Tutor

Dr. Javier Vicente Saurina Purroy

Departament d'Enginyeria Química i Química Analítica

Grau de Química

Treball Final de Grau

Determination of biogenic amines in wines and sparkling wines by liquid chromatography with precolumn derivatization

Determinació d'amines biogèniques en mostres de vins i caves mitjançant cromatografia de líquids amb derivatització pre-columna

Aina Mir Cerdà

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Voldria agrair-li al meu tutor Xavier Saurina tota l'ajuda i assessorament que m'ha ofert. També, l'hi he d'agrair a l'Aina Queral, la meva companya en aquest projecte.

Als totes les persones que he conegut durant aquest camí, que han fet que tot sigui més fàcil i s'han convertit en persones molt importants per mi.

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REPORT

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1.SUMMARY

Biogenic amines are often present in different types of food, but they are especially abundant in wines, cheeses, meat and fish as well as in spoiled products. Besides, the presence of a high content of biogenic amines in food can be an indicator that it has been processed under poor hygiene conditions. These amines are generated mainly from the decarboxylation of the respective amino acids.

In moderate concentrations, these compounds can be beneficial for the body as they participate in different cellular functions but when the intake of these compounds is too high for the body, toxicological problems such as migraines, headache, hypo- or hypertension, effects on the vascular or nervous system and anaphylactic shocks can result. Therefore, given the harmful effects that biogenic amines can cause in an organism, it is necessary to establish an effective and selective methods to determine these amines in food in order to avoid the commercialization of any food with high concentration of these compounds. Consequently, research is also needed to establish legal limits on the contents of different biogenic amines in food products. This last point is more complicated because the content of biogenic amines that an organism can tolerate depends on it, in turn, on the catabolic pathways of each individual. Thus, a given concentration of amines can be tolerated by an organism with a normal catabolism, but it can cause harmful effects on a sensitive organism.

In wines, the most important biogenic amines are histamine, tyramine, putrescine and cadaverine because they are the most abundant and can cause the greatest number of toxicological problems. Among the different types of wines, red wines have the highest content of biogenic amines.

Also, in wines, the presence of ethanol increases the toxicological problems that can cause these biogenic amines because this alcohol inhibits or decreases the activity of amino oxidases responsible for their metabolism.

In this project, the following biogenic amines have been considered: ethylamine, methylamine, dimethylamine, ethanolamine, butylamine, isobutylamine, isopentylamine and hexylamine. Although these amines are present at trace level, their study is important as possible descriptors of the health quality of food. To separate and determine them adequately the following studies have been carried out:

- A pre-column derivatization reaction has been performed with the 1,2-naphthoquinone-4-sulfonate reagent (NQS) to attach a chromophore group to the initial amines so that they could be detected with an ultraviolet-visible absorption detector.
- A liquid-liquid extraction process with chloroform as the solvent and subsequent evaporation until dryness has been used to obtain simpler chromatograms removing efficiently amino acid derivatives and other polar compounds that often interference with the chromatographic separation of biogenic amines, in addition to preconcentrating the extracts obtained. Combination of both derivatization and extraction procedures before the HPLC separation results in an effective clean-up of samples and affords a sensitive determination of biogenic amines.
- HPLC has been used as a technique for separating different amines under reversed-phase mode, optimizing the efficiency and resolution by modifying some variables such as the chromatographic column, the elution gradient or the separation temperature.

It should be pointed out that due to the consequences of the global pandemic caused by the SARS-CoV-2 virus, significant parts of method optimization and validation studies as well as the analysis of wine and cava samples have not been finished.

Keywords: biogenic amines, toxicological problems, wines, derivatization, 1,2-naphthoquinone-4-sulfonate, high performance liquid chromatography

2. RESUM

Les amines biògenes acostumen a estar presents en diferents tipus d'aliments però són especialment abundants en vins, formatges, carn i peix així com també en productes que s'han fet malbé. A més, la presència d'un alt contingut d'amines biògenes en aliments pot ser un indicador de què aquests han estat processats sota unes condicions de higiene deficients. Aquestes amines són generades, principalment, a partir de la descarboxilació dels respectius aminoàcids.

En concentracions moderades, aquests compostos poden ser beneficiosos per a l'organisme ja que participen en diferents funcions cel·lulars però quan la ingesta és massa elevada, poden derivar-se'n problemes toxicològics com per exemple migranya, hipo- o hipertensió, efectes sobre el sistema vascular o nerviós i xocs anafilàctics. Per tant, tenint en compte els efectes nocius que les amines biògenes poden ocasionar en un organisme, és necessari establir un mètode eficaç i selectiu per determinar el seu contingut en els aliments per tal de no posar al mercat cap aliment que contengui una concentració elevada d'aquests compostos. Conseqüentment, també es fa necessari la investigació per tal d'establir uns límits legals sobre la concentració màxima de les diferents amines biògenes que poden contenir els aliments. Aquest últim punt es fa més complicat degut a què el contingut d'amines biògenes que pot tolerar un organisme depèn d'aquest, concretament, de les rutes catabòliques de cada un. Així doncs, una mateixa concentració d'amines pot ser tolerada per un organisme amb un catabolisme normal però pot causar efectes nocius sobre un organisme sensible.

En els vins, les amines biògenes més importants són la histamina, tiramina, putrescina i cadaverina degut a què són les més abundants i les que provoquen un major nombre de problemes toxicològics. D'entre els diferents tipus de vins, el vi negre és el que presenta un major contingut d'amines biògenes.

A més, en el cas dels vins, la presència del etanol augmenta els problemes toxicològics que puguin provocar aquestes amines biògenes degut a què aquest alcohol inhibeix o disminueix l'activitat de les monoaminooxidases o diaminooxidases, responsables del metabolisme de diferents tipus d'amines biògenes.

En aquest projecte, s'ha treballat amb les següents amines biògenes: etilamina, metilamina, dimetilamina, etanolamina, butilamina, isobutilamina, isopentilamina i hexilamina. Encara que

són amines presents a nivell de traça, el seu estudi és important com a possibles descriptors de la qualitat sanitària dels aliments. Per tal de separar-les i determinar-les adequadament:

- S'ha dut a terme una reacció de derivatització pre-columna amb el reactiu NQS per tal d'enganxar un grup cromòfor a les amines inicials i així poder ser detectades amb un detector d'absorció ultravioleta-visible.
- S'ha utilitzat un procés d'extracció líquid-líquid amb el cloroform com a dissolvent orgànic i una posterior evaporació fins a la sequedat d'aquest per tal d'obtenir cromatogrames més simples eliminant possibles interferències com podrien ser derivats d'aminoàcids i altres compostos polars, que sovint interfereixen en la separació cromatogràfica de les amines biògenes, a més de preconcentrar els extractes obtinguts. La combinació de procediments d'extracció i derivatització abans de la separació amb HPLC dona lloc a un *clean-up* efectiu de les mostres i permet una determinació sensible d'amines biogèniques.
- S'ha emprat la cromatografia de líquids com a tècnica de separació de les diferents amines. La cromatografia de líquids s'ha fet amb el mode de fase invertida, optimitzant l'eficàcia i la resolució modificant alguns aspectes com són la columna cromatogràfica, el gradient d'elució o la temperatura de la separació.

Cal assenyalar que a causa de les conseqüències de la pandèmia global causada pel virus SARS-CoV-2, no s'han acabat parts significatives dels estudis d'optimització i validació de mètodes, així com l'anàlisi de mostres de vi i cava.

Paraules clau: amines biògenes, problemes toxicològics, vins, derivatització, 1,2-naftoquinona-4-sulfonat, cromatografia de líquids.

