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Evolution of Ethyl Carbamate During Madeira Wine Ageing by GC-MS

A new methodology

MASTER DISSERTATION

João Micael da Silva Leça

MASTER IN APPLIED BIOCHEMISTRY

UNIVERSIDADE da MADEIRA

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SUPERVISOR
José Carlos Antunes Marques

CO-SUPERVISOR
Vanda Nulita Gomes Pereira

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RESUMO

Recentemente o carbamato de etilo (CE) foi reclassificado pela Agência Internacional de Pesquisa sobre o Cancro (IARC) como "provavelmente carcinogénico para humanos" e este ocorre sobretudo em bebidas fermentadas, tendo vários países definido valores limite de CE em bebidas alcoólicas. Neste seguimento e tendo em conta as baixas concentrações encontradas em bebidas alcoólicas, a comunidade científica tem mostrado interesse para o desenvolvimento de novas metodologias analíticas, pelo que a sua simplificação tem um papel relevante no seu controlo e prevenção.

Primeiramente, foi desenvolvida uma metodologia simples, rápida e sensível para a quantificação do CE em vinhos fortificados, através da microextração em sorvente empacotado (MEPS) com cromatografia gasosa acoplada a um detetor de espectrometria de massas (GC-MS). Esta metodologia apresentou uma boa linearidade (R^2 = 0,999) e sensibilidade (LOD = 1.5µg/L). A precisão da metodologia foi avaliada através da repetibilidade e reprodutibilidade (RSD < 7%). Além disso, ficou demonstrada uma boa recuperação (97 - 106%) e sua aplicabilidade (16 vinhos fortificados). Neste seguimento, a metodologia desenvolvida revelou ser uma excelente abordagem para a quantificação rotineira do CE em vinhos fortificados.

A evolução do CE foi também acompanhada durante um ano e meio de envelhecimento de vinhos Madeira sujeitos aos dois métodos tradicionais de envelhecimento, a estufagem e o canteiro, com o objetivo de avaliar as cinéticas de formação. Os resultados obtidos revelaram que o processo de estufagem aumenta a cinética de formação do CE e promove um aumento linear da sua concentração ($R^2 \geq$ 0,977), proporcional ao tempo de envelhecimento (4 meses). No entanto, os vinhos estufados quando sujeitos ao envelhecimento por canteiro apresentam valores de CE praticamente constantes durante os restantes 14 meses. Os resultados obtidos sugerem que a estufagem não parece ser o fator crítico na formação do CE, mas sim a quantidade de precursores no meio.

Palavras-chave: Carbamato de etilo; Vinhos; Microextração em sorvente empacotado (MEPS); Envelhecimento; Processo de aquecimento.

SUMMARY

Recently, ethyl carbamate (EC) was reclassified by the International Agency for Research on Cancer (IARC) as "probably carcinogenic to humans" and occurs mainly in fermented beverages. Nowadays many countries have set limit values for EC in alcoholic beverages. In this sense and taking into account the low concentrations found in alcoholic beverages, the scientific community has shown interest for the development of new analytical methods, whereby its simplification plays an important role in the EC control and prevention.

Firstly, a simple, rapid and sensitive methodology was developed for the EC quantification in fortified wines by microextraction by packed sorbent (MEPS) with gas chromatography coupled with a mass spectrometer detector (GC-MS). This method showed good linearity (R^2 = 0.999) and sensitivity (LOD = 1.5 µg/L). The accuracy of the method was assessed by means of repeatability and reproducibility (RSD < 7%). Moreover, a good recovery has been demonstrated (97 – 106%) as well as its applicability (16 fortified wines). Thus, the developed methodology has proven to be an excellent approach for routine quantification of EC in fortified wines.

The EC evolution was also evaluated during a year and half of Madeira wine ageing submitted to two traditional ageing methods, *estufagem* and *canteiro*, in order to evaluate the formation kinetic. The results revealed that *estufagem* process increased the formation kinetic and promoted a linear increase of the EC concentration ($R^2 \geq 0.977$), proportionally to the ageing time (4 months). However, when the wines are firstly submitted to *estufagem* and then undergo *canteiro* ageing, the EC values remain almost constant during the following 14 months. The results suggest that *estufagem* does not seem to be the critical factor in the EC formation, but instead the amount of precursors in the medium.

