Central Oenology Laboratory

Department of Chemical Engineering

Oenological Performance Evaluation of Yeast Strains – Implementation of an Analytical Method for Biogenic Amines in Wines by HPLC-Fluorescence

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Integrated Master in Bioengineering
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Sogrape Vinhos

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Abstract

Biogenic amines are low molecular weight organic compounds derived from amino acids that may be formed throughout the winemaking process. Their presence in wine is undesirable due to the hazardous effects they may cause on human health.

This Thesis presents the implementation of a method for analysis of levels of five of the main biogenic amines (histamine, tyramine, phenylethylamine, putrescine and cadaverine) in wine. In order to accomplish that, the method makes use of an online pre-column derivatization of the samples with ortho-phthalaldehyde (OPA). An elution gradient of 50 minutes allows the separation of the amines for consequent quantification. Regarding the limits of detection and quantification, 1.5 ± 0.9 mg/L and 5 ± 3 mg/L, respectively, were obtained while achieving an average recovery percentage and precision (CV%) of 85 ± 15% and 9 ± 4%, respectively.

The developed method was applied to 26 wines.

Samples from malolactic fermentation trials revealed that this phase has a detrimental effect on the contents of biogenic amines registering increases in histamine, putrescine and specially tyramine (from not detected to 29 ± 16 mg/L) when compared to wine samples in which malolactic fermentation did not take place. However, the results are not consistent with the values found for other red wines that also experienced malolactic fermentation. Therefore, other features are taking part in biogenic amine formation.

For the white wines tested the variety effect seems to override strain specific production of biogenic amines. Absolute values of biogenic amines in these wines vary from non detected in most cases for tyramine to a maximum of 3.9 mg/L found for histamine.

Another part of the work consisted on making an evaluation of the oenological performance of five yeast strains previously selected from other FERMDIF projects (386, 496, 666, 765 and QA23) over three white grape varieties (*Loureiro*, *Viosinho* and *Encruzado*).

The strain with best performance regardless of the variety was strain 666. For varieties Viosinho and Encruzado the worst strain was strain 765 while for Loureiro strain 496 has given the worst results. Biogenic amine production evaluated in these combinations of strain and variety resulted in the lowest values attributed to strain 765, average for strains 666 and QA23 and the highest for strains 386 and 496. Viosinho was the variety for which overall biogenic amine production was lower.

Key Words: Biogenic amines, HPLC, OPA, wine

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Notation and Glossary

List of Acronyms

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
CV%	Coefficient of Variation (expressed in percentage)
FERMDIF	*Fermentação Diferida* (Deferred fermentation)
GUI	Graphical User Interface
HPLC	High Performance Liquid Chromatography
HPLC-FL	High Performance Liquid Chromatography with Fluorescence detection
HPLC-MS	High Performance Liquid Chromatography associated with Mass Spectrometry
HPLC-RI	High Performance Liquid Chromatography with Refraction Index detection
LOD	Limits of Detection
LOQ	Limits of Quantification
MLF	Malolactic Fermentation
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate (oxidised form)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
n.d.	Not Detected
NIR	Near Infrared
OIV	*Organisation Internationale de la Vigne et du Vin* (International Organisation of Vine and Wine)
OPA	Ortho-phthalaldehyde
STD	Standard Deviation
TAV	*Titre Alcoométrique Volumique* (Alcoholic strength by volume - % vol.)
UV	Ultraviolet Light

1 Introduction

1.1 Background and Presentation of the Project

This work is a Master's Thesis project included in the Dissertation subject programmed to the second semester of the fifth year of studies of the Master in Bioengineering, Branch of Biological Engineering which was undertaken in business environment during an internship in the Central Oenology Laboratory of Sogrape Vinhos SA at the company's headquarters facility in Freguesia de Avintes, Vila Nova de Gaia.

The objective of this work was to develop, validate and implement a method for analysis of biogenic amines which turned out to make use of a High Performance Liquid Chromatography technique coupled with Fluorescence detection (HPLC-FL) as well as a sample pre-treatment consisting of a derivatization reaction with ortho-phthalaldehyde (OPA). The developed method was then used to analyze samples from an ongoing FERMDIF (*Fermentação Diferida*, Portuguese expression for deferred fermentation) project carried out in Quinta dos Carvalhais, property of Sogrape Vinhos.

The latter project is on the subject of the analysis of oenological and fermentative performance of several strains originating from different demarcated regions (Vinho Verde, Douro and Dão). These strains were used in three white-grape varietal musts: Loureiro from Quinta de Azevedo, Viosinho from Quinta do Cavernelho and Encruzado from Quinta dos Carvalhais. The strains tested were the *Saccharomyces* strains 386, 496 and 666, non-*Saccharomyces* strain 765 and the commercial *Saccharomyces* QA23 as control.

It was also part of this work to appraise glucose and fructose contents of samples from previously accomplished fermentation trials. Results were obtained by HPLC-RI (HPLC with refraction index detection) and, together with provided results from NIR spectroscopy (Near-Infrared) analysis of the alcoholic content, evaluation of the oenological performance of the given strains was also made.

Additionally, the wines resulting from the fermentation trials integrate the set of samples appointed for biogenic amines analysis. The mentioned strains were previously selected from precedent projects under the auspices of Sogrape Vinhos regarding a vast collection of strains isolated from properties/vineyards owned by the company.

1.2 Contributions of the Work

From the two components of this work the biogenic amine analysis has never been done within the company so, an entirely new method was developed using the OIV method as guideline. The FERMDIF project, on the other hand, was already in progress when the work for this Thesis began. The sampling for the analysis of the sugar contents was previous to the present work and the analyses of the alcoholic content were made in parallel by a third party and only the results were provided. Additionally, the Central Oenology Laboratory of Sogrape Vinhos already had an implemented HPLC-RI method for glucose and fructose analysis. Therefore, the existing method was adapted to better fit the purposes of this work.

1.3 Organization of the Thesis

To begin with, an overview of the projects embraced is made in the introduction. Next, a comprehensive description of the state of the art is made regarding biogenic amines including some focus on general chemical and biological information, their formation during winemaking processes, the amounts usually found in wines and their quantitative analysis.

Then, a description of the methods used for both biogenic amine analysis and glucose and fructose analysis is made. To follow, there is a section in which results are presented and discussed. The method development steps are presented for both biogenic amines and sugars and the results concerning each one are discussed. Within the latter section a global analysis of the results was tried to be preformed. Afterwards, a conclusion of the work was written. Finally, additional work accomplished concerning this Thesis is shown and the references used are pointed out.

2 State of the Art

2.1 Biogenic Amines

Biogenic amines may alter wine properties and aromas and when present in large concentrations may have undesired effects on human health. Therefore, knowledge of such effects together with the general awareness over food and beverages composition, driven by the search for healthier products, raised concerns between costumers (Cecchini and Morassut, 2010). Moreover, customers value authenticity, quality and safety of the products (Herbert *et al*., 2006). Consequently, they are more demanding concerning controls of product characteristics. The first entity to be held responsible for this control is the producer. Since the analysis of biogenic amines was not yet implemented in *Sogrape Vinhos*, this work brings the development, validation and application of such method.

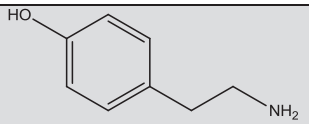
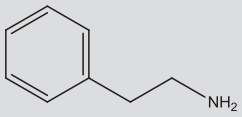
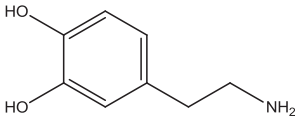
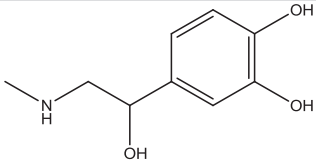
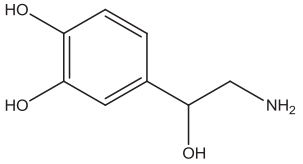
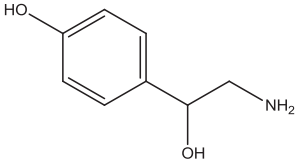
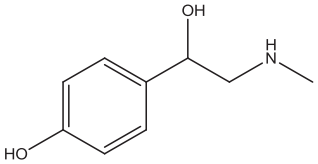
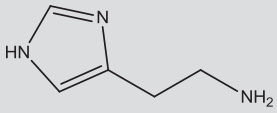
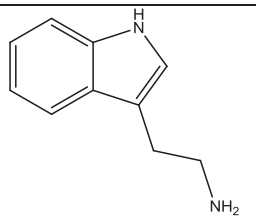
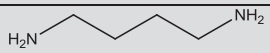
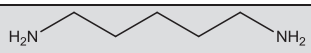
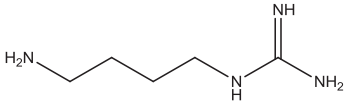
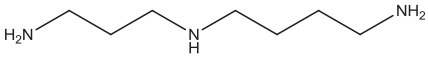
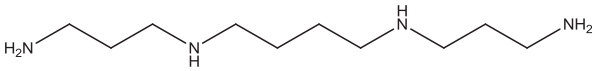
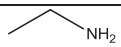
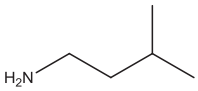
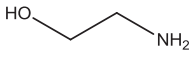
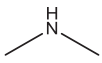
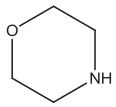
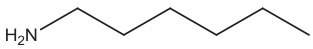
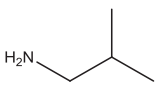
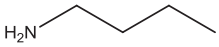
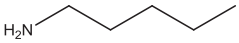
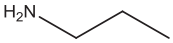
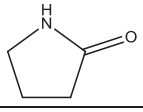
2.1.1 Definition and Classification

Biogenic amines are low molecular weight organic compounds which occur naturally in many microorganisms, plants and animals as part of their metabolism. Therefore, amines are found in several foods and beverages, usually at trace levels (Mafra *et al*., 1999; Anli and Bayram, 2009; Cecchini and Morassut, 2010; Ladero *et al*., 2010; Pena-Gallego *et al*., 2012).

These amines have one or more NH_2 characteristic groups and can be classified according to their structure in aromatic, heterocyclic or aliphatic amines and, within the latter class, in polyamines or volatile amines (Table 2-1).

This classification is not yet well established among literature since there are some authors that assume aromatic and heterocyclic amines in the same group while others do not make distinction between amines within the aliphatic group (Mafra *et al*., 1999; Pena-Gallego *et al*., 2012).

Table 2-1 - Amine classification according to chemical structure and examples of amines belonging to each group* (Cuskey *et al.*, 1987; Mafra *et al.*, 1999; Pena-Gallego *et al.*, 2012) – Figures were designed with ChemBioDraw Ultra version 12 from CambridgeSoft®

Group	Amines		Amines	
Aromatic amines	**Tyramine**		**Phenylethylamine**	
	Dopamine		Epinephrine	
	Nor-epinephrine		Octopamine	
	Synephrine			
Heterocyclic amines	**Histamine**		Tryptamine	
Aliphatic polyamines	**Putrescine**		**Cadaverine**	
	Agmatine		Spermidine	
	Spermine			
Aliphatic volatile amines	Ethylamine		Methylamine	
	Isoamylamine		Ethanolamine	
	Dimethylamine		Morpholine	
	Hexylamine		Isopropylamine	
	Isobutylamine		N-butylamine	
	N-amylamine		N-propylamine	
	2-Pyrrolidone			

* Shaded biogenic amines were the ones selected for this study.

2.1.2 Biological Activity

All biogenic amines have biological activity (Ladero *et al*., 2010). Nevertheless, depending on the organism, they may have radically different functions. For instance, several microorganisms take advantage from the amine structural group of biogenic amines with the purpose of using them as part of their defence mechanism against acidic environments (Anli and Bayram, 2009). Furthermore, amines may be used as part of an additional energy generation mechanism when other sources cease to be available (Coton *et al*., 2010; García-Marino *et al*., 2010).

On the other hand, in humans, the same amines usually act as neurotransmitters and each amine is related to a different physiological response. Therefore, biogenic amines interfere in the metabolism taking part in critical functions of the human body such as brain activity, body temperature regulation, as well as stomach volume, pH and gastric fluids secretion regulation and even influence immune response, cell growth and cell differentiation (Mafra *et al*., 1999; Anli and Bayram, 2009; Ladero *et al*., 2010).

Even though biogenic amines play such important roles in the human body, an imbalance of the amines homeostasis caused by ingestion of large amounts of one or several of these amines can cause hazardous effects in human health.

The uptake of biogenic amines in the human body is made through ingestion. There are specific enzymes (diamine oxidases) in the human gastrointestinal tract that are able to metabolize these amines. Nevertheless, if too high concentrations are to be ingested, part of them enters the blood stream (Anli and Bayram, 2009).

The toxicological effects of biogenic amines include headaches, nausea, rashes, allergenic disorders, hypotension, hypertension, cardiac palpitation, oedema, vomiting, diarrhoea, respiratory distress, renal intoxication, loss of sensorial properties, kidney and vascular failure, intracerebral haemorrhage and even death (Herbert *et al*., 2006; Hernández-Orte *et al*., 2006; Anli and Bayram, 2009; Cecchini and Morassut, 2010; García-Marino *et al*., 2010; Ladero *et al*., 2010).

The effects caused by biogenic amines ingestion depend on the type of amine and amount ingested. Yet, sensitivity of each individual to biogenic amines varies depending on their detoxification capacity (García-Marino *et al*., 2010). Moreover, combined ingestion with alcohol or acetaldehyde enhances the toxicological effects since these components hinder the detoxification system by diamine oxidase (Anli and Bayram, 2009). In such cases smaller amounts of amines are needed to produce the same adverse effects.

Some aliphatic amines (both polyamines and volatile amines) such as putrescine, cadaverine, agmatine, spermidine, spermine, ethylamine, methylamine, isoamylamine and ethanolamine, even though they are not toxic by themselves, they present similar effects to alcohol and acetaldehyde, inhibiting amine oxidation, thus acting synergistically with other amines, enhancing their toxicological effects (Landete *et al*., 2005a; Herbert *et al*., 2006; Hernández-Orte *et al*., 2006; Pramateftaki *et al*., 2006; Coton *et al*., 2010; García-Marino *et al*., 2010; Pena-Gallego *et al*., 2012).

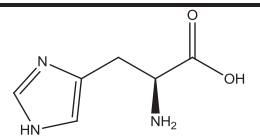
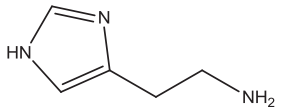
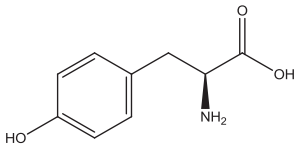
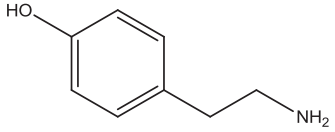
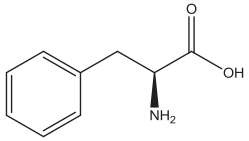
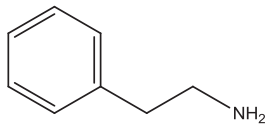
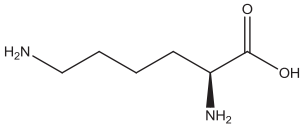
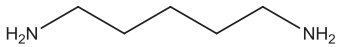
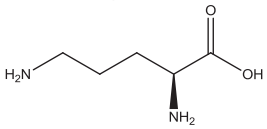
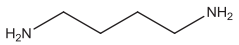
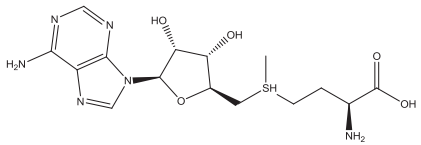
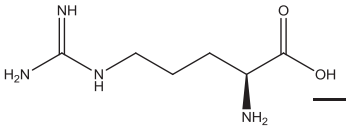
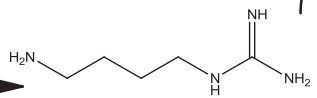
In particular, and besides what was stated before, amines such as tyramine, putrescine and cadaverine may react with nitrites to form carcinogenic nitrosamines. Also, tyramine and phenylethylamine may alter vascular tone, induce migraine and cause hypertension. Histamine may cause fever, sweating and bronchoconstriction and volatile aliphatic monoamines such as methylamine and ethylamine are irritants (Hernández-Orte *et al*., 2006; Pramateftaki *et al*., 2006; Anli and Bayram, 2009; Coton *et al*., 2010; Pena-Gallego *et al*., 2012).

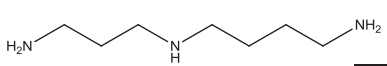
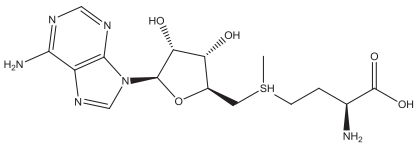
2.1.3 Formation

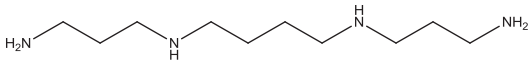
Biogenic amines are mainly formed by the removal of the carboxylic group directly linked to the alpha-carbon of an amino acid. Depending on the amino acid, different amines may be formed (Table 2-2) (Ladero *et al*., 2010; Pena-Gallego *et al*., 2012)

Additionally, some amines result from the transformation of other biogenic amines, e.g., the formation of spermidine from putrescine and spermine from spermidine which is achieved through the reaction with S-adenosyl-L-methionine (Table 2-2) (Caspi, 2005a, 2005b; Pena-Gallego *et al*., 2012).

Table 2-2 – Biogenic amines and corresponding amino acid precursor (Moreno-Arribas *et al.*, 2003; Caspi, 2005a, 2005b; Herbert *et al.*, 2006; Hernández-Orte *et al.*, 2006; Anli and Bayram, 2009; Ladero *et al.*, 2010) – Figures were designed with ChemBioDraw Ultra version 12 from CambridgeSoft®

Precursor Amino Acid	Biogenic Amine
 Histidine	 Histamine
 Tyrosine	 Tyramine
 Phenylalanine	 2-phenylethylamine
 Lysine	 Cadaverine
 Ornithine	 Putrescine +  S-adenosyl-L-methionine
 Arginine →  Agmatine	

 Spermidine +  S-adenosyl-L-methionine

 Spermine

The presence of biogenic amines in food and beverages has long been associated with fermented products as well as with lack of hygienic conditions and spoilage of the raw materials and during processing (Anli and Bayram, 2009; Del Prete *et al*., 2009; Cecchini and Morassut, 2010). This presence results from decarboxylation of amino acids by several microorganisms. Additionally, some volatile biogenic amines such as methylamine, ethylamine and isoamylamine may be formed by reductive amination of non-nitrogen compounds such as aldehydes or ketones or even transamination of aldehydes from amino acids (Soufleros *et al*., 2007; Pena-Gallego *et al*., 2012).

Some food products such as cheese, meat and fish and beverages such as beer and wine are known for the presence of biogenic amines. (Cecchini and Morassut, 2010) In wine, these compounds are mainly formed by the action of decarboxylase-positive microorganisms. However, this production of biogenic amines is only possible if free amino acids are available in the medium and right environmental conditions are present (Pena-Gallego *et al*., 2012).

Numerous environmental factors play decisive roles in the definition of the amount and type of biogenic amines formed in wine. To begin with, the geographical position of the vineyards, also related with climactic conditions, affect grape growth which has influence in the biogenic amine content, not only as natural constituents but also in the type of microorganisms that may naturally develop as well as other components such as sugar levels (Soufleros *et al*., 2007; Cecchini and Morassut, 2010; Coton *et al*., 2010). In line with the previous factors, grape degree of maturation, vintage and hygienic conditions of grapes, i.e., presence of rotten grapes (e.g., noble rot) also influence biogenic amine content being verified higher amounts of amines when rotten grapes are present (Herbert *et al*., 2005; Soufleros *et al*., 2007; Cecchini and Morassut, 2010). Still regarding the vineyard, variety, soil type and composition and nitrogen added in the form of fertilisers contribute to biogenic amine formation (Herbert *et al*., 2005; Soufleros *et al*., 2007; Cecchini and Morassut, 2010). For instance, the lack of potassium in the soil has been associated with increased levels of putrescine in grapevines while nitrogen fertilizers can highly increase biogenic amine production (Landete *et al*., 2007; Soufleros *et al*., 2007; Arena *et al*., 2008). Moreover, too long maceration processes, extended contact with yeast lees (liberation of yeast contents to the wine through autolysis) and inappropriate hygienic conditions during winemaking processes cause levels of biogenic amines to rise (Soufleros *et al*., 2007). Another very important aspect is the fermentation process. The effect of the microorganisms responsible for alcoholic and malolactic fermentation in biogenic amine content is going to be discussed later on. Nevertheless, fermentation conditions such as pH, temperature, fermentation length and

contents of nitrogen, amino acids, alcohol, reducing sugars, SO_2 and organic acids also take part in biogenic amine formation (Herbert *et al*., 2006; Soufleros *et al*., 2007; Pena-Gallego *et al*., 2012). Additionally, some oenological treatments, e.g. bentonite addition, reduce biogenic amine content, while storage and ageing allow more biogenic amines to develop (Soufleros *et al*., 2007).

Regarding free amino acid contents, even though they are precursors to biogenic amines, they have crucial effect on wine quality since they interfere with aroma compounds and other trace compounds related to wine authenticity (Herbert *et al*., 2006).

2.1.4 Fermentation Phases

Lactic acid bacteria decarboxylate free amino acids present in musts being the main responsible for biogenic amine production in wine. (Hernández-Orte *et al*., 2006; Anli and Bayram, 2009; Coton *et al*., 2010). The population of microorganisms in wine is complex and it changes during fermentation processes causing variable production of biogenic amines (Landete *et al*., 2005a).

Alcoholic fermentation is the main fermentative process in which yeasts, usually from *Saccharomyces* genus, convert sugar into alcohol (Landete *et al*., 2007). Among lactic acid bacteria, lactobacilli population, which are the main type of bacteria present in grapes, rapidly decreases giving place to other microorganisms such as pediococci, *Leuconostoc mesenteroides* and *Oenococcus oeni* (Moreno-Arribas *et al*., 2003).

Even though these bacteria are present, their numbers are overwhelmed by yeast population. Moreover, the conditions found by microorganisms during alcoholic fermentation are not favourable for biogenic amine formation by lactic acid bacteria (Del Prete *et al*., 2009; Pena-Gallego *et al*., 2012). Nevertheless, biogenic amines may be formed by the action of several yeasts such as *Saccharomyces cerevisiae*, *Brettanomyces bruxellensis*, *Kloeckera apiculata* which are able to produce histamine, ethanolamine, phenylethylamine, agmatine and cadaverine (García-Marino *et al*., 2010).

Malolactic fermentation may occur following alcoholic fermentation. For the majority of white wines this phase does not take place. On the contrary, it is indispensible for most red wines since many organoleptic features are changed during this phase. Moreover, there is a deacidification (rise in pH) and decomposition of some undesired compounds such as malic

acid, sugars and citric acid (Pramateftaki *et al*., 2006). These changes in fermentation conditions allow lactic acid bacteria to thrive during this phase. Consequently, the major biogenic amine production occurs during malolactic fermentation resulting in greater biogenic amine contents in red wines than in white wines (Cecchini and Morassut, 2010; García-Marino *et al*., 2010; Pena-Gallego *et al*., 2012).

Regarding the main biogenic amines produced during this phase, there seems to be some disagreement between authors. Differences on key factors and conditions such as the ones previously presented may be at the source of such incongruities. Cecchini and Morassut (2010) and Coton *et al*. (2010) state that histamine, putrescine and tyramine are the most produced amines. On the other hand, Landete *et al*. (2007) has not reported increases of putrescine, cadaverine and tryptamine but instead of phenylethylamine, tyramine and histamine. Moreover, Pramateftaki *et al*. (2006) describes putrescine, tyramine and phenylethylamine as the main biogenic amines formed while denying increases in histamine, methylamine and ethylamine. Furthermore, Pena-Gallego *et al*. (2012) and Moreno-Arribas *et al*. (2003) indicate tyramine as the biogenic mine most found in wine while Arena *et al*. (2008) considers putrescine the most abundant.

During malolactic fermentation, bacterial population in wine is a complex mixture of lactic acid bacteria in which species from *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Oenococcus* genera are present (Herbert *et al*., 2005; Coton *et al*., 2010). Among them, *O*. *oeni* is the predominant species (Moreno-Arribas *et al*., 2003; Landete *et al*., 2005a; Pramateftaki *et al*., 2006; Del Prete *et al*., 2009). Yet again, in some cases a single-species population of *O. oeni* may be formed especially during spontaneous malolactic fermentation resulting from higher tolerance to harsh conditions formed in wine after alcoholic fermentation (Moreno-Arribas *et al*., 2003; Pramateftaki *et al*., 2006). Besides lactic acid bacteria, acetic bacteria are also present during malolactic fermentation. However none of these species were found to produce biogenic amines (Landete *et al*., 2007)

More controlled malolactic fermentations begin to take place by selecting starter cultures. However, in many cases spontaneous fermentation is preferred since autochthonous species are better adapted to the local winery conditions (García-Marino *et al*., 2010)

The variability observed in lactic acid bacteria population influenced by a multitude of environmental factors already described, may be the cause for such diverse opinions and findings reported in literature. Moreover, Pramateftaki *et al*. (2006) and Coton *et al*. (2010) highlight the possibility of biogenic amine production being strain dependent. This idea is

reinforced by Coton *et al*. (2010) and Del Prete (2009) reporting loss of biogenic amine production capabilities justified by the presence of the corresponding genes on unstable plasmids. These findings further suggest the horizontal transfer of amine producing capabilities making possible the production (or lack of it) of different biogenic amines by the same species in identical situations (Coton *et al*., 2010).

At the end of malolactic fermentation, microbial stability of wine is increased due to depletion of nutrients and release of some antimicrobial compounds (Pramateftaki *et al*., 2006). Even though microbial stability is higher, several authors have reported increases of biogenic amines contents during storage and ageing of wine. During these phases some lactic acid bacteria can survive as resting cells thus contributing for late biogenic amine production (Arena *et al*., 2008; Pena-Gallego *et al*., 2012). The main microorganisms responsible for this formation seem to be lactobacilli (Arena *et al*., 2008).

Despite the verified disagreements between authors, there are several amines that stand out not only for their frequency and high amount but also for their adverse effects on human health. They are histamine, tyramine and putrescine. In the present work these are the amines that are intended to be tested in wines plus phenylethylamine and cadaverine which were chosen due to their relation to tyramine and putrescine respectively, the frequency and amounts they are usually found in wine and their effects on human health.

2.1.4.1 Histamine

Histamine is an exogenous heterocyclic amine that is involved in many food-borne intoxications and has strong biological activity, influencing important body functions such as motor and sensorial neuron signalling (Landete *et al*., 2005a; Pena-Gallego *et al*., 2012). Besides, it is the most toxic biogenic amine therefore being the most studied and notorious one (Anli and Bayram, 2009; García-Marino *et al*., 2010).

Biogenic amines undergo complex interactions among them and with other compounds thus making it difficult to establish maximum limits for their presence in foodstuff (García-Marino *et al*., 2010). Consequently, no biogenic amine has established limits. In the case of histamine, the interest in limiting its presence lead to the creation of recommended maximum limits by several countries. Each country has distinct demands regarding histamine content in wine (Table 2-3). These limits are very difficult to establish and, since the effects of histamine vary depending on the subject, most of the times they are based on previous cases

of intoxication. For alcoholic beverages, 2-10 mg/L of histamine is suggested as toxic levels (Soufleros *et al*., 2007).

Table 2-3 – Histamine maximum recommended limits by country (Landete *et al*., 2005a; Hernández-Orte *et al*., 2006)

Country	Histamine limit in wine (mg/L)
Germany	2
Belgium	5-6
Switzerland	10
Austria	10
France	8
Netherlands	3

There are several species that can contribute to histamine formation in wine. Histamine-producing strains of *Oenococcus oeni* are the most frequent in wine, but with low production capability. On the contrary, strains belonging to *Pediococcus* genus, found with low frequency and in low proportions, are held the main responsible for histamine levels due to the production of very high amounts of histamine (Landete *et al*., 2005a; Landete *et al*., 2007). Nevertheless, *O. oeni, Lacobacillus hilgardii, Lactobacillus mali, Leuconostoc mesenteroides, Pediococcus damnosus* and *Pediococcus parvulus* contribute for histamine synthesis in wine (Landete *et al*., 2005a; Landete *et al*., 2007; Coton *et al*., 2010).

The genetic aspects of biogenic amine production fall out of the scope of the current work and therefore they are not going to be herein discussed. However, it is worthwhile mention that for histamine there is a 100% correlation between hdc gene presence in the microorganism and its ability to produce histamine. Moreover, several strains have been found to possess multiple genes identified to code for enzymes responsible for amino acid decarboxylation thus allowing the same strain to produce several biogenic amines (Del Prete *et al*., 2009; Coton *et al*., 2010). It is also relevant the abovementioned fact that the ability to produce amines may be strain-dependent which may be responsible for some inconsistencies among literature.

2.1.4.2 Tyramine

Likewise histamine, tyramine can be produced by strains of *O*. *oeni, L*. *hilgardii* and *L*. *mesenteroides* (Moreno-Arribas *et al*., 2003; Landete *et al*., 2007). The latter is the main tyramine producer but other microorganism such as *Lactobacillus brevis*, *Lactobacillus plantarum* and several *Leuconostoc* strains also contribute for tyramine levels (Moreno-Arribas *et al*., 2003; Landete *et al*., 2007; Coton *et al*., 2010).

One interesting characteristic was detected regarding *L*. *hilgardii*. Every time this species was detected as tyramine producer it was also able to produce phenylethylamine. *L*. *brevis* is also able of phenylethylamine production (Landete *et al*., 2007).

2.1.4.3 Phenylethylamine

Phenylethylamine production is often associated with tyramine production by lactic acid bacteria since phenylalanine is a common substrate to tyrosine decarboxylase which produces phenylethylamine as secondary reaction (Landete *et al*., 2007).

The main producers of phenylethylamine are *L*. *hilgardii* and *L*. *brevis* strains. (Landete *et al*., 2007)

2.1.4.4 Putrescine and Cadaverine

Putrescine production can be achieved through two different pathways: ornithine decarboxylation or decarboxylation of arginine to agmatine and removal of urea from the latter (Moreno-Arribas *et al*., 2003) (Table 2-2).

The optimum temperature and pH for putrescine production are 37 °C and 4.5 respectively. Additionally, putrescine production increases when the ethanol content is low. However, influence of ethanol in biogenic amines formation is not well established. Moreover, and surprisingly, high concentrations of arginine (precursor amino acid) diminish putrescine formation. On the other hand, the presence of L-lactic and tartaric acids enhanced putrescine formation. Furthermore, the addition of fructose and glucose was verified to inhibit putrescine formation while the presence of lactic acid was positively correlated with putrescine synthesis. Unlike the majority of biogenic amines, putrescine is positively correlated with malic and citric acids (Arena *et al*., 2008).

The main producers of putrescine are *L. hilgardii* and *O*. *oeni* (Landete *et al*., 2007).

Many authors fail to identify cadaverine-producing strains. Nevertheless, cadaverine is associated with putrescine and it is very commonly found in wines (Coton *et al*., 2010).

2.1.5 Biogenic Amine Levels in Wines

Biogenic amine levels reported in literature are very variable. Such differences can be attributed to the diversity of conditions under which biogenic amines are tested. Additionally, this situation reflects the divergent purposes from each author/paper over the analysis of biogenic amine contents.

Authors often choose wines from a certain country and work on other conditions from that point, e.g., Soufleros *et al*. (2007) analysed Greek wines while Landete *et al*. (2005b) and García-Villar *et al*. (2006) focused on Spanish wines and Mafra *et al*. (1999) and Herbert *et al*. (2005) evaluated Portuguese wines. Noteworthy when judging different papers, besides different considerations, is the effect of geographical position that may prove significant when comparing biogenic amine levels. Paper reviews, on the other hand, try to fill that gap by publishing comparisons between results from different authors and different countries (Anli and Bayram, 2009; Pena-Gallego *et al*., 2012).

Other aspect that is certainly different is the microbial population acting during winemaking processes which may also introduce variability to the results.

Still, some common ground may be found since some authors such as García-Marino *et al*. (2010), Marcobal *et al*. (2005) and Soufleros *et al*. (2007) search for biogenic amines in commercial wines intending to use them as reference values for the results of their experiments.

Furthermore, some papers address specifically a particular factor and its effect on biogenic amine contents. For instance, Del Prete *et al*. (2009) and García-Marino *et al*. (2010) analyze biogenic amine levels during different stages of the winemaking process and study the effect of fermentations phases. Landete *et al*. (2005b), on the other hand, attempts to establish a relationship between the region and grape variety of three Spanish wines, while Cecchini and Morassut (2010) search for influences of grape storage on biogenic amines just in musts. Additionally, ageing effect is explored by García-Villar (2006) whereas the influence of the type of wine (white, red or rose) is assessed by Soufleros *et al*. (2007) and Herbert *et al*. (2006).

Taking into account the biogenic amines analyzed in this work, the extreme values found for each one were taken from the papers gathered (Table 2-4).

Table 2-4 – Extreme levels of biogenic amine found in wines from several regions and varieties, different storage and ageing times and different wine types (Mafra *et al.*, 1999; Landete *et al.*, 2005b; Marcobal *et al.*, 2005; García-Villar *et al.*, 2006; Herbert *et al.*, 2006; Soufleros *et al.*, 2007; Anli and Bayram, 2009; Del Prete *et al.*, 2009; Cecchini and Morassut, 2010; García-Marino *et al.*, 2010; Ladero *et al.*, 2010; Pena-Gallego *et al.*, 2012).

Biogenic Amine	Range mg/L		
Histamine	n.d.	–	25.00
Tyramine	n.d.	–	19.00
Putrescine	n.d.	–	99.90
Phenylethylamine	n.d.	–	18.40
Cadaverine	n.d.	–	98.50

Note: "n.d." stands for not detected.

Additionally, and since the effect of the variety is intended to be herein evaluated, a compilation of biogenic amines levels found for different varieties was made in the attempt to take some conclusions that might be compared to the results obtained (Table 2-5).

Table 2-5 - Compilation of the biogenic amines levels found for different varieties (Landete *et al*., 2005b; Soufleros *et al*., 2007)

Variety	n	Histamine mg/L	Tyramine mg/L	Phenylethylamine mg/L	Putrescine mg/L	Cadaverine mg/L
Agiorgitiko	9	0.07	0.45	**1.42**	1.35	0.54
Asyrtiko	5	0.73	0.35	0.04	1.31	1.03
Bobal	18	**2.3**	**2**	0.8	3.5	-
Cabernet Sauvignon	6	0.12	0.03	0.09	0.14	0.15
Chardonnay	3	0.04	0.33	0.33	0.9	0.72
Debina	3	0.63	0.47	n.d.	0.97	**1.75**
Grenache rouge	11	1.0	0.6	0.5	**6.0**	(n=3) 0.86
Limnio	2	0.29	0.64	n.d.	0.88	1.02
Malagouzia	2	0.06	0.29	0.1	0.2	0.08
Mantilaria	3	1.08	**1.75**	0.02	3.27	0.82
Merlot	3	0.51	0.07	0.4	0.75	0.12
Moshofilero	3	n.d.	0.04	0.05	1.22	0.2
Muscat d'Alexandrie	7	1.09	0.85	0.15	1.22	**2.34**
Muscat de Setúbal	2	0.90	0.30	**1.01**	2.06	0.49
Muscat Hamburg	2	0.04	0.15	n.d.	0.49	0.36
Muscat white	4	0.37	0.44	n.d.	1.16	0.18
Roditis	14	0.35	0.42	**1.05**	0.84	0.75
Syrah	4	0.61	0.76	0.19	2.06	0.78
Tempranillo	46	**2.5**	**2.6**	**1.2**	**7.6**	-
Xinomavro	19	0.53	0.28	0.52	0.68	0.37

Note: "n.d." stands for not detected; "-" sign indicates that the biogenic amine was not tested.

