)	1398568	1,86	1134755	10,20
Valine (Val)	1442393	2,09	1423262	2,09
Phenylalanine (Phe)	2507042	1,60	2417399	2,52
Tryptophan (Trp)	1530675	1,61	1538998	1,73
Cystine (Cys-Cys)	3701553	2,32	4025503	4,75
Isoleucine (Ile)	1437732	1,97	1411021	2,11
Leucine (Leu)	1561890	1,10	1527897	1,50
Methylamine (Meta)	1325045	1,17	1326359	1,25
Tyramine (Tyrn)	1128478	1,82	1183131	2,76
Histidine (His)	2155763	2,18	2211194	2,97
Cysteine + Lysine (Lys + Cys))	2159113	3,24	2060487	4,24
Putrescine + Histamine (Put + Hist)	3718386	2,34	3993744	3,96
Cadaverine (Cad)	968541	3,31	1002457	3,45
Spermidine (Spermd)	3009058	2,65	2991577	2,47
Spermine (Sperm)	4864131	33,84	6867682	36,74

Table 6 - Precision of the method in relation to peaks area

(c.v. = coefficient of variation)

Detection and quantification limits were determined and the results obtained are shown in table 7.

	L.O.D. (mg/L)	**L.O.Q. (mg/L)**
Arginine (Arg)	0,72	2,16
Hydroxyproline (HydPro)	0,53	1,60
Asparagine (Asn)	0,31	0,93
Glutamine (Gln)	0,81	2,42
Citrulline (Cit)	0,81	2,42
Serine (Ser)	0,27	0,81
Aspartic acid (Asp)	0,96	2,88
Glutamic acid (Glu)	0,89	2,66
Threonine (Thr)	0,27	0,81
Glycine (Gly)	0,09	0,26
Alanine (Ala)	0,29	0,87
Tyrosine (Tyr)	0,22	0,67
Proline (Pro)	0,15	0,44
Methionine (Met)	0,28	0,84
γ-Aminobutyric acid (Gaba)	0,30	0,89
Valine (Val)	0,24	0,71
Phenylalanine (Phe)	0,20	0,61
Tryptophan (Trp)	0,21	0,62
Cystine (Cys-Cys)	0,36	1,08
Isoleucine (Ile)	0,22	0,67
Leucine (Leu)	0,30	0,89
Methylamine (Meta)	0,10	0,30
Tyramine (Tyrn)	0,17	0,51
Histidine (His)	1,07	3,21
Cysteine (Cys)	0,31	0,92
Lysine (Lys)	0,19	0,56
Histamine (Hist)	0,19	0,57
Putrescine (Put)	0,15	0,44
Cadaverine (Cad)	0,13	0,38
Spermidine (Spermd)	0,17	0,52
Spermine (Sperm)	0,34	1,02

Table 7 - Limit of quantification (L.O.Q) and limit of detection (L.O.D.) calculated using Luna column

Limit of detection and quantification are good, because the values are less than 1 mg/L for many compounds, suitable for wine composition.

The method was then applied to the analysis of 4 Italian wines.

Content of amino acids and amines showed in table 8 is different among the wines, but there aren't statistical differences.

	Trentin white wine (mg/L)	**Tuscan red wine (mg/L)**	**Sicilian red wine (mg/L)**	**Tuscan red wine (mg/L)**
Arginine (Arg)	39,9	20,9	36,5	19,7
Hydroxyproline (HydPro) + Asparagine (Asn)	n.d.	n.d.	n.d.	n.d.
Glutamine (Gln)	n.d.	n.d.	n.d.	n.d.
Citrulline (Cit)	7,75	3,87	3,66	3,71
Serine (Ser)	5,39	4,02	2,60	2,87
Aspartic acid (Asp)	6,34	3,54	n.d.	n.d.
Glutamic acid (Glu)	8,04	4,33	3,88	3,30
Threonine (Thr)	4,01	3,85	3,68	3,17
Glycine (Gly)	6,12	10,94	5,79	6,02
Alanine (Ala)	10,70	6,24	4,01	3,52
Tyrosine (Tyr)	7,97	2,71	1,73	2,54
Proline (Pro)	496	554	513	547
Methionine (Met)	3,64	2,89	3,47	3,09
γ-Aminobutyric acid (Gaba)	10,33	2,58	4,48	2,58
Valine (Val)	4,85	3,63	2,60	2,25
Phenylalanine (Phe)	13,88	7,45	6,85	4,70
Tryptophan (Trp)	1,46	1,21	1,24	0,77
Cystine (Cys-Cys)	4,90	4,01	3,37	3,26
Isoleucine (Ile)	0,92	2,51	1,17	n.q.
Leucine (Leu)	8,35	2,87	3,02	2,43
Methylamine (Meta)	1,15	0,95	n.d.	0,86
Tyramine (Tyrn)	n.q.	7,99	5,08	2,63
Histidine (His)	5,36	4,02	5,49	4,24
Cysteine + Lysine (Cys + Lys)	6,49	1,96	1,20	1,30
Putrescine + Histamine (Put + Hist)	1,20	8,49	4,52	3,17
Cadaverine (Cad)	n.d.	0,38	n.d.	n.d.
Spermidine (Spermd)	0,67	n.d.	n.d.	n.d.
Spermine (Sperm)	n.d.	n.d.	n.d.	n.d.

Table 8 – Amino acids and amines content in different wines

Results are the mean of triplicate analyses

(n.d. = not detected (under L.O.D))

(n.q. = not quantified (under L.O.Q))

Amino acids content is higher in white wine and this could be due to the different must composition, winemaking process and yeast activity. The prevailing amino acids are proline (marker for wine genuineness) that is around 500 mg/L and arginine around 20-30 mg/L.

Regarding biogenic amines, tyramine, putrescine and histamine are higher in the red wines that had malolactic fermentation and this is due to growth of lactic bacteria that induces an increase of amines content in wines (Soufleros E. *et al.,* 1998).

However, in these wines, the content in total biogenic amines was low and did not represent a toxicological hazard for human health.

1.11.4 Conclusion

In conclusion,

a) pH = 8,25 has been chosen as best pH of derivatization;
b) derivatization time of 2 minutes is the best choice for the simultaneous determination of amino acids and amines;
c) to achieve the best conditions of derivatization with wine sample, borate buffer at pH = 9,00 and a further addition of 0,1 N NaOH were used;
d) linearity of calibration curves and coefficient of variation are good;
e) the separation of compounds is good even if there are still three coelutions;
f) precision and accuracy of the method are high, except for spermine; however, amines are usually present in wine at very low levels;
g) detection and quantification limits are suitable for wine matrix;
h) time analysis is still a bit long (110 min), therefore the further step could be reducing time analysis.

1.12 Validation of a HPLC method for simultaneous determination of amino acids and amines with precolumn-derivatization with 9-Fluorenyl-methoxycarbonyl chloride (FMOC-Cl) and UV detection using a monolithic column

1.12.1 Aim of work

In the previous study we applied experimental design to optimize the derivatization reaction of biogenic amines with FMOC-Cl, as a prior step to their HPLC determination. The influence of pH, time of derivatization, gradient eluition were studied. In order to improve the resolution among amino acids and amines to be separated and reduce the time of analysis, we studied the influence of different columns (traditional and monolithic) and eluition flow rates in the chromatographic separation.

The reliability of the method in terms of accuracy, repeatability and linearity has been studied.

1.12.2 Experimental

Samples

Application of this method was carried out on 3 samples: 2 red wines and 1 white wine. These samples were treated and analyzed as of the standard solution.

Apparatus

The chromatographic system consisted of two PU-1580 pumps (Jasco, Tokyo, Japan), a DAD MD-1510 detector (Jasco, Tokyo, Japan), a 7161 valve (Rheodyne, Cotati, CA, USA) equipped with a 20 μL loop. The detection wavelength was set at 263 nm.

Data acquisition and handling were carried out using the Borwin Chromatography software 1,5 version (JMBS Developments, Grenoble, France).

Firstly, a C18 Luna column (Phenomenex, Torrance, USA), (150 x 4,6 mm, 3 μm) was used and later two monolithic Chromolith columns RP-18e, 100 x 4.6 mm (Merck, Darmstad, Germany) in series and a precolumn RP-18e, 10 x 4,6 mm (Merck, Darmstad, Germany).

Traditional columns have a packing characterized by spherical or irregular particles of silica with a variable size (3, 5, 10 mm), derivatized with polymers containing 18 atoms of C. They have a length from 100 to 250 mm and an internal diameter variable from 2 to 4,6 mm.

Monolithic columns are packed with a single rigid block of silica, which has an internal macroporic and mesoporic structure, derivatized with polymers containing 18 atoms of C. They are commercially available in two lengths (50 and 100 mm) with a fixed diameter of 4,6 mm.

Eluents

The separation was optimized using a mobile phase consisting two eluents.

Eluent A: sodium acetate 50 mM (Carlo Erba, Milan, Italy) in H_2O (Merck, Darmstad, Germany) adjusted to pH = 4,2 with glacial acetic acid (Carlo Erba, Milan, Italy).

Eluent B: CH_3CN (Merck, Darmstad, Germany).

Eluents were filtered through a 0,22 μm nylon membrane filter.

Two different gradient profiles were used, according to each type of column chosen. The gradient profile was studied by varying the time intervals and the flow rate of the mobile phase. The gradients were constructed as described in table 9.

Luna column		**Chromolith column**	
Time (min)	**A (%)**	**Time (min)**	**A (%)**
0	68	0	82
3	68	1	82
14	59	38	64
40	42	50	48
45	35	62	30
90	0	68	0
100	0	72	0
105	68	75	82
110	68		

Table 9 - HPLC elution programs for amino acids and biogenic amines analysis using Luna and Chromolith columns.

The flow-rate was 0,5 mL/min for the Luna column and 2 mL/min for the monolithic columns.

Biogenic amines were identified on the basis of their retention time and by spiking the samples with known amounts of standards.

Reagents

- borate buffer: 1,24 g boric acid (Merk, Germany) in 100 mL of water adjusted to pH = 8,25 with concentrated NaOH;
- 9-fluorenyl-methoxycarbonyl chloride (FMOC-Cl) (Sigma-Aldrich, Milan, Italy) 0,015 M in 10 mL of acetonitrile (Merck, Darmstad, Germany);
- 1-ammino amantadine (ADAM) (Sigma-Aldrich, Milan, Italy) 0,03 M in 10 mL of water acetonitrile (H_2O/CH_3CN) (1:1, v/v);
- chloridric acid (Carlo Erba, Milan, Italy).

Standard solution

A 1 mM standard solution of 25 amino acids and 8 amines (glutamine (Gln), hydroxiproline (HydPro), asparagine (Asn), arginine (Arg), aspartic acid (Asp), citrulline (Cit), serine (Ser), glutamic acid (Glu), glycine (Gly), threonine (Thr), alanine (Ala), agmatine (Agm), proline (Pro), tyrosine (Tyr), valine (Val), γ-aminobutyric acid (Gaba), methionine (Met), methylamine (Meta), tryptophan (Trp), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), cystine (Cys-Cys), tyramine (Tyrn), histidine (His), cysteine (Cys), ornithine (Orn), lysine (Lys), histamine (Hist), cadaverine (Cad), putrescine (Put), spermidine (Spd), spermine (Spm)), in HCl 0,01 M was prepared (Sigma-Aldrich, Milan, Italy).

Derivatization procedure

The derivatization was carried out by adding 20 mL of borate buffer 0,2 M to 10 mL of wine and adjusted to pH = 8,25 with concentrated sodium hydroxide. Then, 900 μL of this mixture were added to 600 μL of FMOC-Cl 15 mM in CH_3CN.

After 2 minutes 600 μL of ADAM 0,3 M in a solution of H_2O/CH_3CN (1:1, v/v) were added. After 2 minutes the derivatizated sample was filtered through a 0,22 μm nylon and immediately injected.

Validation

Precision of the method was estimated. Repeatability were assessed by injecting 5 repeated times the same derivatized solution of amino acids and amines during the same day (intraday) and in different days (interday).

The linearity of the method was determined with 3 derivatizations of 5 different concentrations of amino acids standard, ranging from 2 mg/L to 50 mg/L for each amino acids, ranging from 40 mg/L to 650 mg/L for proline and from 1 mg/L to 14 mg/L for amines.

To evaluate accuracy, recoveries were determined by spiking a red wine sample (already contaminated with a known amount of biogenic amines) with known amounts in amino acids and amines.

The limits of detection were calculated from the concentration of each amines required to give a signal-to-noise ratio of 3 (S/N = 3).

1.12.3 Results and discussion

Simultaneous chromatographic separation of 24 amino acids and 7 amines was initially carried out on the Luna column.

The HPLC gradient had permitted to separate 31 compounds in a very long time (110 min) and despite several attempts to optimize the separation, problems of co-elution (hydroxyproline and asparagine, lysine and cystine, putrescine and histamine) had not been solved.

In order to improve the chromatographic separation, a monolithic column was used. This column has two peculiar characteristics: it can operate to higher flow-rate than traditional column (Bidlingmaier B. *et al.,* 1999; Castellari M. *et al.,* 2002) and can reduce retention times with the same efficiency of separation than conventional columns. With an adequate modification of the eluition gradient previously utilized (Table 9), it was possible to improve noticeably the chromatographic separation and 33 compounds were separated in only 75 min instead of 110 min. Figure 2 shows a typical chromatogram obtained using the monolithic column.

In order to improve the performance of separation and theefficiency of the column, the number of total theoretical plates has been increased and two monolithic columns in series were used.

The optimal flow was chosen after several attempts at different flows of mobile phase between 1,6 and 4,0 mL/min. The optimal flow value of the mobile phase corresponding to 2 mL/min.

Peaks identification occurred through a single injection of each compound and subsequent comparison with the retention times. Using monolithic columns, compounds were eluted with a different order from that obtained by traditional column. This is due to the monolithic column structure which may influence, for steric and mechanical factors, the affinity of solutes with mobile phase.

Linearity was examinated (Table 10). Calibration curves in the range 2-50 mg/L for amino acids a part from proline (40-650 mg/L) to quantificate single compounds were constructed. For the amines the range for calibration was 1-14 mg/L. Determination coefficients (r^2) obtained for each compound were quite good. The values are next to 1 and slightly lower for spermidine and spermine (0,9859 % and 0,9809 % respectively).