Keywords: Ethyl carbamate; Wines; Microextraction by packed sorbent (MEPS); Ageing; Heating process.

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ESTUFA – Madeira wine estufagem monitorization. Funding through FCT.

LIST OF PUBLICATIONS

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Leça, J. M., V. Pereira, A. C. Pereira and J. C. Marques (2014). "Rapid and sensitive methodology for determination of ethyl carbamate in fortified wines using microextraction by packed sorbent and gas chromatography with mass spectrometric detection." Analytica Chimica Acta 811(0): 29-35. Impact factor: 4.31

POSTER COMMUNICATIONS:

Carvalho, M.J., **Leça, J.M.**, Miranda, A., Pereira, V., Pereira A.C., Albuquerque, F., Marques, J.C. Evaluating the polyphenolic content and colour of Tinta Negra must at different fermentation extensions, XX ENCONTRO LUSO-GALEGO DE QUÍMICA, Porto, Portugal, 26–28 Novembro, 2014

Leça, J.M., Pereira, V., Albuquerque, F., Marques, J.C. Effect of winemaking process on the low weight polyphenols content of Madeira wines produced from the traditional varieties, EUROANALYSIS XVII, Varsóvia, Polónia, 25-29 agosto, 2013

Miranda, A., **Leça, J.M.**, Pereira, V., Albuquerque, F., Marques, J.C. Volatile profile comparison between Madeira traditional varieties during winemaking process, EUROANALYSIS XVII, Varsóvia, Polónia, 25-29 agosto, 2013

Leça, J.M., Pereira, V., Albuquerque, F.M., Marques, J.C. Rapid and sensitive method for determination of ethyl carbamate in fortified wines using microextraction by packed sorbent (MEPS)

with gas chromatography and mass spectrometric detection (GC-MS), IN VINO ANALYTICA SCIENTIA, Reims, França, 2-5 julho, 2013

Pontes, M., Pereira, V., **Leça, J.M.**, Albuquerque, F., Marques, J.C. Rapid screening tool for 5-hydroxymethylfurfural determination in Madeira wines by UV spectroscopy and chemometrics, IN VINO ANALYTICA SCIENTIA, Reims, França, 2-5 julho, 2013