In Table 2-5 the highest levels for each biogenic amine were signalled in bold and the lowest ones were underlined. From these values it can be observed that Tempranillo had the highest values for all biogenic amines tested. Additionally, Bobal, Grenache rouge and Mantilaria varieties also present high amounts of total biogenic amines. On the other end of the scale, Cabernet Sauvignon and Malagouzia are the varieties with lower registered biogenic amines formation. These values do not reflect the effect of the fermentation conditions and strains used as well as other abovementioned parameters that could result in different biogenic amines values for the same variety.

The varieties tested in this work cannot be found among those presented.

Another parameter that is often considered for evaluation is the type of wine (Table 2-6).

Table 2-6 – Average biogenic amine levels described in literature grouped by wine type (Mafra *et al.*, 1999; Herbert *et al.*, 2001, 2006; Landete *et al.*, 2005b; Marcobal *et al.*, 2005; García-Villar *et al.*, 2006; Soufleros *et al.*, 2007; García-Marino *et al.*, 2010)

Biogenic Amine	Red (n=480) mg/L	Rose (n=8) mg/L	White (n=148) mg/L
Histamine	3.78	1.03	2.87
Tyramine	2.04	0.36	0.65
Phenylethylamine	0.67	0.32	0.31
Putrescine	13.00	0.74	2.90
Cadaverine	1.27	1.2	1.21

This comparison, only based on the type of wine, is a very rough classification that has large uncertainties associated with the values. In most of the cases, the standard deviation is higher than the value itself. This is probably caused by the great variability of the wine characteristics that may be found within each group (aspects that may influence biogenic amine formation other than the type of wine were already described in section 2.1.3).

In order to better assess biogenic amine formation in the different wine types, their origin was included in the analysis and the cases in which such information is unavailable were disregarded (Table 2-7).

Table 2-7 – Compilation of biogenic amines levels of wines from different origin and respective distinction between wine type

Origin	Type	n	Histamine mg/L	Tyramine mg/L	Phenylethylamine mg/L	Putrescine mg/L	Cadaverine mg/L	References
Greece	Red	45	0.31	0.43	0.59	1.17	0.52	(Soufleros *et al.*, 2007)
Greece	Rose	8	1.03	0.36	0.32	0.74	1.2	(Soufleros *et al.*, 2007)
Greece	White	47	0.41	0.42	0.5	0.98	0.81	(Soufleros *et al.*, 2007)
Alentejo (Portugal)	Red	55	7.4	3.0	n.d.	14.1	2.1	(Herbert *et al.*, 2001, 2006)
Alentejo (Portugal)	White	54	7.0	1.0	n.d.	6.1	1.8	(Herbert *et al.*, 2001, 2006)
Madeira (Portugal)	Red	12	0.50	1.74	0.18	1.10	0.33	(Mafra *et al.*, 1999)
Portugal	Red	8	1.13	0.68	3.91	3.22	0.6	(Mafra *et al.*, 1999)
Portugal	White	6	1.2	0.17	0.75	2.14	0.63	(Mafra *et al.*, 1999)
Campo de Borja (Spain)	Red	4	5	-	2.4	24.8	2.3	(García-Villar *et al.*, 2006)
Cariñena (Spain)	Red	4	9.3	-	5.15	29.9	4.8	(García-Villar *et al.*, 2006)
Jumilla (Spain)	Red	4	5.4	-	0.11	20.4	3.1	(García-Villar *et al.*, 2006)
La Mancha (Spain)	Red	8	2.65	-	0.33	16.35	9.4	(García-Villar *et al.*, 2006)
La Rioja (Spain)	Red	53	8.2	1.6	1.0	44.9	2.8	(Landete *et al.*, 2005b; García-Villar *et al.*, 2006)
Ribera del Duero (Spain)	Red	4	9.2	-	0.83	22.6	10.3	(García-Villar *et al.*, 2006)
Tarragona (Spain)	Red	12	4.5	1.8	0.8	34.1	-	(Landete *et al.*, 2005b)
Utiel-Requena (Spain)	Red	46	2.4	2.3	1	7.5	-	(Landete *et al.*, 2005b)
Valdepeñas (Spain)	Red	4	6.1	-	0.2	13.7	5	(García-Villar *et al.*, 2006)
Valencia (Spain)	Red	4	2.9	-	0.06	10.5	1.9	(García-Villar *et al.*, 2006)
Spain	Red	92	4.5	3.7	0.1	11.8	0.2	(Marcobal *et al.*, 2005; García-Marino *et al.*, 2010)

Note: "n.d." stands for not detected; "-" sign indicates that the biogenic amine was not tested.

From these values it is noteworthy the lower overall amounts of biogenic amines present on Greek wines when compared to the Iberic ones. Yet, it is not surprising higher similarities between Portuguese and Spanish wines since the proximity of the two countries makes it more likely to have vineyards with similar conditions (for instance weather and soil conditions). Moreover, and maybe more importantly the varieties used tend to be the same or at least with similar characteristics (a consequence of the similar weather and soil). An exception to this tendency is putrescine that has a strong presence in Spanish wines.

Phenylethylamine is the biogenic amine found at lower levels having several cases (more than 100) in which this biogenic amine was not detected.

2.1.6 Analysis

Analysis of biogenic amines proved to be difficult for several reasons. Most of the times, the amounts that exist in wines are very low, many amines can be found at concentrations lower than 1 mg/L (Mafra *et al*., 1999). Moreover, wine's strong matrix interference and biogenic amines structural properties, in particular their similarities to amino acids, further hinder their analysis (Mafra *et al*., 1999; Hernández-Orte *et al*., 2006; Del Prete *et al*., 2009).

Hereupon, the analysis of biogenic amines is commonly made through HPLC-FL or HPLC-UV (Ultraviolet light detector). Direct injection of the samples is problematic since amines absorb radiation around 190-200 nm which corresponds to the wavelength in which most functional groups also absorb (Pena-Gallego *et al*., 2012). As for sample preparation several extraction methods were attempted and tested. However, none achieved extraction of all biogenic amines of interest in a single step (Hernández-Orte *et al*., 2006).

Since direct methods could not be used and extensive extraction procedures are undesirable, the alternative found was the derivatization of biogenic amines.

Derivatization is based on the use of specific reagents so as to produce colourful or fluorescent compounds. It allows much better sensitivity of the method and it can be used both online and offline. Offline derivatization is simple and inexpensive however the derivatization reaction, most of the times, is incomplete thus needing to be done in a short period of time. Furthermore, this type of derivatization may raise concerns about reproducibility of the derivatization reactions and injections.

On the other hand, online derivatization allows better flexibility since pre- or post column derivatization is possible allowing increased sensitivity through reduction of interferences, i.e., before or after chromatographic separation (García-Marino *et al*., 2010). Either of these possibilities requires extra pieces of equipment such as automatic injection, additional pumps, a mixing chamber or even a reactor. The post column derivatization has the particularity of diluting the mobile phase with the derivatization reagent which causes a decrease in sensitivity when compared to pre-column derivatization (Pena-Gallego *et al*., 2012). More to the point, the choice of derivatization reagent greatly influences the method. A good derivatization agent should form unique products upon reaction with the sample, the derivative compounds should be stable and no interference should be experienced from subproducts formed by reaction during chromatographic analysis (Pena-Gallego *et al*., 2012). Unfortunately, such behaviour is not always achieved or even achievable. For that reason a multitude of derivatization agents have already been tested using both UV (ultra-violet light) detection and fluorescence detection (Table 2-8).

Table 2-8 – Derivatization agents that may be used depending on the detection method (García-Marino *et al*., 2010; Pena-Gallego *et al*., 2012)

FL detection	UV detection
6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate	Dabsyl chloride
9-Fluorenylmethoxycarbonyl chloride	Dansyl chloride
Dansyl chloride	Diethyl ethoxymethylenemaloate
2-[2-(Dibenzocarbazole)-ethoxy] ethyl chloroformate	1,2-Naphthoquinone-4-sulfonate
Fluorescamine	Ninhydrin
2-Naphthalenoxycarbonyl chloride	p-Nitrobenzyloxycarbonyl chloride
8-Phenyl-(4-oxy acetic acid N-hydroxysuccinimide ester)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a,-diaza-S-indacene	Phenyl isothiocyanate
o-Phthalaldehyde	
6-Oxy-(N-succinimidyl acetate)-9-(2'-methoxycarbonyl) fluorescein	

The most common analysis procedure of biogenic amines involves a reverse phase C18 chromatographic column and pre-column sample derivatization. If more advanced techniques such HPLC-MS (hyphenated technique making use of high performance liquid chromatography and mass spectrometry) or capillary electrophoresis were to be used, samples could be injected directly (Pena-Gallego *et al*., 2012).

2.1.6.1 Available Methods Compilation

One of the objectives of this work was to develop a method for analysis of biogenic amines. To start with, a screening of the existing methods for biogenic amine analysis in wines and/or musts was made in order to gather enough data to set up a new method. The method was intended to be used as routine procedure for the analysis of wine and must samples. Therefore, it should not be too time consuming and sample preparation steps should be minimized. Ideally, samples should be injected directly, i.e., without pre-treatment. Furthermore, the method must be reliable and robust in order to guarantee that the wine follows each customer's demands. Proficiency tests should be made in order to give credit to the method and once again assure its reliability. More to the point, the method must allow simultaneous detection of all the amines of interest since the application of different methods for different amines would not be feasible taking into account its/their applicability as routine procedure(s).

Another conditioning variable for method development is, of course, the availability of equipment in the laboratory. In this case, the method for biogenic amines detection was designed for HPLC-FL. So, bearing in mind the available equipment, the most promising methods found in the literature were compiled. Comparisons were made regarding the equipment and columns used as well as chromatographic conditions and applicability of the method (i.e., which and how many amines can be analyzed by each method) (Tables 2–9 to 2–12).

The majority of the methods found in literature make use of ortho-phtalaldehyde (OPA) derivatives combined with fluorescence detection. This situation occurs, presumably, due to its high selectivity and sensitivity towards the main biogenic amines allied with a short derivatization period (García-Marino *et al*., 2010). Nevertheless, it is noteworthy the variety of conditions presented particularly concerning mobile phase compounds.

The method from *Organisation Internationale de la Vigne et du Vin* (OIV) is highlighted in bold and it is going to discussed be later on.

Table 2-9 – Compilation of methods for biogenic amines analysis and main features comparison

#	Reference	Derivatzation agents	Detection	Excitation (nm)	Emission (nm)	Column	Time (min)	Eluent A	Eluent B
1	**OIV**	**OPA**	**Fluorescence**	**356**	**445**	**C18 - 5 µm**	**95**	**Dibasic sodium phosphate buffer (11.12 g ± 0.01 g in 2 litres)**	**Acetonitrile**
2	(Cecchini and Morassut, 2010)	OPA	Fluorescence	340	450	C18 - 5 µm Silica Alltima	60	1.224 g sodium acetate trihydrate; 500 ml water; 0.09 ml of triethylamine; 1.5 ml tetrahydrofuran	1.088 g sodium acetate trihydrate; 100 ml water; 200 ml acetonitrile; 200 ml methanol
3	(Del Prete *et al.*, 2009)	OPA	Fluorescence	340	450	C18 - 5 µm Silica Alltima	65	1.224 g sodium acetate trihydrate; 500 ml water; 0.09 ml triethylamine; 1.5 ml tetrahydrofuran	1.088 g sodium acetate trihydrate; 100 ml water; 200 ml acetonitrile; 200 ml methanol
4	(Herbert *et al.*, 2001, 2005, 2006)	OPA FMOC-Cl	Fluorescence	340/237	450/340	C18 - 5 µm Silica gel Merck	138	20 mM sodium acetate solution; 0.018% (v/v) triethylamine; 0.3% (v/v) tetrahydrofurane; 0.010% (v/v) of 4% (m/v) solution EDTA (pH adjusted to 7.20 with 0.1% (v/v) solution acetic acid)	20% (v/v) of sodium acetate solution (100 mM, pH 6.0); 40% (v/v) acetonitrile; 40% (v/v) methanol; 0.018% (v/v) triethylamine
5	(Hernández-Orte *et al.*, 2006))	AQC	Fluorescence	250	395	C18 - 5 µm Bonded silica Phenomenex	48	140 mM solution sodium acetate trihydrate; 17 mM of triethylamine (pH adjusted to 5.05)	Methanol
6	(Mafra *et al.*, 1999)	OPA MCE	Fluorescence	340	450	C18 - 5 µm Silica-gel	60	sodium acetate trihydrate 0.05M; tetrahydrofuran (99:1 v/v), pH 7.2	Methanol; sodium acetate trihydrate 0.5 M (90:10 v/V), pH 9.5
7	(García-Marino *et al.*, 2010)	OPA	Fluorescence	340	426	C18 - 4 µm Silica Phenomenex	46	sodium acetate 0.05 M adjusted to pH 6.6; tetrahydrofurane (99:1)	Methanol; acetonitrile (50:50)
8	(Pereira *et al.*, 2008)	OPA	Fluorescence	335	440	C18 - 4 µm Silica-based Waters	80	10 mM sodium; phosphate buffer (pH 7.3); methanol; tetrahydrofuran (91:8:1)	Methanol; phosphate buffer (80:20)

#	Reference	Derivatzation agents	Detection	Excitation (nm)	Emission (nm)	Column	Time (min)	Eluent A	Eluent B
9	(Zotou *et al.*, 2003; Soufleros *et al.*, 2007)	DnS-Cl	UV	254	-	C18 - 5 µm Silica GL Sciences	35	Water (deionized)	Acetonitrile
10	(Pramateftaki *et al.*, 2006)	OPA	Fluorescence	-	-	5 µm	-	-	-
11	(Marcobal *et al.*, 2005; Garai *et al.*, 2007)	OPA	Fluorescence	340	425	C18 - 4 µm Silica-based Waters	60	$Na_2HPO_4 \cdot 12H_2O$; (3.6 mg/l, 10 mM)	1% 2-octanol in acetonitrile; eluent A (70:30 v/v)
12	(Landete *et al.*, 2004, 2007; Landete *et al.*, 2005a; Landete *et al.*, 2005b; Arena *et al.*, 2008)	OPA	Fluorescence	335	450	C18 - 5 µm Silica gel Merck	45	2.268 g KH_2PO_4; 14.968 g $Na_2HPO_4 \cdot 12H_2O$ (adjusted to pH 5.8 with H_3PO_4); (1 litre of water)	Methanol
13	(Moreno-Arribas *et al.*, 2003)	OPA	Fluorescence	340	425	C18 - 4 µm Silica-based Waters	-	-	-
14	(Brückner *et al.*, 2012) (for tea)	FMOC-Cl	Fluorescence	263	313	C8 - 4 µm Silica gel Merck	80	0.1 M sodium acetate buffer	Acetonitrile
15	(Gómez-Alonso *et al.*, 2007; Cejudo-Bastante *et al.*, 2010)	DEEMM	UV	280, 269, 300	-	C18 - 5 µm Silica ACE	85	25 mM acetate buffer (pH 5.8); 0.02% sodium azide	Acetonitrile; Methanol 80:20
16	(Yildirim *et al.*, 2007; Özdestan and Üren, 2009)	OPA	Fluorescence	340	420	C18 - 10 µm Silica Phenomenex	40	[0.050 M acetate buffer/tetrahydrofuran (96/4)]:methanol 60:40	Methanol

#	Reference	Derivatzation agents	Detection	Excitation (nm)	Emission (nm)	Column	Time (min)	Eluent A	Eluent B
17	(Proestos *et al*., 2008)	DnS-Cl	Fluorescence	320	523	C18 - 5 µm Silica based Phenomenex	35	water	Acetonitrile
18	(Yeğin and Üren, 2008; Özdestan and Üren, 2009)	benzoyl chloride	UV	254	-	C18 - 10 µm Silica Hichrom	30	0.05 M acetate; buffer:methanol (60:40)	Methanol
19	(Tang *et al*., 2009; Kim *et al*., 2011)	CNBF	UV	254	-	C18 - 5 µm Bonded silica Agilent	22	Acetonitrile	Acetic acid –Sodium acetate; buffer (0.1 M, pH 6.2)

Table 2-10 – Lable to Table 2-9

Short form	Derivatization Reagent
OPA:	o-phthaldialdehyde
FMOC-Cl:	9-fluorenylmethyl chloroformate
AQC:	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate solution
MCE:	2-mercaptoethanol
NQS:	1,2-naphthoquinone-4-sulfonate
DnS-Cl:	dansyl chloride
DEEMM:	diethyl ethoxymethylenemalonate
CNBF:	4-chloro-3,5-dinitrobenzotrifluoride

Table 2-11 – Applicability of the described methods to the analysis of multiple biogenic amines

	HIS	TYR	PHE	PUT	CAD	AGM	BUT	1,6-DIA	ETH	ETHYL	HEX	ISOA	ISOB	ISOPRO	MET	2-MET	3-MET	PRO	SER	SPD	SPM	TRY
1																						
2																						
3																						
4	*	*	*																			
5																						
6																						
7																						
8																						
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17																						
18																						
19																						
	HIS	TYR	PHE	PUT	CAD	AGM	BUT	1,6-DIA	ETH	ETHYL	HEX	ISOA	ISOB	ISOPRO	MET	2-MET	3-MET	PRO	SER	SPD	SPM	TRY

* Biogenic amines analyzed by the same method reported in a different paper

Table 2-12 – Lable to Table 2-11

Short form	Amine
HIS	Histamine
TYR	Tyramine
PHE	Phenylethylamine
PUT	Putrescine
CAD	Cadaverine
AGM	Agmatine
BUT	Butylamine
1,6-DIA	1,6-Diaminohexane
ETH	Ethanolamine
ETHYL	Ethylamine
HEX	Hexylamine
ISOA	Isoamilamine / Isopentylamine
ISOB	Isobutylamine
ISOPRO	Isopropylamine
MET	Methylamine
2-MET	2-Methylbutylamine
3-MET	3-Methylbutylamine
PRO	Propylamine
SER	Serotonin
SPD	Spermidine
SPM	Spermine
TRY	Tryptamine

2.1.6.2 OPA reaction

The derivatization reaction of biogenic amines with OPA makes use of a third reagent such as mercaptoethanol and occurs through combination of three molecules as pointed out in Figure 2-1.

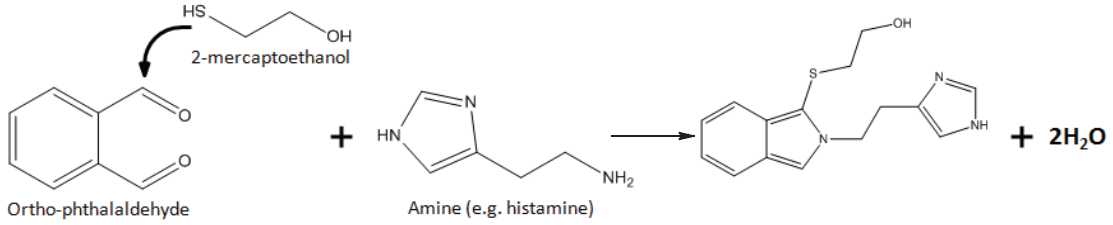


Figure 2-1 – Derivatization reaction mechanism with OPA and mercaptoethanol (based on (Csámpai *et al.*, 2004)) – Figures were designed with ChemBioDraw Ultra version 12 from CambridgeSoft®

Derivatization reactions may also take place with components other than mercaptoethanol such as, e.g., 3-mercaptopropionic acid or N-acetyl-L-cysteine (Csámpai *et al.*, 2004).

2.1.6.3 OIV method

The method adopted by the OIV for the analysis of biogenic amines was set as basis for the development of the present method.

The OIV method (Type II – Method OIV-MA-AS315-18 – Resolution OIV-Oeno 346/2009) can be applied to both musts and wines and it is capable of simultaneous detection of a great variety of biogenic amines, as shown in Table 2-11.

From the list presented, the biogenic amines intended to be evaluated by the method in development are histamine, tyramine, phenylethylamine, putrescine, and cadaverine (as mentioned in section 2.1.4). These amines are the most prone to be found in wine and musts and the most influenced by winemaking practices.

This method describes a 95 min gradient elution (Table 2-13) of a two component mobile phase composed by a buffer of disodium hydrogen phosphate (eluent A) and acetonitrile (eluent B). The mixture of these eluents should be pushed through the HPLC system at 1 mL/min and the fluorescence signals are detected using an excitation wavelength

of 356 nm and an emission wavelength of 445 nm. The column used should be a C-18 at 35 °C and samples should undergo an OPA derivatization before injection (1 µL injection volume).

Table 2-13 – Gradient elution used for biogenic amines analysis

Time (min)	%A	%B
0	80	20
15	70	30
23	60	40
42	50	50
55	35	65
60	35	65
70	80	20
95	80	20

The detection and quantification limits given in Table 2-14 were retrieved from the method document. In Table 2-14 diaminobutane appears as synonym to putrescine and diaminopentane to cadaverine.

Table 2-14 – Limits of detection and quantification presented in the OIV method.

	LOD (in mg/l)	LOQ (in mg/l)
Histamine	0,01	0,03
Methylamine	0,01	0,02
Ethylamine	0,01	0,03
Tyramine	0,01	0,04
Phenylethylamine	0,02	0,06
Diaminobutane	0,02	0,06
2-methylbutylamine	0,01	0,03
3-methylbutylamine	0,03	0,10
Diaminopentane	0,01	0,03

The interlaboratory conditions used for the attainance of these values are presented in Appendix A, A–1.

The reliability of the method will also be compared to the one developed. The values of repeatability and reproducibility can be found in Appendix A, A–2.

As for the values of reproducibility, the range for the maximum acceptable standard deviation of 5 mg/L standards finds a low value of 1.73 mg/L phenylethylamine and goes up to 2.86 mg/L for histamine.

Even though one could argue that these values are too high, the reason for later changes to the method relies on the extended analysis period.

3 Materials and Methods

3.1 Samples

Samples were periodically obtained from fermentation trials using the several possible combinations between the varieties Encruzado, Loureiro and Viosinho and the strains 386, 496, 666, 765 and QA23. The wines resulting from these fermentations constitute the samples for biogenic amine analysis. Additionally, samples for biogenic amines also included red and red Port wines from Quinta do Seixo, rose Port wines from Quinta do Sairrão, red wines from Quinta da Lêda and red wines for malolactic fermentation studies (vineyards owned by Sogrape Vinhos).

In the case of glucose and fructose, preserved (frozen) samples from the same trials, regularly withdrawn throughout the fermentation process, were analyzed.

3.2 Winemaking Process and Sampling

Clarified must (must to which solid particles in suspension have been removed) from each variety was collected and, after adjusting readily assimilable nitrogen to 220 mg/L with diammonium phosphate, it was sterilized through cartridge pre-filtration and filtration using 0.45 μm filters. Then, filtrate was transferred to demijohns previously cleansed with *aguardente vínica* with an alcoholic content of 77% vol.. Finally, the selected strains were inoculated having each strain/variety group been used in triplicate in independent demijohns with approximately 8 L of must each. Fermentation temperature was controlled and fixed to 15 °C and, every other day until the end of the fermentation, samples were taken from each demijohn and frozen for later analysis. Sample freezing stops fermentation so must characteristics of the moment of sampling are preserved.

3.3 Fructose and Glucose Analysis

3.3.1 Chemicals and Reagents

D (-) – Fructose and D (+) – Glucose standards were purchased from Merk, the acetonitrile with purity over 99.9% was purchased from VWR® - BDH Prolabo and the water used was treated in Millipore Elix Advantage 5 water purification system and in a Milli-Q Adavantage A10 Ultrapure water purification system.

3.3.2 Standards Preparation

Five standards of fructose and glucose were prepared with water in 100 mL volumetric flasks by direct weight of the mass of each component (Table 3-1).

Table 3-1 – Glucose and fructose standards for HPLC-RI analysis

Standard	Glucose g/L	Fructose g/L
P1	0.254 ± 0.001	0.249 ± 0.001
P2	1.499 ± 0.002	1.506 ± 0.002
P3	5.001 ± 0.005	5.000 ± 0.005
P4	10.00 ± 0.01	10.00 ± 0.01
P5	15.00 ± 0.02	15.02 ± 0.02

3.3.3 Sample Treatment

Frozen samples were put into a bath with cold water (between 5 and 10 °C) in order to achieve defrost while assuring that must fermentation did not restart which would alter the fructose, glucose and alcoholic contents. Prior to analysis, each sample was filter sterilized using a Sterile Syringe Filter with a 0.2 µm Cellulose Acetate Membrane. Afterwards, because of the high amounts of sugar expected from must samples, several dilutions (in ultra pure water) were made with the intention of getting at least one condition that would fall within the limits of the calibration curve after analysis by HPLC-RI. Dilution factors varied between 5, 10, 20 and 50 depending on an educated guess of the glucose and fructose contents of the

sample based on the fermentation stage (sampling day) and the results obtained from other samples. Diluted samples were left to rest for at least one hour prior to injection so that they have enough time to stabilize.

3.3.4 Chromatographic Analyses

3.3.4.1 Equipment and Software

The HPLC apparatus used for fructose and glucose analysis consisted of a Jasco 880-PU pump, a Jasco AS 2057 Plus autosampler, a Purosphere® LiChroCART 250-4 STAR NH_2 (5µm) chromatographic column, a Jasco 2067 Plus column oven and a Jasco 830-RI refraction index detector.

Chromatogram peak areas were calculated using the software tools from Varian Star Chromatography Workstation System Control version 6.41 and Varian Star Chromatography Workstation Interactive Graphics version 6.41.

3.3.4.2 Operational Conditions

For measurements, a 17 minute isocratic elution of a mixture of acetonitrile / water (85:15), a 1 mL/min flow rate, a 20 µL of sample injection volume and a column temperature of 30 °C were used.

3.3.5 Enzymatic Analyses

3.3.5.1 Equipment and Software

Besides the HPLC-RI, an automatic analyzer was also used to assess the glucose and fructose contents of the samples. The equipment is an Y15 from BioSystem Enology and makes use of enzymatic reagent kits also from BioSystems. (Appendix B). The equipment monitors the enzymatic reactions through absorbance readings at 340 nm and gives the results indirectly through the use of a one point calibration. The solution for calibration was also provided by

BioSystems. Results were retrieved from a software named after the equipment, Y15 version 4.6.1.

3.3.5.2 Enzymatic Reactions

There are three enzymatic reagents responsible for glucose and fructose conversion to gluconate-6-phosphate which is the component that is measured, hexokinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase (Figures 3–1 to 3–3). So, the sum of glucose and fructose content is obtained. In order to get the individual values for each sugar, samples are reanalyzed absent the isomerase. Therefore only glucose is measured and fructose levels are attained through the difference of the two values.

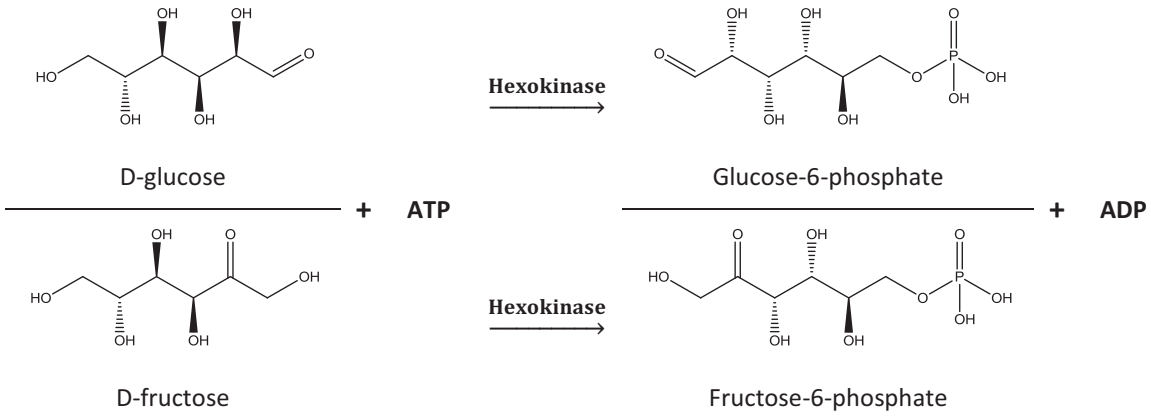


Figure 3-1 – Enzymatic conversion of D-glucose to glucose-6-phosphate by hexokinase – Figures were designed with ChemBioDraw Ultra version 12 from CambridgeSoft®

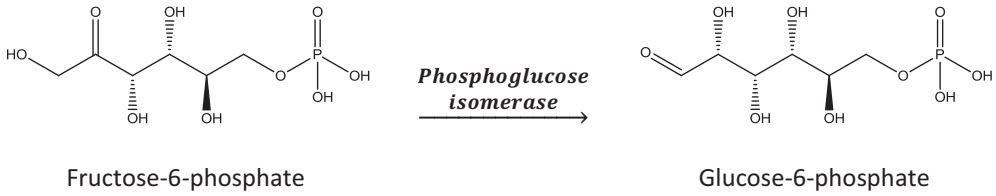
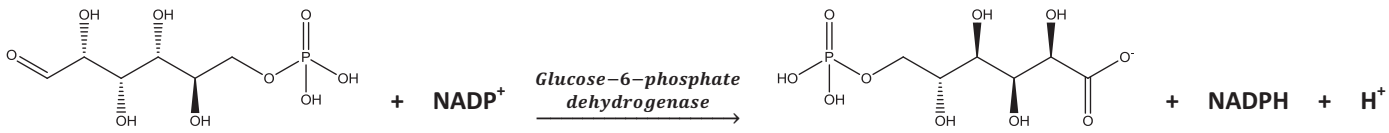


Figure 3-2 – Enzymatic conversion of fructose-6-phosphate to glucose-6-phosphate by phosphoglucose isomerise – Figures were designed with ChemBioDraw Ultra version 12 from CambridgeSoft®



Glucose-6-phosphate Gluconate-6-phosphate

Figure 3-3 – Enzymatic conversion of glucose-6-phosphate to gluconate-6-phosphate by glucose-6-phosphate dehydrogenase – Figures were designed with ChemBioDraw Ultra version 12 from CambridgeSoft®

3.4 Biogenic Amines Analysis

3.4.1 Chemicals and Reagents

Biogenic amines standards tyramine, phenylethylamine, putrescine and cadaverine were purchased from Aldrich®. Histamine standard and 2-mercaptoethanol were bought from Sigma® and the sodium borate decahydrate from Sigma-Aldrich®. Moreover, ortho-phthalaldehyde, disodium hydrogen phosphate (Na_2HPO_4) as well as hydrochloric acid 0.1 N solution came from Merck. Additionally, methanol was obtained from Panreac. The water used was treated with both purification systems from Millipore, previously described in section 3.3.1..

3.4.2 Standards Preparation

Each standard was prepared so as to match a multiple concentration of the calibration mix suggested in the OIV method (Table 3-2) ranging from 0.05 and 3 times the values specified (Table 3-3). Calibration standards were prepared in 0.1 M HCl.

Table 3-2 – Calibration mix from OIV reference method

	Indicative final concentration in the calibration mix in mg/l
Ethanolamine	5
Histamine	5
Methylamine	1
Serotonin	20
Ethylamine	2
Tyramine	7
Isopropylamine	4
Propylamine	2.5
Isobutylamine	5
Butylamine	5
Tryptamine	10
Phenylethylamine	2
Putrescine	12
2- Methylbutylamine	5
3- Methylbutylamine	6
Cadaverine	13
1.6 Diaminohexane	8
Hexylamine	5

Table 3-3 – Biogenic amine standards for HPLC-FL analysis

Standard	Histamine mg/L	Tyramine mg/L	Phenylethylamine mg/L	Putrescine mg/L	Cadaverine mg/L
P1	0.250 ± 0.004	0.350 ± 0.004	0.120 ± 0.004	0.600 ± 0.004	0.650 ± 0.005
P2	1.00 ± 0.02	1.40 ± 0.02	0.482 ± 0.009	2.40 ± 0.02	2.60 ± 0.02
P3	2.50 ± 0.03	3.50 ± 0.03	1.20 ± 0.03	6.00 ± 0.03	6.50 ± 0.03
P4	5.00 ± 0.05	7.00 ± 0.05	2.41 ± 0.05	12.00 ± 0.05	13.00 ± 0.05
P5	10.00 ± 0.09	14.02 ± 0.09	4.82 ± 0.08	24.0 ± 0.2	26.0 ± 0.2
P6	15.0 ± 0.2	21.0 ± 0.2	7.2 ± 0.2	36.0 ± 0.2	39.0 ± 0.2

3.4.3 Sample Treatment

Sample treatment comes down to filter sterilization of the sample using a Sterile Syringe Filter with a 0.2 µm Cellulose Acetate Membrane followed by a 1:1 dilution with methanol.

3.4.4 Chromatographic Analyses

3.4.4.1 Equipment and Software

The equipment used for biogenic amines analysis consisted of two Jasco 880-PU pumps and a Jasco 880-31 solvent mixing unit used for gradient elution, a Jasco AS 2057 Plus autosampler for sample derivatization and injection and a Jasco CO 2067 column oven which supported a LiChroCART® 150-4.6 Purospher® STAR RP-18e (5µm) chromatographic column. Fluorescence was measured by a Jasco 821-FP spectrofluorometric detector. The chromatogram peak areas were calculated using the software tools from Varian Star Chromatography Workstation System Control version 6.41 and Varian Star Chromatography Workstation Interactive Graphics version 6.41.

3.4.4.2 Operational Conditions

The two Jasco 880-PU pumps worked in a master and slave system. The master pump controls the flow of both pumps in order to gather varying proportions of each eluent and making them flow into the mixing unit at the specified flow rate therefore creating the desired gradient. The mixing module Jasco 880-31, as suggested, mixes the eluents coming from the pumps and drives the mixture into the system. For elution, an aqueous solution of 5.56 g/L of sodium phosphate dibasic (Na_2HPO_4) (filtered through 0.45 µm) was used as eluent A while acetonitrile was used as eluent B (in slave pump). Table 3-4 shows the gradient elution which was used at a 1 mL/min flow rate.

Table 3-4 – Gradient elution used for biogenic amines analysis

Time (min)	%A	%B
0	80	20
20	60	40
40	35	65
45	80	20
50	80	20

The injection volume was set to 20 µL, the column was heated to 35 °C and using the excitation and emission wavelengths used for spectrofluorimectric detection were 356 nm and 445 nm respectively.

3.4.4.3 Preliminary derivatization

Samples undergo an online and pre-column derivatization process which is conducted by the autosampler. The derivatization mixture is composed by a daily prepared 0.4 g/L OPA solution (in methanol), a weekly prepared 0.1 M sodium borate decahydrate aqueous buffer (pH adjusted to 10.5 with concentrated sodium hydroxide) and 2-mercaptoethanol in a 10:10:3 proportion.

The autosampler takes 184 µL of this mixture and pours it into a mixing vial along with 16 µL of sample. Noteworthy is the ability of the autosampler of not allowing contact between sample and derivatization reagents while pipetting.

