TD

Ethyl Carbamate in Fortified Wines
Mitigation and control

DOCTORAL THESIS

João Micael da Silva Leça

DOCTORATE IN CHEMISTRY

UNIVERSIDADE da MADEIRA
A Nossa Universidade
www.uma.pt

February | 2021

Cofinanciado por:

MADEIRA 14-20
REGIÃO AUTÓNOMA DA MADEIRA
PORTUGAL 2020
UNIÃO EUROPEIA
Fundo Social Europeu
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ORIENTATION
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Financial Support:

arditi
agência regional para o
desenvolvimento da investigação
tecnologia e inovação

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Dedicado à minha mãe, à minha avó e à Nicole

AGRADECIMENTOS

Quero começar por agradecer à minha família e em especial à minha mãe, Teresa Silva, à minha avó, Teresa Teixeira e à minha madrinha, Sandra Nunes, por todo o amor e apoio que sempre me deram ao longo da vida.

À Nicole por todo o amor, pelo apoio incondicional na vida e no trabalho e por todos os momentos bons que partilhámos. Amo-te muito.

Um enorme obrigado ao Doutor José Manuel Borges por toda a dedicação e apoio, um ser humano e um profissional de muito valor.

E porque a vida não tem valor sem ser partilhada, um grande obrigado aos meus amigos mais próximos: João Mendes, Vítor Correia, Catarina Lume, Vânia Silva, António Miguel Ornelas, Tiago Mendonça, Ayrton Andrade, Noélia Mendonça, Pedro Leme, Nélio Ponte, João Silva, Isabel Caetano, Hugo Leme, Marco Franco, Nuno Pestana, Paulo Silva, Luís Freitas, Miguel Nunes, Fernanda Garcês, David Nunes, Zé Manel e Beatriz Dias.

Por outro lado, quero agradecer às pessoas que de forma mais direta contribuíram para a conclusão desta etapa:

Quero expressar a minha gratidão ao meu orientador principal, Professor Doutor José Carlos Marques, por me ter dado a oportunidade de trabalhar sob a sua orientação e com isso engrandecer a minha formação pessoal e profissional.

Queria também estender o meu agradecimento ao meu coorientador Professor Doutor José Luis Vilchez Quero, que me recebeu em Granada (Espanha) e me fez sentir em casa. Uma pessoa excecional que me deu todo o apoio, suporte e condições para desenvolver o meu trabalho, um grande obrigado a toda a sua equipa e família.

Queria agradecer à Doutora Vanda Nulita Pereira e à Doutora Ana Cristina Pereira, pela partilha de conhecimento e pelo apoio constante no trabalho a ser desenvolvido, desde o início até ao fim do doutoramento. Um especial obrigado à Doutora Vanda Nulita Pereira que me ajudou a preparar este projeto, que foi submetido com a mesma como coorientadora e, apesar de não o poder ter sido de forma oficial, acompanhou e orientou o trabalho de forma muito

dedicada. Um obrigado especial também à Doutora Ana Cristina Pereira pelo apoio no tratamento estatístico de resultados necessário para o desenvolvimento do presente estudo.

Aos meus colegas do Laboratório de Qualidade e Segurança Alimentar (QSALab): Andreia Fátima Miranda, Ana Isabel Freitas, Carolina Leal Vieira, Maria João Carvalho, Anísia Martins, Dayana Aguiar, Marcelo Gaspar, Gabriel Pinto, Rita Fialho e Simone Cavenati, pela amizade, pela partilha constante de conhecimento e pelo companheirismo.

Queria agradecer em especial ao Doutor Alberto Zafra Gómez e ao Doutor Samuel Cantarero Malagón que, durante a estadia em Granada, me apoiaram no desenvolvimento do trabalho prático. Um enorme obrigado também à Doutora Laura Martín Pozo e à Yolanda Gálvez Ontiveros pela amizade e companheirismo.

Quero agradecer o suporte financeiro através de uma bolsa de doutoramento da ARDITI (Agência Regional para o Desenvolvimento da Investigação, Tecnologia e Inovação), projeto M1420-09-5369-FSE-000001. Esta investigação foi financiada por fundos FEDER/OE através dos projetos AROMA (CENTRO-01-0145-FEDER-031568, PTDC/EEI-EEE/31568/2017) e IMPACT III (M1420-01-0247-FEDER-000020).

Este trabalho foi desenvolvido dentro do âmbito dos projetos I3N UIDB/50025/2020 e UIDP/50025/2020, financiado com fundos nacionais através da Fundação para a Ciência e a Tecnologia (FCT)/MEC. Um especial agradecimento ao Professor Doutor João Lemos Pinto e à Doutora Marta Ferreira do I3N Aveiro.

À Faculdade de Ciências Exatas e da Engenharia da Universidade da Madeira e ao Departamento de Química Analítica da Universidade de Granada que me acolheram e deram todas as condições para desenvolver o presente trabalho.

Às empresas de vinho Madeira pelo fornecimento de amostras e um especial obrigado à Madeira Wine Company e ao Engenheiro Francisco Albuquerque.

RESUMO

O carbamato de etilo (CE) é um composto que ocorre naturalmente em bebidas alcoólicas. A sua toxicidade aguda é baixa, mas o consumo continuado levanta algumas preocupações, razão pela qual a indústria de bebidas alcoólicas se comprometeu a reduzir os níveis de CE tanto quanto possível. Este estudo pretende desenvolver e avaliar estratégias para mitigar a formação de CE em vinhos fortificados, com o vinho Madeira como caso de estudo.

Primeiramente, uma extração líquido-líquido miniaturizada seguida por cromatografia líquida de fase reversa com deteção por espectrometria de massa em tandem foi desenvolvida com um bom limite de quantificação. Esta metodologia permitiu a determinação simples e eficiente de CE em vinhos fortificados. Foi utilizada para analisar um conjunto representativo de amostras de vinho Madeira, cujos resultados evidenciaram que os vinhos mais velhos tendem a apresentar concentrações mais elevadas de CE.

A evolução do CE e dos seus principais precursores foi estudada numa amostragem de vinhos doces e secos de castas tintas e brancas, submetidos a estufagem e canteiro. Os vinhos doces Tinta Negra estufados apresentaram a maior propensão para formar CE e concluiu-se que outros compostos ou características físico-químicas não reportadas podem influenciar a ocorrência do CE.

A arginina é apresentada como um novo precursor de CE, menos reativo que a ureia e a citrulina, mas relevante devido aos significativos níveis residuais em vinhos. Os principais açúcares no vinho, glucose e frutose, suprimem a formação de CE pelas vias dos precursores mais reativos, em até 26%. A fortificação alcoólica, utilizada para interromper a fermentação, não é um processo crítico para a ocorrência dos precursores de CE. O impacto da inoculação de leveduras indígenas não-*Saccharomyces* foi avaliado: *Pichia terricola* e *Starmerella bacillaris* revelaram o menor potencial para formar CE e podem ser ferramentas úteis para a sua mitigação em vinhos fortificados.

Palavras chave: Carbamato de etilo; Vinho; Açúcares; Fortificação; Leveduras não-*Saccharomyces*.

SUMMARY

Ethyl carbamate (EC) is a natural occurring compound in alcoholic beverages. Its acute toxicity is low, but its continued consumption raises some toxicological concerns, reason why the industry of alcoholic beverages committed to decrease EC levels as far as possible. This study aims to develop and evaluate strategies to mitigate EC formation in fortified wines, with Madeira wine as a study case. Madeira wine is a well-established product with a known reputation that results from unique edaphoclimatic characteristics and maturation processes.

Firstly, a miniaturized liquid-liquid extraction followed by reversed-phase liquid chromatography-tandem mass spectrometry detection was successfully developed with a good limit of quantification. This methodology was important for the simple and efficient determination of EC in fortified wines. It was used to analyse a representative sample set of Madeira wines and the results highlighted that older wines tend to have higher EC concentrations.

The evolution of EC together with its main precursors was studied in a sampling of sweet and dry wines from red and white grape varieties, submitted to *estufagem* and *canteiro*. Tinta Negra sweet wines submitted to *estufagem* exhibited the highest propensity to form EC and we concluded that unreported compounds or physicochemical characteristics may also influence EC occurrence.

Arginine is here presented as a new EC precursor, less reactive than urea and Citrulline, but important, due to its usually high residual levels in wines. The wine main sugars, glucose and fructose, suppress EC formation from its most reactive precursors up to 26%. The alcoholic fortification step, normally used to stop fermentation, is not a critical process for EC precursors' occurrence. The impact of indigenous non-*Saccharomyces* yeasts on EC formation was evaluated: *Pichia terricola* and *Starmerella bacillaris* revealed the lowest potential to form EC and, therefore, both species can be useful for its mitigation in fortified wines.

Keywords: Ethyl carbamate; Wine; Sugars; Alcoholic fortification; non-*Saccharomyces* yeasts.

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List of abbreviations

Arg – arginine

BC - butyl carbamate

Cit - citrulline

DoE - design of experiments

EC - ethyl carbamate

ESI - electrospray ionization

EU - European Union

FW - fortified wine

FAO – Food and Agriculture Organization of the United Nations

FDA - Food and Drug Administration

FLD - fluorescence detector

Fru – fructose

GC - gas chromatography

GC-MS - gas chromatography coupled with mass spectrometry

Glc - glucose

GYP - glucose yeast peptone agar

HPLC - high-performance liquid chromatography

HS-SPME - headspace solid-phase microextraction

IARC - International Agency for Research on Cancer

IDA - iodoacetic acid

PCR-RFLP - polymerase chain reaction - restriction fragment length polymorphism

IVBAM - *Instituto do vinho, do bordado e do artesanato da Madeira*

LC - liquid chromatography

LLE - liquid-liquid extraction

LOD - limit of detection

LOQ - limit of quantification

mLLE - miniaturized liquid-liquid extraction

MCE - 2-mercaptoethanol

ME - matrix effect

MEPS - microextraction by packed sorbent

MRM - multiple reaction-monitoring

MS/MS - tandem mass spectrometry

m/z - mass-to-charge ratio

OIV - International Organisation of Vine and Wine

OPA - orthophthalaldehyde

PTFE - hydrophilic polypropylene

PVPP - polyvinylpolypyrrolidone

QuEChERS - quick, easy, cheap, effective, rugged, and safe

RP - reverse phase

RP-LC-MS/MS - reversed phase liquid chromatography-electrospray tandem mass spectrometry

Rpm - rotations per minute

RSD - relative standard deviation

R^2 - R-squared

SD - standard deviation

SERS - surface-enhanced raman spectroscopy

SPE – solid-phase extraction

SPME - solid-phase microextraction

TEA - thermal energy analyser detector

TNDry - Tinta Negra dry

TNSweet - Tinta Negra sweet

USAEME - ultrasound-assisted emulsification–microextraction

UV-vis – ultraviolet-visible spectroscopy

WHO - World Health Organization

COLLABORATION IN PROJECTS

IMPACT II – Impact of production technologies in Madeira wine Quality II. Funding by FEDER - Intervir+, QREN.

VALIMED – Validation of analytical methods and determination of uncertainties. Funding by FEDER - Intervir+, QREN.

ESTUFA – Madeira wine *estufagem* monitorization. Funding by FCT.

IMPACT III – Impact of production technologies in Madeira wine Quality III. Funding by FEDER - Intervir+, QREN.

AROMA – New fiber optic sensor designs for the detection of AROMA compounds. by through FCT.

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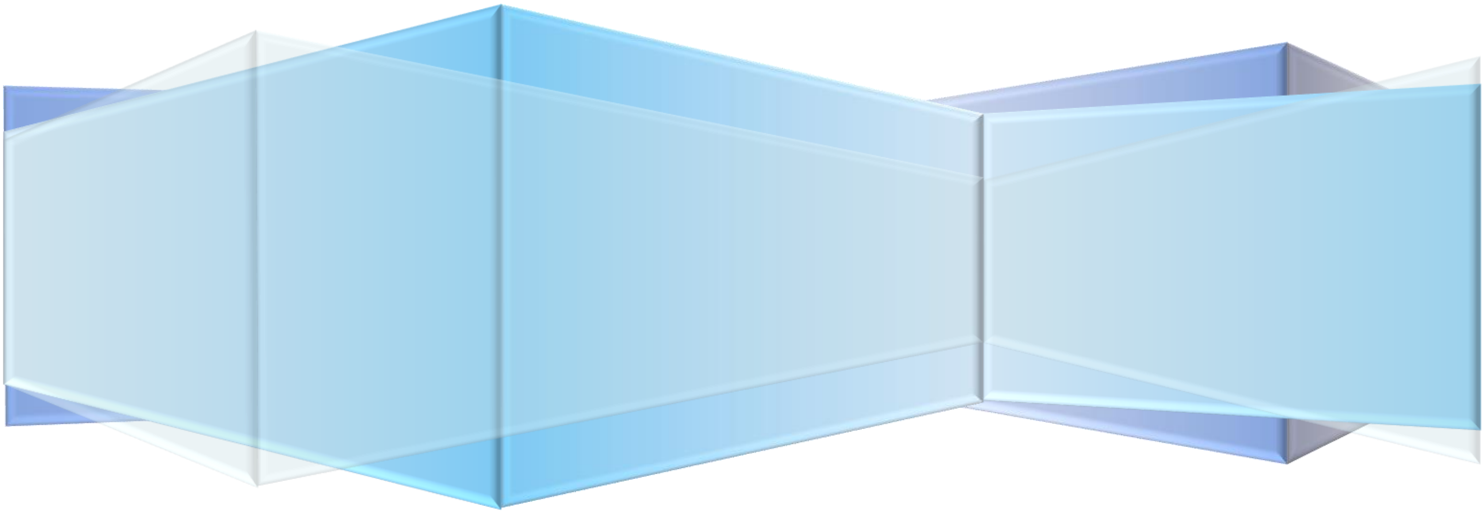
PART I – GENERAL INTRODUTION



1. OVERVIEW AND OBJECTIVES

In general, this work aims to detect, understand, and mitigate the formation of ethyl carbamate (EC) in fortified wines, with Madeira wine as a study case. In the initial part of this study, the purpose was to develop a simple and fast method, which would allowed to quantify EC, almost at the moment of its formation, with a limit of quantification (LOQ) of 0.52 µg/L. Regarding the extraction procedure, ecological approaches that make possible the preparation of several samples simultaneously were prioritized. During this study, we also aimed to simplify the determination of the main EC precursors.

Taking into account the high alcohol contents, ageing processes under considerable temperatures and long periods of maturation, a representative Madeira wines sample set of 108 samples with different ageing times, varying from 3 to 41 years was selected, in order to assess the presence of EC. Once we verified that, in some wines, the EC content was above the legal limits of some markets, we then studied its evolution over three years, together with its main precursors. The study sampling included sweet and dry wines from red and white grape varieties, that were submitted to the two traditional maturation processes, *estufagem* and *canteiro*, for comparison purposes. After analysing the results, the sweet red wines submitted to *estufagem* are the ones that have more propensity to form EC (reaching up to 149 ±11 µg/L) and the study focused particularly on the Tinta Negra, the results also suggested the existence of other EC precursors not reported in the literature, with impact on long-term ageing.

The main features that characterize a fortified wine, such as Madeira wine, are the considerable amount of sugars in the medium and the high ethanol content, both influenced by a process called alcoholic fortification, where the addition of alcohol stops fermentation. Thus, another objective of our study was to evaluate the impact of main sugars on the occurrence of EC. High residual sugars are normally present in fortified wines, influenced by the interruption of the alcoholic fermentation at initial or intermediate stages.

The obtained results indicated that neither the presence of sugars nor the fortification process, in a generalised way, is problematic to the EC occurrence. Therefore, the fermentation process was highlighted, and, following a current trend, we evaluated the impact of indigenous non-*Saccharomyces* yeasts in the occurrence of EC and its precursors. From the studied yeasts, *Pichia terricola* and *Starmerella bacillaris* revealed low potential to form EC and both species can be useful tools for its mitigation in wines.

Finally, several oenological treatments were applied to aged wines, and their ability to remove EC from wine medium was studied. None of the wine treatments tested could remove EC from the analysed fortified wines.

2. ETHYL CARBAMATE

EC, also known as urethane (CAS number 51-79-6), is the ethyl ester of carbamic acid, a naturally occurring compound in fermented foods and alcoholic beverages. EC is formed by the reaction of nitrogen compounds with alcohol. Before it became common knowledge that EC is a probable genotoxic it was used in the food industry, namely as a co-solvent of pesticides and a preservative in alcoholic beverages (2, 3). EC was also used in the medical field, as a treatment for delirium, insomnia, varicose veins and as a topical bactericide (2). In oncology, its anti-neoplastic properties were tested with low success in patients with breast cancer, leukaemia and multiple myeloma (2, 4). Later, from common observations during these treatments it was concluded that long-term therapy with EC causes hepatic necrosis, leukopenia and anaemia (5), which caused its use in the medical field to be abandoned.

The acute toxicity of EC is low, but the continued administration promotes carcinogenesis and biological dysfunctions (6). The carcinogenicity of EC was recognized by Nettleship *et al* (1943), when the authors verified a high incidence of lung cancer in animals that were anesthetized with EC. Several experiments were carried out in animals exposed to EC and the development of different tumours was verified, which proved its carcinogenic effect on the tested species (8). The first study about the presence of EC in wines associated this compound with diethyl carbonate, used at the time as an additive to control microbiological activity and was afterwards prohibited (9). Years later, Ough (1976) proved that EC is a naturally occurring compound in fermented foods and alcoholic beverages. Consecutively, Schmahl *et al* (1977) developed a toxicological study that was used by Canadian authorities in order to devise legislation to limit EC levels in alcoholic beverages. More recently, in 2007, the International Agency for Research on Cancer (IARC) reclassified EC from “possibly carcinogenic” (Group 2B) to “probably carcinogenic to humans” (Group 2A) (12).

Canadian authorities published an official report in 1985 revealing high concentrations of EC in commercial alcoholic beverages, especially those with higher alcohol content. Based on toxicological concerns raised by previous studies, Canada imposed for the first time a legislation that limited EC concentration in specific alcoholic beverages (13). The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) organized a Codex Alimentarius Commission in 2005 that considered EC an important issue. Excluding the consumption of alcoholic beverages, the presence of EC in the human diet is minimal. Therefore, exposure of humans to EC occurs mainly through the consumption of alcoholic beverages and, in this sense, strategies to mitigate this compound have been developed and applied over the last decades, with recognized success (6).

EC itself is not carcinogenic and it is estimated that over 90% of EC is hydrolysed by microsomal esterases and amidases to ethanol, ammonia and carbon dioxide (14, 15). After hydroxylation and conjugation, around 5% of EC is excreted in the urine. A small percentage of EC is alternatively metabolised in the liver, via cytochrome P4502E1 (CYP2E1). This reaction originates N-hydroxyethyl carbamate, 2-hydroxyethyl carbamate, and vinyl carbamate (0.5%). The latter is then converted to vinyl carbamate epoxide, which exerts a carcinogenic effect (15-17). Thus, hepatic metabolism of EC by cytochrome P4502E1 (CYP2E1) is required for its probable carcinogenesis (17).

2.1. Formation pathways

The study of the main precursors of EC and its formation pathways, as well as the evaluation of the impact of environmental factors on each pathway, have been crucial for the mitigation and control of this compound (6). The main ways for EC formation in alcoholic beverages have already been identified (Fig. 1) and the correspondent precursors are carbamoyl and cyanide compounds. The cyanic acid pathway is associated with the formation of EC in distilled spirits. The carbamoyl compounds urea and citrulline (Cit) are considered the main precursors of EC in wines (1). Urea and Cit are mainly produced by the metabolism of arginine (Arg), which is carried out by fermentative organisms, such as *Saccharomyces cerevisiae* and lactic acid bacteria. Inside the intracellular compartment, Arg is simultaneously catabolised and anabolised during the fermentation process (1, 13).

Urea is a compound typically found in fermented foods, but its concentration tends to be higher in alcoholic beverages, up to the order of mg per litre. In wines, the presence of urea is associated with the catabolism of Arg by yeasts in the fermentation step. This reaction is catalysed by arginase and consists in the hydrolysis of Arg to ornithine and urea (18-20). The EC formation from the urea pathway is influenced by external factors such as temperature (21).

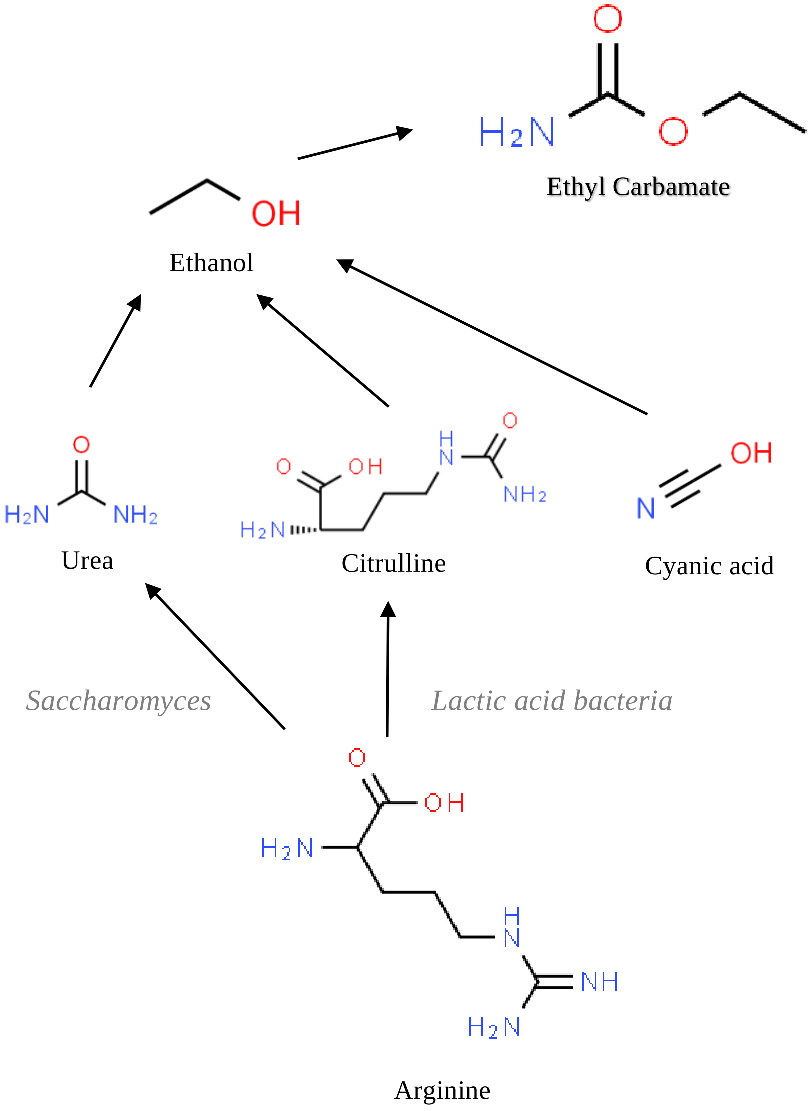


Fig. 1 - Pathways of EC formation in alcoholic beverages (1).

Cit, the other main EC precursor, is found in low concentrations in grape juice. During wine production it is also generated in the fermentation step by *Saccharomyces cerevisiae* through Arg anabolism, wherein carbamoyl phosphate and ornithine react to form Arg, being Cit an intermediate compound of this metabolic reaction (22). Note that Cit occurrence is more associated with malolactic fermentation performed by lactic acid bacteria (18). EC formation through the reaction of Cit with ethanol is also influenced by temperature (21).

The cyanic acid pathway is associated with beverages that undergo the distillation process, especially stone-fruit brandies. Cyanogenic glycosides are present in several species and are decomposed to form cyanohydrin by thermal cleavage. In turn, cyanohydrin decomposition forms hydrocyanic acid that ultimately oxidizes to cyanic acid (23, 24). There are several factors that can influence this EC forming pathway, such as temperature, ethanol content, presence of metallic species, light and pH (13, 25).

2.2. Legislation

An official report published in 1985 by the Liquor Board of Ontario in Canada raised concerns about the high levels of EC in alcoholic beverages (26). Based on the EC toxicity study developed by Schmahl *et al* (1977) and on the data regarding the average consumption rate for each beverage type, Canadian authorities developed the first legislation on this topic, which set EC limit values as follows: wine 30 μg/L, fortified wine 100 μg/L, distilled spirits 150 μg/L, sake 200 μg/L and fruit brandies 400 μg/L. The application of this legislation raised some problems because the producers did not know how to detect and control EC occurrence in their beverages. Additionally, the sensitivity needed to quantify this compound was not accessible to most quality control laboratories (12).

Nowadays, other countries have established their own legislation (Table 1), being Czech Republic the only country that adopted the Canadian one. France applied a legislation only to distilled spirits and fruit brandies, while Germany and Switzerland imposed a maximum residual level of EC just to fruit brandies. South Korea limited the levels of EC in table wines and Brazil did the same for *cachaça*, a distilled spirit. Considerable levels of EC can be found in Chinese rice wines, but no regulation was established by Chinese authorities to this date (13, 27).

Legislation on the EC concentration limit in alcoholic beverages is not consensual. It should be noted that the average intake of EC from food is approximately 15 ng/kg per day and that the lower limit of the reference dose is 0.3 mg/kg per day. It is also important to mention that highly consumed alcoholic beverages, such as spirits, have a limit for EC content higher than the one for fortified wines (11, 12, 28, 29).

Table 1 - Maximum levels of EC in alcoholic beverages (12, 13).

Country	**Table wine** (µg/L)	**Fortified wine** (µg/L)	**Distilled spirit** (µg/L)	**Sake** (µg/L)	**Fruit brandy** (µg/L)
Canada	30	100	150	200	400
Czech Republic	30	100	150	200	400
France	n.r.	n.r.	150	n.r.	1000
Germany	n.r.	n.r.	n.r.	n.r.	800
Switzerland	n.r.	n.r.	n.r.	n.r.	1000
Brazil	n.r.	n.r.	150	n.r.	n.r.
South Korea	30	n.r.	n.r.	n.r.	n.r.

n.r. - no regulation

2.3. Determination

Lofroth *et al* (1971) observed the formation of EC from the degradation of diethyl pyrocarbonate on the first study that quantified EC in alcoholic beverages. Walker *et al* (1974) started by developing an analytical method to quantify EC by gas chromatography (GC), however, the limit of detection (LOD) was too high, and the extraction step was laborious and complex. Later, Ough (1976) developed a GC methodology that made it possible to quantify EC concentrations of 10 µg/L. The legislation imposed by Canadian authorities raised the need to develop simpler and more sensitive (in the order of µg/L) methods to quantify EC in alcoholic beverages. The emergence of new extraction techniques, separation apparatuses, and detectors played an important role in this matter (12).