3. INTRODUCTION

3.1. BIOGENIC AMINES

Biogenic amines (BAs) are low molecular nitrogenous compounds that can be found in foods such as fish, meat, vegetables and fruits but especially in fermented products as cheeses, wine or beer [1-3]. In wines, Bas are mainly formed during malolactic fermentation (MLF) by the action of lactic acid bacteria causing decarboxylation of free amino acids [4]. According to their structures they can be classified in aliphatic (cadaverine, putrescine, spermine, spermidine), aromatic (tyramine, histamine) and heterocyclic (tryptamine) amines [5]. Biogenic amines can also be classified as monoamines (e.g. serotonin, phenylethylamine, ethanolamine, histamine, tyramine and tryptamine), diamines (putrescine and cadaverine) and polyamines (spermine and spermidine) according to the number of amino groups in their structures [6].

Histamine, tyramine and cadaverine are known to be the foremost representative ones in wines. Other amines such as putrescine, ethylamine, 2-phenylethylamine, spermine, spermidine are also present in the grape [7].

High concentrations of some BAs, especially histamine, tyramine, putrescine and cadaverine, are often found in spoiled foods and products processed within poor hygienic conditions [6]. The ingestion of high concentration of these compounds can cause toxicological effects. The removal of these compounds after its formation is incredibly complicated, hence the simplest option would be to stop the formation of those biogenic amines in food [8]. Table 1 shows the content of the main biogenic amines in different foods and beverages.

Table 1
Concentrations (mg L^{-1}) of the main biogenic amines in different types of food and beverages [9]

Food sample	Histamine	Tyramine	Putrescine	Cadaverine
Cabbage	1.0	18.6	11.6	7.6
Grapes	n.d.-5.8	n.d.-2.4	n.d.-8.0	n.d.-3.4
Apples	n.d.	n.d.	5.1	-
Pork meat	0.9-2.3	n.d.-29.0	n.d.-66.5	1.0-145.4
Chicken meat	0.2	n.d.-221.6	n.d.-45.9	n.d.-33.5
Beef meat	0.4-7.4	n.d.-17.4	n.d.-202.5	n.d.-221.4
Salted anchovies	n.d.-2.0	n.d.-22.3	0.1-8.0	0.1-12.0
Fresh mackerel	9.3-12.4	n.d.-0.4	0.-2-2.0	1.2-6.6
Canned tuna	1.3-110.3	n.d–14.5	2.8-94.1	0.1-164.0
Milk	n.d-0.7	n.d.	n.d.	n.d.- 0.1
Brewed coffee	n.d-1.6	n.d.-19.7	0.4-2.3	0.2-9.1
Orange juice	n.d.-0.04	n.d.-0.06	0.1-2.2	-
Chocolate	0.3-2.0	3.1-8.1	0.8	0.8
Chorizo	n.d.-4.5	3.1-186.1	n.d.-178.3	n.d.-52.0
Sausages	n.d.-514.5	n.d.-509.9	n.d.-505.3	n.d.-689.8
Yogurt	n.d.-13	n.d.-6.3	n.d.-26.1	n.d.-4.3
Red wine	0.5-27.0	0.1-37.3	2.9-122	n.d.-3.3
White wine	n.d.-3.4	n.d.-6.8	0.8-12.8	n.d.-2.5
Rice wine	n.d.-72.1	n.d.41.4	n.d.-32.3	n.d.-63.5
Beer	n.d.-0.3	0.4-5.9	2.1-12.8	0.2-1.4
Vinegar	n.d.-0.3	n.d.-5.0	n.d.-3.2	n.d.-0.1
Cider	n.d.-6.9	n.d.-5.0	n.d.12.3	-

n.d: non detected/non determined

Winemaking process

In view of the fact that wines are our samples, we are going to talk about it and its making process [10]. Wine is a fermented grape juice. The vinification starts on the vineyard where the maturity of grapes is evaluated. To proceed with it, instrumental devices can be used, such as a refractometer, which determines the amount of sugar.

Grape seeds must be brown and sugar level enough (between 200 to 250 g L^{-1}) to contemplate that grapes are properly ripened. There are two different methods to carry out the picking: one of them is done by hand to preserve the integrity of the grapes, and the other one is by a machine which is convenient if your preference is to be fast and economical. At this moment, they are transported to the wine cellar once they have been efficiently packed.

Steps involving in the wine cellar:

1) **Pressing:** the juice of grapes is extracted from the pulp after being loaded in presses. This step is essential because the juice must be extracted in the gentlest way possible without removing the bitterness contained in the grape bunches. This process takes approximately 4 hours.
2) **Settling:** During this step, the "liquid" phase (grape juice) is separated from the "solid" phases (skin, remaining pulp, etc.) obtaining a clean juice. It can be left to decant at ambient temperature or within the cold for 24 to 48 hours to get two distinct phases. Then, the clear grape juice is filled into tanks the subsequent step.
3) **Alcoholic fermentation:** The sugar contained in the grape juice becomes alcohol by the action of yeasts. Aromas attributed to the sugar of the grape juice are consumed by yeast, which can then be released helping the most aromatic potential of wine. Different conditions are a crucial aspect within the development of microorganisms like temperature or acidity.
4) **Malolactic fermentation:** In this step, there is a second fermentation, where malic acid (two carboxylic groups) is transformed into lactic acid (one carboxylic group). Therefore, malolactic fermentation is the decarboxylation of L-malic into L-lactic, releasing CO_2 that it can be visualized as small bubbles in the wine. This is a form of natural deacidification made by lactic bacteria owing to a high amount of malic acid in grapes, which gives it a strong and harsh taste. It is necessary to find the right balance between

acidity, sugar and alcohol levels, among other parameters. Winemakers taste their wines continually during this course to make sure they find this balance, and to also know when it is the right moment to stop the malolactic fermentation or let it finish if they are looking for a dry wine.

5) **Racking:** is another step of settling, carried out after fermentation. Wine is separated from the lees, which are residues of dead yeasts. The "clear" wine is then relocated to another tank for aging. To make the wine more intense and complex, the fine lees are sometimes pumped with the clear wine. Coarse lees could cause undesired odours for instance rotten egg, cabbage, or onion. They will be transferred to the distillery to regain alcohol and acids for future use, for example in cosmetics.
6) **Aging:** The aim is to preserve the wine. Winemakers may either bottle the wine or let it age for several months in tanks or barrels to attain the right balance between the aromatic side of the grape, the soil on which it's grown and therefore the taste of consumers. Once this balance has been made, winemakers will wait patiently for the wine to age.

Biogenic amines in wines

Although traces of amines are already found in grapes, the alcoholic fermentation (AF) by yeasts, the malolactic fermentation by lactic bacteria and the wine aging in barrels have been recognized as the principal winemaking processes that participate with the formation of BAs [3].

As it has been explained previously, the production of BAs in wines is quite inevitable. Some biogenic amines might be produced in the grape or the musts (e.g., putrescine, cadaverine, and phenylethylamine) or may be formed by yeast during alcoholic fermentation (e.g., ethylamine and phenylethylamine), although quantitatively only very low concentrations get in these process (less than 3 mg L^{-1}).

Malolactic fermentation, which rises the microbiological stability of wine and the complexity of its aroma, is the main mechanism of biogenic amines formation.

In general, metabolic energy can be generated by lactic acid bacteria and and/or increase their acid resistance by using catabolic pathways that transform amino acids into amine-containing compounds such as BAs. Due to amino acids are metabolized by lactic acid bacteria, their concentrations decrease and biogenic amines concentration increase during MLF [11].

It has been determined over 15 amines in wines in a total concentration form a few ng L^{-1} to almost 50 mg L^{-1}, which it is depend on the wine's quality [12].

In wines, the foremost abundant biogenic amine is putrescine, which might be found in levels from 5 to 60 mg L^{-1} approximately, levels that correspond to ca. 50% of the total amine content in wines. Tyramine is commonly present at concentrations of 1-10 mg L^{-1}.