After that, air is blown twice into the vial (80 µL/s) with the purpose of homogenizing the mixture in which sample derivatization is taking place. Before injection, the vial is checked to assure no bubbles remained from the air mixing step. The vials that host the derivatization reaction are smaller than the ones carrying the sample and derivatization mixture (300 µL capacity opposed to 2 mL) so that the liquid becomes high enough to have the autosampler's needle completely submerged when pipetting the derivatized sample for injection. There is an autosampler option to set a time during which the sample is left to react (reaction time). Even though this time was set to zero, the time of contact of the 2 components prior to injection is about 3 minutes and 45 seconds.

4 Results and Discussion

4.1 Biogenic Amines Analysis

The analysis of biogenic amines content is a pressing concern that must be taken into account when choosing the strain to inoculate the must with. The inexistence of an established analytical method for their analysis in Sogrape Vinhos Central Oenology Laboratory triggered the necessity for its development which is conducted in this work. The method relies on an HPLC-FL analysis that is preceded by a derivatization reaction. Further details regarding the method and the results obtained are going to be explored in sections to follow.

Results presentation does not follow chronological order. Instead, a thematic organization of the results was made in the attempt to allow better understanding of the work done. Moreover, it is noteworthy the advanced age of the HPLC equipment. As a result the conditions in which the analyses were made may not be ideal. With more recent equipments better performance can be achieved.

4.1.1 Biogenic Amines Method Development

Prior to the present work no biogenic amine tests have ever been made in Sogrape Vinhos. Therefore, the method was built from scratch using OIV method as guide. It was intended to reduce the analysis time with the purpose of developing a method for routine analysis. To begin with, the conditions of the OIV method were recreated to the possible extent and from there the method was shaped in line with the company's interests.

At the time the work started the only available column susceptible of being used for biogenic amine determinations was a Chromolith® Performance RP - 18e, 100 - 4.6. This column, in the place of silica spheres, has silica monoliths (monolithic column). Therefore, retention times are expected to be lower. Besides, reduction of the retention times is further influenced by the compact dimensions (100 mm long) when compared to the column described in the OIV method (250 mm long). These features should allow faster analysis times if proper conditions for biogenic amines are found and if resolution turns out not to be an issue.

Some parameters such as eluent composition, flow rate, column temperature and detection wavelengths were not object of optimization within this project and therefore maintained as a copy from the conditions presented in the OIV method.

4.1.1.1 Mobile Phase Gradient Optimization

The first chromatographic tests performed made use of the 95 minute long gradient available from OIV (Figure 4-1 and Table 2-13 section 2.1.6.3). As the method is intended to be used routinely, this elution was given as too time consuming and a different, shorter gradient was tried out.

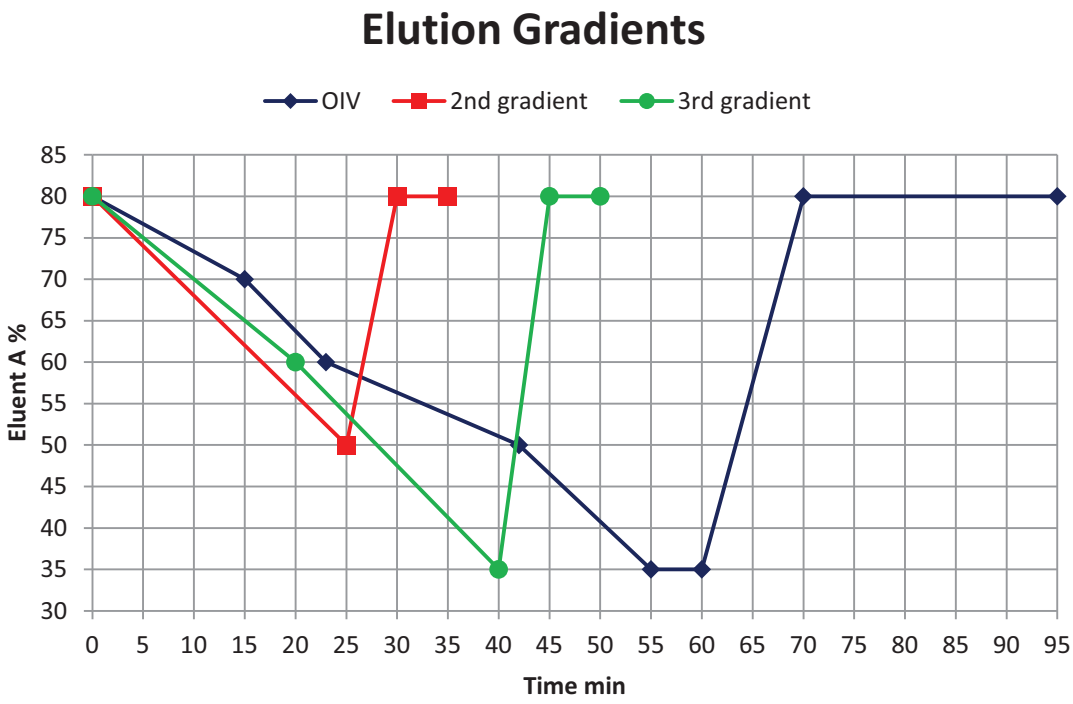


Figure 4-1 – Comparison of the elution gradients tested

Bearing in mind column characteristics and after verifying that histamine was eluted close to the beginning of the chromatographic run, 12 minutes past, (Figure 4-6 section 4.1.1.3) the gradient length was reduced to 35 minutes, concentration drop and rise (i.e., variation of the percentage of the eluents) were made steeper and the final stabilization period was reduced (Figure 4-1).

Later on, product accumulation in the column (Figure 4-2) as well as an inability to identify phenylethylamine peak (Figure 4-3) revealed to be problematic so the gradient was once again changed. This time, the gradient was made closer to the original one. The length was increased to 50 minutes in order to give time to further decrease the eluent A percentage to the lowest value used in the OIV method. The concentration drop was not made steeper in order to achieve better amine separation (Figure 4-1).

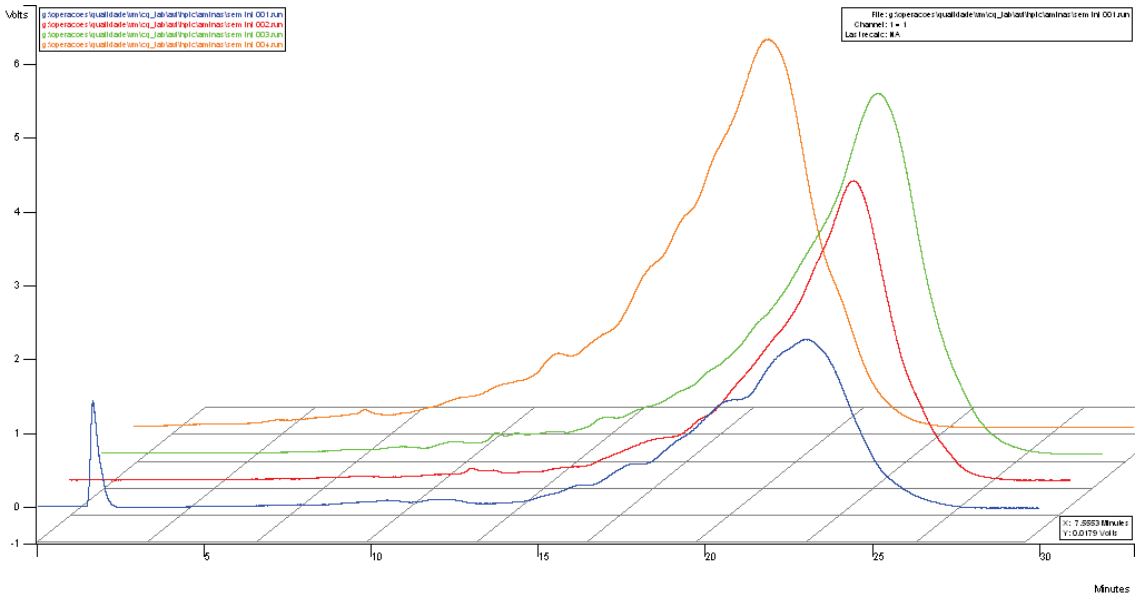


Figure 4-2 – Four replicate chromatograms of gradient run without any injection

The accumulation of products in the column was revealed by the high peaks that appeared in chromatographic runs in which no injection has been made (Figure 4-2). On the other hand, in consecutive injections of phenylethylamine with increasing concentrations ranging from 2.41 mg/L to 482 mg/L no chromatogram revealed proportional increases in any peak (Figure 4-3).

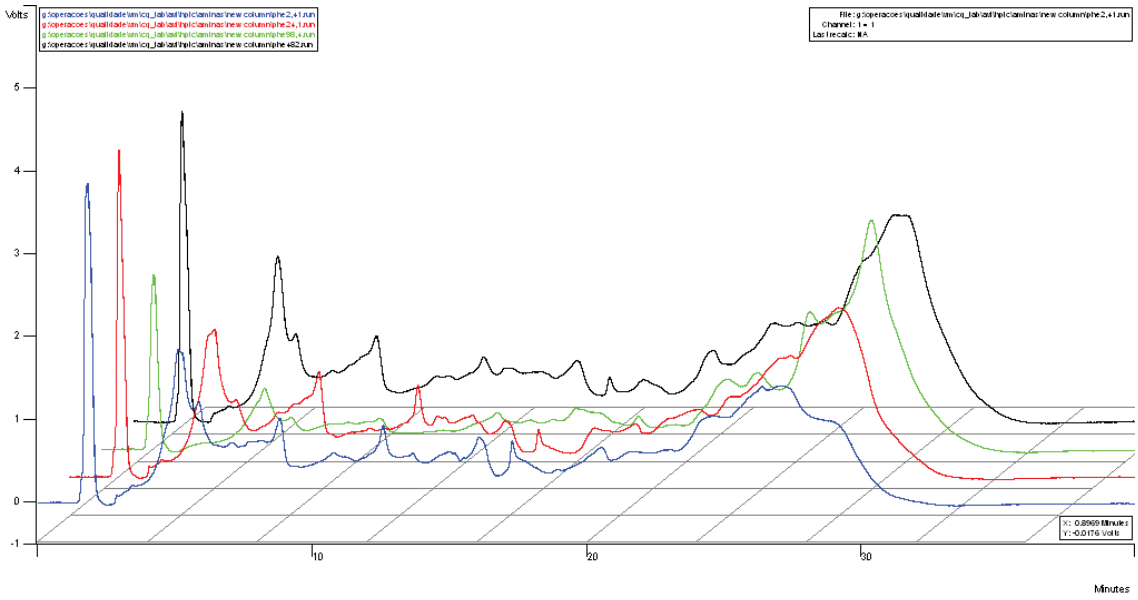


Figure 4-3 – Four phenylethylamine injections with increasing concentrations (blue – 2.41 mg/L, red – 24.1 mg/L, green – 98.4 mg/L and black – 482 mg/L)

The 50 minutes gradient did not presented any of the abovementioned problems so it was the chosen gradient elution to be used in the consequent analysis.

4.1.1.2 Column Exchange

Results obtained with the Chromolith® column were not satisfactory. Most of the times, from replicate injections very different results were obtained. Figures 4–4 and 4–5 stand as examples of the inconsistencies faced.

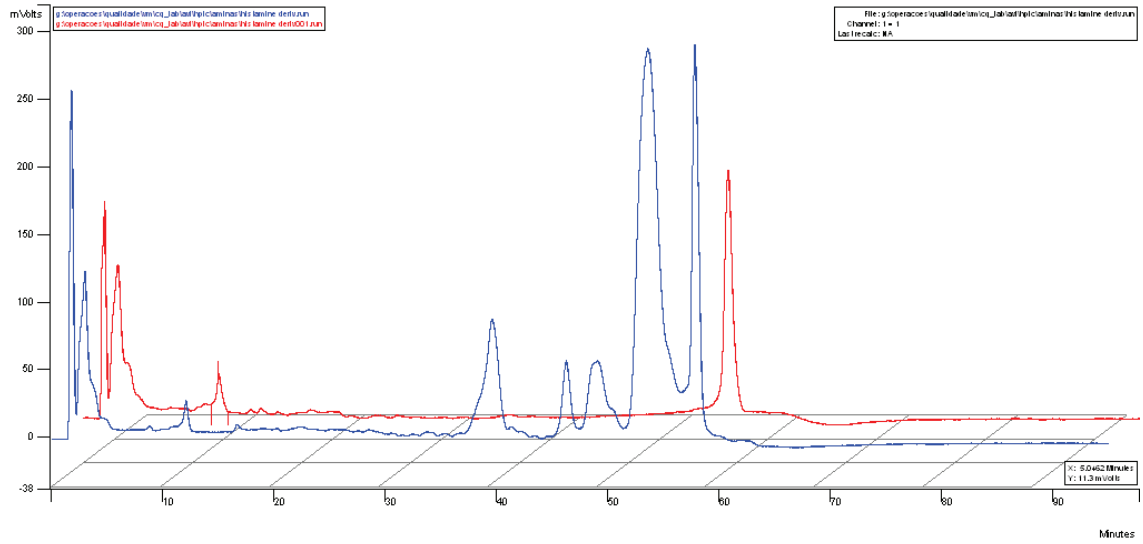


Figure 4-4 – Example of replicate inconsistency

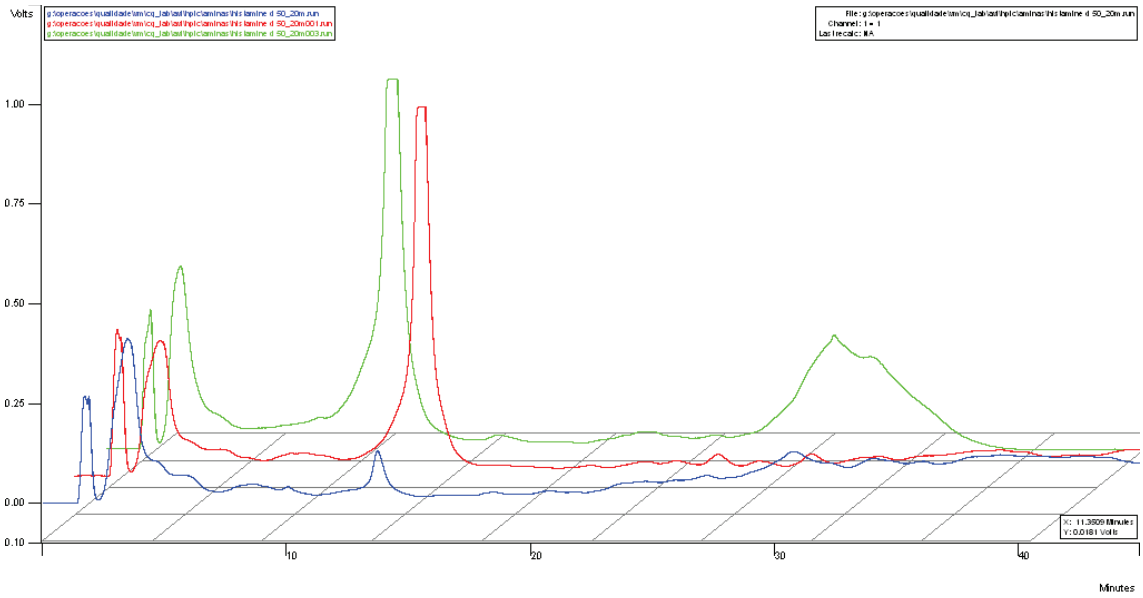


Figure 4-5 – Example of replicate inconsistency

The inability to get consistent consecutive replicates has lead to a change of the chromatographic column. The Chromolith® column was replaced by a LiChroCART® 150-4.6 Purospher® STAR RP-18e (5µm) column that meanwhile became available. Furthermore, the latter presents characteristics closer to the ones of the column used in the OIV method.

4.1.1.3 Derivatization Time Optimization

This project is pioneer concerning the use of derivatization procedures in Sogrape Vinhos. Therefore, the first step towards the optimization of the derivatization was to verify its effect. The same sample was injected with and without derivatization and it could be verified the appearance of new chromatographic peaks including, as expected, the peak of the biogenic amine tested, histamine, only in the derivatized sample (Figure 4-6).

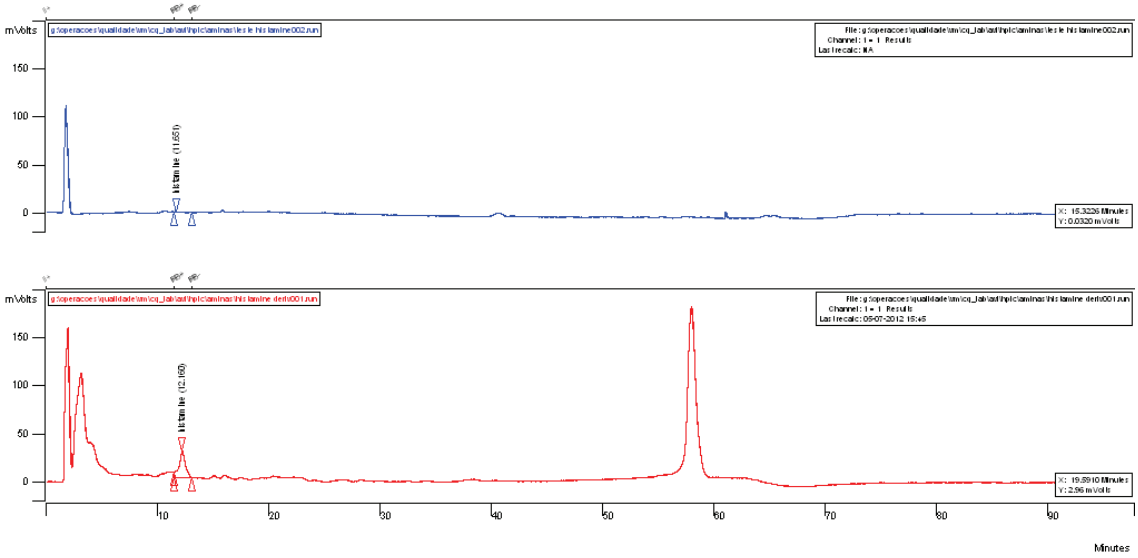


Figure 4-6 – Comparison of the same sample injected with (in red) and without (in blue) derivatization.

The derivatization procedure makes use of the OPA solution, mercaptoethanol and borate buffer. A mixture of these components was prepared every day maintaining the proportions of each component according to the OIV method (10:3:10). In it, an offline derivatization procedure is described. However, in this project the derivatization was carried out online and pre-column through the use of the autosampler features.

The autosampler allows mixing the sample with up to two reagents (i.e., reagent solutions coming from two different vials). In this case, the reagent mixture was made separately and the derivatization was programmed to mix one reagent with the sample. Moreover, the maximum volume authorized is a total of 215 µL (sum of sample and reagents). In order to ease proportion calculus and maintain the proportion described in the OIV method, the total volume of derivatization was fixed to 200 µL - 16 µL of sample were used to react with 184 µL in a mixing vial. These vials have a smaller capacity and conical shape in order to

provide enough liquid height to guarantee total needle submersion when pipetting derivatized samples. The derivatization reaction is incomplete so it must occur in a short period of time and injection should occur immediately afterwards. With the purpose of having better control over the derivatization time an autosampler option has been enabled which assures no contact between the sample and the reagent takes place within the needle. Therefore, the reaction is restricted to the time of contact within the mixing vial. Still, there are three parameters that may yet influence derivatization. After pouring the sample and reagent solution into the same mixing vial, the mixture of the components is homogenized by a flow of air inserted in the vial. The number of times air is used to mix the contents of the mixing vial, the speed at which air is blown and the time during which the autosampler is idle waiting for derivatization reaction to occur are optimizable.

The air mixing speed was left at the default value of 80 µL per second and it was not object of optimization. On the other hand, the effect of both the number of mixes and the reaction time was tested. Noteworthy, is that low reaction times are desired since they take place after the derivatization reagents are mixed with the sample and no action takes place during the specified time. Besides, immediate injection is suggested in the OIV method. Nonetheless, a series of injections were made maintaining the number of mixes constant and varying the reaction time Table 4-1.

Table 4-1 – Effect of reaction time on the peak area of an histamine standard at 5 mg/L

Reaction time min	Peak Area $\times 10^6$
0	3.0 ± 0.6
0.5	1.8 ± 0.6
1	2.4 ± 0.6
2	5.6 ± 0.6

The highest peak area obtained from these tests corresponds to the injection with two minutes of reaction time. However, the total time of derivatization (contact between sample and reagent mixture including the duration of the mixture) was over 6 minutes. This was considered too long. During this time, the derivatization reaction develops giving opportunity to possible secondary reactions. Therefore, a shorter time was considered that could also lead to relatively high sensitivity. The tested value that best fitted these conditions was the zero reaction time case. Consequently, this was the adopted value.

Additionally, the influence of the number of mixes, i.e., the number of times air is inserted in the vial for homogenizing the derivatization solution with the sample, was tested. This parameter influences both the mixture and the derivatization time, i.e., the time during which the sample and the derivatization mixture are in contact.

Table 4-2 – Influence of the number of mixtures in derivatization performance

Number of Replicates	Number of Mixes	Average Peak Area $\times 10^6$	Standard Deviation $\times 10^6$	CV %
1	0	1.1	-	-
7	1	3	4	107%
3	2	2.6	0.9	32%
5	3	4	3	72%
2	4	3	2	53%
1	5	3.2	-	-

The results obtained should be analysed bearing in mind that a deficient mixture may introduce many errors in the system and hinder reliability of the results while with extended mixtures the homogenization is not an issue but the derivatization time is also longer thus shifting the intentions of having short derivatization periods.

With no mixture taking place, the value obtained was lower than any other presumably due to insufficient mixture. This suggests that pouring the sample and reagent mixture into the vial is not enough for proper homogenization and derivatization of the sample. Insufficient time of contact does not seem to be an issue since, even without mixture or reaction time, over 3 minutes have passed from the first contact of the two solutions and the injection moment.

When mixing exists results are higher however they also present very high values of variability coefficient (CV%) especially with one mix, 107%. In terms of absolute values the higher achieved were obtained with three mixes. However, CV% is still 72 %. In this case, higher values were abdicated in favour of lower variability as verified with 2 mixes, 32%.

It would be interesting to verify the tendency of variability evolution with increasing number of mixes. However, the equipment only accepts a maximum of 5 mixtures. On the other hand, too long derivatization time could influence the results with higher number of mixes.

The air driven mixture has a problem though. After air insertion on the vial an air bubble may be formed inside the vial. This bubble occurs occasionally and it usually stays trapped near the bottom of the vial in the area the needle captures the solution for injection. This can cause erroneous results later on. Therefore, as precaution, before every injection, as soon as the mixing procedure is finished, the vial is removed from the sampling rack to check for bubble presence. If it appears, the vial is slightly tilted to remove it being promptly placed back in its place in time to be injected without having to stop or repeat the process.

4.1.1.4 Injection Volume Optimization

The volume of derivatized sample set to be injected in the system also has a great influence on the final results. In the first injections made this volume was set to 1 µL after the OIV method. However, in order to increase sensitivity of the results, the injection volume was first elevated to 10 µL and later to 20 µL. Figure 4-7 represents the variation in the results originating from the modification of this parameter.

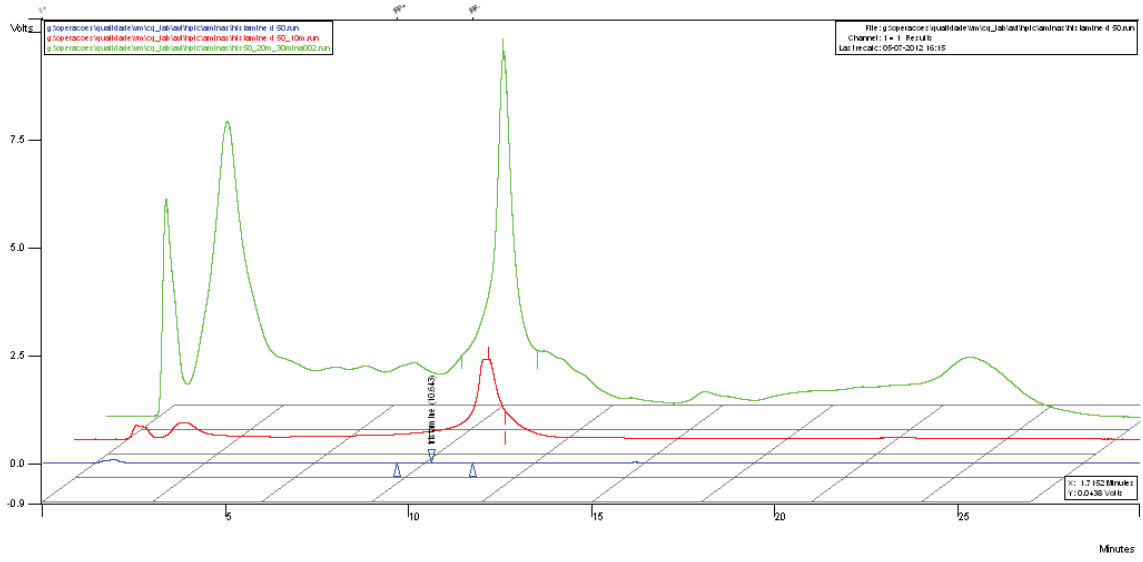


Figure 4-7 – Effect of the injection volume on the results of an histamine standard at 50 mg/L (blue – 1 µL; red – 10 µL; green – 20 µL).

As expected, with higher injection volume much higher sensitivity was achieved. For instance, the signal obtained with 20 µL injection is more than two times higher than the one

with 10 µL. Besides, the baseline noise can be more easily distinguished from the actual peak (zoom is needed to see such effect).

4.1.1.5 Optimum Conditions for Analysis

After gathering the best value for each of the discussed parameters an optimum set of conditions was achieved.

The pump pair was programmed to deliver a 1 mL/min flow of a 50 min gradient elution regarding eluents A, phosphate buffer, and B, acetonitrile. The autosampler was set to join the sample with the reagents mixture in the same proportions as described in the OIV method. Additionally, the number of mixes was fixed in 2 and the reaction time in 0.0 min. The mixing speed was left at the default value of 80 µL/s. With these conditions, the total time of derivatization is 3 minutes and 45 seconds. Finally, the volume of injection was raised to 20 µL while the column temperature and the excitation and emission wavelengths of the detector were set to the values presented in the OIV method, 35 °C, 356 nm and 445 nm, respectively.

4.1.2 Identification of Biogenic Amines Retention Times

After setting the analysis conditions an individual identification of the peaks of each biogenic amine was made in order to get the retention times for each one and allow their identification in wine samples.

The first step in the identification of the peaks of each biogenic amine was to learn the system's response to the blank injection. It is prepared similarly to the standards but without any biogenic amine present, i.e., a derivatized mixture of HCL 0.1 N and methanol (Sections 3.4.2 and 3.4.3).

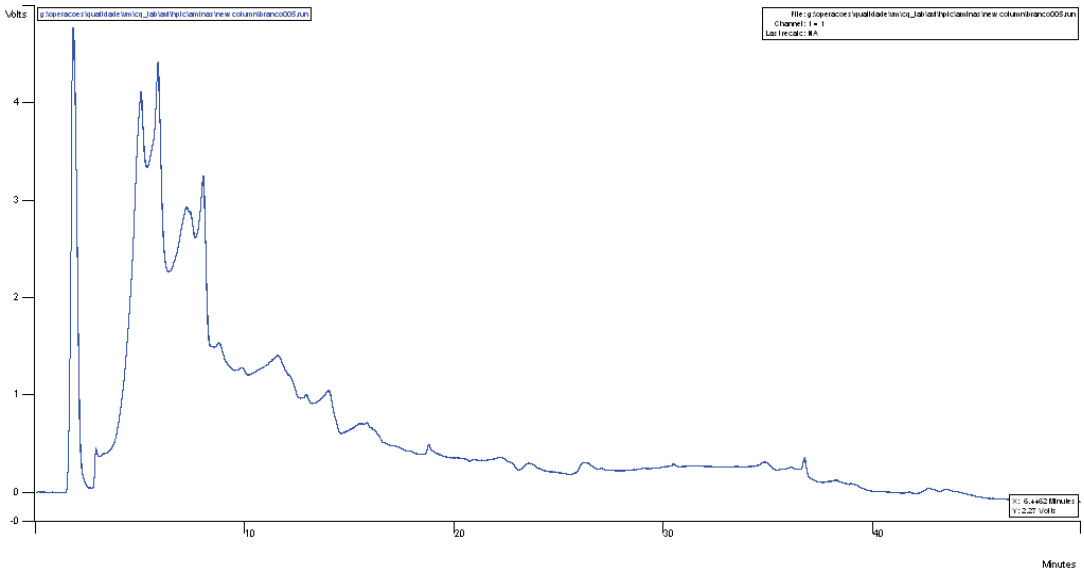


Figure 4-8 – System response to blank injection

Next, each biogenic amine was injected separately (Figures 4–9 to 4–13).

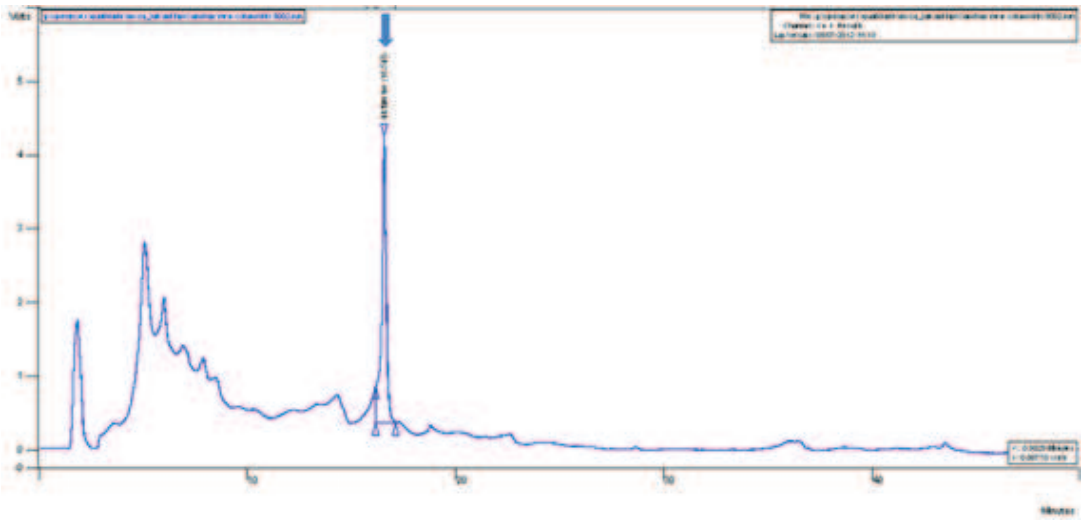


Figure 4-9 – Chromatogram of a histamine standard at 5 mg/L

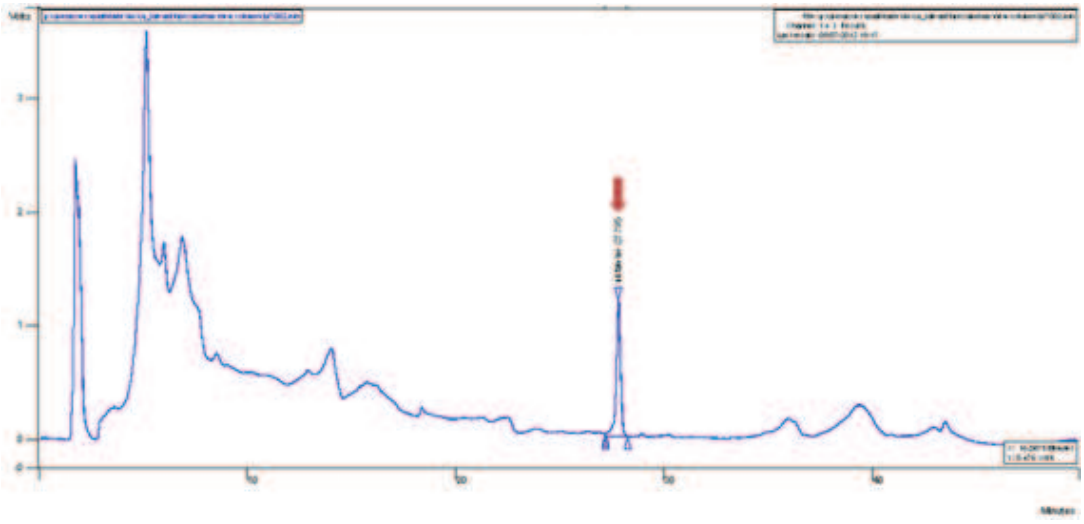


Figure 4-10 – Chromatogram of a tyramine standard at 7 mg/L

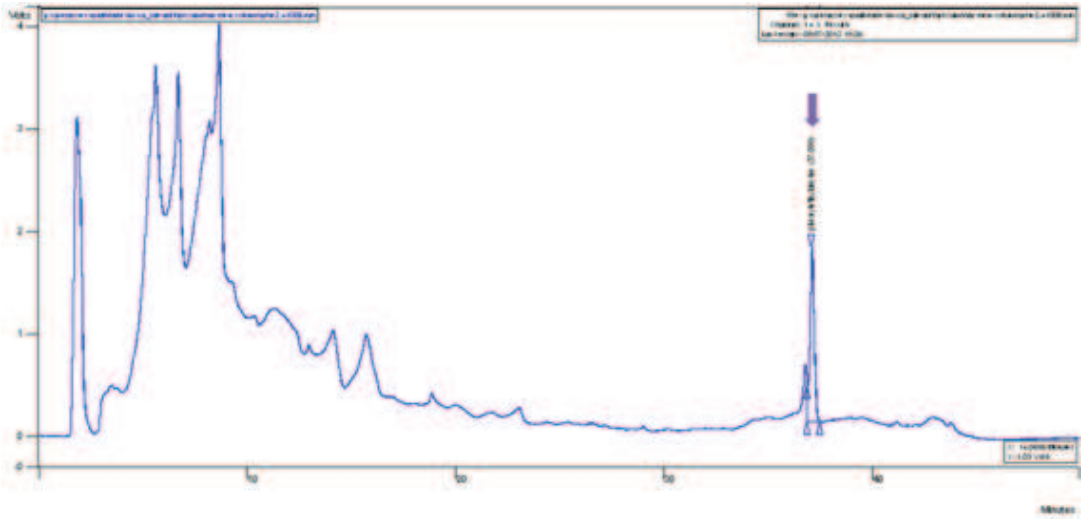


Figure 4-11 - Chromatogram of a phenylethylamine standard at 2.41 mg/L

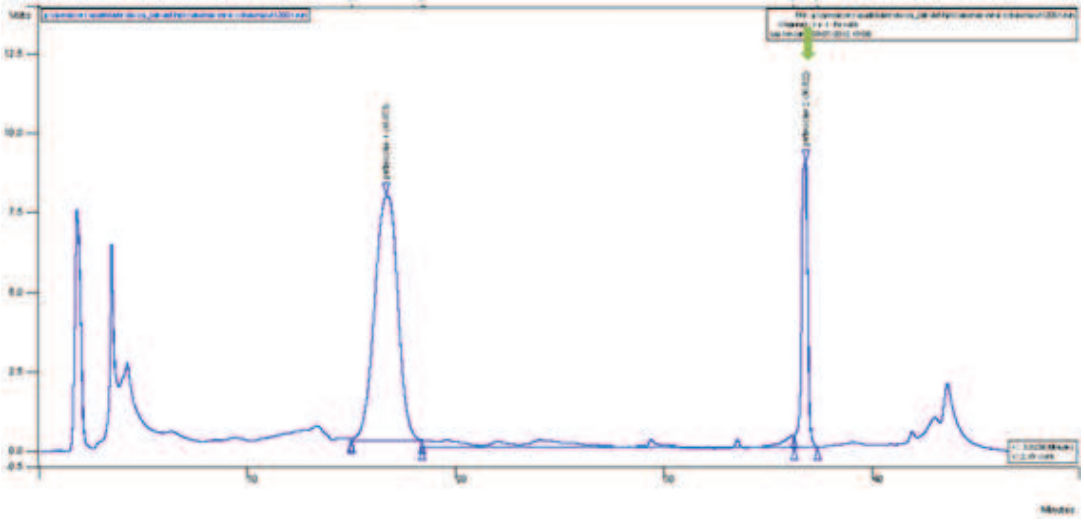


Figure 4-12 - Chromatogram of a putrescine standard at 12 mg/L

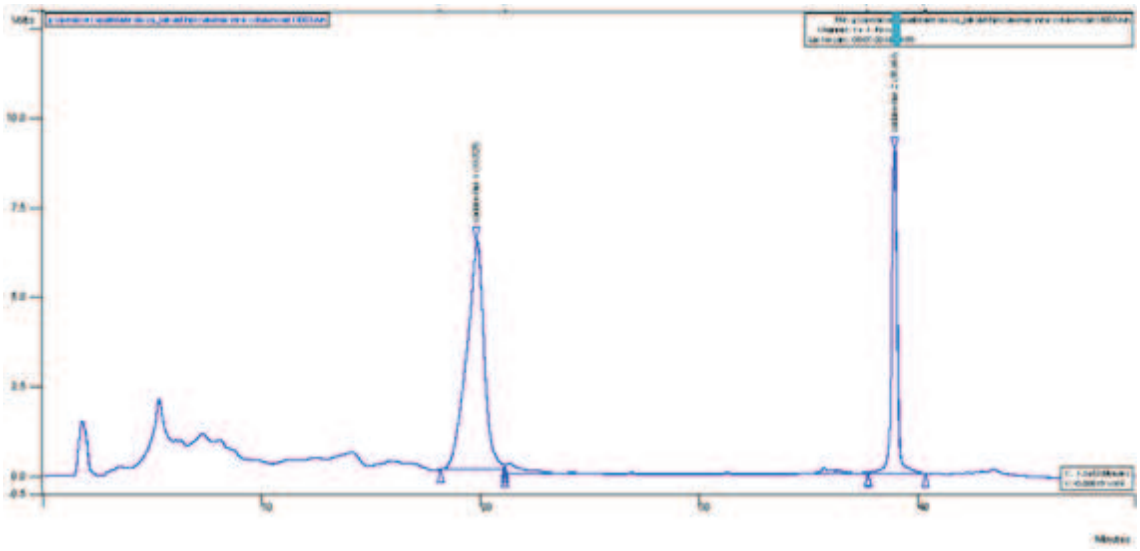


Figure 4-13 - Chromatogram of a cadaverine standard at 13 mg/L

In the isolated injections all the biogenic amines were easily identified since they appear far from the initial solvent peaks and with high response signals even at relatively low concentrations. In particular, histamine co-elutes with minor solvent peaks making it harder to identify the beginning of the peak while putrescine and cadaverine form two peaks each. From these only the later peaks are used for quantification purposes (arrow pointed) due to the higher definition of the peak and uneven form of the former peaks observed in replicate injections (not shown). Moreover, the calibration with the later peaks evidenced a linear relation of the peak area with the putrescine and cadaverine concentration (see section 4.1.3.1). The similar behaviour of cadaverine and putrescine may result from the close structure relation between them. The same cannot be said regarding tyramine and phenylethylamine since the OH group that distinguish them has a great effect on the affinity of the compound to the chromatographic column.