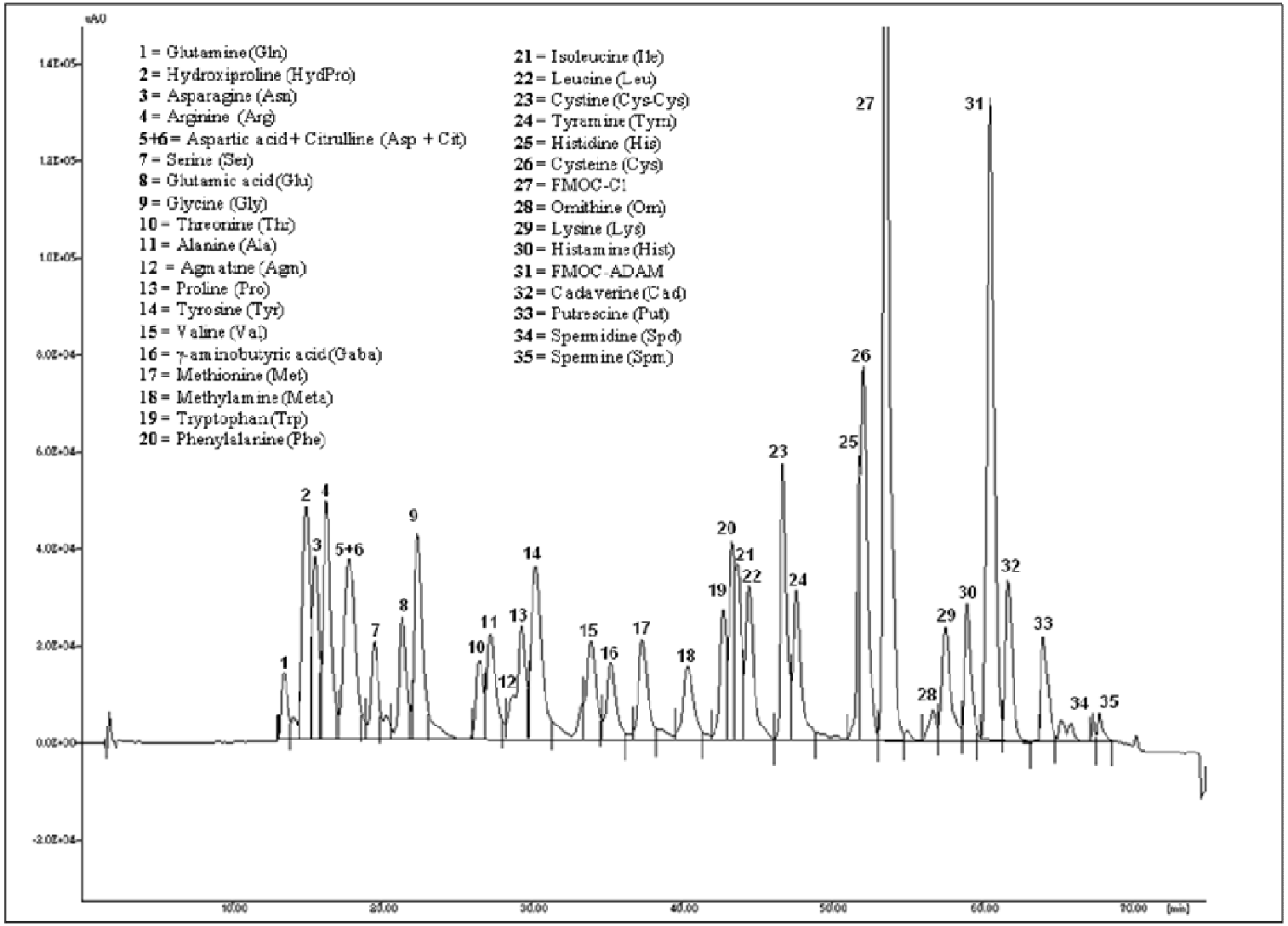


Figure 2 – Chromatographic separation of a biogenic amines and amino acids standard solution with two Chromolith columns in series

y = ax + b	a	b	r^2
Glutamine (Gln)	29176	661320	0,9939
Hydroxyproline (Hyp)	18650	672589	0,9943
Asparagine (Asn)	17112	4518	0,9985
Arginine (Arg)	13378	-81776	0,9973
Aspartic acid (Asp)	6211	72487	0,9983
Citrulline (Cit)	36235	-140589	0,9968
Serine (Ser)	6690	18246	0,9940
Glutamic acid (Glu)	36923	-206495	0,9947
Glycine (Gly)	6448	67360	0,9979
Threonine (Thr)	20154	-231037	0,9915
Alanine (Ala)	12266	13605	0,9972
Agmatine (Agm)	29888	78818	0,9966
Proline (Pro)	93225	1000000	0,9969
Tyrosine (Tyr)	45457	4696	0,9881
Valine (Val)	18086	-131421	0,9949
γ-Aminobutyric acid (Gaba)	15670	-56781	0,9989
Methionine (Met)	21471	-232401	0,9931
Methylamine (Meta)	156831	-44870	0,9994
Tryptophan (Trp)	19067	-166225	0,9948
Phenylalanine (Phe)	19034	-186470	0,9906
Isoleucine (Ile)	18121	-111909	0,9952
Leucine (Leu)	12671	-35165	0,9941
Cystine (Cys-Cys)	21621	-190879	0,9998
Tyramine (Tyrn)	35288	-3314	0,9960
Histidine (His)	24494	233461	0,9920
Cysteine (Cys)	25147	232307	0,9944
Ornithine (Orn)	108728	37014	0,9905
Lysine (Lys)	24263	273035	0,9967
Histamine (Hist)	73253	-37874	0,9959
Cadaverine (Cad)	57922	-40763	0,9945
Putrescine (Put)	68941	-22095	0,9902
Spermidine (Spermd)	6536	-3504	0,9859
Spermine (Sperm)	5006	4205	0,9809

Table 10 - Linearity and determination coefficients (r^2)

To validate the HPLC method also precision and accuracy were determined. Table 11 shows the results obtained for precision calculated on the basis of retention times of each compound evaluated on the same day (intraday) and in different days (interday).
Coefficients of variation percentage are good for each compounds (c.v. < 1,0 %).

	mean time (min)	**intraday c.v. (%)**	**mean time (min)**	**interday c.v. (%)**
Glutamine (Gln)	13,41	0,4	14,46	1,44
Hydroxyproline (Hyp)	14,59	0,51	14,93	1,49
Asparagine (Asn)	15,15	0,46	15,35	2,01
Arginine (Arg)	15,5	0,45	16,21	1,82
Aspartic acid (Asp) + Citrulline (Cit)	16,73	0,44	17,14	1,39
Serine (Ser)	17,57	0,52	17,75	1,23
Glutamic acid (Glu)	19,45	0,39	19,7	1,28
Glycine (Gly)	21,3	0,49	21,39	1,16
Threonine (Thr)	25,21	0,38	25,65	1,15
Alanine (Ala)	26,6	0,28	26,84	1,1
Agmatine (Agm)	28,22	0.25	28,51	0.35
Proline (Pro)	29,30	0,34	29,44	0,56
Tyrosine (Tyr)	30,21	0,29	30,66	1,2
Valine (Val)	33,89	0,29	33,49	0,77
γ-Aminobutyric acid (Gaba)	35,20	0,08	35,62	1,04
Methionine (Met)	37,30	0,18	37,77	1,09
Methylamine (Meta)	40,3	0,14	40,38	0,6
Tryptophan (Trp)	42,72	0,13	42,23	0,77
Phenylalanine (Phe)	43,3	0,28	43,15	0,66
Isoleucine (Ile)	43,84	0,1	43,94	0,79
Leucine (Leu)	44,41	0,09	44,65	0,62
Cystine (Cys-Cys)	46,68	0,04	46,9	0,65
Tyramine (Tyrn)	47,6	0,06	47,9	0
Histidine (His)	50,19	0,07	50,45	0,33
Cysteine (Cys)	51,08	0,03	51,46	0,45
Ornithine (Orn)	55,52	0.05	55,8	0.15
Lysine (Lys)	56,69	0,02	56,2	0,39
Histamine (Hist)	58,97	0,03	59,85	0,24
Cadaverine (Cad)	61,69	0,03	61,76	0,24
Putrescine (Put)	64,04	0,04	65,44	0,17
Spermidine (Spermd)	66,86	0,33	66,99	0,13
Spermine (Sperm)	67,36	0,09	67,43	0,12

Table 11 - Precision of the method in term of retention times of each compound (c.v. = coefficient of variation)

Table 12 shows the results obtained from the precision calculated on the basis of peaks area, obtained using the same procedure adopted for the retention times. In this case, precision resulted under 5 % except for spermine (c.v. = 10,82) whose derivatization is hardly reproducible.

	mean area (μAU x s)	**intraday c.v. (%)**	**mean area (μAU x s)**	**interday c.v. (%)**
Glutamine (Gln)	465792	1,78	396672	2,22
Hydroxyproline (Hyp)	462778	1,13	429322	1,97
Asparagine (Asn)	389244	1,41	435468	2,44
Arginine (Arg)	529558	1,65	519554	3,14
Aspartic acid (Asp) + Citrulline (Cit)	1173841	3,27	1190337	4,33
Serine (Ser)	421267	5,15	416000	2,41
Glutamic acid (Glu)	351093	2,29	366514	1,76
Glycine (Gly)	505290	5,18	495908	4,44
Threonine (Thr)	533506	1,86	614743	3,65
Alanine (Ala)	466094	2,95	481319	3,98
Agmatine (Agm)	311936	2,42	325366	3,15
Proline (Pro)	532140	1,45	536376	1,62
Tyrosine (Tyr)	633800	1,93	666015	1,20
Valine (Val)	548000	4,81	595311	4,59
γ-Aminobutyric acid (Gaba)	616000	1,57	578743	3,80
Methionine (Met)	475912	3,13	465935	3,09
Methylamine (Meta)	535726	1,11	492204	4,75
Tryptophan (Trp)	624415	0,93	609159	2,20
Phenylalanine (Phe)	544504	0,69	546960	3,57
Isoleucine (Ile)	495364	0,87	518245	1,42
Leucine (Leu)	623822	4,11	569245	3,81
Cystine (Cys-Cys)	609591	3,78	665345	2,45
Tyramine (Tyrn)	463756	0,96	483335	1,61
Histidine (His)	1560379	3,46	1444576	2,91
Cysteine (Cys)	954643	2,20	948278	1,11
Ornithine (Orn)	409909	2,78	415530	3,20
Lysine (Lys)	1193018	4,19	1203240	2,42
Histamine (Hist)	760335	4,21	797789	4,01
Cadaverine (Cad)	787584	5,03	795924	3,27
Putrescine (Put)	422449	1,13	419557	2,05
Spermidine (Spermd)	354960	3,97	320297	2,82
Spermine (Sperm)	345855	10,82	318544	3,47

Table 12 - Precision of the method in relation to peaks area of each compound (c.v. = coefficient of variation)

This method allows good recoveries for most of the analyzed compounds (Table 13).

	Mean recovery (%)	**c.v. (%)**
Glutamine (Gln)	99,5 ± 5,2	2,5
Hydroxyproline (Hyp)	102,4 ± 3,2	3,4
Asparagine (Asn)	99,2 ±1,5	0,8
Arginine (Arg)	95,8 ±1,2	0,8
Aspartic acid (Asp)	101,3 ± 4,5	3,2
Citrulline (Cit)	97,6 ± 4,8	3,5
Serine (Ser)	96,5 ± 2,4	2,5
Glutamic acid (Glu)	100,9 ± 1,8	1,5
Glycine (Gly)	101,5 ± 2,3	2,2
Threonine (Thr)	98,2 ± 2,1	2,3
Alanine (Ala)	99,5 ± 5,6	5,5
Agmatine (Agm)	98,3 ± 4,4	3,2
Proline (Pro)	100,2 ± 7,6	4,5
Tyrosine (Tyr)	99,8 ± 3,2	3,1
Valine (Val)	97,4 ± 2,4	1,9
γ-Aminobutyric acid (Gaba)	96,8 ± 1,9	1,7
Methionine (Met)	96,2 ± 4,9	3,2
Methylamine (Meta)	93,5 ± 7,5	4,7
Tryptophan (Trp)	103,5 ± 8,5	6,5
Phenylalanine (Phe)	100,2 ± 6,5	5,4
Isoleucine (Ile)	93,5 ± 7,2	5,2
Leucine (Leu)	90,2 ± 6,5	3,2
Cystine (Cys-Cys)	88,7 ± 8,2	3,3
Tyramine (Tyrn)	99,6 ± 2,1	2,8
Histidine (His)	95,6 ± 5,6	4,4
Cysteine (Cys)	103,1 ± 13,3	10,5
Ornithine (Orn)	95,5 ± 5,8	4,2
Lysine (Lys)	96,5 ± 4,5	4,2
Histamine (Hist)	98,2 ± 4,1	3,8
Cadaverine (Cad)	88,5 ± 7,5	2,8
Putrescine (Put)	90,2 ± 13,0	12,6
Spermidine (Spermd)	89,5 ± 11,5	11,2
Spermine (Sperm)	90,3 ± 15,6	14,3

Table 13 - Accuracy for amino acids and amines determination

(c.v. = coefficient of variation)

Accuracy ranges from 88,5 to 103,5 %. Only for the cysteine, putrescine, spermidine and spermine unsatisfactory recoveries were obtained; probably because of derivatization problem or instability of the derivatized compounds.

For each compound limit of detection (L.O.D.) values were calculated (Table 14).

	L.O.D. (mg/L) traditional column	**L.O.D. (mg/L) monolithic column**
Glutamine (Gln)	0,81	0,18
Hydroxyproline (Hyp)	0,53	0,11
Asparagine (Asn)	0,31	0,11
Arginine (Arg)	0,72	0,21
Aspartic acid (Asp)	0,96	0,08
Citrulline (Cit)	0,81	0,1
Serine (Ser)	0,27	0,12
Glutamic acid (Glu)	0,89	0,2
Glycine (Gly)	0,09	0,07
Threonine (Thr)	0,27	0,13
Alanine (Ala)	0,29	0,11
Agmatine (Agm)	-	0,22
Proline (Pro)	0,15	0,04
Tyrosine (Tyr)	0,22	0,02
Valine (Val)	0,24	0,12
γ-Aminobutyric acid (Gaba)	0,30	0,14
Methionine (Met)	0,28	0,21
Methylamine (Meta)	0,10	0,11
Tryptophan (Trp)	0,21	0,24
Phenylalanine (Phe)	0,20	0,15
Isoleucine (Ile)	0,22	0,12
Leucine (Leu)	0,30	0,13
Cystine (Cys-Cys)	0,36	0,11
Tyramine (Tyrn)	0,17	0,17
Histidine (His)	1,07	0,09
Cysteine (Cys)	0,31	0,06
Ornithine (Orn)	-	0,14
Lysine (Lys)	0,19	0,07
Histamine (Hist)	0,19	0,1
Cadaverine (Cad)	0,13	0,11
Putrescine (Put)	0,15	**0,23**
Spermidine (Spermd)	0,17	**0,32**
Spermine (Sperm)	0,34	0,31

Table 14 – Comparison between detection limits (L.O.D.) for traditional and monolithic columns

Monolithic column showed L.O.D. (min 0,04 and max 0,61 mg/L) generally lower than traditional column (min 0,09 and max 1,07 mg/L).

The influence of flow-rate of the mobile phase on the monolithic column separation was also studied. Table 15 shows the capacity factors (k) calculated for each compound obtained at 5 different flow-rates. High capacity factors increase the time of analysis and determine an enlargement of peaks. The lowest k values are at flow 1,8 followed by 2,0 mL/min; however, the optimal flow was chosen at 2 mL/min, in order to reduce time analysis.

	K values				
Flow (mL/min)	**F = 1,6**	**F = 1,8**	**F = 2,0**	**F = 2,8**	**F = 4,0**
Glutamine (Gln)	8,39	7,27	7,18	10,08	12,02
Hydroxyproline (HydPro)	8,70	7,53	7,53	10,49	12,48
Asparagine (Asn)	9,04	7,84	7,80	11,25	13,33
Arginine (Arg)	9,04	7,84	7,88	11,25	13,33
Aspartic acid (Asp)	9,94	8,71	8,74	12,41	14,70
Citrulline (Cit)	9,94	8,71	8,74	12,92	15,33
Serine (Ser)	10,08	8,92	8,90	12,92	15,33
Glutamic acid (Glu)	10,93	9,71	9,74	14,49	17,23
Glycine (Gly)	12,02	10,75	10,77	16,43	19,50
Threonine (Thr)	12,50	11,25	11,30	16,94	20,26
Alanine (Ala)	14,85	13,40	13,63	16,94	29,00
Agmatine (Agm)	15,87	14,35	14,55	19,66	29,59
Proline (Pro)	16,25	14,73	14,89	21,59	29,00
Tyrosine (Tyr)	16,98	15,61	15,73	25,79	29,48
Valine (Val)	19,00	17,50	17,82	27,24	41,47
γ-Aminobutyric acid (Gaba)	19,67	18,28	18,60	29,25	44,71
Methionine (Met)	20,84	19,49	19,78	31,56	47,79
Methylamine (Meta)	22,22	21,03	21,51	33,65	50,11
Tryptophan (Trp)	22,89	22,29	22,95	34,92	56,24
Phenylalanine (Phe)	23,10	22,55	23,31	41,05	57,10
Isoleucine (Ile)	23,41	22,83	23,51	42,02	57,50
Leucine (Leu)	23,73	23,22	23,98	43,10	58,65
Cystine (Cys-Cys)	24,71	24,53	25,55	48,08	62,85
Tyramine (Tyrn)	25,31	24,84	25,86	48,08	62,85
Histidine (His)	26,92	26,48	27,60	51,30	67,10
Cysteine (Cys)	27,29	27,12	28,25	55,87	69,29
Ornithine (Orn)	28,02	27,89	29,09	55,53	71,22
Lysine (Lys)	28,19	28,06	29,27	55,87	71,65
Histamine (Hist)	31,92	31,68	33,14	65,16	80,53
Cadaverine (Cad)	32,66	32,44	33,95	66,83	68,81
Putrescine (Put)	34,14	34,61	36,36	72,14	88,19
Spermidine (Spermd)	34,73	35,39	37,26	75,37	90,33
Spermine (Sperm)	34,94	35,63	37,50	76,06	90,93

Table 15 - Comparison of capacity factors at different flow-rates of the mobile phase ($k = (t_r - t_m) / t_m$; t_r = retention times; t_m = dead times)

Three wines were assayed to test the applicability of the method on the specific matrix.