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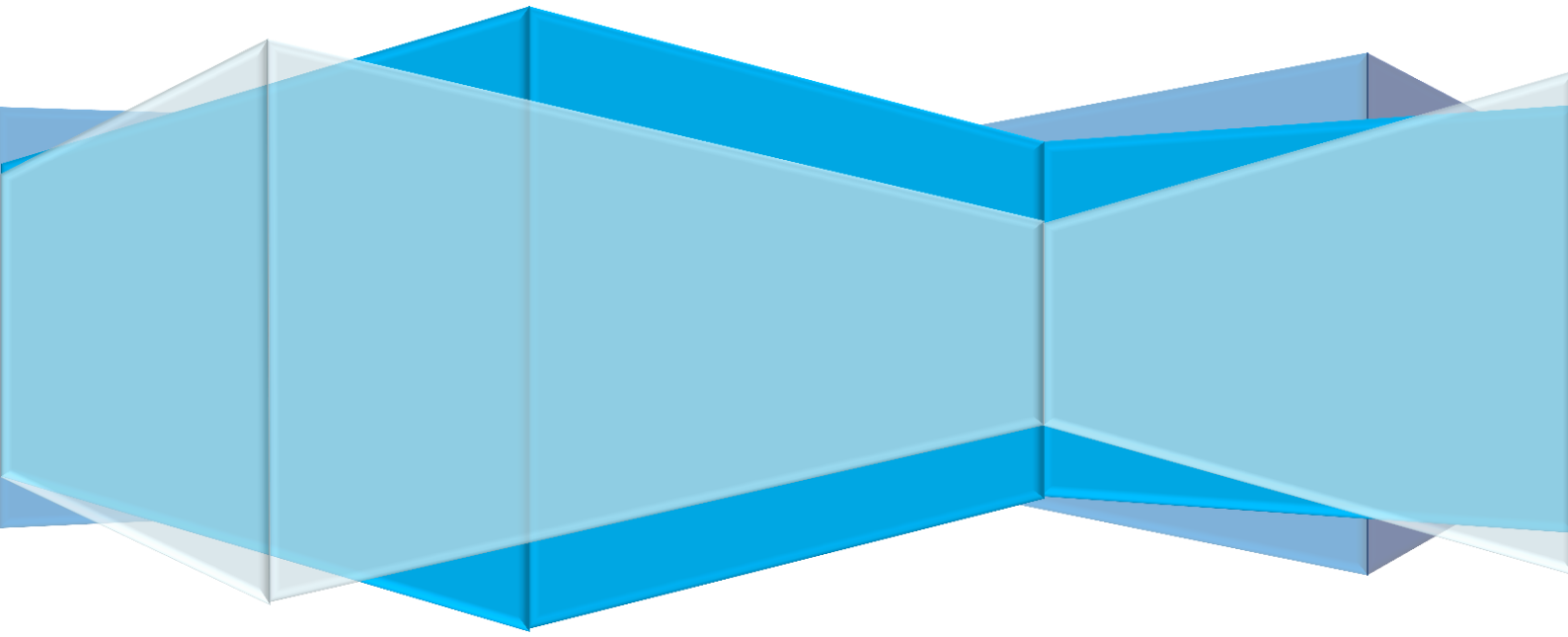
Leça J.M., Pereira V., Albuquerque F., Marques J.C. Evolução dos polifenóis durante o processo de vinificação do vinho Madeira, 11º ENCONTRO DE QUÍMICA DOS ALIMENTOS, Bragança, Portugal, 16–19 Setembro 2012

PART I – GENERAL INTRODUTION



1. SCOPE AND OBJECTIVES

Grapes and wines contain several organic and inorganic forms of nitrogen-containing compounds. The main organic forms are amines, amides, amino acids, pyrazines, nitrogen bases, pyrimidines, proteins and nucleic acids. On the other hand, the inorganic forms include ammonia (in the form of ammonia salts) and nitrates. These group of compounds define the total nitrogen content of grapes and wines (1, 2).

Nitrogenous compounds are indispensable nutrients for yeasts, during alcoholic fermentation. Furthermore, the amino acid composition plays an important role on the wine's aromatic complexity (2, 3). Nitrogen deficiency in must can occur, causing sluggish or stuck fermentations, which leads to the production of hydrogen sulphide in trace amounts, imparting highly offensive odours. Consequently, wine quality can be compromised and that's the reason why the addition of ammonium salts to the must has become a common practice in some wineries, preventing the lack of nutrients for yeasts (4).

The quantity and the form of the available nitrogen not only affects the fermentation but also the composition of the reaction products and therefore the wine quality (5). In general, total nitrogen assays (usually by *Kjeldahl* method) are standard techniques used in wineries to support the winemaking. Nitrogen composition varies between varietals and vineyards and normally the red wines have, on average, twice the concentration of total nitrogen compared to white wines. This variation is related to the differences between the winemaking practices: red wine musts are submitted to higher times and temperatures of maceration, which promotes the dissolution of nitrogenated substances (6).

In recent years, the bioactive potential of nitrogen compounds in wine has raised some concerns and a lot of interest in the scientific community, namely nitrogenated pesticides, which are applied in vineyards and are found in wines, representing a significant issue to the human health. Additionally, amino acids like histidine and tyrosine, in the presence of some enzymes, produced by wine bacteria, originate the corresponding biogenic amines that might cause headaches, nausea, and allergic-type reactions after wine ingestion (4). Ochratoxin A, another nitrogenous compound, is a mycotoxin produced by fungi, mainly by *Aspergillus* species, affecting human health by mycotoxicoses (7).