The official methodology of the International Organisation of Vine and Wine (OIV) consists of GC coupled with mass spectrometry (GC-MS) and determination in the selected ion monitoring mode, preceded by a time-consuming extraction step using a diatomaceous earth solid-phase extraction (SPE) with propyl carbamate as the internal standard (31). In turn, the Association of Official Analytical Chemists International (AOAC) defined two different methods to quantify EC in different matrixes: one by GC-MS and the other by GC coupled with a thermal energy analyser detector (TEA) (32, 33).

EC determination protocols typically include two phases: a pre-treatment - usually an extraction stage, but alternatively a derivatization or an alcohol distillation - and the instrumental analysis. Over the last decades, GC-MS became the most commonly used analytical instrument to identify and quantify EC. Usually, the MS detector operates in the selected ion monitoring mode and the typical ions of EC, m/z 89, 74, and 62, are used to identify and quantify this compound. For the internal standard, the tendency is to use EC labelled with deuterium, although some authors continue to use propyl carbamate and butyl carbamate (BC) with viable results (12, 13, 34). High-performance liquid chromatography (HPLC), coupled with a fluorescence detector (FLD) or with a tandem mass spectrometry detector (MS/MS), have also been used, but with the previous derivatization of the samples using 9-xanthydrol (35-37). It is important to note that the use of MS/MS detection improves the selectivity and the sensitivity of the methodologies (38, 39).

In terms of samples pre-treatment, liquid-liquid extraction (LLE), SPE and solid-phase microextraction (SPME) are the most common techniques used to extract EC. LLE methods, in general, are time-consuming and use large amounts of environmental toxic organic solvents (1). The SPE technique reduces the interference of co-extracted compounds and improves the selectivity and sensitivity of EC determination, but still is a laborious methodology that requires hazardous organic solvents (34). On the other hand, SPME is a solvent-free technique easy to apply and can be automatized with good sensitivity and selectivity. Additionally, the amount of sample required for analysis is minimal. The only downside of this technique is its economic burden (12, 27, 39-41).

Emergent alternative extraction techniques have been developed to quantify EC without the time-consuming procedures and laborious analyses necessary for the conventional methods, combining both precision and high sensitivity. Liao *et al* (2013) developed a sensitive ultrasound-assisted emulsification–microextraction (USAEME) methodology, that uses 200 µL of chloroform per extraction, followed by a GC-MS analysis to quantify EC in alcoholic beverages. Later, Leça *et al* (2014) used the microextraction by packed sorbent (MEPS) technique, also followed by GC-MS, to determine the concentration of EC in fortified wines with high sensitivity and small volumes of sample and extraction solvent, 300 μL and 100 μL, respectively. Yang *et al* (2013) developed a method based on surface-enhanced Raman spectroscopy (SERS), using silver-coated gold nanoparticle colloids to quantify EC in alcoholic beverages with high sensitivity, while Lu *et al* (2015) optimized a simple and highly sensitive enzymatic method with ultraviolet-visible spectroscopy (UV-Vis) detection. Differently, the determination of EC in Chinese rice wine was achieved through an electrochemical approach making use of an amperometric biosensor (46).

2.4. Prevention and control

Even though the alcoholic beverage industry has obtained good results in the prevention and control of EC in the last decades, it is important to maintain and develop new EC mitigation preventive strategies. The scientific community that focused on this issue accomplished the reduction of EC accumulation in different types of alcoholic beverages over the last decades. The identification of the main precursors, the corresponding formation pathways, and the understanding of the impact of external factors (temperature and time of storage, for example) played an important role in its mitigation (6, 12, 47). The methods developed to mitigate EC can be divided into chemical, physical, enzymatic and metabolic engineering (47).

In the case of stone-fruit brandies, the de-stoning process before mashing is an effective physical process to reduce EC since fruit stones are rich in cyanogenic glycosides (48). However, this process has a significative impact on the typical and popular stone flavour (49). Concerning grape wine, the supplementation of grape must with diammonium phosphate can decrease the amount of EC when using specific yeast strains (50).

According to the Food and Drug Administration (FDA) guidelines, the most relevant reaction for EC formation in wine is between urea and ethanol, being the developed prevention manual more focused on table wines. However, producers of fortified wines must consider that the fortification step itself increases urea content in the medium. Urea passes from the intracellular compartment of yeasts cells to the extracellular medium, due to the addition of vinous alcohol. Moreover, soils with excessive nitrogen content and the fertilization of vineyards with ammonia, urea, or other N-fertilizers have a direct influence on the nitrogen content in musts, which can potentiate the formation of EC precursors. Different grape cultivars have marked variations in nitrogen uptake and, so, may also be necessary to measure the nutritional status of each grape juice. The concentration of nitrogen in solution should only be enough to provide nutrients to fermentative yeasts, avoiding sluggish or stuck fermentations. Some products, such as Madeira wine, are derived from spontaneous fermentations, which have a positive impact on their typical characteristics. However, in these cases, fermentations should be monitored since indigenous yeasts strains will cause variability in the occurrence of EC precursors. Besides that, the impact of natural fermentations on EC final levels has not been thoroughly investigated.

The use of commercial yeasts species with low urea excretion is indicated as a possible approach to reduce EC concentration in wines. Regarding enzymatic treatments, FDA considers as a possible solution the addition of acid urease to hydrolyse urea (51). The industrial application of acid urease is generally considered beneficial but is still limited due to lengthy and expensive procedures, having an impact on the organoleptic characteristics of the product (1, 47).

Metabolic engineering tools are also applied to reduce EC in alcoholic beverages and are based on urea reduction in the wine medium at the end of fermentation, either by repressing urea production through silencing of the arginase gene (*CAR*1) or by enhancing the urea metabolic capacity of yeasts through upregulation of the genes involved in urea metabolism and transport (*DUR*1, 2 and 3) (Fig. 2). The first target of metabolic modification was the *CAR*1 gene in *Saccharomyces cerevisiae*, since it encodes arginase, the enzyme responsible for the hydrolysis of Arg into urea. The elimination of arginase activity by *CAR*1 disruption can lead to no urea production during fermentation (52). In addition to the genetic engineering techniques that silence *CAR*1, the enhancement of urea metabolism and transportation have gained attention from researchers since the year 2000, as an attempt to reduce urea accumulation. These new approaches focus on genes that encode urea amidolase (*DUR*1, 2), an enzyme that catalyses the conversion of urea to ammonium, or urea permease

(*DUR*3), which promotes urea diffusion and increases its metabolism in yeasts. Despite these methods being able to reduce EC up to 89%, they promote the accumulation of ammonium with impact on fermentation and significant changes in the flavour of the corresponding beverages (1, 47).

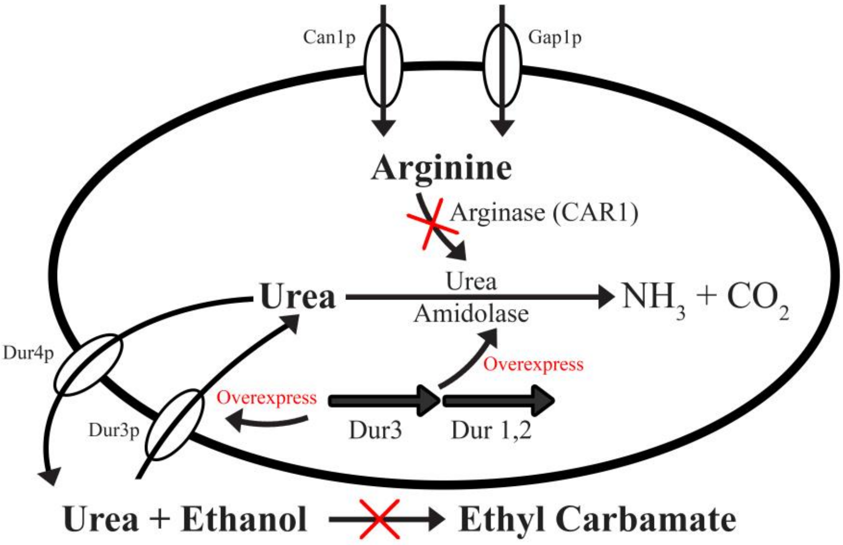


Fig. 2 – Diagram of the two metabolic engineering methods for EC reduction. Inhibition of arginase (*CAR*1 gene) expression and upregulation of genes involved in urea metabolism and transport (*DUR*1, 2 and 3) (based on (47)).

3. MADEIRA WINE

Fortified wines are also known as liquor wines, dessert wines or *generoso* wines and they are produced from unfermented or partially fermented grape must enriched with wine-derived spirit. European Union regulation No. 479/2008 defines liquor wines as those having an acquired alcohol content between 15 and 22% v/v (53).

A wide range of fortified wines is produced in several regions with different styles, from distinct grape varieties, with characteristic attributes that result from unique winemaking and ageing processes. Besides Madeira wine from Portugal, the most well-known fortified wines are Port, also from Portugal, Sherry from Spain, and Marsala, produced in Sicily, Italy. All of these wines are submitted to an alcoholic fortification step, that consists of adding alcohol to increase the total alcohol content up to 22%, stopping the fermentation and generating fortified wines with different levels of sweetness (54).

Madeira wine has an alcoholic content between 17 and 22% v/v, as a result of the addition of vinous alcohol, a process named alcoholic fortification, as already mentioned (55). Historically, the fortification step was introduced to avoid wine spoilage in the course of long sea expeditions, during the Age of Exploration. Then, it was verified that the heat exposure in tropical routes changed wine flavour, improving its quality. This finding led to the development of the ageing process called *estufagem*, in which the wine is heated to a maximum of 50 °C to simulate maturation during those long sea voyages (56, 57).

Madeira island, located in the Atlantic Ocean (at approximately 970 km southwest from Lisbon, Portugal, and 600 km from the African coast), is part of the Madeira archipelago, also composed by Porto Santo, Desertas and Selvagens islands. The Madeira wine distinguishing features are also influenced by the island's characteristics: fertile soils near the sea from volcanic origin, with high acidity, rich in organic matter and mineral content such as magnesium and iron; moderate climate with mild winters, humid hot summers and abundant rainfall in autumn and winter (58, 59). Agricultural lands are characterised by small terraces in very sharp slopes, which is the reason why mechanisation is almost impossible.

Nowadays, Madeira wine commercialization is still a significant source of income for this Autonomous Region, with a marked impact on its touristic promotion. Therefore, improvement of Madeira wine quality continues to be an important concern of producers and trade companies. IVBAM (*Instituto do Vinho, do Bordado e do Artesanato da Madeira*) is the regulatory institution of its production and commercialization (60). The resulting wines are normally served as an aperitif or dessert wine, and are characterized by a marked and intense flavour, being also known for their longevity (61).

In the next subsections, a deeper insight into Madeira wine production and characteristics will be provided to facilitate the understanding of this study.

3.1. Grape varieties

Only *Vitis Vinifera* L. grape varieties are legally authorised to be used in Madeira wine production and, according to the official classification, grape varieties are divided in recommended and authorised categories. In the group of white grape varieties, the recommended are Sercial, Verdelho, Boal, Malvasia Candida and Terrantez and the authorized are Listrão, Caracol, Malvasia de São Jorge, Carão de Moça, Valveirinho, Rio Grande, Malvasia Babosa and Moscatel de Málaga. Regarding red grape varieties, the recommended are Tinta Negra, Bastardo, Tinta da Madeira, Verdelho Tinto and Malvasia Cândida Roxa and the authorized varieties are Complexa, Tinto Negro, Deliciosa and Triunfo (55). However, the most used grape varieties are Sercial, Verdelho, Boal, Malvasia and Tinta Negra, the latter with a higher production than the rest together (53).

Tinta Negra or Tinta Negra Mole is the most used grape variety to produce Madeira wine and it is essentially cultivated in Câmara de Lobos and São Vicente (60). It is a versatile variety, since it can originate sweet, medium-sweet, dry, and medium-dry wines. Its grapes have fine and soft skins, are black with a light pulp and exhibit a small to medium-size and an elliptic-globose shape. This variety is known to have adapted easily to the island's features and for being quite resistant to some pests (60, 62, 63).

Malvasia is the white grape variety that traditionally produces sweet wines. The resultant wines are considered the smoothest ones, retaining a high acidity that is well balanced with the sweetness (64). Malvasia wines often acquire a dark colour and tend to be rich in aromas. This grape variety is mainly produced in Santana and Câmara de Lobos and is usually planted from 150 to 200 meters above sea level, at sunny locations. The berries are big with elliptic or globe-elliptic shapes and golden when ripe; bunches are conical with medium to large sizes (60, 63, 64).

Boal or Bual, also known as Malvasia Fina, is frequently found in warm locations such as Calheta, Câmara de Lobos and Ribeira Brava. This variety is cultivated normally between 100 to 300 meters of altitude and its berries have a resistant skin with an elliptic shape and a greenish-yellowish or golden colour when ripe. The brunches are dense with a medium-large size. The obtained medium-sweet wines are characterized by their fine balance between acidity and sweetness and by their dark colours with raisin notes at tasting (60, 63, 64).

Verdelho variety has a reputation of being difficult to reproduce and it generates medium-dry wines. It is usually cultivated at 400 meters above the sea; the grapes are characterized by its resistant skin with golden colour and the bunches have a small to medium size. Resultant wines are considered highly aromatic with nutty and caramel notes and are normally served as aperitive (60, 63, 64).

Sercial is considered difficult to grow and it is only cultivated in some specific places in Porto Moniz and Câmara de Lobos, with vineyards located at altitudes up to 200 meters and 600 meters, respectively. This variety produces wines with the lowest residual sugar levels. The greenish-yellow grape berries are very acidic, and bunches are compact with a pinecone shape. The obtained wines must maturate during long periods to produce excellent dry wines due to its high acidity and astringency. These wines normally exhibit golden to very pale colours with almond notes. (59, 60, 63, 64).

3.2. Winemaking process

The winemaking process starts with harvesting, that normally occurs from mid-August to October and, as defined by IVBAM, grapes must have a minimum of 9% of potential alcohol. At wine cellars it is confirmed if grapes have achieved the required levels of fermentable sugars and phytosanitary conditions are verified. The grape selection is made according to the variety and the desired wine style (53, 60). The addiction of pectolytic enzymes is an option to some producers (65). With mechanical help from a crusher/destemmer, the stems are separated from grapes and then crushed to obtain the free-run juice. At this stage, sulfite is commonly added to inhibit unwanted yeasts and bacteria (53).

The fermentation of Madeira wine can occur through two different fermentative processes, *bica aberta* or *curtimenta*. In *bica aberta*, free-run juice fermentation is performed without contact with skin and seeds and pressing occurs as soon as grapes are crushed. In *curtimenta*, the fermentation is carried out in the presence of the grape solids and must is only pressed at the end of fermentation. It is important to mention that the fermentation process occurs in the absence of commercial yeasts; in fact, it starts naturally and spontaneously with indigenous yeasts existing in grapes' surface and in the fermentation environment (65, 66). Fermentative stages take place in stainless steel tanks and the temperature is usually kept below or around 25°C. Wine density is measured during this process, which allows the producer to define the optimum moment to interrupt the fermentation with the alcoholic fortification step, during which vinous alcohol is added depending on the type of wine being produced (sweet, medium-sweet, dry or medium-dry). The vinous alcohol contains 95% (v/v) of ethanol and it is used to raise the alcohol content to about 17% (v/v), that later can be readjusted during maturation (60, 65, 66).

After fortification, wines clarification and stabilization are achieved through bentonite clays and/or gelatines or simply by racking/decanting processes. These agents combine with suspended particles and make them precipitate. Finally, wines are racked or filtered into casks or other tanks (53, 66).

Regarding ageing processes, Madeira wine can follow one of two traditional ageing processes, *canteiro* or *estufagem*, the latter being an accelerated ageing process applied to most Madeira wines. In this approach, wines are heated in special stainless-steel closed tanks sealed by IVBAM, where they are kept for at least three months, with a maximum legal temperature of 50°C (53). Tinta Negra wines are the ones typically aged by *estufagem* and this thermal process is usually executed at about 45°C for three to four months. After *estufagem*, the wine is cooled for three to four weeks, clarified again, and then placed in oak casks for at least another three months. This process accelerates the development of the typical bouquet of Madeira wine and of its traditional colour (53, 60, 66).

Other wines, especially those obtained from white varieties and produced in limited quantities, are only aged by *canteiro*. The maturation is gentler and takes much longer, at least two years. After the post-fermentation treatment, wine is placed in oak casks, typically in the lofts of wine cellars or in another suitable warm place with high temperatures. This ageing process is considered to be more appropriate for the varietal flavours of white grapes varieties (53). In casks' headspace a certain amount of air is left deliberately to promote oxidative ageing and often up to 5% of wine per year is lost due to evaporation. The final wines can vary from sweet (>96 g/L of total sugars) to extra dry wines (<49 g/L of total sugars) with colours ranging between dark brown and pale gold (53, 60).

3.3. Fermentative yeasts

Alcoholic fermentation consists in the transformation of grape sugars, mainly fructose (Fru) and glucose (Glc), into ethanol and carbon dioxide with other secondary metabolites being also produced. This process is carried out by fermentative yeasts and, despite their wide variety, only fourteen genera, approximately, are associated with the winemaking process, namely: *Saccharomyces*, *Hanseniaspora*, *Pichia*, *Brettanomyces/Dekkera*, *Candida*, *Saccharomycodes*, *Schizosaccharomyces*, *Cryptococcus*, *Debaryomyces*, *Kluyveromyces*, *Rhodotorula*, *Kloecker*, *Metschnikowia* and *Zygosaccharomyces*. These microorganisms can be divided in non-*Saccharomyces* and *Saccharomyces* yeasts. *Saccharomyces* are known by their fermentative capacities, while non-*Saccharomyces* yeasts are present during fermentation and can impact quality either in a positive or negative way (67-70).

During alcoholic fermentation, the microbiological composition changes in regards of species and concentrations. The beginning of fermentation is normally a responsibility of anaerobic genera such as *Hanseniaspora*, *Kloeckera* and *Candida*; then, species from *Pichia*, *Metschnikowia* and *Hansenula* will proliferate and, at the end, *Saccharomyces* dominates the fermentation due to its alcohol tolerance and fermentative vigour (71-74).

Hanseniaspora non-*Saccharomyces* yeasts, specially *Hanseniaspora uvarum*, are normally found in the highest numbers in grape musts. Together with *Hansenula*, these are known for their weak fermentative capacity but, on the other hand, important contribution to positive wine volatile composition. The low predominance of these yeasts in some wines is often associated with a lack of aroma complexity. However, some species of these genera can produce undesirable compounds, such as acetic acid and ethyl acetate, increasing volatile acidity (69, 75).

Some *Pichia* species are responsible for the production of aldehydes with a consequent increase of off-flavours and changes in wine quality. Others, like *Pichia kluyveri*, have been reported to extract flavour precursors from grape must and to produce higher levels of varietal thiols with an overall positive impact in aroma (69, 75, 76). *Pichia fermentans* and *Pichia terricola*, when individually combined with *Saccharomyces cerevisiae*, improve wine flavour and quality (77, 78).

The *Starmerella bacillaris* or *Candida zemplinina* species, from the *Candida* genus, is considered one of the most promising non-*Saccharomyces* yeasts for winemaking and, when combined with *Saccharomyces cerevisiae*, has been reported to increase wine quality by modulating the production of target metabolites in different types of wine. *Starmerella bacillaris* can also contribute to acetic acid reduction in sweet wines (68, 79).

Brettanomyces produces flavours that are acceptable and sometimes desirable in some alcoholic beverages such as Lambic beer. However, in wine, this genus is associated with poor sanitary conditions, promoting volatile acidity and the prevalence of off-flavours, mainly vinyl phenols and ethyl phenols. In some wines, low levels of *Brettanomyces* can be desirable to improve their complexity. *Zygosaccharomyces* are fermentative yeasts associated with good oenological properties, however, they have some resistance to oenological preservatives and antiseptics, can survive at low pH (< 2.4) and tolerate ethanol levels above 15% (v/v) (69).

Pereira (2012), in her doctoral thesis, presented some preliminary results about yeasts frequency during the fermentation of Madeira wines made from the main white grape varieties. In Malvasia and Boal wines the predominant specie is *Hanseniaspora* uvarum, in Verdelho the *Pichia terricola* and in Sercial, *Torulaspora delbrueckii* (Saccharomycetales) and *Pichia guilliermondii*. At the end of fermentation, *Saccharomyces* yeasts become predominant and, for Malvasia, the main species present in wine medium was *Saccharomyces bayanus.* In the remaining white varieties, *Saccharomyces cerevisiae* is prepotent at this stage.

3.4. Scientific research and technology

The emerging trends in fortified wines have been subject of a recent review (53). *Estufagem* is a unique maturation process that has been largely studied. Historically, the first known *estufa* was developed by Pantaleão Fernandes around 1794 and it took some time and a lot of burned wine until the technique was perfected and effective for its purpose, the simulation of long sea voyages in routes with hot climates. This technique was very important for merchants who had to respond to the great demand for Madeira wine (57).

Nogueira *et al* (1999) analyzed for the first time the physicochemical and sensorial parameters of Madeira wine from different styles and ages, concluding that some parameters were bellow some national and international regulations. After that, Câmara (2004), in his doctoral thesis, performed the aromatic characterization of Madeira wine with several scientific articles published (83-86). Pereira (2011), deeply studied the effect of the *estufagem* process on the chemical constituents and quality of Madeira wines, also with a relevant published bibliography (88-91). Later, Pereira (2012) used advanced multivariate statistical techniques to predict Madeira wines age and evolution (92-95). Finally, the study of other natural occurring compounds with interest on safety of alcoholic beverages, such as EC, was started (39, 43, 96, 97).

A relevant advance in the *estufagem* process was the definition of its optimum temperature at 45 °C by Oliveira *et al* (2008). The defined temperature was also considered adequate to control 5-hydroxymethylfurfural (HMF), which has raised some toxicological concerns in the food industry (88). Although the legislation allows higher temperatures (60), 45 ºC has gradually been defined for the *estufagem* process by Madeira wine producers and researchers (53). Nowadays, wines are heated in special vessels that consist of stainless-steel closed tanks, and the heating is achieved through circulation of hot water in a heating coil inside the tank or, more recently, using a hot-water jacket, that avoids hot spots near the heating coil (53, 60). The implementation of this advanced technology is expected to improve Madeira wine quality which can probably mitigate EC levels.

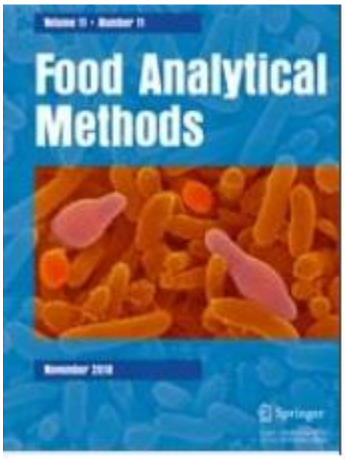
A relevant number of scientific publications has been made about Madeira wine and studies are mainly focused on both traditional ageing processes, *canteiro* and *estufagem*. Most of them are based on chemical characterization, process control and chemometrics with volatiles and polyphenols as the main topics (53).

Despite not published, a trend was observed from the contact with wine producers in the last decade, with greater use of *curtimenta* during the fermentation of all types of wines and some attempts to replace bentonite clays and/or gelatines with ultra-filtration for post-fermentation treatments.

PART II - DEVELOPED METHODOLOGY

This part of the study is based on the following publication:

Leça JM, Pereira V, Pereira AC, Marques JC. A sensitive method for the rapid determination of underivatized ethyl carbamate in fortified wine by liquid chromatography-electrospray tandem mass spectrometry. Food Analytical Methods. 2018;11:327-33.



A Sensitive Method for the Rapid Determination of Underivatized Ethyl Carbamate in Fortified Wine by Liquid Chromatography-Electrospray Tandem Mass Spectrometry

4. DETERMINATION OF ETHYL CARBAMATE IN FORTIFIED WINE BY RP-LC-MS/MS

This section presents an optimization of a miniaturized liquid-liquid extraction (mLLE) followed by reversed-phase liquid chromatography - electrospray tandem mass spectrometry detection (RP-LC-MS/MS) for the determination of EC in fortified wines, without using derivatizing agents. The mLLE was optimized by an experimental design. Thus, 15 mL of wine and 8 mL of ethyl acetate were used for the extraction. After concentration, each extract was injected into the LC-MS/MS equipment and the characteristic secondary ion transition of EC ($m/z = 90.10 \rightarrow 62.05$) was used for quantification purposes. The proposed method presented a good linearity (R^2=0.9999) and a high sensitivity with low LOD and LOQ values, 0.17 µg/L and 0.52 µg/L, respectively. The precision (repeatability and reproducibility) never exceeded 8% of variation and the recoveries varied between 93 and 114%. The applicability of the method was checked through the analysis of 24 fortified wines, with EC values ranging between 23 ±1 µg/L and 194 ±5 µg/L. All chromatograms revealed good peak resolutions and this new method is efficient for the simple, fast and reliable determination of EC in fortified wines, providing great sensitivity without using derivatizing agents or large volumes of organic solvents.

4.1. Introduction

GC-MS has become the most used technique for EC quantification and propyl carbamate, BC and EC labelled with deuterium (^{13}C or ^{15}N isotopes) have been used as internal standards (12, 13). Different approaches have been used to extract EC before the chromatographic separation. The official method of the OIV involves a complex and time-consuming extraction procedure, using a diatomaceous earth SPE column, prior to the GC-MS analysis (99). Thus, several efforts have been done to develop methodologies to determine EC without using long procedures and laborious analyses. In this regard, headspace solid-phase microextraction (HS-SPME) has gained a notorious visibility (39-41). USAEME and

MEPS have also been reported as feasible and easy-to-use extraction techniques to determine EC in alcoholic beverages (42, 43).

The LLE methods found in literature to determine EC use significant volumes of sample and organic solvents along with time-consuming extraction procedures or derivatization agents. Fauhl *et al* (1992) determined EC by continuous LLE through a Soxhlet apparatus followed by GC-MS analysis. Most recently, EC derivatized with bis-(trimethylsilyl)trifluoroacetamide was extracted by LLE and quantified by GC-MS (101). Valente *et al* (2014) used salting-out assisted LLE to analyse EC derivatized with 9-xanthydrol followed by HPLC-FLD. These strategies have emerged as attempts to improve conventional methodologies used for EC determination.

The aim of this part of the study was the development of a simple, efficient and sensitive method to quantify EC in fortified wines, without using derivatizing agents, minimizing the reagent consumption and reducing the use of toxic organic solvents, through the optimization of a mLLE procedure combined with RP-LC-MS/MS.