The derivatization of the samples was the same of the standard solutions.

The correction of the acid pH of wine has been carried out adding 1 N NaOH and then adding boric buffer.

Table 16 shows the contents of amino acids and amines in some wine samples.

	white wine	**red wine 1**	**red wine 2**
Glutamine (Gln)	2,56	6,11	4,95
Hydroxyproline (Hyp)	10,0	20,7	16,6
Asparagine (Asn)	2,44	7,46	8,47
Arginine (Arg)	6,03	11,35	n.d.
Aspartic acid (Asp) + Citrulline (Cit)	3,75	16,33	17,93
Serine (Ser)	5,19	21,62	9,46
Glutamic acid (Glu)	n.d.	20,4	40,4
Glycine (Gly)	6,80	8,15	20,44
Threonine (Thr)	28,6	24,0	20,4
Alanine (Ala)	4,24	11,61	21,9
Agmatine (Agm)	n.d.	1,25	n.d.
Proline (Pro)	408	534	615
Tyrosine (Tyr)	n.d.	66,1	15,1
Valine (Val)	n.d.	47,8	63,5
γ-Aminobutyric acid (Gaba)	43,65	7,08	n.d.
Methionine (Met)	n.d.	4,61	n.d.
Methylamine (Meta)	7,03	6,12	n.d.
Tryptophan (Trp)	3,41	7,36	n.d.
Phenylalanine (Phe)	n.d.	1,34	n.d.
Isoleucine (Ile)	n.d.	11,79	n.d.
Leucine (Leu)	n.d.	56,1	n.d.
Cystine (Cys-Cys)	n.d.	55,1	n.d.
Tyramine (Tyrn)	n.d.	77,9	n.d.
Histidine (His)	n.d.	n.d.	n.d.
Cysteine (Cys)	4,46	3,44	5,52
Ornithine (Orn)	n.d.	n.d.	n.d.
Lysine (Lys)	0,95	0,62	1,55
Histamine (Hist)	13,88	5,50	16,67
Cadaverine (Cad)	18,27	6,17	6,19
Putrescine (Put)	9,67	n.d.	6,31
Spermidine (Spermd)	5,74	5,70	n.d.
Spermine (Sperm)	5,43	6,35	n.d.

Table 16 - Amino acids and amines content (mg/L) in wines

Results are the mean of triplicate analyses

(n.d. = not detected)

Profile of amino acids and amines is not particularly characterizing the type of sample, due to the low number of samples.

1.12.4 Conclusion

This method appears to be suitable for the determination of 25 amino acids and 8 amines in wines.

The optimization of the chromatographic conditions leads to the separation of 33 compounds.

The use of monolithic column reduced the time of analysis by 35 minutes and implies a higher amount of solvent because of higher flows, but can reduce the time of analysis increasing sensitivity.

1.13 A survey of amino acids and biogenic amines in wines produced in the Emilia Romagna region

1.13.1 Aim of work

The evaluation of biogenic amines level in red and white wines produced in the Emilia Romagna region was the main object of the present study.

1.13.2 Experimental

Samples

27 white wines and 37 red wines from 16 different cellars of the Emilia Romagna region were analyzed. 13 white and 33 red wines had a total malo-lactic fermentation and 6 white wines had a partial malo-lactic fermentation.

Chemical analysis

Were performed the following chemical analysis:

- pH, total and volatile acidity, alcohol, malic and lactic acids (Off. J. Eur. 2676/1990);
- reducing sugars (Lane J.H. and Eynon L., 1923);
- total SO_2 (Ripper M. and Schmitt E., 1896);
- tartaric and citric acid (Castellari M. *et al*., 2000);
- amino acids and amines (method up cited).

1.13.3 Results and discussion

64 samples were divided in two groups: white and red wines. The chemical characterization of these samples is shown in table 17. Some statistical differences have been found between the two groups of samples.

		white wines				red wines			
		min	max	mean*		min	max	mean**	
Alcohol	vol %	10,32	14,60	11,76	n.s.	9,67	13,75	12,17	n.s.
Reducing sugars	g/L	< 0,50	59,7	6,67	**b**	< 1,00	4,60	2,16	**a**
pH		2,96	3,57	3,22	**A**	3,09	3,77	3,36	**B**
Total acidity	g/L	3,99	7,85	5,78	n.s.	4,29	9,29	5,89	n.s.
Volatile acidity	g/L	0,14	0,55	0,27	**A**	0,22	0,59	0,40	**B**
Total SO_2	mg/L	23	131	64	n.s.	31	86	60	n.s.
Tartaric acid	g/L	1,40	3,27	2,34	ns	1,68	3,13	2,42	n.s.
Malic acid	g/L	< 0,10	3,48	0,96	**b**	< 0,10	4,16	0,48	**a**
Lactic acid	g/L	< 0,05	3,88	0,93	**a**	< 0,05	3,34	1,36	**b**
Citric acid	g/L	< 0,02	0,42	0,20	n.s.	< 0,02	0,46	0,17	n.s.

Table 17 - Composition of Emilia Romagna wines

Means not identified by the same letters are significantly different at p = 0,05 (small letters) or p = 0,01 (capital letters).

(* = means of 27 samples; ** = means of 37 samples; n.s = not significant)

These differences are due to the different winemaking process, in particular alcoholic fermentation with grape pomace followed by malolactic fermentation for the red wines and a fermentation under controlled temperature and without solid part not followed by malolactic fermentation for the white wines. Reducing sugars are higher in the white wines because of some sweet wines while pH is higher in red wines because malolactic fermentation leads to an increase of pH. Volatile acidity is higher in red wines and this can be due to the higher temperature of fermentation in red wines that determines a different yeast metabolism and an higher production of secondary volatile compounds.

Regards malic and lactic acids, they are affected by malolactic fermentation, which decreases malic acid and increases lactic acid content.

Concerning amino acids and biogenic amines content, the results of the survey are showed in table 18.

	white wines (mg/L)				red wines (mg/L)			
	min	max	mean*		min	max	mean**	
Amino acids								
Glutamine (Gln)	< 0,09	27,8	10,7	n.s.	< 0,09	29,4	14,0	n.s.
Hydroxyproline (HydPro)	3,17	44,3	20,3	n.s.	1,12	42,9	20,3	n.s.
Asparagine + Arginine (Asn + Arg)	< 0,08	105	20,8	n.s.	< 0,08	246	16,9	n.s.
Aspartic acid + Citrulline (Asp + Cit)	< 0,04	16,3	4,7	n.s.	< 0,04	16,1	3,7	n.s.
Serine (Ser)	< 0,06	24,3	5,3	**B**	< 0,06	24,2	1,7	**A**
Glutamic acid (Glu)	< 0,10	44,9	12,5	n.s.	< 0,01	129	16,5	n.s.
Glycine (Gly)	< 0,03	66,8	7,1	n.s.	< 0,03	7,8	2,1	n.s.
Threonine (Thr)	0,06	66,5	31,4	**b**	6,74	40,5	22,8	**a**
Alanine (Ala)	< 0,06	49,1	20,1	**A**	< 0,06	86,2	32,4	**B**
Proline (Pro)	245	1327	490	**A**	280	1197	725	**B**
Tyrosine (Tyr)	15,55	62	34,9	n.s.	4,99	46,8	26,4	n.s.
Valine (Val)	< 0,06	23,2	3,9	n.s.	< 0,06	30,4	6,7	n.s.
γ-Aminobutyric acid (Gaba)	< 0,07	6,5	0,6	n.s.	< 0,07	9,3	0,5	n.s.
Methionine (Met)	< 0,10	21,6	3,4	n.s.	< 0,10	13,8	1,4	n.s.
Tryptophan (Trp)	< 0,12	22,7	6,1	n.s.	< 0,12	19,5	5,4	n.s.
Phenylalanine (Phe)	< 0,07	6,9	1,1	n.s.	< 0,07	7,4	0,9	n.s.
Isoleucine (Ile)	< 0,06	5,5	0,6	n.s.	< 0,06	9,4	0,4	n.s.
Leucine (Leu)	< 0,06	23,7	6,9	**b**	< 0,06	26,3	3,3	**a**
Cysteine + Cystine (Cys + Cys-Cys)	< 0,04	4,2	0,4	n.s.	< 0,04	3,3	0,2	n.s.
Histidine (His)	4,49	163	41,3	n.s.	4,25	105	47,8	n.s.
Lysine (Lys)	0,06	10,9	5,2	n.s.	1,22	13,4	4,9	n.s.
Ornithine (Orn)	< 0,03	18,9	7,2	n.s.	< 0,03	49,0	8,1	n.s.
Amines								
Tyramine (Tyrn)	< 0,08	26,9	3,5	n.s.	< 0,08	11,9	1,1	n.s.
Cadaverine (Cad)	< 0,05	157	14,1	n.s.	< 0,05	97,9	26,9	n.s.
Histamine (Hist)	0,05	45,9	17,8	n.s.	7,07	68,6	19,8	n.s.
Agmatine (Agm)	< 0,01	66,9	8,7	n.s.	< 0,01	39,4	4,4	n.s.
Methylamine (Meta)	< 0,10	< 0,10	< 0,1	n.s.	< 0,10	< 0,10	< 0,1	n.s.
Spermine (Sperm)	< 0,34	< 0,34	< 0,3	n.s.	< 0,34	< 0,34	< 0,3	n.s.
Spermidine (Spermd)	< 0,17	< 0,17	< 0,2	n.s.	< 0,17	< 0,17	< 0,1	n.s.

Table 18 - Contents in amino acids and biogenic amines of Emilia Romagna wines

Means not identified by the same letters are significantly different at p = 0,05 (small letters) or p = 0,01 (capital letters)

(* = means of 27 samples; ** = means of 37 samples; n.s.= not significant)

The content of alanine and proline was higher in red wines. This difference could be due to the longer contact of solid parts with must, that caused a higher extraction of amino acids from the grape pomace, according to Ough C.S., 1971. Also serine, threonine and leucine showed significance differences among red and white wines. This result can be explained taking to account the different winemaking process and the different wine microorganisms, mainly yeast and lactic bacteria.

A variable production of some biogenic amines was found, as presented in figure 3. This is the case of tyramine, histamine, cadaverine and agmatine. The content of tyramine, histidine,

cadaverine and agmatine was low, while methylamine, spermine and spermidine were not detected in all the samples.

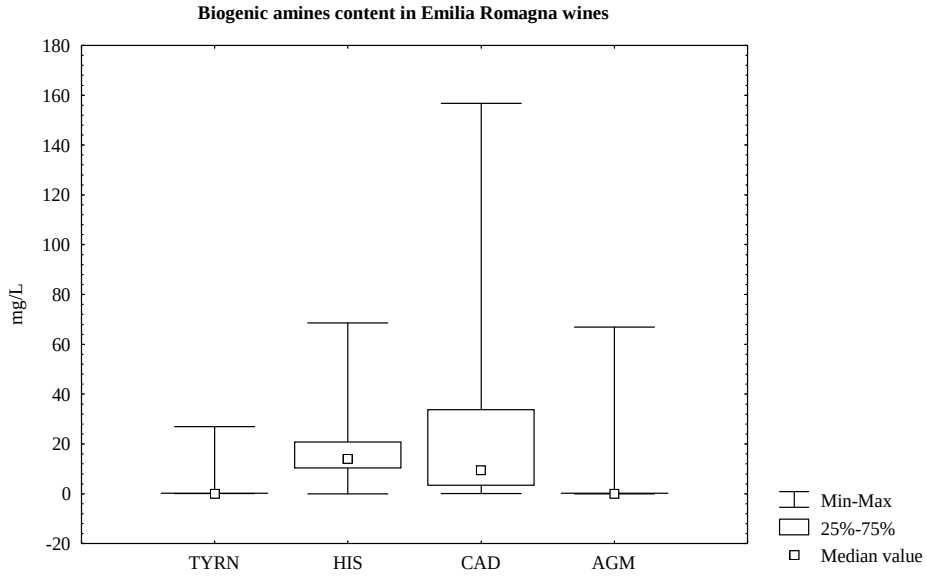


Figure 3

Correlation between malolactic fermentation and amines content was studied. The data of this study didn't show a correlation between lactic acid and biogenic amines content and do not support the opinion that malolactic fermentation has a direct effect on biogenic amine contents of wines, according to Cerutti G. *et al.*, 1987. This study suggests that the biogenic amines formation is related not only to the lactic acid bacteria, in accord to Soufleros E. *et al.*, 1998, but also to yeast strains, according to Torrea Goni D. and Ancin Azpilicueta C., 2001.

At the same time a correlation between ethanol and biogenic amines content was studied. The hypothesis that the biogenic amine content depends on ethanol content was not confirmed by the results.

1.13.4 Conclusion

In conclusion, the investigation on amino acids and amines in wines produced in the Emilia Romagna region permitted to discriminate between red and white wines. Biogenic amines content in Emilia Romagna wines does not represent a possible toxicological problem for human health. Amino acids content is related to the winemaking process, in particular to the presence of grape pomace and malolactic fermentation, according to other Authors (Ough C.S., 1971; Vidal Carou M.C. *et al.*, 1990c.

1.14 Determination of biogenic amines in red wines: influence of enological practices and wine composition

1.14.1 Aim of work

The aim of this work was to evaluate the influence of technological practices on biogenic amines content in red wine. The influence of wine composition (amino acids precursors and pH) and use of different malolactic starters were determined.

Even though the toxicological significance of biogenic amines in wine is still not well established and their exact toxic threshold is difficult to determine, it is prudent to prevent any accumulation in wine. Therefore, an accurate microbiological control is necessary to eliminate or to minimize the activity of the spoilage strains.

1.14.2 Experimental

Material

The study was carried out on cultivar Sangiovese grapes. After the harvest and crushing-destemming of grapes, the product was divided in two parts to evaluate the influence of pH. The pH of the first part was 3,18 (wine A); the pH of the other was adjusted to 3,62 (wine B). Alcoholic fermentation was carried out adding SO_2 (50 mg/L) and selected yeasts (*S. cerevisiae* strain 404 I.M.I.A.). After 7 days of maceration, devatting, pressing and racking of the two wines were carried out. Wine A and wine B are divided in two parts.

In order to study the effect of free amino acids on amines content, a mix of amino acids (lysine: 15 mg/L; arginine: 100 mg/L; histidine: 160 mg/L; phenylalanine: 15 mg/L; ornithine: 50 mg/L; tyrosine 20 mg/L) was added to one part of wine A (+ AA) and to one part of wine B (+ AA). The remaining two parts have not been added.

The amino acids were added instead of having a long contact with yeast lees which increase the extraction of these compounds from the solid parts.

To study the influence of lactic bacteria strain, the 4 samples were divided in two parts, inoculated with different bacteria strains (strain1: *Oenococcus oeni* isolated by C.R.I.V.E. microbiological section with potential skill to produce amines; strain2: MLF Quick *Oenococcus oeni* Oliver Ogar Italy). The trial was made in duplicate (totally 16 wines). The experimental plan was reported in figure 4 (a and b).