Other amines known as phenylethylamine and cadaverine generally occur at concentrations below 1 mg L^{-1}. Beyond the quantification of individual amines, a generic biogenic amine index (BAI) can be used as an indicator of food freshness or spoilage due to abundant amounts of several biogenic amines may be identified in food as a results of the poor quality of raw materials, contamination and inappropriate conditions during food processing and storage [3].

Red wines usually involve malolactic fermentation to reduce their higher acidity [13]. Red wines have clearly shown to have a higher biogenic amine content (especially of histamine, tyramine and putrescine) than rose and white wines, in which malolactic fermentation is not used or occurs in a lesser degree [4].

In winemaking, the Oenococcus, Lactobacillus and Pediococcus genus are the main lactic bacteria responsible for malolactic fermentation. *Oenococcus oeni* is the species best adapted to wine conditions such like low pH, high ethanol content and low nutrient content, and it is used as a starter in commercial winemaking to induce MLF in red and white wines [14]. The high concentrations of free amino acids combined with the presence of decarboxylase-positive microorganisms, and favourable environmental conditions that affect the growth of microorganisms help to the formation of BAs [13].

BAs in wine plays an important role. Not only the fact that the high levels of BAs can cause different human symptoms, but also, they are a sign of wine spoilage [1]. For this reason, it is vital to monitor BA levels in foods. High levels of biogenic amines are related to other wine spoilage components such as butyric acid, lactic acid, acetic acid, ethyl acetate and diethyl succinate, which is the reason why wines with higher levels of BAs normally also contain higher levels of volatile acids [15]. Nevertheless, traces BAs are determined as necessary compounds in the organism, due to their physiological functions in biological cells. BAs are biologically active molecules that participate in multiple cellular functions. Within others, monoamines are involved in neurotransmission and the regulation of blood pressure and body temperature. Polyamines are

essential for cellular proliferation and differentiation as they contribute in the synthesis of DNA, RNA and proteins [1,6].

Amino acid concentration in grapes can be influenced by fertilization treatments and, in wines, by winemaking treatments, such as time of maceration with skins, the addition of nutrients and racking protocols. Moreover, the concentration of biogenic amines in wines depends on the presence and the concentration of yeast and lactic acid bacteria with decarboxylating activity in addition to the precursors [8]. The sort and degree of ripeness of the grapes, the climate and soil of the viticulture area and the vinification techniques also could contribute to the content biogenic amines in wine [14]. In contrast, the use of different clarification substances, such as bentonite and polyvinylpolypyrrolidone (PVPP), can decrease biogenic amines content due to adsorption processes. Furthermore, in some studies, it has been noticed that sulphur dioxide, which is obtained in the reduction of lactic acid bacteria, avoids the biochemical formation of BAs [13].

In 2011, the "OIV code of good vitivinicultural practices" was published by the International Organization of Vine and Wine (OIV) to reduce the existence of biogenic amines in products related to wine. According to the OIV Guide, the conditions in vineyards and cellars leading to moderate presence of biogenic amines in wines are the type of soil and nitrogenous fertilization, the poor state of health of the grapes combined with mold, a high must pH and the development of certain yeasts during alcoholic fermentation [7].

Even though it has been shown that BAs can cause toxicologic effects, there is no legislation dealing with limits in the biogenic amine contents in wines, although some countries have established recommendations for histamine. The recommended upper limit is 8 mg L^{-1} in France, 3.5 mg L^{-1} in the Netherlands, 6 mg L^{-1} in Belgium and 2 mg L^{-1} in Germany. Switzerland has determined a limit of 10 mg L^{-1} as a tolerable value for histamine in wines [1,7-9,17].

3.2 THE IMPORTANCE OF THE DETERMINATION OF THESE AMINES

In these last years, the research for compounds that can be harmful for human health has increased with the help of new currents in food safety joined with the consumer demand for good quality and healthier products. As a result, innovative food analysis methods searching for trace compounds that can affect human health are getting more attention. Among other bioactive

compounds, biogenic amines are particularly important since they are frequently present in fermented and spoiled foods and may provoke health disorders in sensitive humans [4,18].

The fact that food contain BAs is an interesting research for two reasons. Firstly, food which contain high levels of biogenic amines can occasionally cause a health hazard to sensitive individuals because of their potential toxicity. Secondly, the presence of biogenic amines in some foods may be adequate as an indicator of undesired microbial activity, and alongside for the evaluation of good manufacturing practices (GMP) [19].

Under normal conditions, high levels of amines ingested as part of the diet are absorbed from the food and quickly detoxified in the organism by amine oxidases or through conjugation. Enzymes like monoaminooxidase (MAO), diaminooxidase (DAO) and polyaminooxidase (PAO) take part in the degradation of mono-, di-, and polyamines, respectively. However, when normal catabolic routes of amines are inhibited or the amount ingested is too large, some physiological effects can occur, for example migraine, headaches, nausea, hypo- or hypertension, cardiac palpitations and anaphylactic shock [6,11].

Most foodborne intoxication caused by biogenic amines is related to histamine. This amine can cause headaches, hypotension and digestive problems and for this reason, histamine is the most extensively studied amine. Some amines as tyramine, histamine and serotonin can affect, directly or indirectly, to the human vascular and nervous system. Aromatic amines (tyramine and phenylethylamine) are related to migraines and hypertension. Besides, tyramine, tryptamine and β-phenylethylamine show a vasoconstrictor action while others (histamine and serotonin) present a vasodilator effect. Moreover, tyramine and histamine also act as hormonal mediators in humans and animals. Psychoactive amines, like dopamine and serotonin, are neurotransmitters of the central nervous system. Furthermore, some biogenic amines can react with nitrite to generate carcinogenic nitrosamines. Other biogenic amines such as putrescine and cadaverine, although they do not pose a direct risk, might also play an important role in food because they can potentiate the toxicity of tyramine and histamine because of the interference with the enzymes which metabolize them. Apart from toxicological problems, diamines like putrescine and cadaverine have been identified to change negatively the taste properties of wines as these amines have been associated with dirty and rancid flavours [2-4,13,20].

It has been found that wines increase these adverse effects due to the ingestion of ethanol, which reduces or inhibits the activities of monoamine oxidase and diamine oxidase, enzymes responsible for metabolizing different kinds of amines in the liver and intestine, respectively.

Wantke, Manfred & Jarisch (1994) did a study with 28 patients that previously had presented symptoms of wine intolerance. After intake 125 mL of wine (approximately 50 mg of histamine), these patients have allergy problems; nevertheless, this level is perfectly tolerated by healthy people. Therefore, all the toxic effects of biogenic amines rely on individual sensitivity [21].

Table 2 shows some chemical properties of amines considered in this project (butylamine, dimethylamine, ethanolamine, ethylamine, hexylamine, isobutylamine, isopentylamine, and methylamine) such as molecular structure, molecular weight, pKa value [22].

Table 2
Some important properties of amines that have been considered in this project

Amine	Abbreviation	Molecular formula	Molecular weight (g/mol)	pKa value (at 25 °C)
Butylamine	But	$C_4H_{11}N$	73.14	10.80
Dimethylamine	DiMet	C_2H_7N	45.08	10.73
Ethanolamine	EtOH	C_2H_7NO	61.08	9.50
Ethylamine	Ety	C_2H_7N	45.08	10.87
Hexylamine	Hex	$C_6H_{15}N$	101.19	10.64
Isobutylamine	iBut	$C_4H_{11}N$	73.14	10.68
Isopentylamine	iPen	$C_5H_{13}N$	87.16	10.72
Methylamine	Met	CH_5N	31.05	10.66

3.3 DETERMINATION OF THE BIOGENIC AMINES

Shortly, amine levels will have to be regulated in most countries. For this reason, the establishment of accurate methods to determine these substances is especially interesting. Determination of biogenic amines is challenging because of their structures and the low concentrations that they can be found in a complex matrix such as wine. However, analytical methods for the quantification of amines are focused on satisfying the increasing demand for controls in clinical and food analysis [14,16,18].