In particular, amides are a family of nitrogen-containing compounds found in wines that does not seem to have a significant influence on its flavour but have raised some interest to the scientific community due to toxicological concerns. These substances have an amino group ($-NH_2$) linked to a carbonyl group (-CO). The most common amines found in wines are urea and EC. Urea is produced during yeast fermentation, by the arginine metabolism, and can react directly with ethanol producing EC (1). EC is also formed by the reaction of other nitrogened compounds (precursors) with ethanol and appears to be hazardous to human health, due to its carcinogenic and mutagenic potential (2).

The choice of the EC for the study's main purpose was based on not also about the toxicological concerns but mostly on the legislation limitations established for the EC occurrence in beverages, which already imposed some difficulties to wineries in the exportation of Portuguese fortified wines lots. This leads to economic losses and consequently the image of these wines in the international market can be compromised. In this sense, the first objective of this work was the development of an efficient and effective method to quantify EC in fortified wines using simple, fast and affordable analytical procedures, without needing sophisticated and expensive equipment that is available on the market. The first goal for the method development was the EC monitoring during ageing processes, *estufagem* and *canteiro*, in order to verify the formation kinetic.

2. ETHYL CARBAMATE

EC, also known as urethane (CAS number 51-79-6), is the ethyl ester of carbamic acid (Fig. 1). In the past there are records of its use in industry, medicine and veterinary. For several years, it was industrially produced to be used in the preparation and modification of amino resins and as solvent and co-solvent for pesticides and pharmaceuticals. It was also used as a preservative in the food industry, especially in alcoholic beverages, among other applications. The most common method used at the time to commercially obtain the EC was through the reaction of ethyl chlorocarbonate with ammonia (6, 8).

In the medical field, EC was used in the treatment of insomnia, delirium, varicose veins and as a topical bactericidal (8). In the 1940s, after verifying its anti-neoplastic properties, in mice with Walker 256 carcinoma, it started to be applied in cancer therapies (9).

In an initial phase it has been used in patients with terminal breast cancer, which didn't lead to encouraging results. The utilization of EC was extended to the treatment of other neoplasic diseases such as leukemia and multiple myeloma (8) with low rates of success (10). In fact, chronic therapy with EC caused leukopenia, hepatic necrosis and aplastic anemia (11), so its use in human medicine was continuously abandoned. In the veterinary field the EC is regularly used as an anaesthetic, including when it is desired a long term anaesthesia, in laboratory animals, without major changes in their biological parameters (12).

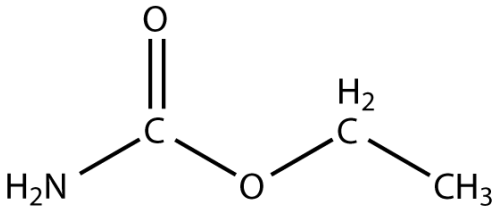


Fig. 1 – Ethyl carbamate.

Since the discovery of its toxicity in 1943, studies regarding the effects of EC in human health have generated an extensive literature about its genotoxicity and carcinogenicity (13). In fact, EC showed to be genotoxic and carcinogenic to various tested species, such as rats, mice, hamsters and monkeys (14). In 2007 the EC was reclassified by the IARC from "possibly carcinogenic" (Group 2B) to "probably carcinogenic to humans" (Group 2A) (15).

EC is formed by the reaction of nitrogen compounds with ethanol and is detected in small quantities, in the order of ng to mg per kg, in fermented foods and beverages such as bread, yoghurt, soy sauce, spirits, wine and beer. Nowadays the consumption of this kind of foods and beverages is the major route of exposure to EC (16). The first reference about the EC presence in alcoholic beverages was in 1971, associated with the degradation of diethyl carbonate, an additive used for the microbial activity control in beverages (17) which was later prohibited. Five years later Ough (18) demonstrated that EC could naturally occur in fermented products, verifying its presence in alcoholic beverages and fermented foods.