Having the retention times of each biogenic amine sorted out, a solution containing all five amines was injected to evaluate their behaviour in a mixture (Figure 4-14).

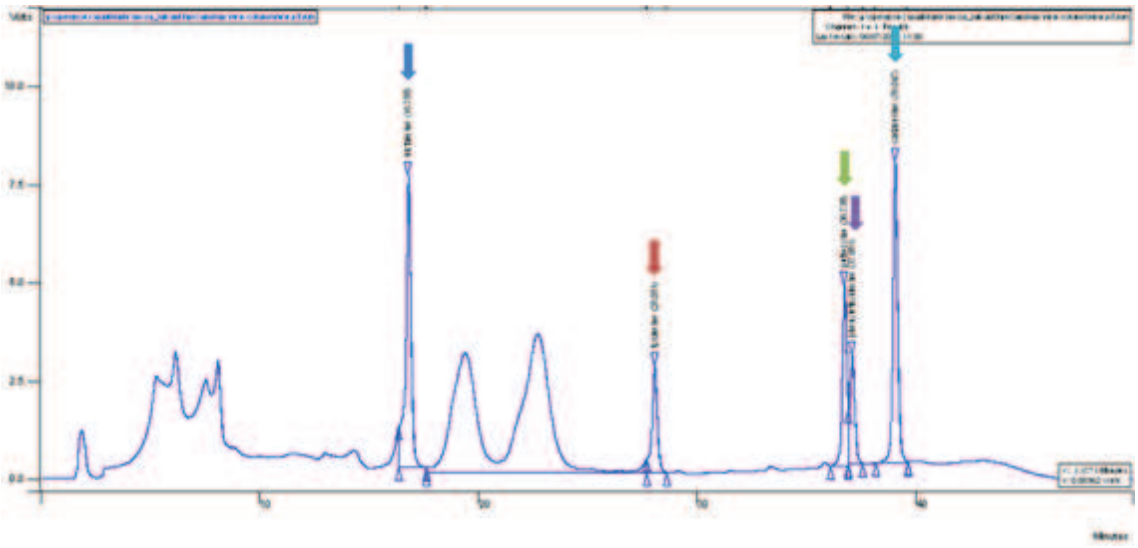


Figure 4-14 – Mixed injection of histamine, tyramine, phenyethylamine, putrescine and cadaverine

In the mixture, phenylethylamine and putrescine peaks elute very close to each other but still separate enough to make use of a peak separation tool that assists in peak area integration. From these results a comparison was made regarding the individual biogenic amine retention time (Table 4-3).

Table 4-3 – Comparison between isolated and mixed injections and recovery percentages

Biogenic Amines	Concentration mg/L	Retention Time min	
		Isolated Response	Mixture Response
Histamine	5	16.545	16.372
Tyramine	7	27.795	27.743
Phenylethylamine	2.41	37.099	36.828
Putrescine	12	36.822	36.547
Cadaverine	13	38.961	38.901

The retention times obtained from both isolated analysis and in mixture were similar for all cases.

The last step in the identification of the biogenic amines was the shift to the wine matrix (Figure 4-15).

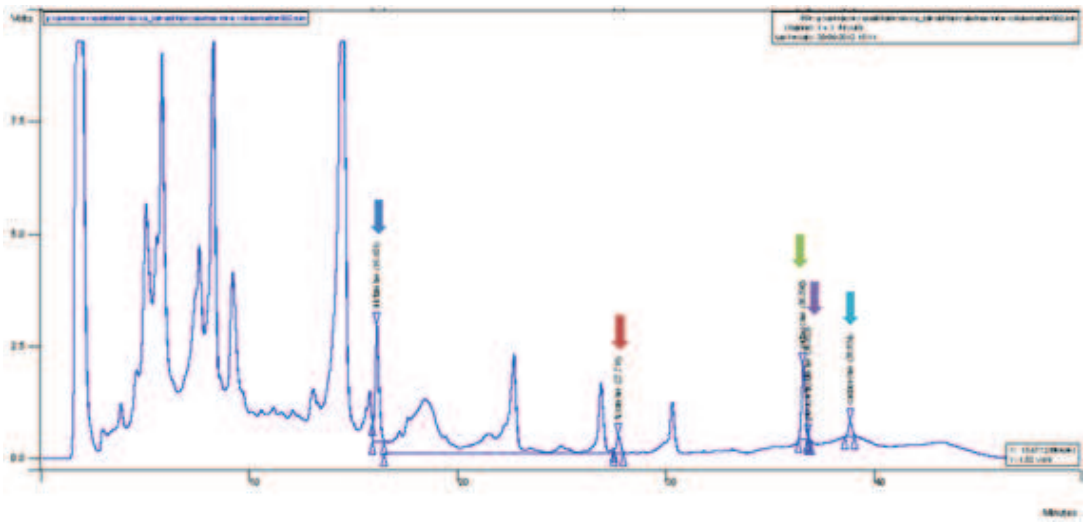


Figure 4-15 – Identification of the biogenic amines in wine – an example

The wine matrix introduces many interferences but their effect is mainly felt in the first 15 minutes of elution. Fortunately, no major interfering compound affects biogenic amine quantification. Recovery was also assayed in this complex matrix by evaluating the difference in peak area obtained when the standard mixture is added to wine (Table 4-4).

Table 4-4 – Recovery values obtained for comparison of wine and wine added a standard mixture

Biogenic amine	Recovery %
Histamine	71%
Tyramine	74%
Phenylethylamine	83%
Putrescine	91%
Cadaverine	108%

Recovery percentages were in line with the desired range between 80 and 120% and the structural similarities go along the results. For instance, putrescine and cadaverine had the highest recoveries.

These results corroborate the applicability of the method to wine and suggest a good correlation between the wine samples and the standards used for calibration which implies that the wine matrix does not have significant effect (interference) over biogenic amine quantification when compared to HCl 0.1 N.

4.1.3 Method Validation

4.1.3.1 Calibration

Biogenic amines calibration was made using six standards with a mixture of each biogenic amine in proportional concentrations (Table 3-3, Section 3.4.2)

A representation of each curve is presented in Appendix C, C.1. From the slopes obtained, the uncertainties and variances were retrieved (not shown) and the limits of detection (LOD) and quantification (LOQ) were calculated (Table 4-5).

Table 4-5 – Limits of detection and quantification

Biogenic Amine	Slope $\times 10^4$	y-intercept $\times 10^4$	R^2	LOD mg/L	LOQ mg/L
Histamine	109 ± 5	-1 ± 32	0.9945	0.864	2.882
Tyramine	27 ± 2	9 ± 13	0.9925	1.408	4.692
Phenylethylamine	78 ± 4	5 ± 13	0.9931	0.466	1.554
Putrescine	26.2 ± 0.9	12 ± 16	0.9962	1.721	5.738
Cadaverine	40 ± 2	-1 ± 38	0.9913	2.825	9.417

LOD and LOQ were calculated using 3 and 10 times the standard deviation obtained for the y-intercept point as the minimum detectable and quantifiable areas, respectively.

The values presented in the OIV method (Table 2-14 Section 2.1.6.3) are much lower than the ones found for the present method. However, the methodology used to calculate such values may have been different since no remarks are made regarding their calculus. Besides, the limits herein found are far too high. The LOD concentrations are similar to the ones of standard P2 which gives response signals just about 1 Volt which should be more than enough to detect and even quantify them given that baseline noise is in the order of milivolt and in some cases microvolt. Derivatization procedures have inherent high variability that may take part in the attainment of high LOD and LOQ values.

Regarding the slopes it is also noteworthy that histamine has the steepest one which indicates the sensitivity of the method towards this biogenic amine. This property is of special notice for histamine since it elutes amongst smaller peaks. Therefore, identification of the

peak boundaries (beginning and end times) is easier. On the contrary, putrescine changes over the peak area produce bigger effects on the values measured.

4.1.3.2 Precision and Accuracy

To assess method accuracy and precision a comparison was made between injections of the same standard mixture. In this case, P5 standard was used (Table 3-3 Section 3.4.2). However, only two replicates were made. Consequently, the results should be carefully analyzed (Table 4-6).

Table 4-6 – Precision and accuracy evaluation

Biogenic Amine	Average Peak Area $\times 10^6$	Concentration mg/L	CV%
Histamine	17 ± 1	15.4 ± 0.9	6%
Tyramine	5.6 ± 0.8	20 ± 3	12%
Phenylethylamine	5.0 ± 0.4	6.3 ± 0.4	6%
Putrescine	11 ± 2	40 ± 5	11%
Cadaverine	17 ± 2	43 ± 3	7%

Note: The conversion of the area values to a concentration was made through the use of the calibration curves (Appendix C, C.1. / Table 4-5 Section 4.1.3.1).

Even though these values might be doubtful, lower CV% values were obtained when compared to the tests performed to identify the best number of mixes for the derivatization procedure. This difference results from the usage of a different column and a different gradient. The previous results, however, do not lose their validity since the comparison between them is made in the same circumstances. A different outcome caused by the derivatization process would also be noticeable in the former column.

Noteworthy is the concentrations obtained with the P5 standard. This is justifiable by the use of a freshly made solution for calibration purposes while this test was undertaken with an old solution (section 3.4.2). Oddly, the results with the new solution were lower than the former ones which cannot be explained by product degradation. This situation was also verified in other cases but no explanation could be found.

Concerning the standard deviation of the concentration, two situations may be evaluated: the sequential replicate injections, i.e., repeatability and the injection of the same

standard in different days, i.e., reproducibility. During this work none of the biogenic amine standards used in the final conditions was injected more than once in the same day. Therefore, no repeatability was calculated. In the case of reproducibility it is usually accepted a minimum of six injections. However, only two were performed. As a result the comparison to the values from the OIV method should be made with reserve. In the latter, the values are obtained through an equation dependent on the levels found (Appendix A, A.2.). The standard variation found for P5 average values may then be compared to the R (upper case) values from OIV method adjusted to the same concentrations (Table 4-7).

Table 4-7 – Comparison of the reproducibility of the results of the present method and the OIV method

Biogenic Amines	P5 Standard Deviation mg/L	P5 OIV Method Reproducibility mg/L
Histamine	0.9	8.1
Tyramine	3	11
Phenylethylamine	0.4	2.2
Putrescine	5	13
Cadaverine	3	16

4.1.4 Results of Wine Samples

A list with all the results form biogenic amine sample analysis is presented in Appendix D. From those distinct characteristics were analyzed separately to evaluate their effect on the final biogenic amine content.

4.1.4.1 Effects of the Malolactic Fermentation

The presence of biogenic amines in wine is told by many authors to be mainly influenced by the malolactic fermentation (see section 2.1.4). Therefore, those premises are going to be herein assessed (Table 4-8).

Table 4-8 – Average biogenic amines levels comparing the effect of the malolactic fermentation

Wine	n	Histamine mg/L	Tyramine mg/L	Phenylethylamine mg/L	Putrescine mg/L	Cadaverine mg/L
With MLF	7	8 ± 7	29 ± 16	0.2 ± 0.3	21 ± 11	1 ± 1
Without MLF	19	2 ± 2	- / n.r	0.9 ± 0.1	2 ± 1	1.2 ± 0.4

The group of wines that underwent malolactic fermentation is formed solely by red wines. In contrast, the wines which did not had a malolactic fermentation phase include 16 white wines, 1 Port rose wine and 2 Port red wines.

From the results, the main aspect clearly noticed is the presence of tyramine that has not been registered for any other wines. Therefore, its presence should originate from malolactic fermentation most likely due to the action of lactic acid bacteria. Moreover, putrescine has an approximate 10 times increase (in average). Histamine levels, for some of the red wines, also registered great increases while for others that was not the case thus achieving high uncertainty of the results. Hence, putrescine and histamine raise may also be attributed to malolactic fermentation. However, some key aspects for histamine formation must be present for that to happen since 3 out of 7 red wines present values of histamine no greater than the ones from white wines. Phenylethylamine, on the other hand, even though associated with the production of tyramine (Landete *et al*., 2007), did not registered higher values after malolactic fermentation. In fact, the average is slightly lower for the red wines. Cadaverine values do not seem to experience significant changes during malolactic fermentation.

The analyses made to malolactic fermentation are in line with the findings of Cecchini and Morassut (2010) and Coton *et al*. (2010). The biogenic amines that were found to have major increases caused by malolactic fermentation were tyramine, putrescine and histamine. However, findings from other authors such as Pramateftaki *et al*. (2006) and Landete *et al*. 2007 showing increases in phenylethylamine were not observed. Instead, the contrary was verified. Furthermore, the former author did not report histamine increases while the latter did not find changes regarding putrescine levels (Pramateftaki *et al*., 2006; Landete *et al*., 2007).

4.1.4.2 Effect of the Wine Type

The effect of the type of wine is complicate to evaluate. All the red wines present higher values of biogenic amines but it is probably caused by the malolactic fermentation they

undergo during winemaking process and not for being red. Moreover the comparison between white wines and the port red and port rose wines does not bear meaning since the amount of samples tested is scarce (Figure 4-16).

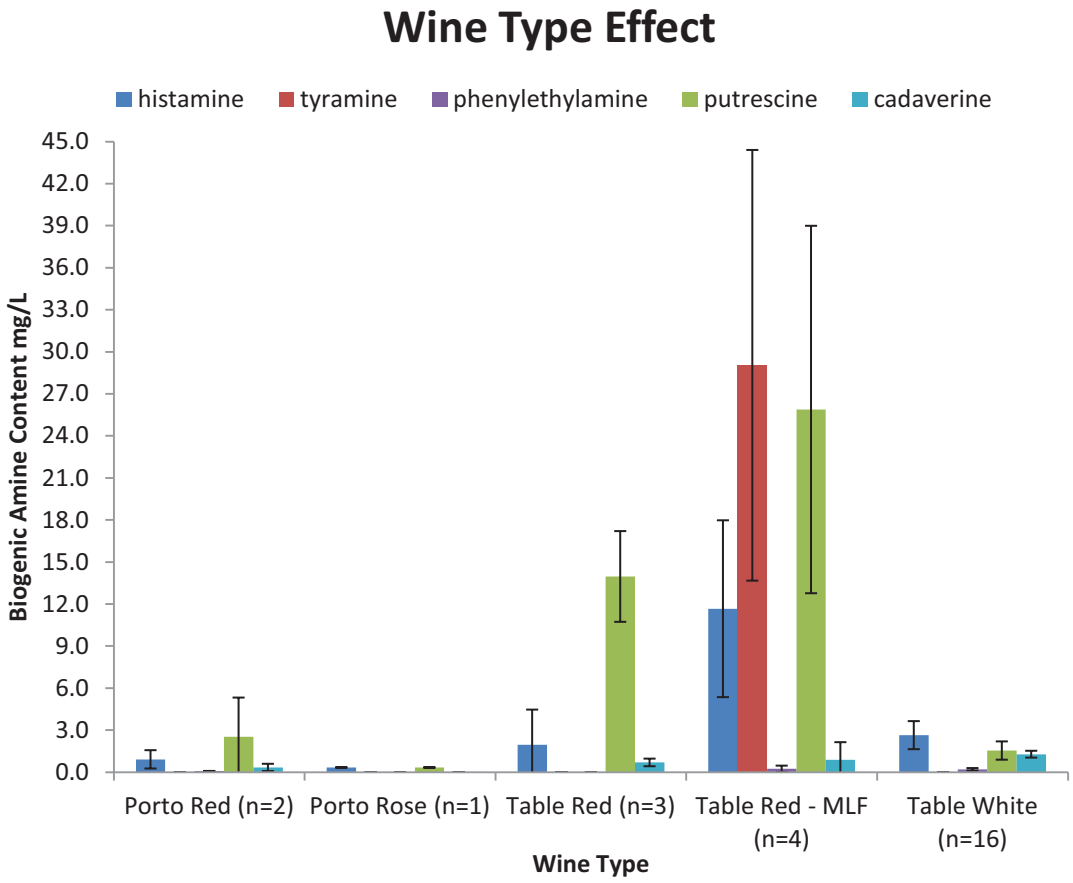


Figure 4-16 – Comparison of the levels of biogenic amines according to the type of wine

Nevertheless, it can be stated that the increases in biogenic amines contents registered for malolactic fermentation trials were not confirmed in the red wine samples analyzed that also underwent malolactic fermentation, except for putrescine. Therefore, other aspects might be influencing biogenic amine formation.

4.1.4.3 Cross Effect of Strain and Variety

These results only refer to white wines from two different varieties, Encruzado and Viosinho. For each one, five strains were separately tested for oenological performance including biogenic amine formation in the final product.

The extended analysis of these results should be made at the light of the results from oenological performance under the pain of losing meaning and even taking erroneous conclusions.

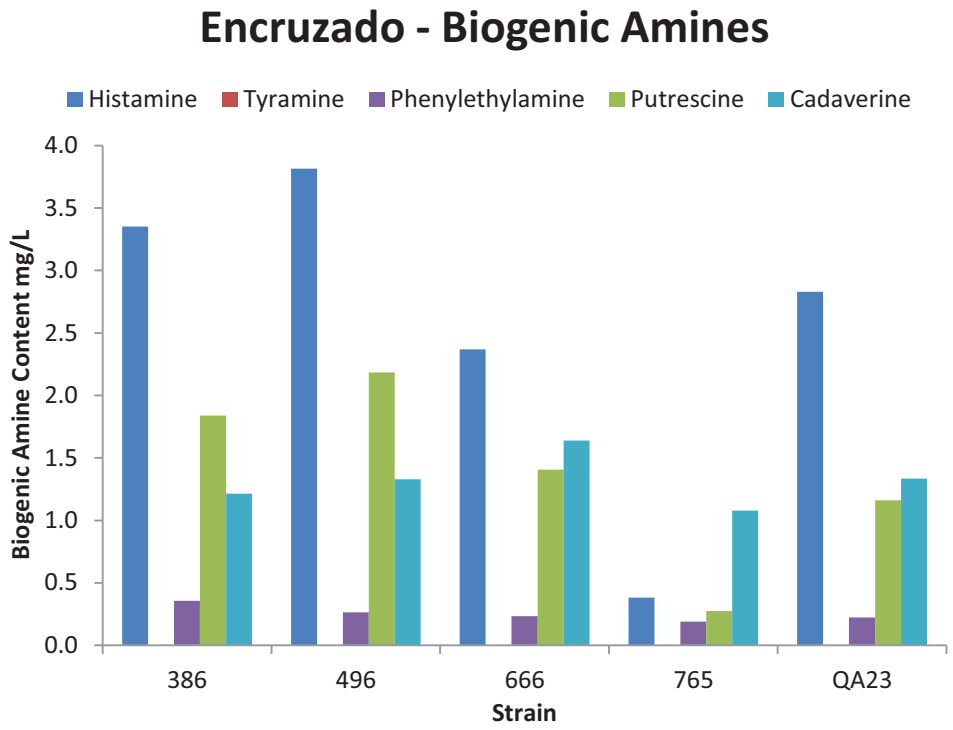


Figure 4-17 – Biogenic amine formation depending on the strain used for fermentation - Encruzado

Biogenic amine production in the Encruzado variety was higher for strains 496 and 386 especially for histamine but also noticeable for putrescine. The strain 666 and QA23 control strain can be said to have overall average biogenic amine content while the 765 non-*Saccharomyces* strain revealed low biogenic amine production. The fact of being from a different genus could not be associated with lower amine production (see section 4.2.3.4).

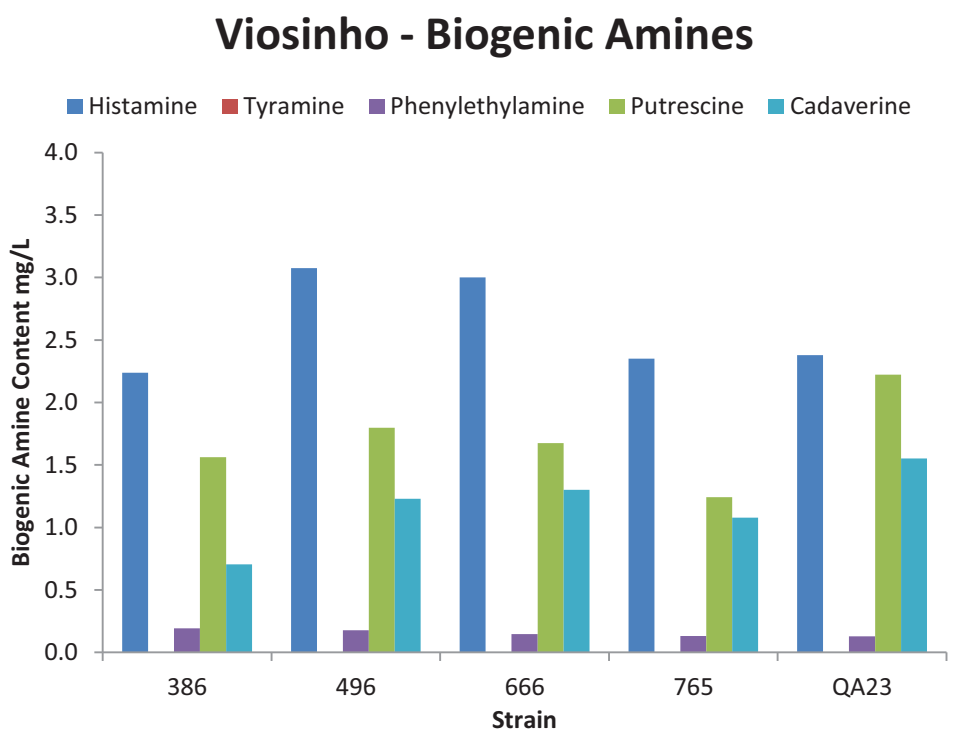


Figure 4-18 – Biogenic amine formation depending on the strain used for fermentation - Viosinho

Within the Viosinho variety all the strains produced equivalent amounts of biogenic amines, perhaps with slightly higher histamine production for 496 and 666 strains and higher putrescine (and cadaverine) production in the control strain QA23.

Phenylethylamine has in both varieties consistently low values regardless of the strain used. This may be caused by residual formation of phenylethylamine by all strains but the low amounts do not allow distinction of the "best" producers. Alternatively, it can originate from the presence of this biogenic amine in the grape which is maintained during and after alcoholic fermentation.

In white wines the variety seems to have a predominant effect over the yeast strain used for alcoholic fermentation in the final biogenic amine content. During malolactic fermentation it remained unclear whether there is an inversion of the relative predominance of the factors (variety and strains present). However, one may speculate that lactic acid bacteria population type and numbers are the major influents during malolactic fermentation since differences, e.g., in the free amino acid pool are not likely to be responsible for about 30 mg/L of tyramine compared to cases in which this biogenic amine was not detected.

That said, generalizations of the biogenic amine formation for all wines regardless of their origin, variety and perhaps strains used may prove to be inconsequential. Furthermore,

even when considering classification of wines in white, red or rose, apart from a greater incidence of biogenic amines in red wines probably caused by malolactic fermentation, the variability of conditions and biogenic amine levels verified within each group is still too high to make proper comparisons and take conclusions from those.

Many of the wines herein tested are white wines from Douro, Vinho Verde and Dão Portuguese wine regions. Hence, results obtained should be equivalent to the ones found for Portuguese white wines presented in Table 2-7, section 2.1.5. In fact, levels of biogenic amines are comparable which favours the hypothesis of an origin influence greater than the yeast strain.

Nevertheless, if the strain does not possess the genetic machinery for production of one or several biogenic amines results would certainly be affected. That might be the reason for the appearance of tyramine in red wines from malolactic fermentation trials but not in other red wines (also experiencing malolactic fermentation).

4.2 Oenological Performance Evaluation

The analysis of sugar contents throughout the fermentation process is an important measurement that provides information regarding substrate consumption. Moreover, alcoholic content assessment allows evaluation of product formation thus enabling a better view over the growth of microflora during winemaking processes.

Analysis of the alcoholic content of samples was not part of this work. Samples were analyzed in different facilities of Sogrape Vinhos in Vila Nova de Gaia using NIR spectroscopy. Hence, method details are omitted and only the results obtained from must and wine analyses are going to be herein presented and discussed. On the contrary, a method for analysis of sugar contents was developed for the purpose of the present work using HPLC equipment, HPLC-RI. Therefore, details regarding the method used and the results obtained are going to be discussed in this section of the work.

In many occasions throughout this work, total sugar and glucose and fructose contents are used interchangeably. In fact, glucose and fructose are not the only sugars present in grapes and consequently in must. Other sugars such as sucrose, raffinose, maltose, galactose and meliobiose may be found. However, fructose and glucose consist of approximately 99% of the total sugar content (Snyman, 2006). For that, their sum is considered the total sugar

amount and that's also why they are the only sugars the musts are tested for. Additionally, there is an interest over the individual consumption of glucose and fructose to evaluate yeast strains preferential consumption which may result in wines with different characteristics, namely the sweetness of the end product.

4.2.1 Fructose and Glucose Method Development

Sogrape Vinhos already had an established HPLC-RI method for fructose and glucose analysis. Therefore, chromatographic conditions were adapted from the pre-existing method in order to better suit the needs of this project.

4.2.1.1 Method Optimization

With the established conditions glucose and fructose peak separation/resolution was deficient. With the aim of improving resolution, the mobile phase consisting of an acetonitrile and water solution at 75:25 was altered so that a higher acetonitrile percentage is available thus further separating the peaks. The proportion of acetonitrile and water of the mobile phase were changed to 85:15.

Furthermore, the pre-established injection volume of 10 µL was increased to 20 µL so as to increase method sensitivity which is especially important for samples from the end of the fermentation process and for those that experienced an excessive dilution.

The column temperature was left unchanged at 30 °C.

4.2.1.2 Calibration

With the new chromatographic conditions the existing calibration curves were no longer applicable. Hence, a new calibration was needed. It was made using five standards comprising a mixture of both sugars in equivalent concentrations (Table 3-1 Section 3.3.2)

A representation of each curve is presented in Appendix C, C.2. From the slopes obtained, the uncertainties and variances were retrieved (not shown) and the limits of detection and quantification were calculated (Table 4-9).

Table 4-9 – Limits of detection and quantification for glucose and fructose

Biogenic Amine	Slope $\times 10^4$	y-intercept $\times 10^4$	R^2	LOD g/L	LOQ g/L
Glucose	7.7 ± 0.4	0.1 ± 3	0.9943	1.098	3.660
Fructose	7.23 ± 0.03	-0.1 ± 0.3	1.0000	0.089	0.296

LOD and LOQ were calculated using 3 and 10 times the standard deviation obtained for the y-intercept point as the minimum detectable and quantifiable areas, respectively.

4.2.2 Sample Analysis

Even with the new adjustments the chromatographic separation of the peaks is not ideal (Figure 4-19). Specific software tools were used to integrate the areas separately.

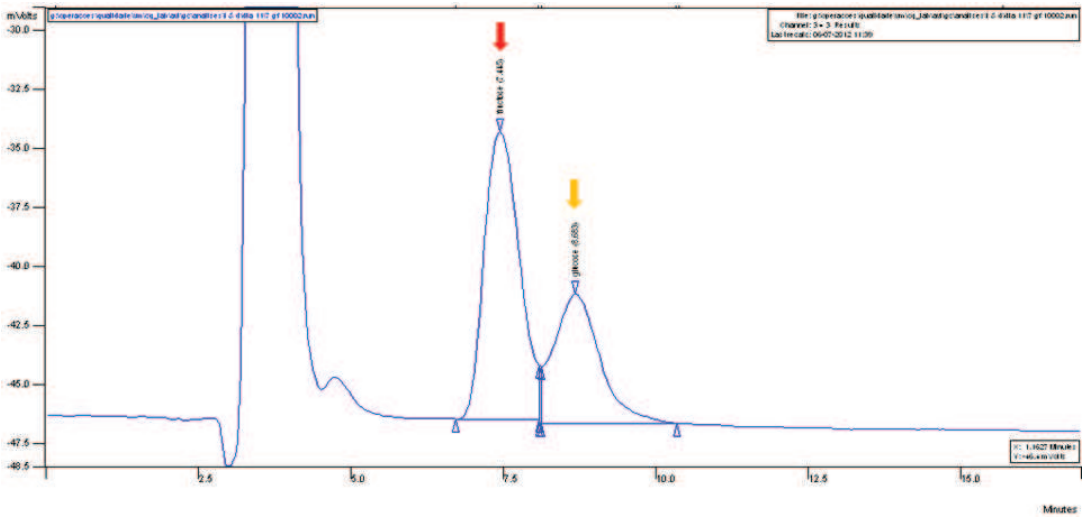


Figure 4-19 – Example of a chromatogram for glucose and fructose analysis

The first peak to appear is from fructose, pointed in red, and the second one, yellow arrow, is the peak corresponding to glucose. As analyses progressed, peak separation between fructose and glucose started to fade. The retention time difference began to shorten and the separation for integration was becoming ever closer to the peak tops (Figure 4-20).

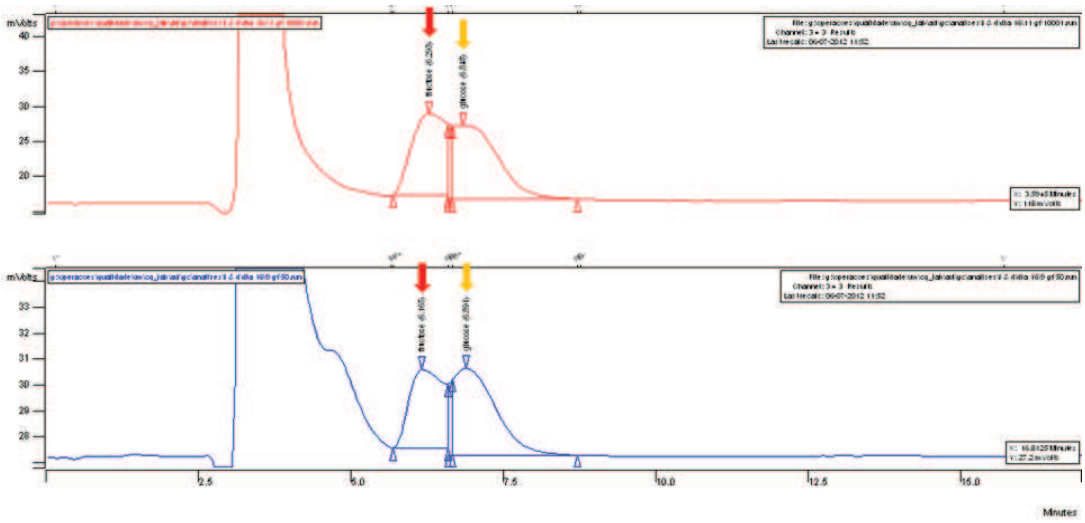


Figure 4-20 – Example of fading difference between fructose and glucose peaks

Eventually peaks started to elute so close to each other that rendered impossible to perform a proper integration of the peak areas (Figure 4-2). At this point the HPLC-RI analyses were stopped. Additional changes on the composition of the mobile phase were no longer a viable option since higher acetonitrile percentages in the acetonitrile/water mixtures would cause the formation of azeotropes which would bring great instability to the system. Such effects are not desirable and since no other column with suitable characteristics to be used in glucose and fructose was available, there was no other option but to stop the analyses.