Since most biogenic amines shown poor molecular features to be detected with spectroscopic techniques (e.g. low UV–vis absorption or native fluorescence), the incorporation of a derivatization step to the procedure is highly recommendable to increase the selectivity and sensitivity of detection. Among other reasons to use a derivatization, first, amine derivatives can easily be separated by reversed-phase mode using standard C18 columns. On the contrary, underivatized amines should be separated by cation-exchange due to their positive charge and this method resulted in poorer separation performance. Second, the flexibility of working with derivatives seems to be superior. Third, with mass spectrometry (MS) detection the sensitivity is, generally, higher using amine derivatives than the underivatized counterparts.

Beyond these important advantages, disadvantages associated with the derivatization process must be mentioned. The most important of them is the presence of additional components corresponding to reagent excess and reaction amine products may generate poorly resolved peaks and interferences [6,16].

Derivatization reactions take place via an amino group. Some of the most popular agents appropriate for spectrophotometric and fluorometric detection are dansyl chloride (dansyl-Cl), o-phthaldialdehyde (OPA) and benzoyl chloride. As an alternative to these commonly derivatization reagents, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatizing reagent has been described to be used in the pre-column derivatization of primary and secondary amino acids and biogenic amines. Moreover, 1,2-naphthoquinone-4-sulfonate (NQS) has been utilized for the pre- and post-column derivatization of amino acids in high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). More recently, it has been adapted the derivatization reaction into a primary and secondary amino group to carry out the quantification of biogenic amines in wines [3,16,18,20].

In this project, a highly sensitive method to determine biogenic amines in wine samples by pre-column derivatization reaction with NQS is described. This reagent reacts with primary, secondary amino groups and aromatic primary amino groups to form amino derivates (see Fig. 1).

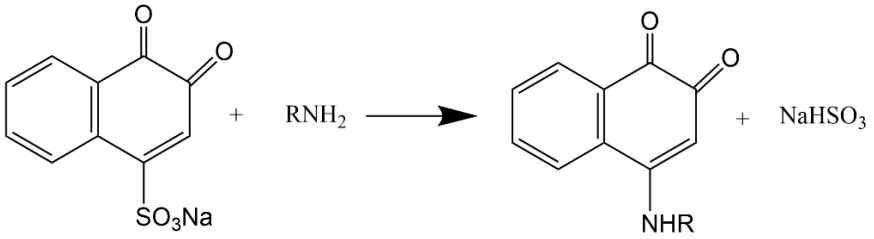

Fig. 1: Scheme of the derivatization reaction of a primary amine with NQS

After the derivatization step, a liquid-liquid extraction in basic medium with organic solvents such as chloroform or diethyl ether has been also applied to achieve preconcentration and clean-up. Alternatively, extracts can be purified by solid-phase extraction (SPE) with C18 or ion-exchange cartridges [3].

The extract purification is essential to avoid interferences from amino acids on the derivatization step seeing that they remain unextracted. In the case of organic extracts, they can be evaporated to dryness and then redissolved in solvents that compose the mobile phase to be ready for the injection into the chromatograph.

In pre-column derivatization modes, components of the wine matrix, other reaction products and reagent excess may produce interfering peaks that should be eliminated. For this purpose, a clean-up step after the reaction may be required to obtain cleaner chromatograms, especially with UV–vis and fluorescence detection.

Sample pre-treatment procedures to be used in the determination of amines in wines may be simpler than those necessary for other food matrices. The pre-treatment is often simplified to filtration. In some cases, the addition of polyvinylpyrrolidone particles to the wine samples can be a useful way of removing polyphenols [3].

High-performance liquid chromatography is the main technique to determine biogenic amines due to its high resolution, sensitivity, great versatility and simple sample treatment. Biogenic amines are typically determined by HPLC using spectrophotometric or fluorometric detection.

The hyphenation of HPLC and mass spectrometry has also been described to increase the performance of the amine analysis. In this case, MS technique is compatible with the detection of underivatized amines even though most of the methods reported in the literature include a derivatization step to simplify the separation and to enhance the sensitivity. Besides, the study of mass spectra procures relevant information about the type of derivatives formed, known as mono and di-derivatives depending on the characteristics of the amines (e.g., monoamines, polyamines and phenylamines). Therefore, determination with MS provide both qualitative and quantitative information about amines [3-5,9,14,18,20,23-27].

Gas chromatography and, especially, capillary electrophoresis have also been proposed in some applications as an alternative to HPLC. Apart from separation methods, the development of simple, rapid and small devices based on immunoassays and enzymatic reactions have received more attention. In addition, commercial kits have been developed, for instance, for the rapid determination of histamine in wine and other food samples [9].

To illustrate what has been previously described, the following graphics have been carried out. The first one, it's about samples and the other one it's about derivatization reagents (see fig 1 and 2). 135 papers have been used to elaborate this graphics searching for *biogenic amines* in the period 2011-2020 on Sci-Finder database.

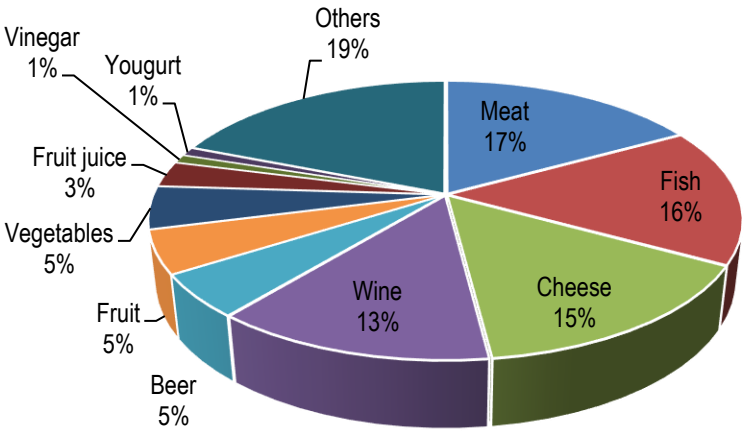

Fig. 2: Scheme of the main food samples analysed on last ten years dealing with the determination of biogenic amines

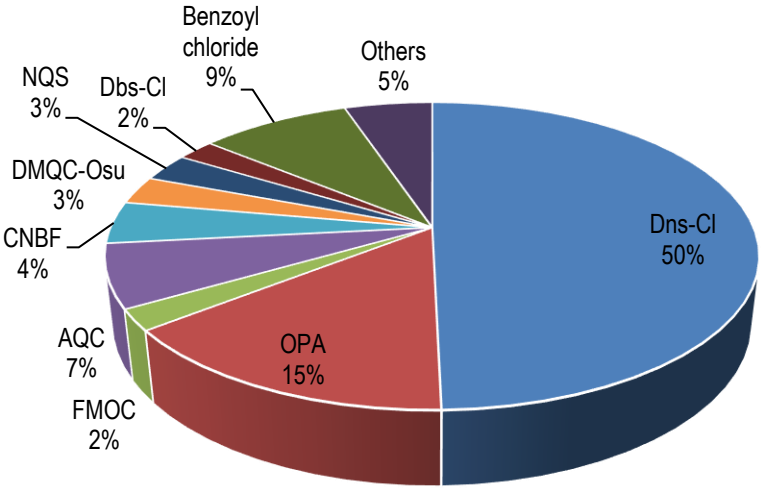

Fig 3: Scheme of the main reagents used on last ten years for the derivatization of the biogenic amines

As the graphics show, the most analyzed samples correspond to meat, fish, or cheese samples. As for the most common reagents to carry out the derivatization reaction are by far the dansyl chloride, and to a lesser extent OPA or benzoyl chloride.