In 1985, an official report published by the Liquor Control Board of Ontario, in Canada, revealed high concentrations of EC in various alcoholic beverages, mainly in beverages with higher ethanol content. The toxicological concerns led Canada to be the first

country to define legislation to EC, in order to establish a concentration limit value in alcoholic beverages. At the present time other countries have followed this example, such as the case of Czech Republic, France, Germany, Switzerland and United States of America (19).

In 2005, the Codex Alimentarius Commission of the United Nations Food and Agriculture (FAO) and the World Health Organization (WHO) concluded that EC is a relevant issue but it is not considered as a high priority problem, by the fact that the exposure to the compound, in a common diet is minimal, excluding the consumption of alcoholic beverages. However, concentrations ingested in diet together with those that come from the consumption of alcoholic beverages have been a matter of concern. In this sense, it is important to continue the preventive and control actions in alcoholic beverages (16).

2.1. Formation pathways

Previous studies regarding the EC control have focused their attention in the identification of the main precursors in alcoholic beverages, as well as understanding the impact of external factors in their formation such as light, reaction time and temperature. In this sense, the study of the formation pathways has been playing a crucial role in the preventive and control actions (16).

The main pathways for the formation of EC in alcoholic beverages are identified in Fig. 2. One part of the main precursors of EC contain a carbamoyl group in its constitution, namely urea, citrulline and carbamoyl phosphate. The other, are the cyanide compounds such as hydrogen cyanide and diethyl pyrocarbonate, despite no longer being used in the industry. Attending to these results, the authors attention has been focused in these two groups of compounds, carbamoyl and cyanide compounds, which originate EC by different pathways (20).