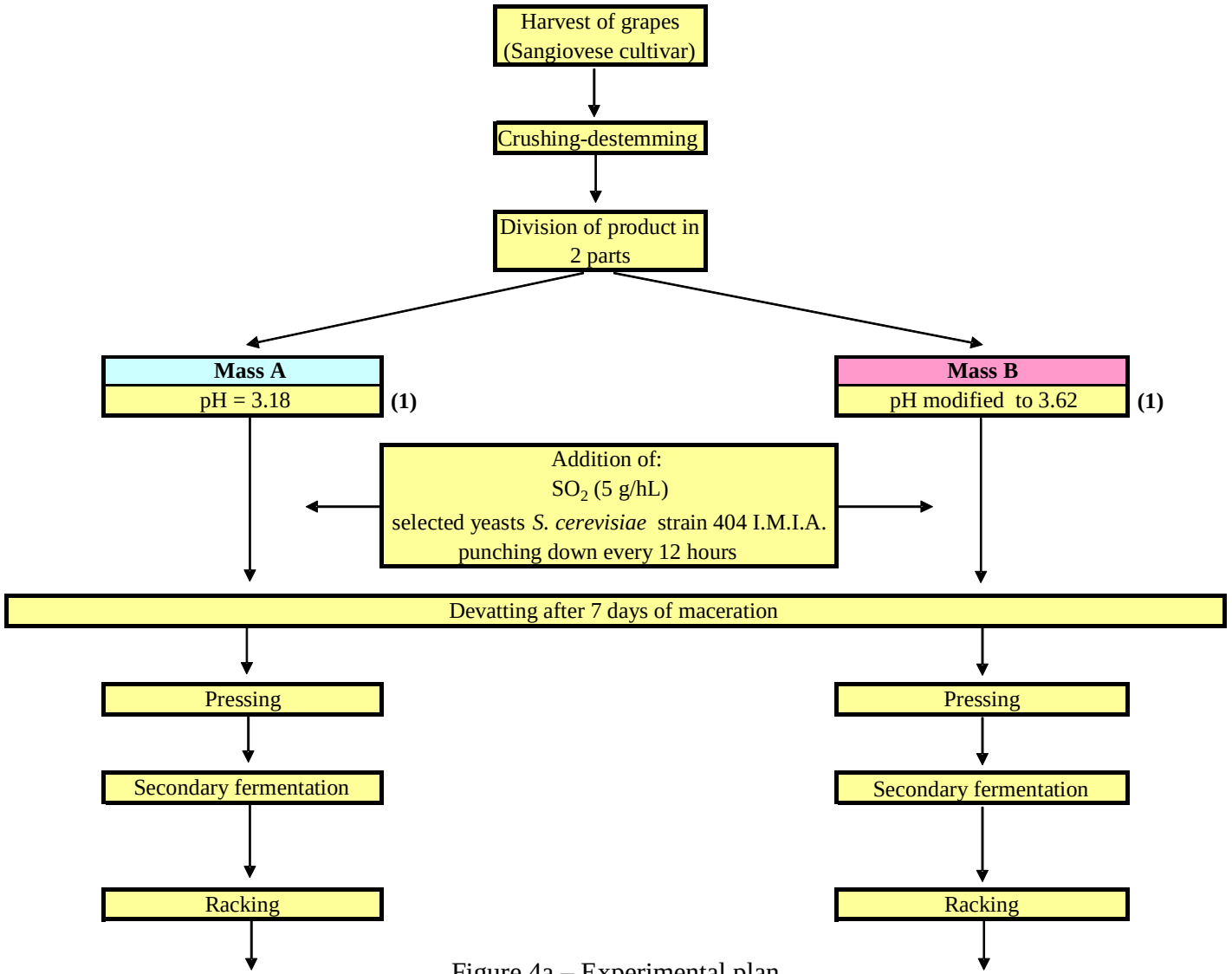


Figure 4a – Experimental plan

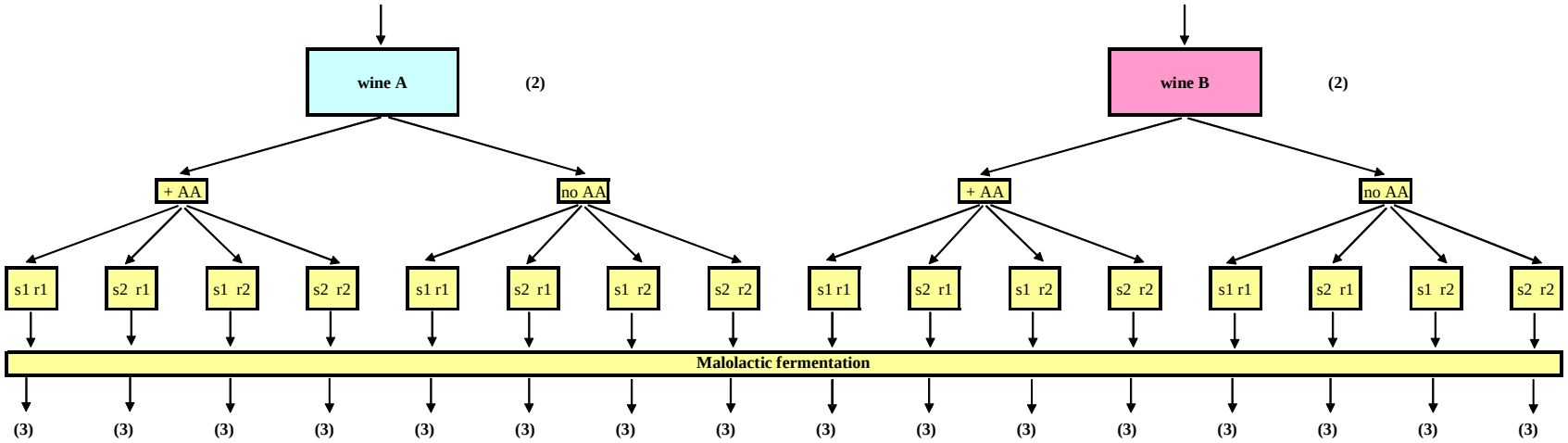


Figure 4b - Experimental plan

1, 2, 3 are the different sampling

+ AA = addition of amino acids

no AA = no addition of amino acids

s1 = strain 1

s2 = strain 2

r1 = repetition 1

r2 = repetition 2

In order to study the differences in the biogenic amines content of wines, 3 different sampling were carried out:

1) the must after inoculation with yeasts and addition of SO_2;

2) the wine at the end of alcoholic fermentation;

3) the wine after malolactic fermentation.

Chemical analysis

The following chemical analysis were performed:

- pH, total and volatile acidity, alcohol, malic and lactic acids (Off. J. Eur., 2676/1990);
- amino acids and amines (method up cited). The method previously described was implemented in order to determine the 2-phenyl ethylamine. The calibration curve and all the parameters able to verify the accuracy and precision of the analytical method were calculated;
- reducing sugars (Lane J.H. and Eynon L., 1923);
- phenolic fraction (Castellari M. *et al.*, 2002);
- total phenols (Ribereau-Gayon P., 1970);
- total and free SO_2 (Ripper M. and Schmitt E., 1896);
- total anthocyans (Margheri G. and Falcieri E., 1972);
- color hue e color intensity (Sudraud P., 1958).

1.14.3 Results and discussion

In this study red wine have been chosen because of the winemaking processes to produce red wine, particularly malolactic fermentation and maceration, affect the development of yeasts and, above all, lactic acid bacteria. Maceration, characterized by diffusion and dissolution phenomena, can create an environment rich in amino acids, increasing the growth of bacteria and decarbossilasic activity.

White wines are less rich in biogenic amines both for their increased acidity (Lonvaud-Funel A., 2001), both for the lower concentration of amino acid precursors due to the intense process of clarification (Guitart A. *et al*., 1998).

As shown in table 19 the masses A and B have a different pH, according to the first phase of the trial which provided the change of pH for the must B, without altering the must composition.

		must A	must B
pH		3,18	3,62
Reducing sugars	g/L	218	219
Total acidity	g/L	5,5	5,6
Total polyphenols	mg/L	423	402
Malic acid	g/L	1,04	1,01

Table 19 - Musts grape composition

Both must A and B have a good sugar content, a good value of total acidity and a balanced amount of malic acid. This value is good to obtain a malolactic fermentation.

The results of the analysis carried out on wines after the alcoholic fermentation on mass A and B are shown in table 20.

		wine A	wine B
Alcohol	% vol	12,97	12,87
Reducing sugars	g/L	2,29	2,51
pH		3,20	3,48
Total acidity	g/L	6,75	5,83
Malic acid	g/L	0,93	0,98
Lactic acid	g/L	0,13	0,20
Total SO_2	mg/L	57	56
Free SO2	mg/L	16	16
Total polyphenols	mg/L	1451	1452
Total anthocyans	mg/L	418	394
o.d. 420 nm		3,65	2,94
o.d. 520 nm		7,22	4,99
Color intensity		10,87	7,93
Color hue		0,51	0,60
Gallic acid	mg/L	15,08	16,13
(+) Catechin	mg/L	3,22	2,83
(+) Epicatechin	mg/L	5,89	5,93
Siringic acid	mg/L	2,98	2,84
Rutin	mg/L	2,53	1,75
Quercetin	mg/L	0,05	0,04
Miricetin	mg/L	2,61	3,20
Cutaric acid	mg/L	0,97	1,04
Caftaric acid	mg/L	9,15	8,06
Amines			
Methylamine (Meta)	mg/L	n.d.	n.d.
Agmatine (Agm)	mg/L	n.d.	n.d.
2-Phenyl ethylamine (Pheta)	mg/L	n.d.	n.d.
Tyramine (Tyrn)	mg/L	n.d.	n.d.
Putrescine (Put)	mg/L	10,44	7,43
Cadaverine (Cad)	mg/L	14,35	11,37
Histamine (Hist)	mg/L	n.d.	n.d.
Spermidine (Spermd)	mg/L	n.d.	n.d.
Spermine (Sperm)	mg/L	n.d.	n.d.
Amino acids			
Glutamine (Gln)	mg/L	45,75	46,08
Hydroxiproline (HydPro)	mg/L	n.d.	n.d.
Asparagine (Asn)	mg/L	11,61	15,00
Arginine (Arg)	mg/L	7,48	6,22
Aspartic acid + Citrulline (Asp + Cit)	mg/L	n.d.	n.d.
Serine (Ser)	mg/L	n.d.	n.d.
Glutamic acid (Glu)	mg/L	n.d.	n.d.
Glycine (Gly)	mg/L	n.d.	n.d.
Threonine (Thr)	mg/L	36,83	41,78
Alanine (Ala)	mg/L	n.d.	n.d.
Proline (Pro)	mg/L	447,3	450,9
Tyrosine (Tyr)	mg/L	9,72	10,05
Valine (Val)	mg/L	n.d.	n.d.
γ-Aminobutyric acid (Gaba)	mg/L	n.d.	n.d.
Methionine (Met)	mg/L	n.d.	n.d.
Tryptophan (Trp)	mg/L	25,22	29,17
Phenylalanine (Phe)	mg/L	9,31	10,17
Isoleucine (Ile)	mg/L	n.d.	n.d.
Leucine (Leu)	mg/L	n.d.	n.d.
Cystine (Cys-Cys)	mg/L	1,31	1,17
Histidine (His)	mg/L	7,21	5,00
Cysteine (Cys)	mg/L	2,97	3,99
Lysine (Lys)	mg/L	37,72	43,83
Ornithine (Orn)	mg/L	n.d.	n.d.

Table 20 - Wines composition after alcoholic fementation

(n.d = not detected)

Analysis performed on phenolic fraction showed a good content in anthocyans, whereas in young wines their concentrations are above 150 mg/L (Margheri G. and Falcieri E., 1972).
The concentration of polyphenols is not very high, as for of Sangiovese *cultivars*.
Wines were obtained by controlling the various stages of the winemaking process and working in good hygienic conditions to prevent the development of wild strains belonging to genera *Pediococcus* that could increase the production of biogenic amines (Aerny J., 1985; Leitao M.C. *et al.*, 2000).
Sample analysis show a low sulphur dioxide content. Sulphiting wasn't carried out after alcoholic fermentation in order to promote malolactic fermentation.
The values of color intensity and color hue are good both in wine A and wine B.
However, it can be see how the value of pH affects the color properties, in fact the wine B, with a higher pH value, has a lower color intensity and higher color hue.
The total anthocyans, responsible for the characteristic red color of wine, have red coloration and their color decreases when the pH increases. This explains why wine A has a higher total anthocyans content than wine B.
A phenolic fraction analysis was carried out to assess the influence of these compounds on the development of lactic acid bacteria, because data found in literature show contradictory results. In 1983, Saraiva R. refers to a stimulation of bacteria by gallic acid, while confirming that different phenolic acids, such coumaric and protocatechic acids, determine an inhibition of the same bacteria (Saraiva R., 1983).
Other data relating to the development of *O. oeni*, have clearly demonstrated an inhibitory effect of vanillic acid and a stimulating effect of gallic acid and free anthocyans (Vivas N. and Lonvaud-Funel A., 1995). Then, the presence of gallic acid seems to encourage the growth of lactic acid bacteria and the start of malolactic fermentation.
The results indicate that the presence of gallic acid, similar in the 2 wines, which could had a positive impact on the grown of lactic acid bacteria.
(+) catechin, (-) epicatechin, syringic acid, rutin, quercetin, miricetin and coutaric and caftaric acids are present in wines in similar quantities.
Regarding the determination of amino acids, the content of each amino acid is very similar, comparing the two wines. This shows that yeast have the same metabolism at the two different pH, according to Charoenchai C. *et al.*, 1998.
Both wines A and B are rich in proline that is, together with arginine, the main amino acid in wines (Ribéreau-Gayon P. *et al.*, 1998b).

The presence of proline in high concentrations can be seen as a marker of wine genuineness authenticity. In fact, instead of the other amino acids, proline is not readily assimilated by yeast during prefermentative phase (cell multiplication) (Fregoni C. *et al*., 2004). After alcoholic fermentation, only putrescine and cadaverine were determined as biogenic amines. Putrescine and cadaverine may be naturally present in grapes (Brodequis M. *et al.,* 1989).

Wines composition after malolactic fermentation (Table 21) confirm its normal evolution.

In both wine A and B (Table 21), pH values increased because of malolactic fermentation, which has its main consequence in "biological deacidification" of wine owing to the transformation of malic acid into lactic acid and carbon dioxide. Data also show that pH has remained different in wines A and B and this result is also supported by statistical data (Table 22).