4. OBJECTIVES

The main objective of this project is to establish an effective and selective method for determining biogenic amines in wine and cava samples. In order to achieve this objective, these steps have been followed:

- The optimization of the pre-column derivatization reaction with the 1,2-naphthoquinone-4-sulfonate reagent, that will be carried out changing some factors such as reaction time and temperature to get the best response.
- The optimization of separation of the different biogenic amines based on reversed-phase liquid chromatography. In this section, different columns, different solvents, different elution gradients and different temperatures will be assayed for the purpose of achieving the best possible chromatographic resolution and efficiency.

5. EXPERIMENTAL SECTION

5.1 REAGENTS, STANDARDS AND SOLVENTS:

Reagents and solvents used for the derivatization reaction were as follows:

- Hydrochloric acid (37% (w/w), Sigma-Aldrich, Steinheim, Germany)
- Sodium hydroxide (Merck, Darmstadt, Germany)
- Sodium tetraborate decahydrate (analytical grade, Carlo Erba, Milan, Italy)
- Sodium 1,2-naphthoquinone-4-sulfonate (NQS) (Carlo Erba, Milan, Italy)
- Chloroform (analytical grade, Merck, Darmstadt, Germany)

The reagent solution consisted of 0.07 M NQS with 0.1 M HCl. The pH buffer solution consisted of a 0.125 M $Na_2B_4O_7 \cdot 10$ H2O and 0.1 M NaOH.

The biogenic amines standards were prepared from the following sources:

- Hexylamine hydrochloride (>98.0%, TCI, Tokyo, Japan)
- Methylamine hydrochloride (>98.0%, TCI, Tokyo, Japan)
- Ethylamine hydrochloride (>98.0%, TCI, Tokyo, Japan)
- Butylamine hydrochloride (>98.0%, TCI, Tokyo, Japan)
- 2-Aminoethanol hydrochloride (>98.0%, TCI, Tokyo, Japan)
- Isopentylamine hydrochloride (>98.0%, TCI, Tokyo, Japan)
- Isobutylamine hydrochloride (>99.0%, TCI, Tokyo, Japan)
- Dimethylamine hydrochloride (>98%, Alfa Aesar, Massachusetts, United States)

These solid standards were dissolved in milli-Q water at a concentration of 1000 mg L^{-1}. A dilution was then made to achieve a concentration of approximately 50 mg L^{-1} of the different amines as the working solutions. It is interesting to have standards solutions of this concentration to be able to inject around 16 mg L^{-1}, a concentration that allows us to obtain suitable peaks. All standards solutions prepared were stored in amber glass vials in the fridge to minimize the decomposition process of the standards throughout time.

These other solvents were used in HPLC to prepare the mobile phase:

- Formic acid (≥95% (w/w), Sigma-Aldrich, Steinheim, Germany)

- Methanol (Reag.Ph. Eur.) for UHPLC Supergradient ACS (PanReac, Barcelona, Spain)
- Acetonitrile (Reag.Ph. Eur.) for UHPLC Supergradient ACS (PanReac, Barcelona, Spain)
- Milli-Q water (Millipore, Milford, MA, USA)

5.2 SAMPLES

Due to the global pandemic caused by the SARS-CoV-2 virus, a disease commonly known as COVID-19, none of the wine and cava samples prepared for this project could be analysed.

5.3 DERIVATIZATION AND LIQUID-LIQUID EXTRACTION PROCESS

Due to the poor absorptivity of these biogenic amines in the UV-vis range, a pre-column derivatization reaction was performed to enhance the sensitivity. This reaction was carried out in a reaction vial mixing 250 µL biogenic amine aqueous standard solution, 250 µL NQS solution and 250 µL borax solution (buffer). The reaction took place at a pH 9.2 and a temperature of 60ºC in a thermostatic water bath (Tectron 473-100, J.P. Selecta, Barcelona, Spain). Next 1mL of chloroform was added to the reaction vial and the solution was shaken (Vortex 3 (IKA) with an accessory VG 3.31 Test tube attachment) in order to get two phases. When the two phases were separated, 800 µL of the organic phase was withdrawn and the solvent was evaporated to dryness using a nitrogen current. Once the is the dry residue was obtained, it was redissolved in 400 µL of acetonitrile/water (20:80, v/v). At this moment, the extract was ready to be injected.

5.4 INSTRUMENTATION

The chromatographic systems used is an Agilent 1100 Series HPLC instrument equipped with a G1312A binary pump, a G1379A degasser, a G1315B diode-array detector provided with a 13 µL flow cell, a G1321A Fluorescence Detector, and an Agilent Chemstation for data acquisition and analysis (Rev. A 10.02), all of them from Agilent Technologies (Waldbronn, Germany). The thermostatted autosampler consisted of two modules, the sampler (G1367A) and the thermostat (G1330A).

5.5 CHROMATOGRAPHIC CONDITIONS

Chromatographic analyses during the work were carried out using a C8 reversed-phase Xterra column (Waters Corporation, Milford, Massachusetts, United States) with length 100 mm, internal diameter 2.1 mm, pore size 125 Å and particle size 3.5 µm. The flow rate used was 0.4 mL/min and the injection volume was 5 µL.

In order to achieve a high chromatographic separation of these biogenic amines, the components of the mobile phase were acetonitrile (solvent B) and water (solvent A) using the elution gradient that is following described in the table 3:

Table 3
Elution gradient used for chromatographic studies

Time (min)	Solvent A (%)	Solvent B (%)
0	97	3
20	20	80
22	20	80
22.2	97	3
27	97	3

The absorbance was measured in the range 190 nm and 700 nm. Chromatograms of derivatives were recorded at 480 nm.

6. DISCUSSION OF RESULTS

6.1 STUDY OF THE DERIVATITZATION REACTION

6.1.1 Preliminary studies

Once all the standard, buffer and reagents were prepared, the derivatization reaction is performed as follows. 250 µL of the standard amine solution, 250 µL of the buffer solution and 250 µL of the derivatization reagent were mixed to react. The solution was heated in the water bath for 20 minutes at 80ºC for each amine. Next, derivatives were extracted with 1 mL of chloroform. 400 µL of the organic phase were withdrawn, the solvent was evaporated until dryness with a nitrogen current and the solid residue was redissolved in 400 µL of MeOH/Water (20:80).

The obtained extracts were injected into the chromatograph with an elution gradient of Water MeOH/Water (97:3) to MeOH/Water (5:95) in 20 min (see experimental section). The aqueous solutions after the derivatization reaction, without extraction, was also injected in order to compare the chromatograms and evaluate the necessity of the liquid-liquid extraction. Two chromatograms recorded at 480 nm that correspond to the injection of the butylamine derivative solution are included in Fig. 3.

The retention times of the derivates with the extraction and without the extraction were very similar. The peak corresponding to the butylamine derivate appeared at 18. min in the two cases. The fundamental difference between both chromatograms is that in the extracted sample, the chromatogram appears cleaner and simpler because the possible peaks corresponding to the excess NQS or other products were eliminated.

These chromatograms show the importance of a liquid-liquid extraction, even more so in the samples where the presence of different peaks from different biogenic amines can make the peak assignation more complicated. Thus, the lower the number of different peaks, the easier it is to assign them. Therefore, from this point on, all the data given correspond to the injection into the chromatograph of the corresponding amine derivative, always applying a liquid-liquid extraction process after the derivatization reaction.