4.2. Materials and methods

4.2.1. Chemicals and samples

EC was purchased from Acros Organics (Geel, Belgium), while BC, used as an internal standard, was obtained from Sigma–Aldrich (Steinheim, Germany). All standards had a purity grade higher than 97%. While absolute ethanol was purchased from Sigma-Aldrich, tartaric acid, formic acid and methanol (UPLC grade) were from Panreac (Barcelona, Spain) and ethyl acetate was from Fisher Scientific (Leicestershire, UK). Ultra-pure water (type 1) was obtained from a Simplicity UV apparatus from Millipore (Milford, MA, USA). EC and BC 1 g/L stock solutions were rigorously prepared in ultra-pure water. Intermediate solutions of 50 mg/L in EC and 10 mg/L in BC were prepared in order to obtain the calibration solutions, which were then used to spike the synthetic wine (containing 6 g/L of tartaric acid, 18% of ethanol and pH adjusted to 3.5, with a 1 M NaOH solution). Each calibration point was extracted in triplicate and injected twice, within the validation range 1.0 – 200 µg/L. To test the method applicability, a sample set of twenty-four fortified wines, with different ages (up to 86 years old) and ethanol content up to 20%, was analysed.

4.2.2. Apparatus and chromatographic conditions

A Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan) consisting of binary LC-30AD pumps, a DGU-20 A5 degassing unit, a CTO-20A column oven, and a SIL-30AC autosampler was used. The LC system was coupled with the Shimadzu triple-quadruple mass spectrometer LCMS-8040, equipped with an electrospray ionization (ESI) module. Sample extracts were separated in the reversed-phase mode (RP) using a Kinetex C18 column, 150 × 2.1 mm, 2.6 µm, 100 Å, from Phenomenex (Torrance, CA, USA) thermostated at 40 ºC. The injection volume was 5 µL and all extracts were injected twice. A gradient elution with methanol (solution A) and ultra-pure water with 0.1% of formic acid (solution B) was used at a 0.4 mL/min flow rate. The gradient started with the solution A maintained at 5% for 4 min, followed by an increase to 30% in 2 min and to 100% in 1 min. Finally, solution A was reduced to 5% in 3 min and held at 5% for 5 min as a preparation for the next injection. All eluents were filtered through a hydrophilic polypropylene (PTFE) 0.2 µm pore size membrane filter (Pall Corporation, Ann Arbor). The column effluent was directed to the detector between 0.5 and 9.0 min and the rest was discarded. The LCMS-8040 ESI was operated in the positive ion mode along with multiple reaction-monitoring (MRM) mode, acquiring data for a single secondary ion transition ($m/z = 90.10 \rightarrow 62.05$) for EC and ($m/z = 118.00 \rightarrow 62.05$) for BC, with an optimal collision energy of -10 and -9 eV, respectively. The desolvation line temperature was maintained at 250 °C and the block heater at 400 °C; the nebulizing gas flow was 2.5 L/min and the drying gas flow 17.5 L/min. The data acquisition and all peaks integration processing were performed with the Labsolutions 5.7 software (Shimadzu).

4.2.3. mLLE optimization

Ethyl acetate was chosen to develop the extraction procedure and was optimized by performing a full factorial design. Experimental design (DoE) methodologies are increasingly more relevant in the optimization of quantification methodologies of various compounds, including EC (43, 103-105). To maximize the LC-MS/MS response of EC, two experimental variables, at 3 levels, were studied: the sample volume at 8, 10 and 15 mL and the extractant volume at 4, 5 and 8 mL. All experiments were made varying the sample and extractant volumes in random order, according to Table 2. A commercial fortified wine was used for the method optimization. The data analysis was performed using the Matlab software, version R2016b, to estimate the factors – levels combination that ensures the maximization of the LC-MS/MS response of EC.

Table 2 - Experiments of the full factorial design.

#	Sample volume (mL)	Solvent volume (mL)
1	8	8
2	15	4
3	15	4
4	8	5
5	10	8
6	10	8
7	8	4
8	8	8
9	11.5	6
10	8	5
11	10	4
12	10	5
13	15	5
14	8	4
15	11.5	6
16	10	5
17	10	4
18	15	5
19	15	8
20	15	8

4.2.4. mLLE optimized procedure

As summarized in Fig. 3, 8 mL of ethyl acetate (extractant solvent) were added to 15 mL of sample/standard solution, in a 50 mL PTFE centrifuge tube, spiked with 50 µL of internal standard (10 mg/L BC solution). After vigorous shaking in vortex for 5 min, the tube was centrifuged at 4400 rotations per minute (rpm) for 5 min to separate the liquid phases. Finally, an aliquot of the upper phase was collected and evaporated using a small nitrogen flow. The residue was redissolved with mobile phase B to a final volume of 1 mL and filtered through Chromafil PTFE 0.2 µm syringe filters (Macherey-Nagel, Düren, Germany). Each sample/standard solution was extracted in triplicate and 5 µL of extract was injected twice into the LC-MS/MS system.

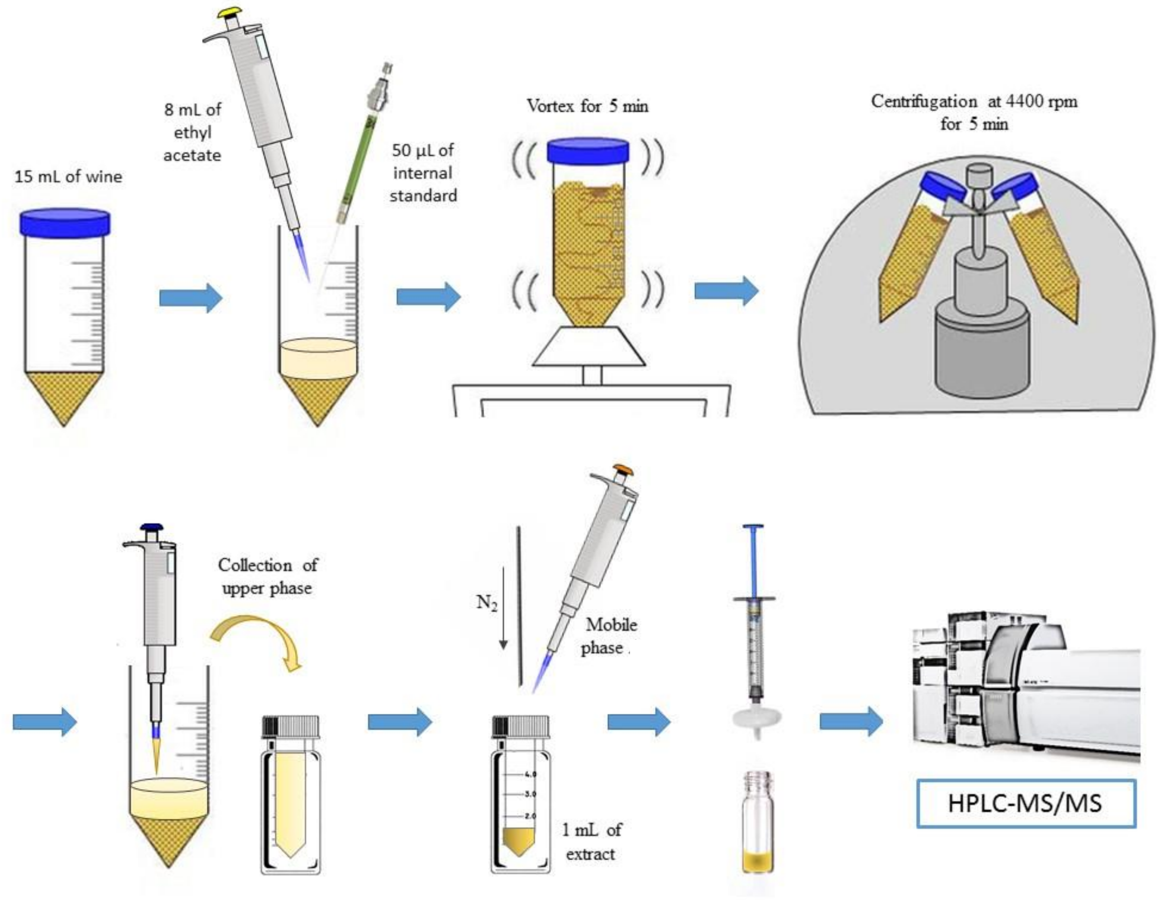


Fig. 3 – Illustration of mLLE optimized procedure.

4.2.5. Method validation

The mLLE/RP-LC-MS/MS method optimized for the determination of EC in fortified wines was validated in terms of selectivity, linearity, sensitivity, matrix effect, precision and accuracy.

Selectivity was appraised by the analysis of five fortified wines to ensure the absence of chromatographic interferences at the retention time of EC (m/z = 90.10 → 62.05) and BC (m/z = 118.00 → 62.05) which could compromise EC quantification.

The working standard solutions were prepared by spiking synthetic wine at eight different concentration levels of EC: 1, 2.5, 5, 12.5, 25, 50, 100 and 200 μg/L with a 50 µL spike of BC (10 mg/L). The calibration curve was obtained by plotting the analyte peak area ratio (EC area/BC area) against the corresponding EC concentration.

The linearity and sensitivity were determined based on the linear regression results. Linearity was determined by the coefficient of correlation, R-squared (R^2), of the generated calibration curve. Sensitivity was evaluated by determining the LOD and LOQ, as follow: LOD=3.3 σ/b and LOQ=10 $\sigma/$b, with σ as the standard deviation (SD) of the y-intercept and b the curve slope (106, 107).

The matrix effect (ME) was assessed through the percentage of the quotient between the slopes of the curves obtained from the standards solutions in synthetic wine and the curves obtained by spiking a dry and a sweet fortified wines with the same concentration of EC, by the following equation (108-112):

$$\%\ \mathrm{ME} = \left[\frac{(\text{slope of fortified wine calibration curve} - \text{slope of synthetic wine calibration curve})}{\text{slope of synthetic wine calibration curve}}\right] \times 100$$

Precision was estimated based on inter-day and intra-day analyses of two standard solutions and one fortified wine. Intra-day repeatability was assessed by the response of ten successive extractions. Inter-day reproducibility was assessed by the results of the analysis of five extractions of the same samples on three different days. These parameters were expressed as a percentage of relative standard deviation (RSD).

The accuracy of the method was evaluated through a recovery study, by spiking a fortified wine in triplicate, with known amounts of EC at four representative concentration levels (25, 50, 100 and 200 μg/L), within the calibration range. Recovery was calculated by comparing the mean values of the three replicates with the theoretical concentrations of each one.

The method was applied to twenty-four different fortified wines, with ages between less than 1 and up to 86 years-old, in order to confirm the applicability of the proposed RP-LC-MS/MS method for determining EC in fortified wines.

4.3. Results and discussion

LC-MS/MS, combined with MRM, was selected for the quantification of EC in fortified wines due to its high loading capacity, sensitivity and selectivity (113). The LC-MS/MS technique was previously used to quantify EC but with a derivatization step or other extraction techniques during samples preparation (37, 114). To optimize the analytical technique, a solution of EC and BC was directly injected into the LC-MS/MS equipment to determine their retention times; 1.9 and 7.8 min, respectively. The optimal collision energy to quantify EC with BC as internal standard was defined through multiple tests, using the Labsolutions 5.7 software. The elution gradient was optimized with real samples to obtain the best separation and peak resolution.

4.3.1. mLLE method optimization

From the previous survey for solvents free of EC, by Leça *et al* (2014), acetonitrile and ethyl acetate were selected, due to their lower density, to firstly perform a QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction procedure. The QuEChERS approach was first reported by Anastassiades *et al* (2003) and is characterized as user-friendly, inexpensive, effective, robust, secure and with high recoveries. This procedure was firstly chosen to extract the target compound from fortified wines since it has been widely applied to various classes of compounds in several matrices (116). The influence of salts and buffers on the extraction yield was studied. Since they did not increase the extraction yield of EC, these were removed from the extraction procedure and the extraction was transformed into a mLLE, with the advantage of being cheaper and more user-friendly. Acetonitrile and ethyl acetate were both tested. Ethyl acetate was chosen as the extractant solvent rather than acetonitrile since it produces chromatograms with less interferences and better peak shapes.

Acetonitrile extracts increased background noise and originated interferences that coeluted with the EC peak.

To optimize the mLLE extraction, a full factorial design was carried out, considering two factors, the sample and extraction solvent volumes. The optimum conditions were those which maximize the EC chromatographic peak area in the LC-MS/MS equipment. The full factorial design was done considering three levels per factor, namely the sample volume at 8, 10 and 15 mL and the extractant volume at 4, 5 and 8 mL. The two variables analysed revealed to be significant, as well as the interaction factor between them (p-values lower than 0.05). As illustrated by Fig. 4, the maximum EC chromatographic peak area, for the mLLE method, can be achieved using 15 mL of the fortified wine sample combined with 8 mL of ethyl acetate.

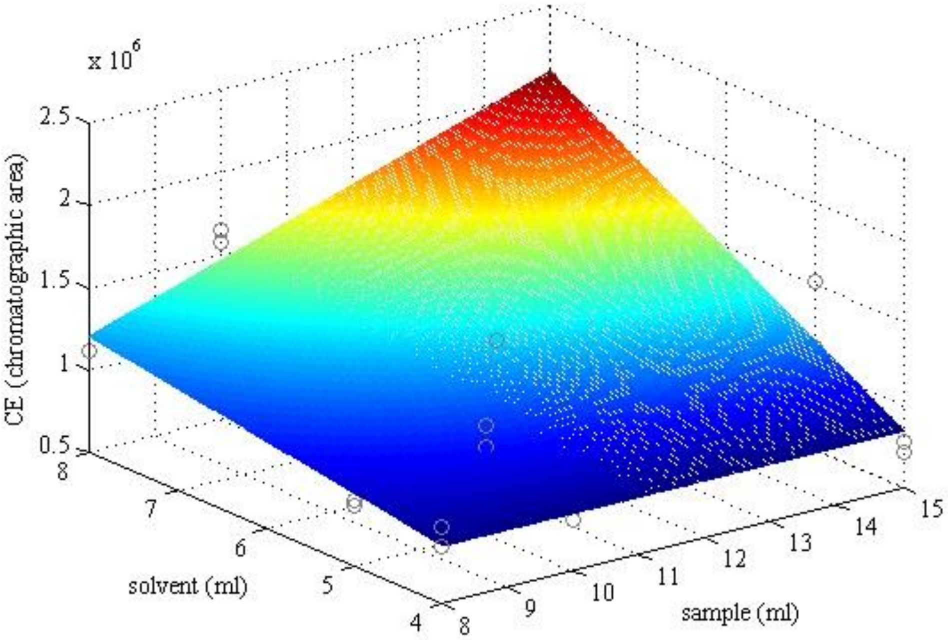


Fig. 4- Full factorial design to optimize the miniaturized LLE procedure with sample volume, extraction solvent volume, and the LC-MS/MS response as variables. The graphic illustrates the variation of the LC-MS/MS response. The raw data that originated this table can be found at the appendix in the Supplementary Table 1.

4.3.2. mLLE method validation

The proposed mLLE/RP-LC-MS/MS method to quantify EC in fortified wines showed good selectivity, verified by the absence of interferences at EC and BC retention times in synthetic and real wine samples, as depicted in Fig. 5.

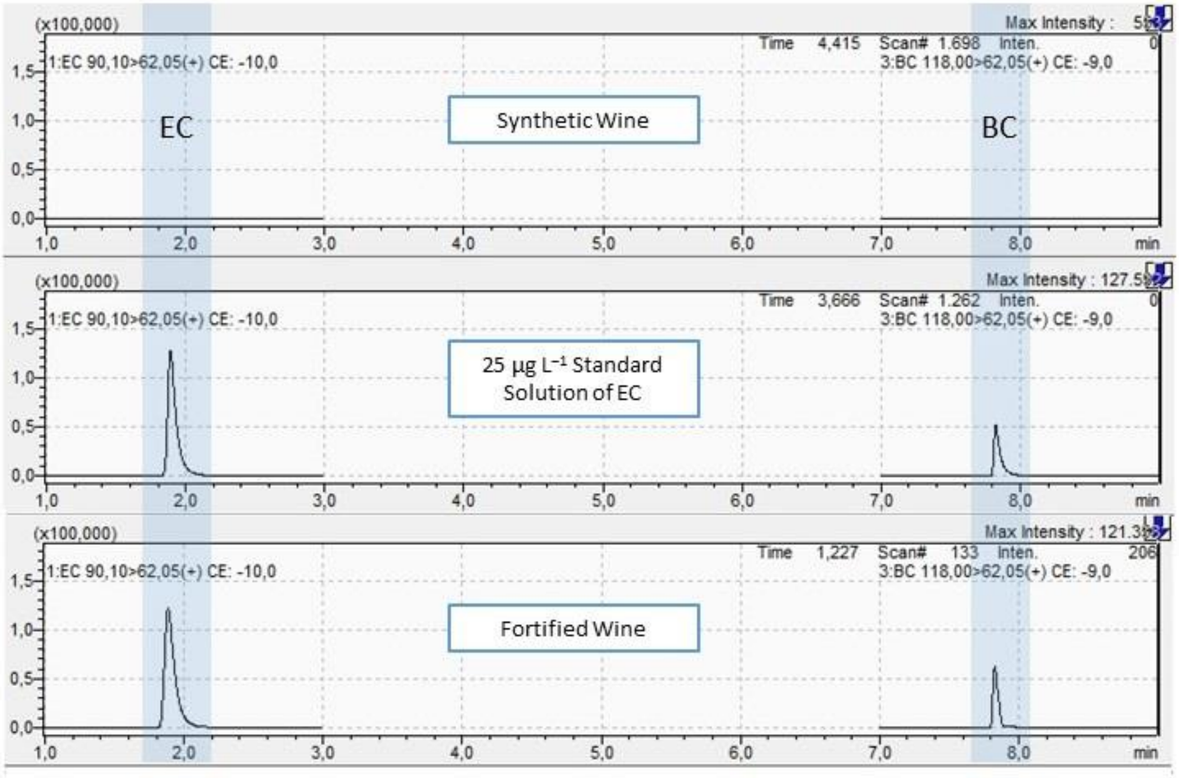


Fig. 5 - Typical chromatograms of synthetic wine used to generate the calibration, 25 µg/L standard solution and fortified wine. EC - ethyl carbamate, BC - butyl carbamate (internal standard).

It is recognized that the matrix effect can compromise the results of an analytical method, especially when the intention is to analyse samples of high complexity, such as fortified wines. Thus, the coefficient of variation between the slopes of the synthetic wine calibration curve and those of the spiked dry and sweet fortified wines was 3.4 and 2.5%, respectively (curves are almost parallel). These results show that the differences between the curves obtained using the synthetic and real wine matrices are negligible.

Table 3 summarizes the method validation results. A single calibration curve was generated by the average response of eight concentration levels, prepared in synthetic wine. Each level was represented by three extracts, injected twice. A good correlation coefficient was obtained, $R^2 = 0.9999$, confirming the linearity of the method. The LOD and LOQ results determined by the calibration curve, 0.17 µg/L and 0.52 µg/L, respectively, are close to and, in most cases, lower than those found in literature (12, 38-41, 43, 101, 117 , 118-120), conferring a good sensitivity to the developed method. The method also revealed a good repeatability (1.6 – 5.6%) and reproducibility (2.1 – 8.0%) that never exceeded 8% of RSD. In turn, the results of the recovery study ranged between 93 and 114%, demonstrating the accuracy of the method.

Table 3 - Validation results obtained for the proposed mLLE/RP-HPLC-MS/MS method.

Parameter	Result
Linear regression	$A_{rel} = 0.1706[EC] + 0.0438$
Linear concentration range	1.0-200 µg/L
R^2	0.9999
LOD (µg/L)	0.17
LOQ (µg/L)	0.52
Recovery	%
FW + 25 µg/L of EC	93
FW + 50 µg/L of EC	113
FW + 100 µg/L of EC	113
FW + 200 µg/L of EC	114

A_{rel} – Relative area (EC peak area / BC peak area); [EC] – EC concentration in µg/L; LOD - limit of detection; LOQ- limit of quantification; FW - fortified wine; EC – ethyl carbamate

Regarding the applicability of the proposed mLLE/RP-LC-MS/MS method for the determination of EC in fortified wines, a set of twenty-four fortified wines were analysed. The results are shown in Table 4. The resolution, selectivity and precision of the obtained chromatograms and corresponding results confirm the applicability of the proposed method to quantify EC in fortified wines. The quantified concentrations varied from 23 ±1 µg/L to 194 ±5 µg/L, showing that the developed method covers the usual concentration range of this compound found in fortified wines (Table 4). Despite the wide range of ages of the analysed sample set, a direct correlation between EC values and wine age was not found. The chosen sample set had methodology validation as its only purpose.

Table 4 - Application of the proposed mLLE/RP-HPLC-MS/MS method for the quantification of EC in 24 fortified wines of different ages.

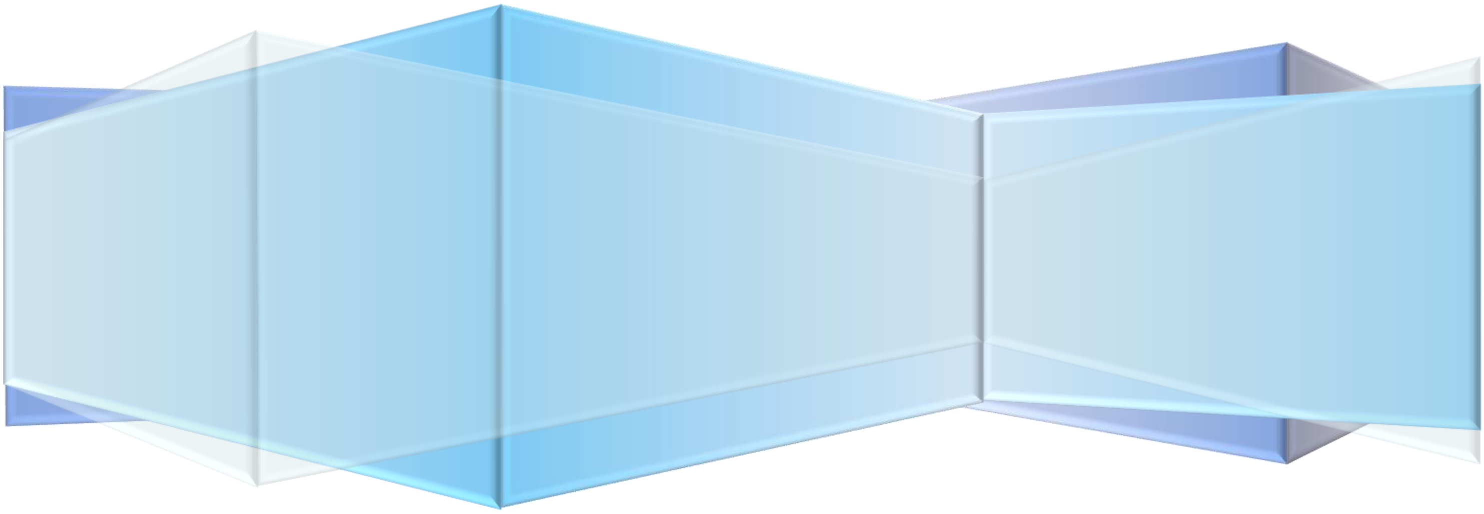
Fortified wine Sample	Wine age (years)	Concentration (µg/L)	SD (n=6)
FW1	unk	194	5
FW2	unk	42	2
FW3	unk	26	1
FW4	< 1	13.4	0.9
FW5	< 1	16.4	0.3
FW6	< 1	32.1	0.6
FW7	< 1	49	2
FW8	3	24	1
FW9	3	23	1
FW10	5	89	2
FW11	3	43.9	0.5
FW12	3	57	2
FW13	3	110	4
FW14	3	103	3
FW15	3	98.7	0.9
FW16	3	23	1
FW17	3	24.7	0.2
FW18	5	131	7
FW19	5	77	2
FW20	5	84	2
FW21	5	75	2
FW22	17	150	3
FW23	20	61.8	0.9
FW24	86	177	11

FW - fortified wine; SD - standard deviation; unk - unknown

4.4. Conclusion

A simple and sensitive method based on mLLE and RP-LC-MS/MS was successfully developed for the determination of EC in fortified wine samples, without resorting to derivatization. The mLLE optimization was achieved, ensuring a good compromise between sensitivity and low usage volumes. The proposed method shows good linearity, sensitivity, selectivity, precision and accuracy. The mLLE provides a favourable extraction of EC in less than 15 min and a LC-MS/MS analysis in 18 min. The method was successfully applied to determine the concentration of EC in twenty-four fortified wines confirming its suitability. This method greatly simplifies EC determination in wine and it will be used for the general EC determinations of the study developed and described in this thesis.

PART III – ETHYL CARBAMATE IN MADEIRA WINE



5. A SURVEY OF ETHYL CARBAMATE IN STORED AND COMMERCIAL MADEIRA WINES

No representative data is available about the presence of EC in Madeira wines. In this study, the EC content was analysed in a representative sample set of Madeira wines, composed by one hundred and eight samples from different producers and with different ageing times, varying from 3 to 41 years, including wines stored in local wineries and others commercially available. The concentration of EC in all samples varied from 12.6 ±0.3 µg/L to 422 ±21 µg/L and the average EC content was: 40.9 µg/L in the group of samples with 3 years of ageing, 69.7 µg/L in samples with 5 years of ageing, 147.8 µg/L in samples aged for 10 years and 271.4 µg/L in the samples with more than 10 years of ageing (from 12 to 41 years). The results highlight that, in average, older wines tend to have higher values of EC and along with maturation time this can also be justified by the strict control and new technologies used nowadays in Madeira wine production. Some wines exceed the values imposed by some markets and, therefore, EC formation in fortified wine should be studied in further detail.

5.1. Introduction

Fortified wines are produced by the fortification of unfermented, partially fermented or completely fermented grape must with wine-based spirit. Madeira wine is a Portuguese fortified wine, made in the Autonomous Region of Madeira through unique and characteristic winemaking and ageing processes (121).

EC is a known genotoxic carcinogen compound that naturally occurs in fermented foods and beverages. A considerable number of studies revealed its presence specially in alcoholic beverages. According to Reader *et al* (2003), in a study using table wine with 12% ethanol content, with common storage temperatures and concentrations of urea, significant amounts of EC can be produced. Factors such as time, temperature, acidity and yeasts species affect the occurrence of this compound (47). Urea and Cit, the most common EC precursors, can be generated during the fermentation step (13). Tegmo-Larsson *et al* (1990) verified that

EC accumulation during storage time of red, white and fortified wines increases with time and temperature. Later, Hasnip *et al* (2004) observed that the formation of EC is proportional to the concentration of urea, Cit and ethanol.

From an alcoholic beverages survey in Zhejiang province (China), it was observed in non-fortified rice wines EC contents up to about 500 µg/L (124). In Sherry wines fermented with *Saccharomyces cerevisiae*, made from Muscat grapes and aged for 28 months in oak casks, EC concentrations up to 156 µg/L were verified (125). In Madeira wine, Perestrelo *et al* (2010) verified, with a non-representative sample set of twenty monovarietal wines from different harvests (1990–2006), that 50% of the analysed samples exceeded the value of 100 µg/L.

The aim of this study was to identify and quantify EC in a representative sample set of Madeira wines, from different producers, including commercial and stored wines from different grape cultivars, with different ageing times and wine styles.