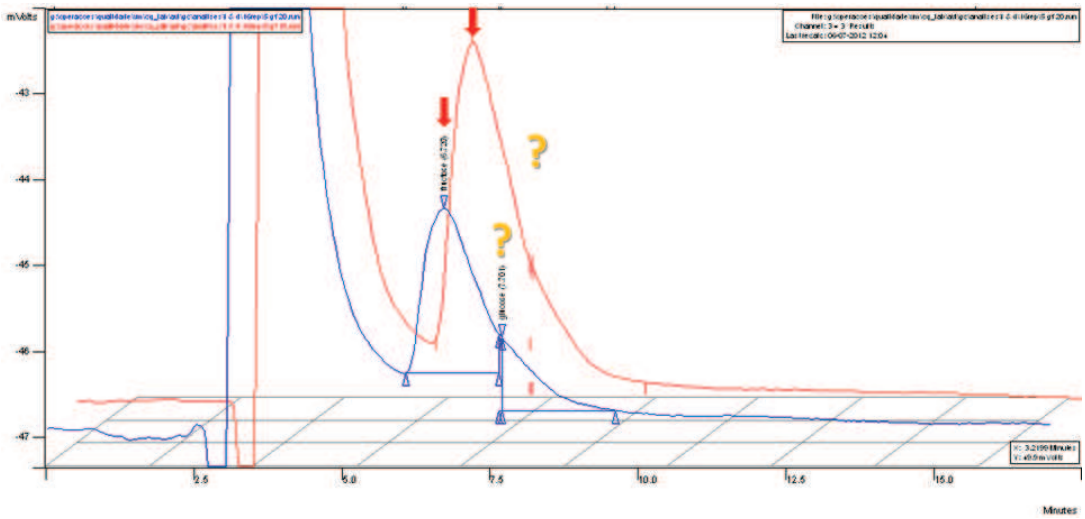


Figure 4-21 – Example of a situation of inability of separation of glucose and fructose peaks

During the progress of the present Thesis a new autoanalyser equipment was installed in the laboratory with the purpose of testing its capabilities and decide over its purchase. Taking advantage from this Y15 Enology BioSystems equipment and respective enzymatic kits provided by the same company, the analysis of fructose and glucose were resumed. Even with this equipment glucose and fructose analyses were far from completion since the enzymatic kits were quickly worn out. Further details regarding the enzymatic tests are addressed in section 3.3.5.

4.2.3 Evaluation of Sugar, Alcohol and Amine Levels

In this section the results of fructose and glucose analysis are appreciated in comparison to the alcoholic content results. Furthermore, extra information regarding biogenic amines can be retrieved through the combined analysis of these three aspects.

Sampling procedures were made before the present work. Therefore, any possible incident was not followed up and no information on that subject was transmitted. Hence, conclusions are going to be taken exclusively from the results.

Loureiro variety samples were not analyzed in terms of biogenic amines content due to a problem in the fluorescence detector which remained unsolved until the end of the period of this Thesis.

4.2.3.1 Strain 386

Regarding total sugar consumption, Viosinho variety exhibited, during the first days, higher sugar consumption than Encruzado and Loureiro both for fructose and glucose. However, the faster sugar uptake was not reflected in the alcohol formation which was equally followed in all varieties. The higher sugar usage did not have significant effect on the biogenic amine content of the wine. In fact, biogenic amine formation in Viosinho variety was slightly lower than in Encruzado.

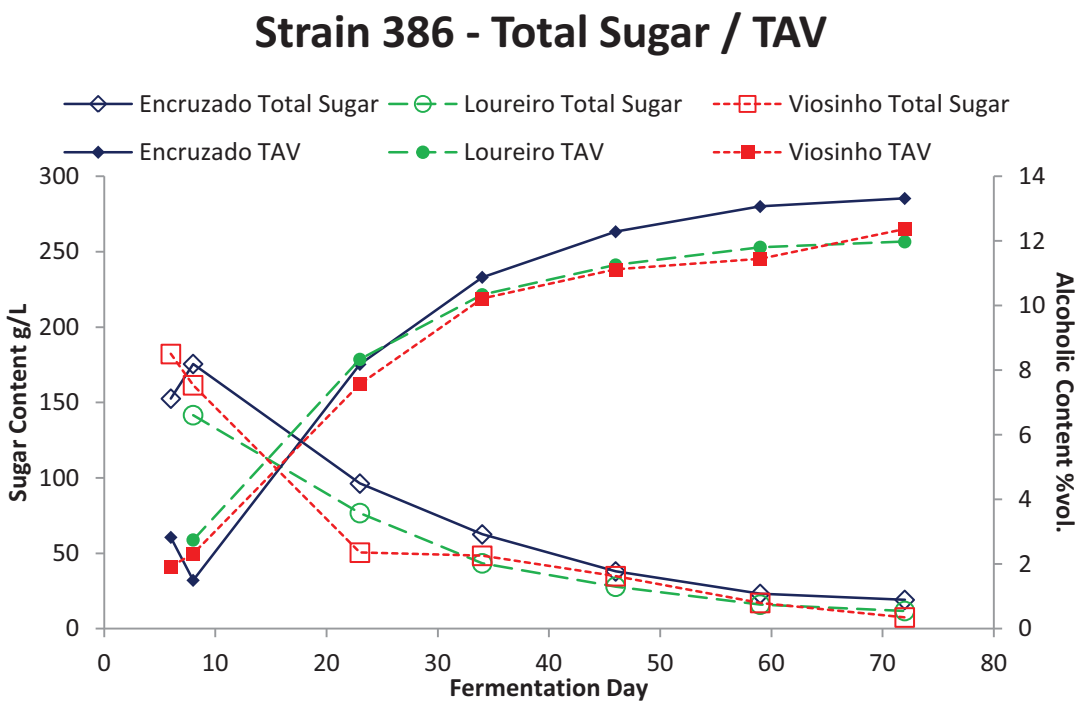


Figure 4-22 – Comparison of total sugar consumption and alcohol formation using strain 386

4.2.3.2 Strain 496

Opposing the results from strain 386, for strain 496 Viosinho variety results were clearly the worst. Glucose and fructose consumption did not accompany the same tendency as Encruzado and Loureiro varieties. Final glucose contents in Viosinho variety were still over 25 g/L compared to 5 g/L and almost zero for Encruzado and Loureiro respectively. Moreover, total sugar content reached no lower than 1/3 of the initial value while alcoholic content did not surpassed 8% vol. having Encruzado and Viosinho reaching 12% vol.

Slightly lower contents in biogenic amines verified for Viosinho seem to result from inappropriate fermentation and not from the strain production capabilities.

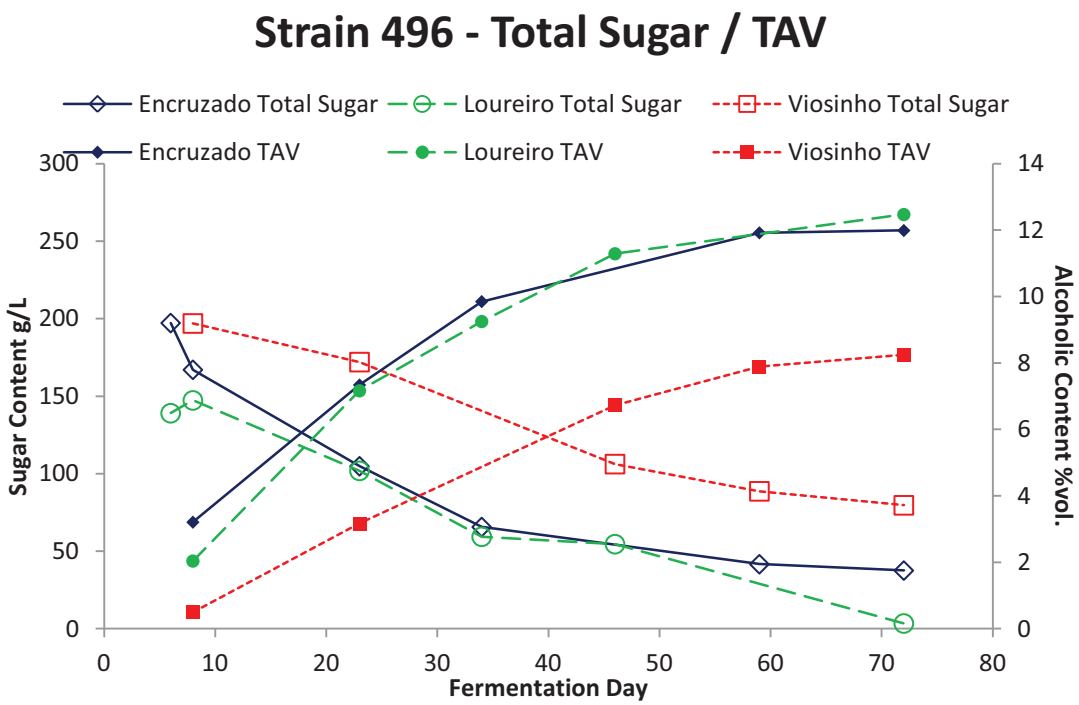


Figure 4-23 – Comparison of total sugar consumption and alcohol formation using strain 496

4.2.3.3 Strain 666

In strain 666 no major differences were revealed between the three varieties neither in the sugar consumption nor in alcohol production. Nevertheless, it is noteworthy a very high initial sugar content in Loureiro variety that was readily pushed towards lower values, close to the ones obtained for the other varieties. The elevated initial sugar content did not affect alcohol formation, though. More to the point, TAV measurements experience some instability near the end of the fermentation that could not be connected to any aspect of the fermentation.

Unfortunately, Loureiro variety could not be tested for biogenic amines contents in order to understand the initial high sugar effect. Concerning the other two strains, results are quite comparable.

It would be interesting to evaluate the effect of the initial sugar content in the fermentation since neither in this case nor in the 386 strain, higher initial sugar consumption resulted in higher alcoholic content or higher biogenic amine production. Besides, such tests could allow assessing the total scope of strain fermentation capacity.

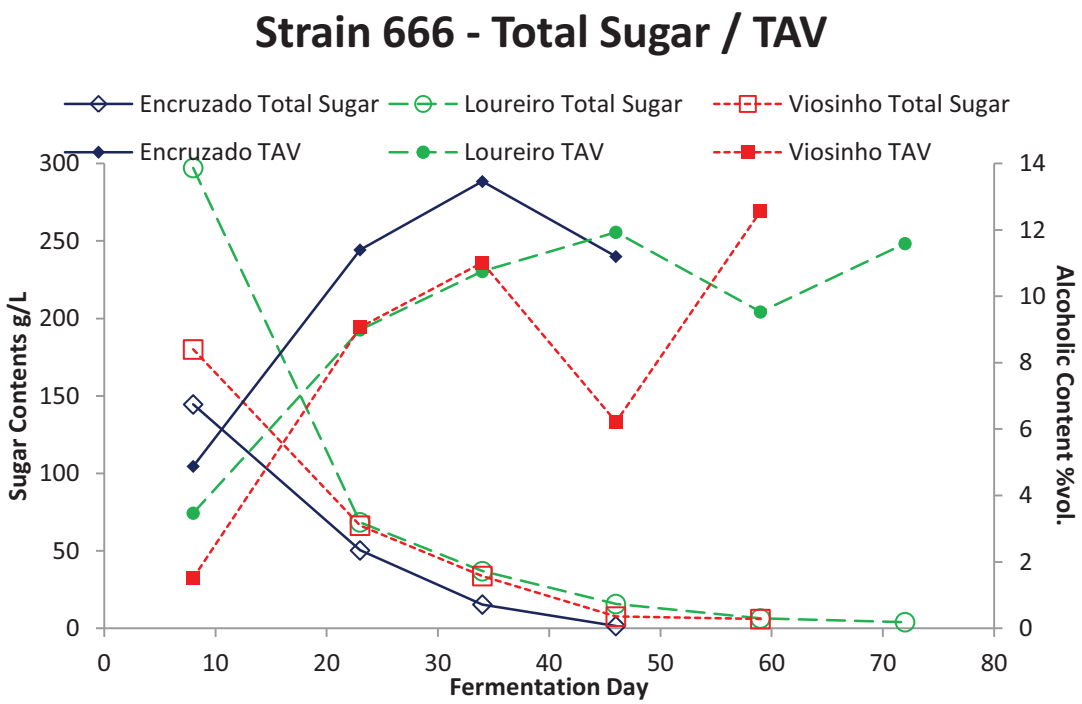


Figure 4-24 – Comparison of total sugar consumption and alcohol formation using strain 666

4.2.3.4 Strain 765

The strain 765 is the only non-Saccharomyces species among the strains tested.

From this strain it is clearly noticed the maintenance of the total sugar levels throughout the fermentation (high variability of the results) using Encruzado variety. This may suggest complete lack of fermentation. However it should occur to some extent since alcoholic content reaches 4% vol.. Perhaps facing the conditions present during fermentation the strain used an alternative carbon source. Greater insight regarding strain features are needed to be able to form a supported hypothesis about what happened.

Contrasting with Encruzado, Loureiro and Viosinho underwent a fermentation process apparently absent of major concerns.

Biogenic amines levels for Encruzado were very low which is consistent with the lack of fermentation hypothesis. On the other hand, this strain may lack the genetic machinery necessary to turn free amino acids into biogenic amines which could have been an alternative energy source. Furthermore, even if no biogenic amine has been produced during this fermentation, the values obtained may originate from the grapes. If that is the case, the influence of the alcoholic fermentation in biogenic amine content can be shown by comparison with other results, specially the Viosinho variety using the same strain.

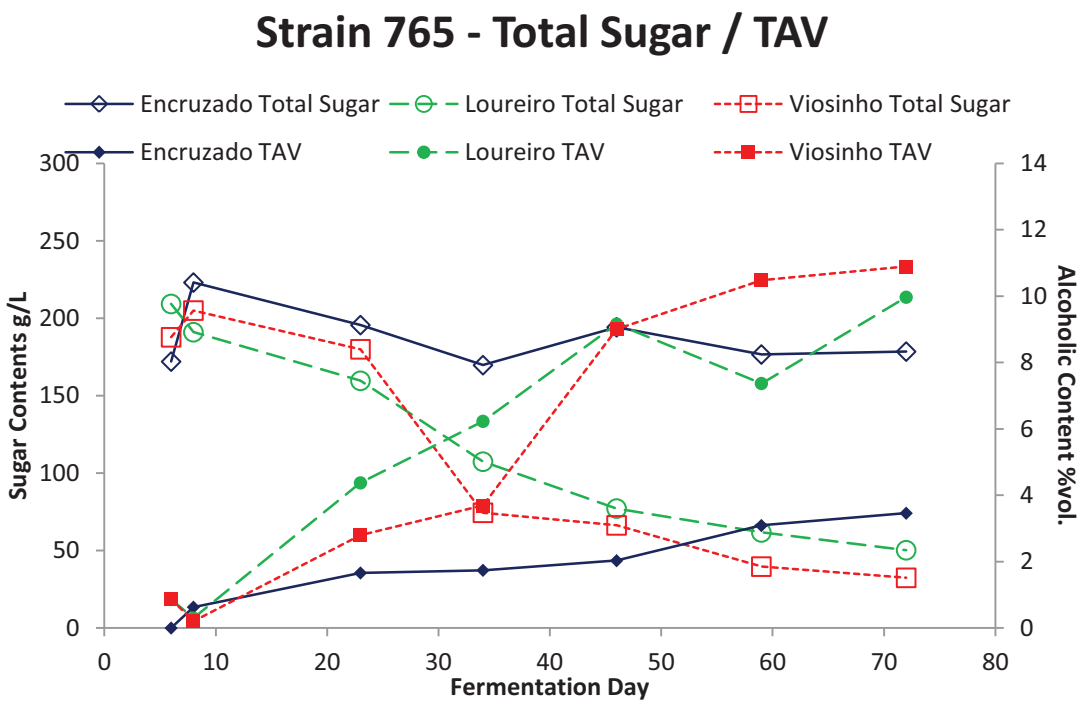


Figure 4-25 – Comparison of total sugar consumption and alcohol formation using strain 765

4.2.3.5 Strain QA23

For the control strain QA23, the gathered data was insufficient to perform a global sugar or alcoholic content analysis. Therefore only the amines may give some insight about this strain. Biogenic amines produced by this strain in Encruzado and Viosinho are similar except the putrescine content that is twice as high in Viosinho variety. This result is somehow odd since Viosinho has a tendency for low biogenic amine levels when compared to Encruzado in the other *Saccharomyces* strains tested.

4.2.3.6 Overall strain and variety cross comparison

Strain 666 revealed to have the best oenological performance for all varieties being observed an early fermentation end with Encruzado and Viosinho varieties. In the case of Loureiro, fermentation goes very similar to strain 386 but the former strain starts fermentation with double initial total sugar content. Moreover, it might be the reason for extended fermentation period when compared to the other varieties. For all varieties the final total sugar concentration obtained with strain 666 is 6 g/L or lower and the alcoholic content stays around 12% vol..

As for the worst combinations of strain and variety, strain 765 combined with Encruzado variety revealed the worst performance followed by strain 496 for Viosinho variety and again strain 765 for Loureiro variety.

Oenological performance results of the best and worst strains of each variety are presented in Figures 4–27 and 4–28. Comparison between these strains regarding biogenic amine formation can be found in Figure 4-26.

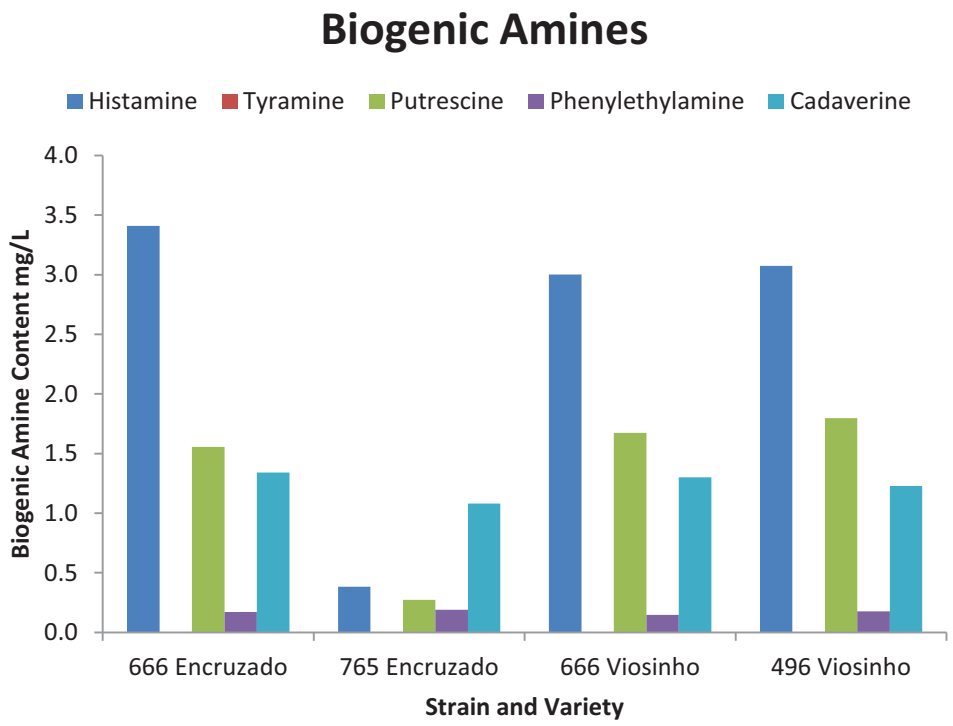


Figure 4-26 – Comparison of the biogenic amine levels formed by the best and worst performing strains for each variety

These results may indicate strain 765 as lowest producer of biogenic amines. However, in this case, fermentation seems not to have occurred. Therefore, the biogenic amines present probably result from their previous existence in grapes. For Viosinho variety, the results are very similar which may be another indicative that biogenic amine formation in white wine is more variety-dependent rather than strain-dependent. In particular, Viosinho variety seems to have an overall lower biogenic amine production (Figures 4–17 and 4–18).

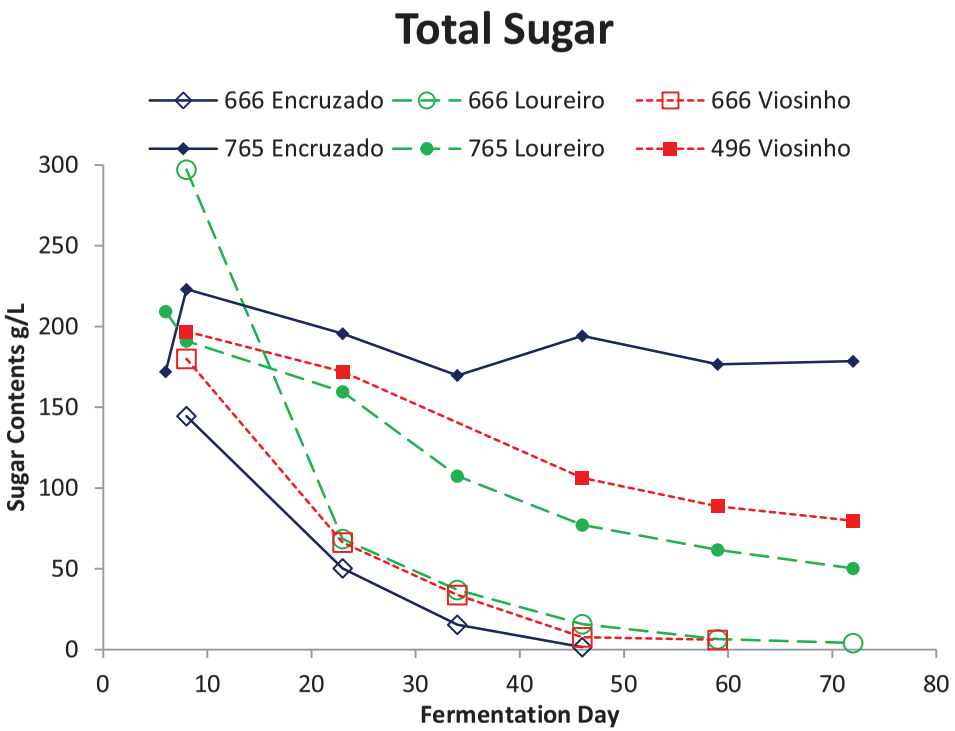


Figure 4-27 – Total sugar comparison for the best and worst strains of each variety

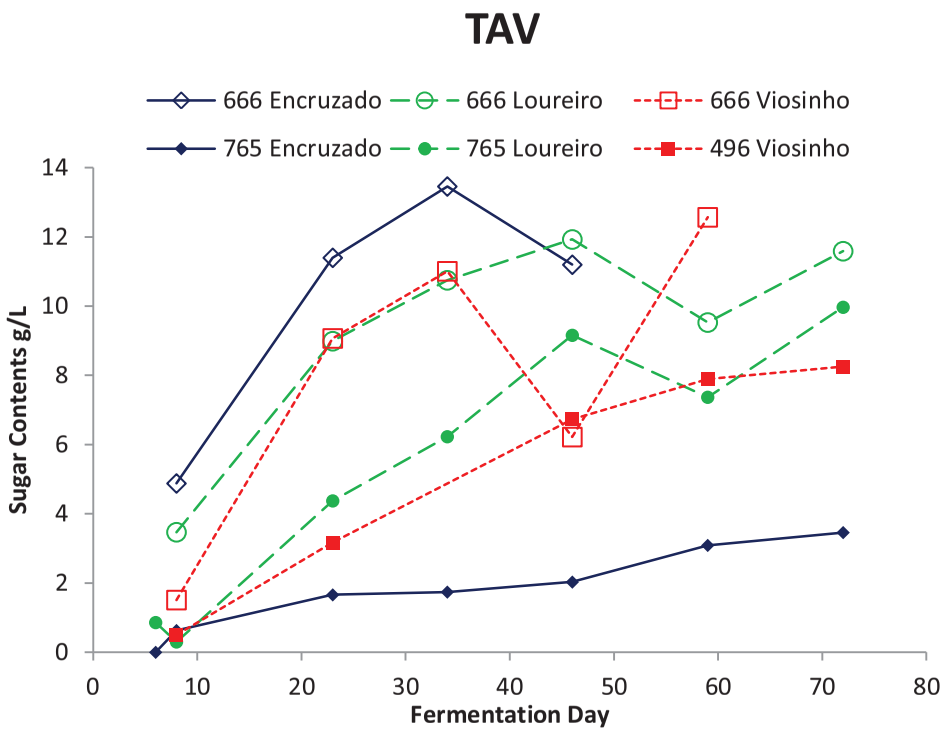


Figure 4-28 – Alcoholic content comparison for the best and worst strains of each variety

4.2.4 Glucose and Fructose Ratio

As aforementioned the sugar preferential consumption may affect wine final characteristics. In all the strains tested seems to exist preferential glucose consumption that is later accompanied by fructose isomerisation to replace consumed glucose.

The behaviour found, in most of the cases herein presented, consists of a similar content of fructose and glucose in the beginning of the fermentation followed by a profile development in which glucose values stay lower but saving the same ratio throughout the fermentation. Near the end of the fermentation, in some cases, fructose and glucose levels are once again drawn together presumably due to the lack of glucose in the media for the yeast to metabolize. Present tests do not allow identifying whether yeast shift their energy and carbon source to fructose or the isomerisation equilibrium is unbalanced by the lack of glucose and the yeasts continue using glucose until all the fructose is transformed.

The only exception is the strain 765 that when used with Encruzado and Viosinho varieties maintains equal proportions of fructose and glucose throughout the fermentation.

5 Conclusions

For this work two major objectives were proposed. To begin with, the development, validation and implementation of a method for analysis of biogenic amines in wines and the evaluation of the oenological performance of several yeast strains, involved in a FERMDIF project, that were used to produce the majority of the wines tested.

Regarding biogenic amines analysis, an HPLC-FL method was used together with an online pre-column derivatization procedure making use of OPA as derivatizing agent. Five of the main biogenic amines, histamine, tyramine, phenylethylamine, putrescine and cadaverine were tested by the present method. Having the OIV method as guideline, the method was modified to achieve more convenient properties. The analysis time achieved was approximately 55 minutes per sample which is close to half the time necessary when using OIV method. However, if many samples are needed to be analyzed, this method may still prove to be too time-consuming for application in routine analysis and even influence the schedule of the analyses requiring HPLC-RI, HPLC-UV or HPLC-FL. On the other hand, if few samples are to be analyzed, daily and weekly solution preparations and a perishable mobile phase are not desirable and they greatly increase the costs for biogenic amines quantification. Additionally, the automatic derivatization is not reliable enough to be left unattended. Prior to every injection the mixing vial had to be checked for bubbles.

Detection and quantification limits were not very satisfying. LOD and LOQ calculated from the variability of the y-interception of the calibration curves resulted in averages of 1.5 ± 0.9 (ranging from 0.466 up to 2.825 mg/L) and 5 ± 3 mg/L (ranging from 1.554 up to 9.417 mg/L), respectively.

As for precision values (CV%) a fairly good average of 9% (ranging from 6 to 12%) was achieved. Following this tendency, reproducibility values found were very low when compared to the ones described in the OIV method: 2 ± 2 against 10 ± 6 mg/L (the ranges of the values were 0.4 to 5 mg/L and 2.2 to 16 mg/L, repectively). For the latter parameters (precision and reproducibility), the results obtained with the present method are relative to just 2 replicate injections that were obtained in different occasions (one week apart). Regarding recovery, values obtained are all close or within the ideal range of 80 to 120% with a minimum of 71%, a maximum of 108% and an average of 85 ± 15%.

The validation was not completed due to insufficient data resulting from the shortage of analyses. However, the results obtained so far are promising.

Biogenic amine analysis was conducted in 26 wines. An overall average of the results does not seem to be reasonable due to the great variability found for different aspects, especially for malolactic fermentation. Tyramine, in particular, was not detected in many of the white wines analyzed and when present the amount was never high enough to allow quantification. On the contrary, for the wines

originating from malolactic fermentation trials, tyramine levels were as high as 29 ± 16 mg/L. However, for red wines external to these trials (also undergoing malolactic fermentation) tyramine was once again not detected or not able to be quantified. Therefore other factors are affecting the final biogenic amine contents in wine. To overcome excessive variability, the results should be presented with coupled parameters such as type and origin or variety. Specifically for the fermentation trials (all white wines with controlled origin: north of Portugal) the average biogenic amines contents were of 3 ± 2 mg/L, 1.5 ± 0.7 mg/L, 0.20 ± 0.09 mg/L and 1.3 ± 0.3 mg/L for histamine, phenylethylamine, putrescine and cadaverine, respectively. Tyramine, as mentioned before was not detected or quantifiable. These results were consistent with the ones found in literature for white Portuguese wines.

Concerning oenological performance of the strains selected from pervious FERMDIF projects, comparison of the total sugar and alcoholic content evolution throughout the fermentation process lead to the conclusion that the strain with best performance is the strain 666 regardless of the variety. As for the worst strain and variety combinations, strain 765 has given the worst results for both Encruzado and Viosinho varieties while strain 496 has proven to be the worst in Loureiro variety fermentations.

Regarding the relation between biogenic amines production of these strains, strain 765 is the one with the lowest amounts. However, since fermentations seem not to have occurred or at least with terrible performances, there was not even opportunity for the formation of biogenic amines. In the case, of strain 666 despite its higher fermentation performance it has overall low production of biogenic amines when compared to strains 386 and 496. In comparison to the control strain QA23 biogenic amine production of strain 666 is equivalent.

An overall analysis of the biogenic amines production in the white wines tested seem to indicate that it is more affected by the variety rather than the strain used. For confirmation of this hypothesis further analysis should be performed.

Glucose and fructose ratios were equivalent for all combinations of strain and variety. A profile of consumption of glucose and fructose was formed maintaining lower amounts of glucose throughout the fermentation that is equalled by fructose levels in the beginning of the fermentation due to the ratio found in grapes and in the end of the fermentation due to the shortage of glucose in the medium. The only exception to these profiles is the strain 765 in the cases in which the fermentation went worse (Encruzado and Viosinho varieties). In these cases, glucose and fructose contents are equivalent throughout the fermentation which suggests a correlation between the ratio between glucose and fructose and oenological performance.

Future work should include the completion of the validation of the method and extended evaluation of the biogenic amines in the wines from the FERMDIF project, especially the ones from Loureiro variety that might have been useful for the analysis herein performed.

Furthermore, the method could be adapted in the attempt to better suit routine analysis and possibly include an internal standard. Additionally, not only to check the method applicability but also to attain some external credibility, proficiency tests could be carried out.

If the strains herein presented are to be used extensively or if there is an interest over them, further fermentation tests could be performed in order to identify the best conditions and the key factors affecting biogenic amines production while still assuring a good fermentative performance.

6 Other Work Conducted

6.1 MATLAB® Glucose and Fructose Results Database

For treatment of the results from glucose and fructose analysis, a database was built. This database was created using MATLAB® version R2012a from MathWorks. This software is a programming environment that makes use of high-level programming language (fourth-generation) named after the software.

The database is based on a series of functions that operate several graphical user interfaces (GUI). This allows easier access to different functions and flexible data treatment with an intuitive interface. On the background several hundreds of code lines are working to manipulate data as desired. Several comments are placed throughout the code, in green, in order to allow a better understanding of the program mechanisms.

Both the results obtained from HPLC analysis and the latter ones from enzymatic analysis were gathered in Microsoft® Excel Worksheets and, from that point, worked out to fit the database in making.

6.1.1 Database

The program is started through the m file “RUN_ME.m”. It holds simple commands that clear existing variables in the current workspace, clear the “Command Window” display and run the function “importexport2.m” (Appendix E, E.1.).

6.1.2 Importing HPLC-RI Results from Microsoft® Excel

From “RUN_ME.m” file order is given to launch “importexport2.m” function. It opens a GUI in which several options are presented to the user. It has five “push button” objects which allow loading the results from “Current Folder” files “Results.xlsx” and “gluc-frut.xlsx” (results from HPLC-RI and enzymatic analysis respectively) to the MATLAB® database, loading a database previously saved in MATLAB® environment also extracted from “Current Folder”, saving the database created to a Microsoft® Excel worksheet with the designation “Results.xlsx” and saving the database created to a MATLAB® file to be stored in “Current Folder” (“GFdata.mat” is just a suggestion). The fifth button on the lower part closes the window and the function “menu2” is started (Figure 6-1).

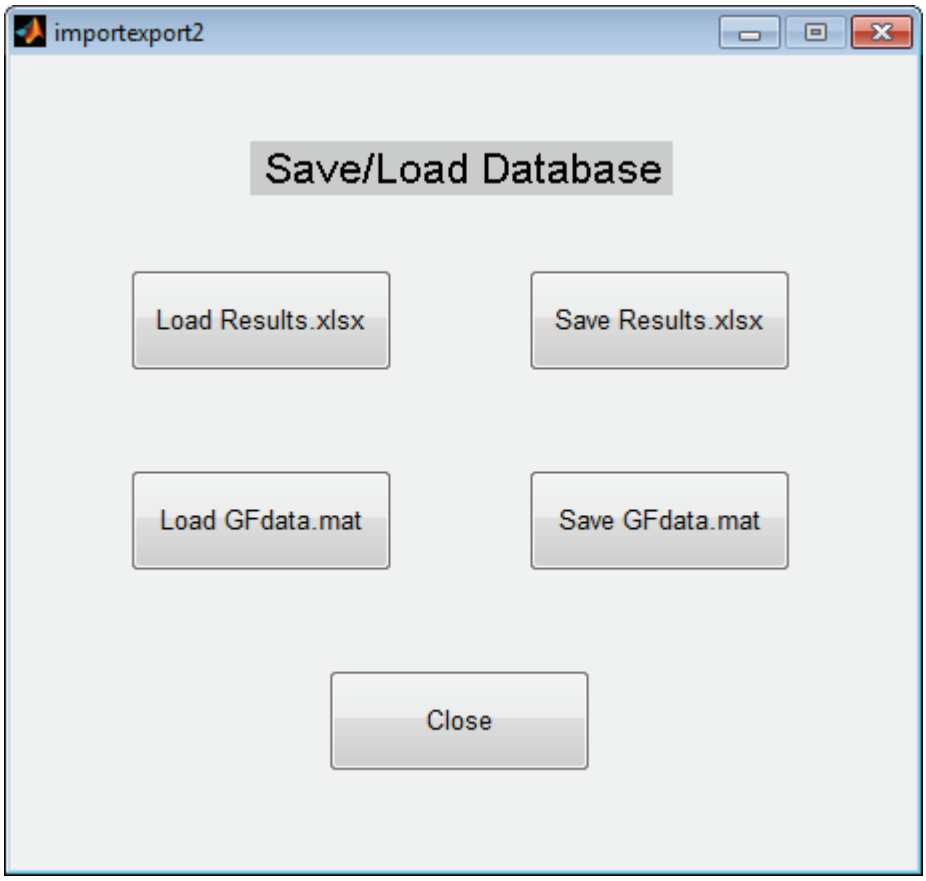


Figure 6-1 – MATLAB® GUI operated when "importexport2.m" function is called

The first part of each function needs to have an exact layout so no additional comments were placed in order to guarantee that all functions work properly. However this might be troublesome regarding program comprehension.

For "importexport2.m" function, the explained code is only present under the "Callback" sub-functions (e.g., "fuction importxlsx_Callback(hObject, eventdata, handles)) that are the ones linked to the action(s) of each button (Appendix E, E.2.).

6.1.3 Menu

The main page of the program is run by the function "menu2.m" which holds six buttons. They allow observation of the results through the use of "viewresults.m" function, accessing the calibration curves for glucose and fructose by means of the function "calibrationcurves.m", editing database entries or add new ones by running "editsample3.m" function, creating new sampling days to add to the list available in the latter function (which makes the database expandable) and saving or loading results through the already discussed "importexport2.m" function (Figure 6-3). Additionally, the "Close" button

quits the program (Figure 6-2). Again the comments are only present under the "Callback" functions (Appendix E, E.3.).