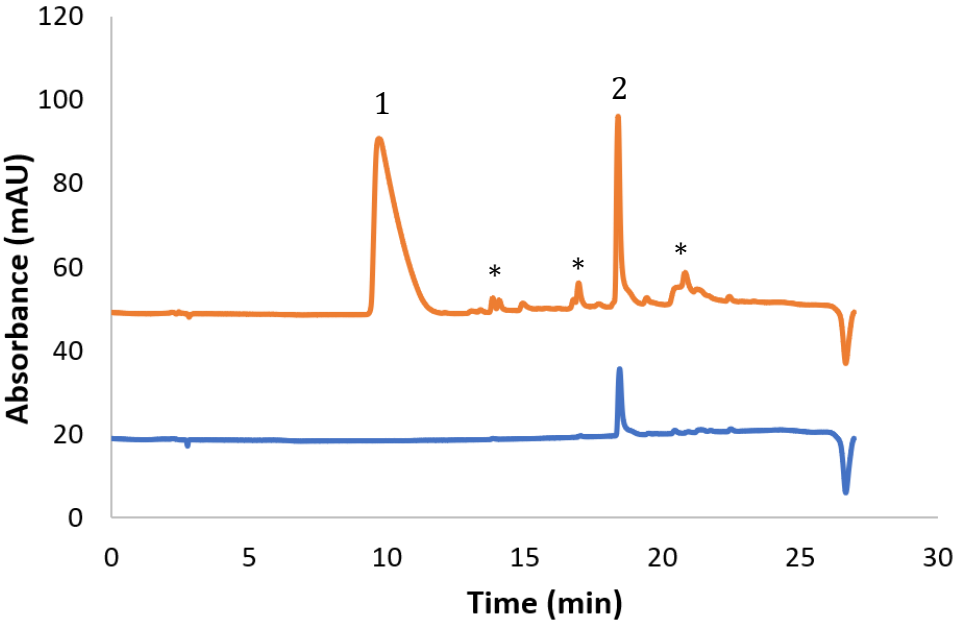

Fig. 3: Chromatogram corresponding to butylamine derivate. Assignation: ● with a liquid-liquid extraction process, ● without a liquid-liquid extraction process. 1= NQS excess, 2= butylamine derivate, *= side products

6.1.2 Optimization of the derivatization reaction

In order to optimize the derivatization reaction, an experimental design approach was done, studying 2 factors (temperature and time of the reaction) at 3 and 4 levels respectively. Fig. 4 show schematically this factorial design.

To make this optimization quicker and easier, butylamine was chosen as the representative standard, displaying intermediate retention time among the 8 studied amines. Peak areas of the obtained signals for each of the 12 combinations of time-temperature were used as the analytical data and the following graphics were drawn in order to evaluate which the optimum conditions leading to the higher response, as well as inspecting whether degradation products occurred (leading to more than one peak corresponding to an amine).

Fig. 5 shows the obtained area for each combination of time-temperature. The higher response was obtained with 20 min of reaction time at 60ºC.

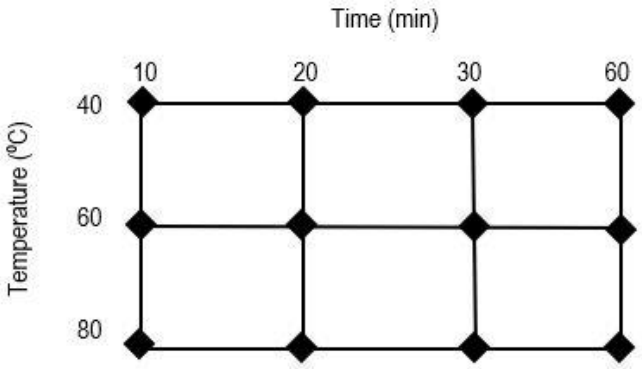

Fig. 4. Scheme of the experimental design applied to study the influence of temperature and time on the reaction.

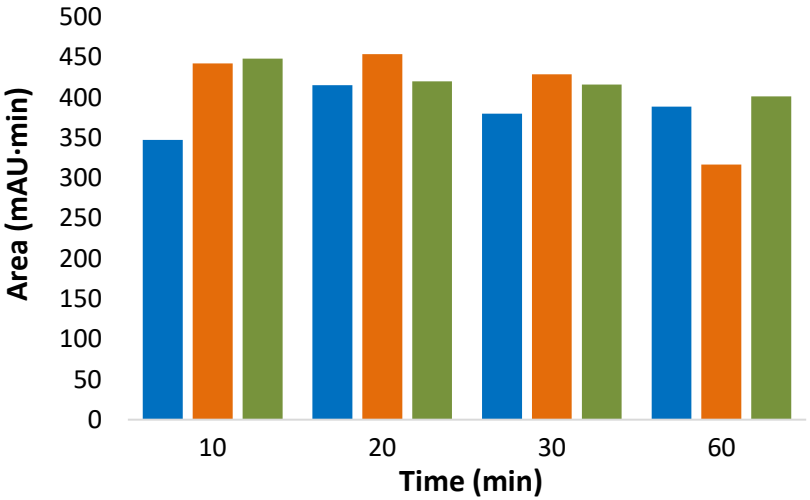

Fig. 5: Study of the influence of temperature and time on the derivatization reaction of butylamine. Assignation: 40ºC, 60ºC, 80ºC.

These results could be also plotted as a 3D response surface (representation of the response of an experiment in front of the different levels of the factors) obtained for the butylamine (see fig. 6). However, the highest responses corresponded to a temperature between 60 and 70ºC and a reaction time of 20 to 30 min.

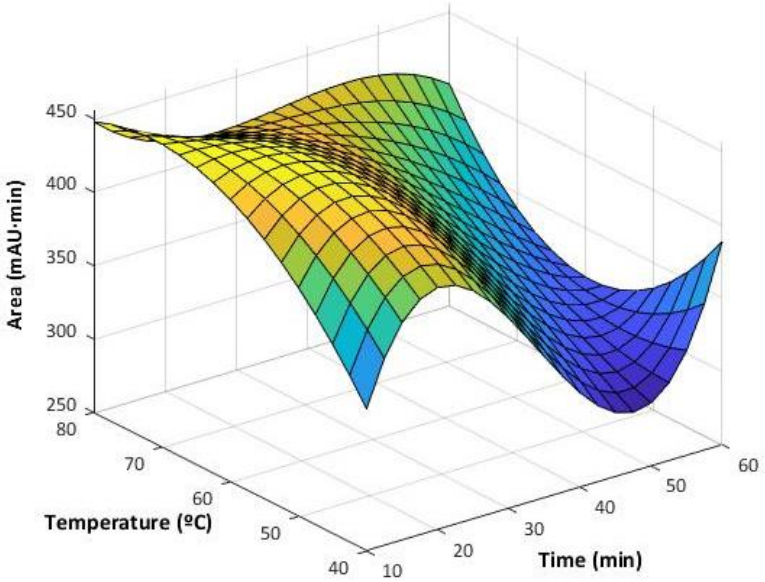

Fig. 6: Response surface of the peak area of the butylamine derivative as a function of the time and temperature.

In order to study more in-depth whether these reaction factors of the derivatization could be significant, an ANOVA (variance analysis) statistical study was performed. The following data about the $F_{calculated}$ and the $F_{tabulated}$ were obtained for each of the two studied factors and are described in the table 4:

Table 4
Statistical study of two factors

	F_{cal}	F_{tab}
Temperature	0.994	5.143
Time	1.237	4.575

In both cases, Ftab > Fcal, which means that, with a 95% confidence level, these factors are not significant for the optimization of the derivatization reaction. Anyway, for practical purposes, a combination of a moderate temperature and short reaction time was finally recommended to carry out the derivatization.

6.2 OPTIMIZATION OF THE CHROMATOGRAPHIC SEPARATION

6.2.1 Preliminary studies

The first experiments concerning the optimization of the separation were some gradient testing. With the initial column (Phenomenex, Kinetex reversed-phase -C18, see table 6 to more information), a mobile phase of MeOH/Water with different initial percentages of MeOH were studied. The elution gradients obtained are depicted in Fig. 7.