5.2. Materials and methods

5.2.1. Ethyl carbamate determination

The methodology used to quantify EC in the studied fortified wines was the one proposed by Leça *et al* (2017), previously described in section 4.

5.2.2. Samples

A total of one hundred and eight samples of Madeira wine from different producers, including sweet, medium-sweet, medium-dry, and dry styles were analysed. This sample set included commercial wines as well as wines stored in local wineries. The wines described were produced in Madeira island from the authorized *Vitis vinifera* L. varieties. The most common white varieties are Sercial, Verdelho, Boal and Malvasia and the most used red variety is Tinta Negra, which represents a big portion of all grape production. These samples include blends and monovarietal wines. Some of the wines were aged only through the *canteiro* process, in which the wines were directly placed into oak casks at wineries, while others were previously submitted to the *estufagem* traditional ageing process. In that process, wines are kept in stainless steel vats for four months and heated at 45 °C, before being moved to the oak casks as mentioned before. The alcohol content of these wines was up to 22% (v/v) and the ageing time varied from 3 to 41 years. This sample set intended to be representative of Madeira wine.

5.3. Results and discussion

As previously mentioned, EC is a naturally occurring compound in alcoholic beverages that accumulates during the fermentation process and subsequent storage. The concentrations of EC in this sample set of Madeira wines are presented in Fig. 6 and were obtained by mLLE with RP-LC-MS/MS separation, identification, and quantification.

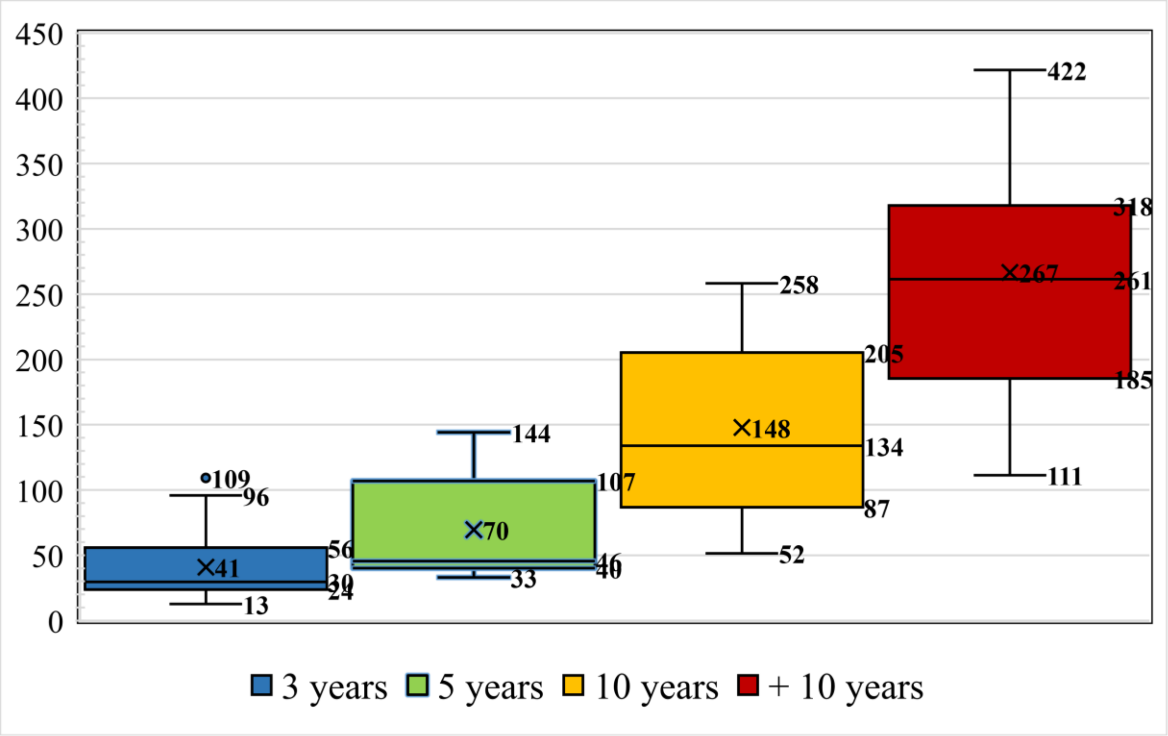


Fig. 6 - Concentrations of EC in stored and commercial Madeira wines with 3 to 41 years of ageing. The raw data that originated this table can be found at the appendix in the Supplementary Table 2.

The concentrations of EC in all one hundred and eight samples varied from 12.6 ±0.3 µg/L to 422 ±21 µg/L. More precisely, in the group of samples with 3 years of ageing, the concentration of EC varied from 12.6 ±0.3 µg/L to 114 ±11 µg/L with a medium value of 40.9 µg/L. In the category of wines with 5 years of ageing, the concentration of EC ranged from 34 ±3 µg/L to 144 ±3 µg/L with a medium value of 69.7 µg/L. In the case of wines that were aged for 10 years, the concentration of EC varied between 52 ±3 µg/L and 258 ±8 µg/L with a medium value of 147.8 µg/L. Furthermore, in the group of samples with more than 10 years of ageing (from 12 to 41 years), the concentration of EC ranged from 111 ±3 µg/L to 422 ±21 µg/L with the highest medium concentration of EC, 271.4 µg/L. In past studies, Ferreira *et al* (1992) analysed three samples of Madeira wine made from Tinta Negra, throughout the *estufagem* process (120 days heated at 45°C), and the EC content reached an overall maximum value of 60.5 µg/L, in the analysed dry wine type. Perestrelo *et al* (2010) analysed twenty monovarietal fortified Madeira wines and the EC concentrations varied from 54.1 to 162.5 µg/L.

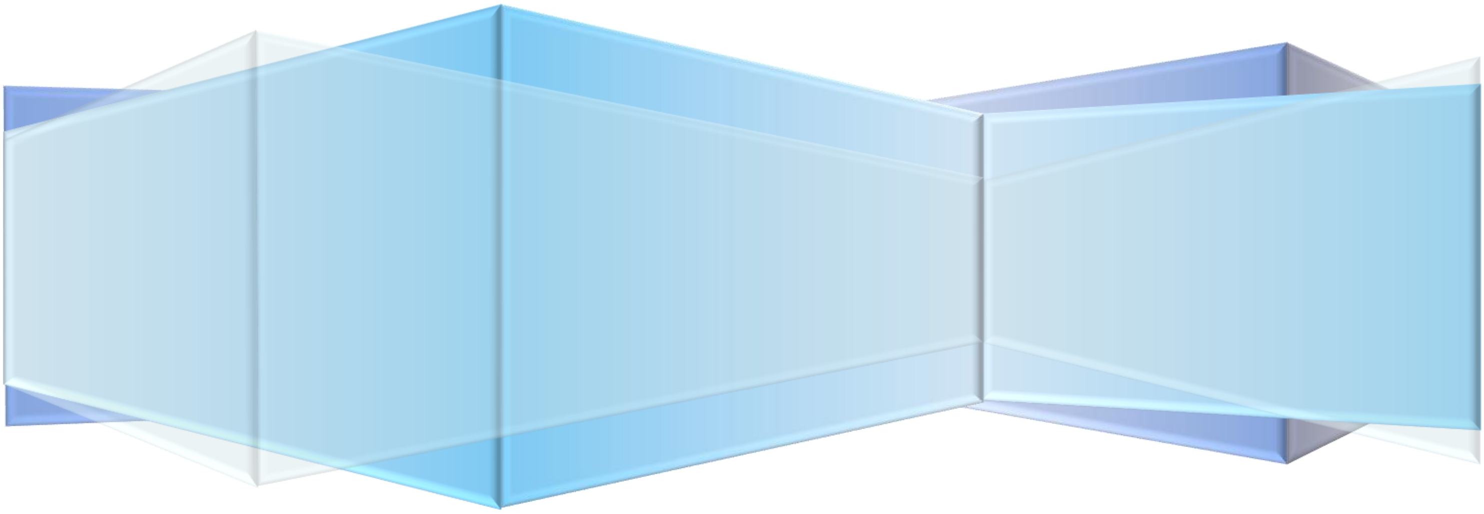
Madeira wine vinification practices have changed over time. This study includes four decades of wine production and takes into account the variability of commercial and non-commercial wines. In Fig. 6 it is possible to observe a tendency towards the increase of EC concentration with the ageing time, despite not linear. These results are in agreement with data obtained in similar studies that concluded that EC concentration increases as a function of time (22, 122, 123). The variation of EC values within samples of the same age can be due to grape cultivar, time, type of ageing and vinification conditions, as statistically verified by Ruiz-Bejarano *et al* (2015). The lower average concentration of EC in the youngest samples should also be associated with the evolution of winemaking practices (as mentioned in section 3.4), which tends to increase the quality of wine and possibly decrease the concentration of EC.

Despite not contemplating the calculations for alcoholic beverages, the benchmark dose lower limit of EC is 0.3 mg/kg per day and the mean intake from food is approximately 15 ng/kg per day. Although Canadian authorities set limits for EC according to the average consumption rate of each beverage type, a legislation on their limit concentrations worldwide is not yet consensual. Although we recognize that some fortified wines with higher levels may appear, most of them are well below the values found in other drinks with currently higher consumption, such as distilled spirits (0.01 to 12 mg/L) (11, 12, 28, 29).

5.4. Conclusion

The present study revealed EC levels in a representative sample set of Madeira wine, which varied from 12.6 ±0.3 µg/L to 422 ±21 µg/L. In the sample groups of wines with 3 and 5 years of ageing the average concentration of EC was below 100 µg/L. In wines that were aged for 10 years and more than 10 years the EC average concentration was 148 µg/L and 271 µg/L, respectively. The results highlight that older wines tend to have higher values of EC and this can be justified by the maturation time, but also by the fact that their production was not monitored as it happens nowadays, in a process that follows strict criteria and is necessary to produce this beverage, that has a considerable alcohol content and a traditional ageing procedure in which temperature is applied. Thus, EC formation in Madeira wine should be studied in further detail. EC occurrence in fortified wines must be understood and mitigated and its main factors and mechanisms should be identified. It is also equally important to develop viable strategies for its control, mitigation and, if possible, its extraction from older wines without impacting their quality.

PART IV – EVOLUTION OF ETHYL CARBAMATE AND ITS PRECURSORS DURING THE AGEING PROCESS: *CANTEIRO* VS *ESTUFAGEM*



6. EVOLUTION OF ETHYL CARBAMATE, UREA, CITRULLINE AND ARGININE IN MADEIRA WINES AGEING: 3 YEARS STUDY

In this study, the evolution of EC, urea, Cit and Arg in Madeira wines were analysed during 3 years of ageing. The wines were produced from the red variety Tinta Negra with two different levels of sweetness: sweet and dry, and from two white varieties: Malvasia (sweet) and Sercial (dry). Each wine was separately aged by the two traditional ageing processes, *estufagem* and *canteiro*. Samples were analysed by in-house validated methods and results revealed that the sweet red wines, when submitted to *estufagem*, are the ones with higher propensity to form EC. Its concentration increases along ageing and the wine fraction that undergoes *estufagem* always has a superior EC content. After the *estufagem*, just one of the six wines exceeded 100 µg/L, at 3 years of ageing. The first year of ageing was the period with more EC formation for all wines, varying between 16.3 ±0.5 µg/L and 114 ±3 µg/L, coinciding with the phase in which urea becomes undetectable or unquantifiable. The studied *Vitis vinifera* L. white varieties, Malvasia and Sercial, promote less EC occurrence and these wines are not so significantly affected by *estufagem*, when compared to Tinta Negra. In some wines, urea and Cit become not detectable or not quantifiable but EC continues to be formed, although in a considerably slower rate. Therefore, other not reported reactional mechanisms or physicochemical characteristics may influence EC occurrence in the long-term ageing of Madeira wines, some of which will be explored in upcoming sections (7 and 8).

6.1. Introduction

EC is a naturally occurring compound in alcoholic beverages, known as a probable carcinogen. A previously detected issue in EC occurrence is the fact that its concentration increases in wines during storage and ageing. Also, storage conditions are known to have an impact on the final EC content at the moment of commercialisation. Tegmo-Larsson *et al* (1990) studied the increase of EC during wine storage, up to 12 months, with different temperatures and verified that, generally, EC increases as a function of time and temperature.

The major reported precursors of EC in wine are urea and Cit, both by-products of fermentation derived from Arg metabolism, performed by *Saccharomyces cerevisiae* and lactic acid bacteria (128, 129). Therefore, Arg is considered a pertinent metabolite to EC occurrence, only when metabolized by fermentative microorganisms (1).

Monteiro *et al* (1989) studied the EC produced by the reaction of urea with ethanol using wines and model solutions. Radioactively labelled urea was used to confirm this formation pathway of EC. It was verified that this reaction is dependent of urea and ethanol concentrations and also of the solution temperature. Azevedo *et al* (2002), in a study conducted in 15% (v/v) ethanol model wines, concluded that EC formation is directly related to the increase of Cit concentration in the medium. Later, Hasnip *et al* (2004) confirmed these results in a 3-year study on the impact of time and temperature on the concentration of EC. Kinetic analyses were performed along the formation of EC which occurred simultaneously to the decay of urea and Cit contents throughout the years. The authors developed an equation to predict EC content in wines for a specific time and temperature profile and assumed that further studies were required to complete its validation.

High alcohol content, application of temperature during the *estufagem* process and long-term ageing and storage are part of the traditional and unique characteristics of the Madeira wine vinification and ageing processes, which can promote the occurrence of EC. The objective of this study was to analyse EC evolution in comparation with its principal precursors, urea and Cit and a relevant compound, Arg, in Madeira wines during 3 years of ageing. The wines analysed were made from the red variety Tinta Negra with two different levels of sweetness, sweet and dry, and two white varieties, Malvasia (sweet) and Sercial (dry). The wines were compared by their level of sweetness and type of ageing processes, *estufagem* or *canteiro*.

6.2. Materials and methods

6.2.1. Ethyl carbamate determination

The methodology used to quantify EC in the studied fortified wines was the one proposed by Leça *et al* (2017), previously described in section 4.

6.2.2. Urea determination

Quantification of urea was based on Zhang *et al* (2014), with some modifications described in more detail in the following subsections. The applied method uses RP-HPLC with FLD, with previous 9-xanthydrol derivatization of urea in the HPLC loop. Three replicates of samples and standards were analysed in duplicate. The calibration range linearity was good ($R^2 = 0.997$) with a LOQ of 0.18 mg/L and recovery rates between 89 and 98%. The intra- and inter-day precision never exceeded 11% of RSD.

6.2.2.1. Standards and reagents

Urea standard (purity > 99%) was obtained from Sigma–Aldrich (St. Louis, MO, USA). 9-xanthydrol (99%) was purchased from Acros Organics (Geel, Belgium), 1-propanol (99.5%) from Lab-Scan (Gliwice, Poland), acetonitrile HPLC gradient grade from Fisher Scientific (Leicestershire, UK) and hydrochloric acid (p.a.) from Riedel-de Haën (Seelze, Germany). Absolute ethanol, tartaric acid (98%), formic acid (98%) and methanol (UPLC grade) were from Panreac (Barcelona, Spain). Ultra-pure water (type 1) was obtained from a Simplicity UV apparatus from Millipore (Milford, MA, USA).

Calibration solutions, 0.25; 0.5; 1; 5 and 10 mg/L, were obtained by successive dilutions of urea stock solution (50 mg/L) in synthetic wine (6 g/L of tartaric acid, 18% of ethanol and pH 3.50). Each calibration point was prepared in triplicate and analysed twice, within the range of 0.25 – 10.0 mg/L. All samples and calibration points were filtered through Chromafil PTFE 0.2 µm syringe filters (Macherey-Nagel, Düren, Germany) and all eluents through a 0.2 µm pore size PTFE membrane filter (Pall Corporation, Ann Arbor).

6.2.2.2. Apparatus and chromatographic conditions

Urea was analysed in an Alliance liquid chromatograph from Waters (Milford, MA, USA) equipped with an auto-injector (Waters 2695, separations module) and a Multi λ Fluorescence detector (Waters 2475). The data acquisition and processing were performed in the Empower Pro software. The derivatization solution was injected and separated in a Kinetex C18 column, 150 × 4.6 mm, 5 μm, 100 Å, from Phenomenex (Torrance, CA, USA), maintained at 30 ºC. The mobile phase was eluted at 1 mL/min, varied in gradient mode, with 1% of formic acid (solution A) and methanol (solution B), as follow: solution A was maintained at 60% for 1 min and then changed to 41% in 8.6 min. In the following 2.4 min, solution A was altered to 0% and maintained for another minute. Finally, solution A was increased to 60% in 1 min to prepare the next injection. Fluorescence excitation and emission wavelengths were set to 213 and 308 nm, respectively.

6.2.2.3. Derivatization

The derivatization reaction was performed in the HPLC injection loop according to the following sequence: 10 μL of sample/standards, followed by 10 μL of 4 g/L 9-xanthydrol solution (dissolved in 1-propanol), 8 μL of 1.5 M hydrochloric acid and 10 μL of acetonitrile. The derivatization mixture was kept into the loop for 15 min and the total reaction volume was then loaded into the column. All solutions used for derivatization were filtered through Chromafil PTFE 0.2 μm syringe filters.

6.2.3. Arginine and citrulline determination

The analysis of Arg and Cit in the sample set of fortified wines was based on Pereira *et al* (2015), which uses an in-loop ortho-phthalaldehyde (OPA)/ mercaptoethanol (MCE)/ iodoacetic acid (IDA) derivatization followed by RP-HPLC-FLD. Some modifications were introduced and are briefly explained bellow. Three replicates of samples and standards were analysed in duplicate. In terms of analytical re-validation of this in-house method, the LOQ was 0.83 mg/L for Arg and 0.46 mg/L for Cit. The Arg linear range has a R^2= 0.999, repeatability and reproducibility that never exceeded 13% of RSD with a recovery between

86 and 99% at different levels of the concentration range. Also, good results were obtained for Cit with a R^2= 0.998, the inter-day and intra-day precision never exceeded the 8% of RSD and the recovery ranged between 86 and 97%.

6.2.3.1. Standards and reagents

Boric acid (99.5%) was purchased from Merck Co. (Darmstadt, Germany). 2-mercaptoethanol (99%) and OPA (p.a.) were obtained from Acros Organics (Geel, Belgium) and hydrochloric acid (p.a.) from Riedel-de Haën (Seelze, Germany). Methanol (UPLC grade), potassium hydroxide (95%), potassium di-hydrogen phosphate (99%), ethanol (p.a.), tetrahydrofuran (99.5%) and iodoacetic acid (99%) were supplied by Panreac (Barcelona, Spain).

Arg and Cit (both with a purity grade > 97%) were obtained from Sigma–Aldrich (St. Louis, MO, USA) and individual stock solutions of 10 g/L were prepared by dissolving each amino acid in 0.1 M HCl. A working solution was prepared by diluting both amino acids to 300 mg/L, which was then used to prepare the calibration points (1, 2.5, 5, 20, 50, 100 and 300 mg/L). All samples and calibration points were filtered through Chromafil PTFE 0.2 µm syringe filters.

6.2.3.2. Equipment and chromatographic conditions

The previously described Waters HPLC system was used. Derivatized standards and samples were separated using a XBridge C18 RP column, 150 × 2.1 mm, 3.5 µm, from Waters (Milford, MA, USA), maintained at 45 ºC. A gradient elution was performed at 0.3 mL/min, using solution A: 1% tetrahydrofuran, 8% methanol and 91% 10 mM phosphate buffer (pH 8.0) and solution B: 100% methanol. The gradient started with the solution A kept at 100% for 14 min, changed to 50% for another 14 min, and, then, decreased to 10% in the next 12 min. Solution A was finally increased to 100% in 3 min, to prepare the next injection. All eluents were filtered through a PTFE 0.2 µm pore size membrane filter (Pall Corporation, Ann Arbor). Fluorescence excitation and emission wavelengths were set to 335 and 440 nm, respectively.

6.2.3.3. Derivatization

Before derivatization, 100 µL of each sample/standard were diluted in 1 mL of 400 mM borate buffer solution (pH 10.5), homogenized and filtered in a PTFE 0.2 µm syringe filter. Samples with Arg concentrations between 300 and 600 mg/L were diluted in half, between 600 and 900 mg/L were diluted for one third and samples with concentrations above 900 mg/L were diluted for one quarter, before being dissolved in borate buffer. The IDA/OPA/MCE derivatization reaction was made in the sample injection loop according to the following sequence: 5 µL of buffered sample followed by 5 µL of IDA solution and 10 µL of OPA/MCE solution. The total reaction volume (20 µL) was kept in the loop for 2 min and then loaded into the column.

6.2.4. Samples

The sampling was conducted with experimental wines obtained from three *Vitis vinifera* L. varieties: one red variety Tinta Negra with two different levels of sweetness, sweet (TNSweet) and dry (TNDry) and two white varieties Malvasia (sweet) and Sercial (dry), as summarised in Fig. 7. Four wines of two different sweetness categories were produced from Tinta Negra: two sweet wines, TNSweet1 and TNSweet2, and two dry wines, TNDry1 and TNDry2. Malvasia was used to obtain a sweet wine and Sercial was used to produce a dry wine, as defined by the regulatory institution of Madeira wine, IVBAM.

At least 1000 kg of grapes were used at a local winery to make each of the previously described wines, according to its own practices. Commercial yeasts were not added during the fermentation process and grape skins, together with the grape juice, followed 24 hours of maceration. Pectinases and diammonium phosphate were added to the musts. A solution of 10% sulphite was also used to obtain a concentration of about 60 mg/L in must solution. After the separation of the skins, the fermentative process was controlled below 25 ºC, in stainless steel tanks. The fermentation process was stopped, at different stages, by the addition of vinous alcohol (containing 95% (v/v) of ethanol), according to the grape variety and the desired wine style, as previously described in section 3.2. The alcohol content was adjusted to about 17% (v/v). In sweet wines, the fermentation was stopped at 1045 mg/L for

TNSweet1, at 1037 mg/L for TNSweet2 and at 1046 mg/L for *Malvasia*. Concerning dry wines, the fermentation was interrupted at 999 mg/L for TNDry1, 1010 mg/L for TNDry2 and 1000 mg/L for *Sercial*.

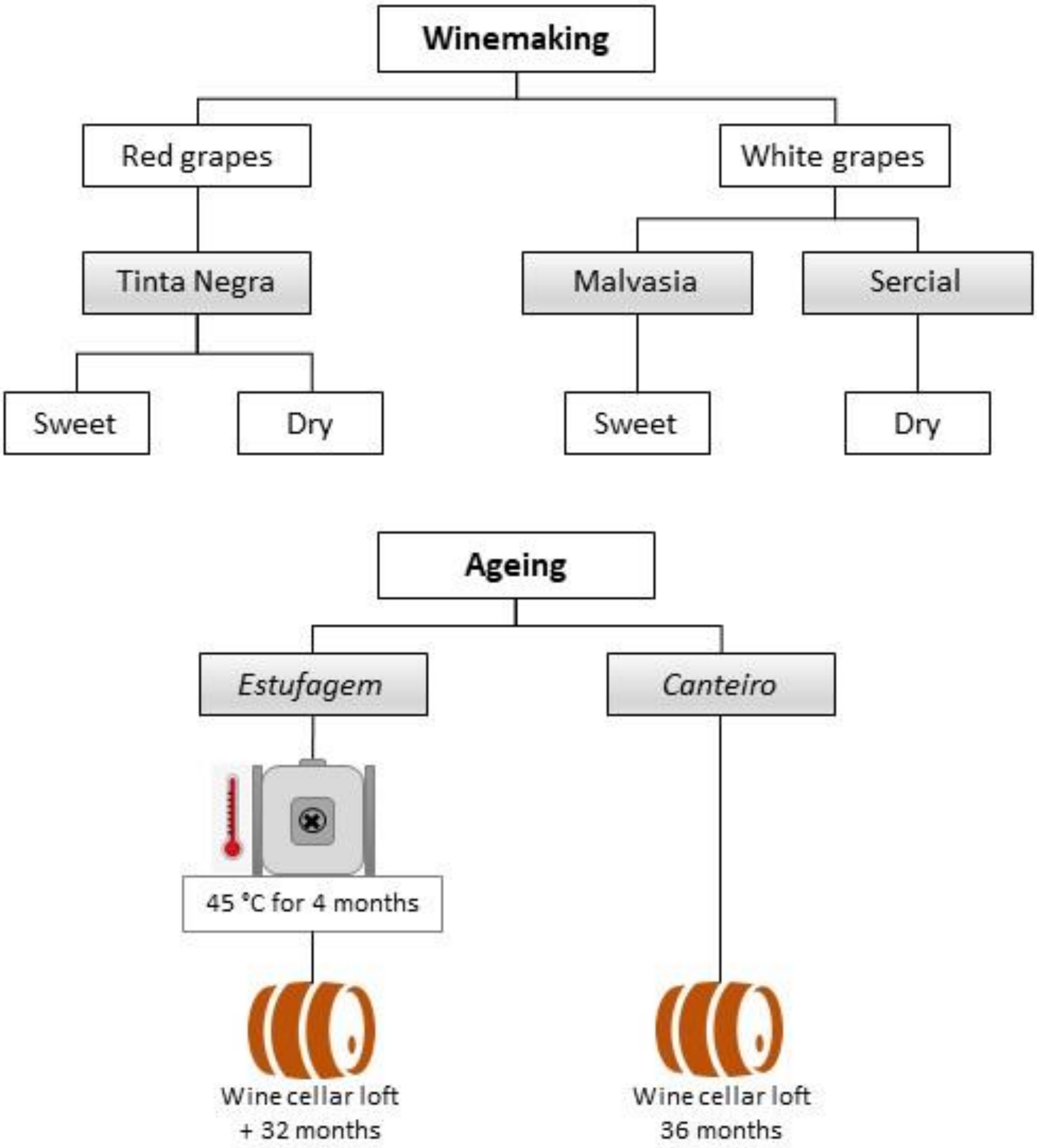


Fig. 7 – Schematic representation of the winemaking and wine ageing processes of the studied Madeira wines.

After fortification, wines were clarified and stabilized through bentonite clays and albuminocol gelatines, common post-fermentation treatments. Each wine previously described was divided in two fractions of 200 L. One fraction was directly stored in oak casks to age through the *canteiro* process, while the other fraction was submitted to *estufagem* (Fig. 7), in a pilot-scale system equipped with 200 L stainless steel vats, maintained at 45 °C for 4 months, before undergoing *canteiro* ageing. The experiment was followed up until 36 months of ageing (3 years) and samples were collected in duplicate at different stages of the process: 0, 6, 12, 24 and 36 months. The samples were then stored at -26 °C before analysis.