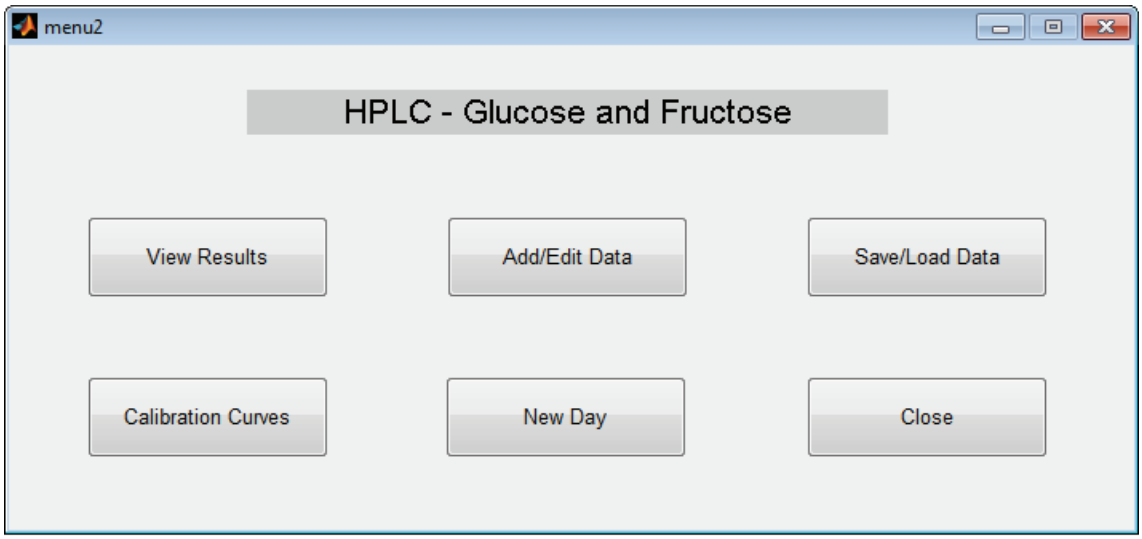


Figure 6-2 – MATLAB® GUI for the main page of the database, "menu2.m" function

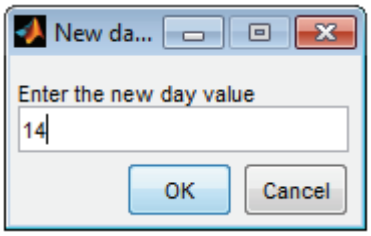


Figure 6-3 – MATLAB® input box for "New Day" option under "menu2.m" function, example of day creation

6.1.4 View Results

This function shows the comparison of the results obtained from fructose and glucose analysis for an editable combination of variety, strain and sample. After choosing these parameters a graphical representation of the evolution of the contents of glucose and fructose is displayed alongside with a table with the actual values (Figure 6-4).

In this case both "Callback" and "CreateFcn" have comments. The latter sub-functions are responsible for the default values that appear when the window is opened and no action has been taken by the user (Appendix E, E.4.).

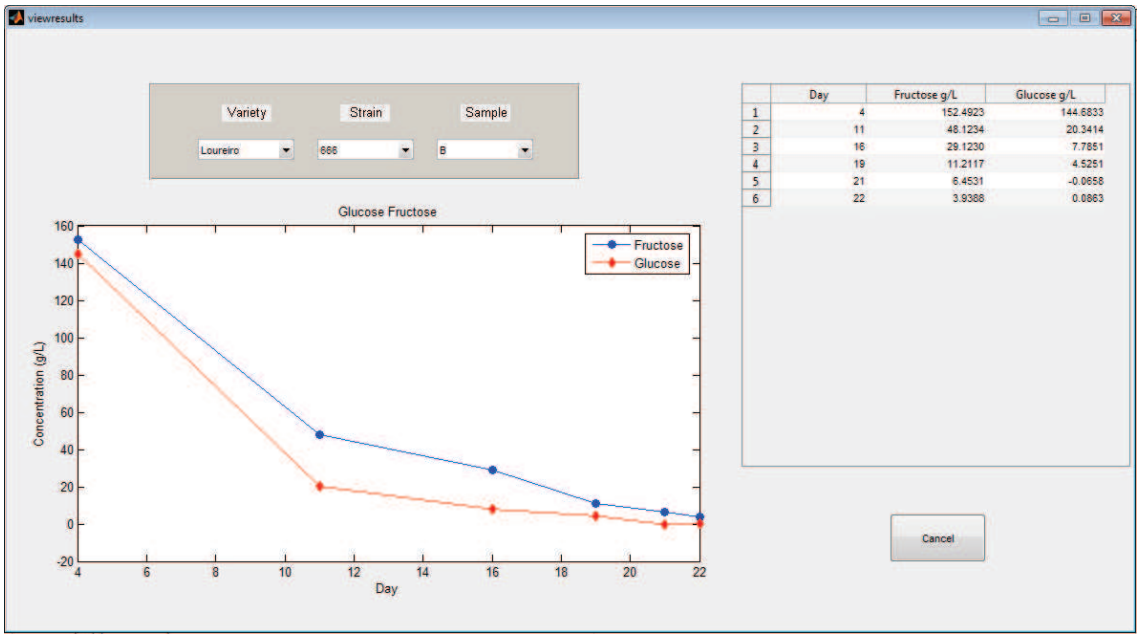


Figure 6-4 – MATLAB® GUI for "viewresults.m" function, example of a possible result evaluation

6.1.5 Calibration Curves

The calibration curves are built with data obtained in the abovementioned "importexport2.m" function. In the GUI there is an option to select the old or new column. However this part of the program is not functional since no chromatographic analysis of glucose and fructose were made with any column other than the one described. The equipment for enzymatic analysis makes use of a single point calibration as specified by the brand. Therefore no calibration curves were built besides the two herein presented (regarding glucose and fructose analysis).

The GUI of this function is merely informative. No actions may be activated by the user since the column switch is disabled. The only action allowed to the user is to close the GUI returning to the menu, "menu2.m" function (Figure 6-5).

This function has the code implemented within the "Opening_Fcn" sub-function since all the contents but the close button are to be presented as static information and therefore need to be displayed as the GUI opens (Appendix E, E.5.).

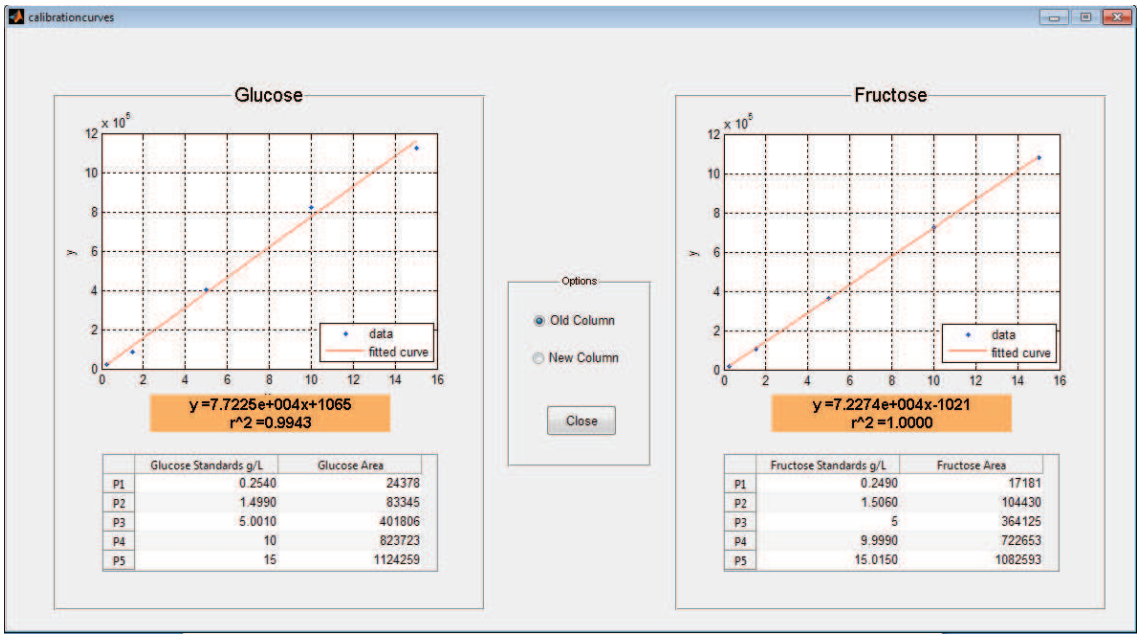


Figure 6-5 – MATLAB® GUI for "calibrationcurves.m" function

6.1.6 Add/Edit Sample

This GUI was designed to give the user the ability to both add new entries to the database and edit previous results. The Identification of the sample is similar to the one used in the "viewresults.m" function but it adds the field day and dilution. For each sample a table is generated with the number of lines equal to the number of replicates specified. If the desired day value is not available the user should return to the main menu ("menu2.m") by clicking on the "Cancel" button and choose the "New Day" option before returning to this GUI. If the sample selected already exists in the database, the values are displayed automatically in the table and are editable. To save changes, either for new data or for pre-existing results, the "Save" button should be pressed. Furthermore, in the case of data already embedded in the database, the user is asked for his/her intents to overwrite the existing values. The choice of the number of replicates has a default value of two but it can be changed to any number (positive integer). More to the point, the replicate number should be the first parameter to adjust since its change causes loss of pre-existing data for the specified sample. This issue was listed to be solved but program development was interrupted (Figure 6-6). The data from enzymatic tests was included in the database with a default value of 100 for the dilution parameter. However, it just serves the purpose of easing calculus and has no relation to the actual dilution used. Moreover, the output of the enzymatic results does not fit in the structure of this GUI.

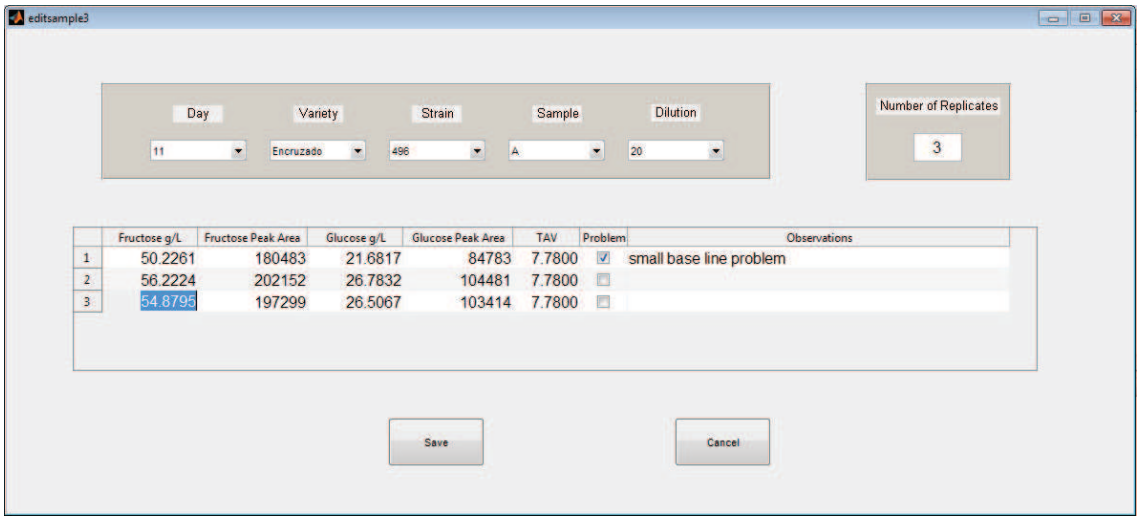


Figure 6-6 – MATLAB® GUI for "editsample3.m" function, example of sample editing

Here the comments to the code are once again under the "Callback" and "Create_Fcn" sub-functions (Appendix E, E.6.).

6.1.7 Selected Results

Besides the main program, a later request was made to have the results selected to be presented in the "viewresults.m" associated GUI in Microsoft® Excel format. To achieve that, a small m-file was created. However, this feature was not integrated as a function of the program (Appendix E, E.7).

7 Limitations

There are several samples both for biogenic amines and for glucose and fructose analyses that were not assayed due to several equipment breakages that could not be replaced or restored in time for the delivery of the present work. Therefore, results availability is limited and from what was obtained the possible best was made to fulfil the objectives of this Thesis.

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Caspi, R., 2005b. Homo sapiens spermine biosynthesis [WWW Document]. HumanCyc. URL http://biocyc.org/HUMAN/NEW-IMAGE?type=PATHWAY&object=ARGSPECAT-PWY [Accessed in 17/07/2012]

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APPENDICES

Appendix A. OIV Method

A.1 Interlaboratory Trials Conditions and Results

Statistical data obtained from the results of interlaboratory trials

The following parameters were defined during an interlaboratory trial. This trial was carried out by the Oenology Institute of Bordeaux (France) under the supervision of the National Interprofessional Office of Wine (ONIVINS – France).

Year of interlaboratory trial: 1994

Number of laboratories: 7

Number of samples: 9 double blind samples

(Bulletin de l'O.I.V. November-December 1994, 765-766, p.916 to 962) numbers recalculated in compliance with ISO 5725-2:1994.

Types of samples: white wine (BT), white wine (BT) fortified = B1, white wine (BT) fortified = B2, red wine n°1 (RT), red wine fortified = R1, red wine (RT) fortified = R2, red wine n°2 (CT), red wine (CT) fortified = C1 and red wine (CT) fortified = C2. fortified in mg/l.

	HistN	MetN	EthN	TyrN	PhEtN	DiNbut	IsoamN	DiNpen
wine B1	wine BT + 0,5	vine BT + 0,12	wineBT + 0,13	wine BT + 0,36	vine BT + 0,15	wine BT + 0,5	wine BT + 0,28	wineBT + 0,25
wine B2	wine BT + 2	wine BT + 0,40	wine BT + 0,50	wine BT + 1,44	wine BT + 0,60	wine BT + 2	Wine BT + 0,1,74	wine BT + 1,04
wine C1	wine CT + 2	wine CT + 0,1	wine CT + 0,18	wine CT + 0,72	wine CT + 0,15	wine CT + 2	wine CT + 0,29	wine CT + 0,26
wineC2	wine CT + 4	wine CT + 0,41	wine CT + 0,50	wine CT + 2,90	wine CT + 0,58	wine CT + 8	wine CT + 1,14	wine CT + 1,04
wine R1	wine RT + 2	wine RT + 0,14	wine RT + 0,13	wine RT + 1,45	wine RT + 0,19	wine RT + 3	wine RT + 0,0,57	wine RT + 0,51
wine R2	wine RT + 5	wine RT + 0,41	wine RT + 0,50	wine RT + 2,88	wine RT + 0,59	wine RT + 10	wine RT + 2,28	wine RT + 2,08

HistN : histamine, MetN : methylamine, EthN : ethylamine, TyrN : tyramine, PhEtN : phenylethylamine, DiNbut : diaminobutane, IsoamN : isoamylamine and DiNpen : diaminopentane.

Figure A–1 – Interlaboratory trials from OIV method

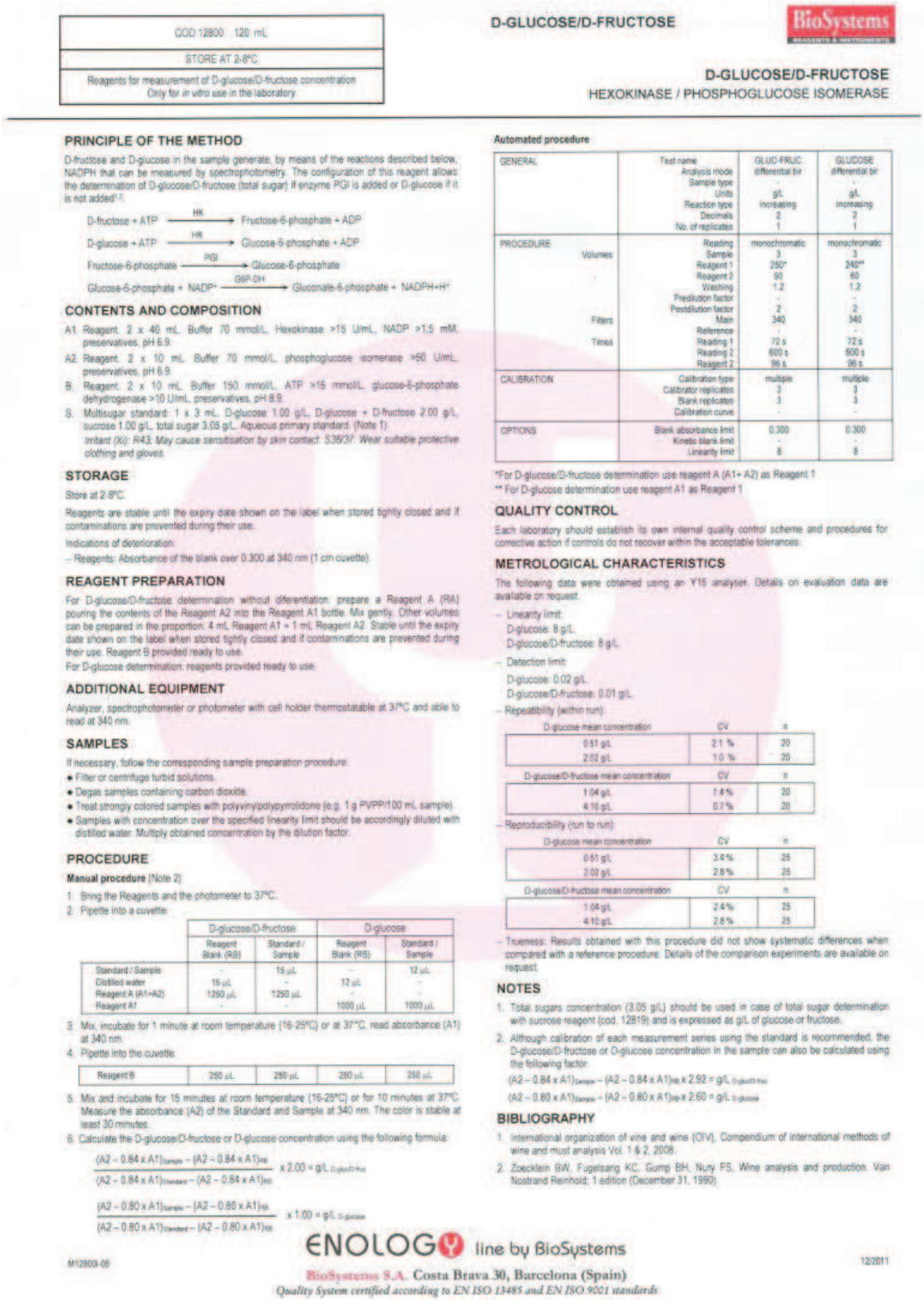
A.2 Repeatability and Reproducibility

Table A–1 – Reliability values for the OIV method (r- repeatability; R –reproducibility)

	r (mg/l)	R (mg/l)
Histamine	0.07x + 0.23	0.50x + 0.36
Methylamine	0.11x + 0.09	0.40x + 0.25
Ethylamine	0.34x - 0.08	0.33x + 0.18
Tyramine	0.06x + 0.15	0.54x + 0.13
Phenylethylamine	0.06x + 0.09	0.34x + 0.03
Diaminobutane	0.03x + 0.71	0.31x + 0.23
2-methylbutylamine et 3-methylbutylamine	0.38x + 0.03	0.38x + 0.03
Diaminopentane	0.14x + 0.09	0.36x + 0.12

In Table A–1 diaminobutane and diaminopentane are used as synonyms to putrescine and cadaverine, respectively.

Appendix B. BioSystems Enzymatic Kit for Glucose and Fructose Analysis



COD 12800 120 mL

STORE AT 2-8°C

Reagents for measurement of D-glucose/D-fructose concentration
Only for *in vitro* use in the laboratory

D-GLUCOSE/D-FRUCTOSE

BioSystems

D-GLUCOSE/D-FRUCTOSE
HEXOKINASE / PHOSPHOGLUCOSE ISOMERASE

PRINCIPLE OF THE METHOD

D-fructose and D-glucose in the sample generate, by means of the reactions described below, NADPH that can be measured by spectrophotometry. The configuration of this reagent allows the determination of D-glucose/D-fructose (total sugar) if enzyme PGI is added or D-glucose if it is not added[1,2]:

D-fructose + ATP —HK→ Fructose-6-phosphate + ADP

D-glucose + ATP —HK→ Glucose-6-phosphate + ADP

Fructose-6-phosphate —PGI→ Glucose-6-phosphate

Glucose-6-phosphate + $NADP^+$ —G6P-DH→ Gluconate-6-phosphate + NADPH+H^+

CONTENTS AND COMPOSITION

A1. Reagent: 2 x 40 mL. Buffer 70 mmol/L, Hexokinase >15 U/mL, NADP >1.5 mM, preservatives, pH 6.9.

A2. Reagent: 2 x 10 mL. Buffer 70 mmol/L, phosphoglucose isomerase >50 U/mL, preservatives, pH 6.9.

B. Reagent: 2 x 10 mL. Buffer 150 mmol/L, ATP >15 mmol/L, glucose-6-phosphate dehydrogenase >10 U/mL, preservatives, pH 8.9.

S. Multisugar standard: 1 x 3 mL. D-glucose 1.00 g/L, D-glucose + D-fructose 2.00 g/L, sucrose 1.00 g/L, total sugar 3.05 g/L. Aqueous primary standard. (Note 1)
Irritant (Xi): R43: May cause sensitisation by skin contact. S36/37: Wear suitable protective clothing and gloves.

STORAGE

Store at 2-8°C.

Reagents are stable until the expiry date shown on the label when stored tightly closed and if contaminations are prevented during their use.

Indications of deterioration:

– Reagents: Absorbance of the blank over 0.300 at 340 nm (1 cm cuvette).

REAGENT PREPARATION

For D-glucose/D-fructose determination without differentiation: prepare a Reagent A (RA) pouring the contents of the Reagent A2 into the Reagent A1 bottle. Mix gently. Other volumes can be prepared in the proportion: 4 mL Reagent A1 + 1 mL Reagent A2. Stable until the expiry date shown on the label when stored tightly closed and if contaminations are prevented during their use. Reagent B provided ready to use.

For D-glucose determination: reagents provided ready to use.

ADDITIONAL EQUIPMENT

Analyzer, spectrophotometer or photometer with cell holder thermostatable at 37°C and able to read at 340 nm.

SAMPLES

If necessary, follow the corresponding sample preparation procedure:

- Filter or centrifuge turbid solutions.
- Degas samples containing carbon dioxide.
- Treat strongly colored samples with polyvinylpolypyrrolidone (e.g. 1 g PVPP/100 mL sample).
- Samples with concentration over the specified linearity limit should be accordingly diluted with distilled water. Multiply obtained concentration by the dilution factor.

PROCEDURE

Manual procedure (Note 2)

1. Bring the Reagents and the photometer to 37°C.
2. Pipette into a cuvette:

	D-glucose/D-fructose		D-glucose	
	Reagent Blank (RB)	Standard / Sample	Reagent Blank (RB)	Standard / Sample
Standard / Sample	-	15 µL	-	12 µL
Distilled water	15 µL	-	12 µL	-
Reagent A (A1+A2)	1250 µL	1250 µL	-	-
Reagent A1	-	-	1000 µL	1000 µL

3. Mix, incubate for 1 minute at room temperature (16-25°C) or at 37°C, read absorbance (A1) at 340 nm.
4. Pipette into the cuvette:

Reagent B	250 µL	250 µL	250 µL	250 µL

5. Mix and incubate for 15 minutes at room temperature (16-25°C) or for 10 minutes at 37°C. Measure the absorbance (A2) of the Standard and Sample at 340 nm. The color is stable at least 30 minutes.
6. Calculate the D-glucose/D-fructose or D-glucose concentration using the following formula:

$$\frac{(A2 - 0.84 \times A1)_{sample} - (A2 - 0.84 \times A1)_{RB}}{(A2 - 0.84 \times A1)_{standard} - (A2 - 0.84 \times A1)_{RB}} \times 2.00 = g/L\ _{D\text{-glucose/D-fruc}}$$

$$\frac{(A2 - 0.80 \times A1)_{sample} - (A2 - 0.80 \times A1)_{RB}}{(A2 - 0.80 \times A1)_{standard} - (A2 - 0.80 \times A1)_{RB}} \times 1.00 = g/L\ _{D\text{-glucose}}$$

Automated procedure

GENERAL	Test name		GLUC-FRUC differential br	GLUCOSE differential br
	Analysis mode		-	-
	Sample type		-	-
	Units		g/L	g/L
	Reaction type		increasing	increasing
	Decimals		2	2
	No. of replicates		1	1
PROCEDURE	Reading		monochromatic	monochromatic
	Volumes	Sample	3	3
		Reagent 1	250*	240**
		Reagent 2	50	60
		Washing	1.2	1.2
		Predilution factor	-	-
		Postdilution factor	2	2
	Filters	Main	340	340
		Reference	-	-
	Times	Reading 1	72 s	72 s
		Reading 2	600 s	600 s
		Reagent 2	96 s	96 s
CALIBRATION	Calibration type		multiple	multiple
	Calibrator replicates		3	3
	Blank replicates		3	3
	Calibration curve		-	-
OPTIONS	Blank absorbance limit		0.300	0.300
	Kinetic blank limit		-	-
	Linearity limit		8	8

*For D-glucose/D-fructose determination use reagent A (A1+ A2) as Reagent 1
** For D-glucose determination use reagent A1 as Reagent 1

QUALITY CONTROL

Each laboratory should establish its own internal quality control scheme and procedures for corrective action if controls do not recover within the acceptable tolerances.

METROLOGICAL CHARACTERISTICS

The following data were obtained using an Y15 analyser. Details on evaluation data are available on request.

– Linearity limit:
D-glucose: 8 g/L.
D-glucose/D-fructose: 8 g/L.

– Detection limit:
D-glucose: 0.02 g/L.
D-glucose/D-fructose: 0.01 g/L.

– Repeatibility (within run):

D-glucose mean concentration	CV	n
0.51 g/L	2.1 %	20
2.02 g/L	1.0 %	20

D-glucose/D-fructose mean concentration	CV	n
1.04 g/L	1.4 %	20
4.16 g/L	0.7 %	20

– Reproducibility (run to run):

D-glucose mean concentration	CV	n
0.51 g/L	3.4 %	25
2.02 g/L	2.8 %	25

D-glucose/D-fructose mean concentration	CV	n
1.04 g/L	2.4 %	25
4.16 g/L	2.8 %	25

– Trueness: Results obtained with this procedure did not show systematic differences when compared with a reference procedure. Details of the comparison experiments are available on request.

NOTES

1. Total sugars concentration (3.05 g/L) should be used in case of total sugar determination with sucrose reagent (cod. 12819) and is expressed as g/L of glucose or fructose.
2. Although calibration of each measurement series using the standard is recommended, the D-glucose/D-fructose or D-glucose concentration in the sample can also be calculated using the following factor:
$(A2 - 0.84 \times A1)_{sample} - (A2 - 0.84 \times A1)_{RB} \times 2.92 = g/L\ _{D\text{-glucose/D-fruc}}$
$(A2 - 0.80 \times A1)_{sample} - (A2 - 0.80 \times A1)_{RB} \times 2.60 = g/L\ _{D\text{-glucose}}$

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BioSystems S.A. Costa Brava 30, Barcelona (Spain)
Quality System certified according to EN ISO 13485 and EN ISO 9001 standards

12/2011

Figure B–1 – General information for glucose and fructose enzymatic kit from BioSystems

Appendix C. Calibration Curves

C.1 Calibration Curves for Biogenic Amines

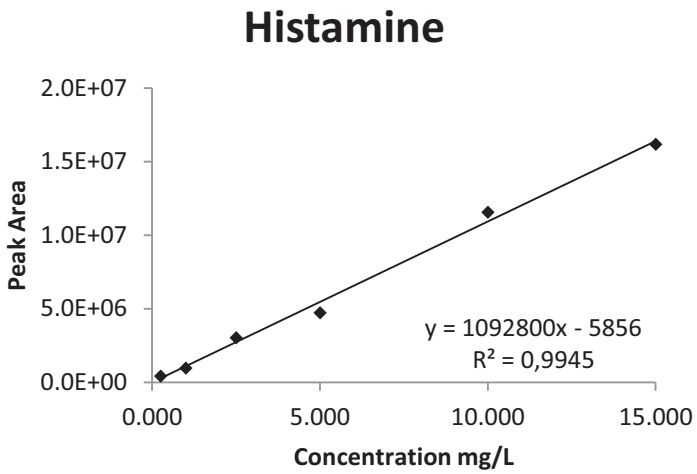

Figure C–1 – Calibration curve and curve fitting equation and correlation obtained for histamine

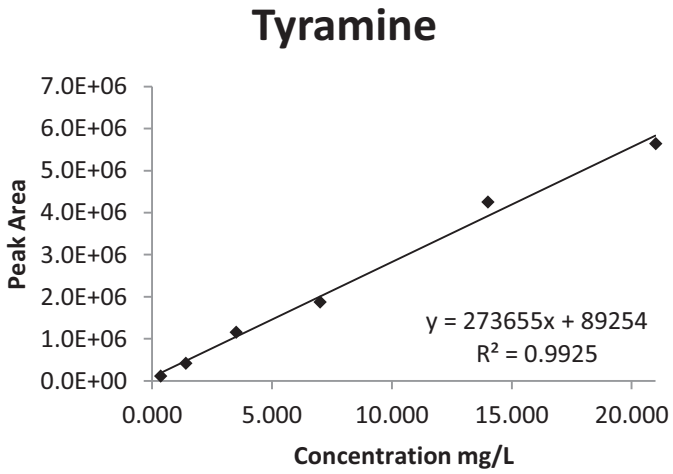

Figure C–2 – Calibration curve and curve fitting equation and correlation obtained for tyramine

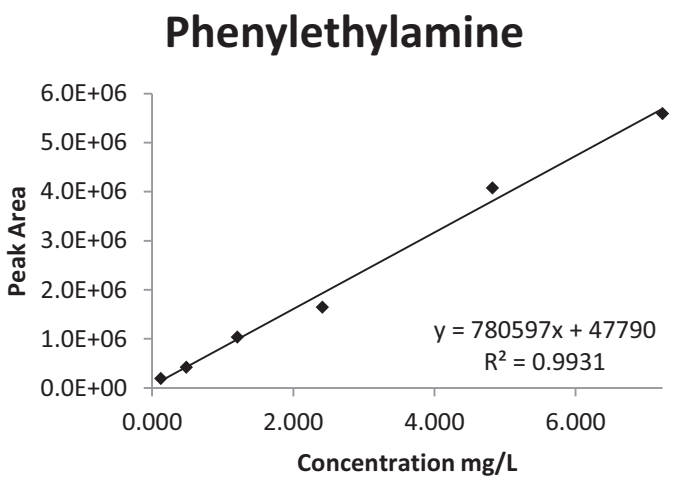

Figure C–3 – Calibration curve and curve fitting equation and correlation obtained for phenylethylamine

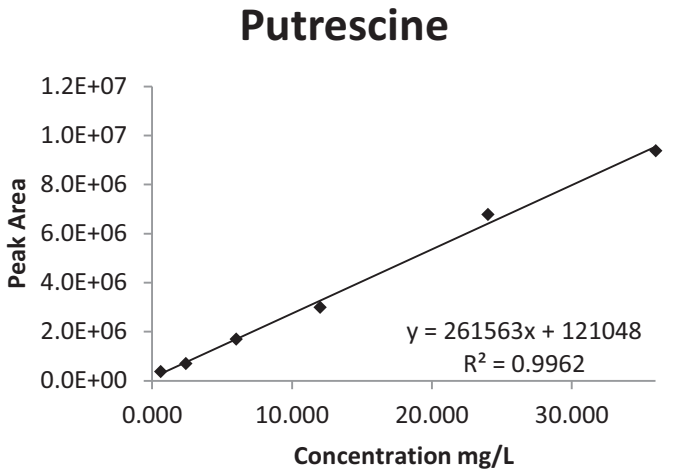

Figure C–4 – Calibration curve and curve fitting equation and correlation obtained for putrescine

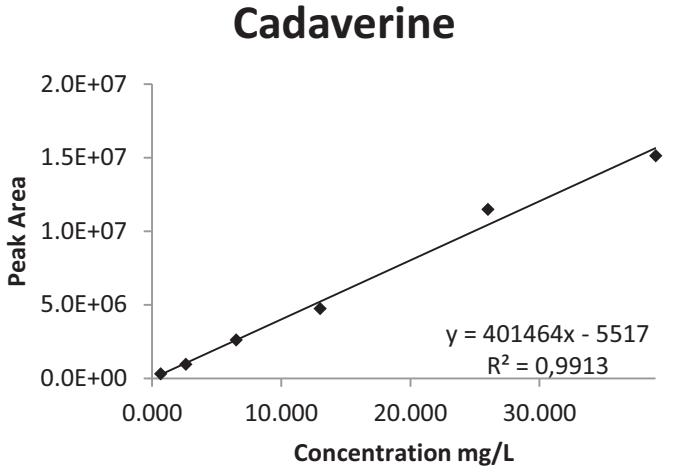

Figure C–5 – Calibration curve and curve fitting equation and correlation obtained for cadaverine

C.2 Calibration Curves for Sugars

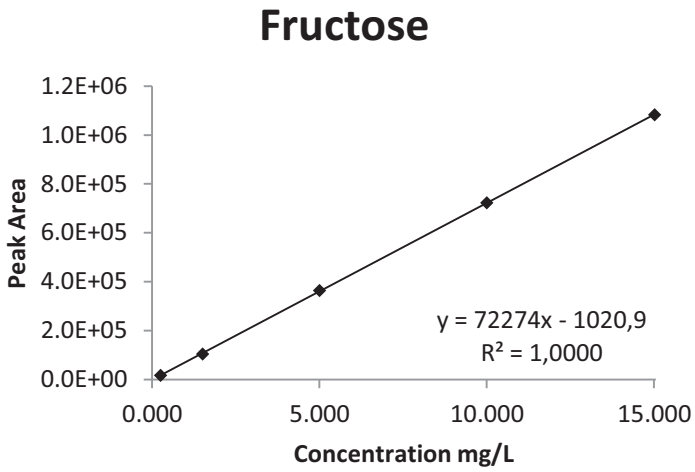

Figure C–6 – Calibration curve and curve fitting equation and correlation obtained for fructose

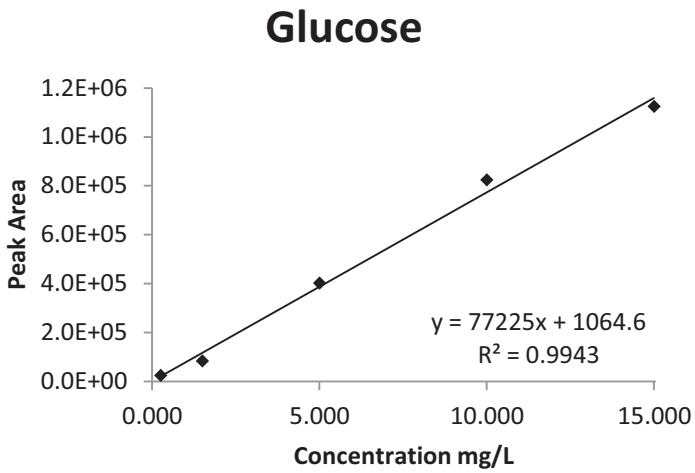

Figure C–7 – Calibration curve and curve fitting equation and correlation obtained for glucose