The chromatographic behaviour of the analytes was compared with other biogenic amines such as histamine, tryptamine and others which, usually, are found in wine samples. These additional amines were chosen because they were spread along the whole chromatogram, being histamine the one with fastest elution (most polar derivative) and the hexylamine the one eluting last. The retention times obtained, expressed in min, for each amine in each elution gradient are shown in the following table 5.

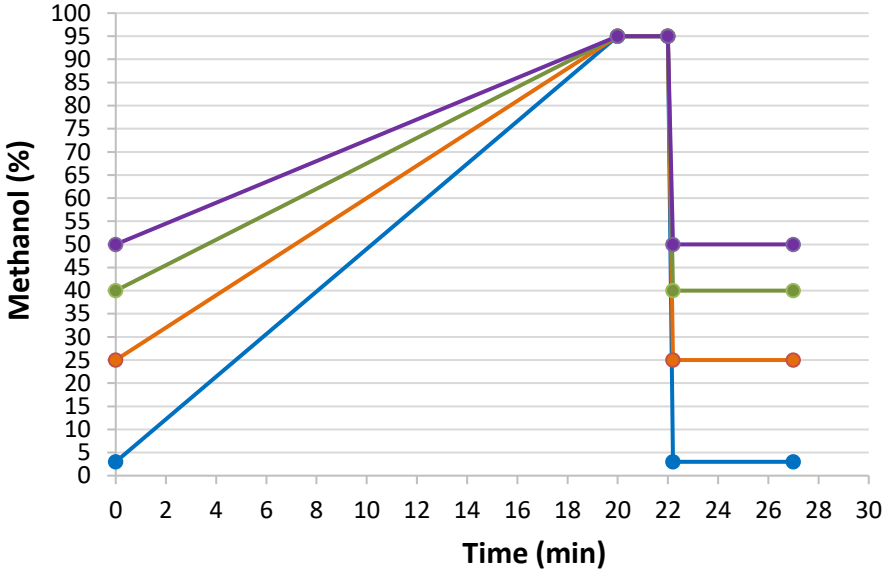

Fig. 7: Elution gradients designed with different percentages of methanol initial: 3% methanol (gradient 1), 25% methanol (gradient 2), 40% methanol (gradient 3), 50% methanol (gradient 4).

Table 5
Retention time of each amine in each elution gradient (see Fig. 7)

	Gradient 1	Gradient 2	Gradient 3	Gradient 4
Histamine	11.39	6.09	3.10	2.64
Methylamine	14.76	10.01	5.57	3.95
Tyramine	15.56	13.82	9.79	6.19
Isobutylamine	18.17	14.85	11.04	7.69
Tryptamine	18.81	16.41	13.17	9.76
Hexylamine	20.84	18.59	15.96	13.19

It is observed that the retention time decreases when the amount of initial organic solvent increases. This is due to the fact that a relatively non-polar stationary phase is used, thus, when

the methanol percentage increases the eluotropic strength also increases. As a result, the mobile phase polarity decreases, making faster the elution of all the compounds. Moreover, because the compounds are structurally similar, the changes in eluotropic strength does not change the order of elution, and, in consequence, the increase in the percentage of methanol only makes the compounds to elute more quickly.

The initial percentage methanol of 40% has been chosen as the optimal one because with lower percentages (3% and 25%) there is too much time between the hold-up time and the retention time of the first compound; in the case of 50%, the peak of the first compound overlaps the hold-up area.

6.2.2 Column election

In order to maximize the chromatographic efficiency, a series of different columns were tested to choose the one giving the narrowest and most symmetric peaks. 6 different columns were tested. The characteristics of these columns are described in Table 6.

Table 6
Characteristic of the columns tested

Column nº	Column	Supplier	Dp (µm)	Por. size (Å)	Di (mm)	L(mm)	Stationary phase	Applications
1	Kinetex	Phenomenex	2.6	100	4.6	100	-C18	Hydrophobic compounds
2	Luna	Phenomenex	3.0	100	4.6	100	Silanol Si-OH	Polar compounds
3	Tracer Excel CN	Teknokroma	3.0	120	2.1	100	-CN	Polar compounds
4	Tracer Excel P	Teknokroma	3.0	120	2.1	100	-Ph	Aromatic compounds
5	Xterra MS	Waters	3.5	125	2.1	100	-C18	Hydrophobic compounds
6	**Xterra RP**	**Waters**	**3.5**	**125**	**2.1**	**100**	**-C8**	**hydrophobic compounds**

Column parameters: Dp, particle diameter; **por. size**, porous size; **Di**, internal diameter; **L**, length

In order to evaluate these columns, the tyramine was chosen as the model biogenic amine to be assayed. The specific chromatographic conditions of each chromatogram and the obtained results are collected in the Table 7:

Table 7
Detail of the chromatographic conditions and parameters obtained in each column tested

Column nº	Flow rate (ml/min)	Hold-up time (min)	Retention time (min)	Peak width at half height (min)	Peak symmetry	Plate number	Plate height (mm/plate)
1	0.4	2.49	9.79	0.116	0.507	39.400	0.0025
2	0.8	1.85	2.25	0.073	0.542	5.200	0.0190
3	0.3	1.09	5.16	0.145	0.509	7.000	0.0143
4	0.3	1.08	6.08	0.098	0.735	21.300	0.0047
5	0.3	0.98	5.80	0.144	0.819	8.900	0.0111
6	0.4	0.74	3.57	0.100	0.829	7.000	0.0142

The chosen column to perform the chromatographic determination is the number six, which corresponds to reversed-phase mode with a bonded stationary phase of alkyl chains of 8 carbons (C8).

Once the column is chosen, the 6 biogenic amines used to optimize the gradient were injected again to see the differences in the retention time, comparing with the initial column (num. 1) and the chosen column (num. 6) (see Table 8). The chromatographic conditions are a MeOH/Water mobile phase with a gradient of 60:40 MeOH/Water until 5:95 MeOH/Water in 20 min. The following table 8 shown the retention time, expressed in min, of the 6 amines used.

Table 8
Comparison of the retention times obtained with the initial column, nº1, and the chosen column, nº6

	Column 1	Column 6
hold-up time	2.50	0.74
Histamine	3.10	0.77
Methylamine	5.57	1.36
Tyramine	9.79	3.59
Isobutylamine	11.04	5.20
Tryptamine	13.17	7.16
Hexylamine	15.96	8.16

An important difference is observed in the retention time of the two columns. Column nº1, with a stationary phase -C18, has a longer retention time than column nº6, with a stationary phase C8. This fact indicates that the analytes have a low polarity, as they interact more with a stationary phase -C18, less-polar, than with a stationary phase -C8, more polar, and therefore are more retained. However, column nº 6 was chosen to carry out the subsequent analyses as it presents a good balance of efficiency-symmetry of the peak.

Next, the elution gradient was redefined due to the observation that reaching 95% methanol was not necessary, because all the compounds were already eluted before 9 min of elution. A final percentage of 75% MeOH was chosen, which is the percentage in which, approximately, the last derivative of the list eluted. In order to better refine this gradient, a two-factor complete factorial experimental design was performed, being the initial percentage of methanol and ramp time of the gradient the factors under study at 3 levels each. The next drawing, Fig.8, shows the combinations done:

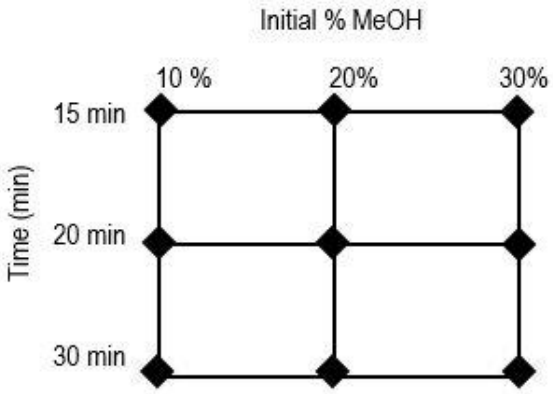

Fig. 8. Two-factor at three-level factorial design for the simultaneous study of the influence of the initial MeOH percentage and the gradient time on the separation of the biogenic amines studied.