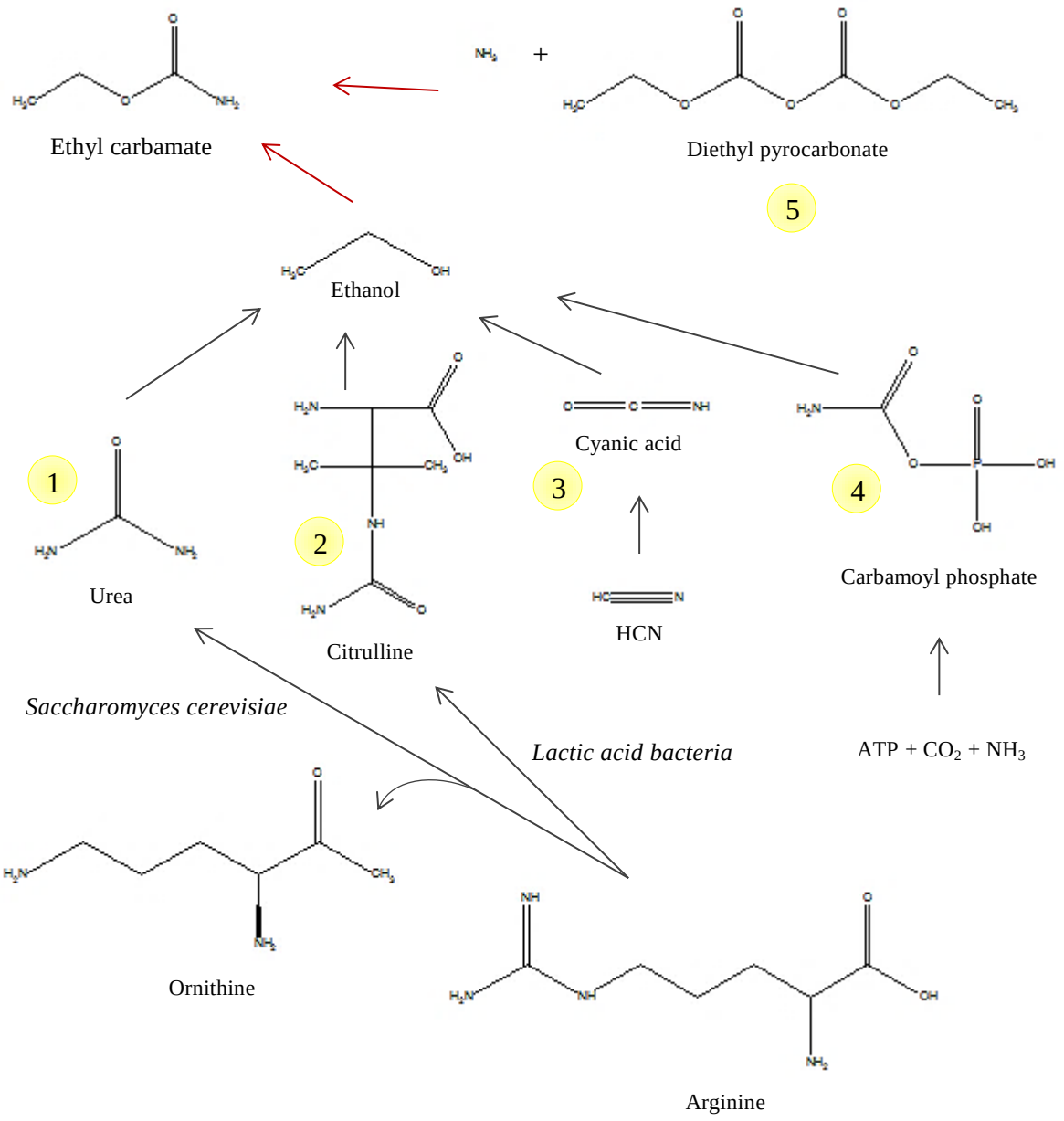


Fig. 2 – Pathways of EC formation in alcoholic beverages. Reaction 1 - urea with ethanol; reaction 2 – citrulline with ethanol; reaction 3 – cyanic acid with ethanol; reaction 4 - carbamoyl phosphate with ethanol and reaction 5 - diethyl pyrocarbonate with ammonia (20).

In wines the most part of EC is formed during or after fermentation, probably by the reaction of carbamoyl compounds with ethanol. In fact, some of these compounds are formed during fermentation. One of the most common ways of EC occurring in acidic medium, such as wines, is the reaction of ethanol with urea. In turn, in distilled alcoholic beverages the major pathway for the formation of EC comes from cyanide volatile compounds, like cyanic

acid, that are able to react in a gas phase or to pass into the distillate. External factors, such as temperature and pH influence the kinetics of these reactions. (19, 20). However, the study of diethyl pyrocarbonate pathway (Reaction 5 of Fig. 2) was taken out of consideration since the prohibition of its use as a food additive (20).

2.1.1. Reaction between urea and ethanol

The abundance of urea in grapes, rice and other fermented sources and its formation during fermentation makes it the most common precursor of EC in food products, by its reaction with ethanol (Reaction 1 of Fig. 2) (20). Urea is a compound found in many fermented foods but its concentration is typically higher in alcoholic beverages or baked products. In alcoholic beverages urea can be found in the order of mg per litre. In wines it is assumed that the most part of urea has origin in the fermentation step, almost exclusively from arginine degradation metabolism by yeast. Arginine is an amino acid, abundantly found in grape juice, which is used as a nitrogen source by the yeast to ensure its growth and cell proliferation (21-23).

The catabolism of arginine (Fig. 3), as the main source of urea, was extensively studied by Davis (24) in *Neurospora crassa* and *Saccharomyces cerevisiae*. This process consists in the hydrolysis of arginine to ornithine and urea, being this reaction catalysed by arginase. Thereafter the formed urea can be metabolized to ammonia and carbon dioxide.