		wine A +AA s1 r1	wine A +AA s1 r2	wine A +AA s2 r1	wine A +AA s2 r2	wine A s1 r1	wine A s1 r2	wine A s2 r1	wine A s2 r2	wine B +AA s1 r1	wine B +AA s1 r2	wine B +AA s2 r1	wine B +AA s2 r2	wine B s1 r1	wine B s1 r2	wine B s2 r1	wine B s2 r2
Alcohol	% vol	13,02	12,83	12,98	12,88	13,00	12,85	13,04	12,91	12,93	12,81	12,98	12,75	12,98	12,73	12,91	12,76
Reducing sugars	g/L	2,12	2,12	2,08	2,09	2,06	2,04	2,11	2,08	2,53	2,23	2,52	2,28	2,54	2,18	2,48	2,23
pH		3,26	3,36	3,25	3,36	3,23	3,34	3,20	3,27	3,68	3,72	3,69	3,70	3,61	3,61	3,62	3,63
Total acidity	g/L	5,53	5,79	5,62	5,85	5,55	5,82	5,59	5,76	4,85	4,45	4,93	4,33	4,84	4,35	4,89	4,38
Malic acid	g/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lactic acid	g/L	1,24	1,15	1,21	1,18	1,18	1,12	1,08	1,14	1,12	1,12	1,10	1,16	0,99	1,06	1,10	1,06
Total polyphenols	mg/L	1401	1395	1446	1431	1455	1407	1455	1443	1431	1428	1443	1440	1416	1407	1434	1440
Total SO_2	mg/L	49	48	51	46	52	52	50	54	46	47	48	51	49	51	53	49
Free SO_2	mg/L	14	11	13	12	12	13	15	11	15	12	12	11	13	15	15	11
Total anthocyans	mg/L	395	369	382	375	386	382	399	373	372	355	385	368	379	359	378	363
Color intensity		10,09	9,89	10,22	10,10	9,82	9,67	10,18	10,08	7,60	7,45	7,46	7,17	7,75	7,99	7,58	7,77
Color hue		0,63	0,63	0,62	0,65	0,62	0,57	0,58	0,58	0,77	0,77	0,78	0,78	0,76	0,74	0,76	0,75
Amines																	
Methylamine (Meta)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Agmatine (Agm)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-Phenyl ethylamine (Pheta)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4,420	1,390	4,970	3,060	n.d.	n.d.	n.d.	n.d.
Tyramine (Tyrn)	mg/L	0,560	0,530	0,560	0,530	n.d.	n.d.	n.d.	n.d.	2,170	3,760	2,130	3,670	n.d.	n.d.	n.d.	n.d.
Putrescine (Put)	mg/L	11,25	14,10	10,07	8,41	10,52	11,10	9,39	8,47	50,86	46,28	44,45	37,82	8,79	8,28	10,07	11,05
Cadaverine (Cad)	mg/L	15,93	17,03	13,33	17,38	15,36	14,99	14,24	13,33	13,09	16,34	12,61	16,73	9,46	8,11	8,38	8,12
Histamine (Hist)	mg/L	1,070	0,550	0,545	0,550	1,200	0,950	1,250	0,750	34,235	33,960	36,008	45,540	0,428	0,250	0,367	0,350
Spermidine (Spermd)	mg/L	3,463	3,550	2,101	2,960	n.d.	n.d.	n.d.	n.d.	2,888	1,670	2,741	1,680	n.d.	n.d.	n.d.	n.d.
Spermine (Sperm)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0,250	n.d.	0,120	n.d.	n.d.	n.d.	n.d.
Amino acids																	
Glutamine (Gln)	mg/L	43,5	48,6	44,5	41,8	44,2	39,4	39,5	43,7	44,7	46,2	45,8	44,2	45,5	45,4	45,1	45,4
Hydroxyproline (HydPro)	mg/L	55,2	60,1	62,3	58,1	63,1	62,1	58,2	54,2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Asparagine (Asn)	mg/L	10,05	8,800	9,90	7,64	7,85	8,34	8,54	7,35	12,60	12,84	9,00	10,32	12,48	11,80	11,40	11,12
Arginine (Arg)	mg/L	99,14	101,60	97,85	101,68	2,52	1,89	1,85	2,31	96,45	94,55	90,44	92,34	1,79	1,89	1,86	1,68
Aspartic acid + Citrulline (Asp + Cit)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Serine (Ser)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glutamic acid (Glu)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glycine (Gly)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Threonine (Thr)	mg/L	34,2	35,3	35,1	38,4	36,2	34,2	36,9	36,2	43,1	42,6	41,2	39,8	40,3	39,0	43,1	41,3
Alanine (Ala)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Proline (Pro)	mg/L	395	409	469	480	462	474	458	454	467	465	456	449	457	456	465	462
Tyrosine (Tyr)	mg/L	30,9	26,3	28,9	26,6	10,2	8,9	10,8	9,1	27,9	28,0	29,0	26,7	11,2	10,6	11,1	9,5
Valine (Val)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
γ-Aminobutyric acid (Gaba)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Methionine (Met)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Tryptophan (Trp)	mg/L	22,13	24,16	22,31	22,14	23,16	19,32	13,65	12,40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenylalanine (Phe)	mg/L	23,21	24,31	25,46	26,33	11,21	10,32	10,00	10,11	19,85	19,24	20,34	18,25	9,96	9,69	10,21	10,03
Isoleucine (Ile)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Leucine (Leu)	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cystine (Cys-Cys)	mg/L	2,05	2,48	1,78	1,32	1,23	0,96	1,12	0,84	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Histidine (His)	mg/L	161,0	169,3	159,2	165,6	5,1	5,5	4,5	4,2	139,2	125,6	126,1	118,5	5,2	5,2	4,5	4,5
Cysteine (Cys)	mg/L	2,14	2,25	2,11	2,19	2,75	2,85	3,51	4,00	2,06	2,74	2,98	2,63	3,52	3,66	3,68	3,18
Lysine (Lys)	mg/L	54,2	50,3	55,3	50,3	41,2	35,2	40,2	35,2	54,3	49,3	54,6	49,3	41,4	37,3	42,1	39,3
Ornithine (Orn)	mg/L	42,5	43,6	45,3	44,3	n.d.	n.d.	n.d.	n.d.	14,1	16,1	15,5	18,1	n.d.	n.d.	n.d.	n.d.

Table 21 - Wines composition after malolactic fermentation

(+ AA = addition of amino acids; s1 = strain 1; s2 = strain 2; r1 = repetititon 1; r2 = repetition 2; n.d. = not detected)

		pH			strain			+ AA		
		wine A	wine B		1	2		yes	no	
Alcohol	% vol	12,94	12,86	n.s.	12,89	12,90	n.s.	12,90	12,90	n.s.
Reducing sugars	g/L	2,09	2,37	n.s.	2,23	2,23	n.s.	2,25	2,22	n.s.
pH		3,28	3,66	**	3,48	3,47	n.s.	3,50	3,44	n.s.
Total acidity	g/L	5,69	4,65	**	5,17	5,17	n.s.	5,19	5,15	n.s.
Malic acid	g/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
Lactic acid	g/L	1,163	1,089	*	1,123	1,129	n.s.	1,160	1,091	*
Total SO_2	mg/L	50	49	n.s.	49	50	n.s.	48	51	n.s.
Free SO_2	mg/L	13,0	13,0	n.s.	13,1	12,5	n.s.	12,5	13,1	n.s.
Total polyphenols	mg/L	1429	1430	n.s.	1418	1442	**	1427	1432	n.s.
Total anthocyans	mg/L	383	370	*	375	378	n.s.	375	377	n.s.
Color intensity		9,995	7,596	**	8,783	8,809	n.s.	8,736	8,855	n.s.
Color hue		0,611	0,765	**	0,686	0,690	n.s.	0,705	0,671	n.s.
Amines										
Methylamine (Meta)	mg/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
Agmatine (Agm)	mg/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
2-Phenyl ethylamine (Pheta)	mg/L	n.d.	1,755	*	0,764	1,041	n.s.	1,755	n.d.	*
Tyramine (Tyrn)	mg/L	0,298	1,491	n.s.	0,903	0,886	n.s.	1,739	n.d.	**
Putrescine (Put)	mg/L	10,4	27,2	*	19,5	18,1	n.s.	27,9	9,7	*
Cadaverine (Cad)	mg/L	15,2	11,6	*	13,8	13,0	n.s.	15,3	11,5	*
Histamine (Hist)	mg/L	0,86	18,89	*	9,1	10,7	n.s.	19,06	0,69	*
Spermidine (Spermd)	mg/L	1,534	1,147	n.s.	1,471	1,210	n.s.	2,632	n.d.	**
Spermine (Sperm)	mg/L	n.d.	0,084	n.s.	0,075	0,059	n.s.	0,084	n.d.	n.s.
Amino acids										
Glutamine (Gln)	mg/L	43	45	n.s.	45	44	n.s.	45	44	n.s.
Hydroxyproline (HydPro)	mg/L	59,2	n.d.	**	30,1	29,1	n.s.	29,5	29,7	n.s.
Asparagine (Asn)	mg/L	8,6	11,4	**	10,6	9,4	n.s.	10,1	9,9	n.s.
Arginine (Arg)	mg/L	51	48	n.s.	50	49	n.s.	96,8	2,0	**
Aspartic acid + Citrulline (Asp + Cit)	mg/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
Serine (Ser)	mg/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
Glutamic acid (Glu)	mg/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
Glycine (Gly)	mg/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
Threonine (Thr)	mg/L	36	41	**	38	39	n.s.	39	38	n.s.
Alanine (Ala)	mg/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
Proline (Pro)	mg/L	450	460	n.s.	448	462	n.s.	449	461	n.s.
Tyrosine (Tyr)	mg/L	19,0	19,2	n.s.	19,2	19,0	n.s.	28,0	10,2	**
Valine (Val)	mg/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
γ–Aminobutyric acid (Gaba)	mg/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
Methionine (Met)	mg/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
Tryptophan (Trp)	mg/L	19,9	n.d.	**	11,1	8,8	n.s.	11,4	8,6	n.s.
Phenylalanine (Phe)	mg/L	17,6	14,7	n.s.	16,0	16,3	n.s.	22,1	10,2	**
Isoleucine (Ile)	mg/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
Leucine (Leu)	mg/L	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.	n.d.	n.d.	n.s.
Cystine (Cys-Cys)	mg/L	1,472	n.d.	**	0,865	0,658	n.s.	0,979	0,544	n.s.
Histidine (His)	mg/L	84	66	n.s.	77	73	n.s.	146	5	**
Cysteine (Cys)	mg/L	2,73	3,06	n.s.	2,75	3,04	n.s.	2,39	3,39	**
Lysine (Lys)	mg/L	45	46	n.s.	45	46	n.s.	52	39	**
Ornithine (Orn)	mg/L	22,0	8,0	n.s.	14,6	15,4	n.s.	29,9	n.d.	**

Table 22 - Wines composition after malolactic fementation: influence of pH, type of lactic bacteria strain and addition of amino acids

(* = $p < 0,01$; ** = $p < 0,05$; n.s.= not significant; n.d. = not detected)

Depending on different pH, total acidity, keeps a different value in wine A and B, according to the initial treatment of pH modification. This is demostrated also by statistical analysis (Table 22). Even color intensity and color hue maintain the differences showed in table 20, because of the influence of pH on total anthocyans content and wine color (Ribéreau-Gayon P. *et al.*, 1998b). This is demostrated also by statistical analysis (Table 22).

Depending on different pH, total polyphenols values show no statistically significant differences. The difference in pH value does not influence the quantity of extracted polyphenols.

On the contrary, the results obtained from the analysis of total anthocyans show statistically significant differences between two wines, in particular there was a greater concentration of these compounds in wines at low pH (wine A).

These results may be explained by increased precipitation of tartaric acid as potassium acid tartrate at higher pH; the potassium acid tartrate can react with free anthocyans and drags them to the bottom of the tank (Ribéreau-Gayon P. *et al*., 1998a). As a matter of fact, the wine with a higher value of pH (wine A), has a lower content in acids, as a result of a higher precipitation of of tartaric acid salts.

The pH affects concentration of some amino acids, as asparagine and threonine, which are higher in wine B. These amino acids were already higher in wine B before malolactic fermentation because of a higher lytic activity of yeasts in this condition.

The amount of hydroxyproline, tryptophan and cystine, present on the contrary only in wines at lower pH (Table 22). This is probably due to an increase of cellular mass of bacteria in wine B (with a more favourable pH) with consequent increased consumption of amino acids. Moreover, cystine could be also consumed by lactic acid bacteria to produce sulphur compounds (Seefeldt K.E. and Weimer B.C., 2000). Tryptophan was not found in wine B probably because it is converted in tryptamine which was not detected by this method.

The pH statistically affects also biogenic amines, as 2-phenyl ethylamine, putrescine, histamine and cadaverine.

2-Phenyl ethylamine is not produced at low pH, in fact this amine is only determined in wine B.

After malolactic fermentation, also putrescine and histamine are higher in wine B. The relation between pH and amines could be explained considering that at higher pH a greater number of bacteria can develop, thus increasing the opportunity to have strains able to producde amines (Soufleros E. *et al.*, 1998).

Cadaverine, is higher in wine A because this amine was already higher in wine A after alcoholic fermentation.

The influence of type of strain was studied (Table 22). The only parameter influenced by the type of strain is the total polyphenols content as result of statistical elaboration of data. These compounds can be used by bacteria in a different way and positively or negatively influence their growth and metabolism (Scalbert A., 1992; Vivas N. *et al.,* 1997).

Chemical data show that amino acids and biogenic amines are not statistically affected by different strain of lactic acid bacteria inoculated as starters for malolactic fermentation (Table 21). Any hypothetical difference between the two strains couldn't be proved. The strain 2 in wine is less than was less active than in a laboratory scale experiment.

In general, the two strains produced very low or not detectable amounts of amines.

The influence of amino acid precursors on wines composition was evaluated (Table 22). Depending on aminoacidic substrate, the statistical analysis showed significant statistically differences in the content of lactic acid, higher in wines added of amino acids (Table 21). When there are nutrients in wine, lactic bacteria can probably grow better, producing more lactic acid.

Table 22 shows statistically significant differences for following added amino acids: histidine, arginine, tyrosine, phenylalanine, ornithine and lysine.

Some of these amino acids have lower concentrations than the one added, and this could be a consequence of activity of lactic acid bacteria.

Cysteine is also statistically different even if it was not added to wines. This could be due to the higher metabolism of lactic bacteria in a more favourable medium than in one poorer in amino acids.

Regarding biogenic amines, 2-phenyl ethylamine is determined only in wine B added of amino acid. These results are confirmed by statistical analysis (Table 21 and Table 22).

The enrichment with amino acids as nutrient also influences the content of histamine, tyramine, putrescine, cadaverine and spermidine which were estimed in wine A and B added with amino acid precursors. These amines are statistically significant because of their amino acids precursors added to wines: histidine, tyrosine, ornithine, lysine and arginine respectively (Table 21 and Table 22).

A statistical analysis of the interaction between pH and strain of lactic acid bacteria was performed (Table 23).

Color intensity was the only statistically significan parameter. This could be related to a different β-glycosidase activity of bacteria in different pH conditions (Grimaldi A. *et al.*, 2005).

The color intensity decreases when the bond anthocyanin-sugar is broken (Iacobucci G.A. and Sweeny J.G., 1983; Jackman R.L. and Yada R.Y., 1987).

		pH strain	pH + AA	strain + AA
Alcohol	% vol	n.s.	n.s.	n.s.
Reducing sugars	g/L	n.s.	n.s.	n.s.
pH		n.s.	n.s.	n.s.
Total acidity	g/L	n.s.	n.s.	n.s.
Malic acid	g/L	n.s.	n.s.	n.s.
Lactic acid	g/L	n.s.	n.s.	n.s.
Total SO_2	mg/L	n.s.	n.s.	n.s.
Free SO_2	mg/L	n.s.	n.s.	n.s.
Total polyphenols	mg/L	n.s.	n.s.	n.s.
Total anthocians	mg/L	n.s.	n.s.	n.s.
Color intensity		*	*	n.s.
Color hue		n.s.	n.s.	n.s.
Amines				
Methylamine (Meta)	mg/L	n.s.	n.s.	n.s.
Agmatine (Agm)	mg/L	n.s.	n.s.	n.s.
2-Phenyl ethylamine (Pheta)	mg/L	n.s.	**	n.s.
Tyramine (Tyrn)	mg/L	n.s.	**	n.s.
Putrescine (Put)	mg/L	n.s.	**	n.s.
Cadaverine (Cad)	mg/L	n.s.	**	n.s.
Histamine (Hist)	mg/L	n.s.	**	n.s.
Spermidine (Spermd)	mg/L	n.s.	n.s.	n.s.
Spermine (Sperm)	mg/L	n.s.	n.s.	n.s.
Amino acids				
Glutamine (Gln)	mg/L	n.s.	n.s.	n.s.
Hydroxyproline (HydPro)	mg/L	n.s.	n.s.	n.s.
Asparagine (Asn)	mg/L	n.s.	n.s.	n.s.
Arginine (Arg)	mg/L	n.s.	**	n.s.
Aspartic acid + Citrulline (Asp + Cit)	mg/L	n.s.	n.s.	n.s.
Serine (Ser)	mg/L	n.s.	n.s.	n.s.
Glutamic acid (Glu)	mg/L	n.s.	n.s.	n.s.
Glycine (Gly)	mg/L	n.s.	n.s.	n.s.
Threonine (Thr)	mg/L	n.s.	n.s.	n.s.
Alanine (Ala)	mg/L	n.s.	n.s.	n.s.
Proline (Pro)	mg/L	n.s.	n.s.	n.s.
Tyrosine (Tyr)	mg/L	n.s.	n.s.	n.s.
Valine (Val)	mg/L	n.s.	n.s.	n.s.
γ-Aminobutyric acid (Gaba)	mg/L	n.s.	n.s.	n.s.
Methionine (Met)	mg/L	n.s.	n.s.	n.s.
Tryptophan (Trp)	mg/L	n.s.	n.s.	n.s.
Phenylalanine (Phe)	mg/L	n.s.	**	n.s.
Isoleucine (Ile)	mg/L	n.s.	n.s.	n.s.
Leucine (Leu)	mg/L	n.s.	n.s.	n.s.
Cystine (Cys-Cys)	mg/L	n.s.	**	n.s.
Histidine (His)	mg/L	n.s.	**	n.s.
Cysteine (Cys)	mg/L	n.s.	n.s.	n.s.
Lysine (Lys)	mg/L	n.s.	n.s.	n.s.
Ornithine (Orn)	mg/L	n.s.	**	n.s.