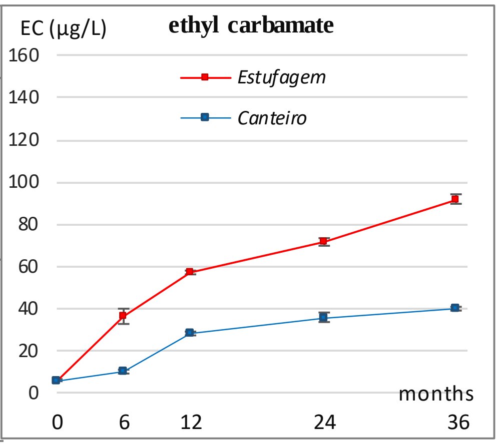
6.3. Results and discussion

In this study, the evolution of EC, urea, Cit and Arg in Madeira wines was evaluated during 36 months of ageing (3 years), with all the samples being analysed at different stages of the process. The wines were separated in sweet and dry categories and each one was aged by the two traditional ageing processes: *estufagem* and *canteiro*. The sweet Madeira wines were made from Malvasia and Tinta Negra, the dry wines were produced with Sercial and Tinta Negra, all *Vitis vinifera* L. varieties.

The results are presented in Fig. 8 and Fig. 9 and it was verified that the sweet red wines submitted to *estufagem* are the ones that have more propensity to form EC, reaching values of 92 ±2 µg/L and 149 ±11 µg/L. Urea, Arg and Cit concentrations decrease along the ageing process. There is a clear tendency to the increase of EC concentration during ageing and the fraction that undergoes *estufagem* always has a superior EC content, which was expected, taking into account that this process accelerates ageing and that temperature increases the EC formation kinetics (21, 123). *Estufagem* occurs during the first four months (at 45 ºC) and, in the analyses performed at 6 months of ageing, the concentration of EC was 9 to 54 µg/L higher when compared to the same wine, only submitted to *canteiro*. However, after the *estufagem* process, most sweet wines and all dry wines do not exceed 100 µg/L at 3 years of ageing. Cit is exclusively detected in red dry wines (Fig. 9), being this the only wine category where this compound influences EC occurrence. Tinta Negra sweet wines have more Arg than dry wines and this can be due to amino acid consumption during fermentation (129).

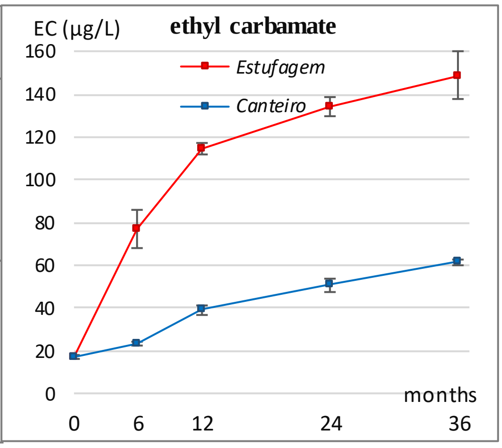
TNSweet1

	months	urea		citrulline		arginine	
		CC (mg/L)	SD	CC (mg/L)	SD	CC (mg/L)	SD
Esfufagem	0	0.81	0.02	n.q.		83.9	0.9
	6	n.d.		n.q.		74.7	0.7
	12	n.d.		n.q.		61	3
	24	n.d.		n.q.		52	1
	36	n.d.		n.q.		50.2	0.4
Canteiro	0	0.81	0.02	n.q.		83.9	0.9
	6	0.58	0.03	n.q.		78	4
	12	n.q.		n.q.		59	1
	24	n.q.		n.q.		59	5
	36	n.d.		n.q.		58	4



TNSweet2

	months	urea		citrulline		arginine	
		CC (mg/L)	SD	CC (mg/L)	SD	CC (mg/L)	SD
Esfufagem	0	0.23	0.02	n.q.		30.6	0.1
	6	n.q.		n.q.		30.0	0.1
	12	n.q.		n.q.		25.9	0.9
	24	n.q.		n.q.		26.5	0.8
	36	n.d.		n.q.		25.6	0.8
Canteiro	0	0.23	0.02	n.q.		30.6	0.1
	6	n.q.		n.q.		28.9	0.1
	12	n.q.		n.q.		20.7	0.2
	24	n.q.		n.q.		20.4	0.1
	36	n.d.		n.q.		19.8	0.5



Malvasia

	months	urea		citrulline		arginine	
		CC (mg/L)	SD	CC (mg/L)	SD	CC (mg/L)	SD
Esfufagem	0	0.38	0.03	n.q.		10.9	0.2
	6	0.19	0.01	n.q.		9.2	0.2
	12	n.q.		n.q.		8.9	0.2
	24	n.d.		n.q.		8.0	0.2
	36	n.d.		n.q.		7.4	0.2
Canteiro	0	0.38	0.03	n.q.		10.9	0.2
	6	n.d.		n.q.		9.1	0.1
	12	n.d.		n.q.		7.9	0.4
	24	n.d.		n.q.		7.5	0.2
	36	n.d.		n.q.		7.1	0.0

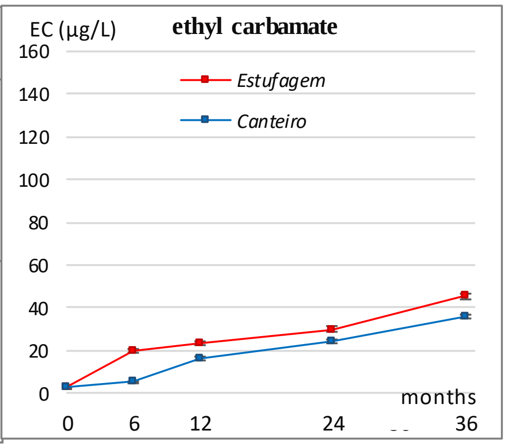
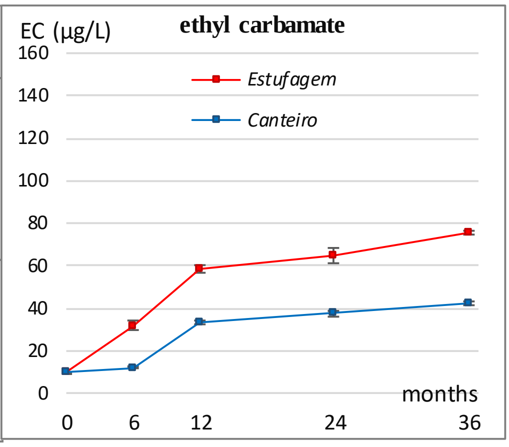


Fig. 8 - Evolution of EC, urea, citrulline and arginine in sweet Madeira wines, made from two *Vitis vinifera* L. varieties, Malvasia (white variety) and Tinta Negra (red variety), aged by the two traditional ageing processes, *estufagem* (120 days heated at 45°C) and *canteiro*, for 3 years. Mean value (n = 6), n.d. - not detected, n.q. - not quantifiable.

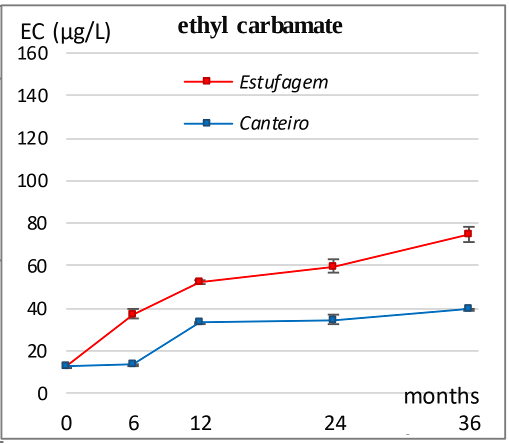
TNDry1

	months	urea		citrulline		arginine	
		CC (mg/L)	SD	CC (mg/L)	SD	CC (mg/L)	SD
Esfufagem	0	0.20	0.01	1.54	0.07	13	1
	6	n.q.		1.5	0.1	10.5	0.2
	12	n.q.		1.5	0.1	10.3	0.2
	24	n.d.		1.19	0.09	10.1	0.1
	36	n.d.		1.10	0.03	10.1	0.2
Canteiro	0	0.20	0.01	1.54	0.07	13	1
	6	n.q.		1.39	0.07	11.1	0.2
	12	n.q.		1.35	0.08	10.5	0.1
	24	n.d.		1.23	0.06	10.3	0.2
	36	n.d.		1.21	0.06	10.1	0.2



TNDry2

	months	urea		citrulline		arginine	
		CC (mg/L)	SD	CC (mg/L)	SD	CC (mg/L)	SD
Esfufagem	0	n.d.		1.0	0.1	10.7	0.1
	6	n.d.		n.q.		5.47	0.07
	12	n.d.		n.q.		4.45	0.04
	24	n.d.		n.q.		4.42	0.08
	36	n.d.		n.q.		4.3	0.1
Canteiro	0	n.d.		1.0	0.1	10.7	0.1
	6	n.d.		n.q.		6.24	0.03
	12	n.d.		n.q.		5.66	0.08
	24	n.d.		n.q.		5.6	0.1
	36	n.d.		n.q.		5.7	0.2



Sercial

	months	urea		citrulline		arginine	
		CC (mg/L)	SD	CC (mg/L)	SD	CC (mg/L)	SD
Esfufagem	0	0.24	0.03	n.q.		3.26	0.08
	6	n.q.		n.q.		2.74	0.03
	12	n.q.		n.q.		2.09	0.03
	24	n.d.		n.q.		1.93	0.07
	36	n.d.		n.q.		1.92	0.02
Canteiro	0	0.24	0.03	n.q.		3.26	0.08
	6	n.q.		n.q.		2.67	0.04
	12	n.d.		n.q.		2.50	0.06
	24	n.d.		n.q.		2.48	0.04
	36	n.d.		n.q.		2.5	0.1

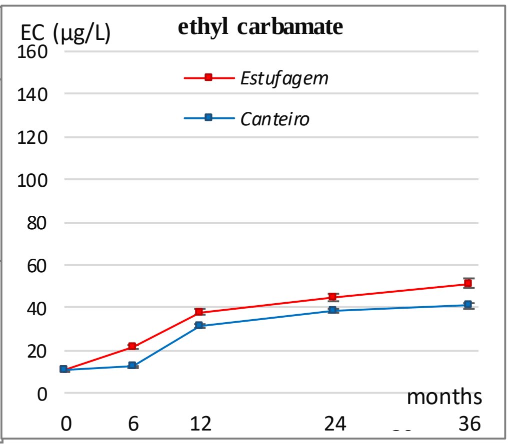


Fig. 9 - Evolution of EC, urea, citrulline and arginine in dry Madeira wines, made from two *Vitis vinifera* L. varieties, Sercial (white variety) and Tinta Negra (red variety), aged by the two traditional ageing processes, *estufagem* (120 days heated at 45°C) and *canteiro*, for 3 years. Mean value (n = 6), n.d. - not detected, n.q. - not quantifiable.

The first year of ageing (12 months) is the period with higher EC formation, from 16.3 ±0.5 µg/L to 115 ±3 µg/L, which coincides with the time when urea becomes not detectable or not quantifiable in all wines. Urea and Cit are assumed as the main precursors of EC in wine but, even when both become not detectable or not quantifiable, EC continues to be formed in all wines. These results showed that other compounds that can act as EC precursors may exist in long term ageing of fortified wines (more than one year of ageing).

In sweet Madeira wines (Fig. 8) it was verified that the white variety, Malvasia, has less tendency to form EC when compared to the red variety, even when the content of urea is higher than those found in TNSweet2, at time 0. The only relevant difference between Malvasia and both Tinta Negra sweet wines is that Arg values are lower in the former. Also, in red wines, the wine that has more urea and Arg (TNSweet1) is not the one with more EC formation. These results rise the possibility of the existence of other compounds or physicochemical characteristics that may influence the EC occurrence in fortified wines.

In dry Madeira wines, TNDry1 starts with 1.54 ±0.07 mg/L of Cit and decreases over 3 years, resulting in 1.10 ±0.03 mg/L with *estufagem*, and 1.21 ±0.06 mg/L with *canteiro*. TNDry2 starts with 0.96 ±0.07 mg/L of Cit and becomes not quantifiable at 6 months, in both ageing processes. Furthermore, urea only appears at the beginning of the ageing process in TNDry1 with 0.20 ±0.01 mg/L. Despite the differences across Tinta Negra dry red wines, it was verified that the evolution of EC is similar in both. Sercial derived wines exhibited more urea and a lower content in Arg and Cit; at 3 years of ageing, those that undergo *estufagem* only contain more 10.2 µg/L of EC, compared to the same at *canteiro*. A similar tendency was observed for sweet Malvasia wines, when comparing *estufagem* and *canteiro*. Overall, white varieties appear to be less problematic in terms of EC occurrence.

6.4. Conclusion

The obtained results indicate that sweet red wines submitted to *estufagem* are the ones that have more propensity to form EC, reaching up to 149 ±11 µg/L. EC concentration increases with the ageing time and wines that undergo *estufagem* always have a superior EC content when compared to the same wines exclusively maturated by the *canteiro* process.

After the *estufagem* process, all dry wines and most sweet wines do not exceed 100 μg/L of EC at 3 years of ageing. Cit only appears in red dry wines and all precursors decrease during ageing. The first year of ageing is the period with more EC formation, up to 114 ±3 μg/L, which coincides with the time interval in which urea becomes not detectable or not quantifiable. White varieties, Malvasia and Sercial, appear to have less tendency to form EC and the *estufagem* does not affect so significantly their EC occurrence as in Tinta Negra wines. The general results reveal that other compounds, besides urea and Cit, or certain specific physicochemical characteristics, may influence EC occurrence in long-term ageing.

PART V – ETHYL CARBAMATE OCCURRENCE IN MADEIRA WINES

Sections 7 and 8 is based on the following publication:

Leça JM, Pereira V, Miranda A, Vilchez JL, Marques JC. New insights into ethyl carbamate occurrence in fortified wines. LWT - Food Science and Technology. 2021.

LWT
Available online 18 May 2021, 111566
In Press, Journal Pre-proof

New insights into ethyl carbamate occurrence in fortified wines

João M. Leça [a,b], Vanda Pereira [b,c], Andreia Miranda [a,b], José Luis Vilchez [d], José C. Marques [a,b,c]

https://doi.org/10.1016/j.lwt.2021.111566

Section 9 is now is based on the following publication:

Leça JM, Pereira V, Miranda A, Vilchez JL, Malfeito-Ferreira M, Marques JC. Impact of Indigenous Non-Saccharomyces Yeasts Isolated from Madeira Island Vineyards on the Formation of Ethyl Carbamate in the Aging of Fortified Wines. Processes. 2021;9:799-809.

Article

Impact of Indigenous Non-*Saccharomyces* Yeasts Isolated from Madeira Island Vineyards on the Formation of Ethyl Carbamate in the Aging of Fortified Wines

João Micael Leça [1,2], Vanda Pereira [2,3,*], Andreia Miranda [1,2,4], José Luis Vilchez [5], Manuel Malfeito-Ferreira [6] and José Carlos Marques [1,2,3]

7. IMPACT OF INDIVIDUAL SUGARS ON ETHYL CARBAMATE FORMATION AND ARGININE AS THE NEW PRECURSOR

The occurrence of EC in fortified wines was studied, testing different model wines under accelerated ageing (45°C for 4 months and 70°C for 1 month), to investigate the possibility of Arg being a direct precursor of EC in fortified wines, comparing it with known major precursors, urea and Cit. Wine main sugars were appraised as possible catalysts, as previously pointed out. Model wines showed that: Arg can induce EC formation under accelerated ageing conditions, even without being previously metabolised to other compounds by microorganisms; although much less reactive, Arg can play a significant role since high residual levels can occur in young fortified wines; Glc and Fru suppress the EC formation from urea and Cit pathways, in about 11 to 26%. Although this was a preliminary study, additional information about the EC formation in fortified wines was obtained, namely for the design of new mitigation strategies, which can pass through the reduction of residual Arg.

7.1. Introduction

In wines, EC is mainly formed after fermentation, by the reaction of carbamoyl compounds with ethanol (13). Urea and Cit have been identified as the most relevant precursors of EC (13, 17, 88, 89). The fermentation conditions impact the concentration of EC precursors in medium, while temperature and maturation time are factors with marked influence on their reaction with ethanol and consequently in the final concentration of EC in wine (17, 89, 90). Hasnip et al (2004) confirmed that EC formation is proportional to the concentration of urea, Cit and ethanol. Urea is mainly formed from Arg metabolization inside of yeasts cells during the early and middle fermentation stages (13). Cit is already present in grape juice, but it can also be generated through Arg anabolism during the fermentation by *Saccharomyces cerevisiae*, wherein ornithine and carbamoyl phosphate react to form Arg, with Cit acting as an intermediate product of the reaction (18).

Thus, Arg has been considered a preponderant metabolite for EC formation only after its metabolization by fermentative microorganisms (46). Arg is commonly one of the most abundant amino acids in grape juice and its concentration changes between different grape varieties and is also proportional to the increase of nitrogen fertilisation in the vineyard. An important decrease in amino acid levels, including Arg, during fortified wines' accelerated ageing was previously reported (87), suggesting its transformation into new ageing-related metabolites.

The alcoholic beverages industry and FDA committed to lowering EC levels as far as possible (46). Thus, researchers have been motivated to develop strategies for the mitigation of EC in fermented foods and beverages (27). These procedures are recommended to minimize EC content especially in table wines and most involve the reduction of storage time and temperature, the use of acidic ureases and genetic engineering tools focused on yeasts genes responsible for urea metabolism. However, these strategies are not guaranteed to be viable solutions for the fortified wine sector without affecting product quality (24, 42, 80).

One of the characteristics of Madeira wine and fortified wines, in general, is the presence of considerable amounts of sugar derived from the interruption of fermentation at initial or intermediate stages (53). Martins (2011), in his master thesis, reported that Glc is a catalyst of the urea pathway of EC formation.

The present study aims to investigate the possibility of Arg being a direct precursor of EC in fortified wines, comparing it with known major precursors, and assess whether wine major sugars (Glc and Fru) influence EC occurrence.

7.2. Materials and methods

7.2.1. Chemicals and ethyl carbamate determination

The methodology used to quantify EC in the studied model wine systems was the one proposed by Leça *et al* (2017), previously described in section 4. Glc and Fru were obtained from Merck Co. (Darmstadt, Germany). Absolute ethanol, urea, Arg and Cit were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *L-(+)*-tartaric acid was from Panreac (Barcelona, Spain). All chemicals had a purity grade higher than 98%.

7.2.2. Model wine systems

Different model wine systems were prepared by individually adding each EC precursor (urea, Cit and Arg) at 100 mg/L to a synthetic wine solution (6 g/L of tartaric acid, 18% (v/v) of ethanol and pH adjusted to 3.5 with a 1 M NaOH solution) for direct comparison. This concentration was set considering it as an intermediate value of the quite discrepant concentrations found in literature for these precursors. Despite never been referenced as a direct precursor of EC, Arg potential influence in its occurrence during wine ageing was studied for the first time. Some model wines were prepared with the addition of wine main sugars, Fru and Glc, at 100 g/L. About 100 mL of each model system was placed into 125 mL amber glass bottles and prepared in quadruplicate. Two replicates were stored at 45 °C for 4 months (experiment 1) and the other two were stored at 70 °C for 1 month (experiment 2) in two ED115 laboratory heating chambers (Binder, Tuttlingen, Germany). Experiment 1 intends to simulate the *estufagem* accelerated ageing process. On the other hand, and considering that temperature is the most important factor for the EC formation kinetics in fortified wines, experiment 2 was used to force the development of EC and to estimate the potential formation risk at long-term ageing, as used in previous studies (89, 91, 130).

For a better understanding on how samples were prepared, please check Table 5 of the "Results and discussion" (section 7.3). After heated storage, EC formation was assessed in all model wines.

7.2.3. Statistics

Significant differences were evaluated by the analysis of variance (one-way ANOVA with the Holm-Sidak method) using the Sigmaplot 11 (Systat Software, Inc. San Jose, California, USA).

7.3. Results and discussion

Table 5 summarizes the EC levels formed in the model systems mimicking fortified wines conditions, studied under accelerated ageing (45°C for 4 months – experiment 1, and 70°C for 1 month – experiment 2). It was verified that EC was formed in the model wines prepared with urea, Cit and Arg, regardless of the temperature tested (experiments 1 and 2). The control model wine did not develop EC in both experiments.

Table 5 – Composition of the prepared fortified wine model systems and the corresponding EC concentration developed under storage at 45 °C for 4 months (experiment 1) and at 70 °C for 1 month (experiment 2).

Model Systems	Percursor (100 mg/L)	Sugar (100 g/L)	EC (µg/L)	
			45 ºC 4M	70 ºC 1M
control - SW	-	-	n.d.	n.d.
Urea-wS	urea	-	2747 ±69 ^{a*}	10554 ±280 $^{a+}$
Urea-Glc	urea	glucose	2173 ±14 ^{b*}	9389 ±70 $^{b+}$
Urea-Fru	urea	fructose	2036 ±8 ^{c*}	9336 ±718 $^{b+}$
Cit-wS	citrulline	-	388 ±14 ^{a**}	2538 ±259 $^{a++}$
Cit-Glc	citrulline	glucose	304 ±11 ^{b**}	2033 ±50 $^{b++}$
Cit-Fru	citrulline	fructose	286 ±8 ^{c**}	1879 ±48 $^{b++}$
Arg-wS	arginine	-	19 ±6 ^{a***}	41 ±8 $^{a+++}$
Arg-Glc	arginine	glucose	18 ±8 ^{a***}	40 ±10 $^{a+++}$
Arg-Fru	arginine	fructose	19 ±12 ^{a***}	43 ±5 $^{a+++}$

Mean value (n = 6) ± standard deviation; n.d. - not detected; wS - without sugar; SW - synthetic wine; each percursor has different notations within each columns that denote statistically significant differences (P<0.05) by Holm-Sidak test

Arg systems at both ageing conditions generated a small amount of EC. Thus, for the first time, it is demonstrated that Arg in synthetic fortified wine medium chemically reacts with ethanol to form EC without being previously metabolised by fermentative microorganisms, as suggested by previous studies (1, 13, 47, 51, 132). A model system with 100 mg/L of Arg leads to the formation of EC levels varying between 18 and 43 µg/L (Table 5). These results show that the conversion percentage of this precursor into EC is low (<1% mole/mole). However, considering that Pereira *et al* (2015) demonstrated that Arg content in young fortified wines was found up to levels of 356 mg/L, EC formed through this pathway during the ageing of fortified wines must be considered. According to rough estimates, the occurrence of Arg at these concentrations can account for substantial amounts of EC, up to about 64 µg/L, at standard ageing conditions (*estufagem*).

In urea and Cit systems at 100 mg/L, the EC concentration varied between 2036 to 10554 µg/L and 286 to 2538 µg/L, respectively (Table 5). Thus, at the same conditions, urea was the precursor with the highest EC formation rate, being on average 5-fold higher than Cit and 201-fold higher than Arg. The higher reactivity of urea, when compared to Cit, was also demonstrated by Stevens *et al* (1993) and Hasnip *et al* (2004) in previous studies. However, is important to note that the urea content was found to vary between 0.005 and 1 mg/L (133) in different types of wine, including fortified ones, considerably lower concentrations than the ones herein tested. Cit occurrence is usually more associated with the malolactic fermentation process and, in white table wines, levels ranging between 9 and 31 mg/L can be found (134). Despite malolactic fermentation not being usually induced in the vinification of Madeira fortified wines, this kind of fermentation can occur spontaneously by the action of naturally occurring bacteria and increases the content of Cit, which in turn can contribute for EC production during wine ageing.

Regarding the effect of wine major sugars, the results herein obtained are not in agreement with the conclusions from an academic study developed by Martins (2011), wherein Glc was pointed out as a catalyst for the reaction between urea and ethanol to form EC. The presence of Glc and Fru in urea and Cit systems promoted a small but important decrease in EC formation (Table 5), tendency that was not observed in the Arg model systems. In the systems containing urea, the depletion varied between 11 to 26%, while in Cit systems it varied between 20 to 26%. No significant differences were detected between Glc or Fru, although lower EC values were in general obtained in Fru model systems. Thus, other unidentified factors must have a higher contribution to EC formation in sweet wines (section 6) than sugars, which actually showed a suppressive effect in the current study.

As expected, the temperature rise impacted EC formation in all model wines (Table 5). However, despite being observed that model wines submitted to ageing at 70 ºC (experiment 2) had higher EC contents than those at 45 ºC (experiment 1), the increase did not happen with the same magnitude: EC concentrations increased more in the model systems containing Cit (about 7-fold), followed by urea (4-fold) and Arg (2-fold) systems.

7.4. Conclusion

This study allows to conclude that Arg can induce the formation of EC during wine ageing, without being previously metabolised by fermentative microorganisms. Despite not being the most reactive EC precursor, significant levels of Arg can be found in fortified wines before ageing, and this amino acid should be considered a contributor for EC occurrence during the ageing process. Thus, this study reinforces the need for industrial control of nitrogen sources during fermentation to avoid an excessive amount of residual Arg. Regarding the amount of Arg in grape must, it should only be the necessary for yeasts consumption, being its concentration at the end of the fermentation as low as possible. It can also be concluded that wine main sugars, Glc and Fru, not favour the EC formation by urea and Cit pathways during wine ageing (in about 11 to 26%), but not by the Arg pathway. The kinetics of EC formation is differently affected by the temperature rise to 70 ºC (extreme conditions): the Cit pathway seems to be more favoured, increasing approximately 7-fold, followed by the pathways of urea (4-fold) and Arg (2-fold).

8. IMPACT OF THE FORTIFICATION STEP ON THE CONCENTRATION OF ETHYL CARBAMATE PRECURSORS IN MEDIUM

In this study, the effect of the alcoholic fortification step, used to stop the fermentation in the production of fortified wines, was evaluated regarding the excretion of EC precursors from the intracellular yeasts space to the extracellular medium. Experimental wines were obtained from the Tinta Negra red *Vitis vinifera* L. variety and all EC precursors in the medium, urea, Cit and Arg were quantified before and after the fortification. Concerning urea, the most reactive EC precursor, only one of the twelve wines analysed exhibited a small rise in its concentration with the alcoholic fortification. In wines where Cit is quantifiable, a decrease of this compound was observed, from 11 to 46%. Alcoholic fortification, as it is traditionally performed, is not a critical process for the transfer of the two most reactive EC precursors, urea and Cit, to the wine medium. For Arg, the must fortification promoted a significant variation, from a decrease of 34% up to a 2.9-fold increase. The concentration of Arg in the must before fortification seems to influence whether fortification will cause a reduction or a rise on its concentration in the medium and an increase only occurs when the values of Arg are lower than 20.3 mg/L, before adding vinous alcohol.