Appendix D. Results from Biogenic Amines Analysis in Wines

Table D–1 – Results from biogenic amines analysis by HPLC-FL

	#	Sample	Histamine	Tyramine	Phenylethylamine	Putrescine	Cadaverine
Wine Samples	1	Seixo Porto Tinto Testemunha	1.3736	n.r.	0.0807	4.5015	0.5172
	2	Seixo Porto Tinto 386	0.4488	n.r.	n.r.	0.5605	0.1512
	3	Sairrão Porto Rosé	0.3302	-	-	0.3287	-
	4	Seixo Douro Tinto Testemunha	4.8398	n.r.	n.r.	13.9541	0.7283
	5	Seixo Douro Tinto 666	0.7064	n.r.	-	17.2053	0.9395
	6	Lêda Douro Tinto 386	0.3286	-	-	10.7291	0.3991
Malolactic Fermentation Trials	7	FML sequencial Testemunha	18.433	46.0204	0.1307	36.7075	0.1516
	8	FML sequencial Inoculação Bactéria	15.0093	30.8085	0.0768	17.6857	0.1418
	9	FML Co-inoculação PROENOL	8.96	30.625	0.4937	37.3625	2.7412
	10	FML Co-inoculação Testemunha	4.2386	8.6759	-	11.7549	0.5116
Samples from FERMDIF Project - Alcoholic Fermentation (Samples are Related to Glucose and Fructose Analysis	11	Viosinho – 386 A	2.2391	n.r.	0.1935	1.561	0.7054
	12	Viosinho – 386 B+C – 5/1/12	2.8694	n.r.	0.0594	1.8376	1.2344
	13	Viosinho – 386 B+C – 27/1/12	2.4225	n.r.	0.2554	16534	1.3602
	14	Viosinho – 496 B+C	3.0744	-	0.1775	1.7968	1.2295
	15	Viosinho – 666 A+C	3.2505	-	0.1321	1.2478	1.3492
	16	Viosinho – 666 B	3.0023	-	0.1468	1.6748	1.3015
	17	Viosinho – 765 A+C	2.3506	-	0.1314	1.2425	1.0778
	18	Viosinho – QA23	2.3794	-	0.1286	2.2229	1.5522
	19	Encruzado – 386 A+B	3.3508	-	0.3566	1.84	1.215
	20	Encruzado – 386 C	3.9375	n.r.	0.4017	2.7562	1.6905
	21	Encruzado – 496 B+C	3.8151	n.r.	0.2635	2.1855	1.3298
	22	Encruzado – 666 A	2.3684	n.r.	0.233	1.4064	1.6396
	23	Encruzado – 666 B+C	3.41	-	0.1718	1.5547	1.3401
	24	Encruzado – 765 A+C	0.4998	-	0.1123	0.1924	1.0079
	25	Encruzado – 765 B+C	0.3832	-	0.1889	0.2744	1.0796
	26	Encruzado – QA23	2.8307	-	0.2233	1.1591	1.3345

Appendix E. MATLAB® Database Code

E.1 MATLAB® Code for "RUN_ME.m" m-file

```
%%%%%%%%%%%%%%%%%%%   Database Glucose/Fructose   %%%%%%%%%%%%%%%%%%%

%%%  Sogrape Vinhos  %%%                 %%% Author:  Nuno Neves  %%%

% % % % % % % % % % % % % % % % % % % % % % % % % % % % % % % % % % %


% Clear Workspace variables
clear
% Clear Command Window
clc

% Open function "importexport2" (it must be present on Current Folder)
importexport2


% From this point forward, the database is built through a sequence of
% functions later on described within each one
```

E.2 MATLAB® Code for "importexport2.m" Function

```
function varargout = importexport2(varargin)
% IMPORTEXPORT2 M-file for importexport2.fig
%      IMPORTEXPORT2, by itself, creates a new IMPORTEXPORT2 or raises
%      the existing singleton*.
%
%      H = IMPORTEXPORT2 returns the handle to a new IMPORTEXPORT2 or
%      the handle to the existing singleton*.
%
%      IMPORTEXPORT2('CALLBACK',hObject,eventData,handles,...) calls
%      the local function named CALLBACK in IMPORTEXPORT2.M with the
%      given input arguments.
%
%      IMPORTEXPORT2('Property','Value',...) creates a new
%      IMPORTEXPORT2 or raises the existing singleton*.  Starting from
%      the left, property value pairs are applied to the GUI before
%      importexport2_OpeningFcn gets called.  An unrecognized property
%      name or invalid value makes property application stop.  All
%      inputs are passed to importexport2_OpeningFcn via varargin.
%
%      *See GUI Options on GUIDE's Tools menu.  Choose "GUI allows
%      only one instance to run (singleton)".
%
% See also: GUIDE, GUIDATA, GUIHANDLES

% Edit the above text to modify the response to help importexport2

% Last Modified by GUIDE v2.5 10-Apr-2012 13:41:18

% Begin initialization code - DO NOT EDIT
gui_Singleton = 1;
gui_State = struct('gui_Name',       mfilename, ...
                   'gui_Singleton',  gui_Singleton, ...
                   'gui_OpeningFcn', @importexport2_OpeningFcn, ...
                   'gui_OutputFcn',  @importexport2_OutputFcn, ...
                   'gui_LayoutFcn',  [] , ...
                   'gui_Callback',   []);
if nargin && ischar(varargin{1})
    gui_State.gui_Callback = str2func(varargin{1});
end

if nargout
    [varargout{1:nargout}] = gui_mainfcn(gui_State, varargin{:});
else
    gui_mainfcn(gui_State, varargin{:});
end
% End initialization code - DO NOT EDIT


% --- Executes just before importexport2 is made visible.
function importexport2_OpeningFcn(hObject, eventdata, handles,
varargin)
% This function has no output args, see OutputFcn.
% hObject    handle to figure
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
```

```
% varargin   command line arguments to importexport2 (see VARARGIN)

% Choose default command line output for importexport2
handles.output = hObject;

% Update handles structure
guidata(hObject, handles);

% UIWAIT makes importexport2 wait for user response (see UIRESUME)
% uiwait(handles.figure1);


% --- Outputs from this function are returned to the command line.
function varargout = importexport2_OutputFcn(hObject, eventdata,
handles)
% varargout  cell array for returning output args (see VARARGOUT);
% hObject    handle to figure
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Get default command line output from handles structure
varargout{1} = handles.output;


% --- Executes on button press in impotxlsx.
function impotxlsx_Callback(hObject, eventdata, handles)
% hObject    handle to impotxlsx (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)


%%% Glucose and Fructose data %%%
   %%% Create database from Microsoft® Excel files "Results.xlsx" and
   % "gluc-frut.xlsx", which have results from HPLC-RI and enzymatic
   % analysis respectively


% The user is asked to confirm his/her intents so as to avoid data
% loss (due to undesired overwriting) in case a button has been
% pushed inadvertently.
confirm=questdlg('Are you sure you want to proceed?',...
    'Confirmation','Yes','No','Yes');

% If the answer is "Yes" the program is resumed. If not, no action
% takes place and the user is faced once again with the initial
% options.
if strcmp(confirm,'Yes')==1


    %%% Clear workspace %%%
    % Each function has its own workspace and in case the same
    % function is used multiple times this step assures there are no
    % variable interferences.
    clear all; clc


    %%% Import Microsoft® Excel worksheets %%% Results.xlsx
    % The entire worksheet with the calibration curves and sample
```

```
% analysis values is retrieved to the variables num, txt, raw,
% numG and numF.
[num,txt,raw]=xlsread('Results.xlsx','Results');
numG=xlsread('Results.xlsx','Calibration Glucose');
numF=xlsread('Results.xlsx','Calibration Fructose');


%%%%%%%%%%%%%%%%%%   Calibration curves   %%%%%%%%%%%%%%%%%%

% The global command will appear repeatedly. It is used to make a
% variable available in all workspaces.
global calibG calibF

% The values needed for the calibration curves are extracted to
% the variables calibG and calibF (glucose and fructose
% calibrations respectively).
calibG=[numG(1:5,1),numG(1:5,2)];
calibF=[numF(1:5,1),numF(1:5,2)];


%%%%%%%%%%%%%%%%%%   Sample Results   %%%%%%%%%%%%%%%%%%

%%% Create variables %%%
% The first entry of the structure s (see ahead) has all values
% set to zero or as an empty string depending on the type of
% variable.
% Later structures with results will not have this entry.
Day1={0};
Variety1='';
Strain1='';
Sample1='';
Dilution1={0};
Fructose1(1,1)=0;
FructoseArea1(1,1)=0;
Glucose1(1,1)=0;
GlucoseArea1(1,1)=0;
TAV1(1,1)=0;
Problem1={false};
Observations1='';

% Build database - s structure.
s=struct('Day',Day1,'Variety',Variety1,'Strain',Strain1,...
    'Sample',Sample1','Dilution',Dilution1,'Fructose',...
    Fructose1,'FructoseArea',FructoseArea1,'Glucose',Glucose1,...
    'GlucoseArea',GlucoseArea1,'TAV',TAV1,'Problem',Problem1,...
    'Observations',Observations1);

% Add results to the database.
for n=2:length(raw(:,1))

    % Define day and variety from line n.
    Day2=raw(n,2);
    Variety2=raw(n,3);

    % Verify if the value that defines the strain from line n is a
    % scalar or a string and change the value type to String in
    % needed, e.g., 496 strain is a scalar and QA23 is a string.
    if isscalar(raw{n,4})==1
        Strain2=num2str(raw{n,4});
```

```
173             else
174                 Strain2=raw{n,4};
175             end
176 
177             % Define sample and dilution from line n.
178             Sample2=raw(n,5);
179             Dilution2=raw(n,6);
180 
181             % Define fructose concentration and peak area from line n.
182             Fructose2=raw(n,8);
183             FructoseArea2=raw(n,9);
184 
185             % Define glucose concentration and peak area from line n.
186             Glucose2=raw(n,11);
187             GlucoseArea2=raw(n,12);
188 
189             % Define TAV, problem and observations from line n.
190             TAV2=raw(n,14);
191             Problem2={logical(raw{n,15})};
192             Observations2=txt(n,16);
193 
194             % Select which data to join as replicates and which data is
195             % used to create new entries.
196             if cell2mat(Day1)==cell2mat(Day2) && strcmp(Variety1,Variety2)
197 ...
198                     && strcmp(Strain1,Strain2) && strcmp(Sample1,Sample2)
199 ...
200                     && cell2mat(Dilution1)==cell2mat(Dilution2)
201 
202                 % Define replicate values.
203                 Fructose1(end+1,1)=Fructose2;
204                 FructoseArea1(end+1,1)=FructoseArea2;
205                 Glucose1(end+1,1)=Glucose2;
206                 GlucoseArea1(end+1,1)=GlucoseArea2;
207                 TAV1(end+1,1)=TAV2;
208                 Problem1(end+1,1)=Problem2;
209                 Observations1(end+1,1)=Observations2;
210 
211                 % Add replicate to the database - the corresponding matrix
212                 % is replaced.
213                 s(end).Fructose=Fructose1;
214                 s(end).FructoseArea=FructoseArea1;
215                 s(end).Glucose=Glucose1;
216                 s(end).GlucoseArea=GlucoseArea1;
217                 s(end).TAV=TAV1;
218                 s(end).Problem=Problem1;
219                 s(end).Observations=Observations1;
220 
221             else
222                 % Define new entry parameters.
223                 Day1=Day2;
224                 Variety1=Variety2;
225                 Strain1=Strain2;
226                 Sample1=Sample2;
227                 Dilution1=Dilution2;
228                 Fructose1=Fructose2;
229                 FructoseArea1=FructoseArea2;
230                 Glucose1=Glucose2;
231                 GlucoseArea1=GlucoseArea2;
232                 TAV1=TAV2;
```

```
            Problem1=Problem2;
            Observations1=Observations2;

            % Add entry to the database.
            s(end+1)=struct('Day',Day1,'Variety',Variety1,...
                'Strain',Strain1,'Sample',Sample1','Dilution',...
                Dilution1,'Fructose',Fructose1,'FructoseArea',...
                FructoseArea1,'Glucose',Glucose1,'GlucoseArea',...
                GlucoseArea1,'TAV',TAV1,'Problem',Problem1,...
                'Observations',Observations1);
        end
    end

    % Remove the first entry (empty) and transpose structure.
    global results
    results=s(2:end)';



    %%%%%%%% Add results from enzimatic tests %%%%%%%%

    %%% Import Microsoft® Excel worksheets %%% gluc-frut.xlsx
    % The data from the enzymatic tests is obtained in a differnt
    % configuration. So, it is preferable to treat the results
    % directly in MATLAB than to have the expense of prior adjustment
    % to the existing Microsoft® Excel worksheet.

    % To begin with similar data extraction needs to be performed.
    [nGF tGF rGF]=xlsread('gluc-frut.xlsx','Gluc-Frut');
    [nG tG rG]=xlsread('gluc-frut.xlsx','Glucose');

    % Create the first entry of the new set of results.
    rG(1,7)={'Day'};rG(1,8)={'Variety'};
    rG(1,9)={'Strain'};rG(1,10)={'Sample'};
    rGF(1,7)={'Day'};rGF(1,8)={'Variety'};
    rGF(1,9)={'Strain'};rGF(1,10)={'Sample'};
    rG(1,11)={'Fructose'};rG(1,12)={'Glucose'};
    rGF(1,11)={'TAV'};rGF(1,12)={'Glucose/Fructose'};

    % Split the string identifying the sample and set the desired type
    % for the data.
    for n=2:length(rG)
        a=cell2mat(rG(n,1));
        b=cell2mat(rGF(n,1));

        % Set sample value.
        rG(n,10)={a(end)};
        rGF(n,10)={b(end)};

        % Get strain and variety values - length of the string
        % influences the position of the desired values.
        if isnan(str2double(a([end-3 end-2 end-1])))==0
            rG(n,9)={a([end-3 end-2 end-1])};
            rG(n,8)={a(end-4)};
        else
            rG(n,9)={a([end-4 end-3 end-2 end-1])};
            rG(n,8)={a(end-5)};
        end
        if isnan(str2double(b([end-3 end-2 end-1])))==0
```

```
292             rGF(n,9)={b([end-3 end-2 end-1])};
293             rGF(n,8)={b(end-4)};
294         else
295             rGF(n,9)={b([end-4 end-3 end-2 end-1])};
296             rGF(n,8)={b(end-5)};
297         end
298
299         % Get day value.
300         if length(a)==7 && length(cell2mat(rG(n,9)))==3 || ...
301                 length(a)==8 && length(cell2mat(rG(n,9)))==4
302             rG(n,7)={a([1 2])};
303             rGF(n,7)={b([1 2])};
304         elseif length(a)==6 && length(cell2mat(rG(n,9)))==3 || ...
305                 length(a)==7 && length(cell2mat(rG(n,9)))==4
306             rG(n,7)={a(1)};
307             rGF(n,7)={b(1)};
308         end
309
310         % Fructose results - obtained by the difference between
311         % combined fructose and glucose and the results for the same
312         % sample just for glucose.
313         rG(n,11)={cell2mat(rGF(n,12)) - cell2mat(rG(n,12))};
314
315         % Change leters identifying the variety to the respective full
316         % name.
317         switch cell2mat(rG(n,8))
318             case 'E'
319                 rG(n,8)={'Encruzado'};
320             case 'L'
321                 rG(n,8)={'Loureiro'};
322             case 'V'
323                 rG(n,8)={'Viosinho'};
324         end
325
326         % Add results to existing database.
327             % Create an extra position.
328         p=length(results)+1;
329
330         % Define the parameters
331         results(p).Day=str2double(rG(n,7));
332         results(p).Variety=cell2mat(rG(n,8));
333         results(p).Strain=cell2mat(rG(n,9));
334         results(p).Sample=cell2mat(rG(n,10));
335         results(p).Fructose=rG(n,11);
336         results(p).Glucose=rG(n,12);
337         results(p).TAV=rGF(n,13);
338
339
340         % Define values to fill the remaining parameters not
341         % applicable to the enzymatic analysis.
342         results(p).Dilution=100;
343         results(p).FructoseArea={0};results(p).GlucoseArea={0};
344         results(p).Problem={0};
345         results(p).Observations={''};
346     end
347
348
349     % Sort results by day, variety, strain, sample and dilution.
350     [tmp ind]=sortrows([{results.Day}',{results.Variety}',...
351         {results.Strain}',{results.Sample}',{results.Dilution}'],...
```

```
        [1 2 3 4 5]);
    results=results(ind);



    %%%%%%%% Choose the best results %%%%%%%%
    % For later data handling it was necessary to select the
    % appropriate results from the bigger pool just created. For
    % instance, in the HPLC-RI results some dilutions made pushed the
    % results out of the calibration curves.

    global zerop
    % Create variables for support in the following steps
    h=1;
    zerop=struct;

    % Creating the zerop structure which is similar to the results
    % structure but excludes the cases in which there have been
    % problems in the chromatogram.
    for e=1:length(results)
        g=0;
        for f=1:length(results(e).Problem)
            if results(e).Problem{f}==false
                g=g+1;
                zerop(h).Fructose(g)=results(e).Fructose(f);
                zerop(h).FructoseArea(g)=results(e).FructoseArea(f);
                zerop(h).Glucose(g)=results(e).Glucose(f);
                zerop(h).GlucoseArea(g)=results(e).GlucoseArea(f);
                zerop(h).TAV(g)=results(e).TAV(f);
                zerop(h).Day=results(e).Day;
                zerop(h).Variety=results(e).Variety;
                zerop(h).Strain=results(e).Strain;
                zerop(h).Sample=results(e).Sample;
                zerop(h).Dilution=results(e).Dilution;
                zerop(h).TAV=results(e).TAV;
            end
        end
        if g~=0
            h=h+1;
        end
    end
    zerop=zerop';


    global t

    % Set additional variables for support.
    j=1;
    a=1;
    b=1;

    % Get the ratio between each of the sugars, fructose and glucose
    % and the respective dilution in order to determine which value to
    % use in later analysis.
    y(b,1)=mean([zerop(j).Fructose{:}])/zerop(j).Dilution;
    u(b,1)=mean([zerop(j).Glucose{:}])/zerop(j).Dilution;

    % Values for the first entry.
    t(a,1).Day=zerop(j).Day;
```

```
411         t(a,1).Variety=zerop(j).Variety;
412         t(a,1).Strain=zerop(j).Strain;
413         t(a,1).Sample=zerop(j).Sample;
414         t(a,1).Dilution=zerop(j).Dilution;
415         t(a,1).TAV=zerop(j).TAV;
416         t(a,1).MeanFD=y;
417         t(a,1).MeanGD=u;
418
419         % Get the average results for each condition tested and place the
420         % entries on a new structure, t.
421         for k=2:length(zerop)
422             if zerop(k).Day==zerop(j).Day && strcmp(zerop(j).Variety,...
423                     zerop(k).Variety)==1 && strcmp(zerop(j).Strain,...
424                     zerop(k).Strain)==1 && strcmp(zerop(j).Sample,...
425                     zerop(k).Sample)==1
426                 b=b+1;
427             else
428                 j=k;
429                 b=1;
430                 a=a+1;
431                 y=[];
432                 u=[];
433             end
434             y(b,1)=mean([zerop(j).Fructose{:}])/zerop(k).Dilution;
435             u(b,1)=mean([zerop(j).Glucose{:}])/zerop(k).Dilution;
436             t(a,1).Day=zerop(j).Day;
437             t(a,1).Variety=zerop(j).Variety;
438             t(a,1).Strain=zerop(j).Strain;
439             t(a,1).Sample=zerop(j).Sample;
440             t(a,1).Dilution=zerop(j).Dilution;
441             t(a,1).TAV=zerop(j).TAV;
442             t(a,1).MeanFD=y;
443             t(a,1).MeanGD=u;
444         end
445
446
447         % For each case select the results that were measured closer to
448         % the centre of the calibration curve.
449         for s=1:length(t)
450             t(s).MeanFD=sort(t(s).MeanFD,'descend');
451             t(s).MeanGD=sort(t(s).MeanGD,'descend');
452             for r=1:length(t(s).MeanFD)
453                 if round(t(s).MeanFD(r))>10
454                     t(s).MeanFD(r)=0;
455                 elseif round(t(s).MeanGD(r))>10
456                     t(s).MeanGD(r)=0;
457                 end
458             end
459             t(s).PointF=max(t(s).MeanFD)*t(s).Dilution;
460             t(s).PointG=max(t(s).MeanGD)*t(s).Dilution;
461             t(s).Total=t(s).PointF+t(s).PointG;
462             t(s).TAV=t(s).TAV{1};
463         end
464
465         % Save structure t variable to the workspace of the main MATLAB
466         % window.
467         assignin('base','t',t)
468
469
470         %%% Variable lists %%%
```

```
% A list for the possible values of each variable identifying the
% sample were made with the intention of using them for dropdown
% boxes in other functions.
global database

% Day Matrix.
database.day(1)=results(1).Day;
for d=1:length(results)
    if any((results(d).Day==database.day(:)))==0
        database.day(end+1,1)=results(d).Day;
    end
end
database.day=sort(database.day);

% Convert to string.
global list_day
for p=1:length(database.day)
    list_day{p}=num2str(database.day(p));
end
list_day=list_day';

% Save list_day to workspace.
assignin('base','list_day',list_day)


% Variety matrix.
database.variety(1)={results(1).Variety};
for v=1:length(results)
    if strcmp(results(v).Variety,database.variety(:))==0
        database.variety(end+1,1)={results(v).Variety};
    end
end
database.variety=sort(database.variety);
global list_variety
list_variety=database.variety;

% Save list_variety to workspace.
assignin('base','list_variety',list_variety)


% Strain matrix.
database.strain(1)={results(1).Strain};
for s=1:length(results)
    if strcmp(results(s).Strain,database.strain(:))==0
        database.strain(end+1,1)={results(s).Strain};
    end
end
database.strain=sort(database.strain);
global list_strain
list_strain=database.strain;

% Save list_strain to workspace.
assignin('base','list_strain',list_strain)


% Sample matrix.
database.sample(1)={results(1).Sample};
for sa=1:length(results)
    if strcmp(results(sa).Sample,database.sample(:))==0
```

```
                database.sample(end+1,1)={results(sa).Sample};
            end
        end
        database.sample=sort(database.sample);
        global list_sample
        list_sample=database.sample;

        % Save list_sample to workspace.
        assignin('base','list_sample',list_sample)


        % Dilution matrix.
         database.dilution(1)=results(1).Dilution;
        for di=1:length(results)
            if any(results(di).Dilution==database.dilution(:))==0
                database.dilution(end+1,1)=results(di).Dilution;
            end
        end
        database.dilution=sort(database.dilution);

        % Convert to string.
        global list_dilution
        for p=1:length(database.dilution)
            list_dilution{p}=num2str(database.dilution(p));
        end
        % Save list_dilution to workspace.
        assignin('base','list_dilution',list_dilution)



        %%% Save other variables to workspace %%%
        % Save results.
        assignin('base','results',results)

        % Save variable list structure - structure containing all variable
        % lists.
        assignin('base','database',database)

        %Confirmation message to inform the user that the results loading
        %process has finished.
        msgbox('The results were loaded.')
        w=waitforbuttonpress;
        if w==0 || w==1
            close(importexport2)
            menu2
        end
    end


    % --- Executes on button press in importmat.
    function importmat_Callback(hObject, eventdata, handles)
    % hObject    handle to importmat (see GCBO)
    % eventdata  reserved - to be defined in a future version of MATLAB
    % handles    structure with handles and user data (see GUIDATA)


    % For results imported from a MATLAB database previously created and
    % saved in a .mat file the process is much simpler.
```

```
% A new window pops up asking the user to select the file pretended to
% be loaded.
file=uigetfile('*.mat','Load Workspace Variables');

% When the file is selected the confirmation question previously used
% is asked.
if file~=0
    confirm=questdlg('Are you sure you want to
proceed?','Confirmation',...
        'Yes','No','Yes');

    % If the answer is "Yes" a general clean is made and new variables
    % are loaded.
    if strcmp(confirm,'Yes')==1
        clear all; clc
        load(file)
        assignin('base','results',results)
        assignin('base','database',database)
        assignin('base','list_day',list_day)
        assignin('base','list_dilution',list_dilution)
        assignin('base','list_sample',list_sample)
        assignin('base','list_strain',list_strain)
        assignin('base','list_variety',list_variety)

        % Confirmation message.
        msgbox('The results were loaded.')
    end
end


% --- Executes on button press in exportxlsx.
function exportxlsx_Callback(hObject, eventdata, handles)
% hObject    handle to exportxlsx (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Here the results in the database are saved to a Microsoft® Exel file
% named "Results.xlsx". To avoid overwriting, the file used to load
% the database should be moved from the "Current Folder".

% First the existence of loaded results is checked and then the
% confirmation of the user intents. If there are no results loaded, an
% error message appears.
global results
if isempty(results)~=1
    confirm=questdlg('Are you sure you want to proceed?',...
        'Confirmation','Yes','No','Yes');
    if strcmp(confirm,'Yes')==1
        a=1;
        b=1;

        % Group the existing variables.
        while b<=length(results)
            for c=1:length(results(b).Fructose)
                sampledataF(a,:)={results(b).Fructose{c},...
                    results(b).FructoseArea{c}};
                sampledataG(a,:)={results(b).Glucose{c},...
                    results(b).GlucoseArea{c}};
                sampleid(a,:)={results(b).Day,results(b).Variety,...
                    results(b).Strain,results(b).Sample, ...
```

```
                    results(b).Dilution};
                samplepo(a,:)={results(b).Problem{c},...
                    results(b).Observations{c}};
                if c==1
                    sampletav(a,:)={results(b).TAV{c}};
                else
                    sampletav(a,:)={};
                end
                a=a+1;
            end
            b=b+1;
        end

        % Positioning of the results in the worksheet.
        posid=horzcat('B2:F',num2str(a));
        status1=xlswrite('Results.xlsx',sampleid,'Results',posid);

        posdataF=horzcat('H2:I',num2str(a));
        status2=xlswrite('Results.xlsx',sampledataF, ...
            'Results',posdataF);

        posdataG=horzcat('K2:L',num2str(a));
        status3=xlswrite('Results.xlsx',sampledataG, ...
            'Results',posdataG);

        pospo=horzcat('O2:P',num2str(a));
        status4=xlswrite('Results.xlsx',samplepo,'Results',pospo);

        postav=horzcat('N2:N',num2str(a));
        status5=xlswrite('Results.xlsx',sampletav,'Results',postav);

        % Verify if positioning was successfully completed and inform
        % user of process completion. If an error occurs during
        % positioning, a message appears asking the user to retry.
        compare=1;
        if isequal(status1,status2,status3,status4,status5,compare)==1
            msgbox('Resuls.xlsx were saved.')
        else
            warndlg({'Results.xlsx were not saved successfully.', ...
                'Please try again.'},'Error while saving')
        end
    end
else
    warndlg('Please load results first','Data not found!','modal')
end


% --- Executes on button press in close.
function close_Callback(hObject, eventdata, handles)
% hObject    handle to close (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% The "Close" button closes the current function and launches
% "menu2.m".
close(importexport2);menu2;


% --- Executes on button press in exportmat.
```

```
function exportmat_Callback(hObject, eventdata, handles)
% hObject    handle to exportmat (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% In this case the there are the same confirmations as in exportxlsx
% but this time the database created is saved in .mat format. The user
% may choose where and under what name the database should be saved.
global results database list_day list_dilution list_sample list_strain
list_variety
if isempty(results)~=1
    confirm=questdlg('Are you sure you want to proceed?', ...
        'Confirmation','Yes','No','Yes');
    if strcmp(confirm,'Yes')==1
        save(uiputfile('*.mat','Save Workspace Variables'))

        % Confirmation message.
        msgbox('The results were saved.')
    end
else
    warndlg('Please load results first','Data not found!','modal')
end
```

E.3 MATLAB® Code for "menu2.m" Function

```
function varargout = menu2(varargin)
% MENU2 M-file for menu2.fig
%      MENU2, by itself, creates a new MENU2 or raises the existing
%      singleton*.
%
%      H = MENU2 returns the handle to a new MENU2 or the handle to
%      the existing singleton*.
%
%      MENU2('CALLBACK',hObject,eventData,handles,...) calls the local
%      function named CALLBACK in MENU2.M with the given input
arguments.
%
%      MENU2('Property','Value',...) creates a new MENU2 or raises the
%      existing singleton*.  Starting from the left, property value
pairs
%      are applied to the GUI before menu2_OpeningFcn gets called.  An
%      unrecognized property name or invalid value makes property
%      application stop.  All inputs are passed to menu2_OpeningFcn
via
%      varargin.
%
%      *See GUI Options on GUIDE's Tools menu.  Choose "GUI allows
only
%      one instance to run (singleton)".
%
% See also: GUIDE, GUIDATA, GUIHANDLES

% Edit the above text to modify the response to help menu2

% Last Modified by GUIDE v2.5 08-Apr-2012 11:37:33

% Begin initialization code - DO NOT EDIT
gui_Singleton = 1;
gui_State = struct('gui_Name',       mfilename, ...
                   'gui_Singleton',  gui_Singleton, ...
                   'gui_OpeningFcn', @menu2_OpeningFcn, ...
                   'gui_OutputFcn',  @menu2_OutputFcn, ...
                   'gui_LayoutFcn',  [] , ...
                   'gui_Callback',   []);
if nargin && ischar(varargin{1})
    gui_State.gui_Callback = str2func(varargin{1});
end

if nargout
    [varargout{1:nargout}] = gui_mainfcn(gui_State, varargin{:});
else
    gui_mainfcn(gui_State, varargin{:});
end
% End initialization code - DO NOT EDIT


% --- Executes just before menu2 is made visible.
function menu2_OpeningFcn(hObject, eventdata, handles, varargin)
```

```
% This function has no output args, see OutputFcn.
% hObject    handle to figure
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
% varargin   command line arguments to menu2 (see VARARGIN)

% Choose default command line output for menu2
handles.output = hObject;

% Update handles structure
guidata(hObject, handles);

% UIWAIT makes menu2 wait for user response (see UIRESUME)
% uiwait(handles.figure1);


% --- Outputs from this function are returned to the command line.
function varargout = menu2_OutputFcn(hObject, eventdata, handles)
% varargout  cell array for returning output args (see VARARGOUT);
% hObject    handle to figure
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Get default command line output from handles structure
varargout{1} = handles.output;


% --- Executes on button press in viewresults.
function viewresults_Callback(hObject, eventdata, handles)
% hObject    handle to viewresults (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% This function works as the main page of the programe and its
"Callback"
% functions are mainly used to launch other functions

% If results have been loaded to the database, "menu2.m" is closed and
% function "viewresults.m" is presented.
global results
if isempty(results)~=1
    close(menu2);viewresults
else
    warndlg('Please load results first','Data not found!','modal')
end


% --- Executes on button press in editdatabase.
function editdatabase_Callback(hObject, eventdata, handles)
% hObject    handle to editdatabase (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% If results have been loaded to the database, "menu2.m" is closed and
% function "editsample3.m" is presented.
global results
if isempty(results)~=1
    close(menu2);editsample3;
else
```

```
113         warndlg('Please load results first','Data not found!','modal')
114     end
115
116
117     % --- Executes on button press in saveloadresults.
118     function saveloadresults_Callback(hObject, eventdata, handles)
119     % hObject    handle to saveloadresults (see GCBO)
120     % eventdata  reserved - to be defined in a future version of MATLAB
121     % handles    structure with handles and user data (see GUIDATA)
122
123     % "menu2.m" is closed and function "importexport.m" is presented.
124     close(menu2);importexport2
125
126
127     % --- Executes on button press in newday.
128     function newday_Callback(hObject, eventdata, handles)
129     % hObject    handle to newday (see GCBO)
130     % eventdata  reserved - to be defined in a future version of MATLAB
131     % handles    structure with handles and user data (see GUIDATA)
132
133     % In "editsample3.m" the choice of samples is limited to the set of
134     days,
135     % varieties, strains and samples already present in the dropdown boxes
136     % which correspond to the variable list created by "importexport.m"
137     % function.
138
139     % In order to allow the database to expand, this feature was created
140     to
141     % add a different sampling day to the list. The other variables are
142     not
143     % meant to be altered since in this work only the discussed strains
144     and
145     % varieties are used and the tests were all done in triplicate so the
146     % sample list does not need to be extended either.
147     % Note: The day corresponds to the nth time that samples were withrawn
148
149     %The confirmation if results have already been loaded is also used
150     global results list_day
151     if isempty(results)~=1
152         n=0;
153         % The user is asked to enter the new day value and the program
154     checks
155         % if the value introduced is a numeric value. Otherwise an error
156         % message is produced and the user has to retry.
157         while n~=1
158             newday=cell2mat(inputdlg('Enter the new day value','New day
159     entry',1));
160             if isnan(str2double(newday))==0 && isempty(newday)==0 && ...
161                     any(strcmp(newday,list_day))==0 && ...
162
163     isequal(str2double(newday),round(str2double(newday)))==1
164                 list_day{end+1}=newday;
165                 for p=1:length(list_day)
166                     list_day_num(p)=str2double(list_day(p));
167                 end
168                 list_day_num=sort(list_day_num);
169                 for q=1:length(list_day_num)
170                     list_day{q}=num2str(list_day_num(q));
171                 end
172                 assignin('base','list_day',list_day)
```

```
            n=1;
        elseif isnan(str2double(newday))==1
            if isempty(newday)==1
                n=1;
            else
                uiwait(warndlg('The value must be numeric', ...
                    'Error - Not a number'))
            end
        elseif isempty(newday)==1
            n=1;
        elseif any(strcmp(newday,list_day))==1
            uiwait(warndlg('The number introduced already exists in
...
                the list','Number already exists'))
        else
            uiwait(warndlg('The number must be an integer', ...
                'Error - Not an integer'))
        end
    end
else
    warndlg('Please load results first','Data not found!','modal')
end

% --- Executes on button press in calibcurves.
function calibcurves_Callback(hObject, eventdata, handles)
% hObject    handle to calibcurves (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% If results have been loaded to the database, "menu2.m" is closed and
% function "calibrationcurves.m" is presented.
global results
if isempty(results)~=1
    close(menu2);calibrationcurves;
else
    warndlg('Please load results first','Data not found!','modal')
end

% --- Executes on button press in close.
function close_Callback(hObject, eventdata, handles)
% hObject    handle to close (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

%The close button quits the program.
close all
```