Table 23 – Statistical data treatment of interaction between pH and strain; interaction between pH and amino acids addition, interation between strain and amino acids addition for wines after malolactic fermentation

(* = $p < 0,01$; ** = $p < 0,05$; n.s.= not significant)

A statistical analysis of the interaction between pH and amino acids substrate was also performed (Table 23).

Color intensity is statistically different and this is probably due to the influence of higher amounts of biogenic amines in wines at higher pH added with amino acids. The amino compounds can react with anthocyans and influence the color intensity (Garcia-Viguera C. and Bridle P., 1999).

Arginine, phenylalanine, histidine and ornithine are statistically different (Table 23): in particular they are higher in wines with added amino acids as a result of their addition in wines (Table 22). They are higher in wine A than in wine B because at higher pH value, there is a greater consumption of amino acid by lactic acid bacteria. The content of these compounds is therefore affected by the type of wine and the addition of amino acids.

Tyrosine and lysine content is higher in wine A with added amino acids (Table 22), but the results are not statistically different (Table 23).

Considering the amino acids previously described, their corresponding amines (spermine, 2-phenyl ethylamine, histamine, putrescine, tyramine and cadaverine) have a higher content in wine B added with amino acids (Table 22) and are statistically significant, except for spermine (Table 23).

A statistical analysis of the interaction between strain of lactic acid bacteria and amino acids substrate was also performed (Table 23). All the parameters considered were not statistically significant. The presence of amino acid precursors does not seem to modify the metabolism of the two inoculated strains.

1.14.4 Conclusion

The results of studing the influence of technologies on wine composition proved important aspects.

pH is a very important factor for wines: wines with pH > 3,5 showed higher concentration of biogenic amines than wines with lower pH, according to Lonvaud-Funel A. (2001). During winemaking it is very difficult to control wine pH reducing the risk of contamination. As a consequence it is important to consider other factors such as vintage, grape variety, yeast and lactic bacteria type, duration of skin maceration and storage with lees.

Original amino acids content influences the concentrations of biogenic amines according to Soufleros E. *et al.* (1998).

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2. Ochratoxin A

Ochratoxin A (OTA) is a mycotoxin of current interest. Its chemical structure consist of a chlorine-containing dihydroisocumarin linked through the 7-carbonyl group to 1-β-phenylalanine (Figure 5).

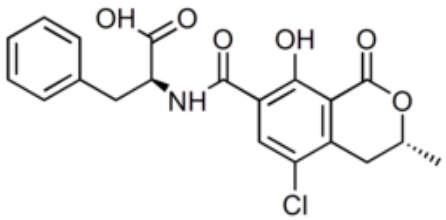

Figure 5 – Structure of ochratoxin A (OTA)

OTA was discovered in 1965 as a secondary metabolite of *Aspergillus ochraceus* strains (Van der Merwe K.J. *et al*., 1965). Later, several other *Aspergillus* and *Penicillium species* were described as producers of this toxin (Varga J. *et al*., 1996).

Aspergillus ochraceus and *Penicilliun verrucosum* are considered the main OTA-producing species. *P. verrucosum* produces OTA in temperate and cold climates and has been reported almost exclusively in cereals and cereal product while *A. ochraceus* is more commonly associated with stored foods in warmer and tropical climates (Pitt J.I. and Hocking A.D., 1997).

However, in the mid-1990s, for the first time, *Aspergillus* section *Nigri* were identified as being able to produce OTA in grapes and wine (Zimmerli B. and Dick R., 1996). Later, within this group, *A. carbonarius* was considered predominantly responsible for the production of OTA in grapes and wine (Bragulat M.R. *et al*., 2001; Battilani P. and Pietri A., 2002; Battilani P. *et al.*, 2006a).

2.1 Natural occurrence of OTA in food

OTA has been widely detected in food of vegetal origin mainly in cereals (barley, wheat, maize, oat, etc.) and their by-products (Speijers C.S. and Van Egmond H.P., 1993; Trucksess M.W. *et al*., 1999), in green coffee (Trucksess M.W. *et al*., 1999) and also in spices (Hubner M. *et al*., 1998).

OTA has also been detected in some drinks as coffee (Bucheli P. *et al*., 1998; Burdaspal P.A. and Legarda T.M., 1998), beer (Jorgensen K., 1998; Bononi M. *et al*., 2004), grapes juices and wines (Zimmerli B. and Dick R., 1996; Visconti A. *et al*., 1999; Cerutti G. *et al.*, 2000; Larcher R. and Nicolini G., 2001; Markaki P. *et al*., 2001; Pietri A. *et al*., 2001; Soleas G.J. *et al*., 2001; Belli N.

et al., 2002 and 2004).
After cereals, wine is considered a major source of daily OTA intake.

2.2 OTA in grape and grape derivates

Several surveys in different countries, Morocco (Filali A. *et al*., 2001), Spain (Burdaspal P.A. and Legarda T.M., 1999, Lopez de Cerain A. *et al*., 2002; Belli N. *et al*., 2004), Italy (Tateo F. *et al.,* 2000; Pietri A. *et al*., 2001; Cecco A. and Bocchi E., 2003; Piracci A. *et al.,* 2005), Switzerland (Zimmerli B. and Dick R., 1996; Argentina, and Brasil (Rosa C.A.R. *et al*., 2002 and 2004; Shundo L. *et al.,* 2006), Japan and Australia (Stockley C.S., 2000; Leong S.L. *et al.,* 2006b) confirmed the contamination of OTA on grape products and wine.
The range of OTA content in wine produced in Europe varied between 0,01 and 3,4 μg/L. Values higher than 0,5 μg/L were reported by several European authors (Burdaspal P.A. and Legarda T.M., 1999; Pietri A. *et al.,* 2001) and also from Morocco (Filali A. *et al.,* 2001).
Only Ospital M. *et al.* (1998) in France and Festas I. *et al.* (2000) in Portugal, reported very low content or absence of OTA, at least in certain good quality wines. A few samples of dessert wine contained between 1 and 3,9 μg/L (Burdaspal P.A. and Legarda T.M., 1999; Pietri A. *et al.,* 2001).
Grape juice, especially from red grapes has been high-lighted as an important source of OTA in children's diets. Values are reported between 1,16 and 2,32 μg/L (Filali A. *et al*., 2001).
Among grape and its derivates, the highest OTA content was measured in dried vine fruits (MAFF, 1997) with more than 40 μg/kg (MacDonald S. *et al*., 1999; MAFF, 1999).
OTA was also found in vinegar, especially balsamic vinegar (Markaki P. *et al*., 2001), although the level was low (0,2 μg/L).
A gradient was observed through regions and wine color; both OTA incidence and concentration were higher in products from southern regions than in wines from northern areas and increased in the order white < rosé < red (Majerus P. and Otteneder H., 1996; Ospital M. *et al.,* 1998; Ottender H. and Majerus P., 2000; Markaki P. *et al*., 2001; Pietri A. *et al*., 2001; Battilani P. *et al.,* 2003a; Mateo R. *et al*., 2007). It is evident that the geographical region of origin has a strong influence on mould contamination and OTA contamination of grapes.
Pietri A. *et al*. (2001) found that wines produced in southern Italy and in the islands were markedly more contaminated than those from the North.

2.3 Sources of OTA contamination in grapes

Fungi responsible for the presence of OTA in grapes have been identified as belonging to the *black aspergilli*, *Aspergillus* section *Nigri*. These species are considered as opportunistic pathogens of grape and may cause bunch rot (sour rot) or berry rots and raisin mould (Varga J. *et al.,* 2004). *Black Aspergilli* usually attack damaged berries. A recent study indicates that *black Aspergilli* are also responsible for vine canker of grapes (Michailides T.J.W. *et al.,* 2002).

Among *black aspergilli*, *Aspergillus carbonarius* is the main producer (Abarca M.L. *et al.,* 2001; Cabanes F.J. *et al*., 2002; Battilani P. *et al*., 2003a). This species is very invasive because colonizes and penetrates berries, even without skin damage.

Apart from *A. carbonarius*, other black aspergilli including the *A. niger* aggregate and *A. aculeatus* have also been found to produce OTA on grapes (Battilani P. *et al.,* 2003b).

Due to their ability to produce OTA at a wide range of temperatures, OTA can be continuously produced in the field. This fact has to be taken into account in commodities such as grapes, raisins and wine, where *A. carbonarius* and member of the *A. niger* aggregate are considered to be the main sources of OTA contamination (Varga J. and Kozakiewicz Z., 2006).

The moulds will develop most rapidly between veraison and maturation. The growth of these moulds is possible at air humidity levels of 70 % to 90 % and temperatures (in the range 12-39 °C, optimum 28 °C). OTA can be found on grapes one month before harvest (Rousseau J., 2004b).

OTA contamination in wine was observed to increase with grape maturity (Rousseau J., 2004b).

In countries with colder temperate climates such as Germany, Northern Hungary, Czech Republic or northern parts of Portugal, France and Italy, *black Aspergilli* have not been isolated from grape berries in spite of the presence of OTA in wine (Abrunhosa L. *et al.,* 2001; Torelli E. *et al*., 2003).

In colder climates, *Penicillium* species were found to be responsible for OTA contamination of several food commodities including cereals (Pitt J.I., 2000). Although *Penicilliun* species are able to grow and produce mycotoxins in must and wine (Moller T. *et al.,* 1997), OTA producing penicillia have rarely been found on grapes. However Battilani P. *et al.* (2001) and Rousseau J. (2004a) identified OTA producing *Penicillium* species from grapes collected in Northern Italy and France suggesting that *Penicillium* species could be responsible for OTA contamination of grapes in these regions (Varga J. and Kozakiewicz Z., 2006).

2.4 Factors influencing production of OTA concentration in wine

Several factors could influence fungal colonization of grapes. Climatic conditions were found to have a significant effect. OTA contamination of grapes and wines were found to vary from year to year even in the same vineyard (Rousseau J., 2004b; Belli N. *et al.,* 2005; Bejaoui H. *et al.*, 2006).

Location of vineyard is also important; the Mediterranean bacin is particularly affected, including southern regions of France and Italy, Greece and certain regions of Portugal and Spain (Rousseau J., 2004b; Belli N. *et al.,* 2005; Tjamos S.E. *et al.,* 2006; Battilani P. *et al.,* 2006b). In a recent study, ochratoxin producing *black Aspergilli* were mainly isolated from vineyard located in southern parts of Portugal characterized by hot and dry summers and hardly any *black Aspergilli* were recovered from vineyards located in northern parts of Portugal where temperatures are moderate during summer (Abrunhosa L. *et al*., 2001; Serra R. *et al.,* 2003). Similarly, OTA producing *black Aspergilli* were only isolated from southern parts of Hungary (Varga J. *et al.*, 2005).

Soil was found to be the main source of inoculation of OTA producing black aspergilli in Australian vineyards. Black aspergilli were isolated more in frequently cultivated soils than in the ones of minimally cultivated vineyards. Additionally, mould counts were higher in soil under vines than in soil between vine rows (Clarke K. *et al.,* 2004).

Health of the grapes is very importat for OTA contamination. Rotted or damaged berries were found to contain more OTA than healthy berries (Rousseau J., 2004b).

Damage can be caused by larvae of grape moth and other insects (*Eudemis, Cochylis* sp.), fungal pathogens and by excessive irrigation or rain damage. Research carried out by the Interprofessionel de la Vigne et du Vin France (ITV France) indicated that larvae of the grape moth (*Eudemis* and *Cochylis* sp.) act as vectors for conidial dispersal of OTA-producing fungi. A positive correlation was observed between the number of perforations caused by these larvae and OTA concentration in grapes (Rousseau J., 2004b).

Recently Battilani P. *et al.* (2004a) found that different grape varieties differ in their susceptibility to *A. carbonarius* colonization and OTA accumulation.

Skin thickness also affect OTA contamination. Grapes with thin and more fragile skin are more susceptible to be contaminated by moulds. This may explain the presence of OTA in wine made with very mature grapes of excellent sanitary quality (Rousseau J., 2004b).

These observations are extremely important, since they open the possibility for breeding grape varieties resistant to *Aspergillus* contamination and OTA accumulation (Varga J. and Kozakiewicz Z., 2006). Climatic factor, water availability (water activity), temperature, grape

varieties, grape bunch shape, susceptibility of vine varieties, aeration level of grape bunch and health status of grapes are the main factors influencing germination, growth and sporulation of these fungi (Magan N. and Lacey J., 1984; Battilani P. and Pietri A., 2002; Mitchell D. *et al.*, 2004).

2.5 Prevention strategies in vineyard

Rousseau J. and Blateyron L. (2002) found that the occurrence of OTA in wine may decrease by about 80 % using appropriate vineyard management.

Battilani P. *et al.* (2004a) suggested that management of *black Aspergilli* in vineyards should focus on the status of berries between early veraison and ripening and on decreasing the incidence of *black aspergilli* in vineyard. Recently Emmett B. *et al.* (2004) have proposed some strategies for management of *A. carbonarius* in vineyards. The preliminary strategies include: 1) producing small loose bunches that are well dispersed through well aerated canopies by the use of vineyard management, vine pruning and irrigation practices; 2) preventing pest damage to berries and bunches, especially between veraison and harvest, 3) minimizing mechanical and environmental damage (e.g. sun burn and rain damages) to berries and bunches; 4) controlling the incidence of *A. carbonarius* in bunches by vineyard floor management (Rousseau J., 2004c). Integration of these strategies were suggested to minimize the development of bunch rot caused by *A. carbonarius* in vineyards, reduce amounts of OTA produced by *A. carbonarius* in grapes and minimize the OTA incidence in wine.

Pest and disease control is very important. Leaves removal could be carried out in the grape cluster zone whilst recognizing the need to limit the risk of sun exposition (this must enable the aeration of clusters). It is also important to: 1) avoid lesions on the berries and skin damage caused by diseases, insects, phytotoxicity and sun exposition; 2) apply vine protection plans in order to control dangerous fungal diseases affecting grape quality (oidium disease, acidic rot); 3) prevent attacks of grape berry moths, grape mealy bugs and grape leafhoppers which promote mould development on damaged berries; 4) apply appropriate and registered protective plans against grape rot and mould. Specific treatment are recommended in all the situations which are favorable to the development of toxin producing species.

A healthy grape harvest can ensure optimal quality and safety of vitivinicultural products. The date of harvest must be decided taking into account grapes ripeness, their sanitary level and forecasted climatic changes and epidemic risk. In OTA high risk areas, it is recommended to advance the harvest date.

To decrease OTA content in wines, removing rotten grapes prior to crushing and pressing should be carried out (Rousseau J., 2004d). Since OTA content of damaged berries was higher than that of the undamaged ones, selecting grapes seems to be the best and natural practice to limit OTA occurrence in wine (Kozakiewicz Z. *et al.*, 2003).

Harvested grapes must be transported as quickly as possible to the winery in order to avoid long waiting, especially for grapes with a high proportion of juice. It is important to clean containers after each load operation, especially in the case of rotten grapes.

2.6 Prevention strategies in winery

Winemaking procedures, which are totally different with respect to red or white wine, can affect OTA concentration in wines. While white grapes are immediately pressed after being picked, red grapes are mashed and skin and juice could be in contact for several days. This stage of the process good conditions exist for mould growth, as long as there is no fermentation and there are aerobic conditions. This thesis is well supported by the observations on red grape juice, which also frequently contains elevated amounts of OTA (Zimmerli B. and Dick R., 1996). This might be due to the fact that the grapes are treated with pectolytic enzymes in order to dissolve natural colorants. During this process there is an intense contact between skin and juice, there is an high temperature as well as no alcoholic fermentation. These factors facilitate mould growth and OTA production.

Gambuti A. *et al.* (2005) evaluated the effect of several enological practices on OTA concentration in wines. They showed that intensive pressing of pomace, prolonged drying of grapes and storage in partially empty tanks increased OTA contamination in wines.