8.1. Introduction

As aforementioned, urea is an important precursor of EC. When the amount of intracellular urea surpasses a specific concentration (usually >0.5 mmol/L), it is progressively excreted by yeasts into the extracellular medium. Yeasts cells can later reabsorb the released urea, decreasing the chances of EC formation. Cit, despite being quantified in a certain concentration in grape juice, is generated in higher quantities through Arg metabolism during the fermentation and its occurrence is more associated with the lactic acid bacteria activity (1, 9, 22). Cit is generated by Arg anabolism during the fermentation of *Saccharomyces cerevisiae* as an intermediate product of this metabolic reaction (22). Arg is commonly the most abundant amino acid in grape juice and is used by yeasts as a nutrient, being actively metabolized during fermentation. The concentration of Arg in grape juice depends on grape variety and it increases with the rise of nitrogen fertilization in the vineyard (51).

According to FDA, producers of fortified wine have to consider that fortification itself may aggravate the urea excretion to the medium (51). Ough *et al* (1988) verified that alcoholic fortification in the presence of yeasts cells promotes the excretion of EC precursors from the intracellular to the extracellular medium, raising the potential to form EC up to 106% (from 287 to 591 µg/L of EC), when compared to wine fortification without yeasts. The authors associated these data with the change of porosity of yeasts cells' membranes and walls, due to the high concentration of alcohol. Considering that alcoholic fortification may increase urea excretion to the medium, the exact timepoint at which it is performed defines the final concentration of urea in wine. Indeed, if the fermentation is interrupted at the moment of maximum urea excretion, it will not be reabsorbed afterwards (1, 51, 135).

This work intended to ascertain the effect of alcoholic fortification in the transfer of EC precursors from yeasts to the extracellular medium, taking as a case study the production of Madeira wine, through the analysis of samples before and after fortification.

8.2. Materials and methods

8.2.1. Ethyl carbamate, urea, citrulline and arginine determinations

The methodology used to quantify EC in the studied model wine systems was the one proposed by Leça *et al* (2017), previously described in section 4, while Arg and Cit determinations in fortified wines were based on Pereira *et al* (2008), explained in section 6.1.2. Urea was determined by HPLC-FLD as previously referred in section 6.1.3, with a procedure based on Zhang *et al* (2014).

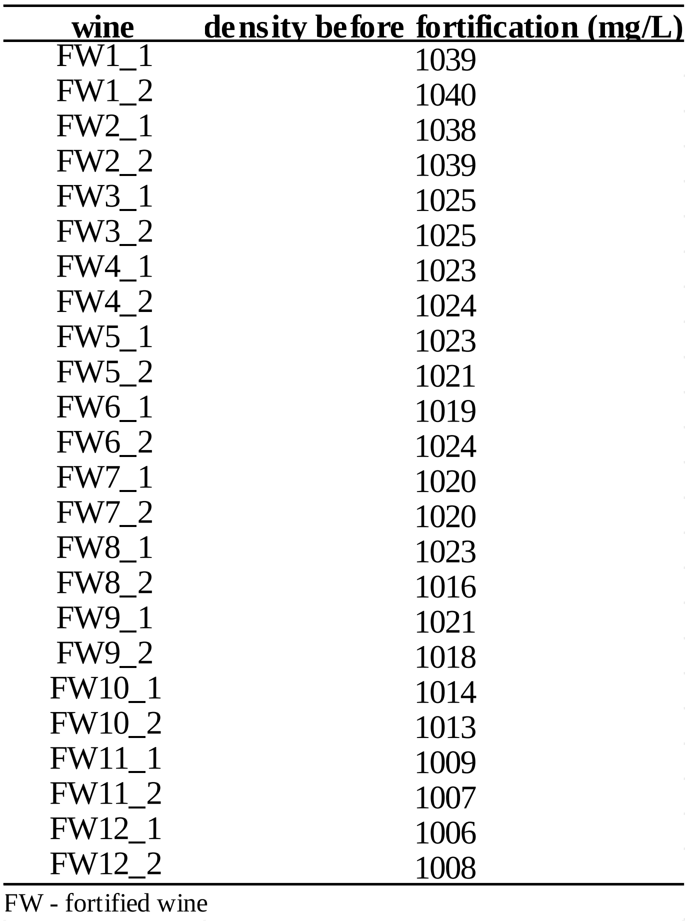
8.2.2. Wine samples

Different trials of fortified wines were studied before and after the fortification process to assess the content of EC precursors. These wines were obtained from Tinta Negra red grapes (*Vitis vinifera* L.) collected at eight different locations of Madeira Island (Portugal). All fortified wines were produced in duplicate from grapes of the same origin, at laboratory scale and according to Madeira wine industrial practices. Amber glass bottles of 3L with enough headspace for carbon dioxide release were used during fermentation. Each microvinification started with the manual separation of grapes from stems and its crushing, with the addition of about 60 mg/L of potassium metabisulfite; no commercial yeasts were added. Pectinases and diammonium phosphate were also included in the mixture. Grape skins, together with the free-run juice, followed 24 hours of maceration before being separated. The alcoholic fermentation process was conducted at about 22 ±3 ºC and stopped at different stages by the addition of neutral vinous alcohol, with densities between 1040 and 1006 g/L (Table 6). The extension of fermentation increases from the fortified wine FW1 to FW12 and the alcohol content was adjusted to about 17% (v/v). Each wine was obtained in duplicate and the respective density was measured using a pycnometer. A 20 mL aliquot of each sample was collected before and after alcoholic fortification for the analysis of urea, Arg and Cit.

8.2.3. Statistics

Significant differences were evaluated by the analysis of variance (one-way ANOVA with the Holm-Sidak method) using the Sigmaplot 11 (Systat Software, Inc. San Jose, California, USA).

Table 6 – Density of wines before fortification with neutral vinous alcohol.



wine	density before fortification (mg/L)
FW1_1	1039
FW1_2	1040
FW2_1	1038
FW2_2	1039
FW3_1	1025
FW3_2	1025
FW4_1	1023
FW4_2	1024
FW5_1	1023
FW5_2	1021
FW6_1	1019
FW6_2	1024
FW7_1	1020
FW7_2	1020
FW8_1	1023
FW8_2	1016
FW9_1	1021
FW9_2	1018
FW10_1	1014
FW10_2	1013
FW11_1	1009
FW11_2	1007
FW12_1	1006
FW12_2	1008

FW - fortified wine

8.3. Results and discussion

The effect of the fortification step on the excretion of EC precursors from the intracellular to the extracellular yeast medium was evaluated, with the addition of approximately 17% (v/v) of ethanol. The EC precursors, urea, Cit and Arg, were individually analysed and their concentrations were determined before and after the fortification step of 12 wines, obtained from the Tinta Negra *Vitis vinifera* L. red grape variety. In general, the concentration of the precursors considerably differed among wines (Fig. 10, Fig. 11 and Fig. 12), but a trend was not verified with regard to the extension of fermentation, which increases from the fortified wine FW1 to FW12. This variation can be justified by different origins of the wine grapes, and therefore, by eventual microbiological differences among wine musts. Madeira Island is characterized by the existence of several microclimates, therefore differentiated treatments can be applied in the vineyards according to the region where parcels are located. Thus, the grapes sampling was made to obtain wines with the highest variability as possible, what allowed to evaluate the impact of fortification on wines with different concentrations of urea, Cit and Arg.

Urea levels were found to vary between 0.21 ±0.02 and 4.8 ±0.6 mg/L in the analysed sample set, which, according to rough estimates, could originate up to 500 µg/L of EC with long-term ageing of the fortified wines (according to Table 5 in section 7.3). The fortification step generally decreased the concentration of the most reactive EC precursor in wines, in 5 to 33% (Fig. 10), contrarily to the possibility raised by FDA that fortification itself could aggravate urea presence in the wine medium (51). This was observed in eight of the twelve fortified wines under study and can be justified by the dilution inherent to ethanol addition. Another possibility is related to the reduced solubility of urea with an increased ethanol concentration as previously suggested by Lee *et al* (1972) and demonstrated by Capuci *et al* (2016). An increase (+22%) was only observed in one of the twelve wines analysed, which can be justified with the porosity change in yeasts cells induced by ethanol addition (129). In the three remaining wines, the change in urea concentration was not statistically significant.

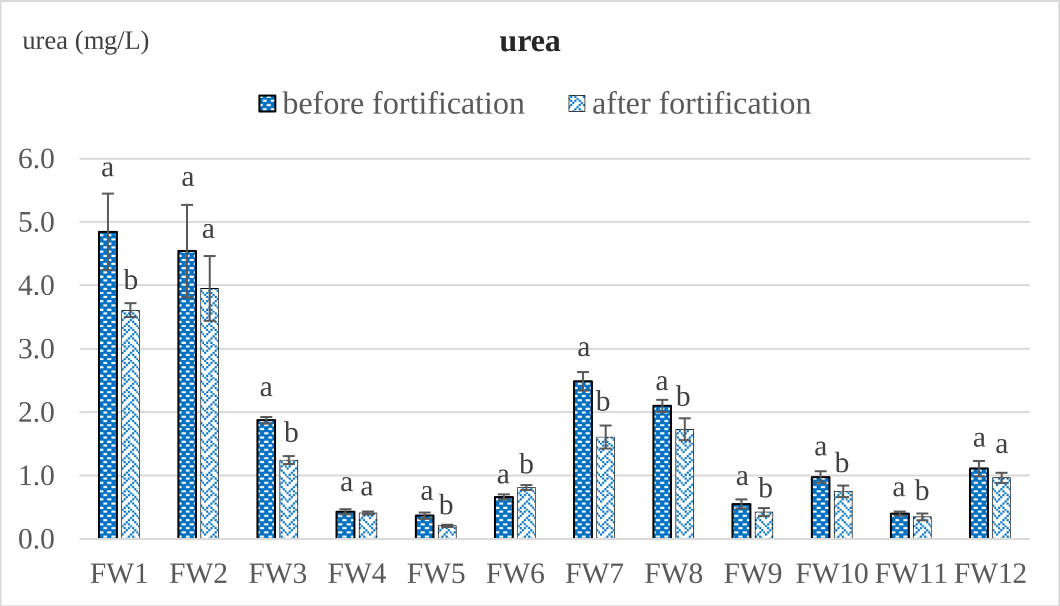


Fig. 10 – Concentration of urea in the fortified wine medium before and after the alcoholic fortification step. Mean value (6) ± standard deviation; FW – fortified wine; different letters within the same wine denote statistically significant differences (P<0.05) by Holm-Sidak test.

Cit was only quantifiable in seven of the twelve wines analysed, with concentrations ranging between 0.21 and 15.1 mg/L (Fig. 11), which, according to rough estimates, could originate up to about 380 µg/L of EC with long-term ageing (according to Table 5 in section 7.3). Cit occurrence is more closely related with the activity of lactic acid bacteria, since in alcoholic fermentation it only comes up as an intermediate of Arg anabolism (1, 18, 22). Thus, the eventual presence of lactic acid bacteria in the medium can justify a higher Cit content in some wines, since malolactic fermentation can naturally occur (138-140). In wines where Cit was quantifiable, its concentration decreased with the fortification step, in between 11 and 46%. This tendency can also be related with the dilution that naturally occurs in the fortification step and with the reduced solubility of Cit as the ethanol content increases (141).

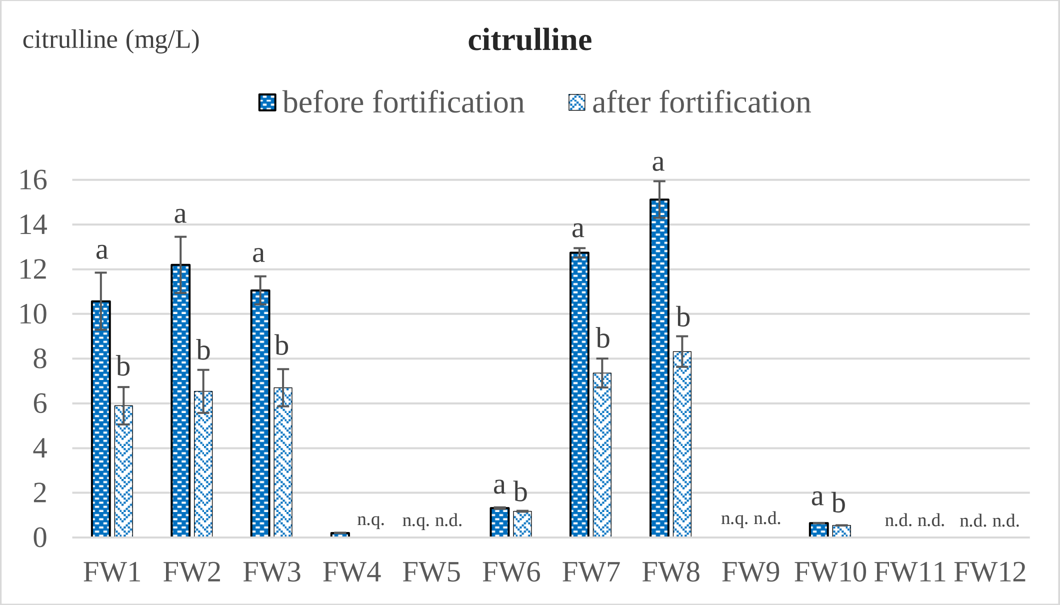


Fig. 11 – Concentration of citrulline in the fortified wine medium before and after the alcoholic fortification step. Mean value (6) ± standard deviation; FW – fortified wine; n.d. - not detected, n.q. - not quantifiable; different letters within the same wine denote statistically significant differences (P<0.05) by Holm-Sidak test.

Arg, the less reactive EC precursor, was also quantified in the wines under fortification and the results are depicted in Fig. 12. Arg levels varied between 1.6 and 901 mg/L, with the fortification step promoting a great variation in Arg contents and trends. When the Arg content in wines before fortification was between 1.6 mg/L and 20.3 mg/L (five out of the twelve wines), there was an increase of up to 2.9-fold on its concentration after alcoholic fortification. The increase of Arg in the medium can be due to the effect of ethanol addition on the porosity of yeasts membranes (129) or even by yeasts autolysis (142). When higher Arg contents were found, namely 115 mg/L and 217 mg/L (2 out of 12 wines), no statistically significant differences were observed while adding vinous alcohol. At levels higher than 687 mg/L, a decrease of Arg up to 34% was detected, which can be explained by evidences recently found by Bowden *et al* (2018) that support that ethanol ascending concentrations lower Arg solubility.

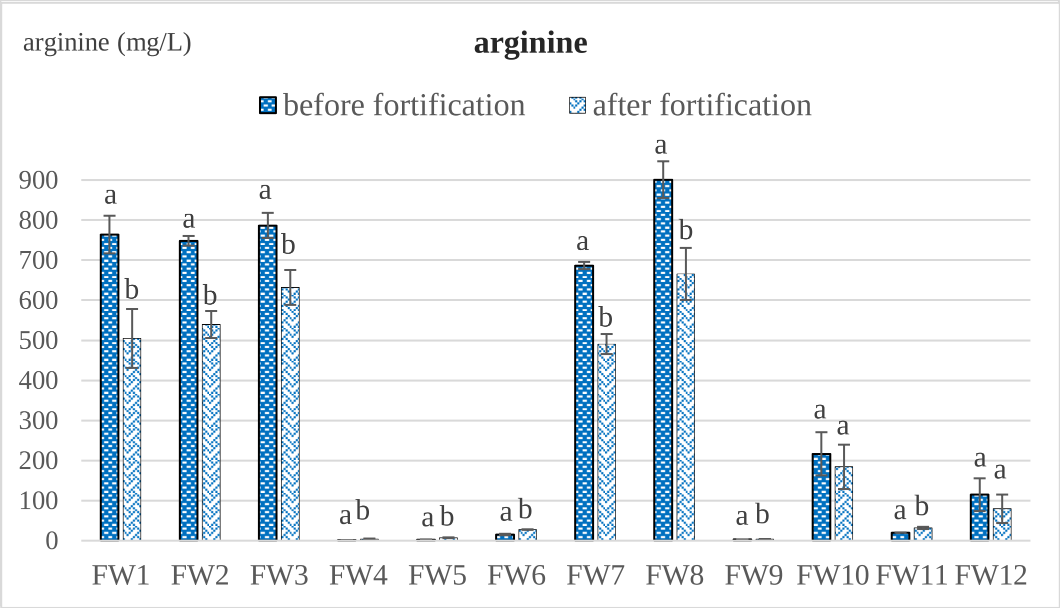


Fig. 12 - Concentration of arginine in the fortified wine medium before and after the alcoholic fortification step. Mean value (6) ± standard deviation; FW – fortified wine; different letters within the same wine denote statistically significant differences ($P<0.05$) by Holm-Sidak test.

8.4. Conclusion

With this study, it was concluded that alcoholic fortification is not a critical process for the transfer of the two most reactive EC precursors, urea and Cit, to the wine medium from the intracellular compartment of yeasts cells. On the contrary, this vinification step affects the transfer of the less reactive precursor, Arg, but only when it occurs in low concentrations in the medium before alcoholic fortification. In wines with different origins there is no specific evolution trend of EC precursors in the medium, regarding the wine fermentation extension, which does not mean that there is no general trend in each individual fermentation/wine. In a perspective of EC mitigation, the process of alcoholic fortification as it is currently done is not problematic and no adjustments are necessary, such as the separation of yeasts before fortification. Also, considering the results of sections 6 and 7, the mitigation of EC should focus on choosing the optimum moments for alcoholic fortification and other measures that can reduce the residual values of its precursors.

9. IMPACT OF INDIGENOUS NON-*SACCHAROMYCES* YEASTS ISOLATED FROM MADEIRA ISLAND VINEYARDS ON THE ETHYL CARBAMATE OCCURRENCE

The impact of selected non-*Saccharomyces* yeasts on the EC occurrence was evaluated for the first time in fortified wines. *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Pichia terricola*, *Pichia fermentans* and *Pichia kluyveri* were inoculated in Tinta Negra musts, after being isolated from Madeira Island vineyards. Urea, Cit and Arg were quantified when the density of musts attained the levels to obtain sweet (1052 ±5 g/L) and dry (1022 ±4 g/L) Madeira wines. Urea concentration varied between 1.3 and 5.3 mg/L, Cit from 10.6 to 15.1 mg/L and Arg between 687 and 959 mg/L. *Pichia terricola* and *Starmerella bacillaris* generated low levels of urea (<2.5 mg/L), Cit (<12.7 mg/L) and Arg (<845.6 mg/L). The five resulting fortified wines, individually fermented by the selected non-*Saccharomyces* yeasts, were exposed to laboratory scale forced ageing, at 70 ºC for 1 month. From the studied yeasts, *Pichia terricola* and *Starmerella bacillaris* revealed low potential to form EC (<100 µg/L), therefore both species can be useful tools for its mitigation in Madeira wines.

9.1. Introduction

The use of selected indigenous starters from a complex biota of specific environments is a new trend in the wine sector (144). Studies on indigenous yeasts adapted to specific grape varieties and regions have grown in number in recent years, not only with the purpose of evaluating biodiversity but also for selecting new indigenous strains associated with “terroir” in order to produce different types and styles of wine (145, 146). Particularly, there is a growing interest in non-*Saccharomyces* yeasts, given that selected strains can have a positive impact on wine quality. These yeasts can be inoculated singularly or included in a mix of yeasts starters to compensate their poor fermenting capacities. Non-*Saccharomyces* yeasts are also known to inhibit undesirable microorganisms (147). Besides being responsible for the production of several flavour compounds that can ensure wine quality, yeasts metabolism also produces other compounds related with off-flavours or food safety concerns, which can compromise wine commercialisation.

As mentioned before, urea and Cit are considered the most relevant precursors of EC (1, 130), but Arg can also induce its formation during wine ageing, without being previously metabolised by fermentative microorganisms. Urea and Cit are mainly formed from Arg metabolization during fermentation. External factors can have a marked influence on the EC final concentration in wine, such as temperature and maturation time (21, 130, 148). The mitigation of EC in wine has been focused on the reduction of urea in the medium and one way to accomplish that is by using selected commercial yeasts strains with low urea excretion (51). Non-*saccharomyces* yeasts may also play an important role in this objective, reducing not only urea but all EC precursors.

An *et al* (1993) studied different yeasts strains in regard to their urea excretion and uptake during fermentation and verified that distinct strains are related with different concentrations of urea in the medium. Additionally, they concluded that differences in must composition also influence urea metabolism during fermentation. Henschke *et al* (1991) observed that an increase of Arg and amino acids in general enhances urea accumulation in musts. The aeration of the must at specific moments during fermentation can also influence the final concentration of urea in the medium. Yeasts with high urea production are the ones with more affinity to convert Arg into urea and with the lowest ability to metabolize urea. Genetic factors can influence the amount of urea that is released and taken up by yeasts cells (51).

Tinta Negra red grape variety is the one with the highest total production in Madeira island (Portugal) and can be used to obtain all Madeira wine styles: sweet, medium-sweet, dry and medium-dry (53, 63, 150). The fermentation process of all Madeira wines is usually conducted by native yeasts present on grapes and its extension depends on the wine style to be produced, being the fermentation halted through the fortification when the desired sweetness is attained, raising the alcohol content usually up to 17% (v/v). Thus, the timing of alcoholic fermentation interruption in drier wines happens at a later stage, while in sweeter wines it happens at an earlier stage. In table wines, the fermentation step is completed to obtain less than 2 g/L of residual sugars, while the denomination of “extra dry” in Madeira wine is applied to those with less than 49 g/L (53).

As far as we are aware, there is no reference in literature about the impact of non-*Saccharomyces* on the occurrence of EC and its precursors in Madeira wines. Therefore, the aim of this study was to assess the impact of indigenous non-*Saccharomyces* isolates from *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Pichia terricola*, *Pichia fermentans* and *Pichia kluyveri*, on the formation of EC and its precursors in fortified wine, when individually inoculated in Tinta Negra musts.

9.2. Materials and methods

9.2.1. Microorganisms and samples

9.2.1.1. Yeasts isolation and identification

Tinta Negra represents the most cultivated grape variety to produce Madeira wine. The indigenous non-*Saccharomyces* yeasts species used in the current study have been selected and isolated from musts of this cultivar from Estreito de Câmara de Lobos, Madeira Island (Portugal). This was achieved by using a glucose-yeast-peptone (GYP) medium (20 g/L of Glc from Sharlau (Barcelona, Spain), 5 g/L of peptone, 5 g/L of yeasts extract and 20 g/L of Nutrient Agar from Himedia (Einhausen, Germany)). The isolates were directly collected from a fresh colony. The polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) was carried using ITS1 primer (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 primer (5’TCCTCCGCTTATTAGATATGC-3’) from STABVida, Portugal. The DNA isolation and the PCR conditions were based on Esteve-Zarzoso *et al* (1999). Amplified DNAs were digested with NZYTaq II DNA Polymerase from NZYTech, Lda – Genes and Enzymes (Portugal). The DNA products and their restriction fragments were separated on a 1.5% agarose gel from Cleaver Scientific (United Kingdom) with 1x TAE buffer. The species were identified based on the internal transcribed spacer (ITS) fragment sequence (STABVida, Portugal), then performed a blast analysis for the sequences obtained.

9.2.1.2. Inoculation and wine production

Tinta Negra grape must was individually inoculated with different non-*Saccharomyces* yeasts, at laboratory scale (in duplicate), in amber glass bottles (10×3L) with a headspace for carbon dioxide release. The yeasts species used in the present study were *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Pichia terricola*, *Pichia fermentans* and *Pichia kluyveri*. The Tinta Negra grape must used (red grape *Vitis vinifera* L.variety) derived from the south of Madeira Island (Câmara de Lobos) with a pH of 3.07 and a density of 1075 g/L, equivalent to about 196 g/L of dissolved sugar according to *VinoLab* and *Vinocalc* online calculator tools.

Bunches were picked, berries were manually separated from stems and crushed. Must was pasteurized at 105 ºC for 5 min in 3 L glass jars. The procedure for yeasts inoculation was based on Benito et al. (2017). Briefly, yeasts species were cultivated in GYP broth at 25 °C for 24 h up to 10^8 CFU/mL and inoculated in must to give an initial count of 10^6 CFU/mL. Fermentations followed the typical Madeira winemaking process, under controlled temperature (22 ±3 ºC) and samples were obtained when density reached the values at which the alcoholic fortification is generally performed to obtain sweet (about 1052 g/L, equivalent to 135 g/L of dissolved sugar) and dry Madeira wines (about 1022 g/L, equivalent to 57 g/L of dissolved sugar), according to Table 7.

Table 7 - Average densities of the samples collected from each inoculation.

Yeast	Sweet wines (g/L)	Dry wines (g/L)
Hanseniaspora uvarum	1054 ± 3	1020 ± 5
Starmerella bacillari	1059 ± 1	1021 ± 1
Pichia terricola	1052 ± 1	1026 ± 1
Pichia kluyveri	1051 ± 1	-
Pichia terricola	1045 ± 1	-

Along the experiments, the density was controlled measuring it with a pycnometer. Experiments made with *Hanseniaspora uvarum*, *Starmerella bacillaris* and *Pichia terricola* yielded sweet and dry wine samples. *Pichia fermentans* and *Pichia kluyveri* showed poor fermenting performance and only sweet wines were sampled.

9.2.1.3. Fortification and sampling

The fermentations were arrested by adding neutral vinous alcohol (95% (v/v) of ethanol) up to 17% (v/v) of ethanol. The alcohol content was adjusted taking into account its determination according to OIV (152). After fortification, the wines were clarified and stabilized through bentonite clays and albuminocol gelatins (Proenol S.A., Portugal), resting 1 month at room temperature. Five different wines were obtained in duplicate, 3 dry wines (*Hanseniaspora uvarum*, *Starmerella bacillaris* and *Pichia terricola*) and 2 sweet wines (*Pichia fermentans* and *Pichia kluyveri*). Each resulting wine was force-aged in a 500 mL glass flask at 70 ºC for 1 month in a ED115 laboratory heating chamber (Binder, Tuttlingen, Germany), mimicking the long-term ageing of Madeira wine (89, 91, 130).

9.2.2. Ethyl carbamate, urea, citrulline and arginine determinations

The methodology used to quantify EC was the one proposed by Leça *et al* (2017), previously described in section 4. The method used to determine Arg and Cit in fortified wines was based on Pereira *et al* (2008), explained in section 6.1.2. Urea was determined by HPLC-FLD as previously referred in section 6.1.3 with a method based on Zhang *et al* (2014).

9.2.3. Statistics

Significant differences were evaluated by the analysis of variance (one-way ANOVA with the Holm-Sidak method) using the Sigmaplot 11 (Systat Software, Inc. San Jose, California, USA).

9.3. Results and discussion

In this study it was evaluated the impact of different non-*Saccharomyces* yeasts, namely *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Pichia terricola*, *Pichia fermentans* and *Pichia kluyveri,* on the occurrence of EC precursors, such as urea, Cit and Arg within the fermentation. The resulting fortified wines were subjected to accelerated ageing in the laboratory (70 ° C for 1 month) and then EC was quantified. Different experimental wines were obtained by the inoculation of the same batch of Tinta Negra grape must. Five fortified wines were produced in duplicate, three of which were dry ones, namely from *Hanseniaspora uvarum*, *Starmerella bacillaris* and *Pichia terricola*. The remaining non-*Saccharomyces* showed poor fermenting performance and only sweet wines could be produced. Samples were collected and analysed once they reached the densities at which alcoholic fortification is generally performed to obtain sweet and dry Madeira wines.