This experimental design was performed with the former 6 analytes used but, for the sake of simplicity, only the data of butylamine derivate will be given. The time corresponds to the ramp time of the gradient, while the % refers to the initial percentage of methanol. The results are shown in the Table 9.

Table 9
Chromatographic parameters obtained in the experimental design applied to study of the influence of the initial MeOH percentage and the gradient time on the separation of the biogenic amines studied.

Time (min)	% MeOH	Retention time (min)	Area (mAU·min)	Peak width at half height (min)	Peak symmetry
15	30	6.31	43.348	0.0953	0.924
15	20	8.97	43.040	0.0851	0.962
15	10	11.08	42.851	0.0724	0.961
20	30	6.48	41.985	0.1095	0.924
20	20	9.80	42.324	0.1005	0.957
20	10	12.52	42.162	0.0853	0.983
30	30	6.74	41.317	0.1223	0.917
30	20	10.99	41.351	0.1299	0.968
30	10	15.11	41.353	0.1178	0.982

In the table 9, it is observed how the retention times of butylamine varied with initial percentage of methanol and the ramp time of the gradient. When the initial percentage of methanol decreased, the retention time increased because the eluotropic strength of the mobile phase was lower. In the case of the ramp time of the gradient, the higher the ramp time, the higher the retention time, because the gradient was not so sharp, and the elution of compounds was more progressive. Furthermore, it was observed that the narrowest peaks were obtained in a 15 min ramp and an initial percentage of methanol of 10%. Since this gradient is the one giving the best efficiency, which will also affect the resolution and symmetry of peaks, it was chosen as the optimal one to do the further chromatographic analysis.

6.2.3 Other factors: solvent, temperature

In order to finish to optimize the separation, other factors that may affect the chromatographic resolution were studied. First, the influence of the organic solvent was studied. Until now, mobile phases were composed of water and methanol in different proportions. A test to check whether the resolution could be improved utilizing a mobile phase of Acetonitrile/Water in an elution gradient from 97:3 to 5:95 of Acetonitrile/Water in 20 min was investigated.

After doing this experiment, it has been possible to check that there is a big difference between the retention time of the last compound (12.6 min) and the chromatographic time (27 min), fact that implies an unnecessary waste of acetonitrile. To correct it, new conditions featuring a gradient with an initial percentage of 3% acetonitrile, arriving to 80% as the final percentage in three different ramps of 15, 20 and 30 minutes were studied. In the following tables 10-12, these elution gradients are described in detail.

Table 10
Gradient A: Ramp of 30 min

Time (min)	Acetonitrile (%)
0	3
30	80
32	80
32.2	3
37	3

Table 11
Gradient B: Ramp of 20 min

Time (min)	Acetonitrile (%)
0	3
20	80
22	80
22.2	3
27	3

Table 12
Gradient C: Ramp of 15 min

Time (min)	Acetonitrile (%)
0	3
15	80
17	80
17.2	3
22	3

The next table 13 contain the obtained chromatographic parameters for the three described gradients including the initial gradient used (an initial percentage of 3% acetonitrile, arriving to 95% as the final percentage in a ramp of 20 min). The data correspond to the butylamine derivative.

Table 13
Comparison of the chromatographic parameters gotten for the butylamine derivative using four different elution gradients

	Retention time (min)	Area (mAU·min)	Peak width at half height (min)	Peak symmetry
Initial gradient	10.697	45.61	0.0483	0.911
Gradient A	14.078	49.25	0.0703	1.016
Gradient B	9.824	45.66	0.0455	0.915
Gradient C	11.304	44.961	0.0536	0.944

From this acetonitrile tests, the gradient with an initial methanol percentage of 3% until 80% in a 20 minutes ramp is chosen, because it is the one giving the narrower peaks and the lower retention time, fact that makes the chromatographic time decrease, increasing the velocity of the analysis. Moreover, the fact that narrower peaks are obtained also affects the resolution, because the narrower peaks may be better separated.

Following, a comparison between the two mobile phases used until now (Acetonitrile/Water and Methanol/Water) is done to find the best organic modifier in terms of the best compromise between resolution and chromatographic performance. The chromatographic parameters obtained are shown in the next table 14, having used in both cases an elution gradient of a percentage of the organic modifier from 3% until a 95% in 20 min. Again, the data correspond to the butylamine derivative.

Table 14
Comparison of the chromatographic parameters obtained for the butylamine derivative using two different mobile phases

	Retention time (min)	Area (mAU·min)	Peak width at half height (min)	Peak symmetry
Acetonitrile/Water	10.697	145.61	0.0483	0.911
MeOH/Water	18.441	135.58	0.0895	0.712

The most important difference was the peak width, being the peak width corresponding to the mobile phase made of Acetonitrile/Water almost half of the peak width corresponding to the one made of Methanol/Water. This fact implies obtaining of narrower peaks and, in consequence, higher effectivity and resolution. In addition, the peak symmetry of the mobile phase of Acetonitrile/Water is also better and, thus, acetonitrile was selected as the organic solvent.

Also, it has been tried to improve the chromatographic resolution increasing the temperature. The peaks were similar to those without an increase in temperature and the only parameter that had changed was the retention time. Therefore, temperature does not contribute to the increase in resolution.

10. CONCLUSIONS

During this project, different studies have been carried out in order to establish a method to determine biogenic amines in wines and cava samples. Due to the special circumstances of the Covid-19 episode, it should be pointed out that the experimental work is unfinished. Some optimization and validation studies are still pending and the final application to wine and cava analysis has not been implemented. From the experiments carried out until 10th March, the following conclusions have been drawn:

- Various tests have been carried out with different time-temperature combinations to find the best conditions for the derivatization reaction. Following these analyses, it has been observed that all combinations are statistically valid, with 95% confidence level. Even then, it has been decided to carry out the derivatization reaction at a moderate temperature and short reaction time (60ºC, 20 min).
- A comparison was made between one chromatogram of the reaction mixture and another after the liquid-liquid extraction process. As expected, the chromatogram of the reaction mixture shows peaks that do not correspond to biogenic amine derivatives, such as excess NQS, possible hydrolysis products that may be formed in the aqueous phase, etc. Therefore, it is important to emphasize the importance of the liquid extraction process to facilitate the determination.
- It has been determined that a mobile phase consisting of Acetonitrile/Water (instead of Methanol/Water) provides better chromatographic efficiency and resolution as narrower peaks are obtained.
- The C8 reversed-phase Xterra column (Waters Corporation) has been selected with a mobile phase Acetonitrile/Water with an elution gradient with an initial percentage of 3% acetonitrile, arriving to 80% as the final percentage in 20 min.
- Finally, the separation of all amines should be studied in order to see if the resolution between them is adequate. On the contrary, the gradient of the Acetonitrile/Water mobile phase must be finished by varying the eluotropic strength, and consequently, the separation of the analytes.
- Once the optimal chromatographic conditions have been decided, a study must be carried out on the stability of the derivatives over time and the influence of pH.

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12. ACRONYMS

BA: Biogenic Amine

CE: Capillary Electrophoresis

GMP: Good Manufacturing Practices

HPLC: High-Performance Liquid Chromatography

MLF: Malolactic Fermentation

MS: Mass Spectrometry

NQS: 1,2-naphthoquinone-4-sulfonate

OIV: International Organization of Vine and Wine

PVPP: bentonite polyvinylpolypyrrolidone

SPE: Solid-Phase Extraction