Since 2001, the ICV has followed the evolution of OTA in wines. OTA was measured in grapes, in fermenting must and during wine ageing before and after bottling. This study allowed to predict the evolution of OTA levels on contaminated wines.

OTA appears during the first days of vatting. After crushing, OTA content rapidly increases and, within 4 days, reaches levels similar to those measured in bottle. The maximum is reached after malolactic fermentation. Afterwards, OTA levels decrease, also after bottling (Rousseau J., 2004b).

The use of appropriate technology in food processing may play an important role for reducing the content of OTA in food and beverages. Several physical, chemical and microbiological methods have been proposed to remove mycotoxin from food commodities, but few of these methods have practical application (Blanc M. *et al.*, 1998; Bata A. and Lasztity R., 1999; Heilmann W. *et al.*, 1999).

To reduce OTA in wine, it important to avoid skin maceration in the case of OTA high risk harvest or carry out short maceration adapting pressing rate to the health status of grapes. In the case of contamination, small volumes, low pressures and quick pressing should be used. In the case of contaminated grapes, use of pectolytic enzymes for racking must or maceration should be avoided. Quick clarification with must filtration, centrifugation and flotation are preferable. Heating treatments and aggressive and prolonged macerations should be avoided.

In the case of contamination, it is preferable to use only a low amount of most effective oenological fining agents in order to avoid loss of aromatic and polyphenolic compounds as the consequence of the treatment.

Enological fining agents have been shown to reduce OTA level in wine during the ordinary clarification practice (Dumeau F. and Trioné D., 2000).

In general, adsorption involves the accumulation of molecules from a solvent into the exterior and interior (i.e. pore) surfaces of an adsorbent. The surface phenomenon is a manifestation of complex interactions (van der Waals, resonance and electrostatic forces and hydrogen bonding) between the adsorbent, the adsorbate and the solvent. To achieve adsorption, the interaction between OTA and adsorbent should be stronger than the one between OTA and solvent. The molecular size and the physicochemical properties of OTA clearly affect the efficiency of the binding action. OTA is a weak acid with a pK_a value for the carboxyl group of the phenylalanine moiety of 4,4 (Valenta H., 1998). This implies that OTA is partially dissociated from the pH of wine (ca. 3,5) and carries a negative charge that may interact with a positively charged surface. In addition OTA may also react by means of phenol moiety and carboxylic group. The phenol group could be adsorbed onto a negatively charged surface through hydrogen bonding and/or charge-transfer complexes (Hamaker J.W. and Thompson J.M..,1972). Moreover, adsorption of phenol onto hydrophobic adsorbent (e.g. carbon) is the result of the interaction of two π-electron orbital (Furuya E.G. *et al.*, 1997). Among many relationship used to characterize the solid-liquid adsorption systems, the Freundlich model (Freundlich H., 1926) is purely empirical but it is widely used to describe monolayer adsorption because of its simplicity and versatility and assumes an infinite number of adsorption sites.

Activated carbon has a good capacity to absorb OTA (Galvano F. *et al.*,1998; Castellari M. *et al.*, 2001; Gambuti A. *et al.*, 2005). Activated carbon is an effective adsorbent having a high surface area per unit mass and its adsorption ability varied depending on the activation process. The chemically activated carbon has an irregular surface compared to steam activated carbon (Mazzoleni V. *et al.*, 1986). Therefore, former type of carbon has highest adsorption surface to bind compounds from the media (Castellari M. *et al.*, 2001)

The treatment efficiency depends both on the initial concentration of OTA and the adsorbent amounts (Silva A. *et al*., 2003). It is well-known that carbon also removes anthocyanins and other colored polyphenols from wine. Using high amounts (> 50 g/hL) of carbon irreversibly changes red wine characteristics (color and organoleptic properties) even with preventive addition of oenological tannins (Silva A. *et al*., 2003; Battilani P. *et al.*, 2004b).

Clear differences can be found considering OTA removal efficiency among different active carbons applied at the same dose. The ICV study showed an OTA reduction rate of 50 to 80 %, which was in tight correlation with the adsorbing power of the activated carbons (Rousseau J., 2004d).

Other authorized enological fining agents display poor efficiency with OTA contaminated red o white wines: bentonite, silica gel, gelatin and tannins, used alone or in sinergy, can reduce OTA only 7 to 14 % (Rousseau J., 2004d; Leong S.L. *et al.*, 2006a).

Silica gel positively charged showed a good affinity versus OTA, whereas the silica gel negatively charged was less effective. In fact, OTA is a weak acid partially dissociated at the pH of wine and carries a negative charge. Silica gels and sols are commonly used to remove haze-active proteins in wine.

Bentonite, a layer-aluminum silicate with a negative charged surface showed a relative efficiency compared to carbons and silica gel. However, bentonite removed a little amount of OTA compared to most active fining agents. As wine proteins are usually adsorbed by bentonite, they may interfere with the removal of OTA from wine (Castellari M. *et al.*, 2001).

Leong S.L. *et al.* (2004) suggested that perhaps the presence of grape proteins in wine may determine the efficacy of bentonite fining. This hypothesis was confirmed in a study of Leong S.L. *et al*. (2006b). The addition of proteinaceous fining agents was less effective than bentonite for removal OTA, as they did not enhance precipitation of the grape proteins to which OTA was already bound. Rather, the fining proteins were likely to merely compete with grape proteins for the binding of OTA.

Gelatin and potassium caseinate, which are proteins positively charged at the pH of wine, showed a good affinity for OTA. According to previous findings (Versari A. *et al.* 1998 and 1999), Castellari M. *et al*., (2001) formulated the hypothesis that wine polyphenols could interfere with the adsorption of OTA by fining agents. In particular, gelatin could interact with the negatively charged polymers (e.g. tannins) through hydrophobic interactions.

Filtration improve the reduction of fining agents, but without exceeding 20 % of total reduction of OTA. These treatments do not allow a reduction of OTA levels of highly contaminated wines (Rousseau J., 2004d).

Yeasts or bacteria which have adsorbent properties for OTA, for alcoholic or malolactic fermentation can be used. Different decontamination procedures using *Saccharomyces* strains have recently been proposed for OTA-removal (Bejaoui H. *et al.*, 2004; Caridi A. *et al.*, 2006).

The yeast cell wall is made up of two principal component: β-glucans and mannoproteins. Mannoproteins constitute 25-50 % of the cell wall of *Saccharomyces* and their degree of glycosilation is variable, as in some cases they can contain up to 90 % mannose, with 10 % of peptides being hypermannosylated. Mannoproteins are partially water-soluble components that are released during and, at the end of alcoholic fermentation. The mannoproteins located in the outermost layer of the yeast cell wall make this structure active and have an important role in controlling wall's porosity, regulating the equilibrium of proteins in the environment. At different pH values, the electrical charge of the parietal yeast mannoproteins is different and for the pH of wine, mannoproteins carry negative charges and, as a consequence, they may establish electrostatic and ionic interactions with the other wine components. In contrast to mannoproteins, phenolic compounds carry no or negligible negative charges, so that electrostatic and ionic forces are not determinant to their physico-chemical reactivity. In several yeasts, including the genus *Saccharomyces*, the glycan portion of mannoproteins is composed not only of neutral oligasaccharides containing mannose and N-acetylglucosamine, but also of acidic oligosaccharides containing mannosylphosphate, in quantities which vary from strain to strain. This structural variability may explain the differences in the binding activity of wine yeasts towards phenolic compounds and OTA. Mannoproteins could be implicated in OTA adsorption from contaminated musts, because of their ability to bind mycotoxins. Moreover, strains' ability to bind OTA in physiological sterile saline solution is quite different to their ability in wine. In wine, mannoproteins adsorb not only OTA, but, at different levels of adsorption, also other components such as phenolic compounds (Caridi A. *et al.*, 2006).

Aging on lees can help in reducing OTA level (Fernandes A. *et al.*, 2007). The risk of this technique related to the organoleptic quality of wine must be evaluated.

Sur-lie ageing with batonnage lead to a stronger reduction of OTA than ageing in bottle of the same filtered wine. A hypothesis for this effect is the adsorption of OTA by the mannoprotein of yeasts wall. However, sur-lie ageing is neither appropriate nor applicable to all wine types (Rouseeau J., 2004d).

In conclusion, during winemaking, grapes are submitted to many processing steps and the final result is a decrease in OTA level.

OTA originally present in grapes is partially released into must during crushing and during maceration. During fermentation (either alcoholic or malolactic), OTA content decreases in the

liquid fraction. The clarification (either natural sedimentation or using of fining agents, racking), contribute to OTA decrease, because of its adsorption on the solid sedimented parts. The decrease in OTA due to natural sedimentation is limited, while a decrease due to the use of fining agents can be relevant depending both on the kind and the amount of fining agents (Silva A. *et al.*, 2003).

Grape selection is a preventive measure to control the contamination and good manufacturing practices in winemaking which can effectively reduce contamination.

Grape crushing is a crucial step and OTA measurement should be done at this stage.

Solid-liquid separation, and the fermentative process could effectively reduce OTA. In order to manage the hazards of OTA in winemaking and to verify if OTA content in wine is lower than the legal limit of 2 μg/L defined by the European Commission (EC regulation n° 123/2005 of 26 January 2005), at the end of alcoholic fermentation, OTA analysis in must and wine should be carried out, since the following phases reduce OTA content (Grazioli B. *et al*., 2006).

2.7 Regulation of OTA levels in grape-derived products.

Maximum level for OTA in dried vine fruits (raisin, currants and sultanas) is 10 μ/kg according to Commission Regulation (EC) No 1881/2006 (Official Journal of the European Union, 2006). In the European Union, currently maximum permitted levels of 2 μg/L have been established for OTA in wines and grape must based drinks (Official Journal of the European Union, 2005).

Furthermore, there are also national laws and regulations in the Member States covering other foodstuffs not regulated by European law or other mycotoxins. Some countries and buyers (e.g. Finland, some British supermarkets) also carry out OTA controls and apply their own limits (sometimes as low as 0,5 μg/Kg).

In order to reduce risk associated with OTA content in wine, preventive and corrective measures were taken into consideration through the application of HACCP (Hazard Analysis Critical Control Point) system in an FP5 EU project WINE-OCHRA RISK (Risk Assessment and Integrated Ochratoxin A) (OTA) Management in Grapes and Wine. Contract n. QLK1-CT-2001-01761). The control programme based on the HACCP approach involves strategies for prevention, control, good manufacturing practices and quality control at all stages of production, from the field to the final consumer (Varga J. and Kozakiewicz Z., 2006).

2.8 Biological effects of OTA

OTA is receiving increasing attention worldwide because of the hazard it poses to human and animal health.

OTA is a mycotoxin that has nephrotoxic, carcinogenic, teratogenic, immunotoxic and possibly neurotoxic and genotoxic properties.
In humans, OTA has been implicated as a causal agent of Balkanic Endemic Nephropathy (BEN), a chronic nephropathy described in several rural region of Bulgaria, Romania, Serbia, Croatia and Bosnia and associated with an increased incidence of tumors of the upper urinary tract (Castegnaro M. *et al.*, 1991).
In the 1993, the International Agency for Research on Cancer (IARC) classified OTA into group 2B as a possible human carcinogenic substance (IARC, 1993).

2.9 Analytical methods for the detection of OTA

The basic steps of OTA analysis include sampling, extraction of the toxin from the matrix, purification of the extract (clean-up) and concentration, separation, detection, quantification and confirmation of positive findings. Clean-up and concentration are usually necessary when low detection limits are required (Festas I. *et al*., 2000). Clean-up can be carried out by liquid-liquid partitioning using aqueous Na-bicarbonate or by solid-phase extraction (SPE) (Valenta H., 1998), but sometimes the cleaning effect is not suitable for the complexity of matrices.
Ospital *et al*. (1998) obtained satisfactory results in term of recoveries and sensitivity, operating a sample clean-up with silica gel SPE cartridges. Among the recent improvements the application of a molecularly imprinted SPE method is notable (Maier N.M. *et al*., 2004). One of the main advantages is that the polymer can be reused in contrast with immunoaffinity columns. Saez J.M. *et al*. (2004) developed a polyethylene glycol based extraction method which is relatively simple, rapid and does not require the use of organic solvents, while Gonzalez-Penas E. *et al*. (2004) developed a micro-extraction method which was suggested to be an inexpensive alternative to immunoaffinity columns.
Monoclonal antibody based immunoaffinity columns (IACs) were developed to substitute the traditional solvent clean-up (Sharman M. *et al*., 1992). The main advantage of these columns is that OTA is bound specifically to the antibody and the matrix interferences can be removed nearly completely. Furthermore, IACs give an optimal performance in terms of precision and accuracy within a wide range of concentrations and they also reduce the use of dangerous solvents (Visconti A. *et al*., 1999). Nowadays different types of immunoaffinity columns are commercially available for the analysis of OTA: OchraTest (Vicam, USA), Ochraprep (Rhone-Diagnostic Technologies, UK), RIDA Ochratoxin (R-Biopharm, Germany) and OchraStarTM Immunoaffinity Columns (Romer Labs Diagnostic GmbH, Austria). Castellari M. *et al*. (2000) compared three immunoaffinity clean-up procedures one direct wine clean-up with IACs and one

IACs clean-up with a preliminary chloroform extraction as in the method proposed by Zimmerli B. and Dick R. (1995). All these procedures gave comparable results in terms of accuracy and precision, limit of detection and quantification for OTA. The time of analysis was reduced if compared with the reference procedure involving a preliminary extraction of OTA with chloroform.

More recently Siantar D.P. *et al.* (2003) compared the performance of IA and SPE columns discovering that IACs give higher recoveries compared to C18 or cross-linked polymer-based SPE columns.

The detection and quantification of OTA can be carried out by conventional reversed-phase HPLC or enzyme-linked immunosorbent assays (ELISA), obtaining different results and detection limits depending on sample matrix complexity. Chromatographic separation has been normally performed using RP-C18 columns and isocratic elution with diluted acidified acetonitrile (Valenta H., 1998) while the analysis includes HPLC with fluorescence detection (Ospital et al. 1998). Later, Brera C. *et al.* (2003) developed an automated HPLC method for OTA determination in wines, while Dall'Asta C. *et al*. (2004) developed a simple reversed-phase HPLC technique which can be applied directly to wine samples without extraction or clean-up.

Leitner A. *et al.* (2002) compared different analytical methods for OTA determination in wine and found that SPE combined with HPLC-tandem mass spectrometric (MS-MS) detection and immunoaffinity clean-up combined with HPLC-fluorescent detection offered comparable good results. A stable isotope dilution assay using HPLC-MS-MS has also been developed recently; this technique is relatively expensive but provides excellent accuracy (Shepard G.S. *et al.*, 2003; Lindenmeier M. *et al*., 2004). Besides, chromatographic techniques and immunochemical methods have also been developed for rapid screening of OTA in food commodities (Barna-Vetrò I. *et al.*, 1996; Yu F.Y. *et al*., 2005; Zheng Z. *et al.*, 2005). The combination of IACs and ELISA detection was found to be effective and in compliance with the 2 μg/L allowable maximum level established by the European Union. In a recent inter-laboratory survey, the ELISA method was successfully used to determine OTA content in wines finding a comparable amount to that obtained by using HPLC (Da Rocha C.A.R. and de Souza C.S., 2005).

Currently, the method recommended for OTA determination in wines and beer (European Standard prEN 14133) uses IACs columns to clean up OTA after dilution of the samples in an aqueous solution of polyethylene glycol and $NaHCO_3$ and the samples are analyzed by HPLC with fluorescent detection (Visconti A. *et al*., 1999).

2.10 Evaluation of different clean-up and analytical methods for the determination of ochratoxin A

2.10.1 Aim of work

The aim of this study was a comparative evaluation of 3 analytical methods for the determination of OTA in wine.

Analytical methodologies were compared in terms of precision and accuracy.

2.10.2 Experimental

Samples

A total of 37 wines were analyzed. 10 of these wines didn't contain OTA while the others were spiked with different concentration of OTA (1,11; 1,31; 1,51; 1,81; 2,01; 3,01; 4,02; 6,03; 8,04 µg/L). The samples were prepared and analyzed in triplicate.

Each sample was analyzed in order to compare the different clean-up.