9.3.1. Analysis of ethyl carbamate precursors

Urea is recognized as the most reactive precursor to form EC in wines (section 7.3). The content of urea found in sweet and dry wines sampled before alcoholic fortification was strongly dependent on the non-*Saccharomyces* yeasts strain inoculated (Fig. 13). In sweeter wines, the urea content varied from 1.26 ±0.08 to 5.3 ±0.6 mg/L, with *Hanseniaspora uvarum* and *Pichia terricola* being responsible for the lower levels of about 1.3 mg/L. *Pichia fermentans* and *Pichia kluyveri* ferments yielded the highest contents, 4.3 ±0.2 and 5.3 ±0.6 mg/L, respectively. This can be justified by the assumption that these yeasts have a higher affinity to convert Arg into urea, as previously pointed out (51). These results suggest that the use of these two non-*Saccharomyces* as yeasts starters can lead to high levels of urea, and therefore, to a higher potential of EC development. In drier wines, urea concentration ranged from 1.9 ±0.6 to 2.5 ±0.2 mg/L. *Pichia terricola* inoculation led to the lowest levels, while *Starmerella bacillaris* resulted in the highest. The sweet wines under study presented lower urea contents than those fermented by *Saccharomyces* yeasts found in literature, while dry wines had values of the same concentration level (153, 154).

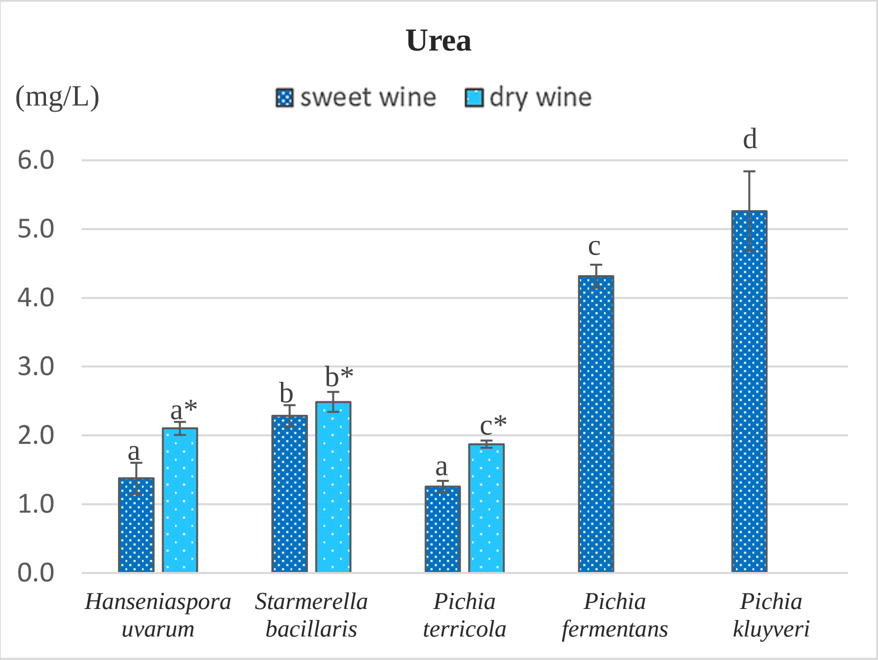


Fig. 13 – Impact of different indigenous non-*Saccharomyces* yeasts on the urea levels found in sweet and dry wines sampled before alcoholic fortification; different letters within the same type of wine denote statistically significant differences (P<0.05) by Holm-Sidak test. Data represent average from duplicate experiments.

Cit is less reactive than urea but is also considered a precursor with high reactivity to form EC (section 7.3). In *Saccharomyces cerevisiae*, Cit is generated through the Arg anabolism in the fermentation process, during which ornithine and carbamoyl phosphate react to form Arg, being Cit an intermediate product of this metabolism (22). Thus, it is expected that this metabolic activity might differ according to the yeasts strain. High levels of Cit in wines are usually associated with the occurrence of malolactic fermentation (18, 155). Although malolactic fermentation is not usually induced in Madeira wine vinification, this kind of fermentation can occur spontaneously by bacteria naturally present and increase the content of Cit, which in turn can contribute for EC development during wine ageing. However, this possibility was discarded in the present study due to must pasteurization. Fig. 14 depicts the results of this EC precursor in the current sample set and it can be confirmed that different species of non-*Saccharomyces* yeasts generated different levels of Cit in the extracellular medium, with values ranging from 10.6 ±1.3 to 15.1 ±0.8 mg/L. *Starmerella bacillaris* and *Pichia terricola* originated the lowest Cit contents in the sampled sweet wines, 11.2 ±1.1 and 10.6 ±1.0 mg/L, respectively. In dry wine style fermentations, *Pichia terricola* showed, once again, the lowest level of Cit (11.0 ±0.6 mg/L), while the highest was obtained from the *Hanseniaspora uvarum* inoculation (15.1 ±0.8 mg/L). The observed values of Cit are comparable or lower than those observed in previous reports (130, 134).

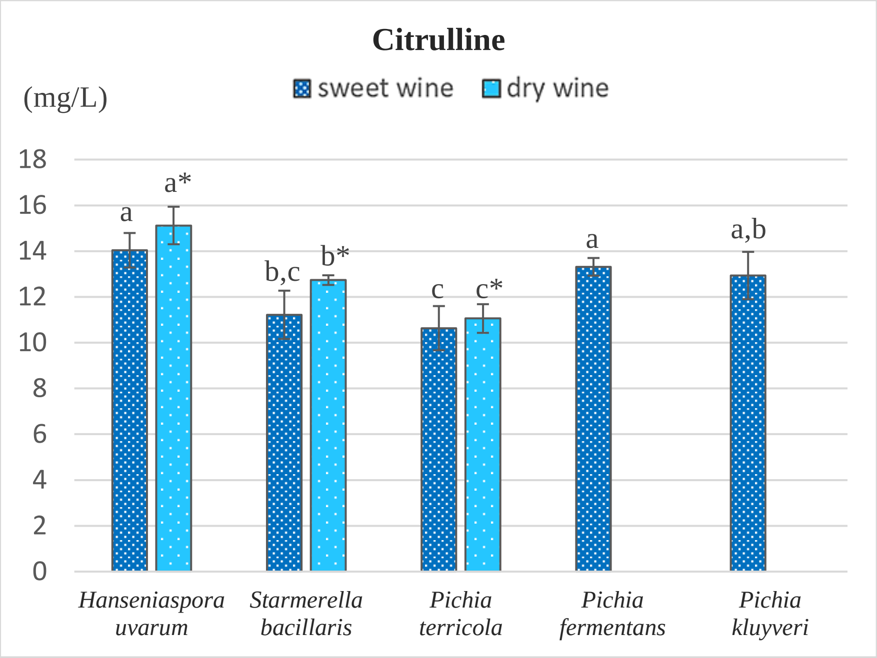


Fig. 14 - Impact of different indigenous non-*Saccharomyces* yeasts on the Cit levels found in sweet and dry wines sampled before alcoholic fortification; different letters within the same type of wine denote statistically significant differences (P<0.05) by Holm-Sidak test. Data represent average from duplicate experiments.

As mentioned in section 7.3, Arg can chemically react with ethanol to form EC under accelerated ageing conditions, although being much less reactive than urea and Cit. Fig. 15 shows that the different indigenous non-*Saccharomyces* yeasts inoculated in this study also influenced the levels of Arg in both styles of wines, varying from 687 ±9 to 959 ±35 mg/L. All wines fermented by *Hanseniaspora uvarum* and *Pichia fermentans* presented higher contents of Arg (in average 930 and 816 mg/L, respectively), while those fermented by *Starmerella bacillaris* presented the lowest (in average 726 mg/L). It is worth mentioning that none of the dry style wines revealed great differences in relation to the corresponding sweet wines. The fact that different yeasts have different affinity for Arg as a nitrogen source can justify these results.

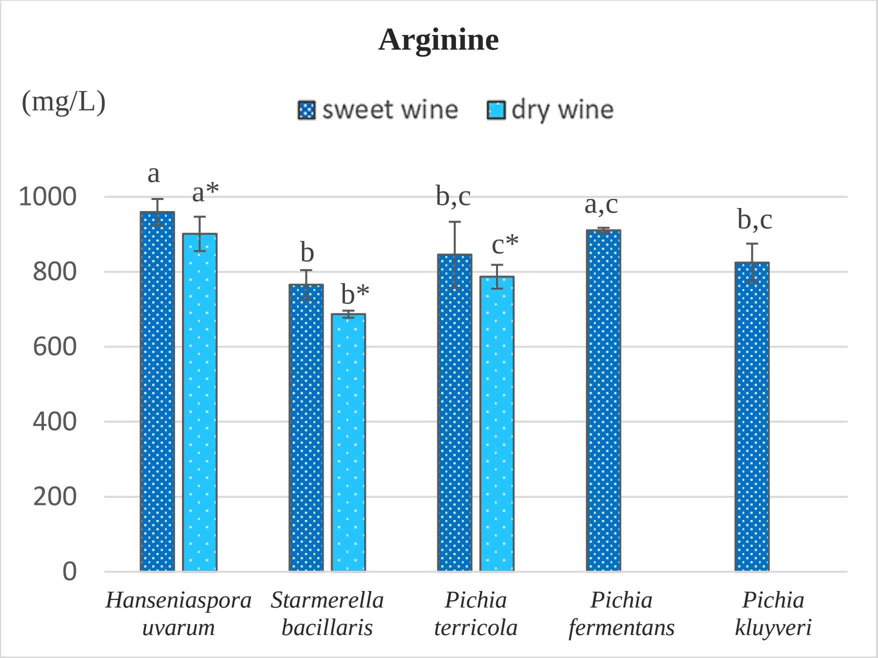


Fig. 15 - Impact of different indigenous non-*Saccharomyces* yeasts on the Arg levels found in sweet and dry wines sampled before alcoholic fortification; different letters within the same type of wine denote statistically significant differences (P<0.05) by Holm-Sidak test. Data represent average from duplicate experiments.

The results obtained for sweet wines are comparable to those of wines in the beginning of the fermentation with *Saccharomyces* yeasts (131, 153), while the ones of the dry style are not. It is recognized that Arg is a good source of nitrogen for *Saccharomyces* yeasts, being commonly observed its significant decrease, often more than 90%. These results seem to indicate that the studied non-*Saccharomyces* yeasts have less affinity to Arg as a source of nitrogen, which is consistent with the low levels of urea detected in sweet wines (Fig. 13), when compared to similar studies previously reported for *Saccharomyces* or native yeasts (131, 153, 156, 157).

9.3.2. Analysis of potential formation of ethyl carbamate

As aforementioned, the resulting wines were fortified and exposed to forced ageing at 70 ºC for 1 month to evaluate the potential of the wines inoculated with each yeasts strain to form EC during ageing. Fig. 16 shows that EC content varied from 78.7 ±0.4 µg/L in wines inoculated with *Pichia terricola* to 178 ±27 µg/L in wines inoculated with *Pichia kluyveri*.

Pichia terricola was the species that led to sweet and dry wines with the lowest amounts of urea and Cit, and resultant wines were those that showed less potential to form EC, after forced ageing. Similarly, wines derived from *Starmerella bacillaris*, which is one of the most promising species for winemaking (68), presented low concentrations of EC precursors and also showed EC concentrations lower than 100 µg/L.

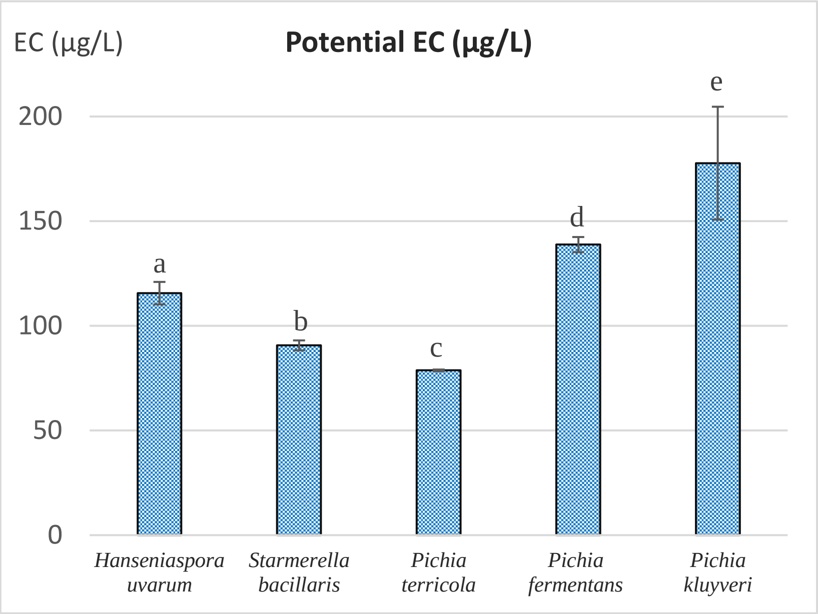
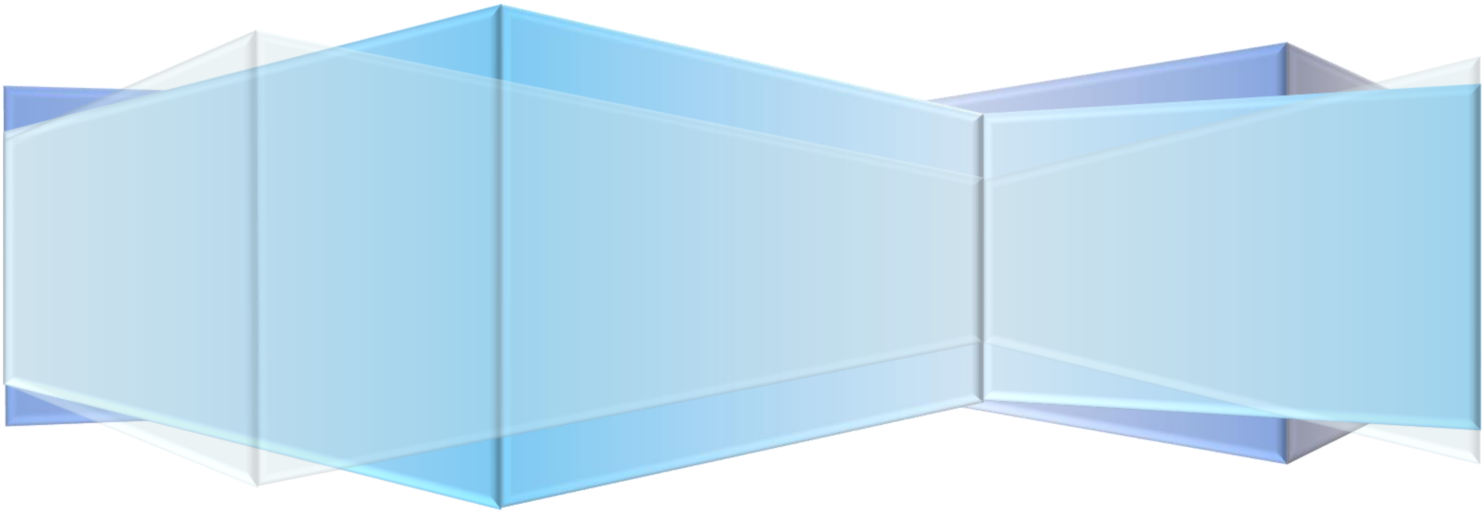


Fig. 16 - EC concentration in fortified wines exposed to forced ageing at 70 ºC for 1 month, and previously fermented by different indigenous non-*Saccharomyces* yeasts isolated from Madeira Island vineyards; different letters denote statistically significant differences ($P<0.05$) by Holm-Sidak test. Data represent average from duplicate experiments.

9.4. Conclusion

This study showed that *Pichia terricola* and *Starmerella bacillaris* are the non-*Saccharomyces* yeasts that generated lower concentrations of the most reactive EC precursors, which can produce safer wines with different degrees of sweetness. The urea concentration in the extracellular medium was less than 1.9 mg/L, while Cit was lower than 12.7 mg/L. The inoculations individually performed by both yeasts generate average Arg levels of 771 mg/L. It was also concluded that these non-*Saccharomyces* yeasts produced sweet wines with lower levels of urea and dry wines with higher levels of Arg, probably due to less affinity to Arg as a nitrogen source when compared to *Saccharomyces* yeasts reported in literature. The resultant Madeira wines, which were fortified and exposed to forced ageing, also revealed low levels of EC, below 100 µg/L. Thus, *Starmerella bacillaris* and *Pichia terricola* showed low potential to form EC in wines. These results provided relevant information for the utilisation of these yeasts for EC mitigation purposes in Madeira wines. Moreover, these species can eventually participate in the elaboration of more differentiated Madeira wines, reflecting the characteristics of this wine region. The requirement for full fermentation dryness is not necessary in these fortified wines and so co-inoculation with *Saccharomyces* starters can be avoided. Therefore, future studies will focus on the detailed evaluation of their fermentation performances, utilisation of nitrogen sources, and volatile compounds to find a good compromise between wine quality and safety.

PART VI – FORTIFIED WINE TREATMENTS TO REDUCE ETHYL CARBAMATE



10. EVALUATION OF POSSIBLE FORTIFIED WINE TREATMENTS FOR THE REDUCTION OF ETHYL CARBAMATE CONTENT

In this section, the impact of different wine treatments on the reduction of EC content was evaluated in Madeira fortified wines. The treatments used were charcoal, silicon dioxide, polyvinylpolypyrrolidone (PVPP) and chitosan. A total of seven wines were studied, both commercial wines and wines stored in local wineries. It is well known that after EC is formed in wine it is very difficult to be removed and, thus, strategies to mitigate EC in wine are focused on the prevention of its occurrence. None of the four different wine treatments tested could remove EC from the analysed fortified wines.

10.1. Introduction

In fermented foods and alcoholic beverages, different precursors are involved in EC formation. Different strategies have been developed to reduce EC and these can be divided into physical, chemical, microbiological, enzymatic, and metabolic engineering. The reduction of storage time and temperature in wines can be a solution to promote the decrease of EC in the medium (123). However, in Madeira wines, the storage time and temperature are essential to their maturation and unique organoleptic characteristics, both of which are the result of oxidation processes.

The trends to minimize EC in table wines, at the fermentation step, are the use of acidic ureases to break urea molecules and the application of genetic engineering strategies on fermentative yeasts to also reduce urea in the medium (27, 47, 123). Once formed, EC is very difficult to decompose and the usual methodologies for its mitigation are focused on preventing its formation. Two studies attempted to use urethanase in Chinese rice wine to directly remove EC. Results from one research showed that 35% of EC can be removed without influencing the flavour while another study showed that 52% of EC can be removed from commercial rice wine, although without evaluating the impact on flavour (158, 159).

Through a charcoal filtration process, Park *et al* (2009) removed up to 47% of EC in diluted spirits and soy sauce, also without assessing the organoleptic impact.

In literature, no treatment was found to remove EC from table or fortified wines. In this sense, the goal of this study was to evaluate the capacity of four different wine treatments, charcoal, silicon dioxide, PVPP and chitosan, to remove EC from Madeira wines.

10.2. Materials and methods

10.2.1. Ethyl carbamate determination

The methodology used to quantify EC in the studied fortified wines was the one proposed by Leça *et al* (2017), previously described in section 4.

10.2.2. Wines and treatments applied

Four different treatments were tested to reduce EC in Madeira wines: charcoal, silicon dioxide, PVPP and chitosan. The concentrations used for the treatments followed the limits imposed by OIV, 200 g/hL for charcoal, 75g/hL for silicon dioxide, 80 g/hL for PVPP and 500 g/hL for chitosan (161). All treatments were made in duplicate and performed by direct addition and manual agitation. After 24 hours, 50 mL of each wine were centrifuged for 10 min at 4400 rpm and the upper wine was collected. Charcoal was obtained from Enartis, while silicon dioxide, PVPP and chitosan were obtained from Sigma–Aldrich (Steinheim, Germany). The samples used for these experiments consisted of a total of seven wines, some of which commercial and others stored in local wineries. EC concentration was analysed in all wines before and after each treatment.

10.3. Results and discussion

Four different wine treatments authorized by OIV were tested in order to remove EC from Madeira wines: charcoal, silicon dioxide, PVPP and chitosan. Charcoal treatment is approved by OIV for providing specific organoleptic characteristics to aromatised wines.

Silicon dioxide is an aqueous colloidal suspension and is commonly used as a fining agent for clarification and, in turn, PVPP decreases the content of polyphenols and tannins, reducing astringency, and is also used for wine colour correction or browning prevention. Wine treatments with chitosan can be used to reduce the concentration of heavy metals, such as iron and copper, possible contaminants, like ochratoxin A, and specific undesirable microorganisms, such as *Brettanomyces* (161).

The obtained results (Fig. 17) revealed that none of the applied treatments can remove EC from Madeira wines.

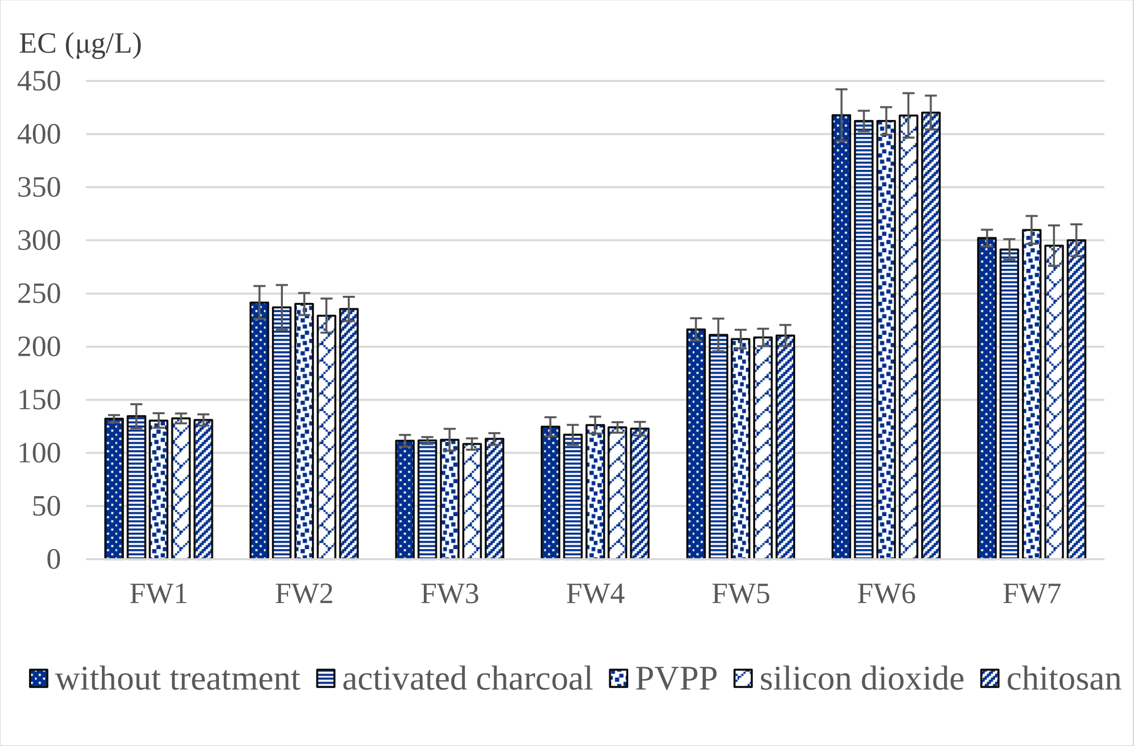
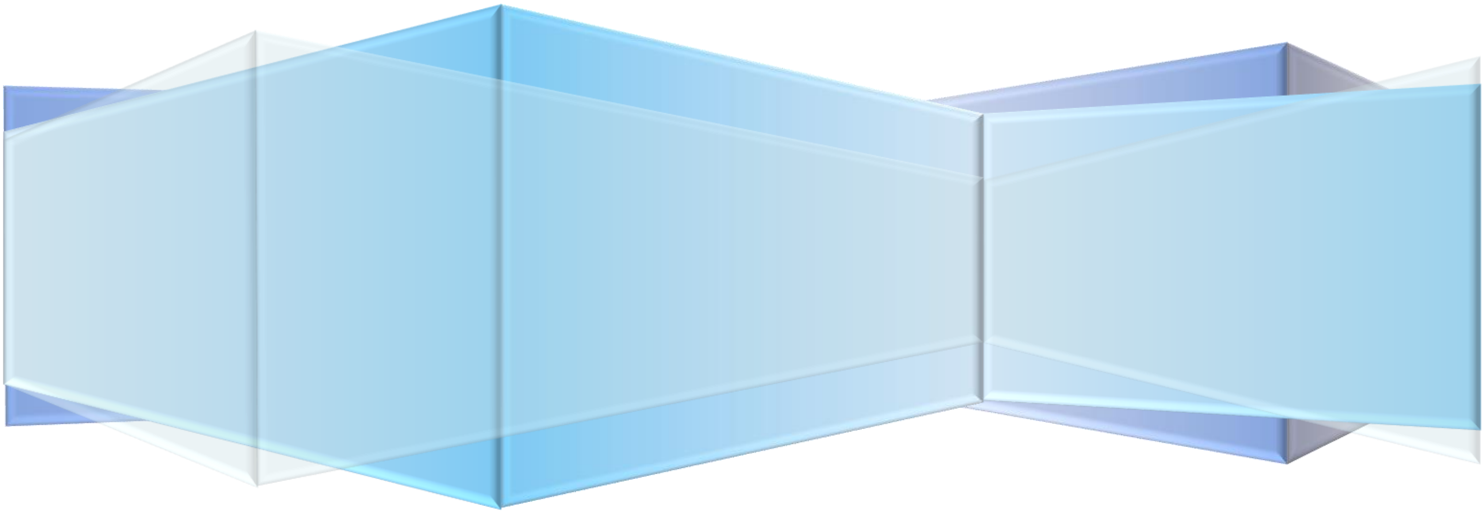


Fig. 17 – Effect of fortified wine treatments in the reduction of EC in the medium; FW – fortified wine.

10.4. Conclusion

As we can verify, the identification of effective oenological treatments for EC removal in Madeira wine is difficult to accomplish, even when testing treatments with some success in other alcoholic beverages. A treatment that proves to be effective in EC removal must be valid in economic terms and, additionally, maintain the quality and typical characteristics of this product. Therefore, and as previously mentioned, the strategies for reduction of EC occurrence must be focused on optimizing the treatment of vineyards and the fermentation process with the possible inoculation of selected indigenous yeasts, adjusted to the grape variety and desired wine style. It is also important to define the ideal moments for alcoholic fortification and strictly control the *estufagem* temperature, if possible, with technology that promotes a uniform temperature diffusion.