E.4 MATLAB® Code for "viewresults.m" Function

```
function varargout = viewresults(varargin)
%VIEWRESULTS M-file for viewresults.fig
%      VIEWRESULTS, by itself, creates a new VIEWRESULTS or raises the
existing
%      singleton*.
%
%      H = VIEWRESULTS returns the handle to a new VIEWRESULTS or the
handle to
%      the existing singleton*.
%
%      VIEWRESULTS('Property','Value',...) creates a new VIEWRESULTS
using the
%      given property value pairs. Unrecognized properties are passed
via
%      varargin to viewresults_OpeningFcn.  This calling syntax
produces a
%      warning when there is an existing singleton*.
%
%      VIEWRESULTS('CALLBACK') and VIEWRESULTS('CALLBACK',hObject,...)
call the
%      local function named CALLBACK in VIEWRESULTS.M with the given
input
%      arguments.
%
%      *See GUI Options on GUIDE's Tools menu.  Choose "GUI allows
only one
%      instance to run (singleton)".
%
% See also: GUIDE, GUIDATA, GUIHANDLES

% Edit the above text to modify the response to help viewresults

% Last Modified by GUIDE v2.5 18-Apr-2012 11:37:07

% Begin initialization code - DO NOT EDIT
gui_Singleton = 1;
gui_State = struct('gui_Name',       mfilename, ...
                   'gui_Singleton',  gui_Singleton, ...
                   'gui_OpeningFcn', @viewresults_OpeningFcn, ...
                   'gui_OutputFcn',  @viewresults_OutputFcn, ...
                   'gui_LayoutFcn',  [], ...
                   'gui_Callback',   []);
if nargin && ischar(varargin{1})
   gui_State.gui_Callback = str2func(varargin{1});
end

if nargout
    [varargout{1:nargout}] = gui_mainfcn(gui_State, varargin{:});
else
    gui_mainfcn(gui_State, varargin{:});
end
% End initialization code - DO NOT EDIT


% --- Executes just before viewresults is made visible.
```

```
function viewresults_OpeningFcn(hObject, eventdata, handles, varargin)
% This function has no output args, see OutputFcn.
% hObject    handle to figure
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
% varargin   unrecognized PropertyName/PropertyValue pairs from the
%            command line (see VARARGIN)

% Choose default command line output for viewresults
handles.output = hObject;

% Update handles structure
guidata(hObject, handles);

% UIWAIT makes viewresults wait for user response (see UIRESUME)
% uiwait(handles.figure1);


% --- Outputs from this function are returned to the command line.
function varargout = viewresults_OutputFcn(hObject, eventdata,
handles)
% varargout  cell array for returning output args (see VARARGOUT);
% hObject    handle to figure
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Get default command line output from handles structure
varargout{1} = handles.output;


% --- Executes on button press in cancel.
function cancel_Callback(hObject, eventdata, handles)
% hObject    handle to cancel (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% This function shows the results for fructose and glucose both a
% graphical comparison and the values in a side table according to the
% specified sample defined by the top dropdown boxes.


%The "Cancel" button closes the window and reopens "menu2.m" function.
close(viewresults);menu2;


% --- Executes on selection change in pvariety.
function pvariety_Callback(hObject, eventdata, handles)
% hObject    handle to pvariety (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Hints: contents = cellstr(get(hObject,'String')) returns pvariety
contents as cell array
%        contents{get(hObject,'Value')} returns selected item from
pvariety

% This sub-function operates the dropdown box entitled "Variety".
global variety
```

```
114
115 % Get the contents of the selected variety entry.
116 contents=cellstr(get(hObject,'String'));
117 variety=contents{get(hObject,'Value')};
118
119 % Display results in gf table if entry already exists.
120 global t strain sample
121 r=[];
122 p=0;
123 for n=1:length(t)
124     if strcmp(t(n).Variety,variety)==1 &&
125 strcmp(t(n).Strain,strain)==1 ...
126             && strcmp(t(n).Sample,sample)==1
127         p=p+1;
128         r(p)=n;
129     end
130 end
131 for q=1:p
132     data_var(q,:)=[t(r(q)).Day,t(r(q)).PointF,t(r(q)).PointG];
133 end
134 if isempty(data_var)==0
135     set(handles.pointtable,'Data',data_var)
136     assignin('base','data_var',data_var)
137
138     % Plot results.
139     hold off
140     x=data_var(:,1);
141     y1=data_var(:,2);
142     y2=data_var(:,3);
143     plot(x,y1,'Marker','o','MarkerFaceColor',[0 0 1],'Color','b')
144     hold on
145     plot(x,y2,'Marker','d','MarkerFaceColor',[1 0 0],'Color','r')
146     xlabel('Day')
147     ylabel('Concentration (g/L)')
148     title('Glucose Fructose')
149     legend('Fructose','Glucose','Location','Best')
150 else
151     warndlg('There are no results for the specified conditions','No
152 results found','modal')
153 end
154
155
156
157
158 % --- Executes during object creation, after setting all properties.
159 function pvariety_CreateFcn(hObject, eventdata, handles)
160 % hObject    handle to pvariety (see GCBO)
161 % eventdata  reserved - to be defined in a future version of MATLAB
162 % handles    empty - handles not created until after all CreateFcns
163 called
164
165 % Hint: popupmenu controls usually have a white background on Windows.
166 %       See ISPC and COMPUTER.
167 if ispc && isequal(get(hObject,'BackgroundColor'),
168 get(0,'defaultUicontrolBackgroundColor'))
169     set(hObject,'BackgroundColor','white');
170 end
171
172 %Set the default variety value
173 global list_variety variety
```

```
set(hObject,'String',list_variety)
contents=cellstr(get(hObject,'String'));
variety=contents{get(hObject,'Value')};


% --- Executes on selection change in pstrain.
function pstrain_Callback(hObject, eventdata, handles)
% hObject    handle to pstrain (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Hints: contents = cellstr(get(hObject,'String')) returns pstrain
contents as cell array
%        contents{get(hObject,'Value')} returns selected item from
pstrain

% This sub-function operates the dropdown box entitled "Strain".
global strain

% Get the contents of the selected strain entry.
contents = cellstr(get(hObject,'String'));
strain=contents{get(hObject,'Value')};

% Display results in gf table if entry already exists.
global t variety sample
r=[];
p=0;
for n=1:length(t)
    if strcmp(t(n).Variety,variety)==1 &&
strcmp(t(n).Strain,strain)==1 ...
            && strcmp(t(n).Sample,sample)==1
        p=p+1;
        r(p)=n;
    end
end
for q=1:p
    data_str(q,:)=[t(r(q)).Day,t(r(q)).PointF,t(r(q)).PointG];
end
if isempty(data_str)==0
    set(handles.pointtable,'Data',data_str)
    assignin('base','data_str',data_str)

    %Plot results.
    hold off
    x=data_str(:,1);
    y1=data_str(:,2);
    y2=data_str(:,3);
    plot(x,y1,'Marker','o','MarkerFaceColor',[0 0 1],'Color','b')
    hold on
    plot(x,y2,'Marker','d','MarkerFaceColor',[1 0 0],'Color','r')
    xlabel('Day')
    ylabel('Concentration (g/L)')
    title('Glucose Fructose')
    legend('Fructose','Glucose','Location','Best')

else
    warndlg('There are no results for the specified conditions','No
results found','modal')
end
```

```
234
235 % --- Executes during object creation, after setting all properties.
236 function pstrain_CreateFcn(hObject, eventdata, handles)
237 % hObject    handle to pstrain (see GCBO)
238 % eventdata  reserved - to be defined in a future version of MATLAB
239 % handles    empty - handles not created until after all CreateFcns
240 called
241
242 % Hint: popupmenu controls usually have a white background on Windows.
243 %       See ISPC and COMPUTER.
244 if ispc && isequal(get(hObject,'BackgroundColor'),
245 get(0,'defaultUicontrolBackgroundColor'))
246     set(hObject,'BackgroundColor','white');
247 end
248
249 %Set the default strain value
250 global list_strain strain
251 set(hObject,'String',list_strain)
252 contents=cellstr(get(hObject,'String'));
253 strain=contents{get(hObject,'Value')};
254
255
256 % --- Executes on selection change in psample.
257 function psample_Callback(hObject, eventdata, handles)
258 % hObject    handle to psample (see GCBO)
259 % eventdata  reserved - to be defined in a future version of MATLAB
260 % handles    structure with handles and user data (see GUIDATA)
261
262 % Hints: contents = cellstr(get(hObject,'String')) returns psample
263 contents as cell array
264 %        contents{get(hObject,'Value')} returns selected item from
265 psample
266
267 % This sub-function operates the dropdown box entitled "Sample".
268 global sample
269
270 % Get the contents of the selected sample entry.
271 contents=cellstr(get(hObject,'String'));
272 sample=contents{get(hObject,'Value')};
273
274 % Display results in gf table if entry already exists.
275 global t variety strain
276 r=[];
277 p=0;
278 for n=1:length(t)
279     if strcmp(t(n).Variety,variety)==1 &&
280 strcmp(t(n).Strain,strain)==1 ...
281             && strcmp(t(n).Sample,sample)==1
282         p=p+1;
283         r(p)=n;
284     end
285 end
286 for q=1:p
287     data_sam(q,:)=[t(r(q)).Day,t(r(q)).PointF,t(r(q)).PointG];
288 end
289 if isempty(data_sam)==0
290     set(handles.pointtable,'Data',data_sam)
291     assignin('base','data_sam',data_sam)
292
```

```
293         % Plot results.
294         hold off
295         x=data_sam(:,1);
296         y1=data_sam(:,2);
297         y2=data_sam(:,3);
298         plot(x,y1,'Marker','o','MarkerFaceColor',[0 0 1],'Color','b')
299         hold on
300         plot(x,y2,'Marker','d','MarkerFaceColor',[1 0 0],'Color','r')
301         xlabel('Day')
302         ylabel('Concentration (g/L)')
303         title('Glucose Fructose')
304         legend('Fructose','Glucose','Location','Best')
305
306     else
307         warndlg('There are no results for the specified conditions','No
308     results found','modal')
309     end
310
311
312
313     % --- Executes during object creation, after setting all properties.
314     function psample_CreateFcn(hObject, eventdata, handles)
315     % hObject    handle to psample (see GCBO)
316     % eventdata  reserved - to be defined in a future version of MATLAB
317     % handles    empty - handles not created until after all CreateFcns
318     called
319
320     % Hint: popupmenu controls usually have a white background on Windows.
321     %       See ISPC and COMPUTER.
322     if ispc && isequal(get(hObject,'BackgroundColor'),
323     get(0,'defaultUicontrolBackgroundColor'))
324         set(hObject,'BackgroundColor','white');
325     end
326
327     %Set the default sample value
328     global list_sample sample
329     set(hObject,'String',list_sample)
330     contents=cellstr(get(hObject,'String'));
331     sample=contents{get(hObject,'Value')};
```

E.5 MATLAB® Code for "calibrationcurves.m" Function

```
function varargout = calibrationcurves(varargin)
% CALIBRATIONCURVES M-file for calibrationcurves.fig
%      CALIBRATIONCURVES, by itself, creates a new CALIBRATIONCURVES
or raises the existing
%      singleton*.
%
%      H = CALIBRATIONCURVES returns the handle to a new
CALIBRATIONCURVES or the handle to
%      the existing singleton*.
%
%      CALIBRATIONCURVES('CALLBACK',hObject,eventData,handles,...)
calls the local
%      function named CALLBACK in CALIBRATIONCURVES.M with the given
input arguments.
%
%      CALIBRATIONCURVES('Property','Value',...) creates a new
CALIBRATIONCURVES or raises the
%      existing singleton*.  Starting from the left, property value
pairs are
%      applied to the GUI before calibrationcurves_OpeningFcn gets
called.  An
%      unrecognized property name or invalid value makes property
application
%      stop.  All inputs are passed to calibrationcurves_OpeningFcn
via varargin.
%
%      *See GUI Options on GUIDE's Tools menu.  Choose "GUI allows
only one
%      instance to run (singleton)".
%
% See also: GUIDE, GUIDATA, GUIHANDLES

% Edit the above text to modify the response to help calibrationcurves

% Last Modified by GUIDE v2.5 06-Apr-2012 17:25:48

% Begin initialization code - DO NOT EDIT
gui_Singleton = 1;
gui_State = struct('gui_Name',       mfilename, ...
                   'gui_Singleton',  gui_Singleton, ...
                   'gui_OpeningFcn', @calibrationcurves_OpeningFcn,
...
                   'gui_OutputFcn',  @calibrationcurves_OutputFcn, ...
                   'gui_LayoutFcn',  [] , ...
                   'gui_Callback',   []);
if nargin && ischar(varargin{1})
    gui_State.gui_Callback = str2func(varargin{1});
end

if nargout
    [varargout{1:nargout}] = gui_mainfcn(gui_State, varargin{:});
else
    gui_mainfcn(gui_State, varargin{:});
end
% End initialization code - DO NOT EDIT
```

```
56
57
58  % --- Executes just before calibrationcurves is made visible.
59  function calibrationcurves_OpeningFcn(hObject, eventdata, handles,
60  varargin)
61  % This function has no output args, see OutputFcn.
62  % hObject    handle to figure
63  % eventdata  reserved - to be defined in a future version of MATLAB
64  % handles    structure with handles and user data (see GUIDATA)
65  % varargin   command line arguments to calibrationcurves (see
66  VARARGIN)
67
68  % Choose default command line output for calibrationcurves
69  handles.output = hObject;
70
71  % Update handles structure
72  guidata(hObject, handles);
73
74  % UIWAIT makes calibrationcurves wait for user response (see UIRESUME)
75  % uiwait(handles.figure1);
76
77
78  % Include calibG and calibF (previously created) in the workspace of
79  this
80  % function.
81  global calibG calibF
82
83  % Identify user's choice over the radio button options "Old Colmn" and
84  "New
85  % Column".
86  old=get(handles.oldcolumn,'Value');
87  new=get(handles.newcolumn,'Value');
88
89  % Deppending on the choice different data sets are presented
90  if old==1 && new==0
91
92      %%% Glucose %%%
93      % Display values of standards and areas on tableG.
94      set(handles.calibtableG,'Data',calibG)
95
96      % Create data linear fit.
97      [fitG,gofG]=fit(calibG(:,1),calibG(:,2),fittype('poly1'));
98
99      % Get coefficients separately.
100     coefG=coeffvalues(fitG);
101
102     % Get R square.
103     r2G=gofG.rsquare;
104
105     % Display equation and R square.
106     set(handles.eqG,'String',{strcat('y =
107 ',num2str(coefG(1),'%6.4e'),...
108         'x',num2str(coefG(2),'%+6.0f'));strcat('r^2 =
109 ',num2str(r2G,'%6.4f'))})
110
111     % Select the correct axes to edit.
112     axes(handles.Gplot)
113
```

```
114         % Plot data and curve fit.
115         plot(fitG,calibG(:,1),calibG(:,2))
116 
117         % Assign properties to the axes.
118         set(handles.Gplot,'XGrid','on','YGrid','on','XLim',[0
119     16],'YLim',[0 1200000])
120         legend(handles.Gplot,'Location','SouthEast')
121         hold on
122 
123 
124         %%% Fructose %%%
125         % Display values of standards and areas on tableF.
126         set(handles.calibtableF,'Data',calibF)
127 
128         % Create data linear fit.
129         [fitF,gofF]=fit(calibF(:,1),calibF(:,2),fittype('poly1'));
130 
131         % Get coefficients separately.
132         coefF=coeffvalues(fitF);
133 
134         % Get R square.
135         r2F=gofF.rsquare;
136 
137         % Display equation and R square.
138         set(handles.eqF,'String',{strcat('y =
139     ',num2str(coefF(1),'%6.4e'),...
140             'x',num2str(coefF(2),'%6.0f'));strcat('r^2 =
141     ',num2str(r2F,'%6.4f'))})
142 
143         % Select the correct axes to edit.
144         axes(handles.Fplot)
145 
146         % Plot data and curve fit
147         plot(fitF,calibF(:,1),calibF(:,2))
148 
149         % Assign properties to the axes.
150         set(handles.Fplot,'XGrid','on','YGrid','on','XLim',[0
151     16],'YLim',[0 1200000])
152         legend(handles.Fplot,'Location','SouthEast')
153         hold on
154     elseif old==0 && new==1
155 
156         % WAINTING FOR VALUES FROM THE CALIBRATION OF A NEW COLUMN
157 
158     else
159         error('Error: Old or New?')
160     end
161 
162 
163     % --- Outputs from this function are returned to the command line.
164     function varargout = calibrationcurves_OutputFcn(hObject, eventdata,
165     handles)
166     % varargout  cell array for returning output args (see VARARGOUT);
167     % hObject    handle to figure
168     % eventdata  reserved - to be defined in a future version of MATLAB
169     % handles    structure with handles and user data (see GUIDATA)
170 
171     % Get default command line output from handles structure
```

```
varargout{1} = handles.output;


% --- Executes on button press in close.
function close_Callback(hObject, eventdata, handles)
% hObject    handle to close (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% The "Close" button closes the calibation curves window and returns
to the
% "menu2.m" GUI.
close(calibrationcurves);menu2
```

E.6 MATLAB® Code for "editsample3.m" Function

```
function varargout = editsample3(varargin)
%EDITSAMPLE3 M-file for editsample3.fig
%      EDITSAMPLE3, by itself, creates a new EDITSAMPLE3 or raises the
%      existing singleton*.
%
%      H = EDITSAMPLE3 returns the handle to a new EDITSAMPLE3 or the
%      handle to the existing singleton*.
%
%      EDITSAMPLE3('Property','Value',...) creates a new EDITSAMPLE3
%      using the given property value pairs. Unrecognized properties
%      are passed via varargin to editsample3_OpeningFcn.  This
%      calling syntax produces a warning when there is an existing
%      singleton*.
%
%      EDITSAMPLE3('CALLBACK') and EDITSAMPLE3('CALLBACK',hObject,...)
%      call the local function named CALLBACK in EDITSAMPLE3.M with
%      the given input arguments.
%
%      *See GUI Options on GUIDE's Tools menu.  Choose "GUI allows
%      only one instance to run (singleton)".
%
% See also: GUIDE, GUIDATA, GUIHANDLES

% Edit the above text to modify the response to help editsample3

% Last Modified by GUIDE v2.5 11-Apr-2012 23:19:12

% Begin initialization code - DO NOT EDIT
gui_Singleton = 1;
gui_State = struct('gui_Name',       mfilename, ...
                   'gui_Singleton',  gui_Singleton, ...
                   'gui_OpeningFcn', @editsample3_OpeningFcn, ...
                   'gui_OutputFcn',  @editsample3_OutputFcn, ...
                   'gui_LayoutFcn',  [], ...
                   'gui_Callback',   []);
if nargin && ischar(varargin{1})
   gui_State.gui_Callback = str2func(varargin{1});
end

if nargout
    [varargout{1:nargout}] = gui_mainfcn(gui_State, varargin{:});
else
    gui_mainfcn(gui_State, varargin{:});
end
% End initialization code - DO NOT EDIT


% --- Executes just before editsample3 is made visible.
function editsample3_OpeningFcn(hObject, eventdata, handles, varargin)
% This function has no output args, see OutputFcn.
% hObject    handle to figure
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)
% varargin   unrecognized PropertyName/PropertyValue pairs from the
%            command line (see VARARGIN)
```

```
56
57  % Choose default command line output for editsample3
58  handles.output = hObject;
59
60  % Update handles structure
61  guidata(hObject, handles);
62
63  % UIWAIT makes editsample3 wait for user response (see UIRESUME)
64  % uiwait(handles.figure1);
65
66
67  % --- Outputs from this function are returned to the command line.
68  function varargout = editsample3_OutputFcn(hObject, eventdata,
69  handles)
70  % varargout  cell array for returning output args (see VARARGOUT);
71  % hObject    handle to figure
72  % eventdata  reserved - to be defined in a future version of MATLAB
73  % handles    structure with handles and user data (see GUIDATA)
74
75  % Get default command line output from handles structure
76  varargout{1} = handles.output;
77
78
79  % --- Executes on button press in save.
80  function save_Callback(hObject, eventdata, handles)
81  % hObject    handle to save (see GCBO)
82  % eventdata  reserved - to be defined in a future version of MATLAB
83  % handles    structure with handles and user data (see GUIDATA)
84
85  % This function may be used to edit existing data or to add new values
86  % to the database.
87  global results day variety strain sample dilution
88
89  % Create empty variable.
90  r=[];
91
92  % Check if specified sample entry exists and define the row intended
93  % to be modified (row m)
94  for n=1:length(results)
95      if results(n).Day==str2double(day) && ...
96              strcmp(results(n).Variety,variety)==1 && ...
97              strcmp(results(n).Strain,strain)==1 && ...
98              strcmp(results(n).Sample,sample)==1 && ...
99              results(n).Dilution==str2double(dilution)
100         r=results(n);m=n;
101     end
102 end
103
104 % Get the matrix with the new values
105 gf=get(handles.gftable,'Data');
106 F=gf(:,1);FA=gf(:,2);G=gf(:,3);GA=gf(:,4);
107 T=gf(:,5);P=gf(:,6);O=gf(:,7);
108
109 % Add new data to the database
110 if isempty(r)==1
111     results(end+1)=struct('Day',str2double(day),'Variety',variety,...
112         'Strain',strain,'Sample',sample','Dilution',...
113         str2double(dilution),'Fructose',{F},'FructoseArea',{FA},...
114         'Glucose',{G},'GlucoseArea',{GA},'TAV',{T},'Problem',{P},...
```

```
115             'Observations',{O});
116
117     % Edit existing database entry - The user is asked if overwriting the
118     % existing results is intended.
119     else
120         confirm=questdlg({'The selected entry already exists.';'';...
121             'Overwrite data?'},'Warning - Overwriting','Yes','No','No');
122         if strcmp(confirm,'Yes')==1
123             r=struct('Day',str2double(day),'Variety',variety,'Strain',...
124                 strain,'Sample',sample','Dilution',...
125                 str2double(dilution),'Fructose',{F},'FructoseArea',...
126                 {FA},'Glucose',{G},'GlucoseArea',{GA},'TAV',{T},...
127                 'Problem',{P},'Observations',{O});
128             results(m)=r;
129         end
130     end
131
132     %Save the modified database to the workspace outside the function
133     assignin('base','results',results)
134
135
136     % --- Executes on button press in cancel.
137     function cancel_Callback(hObject, eventdata, handles)
138     % hObject    handle to cancel (see GCBO)
139     % eventdata  reserved - to be defined in a future version of MATLAB
140     % handles    structure with handles and user data (see GUIDATA)
141
142     % "Cancel" button causes the GUI to shut down and opens the "menu2.m"
143     % function
144     close(editsample3);menu2;
145
146
147     function replicate_Callback(hObject, eventdata, handles)
148     % hObject    handle to replicate (see GCBO)
149     % eventdata  reserved - to be defined in a future version of MATLAB
150     % handles    structure with handles and user data (see GUIDATA)
151
152     % Hints: get(hObject,'String') returns contents of replicate as text
153     %        str2double(get(hObject,'String')) returns contents of
154     % replicate as a double
155
156     % The replicate number influences the number of lines in te table
157     % presented.
158
159     % Get the value in the box presented in the GUI.
160     repnumber=str2double(get(hObject,'String'));
161
162     % Verification if the value (which may be modified by the user) is a
163     % number and if it is a positive integer. In case of error, the
164     % default number 2 is replaced in the box.
165     if isnan(repnumber)==1
166         warndlg('The "Number of Replicates" must be a numeric value!',...
167             'Warning - Input error!')
168         set(hObject,'String','2')
169     else
170         if isequal(round(repnumber),repnumber)==1
171             data=cell(repnumber,7);
172             set(handles.gftable,'Data',data)
173         else
```

```
174             warndlg('The "Number of Replicates must be an integer!',...
175                 'Warning - Input error!')
176             set(hObject,'String','2')
177         end
178     end
179
180
181     % --- Executes during object creation, after setting all properties.
182     function replicate_CreateFcn(hObject, eventdata, handles)
183     % hObject    handle to replicate (see GCBO)
184     % eventdata  reserved - to be defined in a future version of MATLAB
185     % handles    empty - handles not created until after all CreateFcns
186     % called
187
188     % Hint: edit controls usually have a white background on Windows.
189     %       See ISPC and COMPUTER.
190     if ispc && isequal(get(hObject,'BackgroundColor'),
191     get(0,'defaultUicontrolBackgroundColor'))
192         set(hObject,'BackgroundColor','white');
193     end
194
195     % When the GUI is opened the table has to be create according to the
196     % existence of the specified sample.
197     global results day variety strain sample dilution
198
199     %  Check if specified sample entry already exists.
200     r=[];
201     for n=1:length(results)
202         if results(n).Day==str2double(day) && ...
203                 strcmp(results(n).Variety,variety)==1 && ...
204                 strcmp(results(n).Strain,strain)==1 && ...
205                 strcmp(results(n).Sample,sample)==1 && ...
206                 results(n).Dilution==str2double(dilution)
207             r=results(n);
208         end
209     end
210
211     % Asssign the values to the table.
212     if isempty(r)~=1
213         repnumber=length(r.Fructose);
214         set(hObject,'String',num2str(repnumber))
215         data=[r.Fructose,r.FructoseArea,r.Glucose,r.GlucoseArea,r.TAV,...
216             r.Problem,r.Observations];
217         set(handles.gftable,'Data',data)
218     end
219
220
221     % --- Executes on selection change in pday.
222     function pday_Callback(hObject, eventdata, handles)
223     % hObject    handle to pday (see GCBO)
224     % eventdata  reserved - to be defined in a future version of MATLAB
225     % handles    structure with handles and user data (see GUIDATA)
226
227     % Hints: contents = cellstr(get(hObject,'String')) returns pday
228     % contents as cell array
229     %        contents{get(hObject,'Value')} returns selected item from
230     % pday
231
232     % Parameter choice: Day
```

```
global day

% Get contents of the selected day
contents = cellstr(get(hObject,'String'));
day=contents{get(hObject,'Value')};

% Display results in table if entry already exists. If the entry does
% not exist in the database the table is created according to the
% number of replicates specified.
global results variety strain sample dilution
r=[];
for n=1:length(results)
    if results(n).Day==str2double(day) && ...
            strcmp(results(n).Variety,variety)==1 && ...
            strcmp(results(n).Strain,strain)==1 && ...
            strcmp(results(n).Sample,sample)==1 && ...
            results(n).Dilution==str2double(dilution)
        r=results(n);
    end
end
if isempty(r)==0
    repnumber=length(r.Fructose);
    set(handles.replicate,'String',num2str(repnumber))
    data=[r.Fructose,r.FructoseArea,r.Glucose,r.GlucoseArea,r.TAV,...
        r.Problem,r.Observations];
    set(handles.gftable,'Data',data)
else
    repnumber=str2double(get(handles.replicate,'String'));
    data=cell(repnumber,7);
    set(handles.gftable,'Data',data)
end


% --- Executes during object creation, after setting all properties.
function pday_CreateFcn(hObject, eventdata, handles)
% hObject    handle to pday (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    empty - handles not created until after all CreateFcns
% called

% Hint: popupmenu controls usually have a white background on Windows.
%       See ISPC and COMPUTER.
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end

% During the function opening the list with the days is attributed to
% the dropdown box list.
global list_day
set(hObject,'String',list_day)


% --- Executes on selection change in pvariety.
function pvariety_Callback(hObject, eventdata, handles)
% hObject    handle to pvariety (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Hints: contents = cellstr(get(hObject,'String')) returns pvariety
```

```
% contents as cell array
%        contents{get(hObject,'Value')} returns selected item from
pvariety

% Parameter choice: Variety.
global variety

% Get contents of the selected variety.
contents=cellstr(get(hObject,'String'));
variety=contents{get(hObject,'Value')};

% Display results in table if entry already exists. If the entry does
% not exist in the database the table is created according to the
% number of replicates specified.
global results day strain sample dilution
r=[];
for n=1:length(results)
    if results(n).Day==str2double(day) && ...
            strcmp(results(n).Variety,variety)==1 && ...
            strcmp(results(n).Strain,strain)==1 && ...
            strcmp(results(n).Sample,sample)==1 && ...
            results(n).Dilution==str2double(dilution)
        r=results(n);
    end
end
if isempty(r)==0
    repnumber=length(r.Fructose);
    set(handles.replicate,'String',num2str(repnumber))
    data=[r.Fructose,r.FructoseArea,r.Glucose,r.GlucoseArea,r.TAV,...
        r.Problem,r.Observations];
    set(handles.gftable,'Data',data)
else
    repnumber=str2double(get(handles.replicate,'String'));
    data=cell(repnumber,7);
    set(handles.gftable,'Data',data)
end


% --- Executes during object creation, after setting all properties.
function pvariety_CreateFcn(hObject, eventdata, handles)
% hObject    handle to pvariety (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    empty - handles not created until after all CreateFcns
% called

% Hint: popupmenu controls usually have a white background on Windows.
%       See ISPC and COMPUTER.
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end

% During the function opening the list with the varieties is attached
% to the dropdown box list.
global list_variety
set(hObject,'String',list_variety)


% --- Executes on selection change in pstrain.
function pstrain_Callback(hObject, eventdata, handles)
```

```
% hObject    handle to pstrain (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Hints: contents = cellstr(get(hObject,'String')) returns pstrain
% contents as cell array
%        contents{get(hObject,'Value')} returns selected item from
%pstrain

% Parameter choice: Strain
global strain

% Get contents of the selected strain
contents = cellstr(get(hObject,'String'));
strain=contents{get(hObject,'Value')};

% Display results in table if entry already exists. If the entry does
% not exist in the database the table is created according to the
% number of replicates specified.
global results day variety sample dilution
r=[];
for n=1:length(results)
    if results(n).Day==str2double(day) && ...
            strcmp(results(n).Variety,variety)==1 && ...
            strcmp(results(n).Strain,strain)==1 && ...
            strcmp(results(n).Sample,sample)==1 && ...
            results(n).Dilution==str2double(dilution)
        r=results(n);
    end
end
if isempty(r)==0
    repnumber=length(r.Fructose);
    set(handles.replicate,'String',num2str(repnumber))
    data=[r.Fructose,r.FructoseArea,r.Glucose,r.GlucoseArea,r.TAV, ...
        r.Problem,r.Observations];
    set(handles.gftable,'Data',data)
else
    repnumber=str2double(get(handles.replicate,'String'));
    data=cell(repnumber,7);
    set(handles.gftable,'Data',data)
end


% --- Executes during object creation, after setting all properties.
function pstrain_CreateFcn(hObject, eventdata, handles)
% hObject    handle to pstrain (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    empty - handles not created until after all CreateFcns
% called

% Hint: popupmenu controls usually have a white background on Windows.
%       See ISPC and COMPUTER.
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end

% During the function opening the list with the strains is attached to
% the dropdown box list.
global list_strain
```

```
413   set(hObject,'String',list_strain)
414
415
416   % --- Executes on selection change in psample.
417   function psample_Callback(hObject, eventdata, handles)
418   % hObject    handle to psample (see GCBO)
419   % eventdata  reserved - to be defined in a future version of MATLAB
420   % handles    structure with handles and user data (see GUIDATA)
421
422   % Hints: contents = cellstr(get(hObject,'String')) returns psample
423   % contents as cell array
424   %        contents{get(hObject,'Value')} returns selected item from
425   % psample
426
427   % Parameter choice: Sample.
428   global sample
429
430   % Get contents of the selected sample.
431   contents=cellstr(get(hObject,'String'));
432   sample=contents{get(hObject,'Value')};
433
434   % Display results in table if entry already exists. If the entry does
435   % not exist in the database the table is created according to the
436   % number of replicates specified.
437   global results day variety strain dilution
438   r=[];
439   for n=1:length(results)
440       if results(n).Day==str2double(day) && ...
441               strcmp(results(n).Variety,variety)==1 && ...
442               strcmp(results(n).Strain,strain)==1 && ...
443               strcmp(results(n).Sample,sample)==1 && ...
444               results(n).Dilution==str2double(dilution)
445           r=results(n);
446       end
447   end
448   if isempty(r)==0
449       repnumber=length(r.Fructose);
450       set(handles.replicate,'String',num2str(repnumber))
451       data=[r.Fructose,r.FructoseArea,r.Glucose,r.GlucoseArea,r.TAV,...
452           r.Problem,r.Observations];
453       set(handles.gftable,'Data',data)
454   else
455       repnumber=str2double(get(handles.replicate,'String'));
456       data=cell(repnumber,7);
457       set(handles.gftable,'Data',data)
458   end
459
460
461   % --- Executes during object creation, after setting all properties.
462   function psample_CreateFcn(hObject, eventdata, handles)
463   % hObject    handle to psample (see GCBO)
464   % eventdata  reserved - to be defined in a future version of MATLAB
465   % handles    empty - handles not created until after all CreateFcns
466   % called
467
468   % Hint: popupmenu controls usually have a white background on Windows.
469   %       See ISPC and COMPUTER.
470   if ispc && isequal(get(hObject,'BackgroundColor'),
471   get(0,'defaultUicontrolBackgroundColor'))
472       set(hObject,'BackgroundColor','white');
```

```
end

% During the function opening the list with the samples is attached to
% the dropdown box list.
global list_sample
set(hObject,'String',list_sample)


% --- Executes on selection change in pdilution.
function pdilution_Callback(hObject, eventdata, handles)
% hObject    handle to pdilution (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    structure with handles and user data (see GUIDATA)

% Hints: contents = cellstr(get(hObject,'String')) returns pdilution
% contents as cell array
%        contents{get(hObject,'Value')} returns selected item from
% pdilution

% Parameter choice: Dilution.
global dilution

% Get contents of the selected dilution.
contents=cellstr(get(hObject,'String'));
dilution=contents{get(hObject,'Value')};

% Display results in table if entry already exists. If the entry does
% not exist in the database the table is created according to the
% number of replicates specified.
global results day variety strain sample
r=[];
for n=1:length(results)
    if results(n).Day==str2double(day) && ...
            strcmp(results(n).Variety,variety)==1 && ...
            strcmp(results(n).Strain,strain)==1 && ...
            strcmp(results(n).Sample,sample)==1 && ...
            results(n).Dilution==str2double(dilution)
        r=results(n);
    end
end
if isempty(r)==0
    repnumber=length(r.Fructose);
    set(handles.replicate,'String',num2str(repnumber))
    data=[r.Fructose,r.FructoseArea,r.Glucose,r.GlucoseArea,r.TAV,...
        r.Problem,r.Observations];
    set(handles.gftable,'Data',data)
else
    repnumber=str2double(get(handles.replicate,'String'));
    data=cell(repnumber,7);
    set(handles.gftable,'Data',data)
end


% --- Executes during object creation, after setting all properties.
function pdilution_CreateFcn(hObject, eventdata, handles)
% hObject    handle to pdilution (see GCBO)
% eventdata  reserved - to be defined in a future version of MATLAB
% handles    empty - handles not created until after all CreateFcns
% called

```

```
% Hint: popupmenu controls usually have a white background on Windows.
%       See ISPC and COMPUTER.
if ispc && isequal(get(hObject,'BackgroundColor'),
get(0,'defaultUicontrolBackgroundColor'))
    set(hObject,'BackgroundColor','white');
end

% During the function opening the list with the dilutions is attached
% to the dropdown box list.
global list_dilution
set(hObject,'String',list_dilution)
```

E.7 MATLAB® Code for "Selected2xls.m" m-file

```
%%% Create a Microsoft® Excel worksheet with selected results %%%


% The results have already been selected by the database functions.
% This file only takes them and transforms them into a Microsoft®
% Excel file.

% Call the variable holding the desired values.
global t

% Form a matrix with the desired parameters.
x=cell(length(t),8);
for a=1:length(t)
    x(a,:)=[{t(a).Variety},{t(a).Strain},{t(a).Sample},{t(a).Day},...
        {t(a).PointF},{t(a).PointG},{t(a).Total},{t(a).TAV}];
end

% Create line with the column headers
header=[{'Variety'},{'Strain'},{'Sample'},{'Day'},{'Fructose g/L'},...
    {'Glucose g/L'},{'Total'},{'TAV'}];

% Write the headers and the matrix values to "SelectedData.xlsx" file
% that is saved in "Current Folder".
xlswrite('SelectedData.xlsx',x,horzcat('A2:H',num2str(length(x))))
xlswrite('SelectedData.xlsx',header)
```