The comparison between the 3 different clean-up was performed for all the samples, while the corresponding analytical determinations were carried out in HPLC.

A comparison between 2 analytical techniques (HPLC and ELISA) was carried out only on samples extracted with SPE Mycosep and LLE.

The experimental plan is well detailed in figure 6.

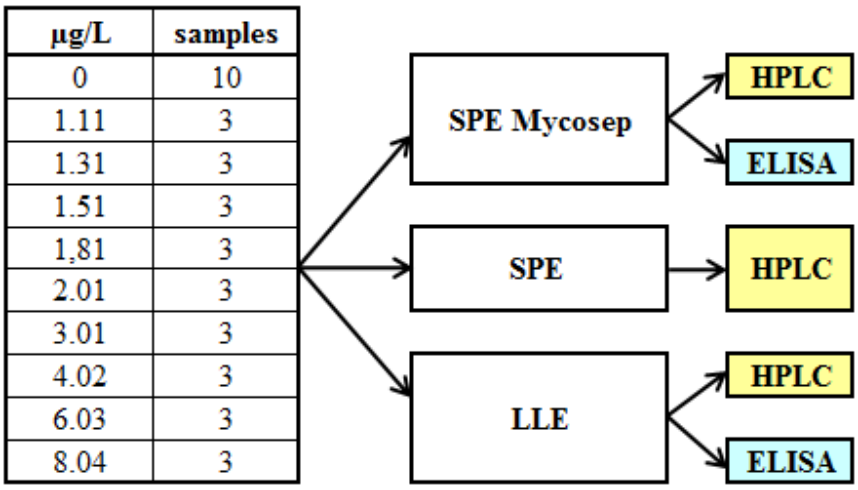

Figure 6 - Experimental plan

Apparatus

LC system - PU-980 pump (Jasco International, Tokyo, Japan) connected to FP-1520 fluorescence detector (Jasco International, Tokyo, Japan). Sample injection was made with a 7725 valve (Rheodyne, Cotati, CA, USA) equipped with a 100 µL loop.

Chromatographic column - Inertsil RP-ODS-2 (GL Science, Tokyo, Japan) column (250 x 4,0 µm I.D., 5 µm) was used. The column was protected by an inline C18 Security Guard (4,0 x 3 mm I.D., 5 µm) cartridge system (Phenomenex, Torrance, CA, USA). The column was kept at 35 °C using a heater 7980 (Jones Chromatography, Hengeod, UK).

Data collection system - Data acquisition and handling were carried out using the Borwin 1,5 software (JMBS Developments, Grenoble, France).

Mobile phase - water-acetonitrile-acetic acid (49,5/49,5/1, v/v/v). Acetonitrile, methanol, water and acetic acid were furnished by Sigma-Aldrich (Merck, Darmstad, Germany).

Extraction cartridges - OchraStar™ Immunoaffinity Columns were taken from Romer Labs Diagnostic GmbH (Austria). MycoSep® 229 Columns were taken from Tecna S.r.l, Trieste, Italy.

Kit I'screen OCHRA ELISA - a quantitative immunoassay for the detection of ochratoxin A was obtained from Tecna S.r.l., Trieste, Italy.

Chemicals and materials

OTA standard - A stock solution of OTA (1000 µg/L) dissolved in benzene-acetic acid (99:1, v/v) was furnished by Rhone Diagnostic Technologies (Glasgow, UK). The purity of this standard was checked by UV at 333 nm in benzene-acetic acid (99:1), considering a molar adsorption coefficient (ε) of 5550 $M^{-1}cm^{-1}$.

OTA standard solutions - The working standard (ranging from 0,05 to 20 µg/L) were prepared by evaporating under nitrogen the stock solution and dissolving the residue in an appropriate volume of mobile phase.

Phosphate-buffered saline (PBS) – The buffer was prepared adding potassium chloride (KCl) (0,2 g), potassium dihydrogenphosphate (KH_2PO_4) (0,2 g), anhydrous disodium hydrogenphosphate (Na_2HPO_4) (1,16 g) and sodium chloride (NaCl) (8,0 g) to 900 mL of distilled water. Then the pH was adjusted to 7,4 and the solution made up to 1 L.

All buffer salts were purchased from Carlo Erba (Milan, Italy).

Water - Purified distilled, deionized in a Milli-Q purification system (Millipore, Bedford, MA).

Sodiumhydroxide 2 M - Was prepared adding 21,198 g in 100 mL of distilled water (Merck, Darmstad, Germany).

Ammonium acetate solution 0,2 M - Was prepared adding 15,4168 in 1 L of distilled water for the washing of columns (Merck, Darmstad, Germany).
Methanol/acetic acid 98/2 (v/v) for the elution of columns (Merck, Darmstad, Germany).
Chloridric acid 1 M - Carlo Erba (Milan, Italy).
D*ichloromethane* - (Merck, Darmstad, Germany).
Sodium bicarbonate solution 0,13 M - Was prepared adding 10,9213 g in 1000 mL of distilled water (Merck, Darmstad, Germany).

Extraction and Cleanup

Extraction and sample cleanup were performed using 3 different methods. These were evaluated for linearity and precision.

1) The first method was a solid-liquid extraction (SPE). 5 mL of wine were combined with 0,2 mL of glacial acetic acid and 15 mL of acetonitrile; the solution was handly shaken for 30 seconds and 5 mL of this solution was transferred into a glass tube; forcing the extract to filter upwards through the packing material of the column (MycoSep). The interferences were adhered to the chemical packing in the column and the purified extract was passed through the membrane (about 1 mL of filtered extract); 400 µL of purified extract were transferred in to a vial; the sample was evaporated under nitrogen at 65 °C; once completely dry, the sample was redissolved in 200 µL of sodium bicarbonate. The dilution factor was 2.

2) The second method was a direct clean-up on OchraStar^{TM} immunoaffinity column (IACs). Each immunoaffinity column was at first washed with 5 mL of PBS at pH 7,4 before use. Then 10 mL of wine adjusted to pH 7,8 using 2 M NaOH were diluted with 10 mL of PBS. 4 mL of this solution were applied directly to the IAC, at flow-rate of about 1-2 drops/s. After the diluted extract had completely passed through, the column was washed with 10 mL of PBS at flow-rate of 3-4 drops/s. Column was successively washed with 2 x 10 mL of 0,2 M ammonium acetate solution at flow-rate of 3-4 drops/s. Any remaining liquid was removed from the column through slight negative pressure from below, while the column was not allowed to dry. The syringe barrel was removed from the IACs and a suitable vial placed under the column for the collection of the elute. For the elution of OTA 2 mL of methanol/acetic acid 98/2 (v/v) were added. The methanol was left on the column for a short period of time before letting it run off. The column was dried under a gentle stream of air. The eluate containing OTA was collected and mixed with 2 mL of mobile phase before the HPLC analysis.

3) The third method was a liquid/liquid clean-up (LLE). 5 mL of HCl 1 M were added to 5 mL of the wine sample and 10 mL of dichloromethane were also added. The solution was shook for 15

min on a low speed shaker (400 rpm) and centrifuged at 2200 g. Two phases were formed: organic (bottom) and aqueous (top). 5 mL of the organic phase were taken and 2,5 mL of the sodium bicarbonate solution (0,13 M) were added; the solution was shook for 15 min on a low speed shaker (400 rpm) and centrifuged at 2200 xg. The aqueous phase was taken and centrifuged again for 15 min at 2200 xg to separate it from any residual solvent. The aqueous phase was diluted in 2 times with the bicarbonate solution (1 mL of aqueous phase + 1 mL of sodium bicarbonate). The dilution factor was 2.

Liquid Chromatography

The extracts were analyzed by a reversed-phase isocratic HPLC with mobile phase water-acetonitrile-acetic acid (49,5/49,5/1, v/v/v) at 0,75 mL/min. Eluent was freshly prepared and filtered (0,22 μm) before use. Detection was made working at an excitation wavelength of 333 nm and an emission wavelength of 460 nm.

For the quantitative analysis, a calibration curve was constructed by injecting seven solutions containing known amounts of the pure standard ranging from 0,05 to 20 μg/L of OTA.

Kit I'screen OCHRA ELISA

The assay is performed in polystyrene micro-wells which have been coated with antibodies (IgG) and anti-IgG of rabbit. OTA standard solution or sample, the enzyme conjugate ochratoxin-HRP and the specific antibody anti-Ochratoxin A were added to the micro-wells. During the incubation, free ochratoxin-A molecules and ochratoxin-HRP compete for the anti-ochratoxin antibodies binding sites. The anti-ochratoxin antibodies are simultaneously bound to the solid phase. Any unbound enzyme ochratoxin-HRP is then removed in a washing step. The bound enzyme (HRP) activity is determined by adding a fixed amount of a chromogenic substrate: the enzyme converts the colorless chromogen into a blue product and the addition of the stop reagent leads to a color change from blue to yellow. The absorbance is measured by a microplate reader at 450 nm. The color development is inversely proportional to the OTA concentration in the sample. The detection limit of the kit I'screen OCHRA in wine and grapes is 0,1 ppb (Brera C. *et al.*, 2004). The result is based on the calculation of the mean absorbance of blank, standards and samples. The mean absorbance value of each standard and sample, subtracted of the mean absorbance value for the blank, is divided by the mean absorbance of maximum binding (B_0) and multiplied by 100. The maximum binding is thus made equal to 100 % and the absorbance values is quoted in percentage.

Absorbance of standard (or sample)/ absorbance of maximum binding x 100 = B/B_0 (%)

The (%) B/B_0 values calculated for each standard against the ochratoxin A standards concentration are entered in a semi-logarithmic system of coordinates and the curve was marked. The B/B_0 value is interpolated for each sample to the corresponding concentration on the calibration curve. The concentration of ochratoxin A in the samples was obtained from the concentration from the calibration curve multiplied by the dilution factor, that for wine was 2.

2.10.3 Results and Discussion

Linearity

Linearity of response (peak area versus the injected analyte amount) was obtained by injecting in HPLC different concentrations of analyte ranging from 1,11 to 8,04 µg/L and reading the absorbance at λ = 460 nm. Only SPE (MycoSep) and LLE extracted samples were also analyzed by the ELISA method. In the last one, linearity of response was determined using the difference of absorbance versus concentration.

To construct a regression curve and calculate the determination coefficients, measurements were done in triplicate at each concentration (Table 24).

Extraction method	**Regression equation**	**r^2**
SPE (MycoSep) HPLC	y = 0.00022533x - 0.4430	0,9997
SPE (IAC) HPLC	y = 0.00002819 - 0.0652	0,9999
LLE HPLC	0.00001407x - 0.0211	0.9964
SPE (MycoSep) ELISA	0,1037x exp -2,5572	0.9048
LLE ELISA	0.0862x exp -1.3594	0.9755

Table 24 - Regression equation and determination coefficients of ELISA and HPLC methods

The immunoaffinity clean-up showed the best linear fit (r^2 = 0,9999), followed by SPE (MycoSep) (r^2 = 0,9997) and LLE. The ELISA method showed very bad results with a linear regression; therefore an exponential equation to obtain an acceptable fitting with SPE clean–up and a good fitting with LLE was used.

Recoveries and Precision

Recoveries were made using spiked samples at different concentration as detailed in the experiment plan, taking to account the known amount of OTA originally present.

The best recoveries were obtained with IACs (88-115 %) both at high and low concentrations of OTA even if also SPE (MycoSep) showed very good recoveries (84-126 %), while LLE obtained lower recoveries (64-96 %), especially for OTA contaminated samples near the legal limit (Table 25), as referred in other works (Gonzalez-Penas E. *et al.,* 2004).

	Spiked concn (µg/L)	Avg. (µg/L)	Standard deviation	Coefficient of variation (%)	Mean recovery (%)
SPE (MycoSep) HPLC	1.11	0.936	0.034	3.6	84
	1.31	1.285	0.064	3.6	98
	1.51	1.307	0.065	5,0	87
	1.81	1.747	0.120	6.9	97
	2.01	2.197	0.076	3.4	109
	3.01	3.381	0.294	8.7	112
	4.02	4.304	0.091	2.1	107
	6.03	6.776	0.328	4.8	112
	8.04	10.128	1.109	10.9	126
SPE (IAC) HPLC	1.11	1.063	0.041	3.9	96
	1.31	1.153	0.037	3.2	88
	1.51	1.334	0.080	6.0	88
	1.81	1.762	0.086	4.9	97
	2.01	1.915	0.131	6.8	95
	3.01	3.331	0.286	8.6	111
	4.02	4.287	0.423	9.9	107
	6.03	5.928	0.543	9.2	98
	8.04	9.281	1.062	11.4	115
LLE HPLC	1.11	0.908	0.006	0.6	82
	1.31	0.870	0.026	3.0	66
	1.51	0.963	0.074	7.7	64
	1.81	1.183	0.061	5.2	65
	2.01	1.399	0.039	2.8	70
	3.01	2.224	0,187	8.4	74
	4.02	3.448	0.068	2.0	86
	6.03	4.944	0.102	2.1	82
	8.04	7.708	1.182	15.3	96
SPE (MycoSep) ELISA	1.11	1.071	0.071	6.6	96
	1.31	0.917	0.042	4.5	70
	1.51	0.998	0.107	10.7	66
	1.81	1.061	0.113	10.6	59
	2.01	1.427	0.040	2.6	71
	3.01	2.043	0.256	12.5	68
	4.02	2.751	0.424	15.4	68
	6.03	4.817	0.733	15.2	80
	8.04	5.770	1.740	30.2	72
LLE ELISA	1.11	0.911	0.094	10.3	82
	1.31	1.232	0.120	9.7	94
	1.51	1.156	0.148	12.8	77
	1.81	1.417	0.051	3.6	78
	2.01	1.790	0.173	9.7	89
	3.01	2.178	0.035	1.6	72
	4.02	3.005	0.241	8.0	75
	6.03	3.253	0.037	1.1	54
	8.04	4.195	0.228	5.4	52

Table 25 - Comparison of different clean-up and determination methods in terms of accuracy and precision.

In HPLC analysis the precision was good (< 10 %) for the samples of each clean-up apart from the ones spiked with the highest amount of OTA.
Analysing with the ELISA method the samples cleaned up with Mycosep and the ones extracted with LLE, recoveries and precision became worse (Table 25).
Particularly, LLE showed the best recoveries (72-94 %) especially with samples spiked with an amount of OTA near the legal limit, while became worse at maximum levels. Using SPE MycoSep similar recoveries were obtained (59-96 %), even if the technique showed lower recoveries at lower concentrations.
Precision was not so good because of the variation coefficients (CV) going from 1,1 to 12,8 % for LLE and from 2,6 to 30,2 % for SPE MycoSep.
Considering the HPLC analysis, the three clean-up procedures, (Table 26) showed a good correlation. The two SPE clean up gave comparable results while LLE underestimated OTA concentrations.

Clean-up method	Regression equation	r^2
SPE (IAC) vs SPE (MycoSep)	y = 1.1155x - 0.1088	0.9878
LLE vs SPE (IAC)	y = 1.1534x + 0.2786	0.9848
LLE vs SPE (MycoSep)	y = 1.2989x + 0.1732	0.9914

Table 26 - Regression equation and determination coefficients of the 3 clean-up analyzed by HPLC

Comparing the samples taken from the clean-up procedures with two different methods of analysis, the ELISA method (Table 27) gave lower results than HPLC analysis, apart from a discrete correlation.

Determination methods	Regression equation	r^2
HPLC vs ELISA (SPE)	y = 0.5634x + 0.2800	0.8685
HPLC vs ELISA (LLE)	y = 0.4550x + 0.9348	0.8997
ELISA (SPE vs LLE)	y = 0.5688x + 0.8109	0.8745

Table 27 - Regression equation and determination coefficients of the 2 clean-up analyzed by HPLC and the ELISA quantification methods

2.10.4 Conclusion

The results above discussed proved that the 2 SPE clean-up are reliable at the same level, while the LLE procedures showed results less good. The ELISA method gave a lower determination and a low reproducibility than HPLC method instead of what evidenced by other Authors (Wilkes J.G. and Sutherland J.B., 1998).

LLE and ELISA could be combined in screening analysis of a great number of samples, because they are faster and less expensive than the SPE/HPLC methods.

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