PART VII – FINAL REMARKS



11. GENERAL DISCUSSION

The methodology developed and addressed in section 4 was of great relevance to this study, because it allowed us to robustly detect EC values in the order of ng/L without matrix effects from a complex matrix such as a fortified wine. The extraction method is ecological (low volume of extraction solvent) and allows the preparation of several samples at the same time. Improving the determination of EC precursors turned out to be more difficult and advances in this direction constitute an ongoing work, discussed later in subsection 13.1.

After the development of the sensitive analytical method aforementioned, the analysis of a representative number of Madeira wine samples was possible, as described in section 5. The results showed a certain tendency towards an increase of EC average concentration over time, although Madeira wines with the same age present very different EC values. The lower levels of EC in younger wines can also be a direct consequence of the research and technological innovation targeted by the sector.

Viticulture has never been the object of this study, nor have wine treatments that may directly impact the composition of the resulting grape musts in terms of amino acids and nitrogen compounds. However, the fact that grapes should only have the required levels of nitrogen for fermentation and, more specifically, of Arg, is reinforced in section 7. The whole study found that strategies for EC mitigation should focus on fermentation; in this step of the winemaking process, both wine density and EC precursors levels should be monitored so that alcoholic fortification is carried out at the right moment to obtain the lowest EC precursors residual levels as possible. In the future, the obtained data can be processed to define optimal fermentation interruption timepoints depending on the grape variety, alcohol potential and desired wine style.

In section 6 we verified that white grape varieties have less propensity to form EC, even when submitted to *estufagem*; from that moment on, the main focus of this study became the Tinta Negra variety, which is also the most used variety in Madeira wine production. An important question to be addressed was the fact that, even when urea and Cit, the main precursors of EC, were not quantifiable or detected, EC continued to be formed (at a slower rate). This observation led to the discovery of Arg as a new EC precursor (section 7), that reacts directly with ethanol. Despite its lower reactivity, Arg is usually the most abundant amino acid, being particularly relevant for wines with longer ageing periods. Other compounds or environmental characteristics may also promote EC formation and, in this sense, iron and copper levels were studied in wines submitted to forced ageing. No impact was verified on EC final levels (Supplementary Table 3, Appendix).

Two relevant issues raised by the bibliographic review, at the beginning of this study, were explored in sections 7 and 8: the alcoholic fortification and the possible role of individual sugars as catalysts of EC formation reactions. The main sugars, Glc and Fru, were tested and its hypothesised catalytic action was not confirmed and a rather suppressing or not relevant effect was observed. Although Fig. 8 and Fig. 9 of subsection 6.3 show that EC values tend to be higher in Tinta Negra sweet wines, this may be due to its fortification moment and not to the presence of sugars. TNSweet1 was fortified with more sugars (density before alcoholic fortification = 1045 mg/L) and when submitted to *estufagem*, showed less tendency to form EC in comparison with TNSweet2 wine, which had less sugars (density before alcoholic fortification = 1037 mg/L), reinforcing the idea that the presence of sugars is not problematic. Alcoholic fortification is known to cause yeasts metabolic inactivation and possible cell lysis, but this procedure was not found to be troublesome regarding EC occurrence, even though it increased Arg levels (the less reactive precursor) in some cases.

Selected indigenous non-*Saccharomyces* yeasts have attracted attention on wine production research and can be an effective approach for EC mitigation in Madeira wines, either added alone or in a set of yeasts. They are known to have a poor fermentative capacity, but that would not be limiting, since Madeira sweet wines have a very short fermentation period and dry wines, which still contain considerable amounts of residual sugars, normally ferment to a much shorter extent than table wines. Therefore, in section 9 we verified that two non-*Saccharomyces* yeasts - *Starmerella bacillaris* and *Pichia terricola* - have the potential to mitigate the occurrence of EC precursors and, consequently, EC itself in wines in which these microorganisms are inoculated.

Finally, in section 10, we found that EC removal with oenological treatments is not an easy task, although attractive. Thus, its mitigation strategies should focus on fermentation and on the continuous transition to the most evolved *estufagem* technologies at the ideal temperature (45ºC). Besides controlling wine treatments, the need for the definition of an ideal yeast (or yeast broth), as well as the control of EC precursors - and not only of wine density during fermentation - is reinforced, which can be adjusted to each variety, wine style and expected ageing period.

12. FINAL CONCLUSIONS

The aim of this study was to detect, understand, and mitigate EC formation in fortified wines, with Madeira wine as a study case. From the overall work, the following conclusions can be drawn:

- A simple and fast method, which allows EC quantification in the order of nanograms per litre was successfully developed. The extraction of EC takes less than 15 min and a LC-MS/MS analysis 18 min, with the possible preparation of several samples simultaneously. The extraction method is ecological (low volume of extraction solvent) without matrix effects from a complex matrix such as a fortified wine. The developed methodology significantly simplifies EC determination in wine and was crucial for EC determinations performed in this study.

- For the first time, a representative set of Madeira wine samples was analysed to assess the presence of EC. The results revealed that older wines tend to have higher EC concentrations, which can be justified by the maturation time, but also by the research and technological advances introduced in the winemaking process that may beneficiate younger wines. In some wine samples, the EC content was above the legal limits of some markets, which justified the detailed study of EC formation in Madeira wine.

- The evolution of EC in Madeira wines was monitored over three years, together with its main precursors. The study included sweet and dry wines from red and white grape varieties, submitted to *estufagem* and *canteiro*, for comparison purposes. We verified that Tinta Negra sweet red wines submitted to *estufagem* are the ones with more propensity to form EC. On the other hand, white varieties, Malvasia and Sercial, have less tendency to form EC even when submitted to *estufagem*. When Urea and Cit stop being quantifiable or detectable, EC continues to be formed, even though at a slower rate. Thus, other precursors, such as Arg, and physicochemical characteristics, not previously reported, may influence EC occurrence.

- Arg can directly form EC by reacting with ethanol during wine ageing, without being previously metabolized by fermentative microorganisms. Arg is the less reactive EC precursor, but it is usually the most abundant amino acid in grape musts, reason why it should be considered an important EC contributor during the ageing process. This fact reinforces the need for strategies to control nitrogen sources during fermentation, as a way to avoid an excessive amount of residual Arg in wines before ageing.

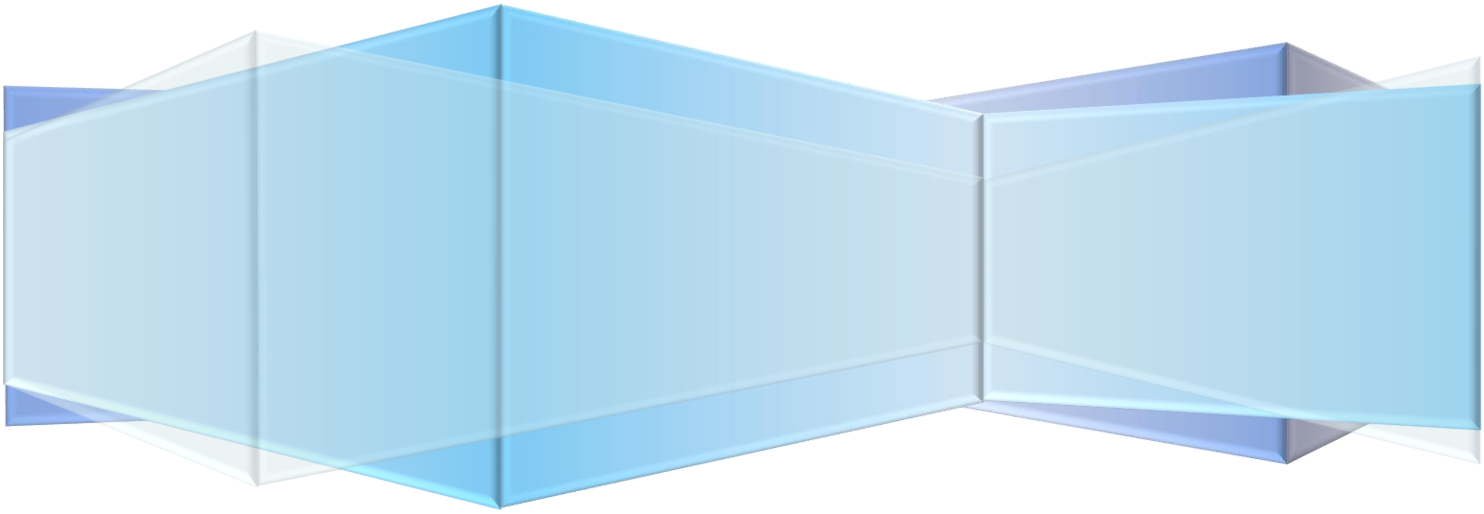
- Wine main sugars, Glc and Fru, suppress the amount of EC formed from its most reactive precursors, urea and Cit, up to 26%. However, the Arg formation pathway is not affected by their presence. The fact that Tinta Negra sweet red wines have more propensity to form EC may be associated with the levels of its precursors in the medium when alcoholic fortification occurs, with the Arg retention capacity of this grape variety, and with the microbiological environment during fermentation.

- Alcoholic fortification causes changes in yeasts' membranes, metabolic inactivation and possibly cell lysis, but it was verified that this process is not critical for the transfer of the two most reactive EC precursors (urea and Cit) to the wine medium. In fact, it only affects the less reactive precursor, Arg, particularly if in low concentrations before fortification. Therefore, the alcoholic fortification step can be executed as it is now, with no further adjustments.

- The fermentation process was targeted by the results obtained during this study, and, following a current trend, the impact of indigenous non-*Saccharomyces* yeasts in the occurrence of EC and its precursors was evaluated. *Starmerella bacillaris* and *Pichia terricola* yeasts produced wines with different degrees of sweetness with the lowest potential to form EC most reactive precursors, and fortified wines with less EC occurrence itself.

- Finally, the identification of an effective oenological treatment for EC removal was difficult to accomplish since none of the tested treatments could remove EC from fortified wines. Furthermore, besides removing EC, a valid treatment must be economically attractive and able to maintain wine typical features and quality. For now, strategies to mitigate EC in Madeira wine must be focused on the prevention and control of its occurrence.

PART VIII - FUTURE PERSPECTIVES



13. PRELIMINARY RESULTS

13.1. Simultaneous determination of ethyl carbamate and its precursors

Madeira wine production brings together natural conditions that can favour EC occurrence at levels close to, or sometimes even higher than, the maximum limits imposed by some markets. This sets the basis for its study as an attempt to control and mitigate its formation in fortified wines. From an analytical point of view, the determination and quantification of EC and its precursors (urea, Arg and Cit) face several obstacles. First, EC is found in wine at very low levels, in the order of µg/L. Also, urea is difficult to ionize and fragment in an ESI-MS detector, especially when using hydrophilic interaction chromatography (HILIC) typical mobile phases. Urea, Arg and Cit cannot be separated in a C18 column without a previous derivatization step, thus increasing the complexity of the analysis and associated errors. Finally, bearing in mind that Arg and Cit derivatization occurs in a basic medium and urea's in acidic conditions, its simultaneous analysis is hampered.

Therefore, the study carried out in the Faculty of Sciences of the University of Granada consisted in the development of an analytical method that simultaneously quantifies EC and its precursors, and a good separation and ionisation of these compounds were achieved in a chromatographic analysis that only requires ten minutes (Fig. 18). This is an ongoing work, at the Faculty of Exact Sciences and Engineering of University of Madeira, and now, the research is focused on the development of an effective and simple extraction method for the target compounds.

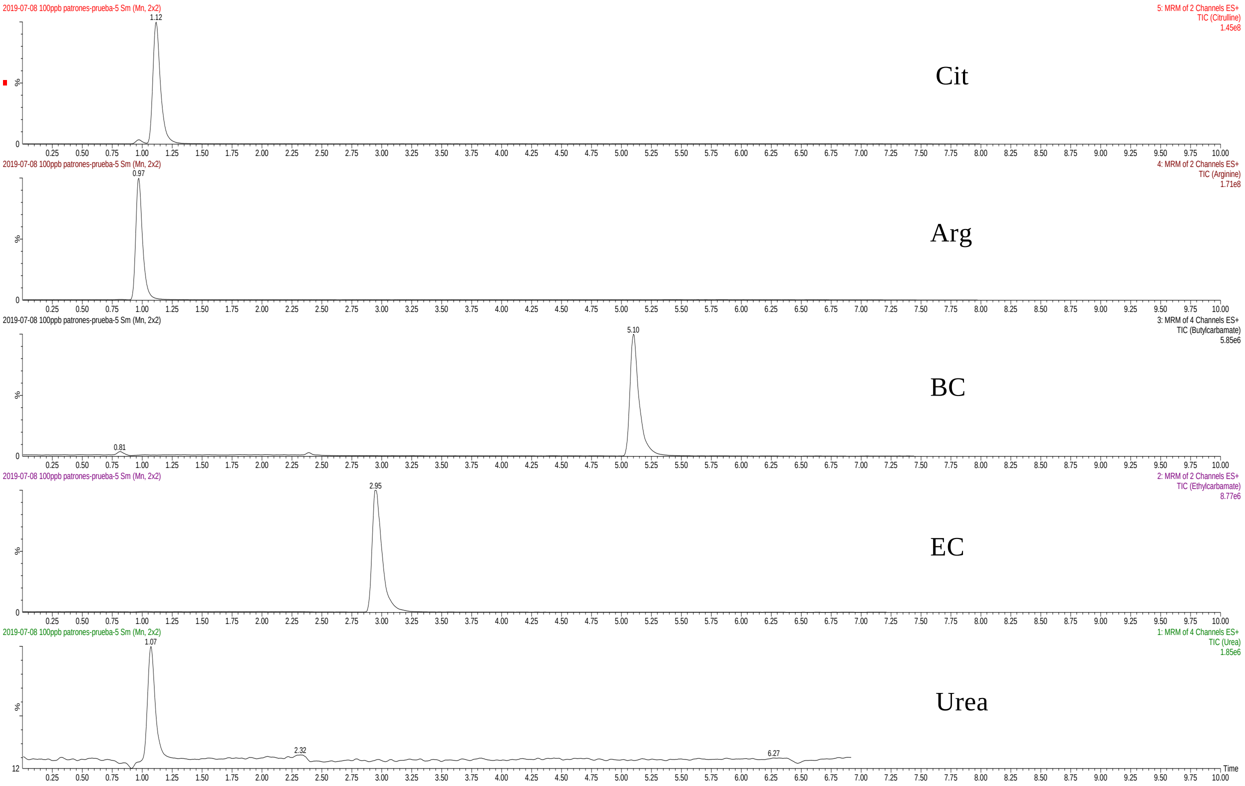


Fig. 18 - LC-MS/MS chromatogram for EC and its precursors: urea, Cit and Arg with BC as internal standard.

13.2. Determination of ethyl carbamate by optical fibre-based sensors

Nowadays, sensors represent an important tool for the food industry process control. Optical fibre-based sensors are also being studied as an alternative technology to classical analytical methods used in wine process control. The interest in optical fibre-based sensors is motivated by its small dimensions, low weight, and real time monitoring. Furthermore, these sensors usually require very low sample volumes, with minimal preparation, being adequate for *in situ*, real time measurements. Most Madeira wine producers, and wine producers in general, do not have internal analytical capacity to determine EC. Obtaining a simple tool for EC determination would be important for producers so that they could more effectively manage the winemaking process in relation to the presence and formation of this compound. Together with the Institute for Nanostructures, Nanomodelling and Nanofabrication (I3N), Aveiro, we are currently developing the first sensors capable of detecting differential concentrations of EC (Fig. 19). Although at a very preliminary stage of development, these devices could constitute a considerable advance for EC determination in fortified wines.

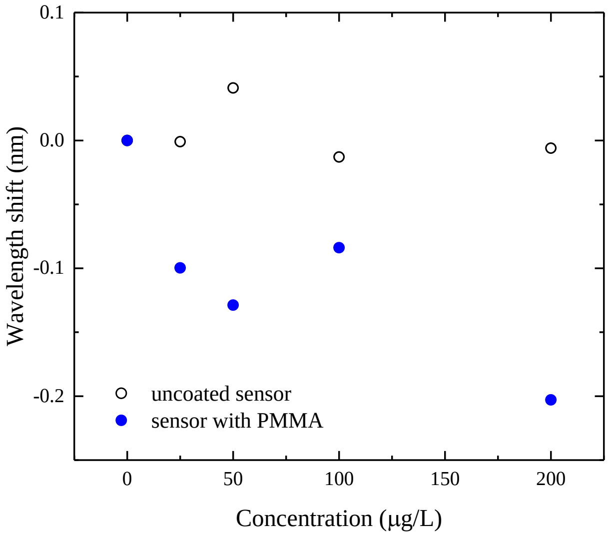


Fig. 19 – Optical fibre-based sensor, with and without coating, based on multimode interference response to different concentrations of EC.

14. GENERAL FUTURE PERSPECTIVES

The main conclusions of this thesis raise new questions that require additional studies to mitigate EC occurrence in fortified wines such as Madeira wine. New tests are fundamental for the development of specific strategies and tools for fortified wine producers. Besides the ongoing work described in sections 13.1 and 13.2, in the future we consider important to:

- Study the vines and grape varieties in terms of nitrogen absorption and Arg accumulation to better adjust vineyard treatments;

- Evaluate the impact of the measurement of total nitrogen or assimilable nitrogen in free-run juice before fermentation, so ammonium phosphate is only added if necessary, thus reducing residual arginine values;

- Define the optimal moments for fortification, depending on the grape variety, the alcoholic potential of grapes and the desired degree of sweetness. For this purpose, EC precursors need to be monitored during fermentation, as well as wine density, to develop helpful predictive multivariate statistical models;

- Evaluate the impact of the indigenous non-*Saccharomyces* yeasts inoculation on the organoleptic characteristics of Madeira wines;

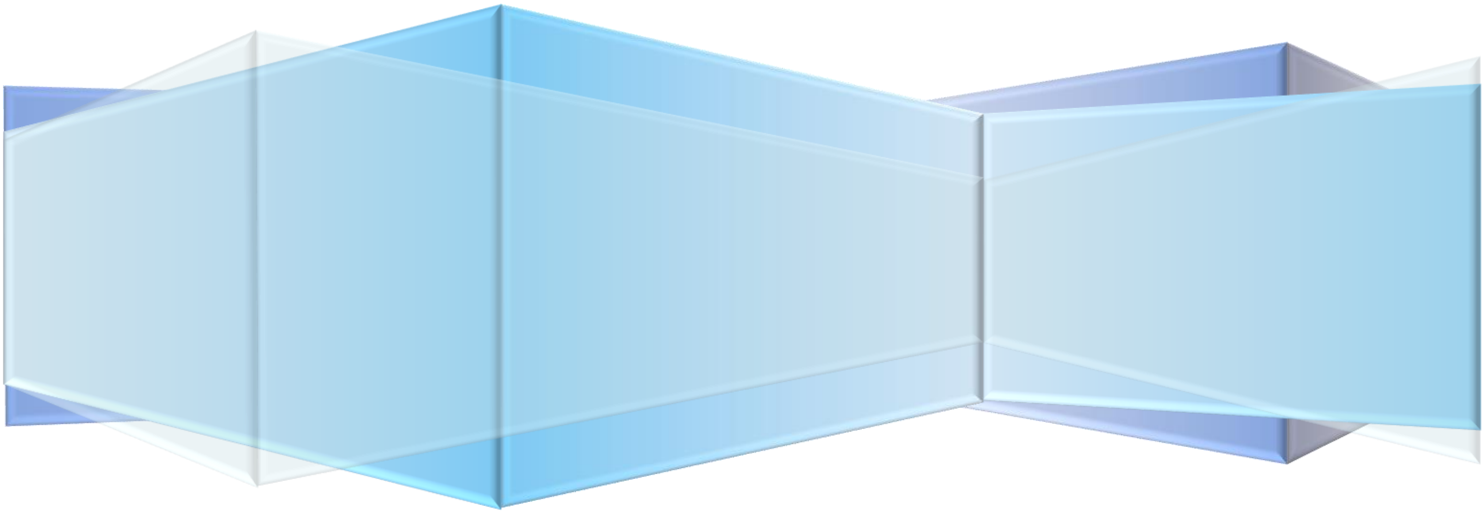
- Survey the presence of EC in the neutral vinous alcohol used in the fortification step to assess its impact on the final EC concentration of Madeira wines;

- Develop new tools for real time and *in situ* measurements of EC and its precursors, giving producers a greater capacity to manage their wines in order to mitigate the occurrence of this compound;

- Increase the knowledge about indigenous yeasts of Madeira wine and use them to produce differentiated wines, adjusted to each variety and desired style, improving their quality and mitigating EC occurrence;

- Finally, despite being an apparently difficult task, develop a polymer with the ability to remove EC from Madeira wine without a major impact on its quality and general characteristics, what would be appealing for producers.

PART IX - REFERENCES



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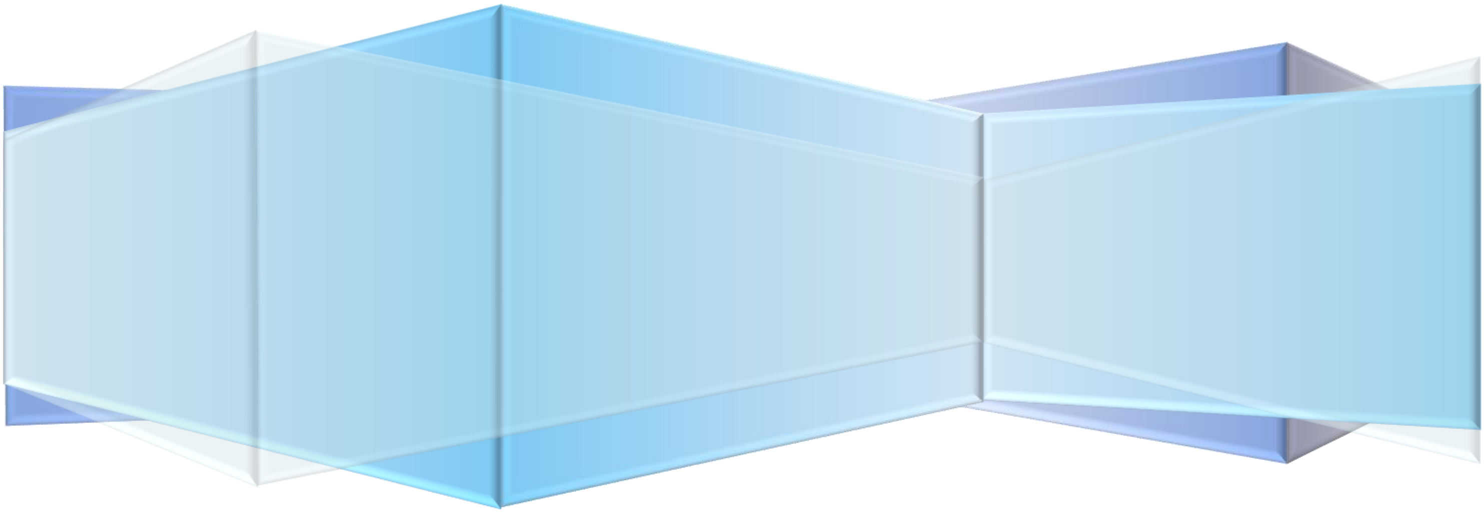
PART X – APPENDIX



Supplementary Table 1 – EC areas used to perform the full factorial design in the miniaturized LLE procedure optimization.

#	**Sample** volume (mL)	**Solvent** volume (mL)	**EC** area
1	8	8	1110440 1121429
2	15	4	733467 700128
3	15	4	777691 788278
4	8	5	998841 802528
5	10	8	1657622 1640575
6	10	8	1579665 1572036
7	8	4	945041 992135
8	8	8	1108007 1122059
9	11.5	6	1290675 1291721
10	8	5	870038 873764
11	10	4	808629 808647
12	10	5	1025015 1017250
13	15	5	1131248 1108255
14	8	4	850365 850365
15	11.5	6	1052178 1075987
16	10	5	1173103 1120181
17	10	4	809796 805344
18	15	5	1521314 1514713
19	15	8	2029131 2007524
20	15	8	2050853 2061622

Supplementary Table 2 - Concentration of EC in stored and commercial Madeira wines with 3 to 41 years of ageing.

#	Years	Concentration of EC (µg/L)	SD
1	3	29	2
2	3	27	2
3	3	30	3
4	3	27	1
5	3	32.8	0.8
6	3	58	4
7	3	31	2
8	3	43	2
9	3	22.9	0.9
10	3	20	1
11	3	20	2
12	3	25	1
13	3	28	3
14	3	12.6	0.3
15	3	33	2
16	3	16	1
17	3	24	2
18	3	26	2
19	3	32	2
20	3	24	1
21	3	22	1
22	3	22.9	0.9
23	3	20	1
24	3	27	2
25	3	75	3
26	3	96	8
27	3	70	3
28	3	109	3
29	3	29	1
30	3	29.7	0.5
31	3	30.4	0.9
32	3	93	8
33	3	56	5
34	3	60	4
35	3	114	11
36	3	17.5	0.7
37	3	31	1
38	3	89	5
39	3	40	3
40	5	41	2
41	5	34	3

42	5	33	3
43	5	56	4
44	5	43	4
45	5	41	2
46	5	36	2
47	5	46	5
48	5	43	3
49	5	85	3
50	5	41	4
51	5	36	3
52	5	36	3
53	5	138	5
54	5	142	8
55	5	129	3
56	5	144	3
57	5	126	5
58	5	96	9
59	5	118	4
60	5	82	3
61	5	40	3
62	5	47	3
63	5	70	6
64	5	40	2
65	6	57	2
66	7	78	6
67	7	20.2	0.9
68	8	93	8
69	10	52	3
70	10	205	3
71	10	258	8
72	10	231	4
73	10	234	5
74	10	134	8
75	10	55	5
76	10	208	19
77	10	194	2
78	10	112	2
79	10	194	9
80	10	158	3
81	10	127	9
82	10	155	6
83	10	127	8
84	10	110	10
85	10	86	6
86	10	87	5
87	10	84	2
88	12	161	17

89	13	296	4
90	13	162	10
91	14	172	16
92	15	239	12
93	15	306	6
94	15	312	20
95	15	324	16
96	18	298	17
97	19	178	21
98	20	262	13
99	20	193	18
100	20	248	18
101	22	111	6
102	25	261	25
103	28	257	7
104	28	379	10
105	30	422	28
106	33	242	14
107	39	382	13
108	41	395	5

Supplementary Table 3 – Impact of iron and copper addition to wine, during accelerated ageing conditions, on EC concentration.

	V0 - After Fortification		Accelerated ageing 70°C for 1 month					
Metal addition	na		na		+ 20 g/L Iron		+ 5 mg/L Copper	
	EC (µg/L)	SD	EC (µg/L)	SD	EC (µg/L)	SD	EC (µg/L)	SD
FW1	11	1	120	14	125	8	118	6
FW2	4.2	0.5	296	21	290	15	300	20
FW3	3.5	0.3	111	3	113	9	105	5
FW4	6.7	0.7	298	15	301	20	295	9