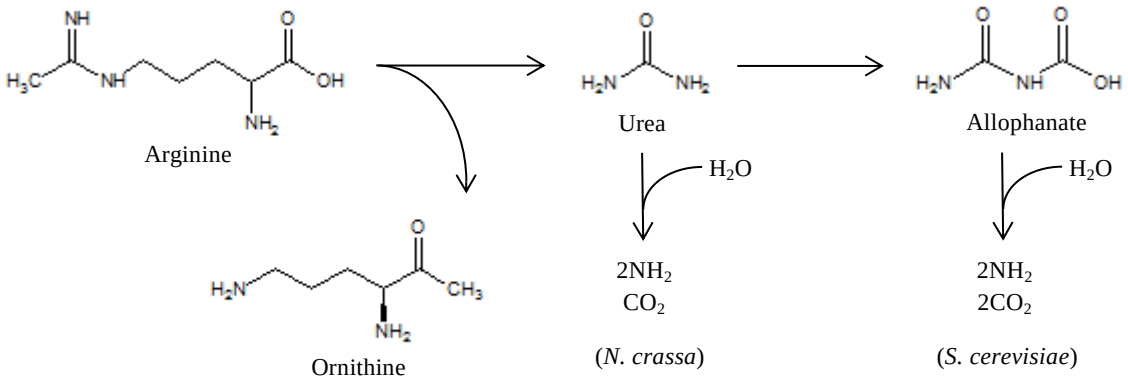


Fig. 3 - Catabolism of arginine in *Neurospora crassa* and *Saccharomyces cerevisiae* (24).

EC production resultant from the reaction of urea and ethanol, at room temperature, has a moderate kinetic, however its formation increases with temperature (in baking, boiling or toasting) (25, 26). Other routes for the formation of EC have also been described, involving the application of temperatures above 60 ºC through isocyanate, cyanate or cyanic acid as products which might react with ethanol (27-29).

The production of fortified wine, such as Madeira wine, involves a step that consists in the interruption of fermentation by the addition of natural grape spirit (containing 95% (v/v) of ethanol). This procedure usually results in an increase of urea in solution. This is due to the ability of ethanol to remove urea from the yeast intracellular space to the solution. The concentration of urea in the yeast also varies during fermentation, so the amount of urea extracted depends on the time in which the fortification occurs (30-32).

2.1.2. Reaction between citrulline and ethanol

Citrulline is found in grape wines and fruit brandies. Despite being quantified in a certain concentration in grape juice, citrulline is generated in larger quantities through arginine anabolism during the fermentation (Fig. 4). Summarized, ornithine and carbamoyl phosphate reacts to form arginine, being citrulline an intermediate product of this metabolism (24). Citrulline is a precursor of EC (Reaction 2 of Fig. 2) and its occurrence is more commonly associated with lactic acid bacteria activity, via malolactic fermentation (20, 23, 24).

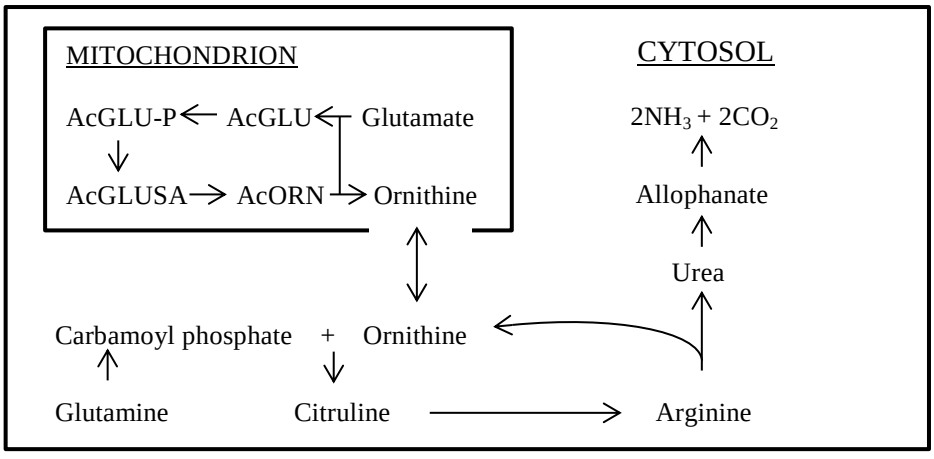


Fig. 4 - Anabolism of arginine in *Neurospora crassa* and *Saccharomyces cerevisiae* (24).

Liu (33) demonstrated that the production of citrulline is correlated with the increase of EC and the degradation of arginine. The catabolic and anabolic metabolism of arginine, by yeasts, occurs simultaneously during fermentation, which can explain this result. In fact, by using fermentation model systems, it was verified that the amount of citrulline produced can be applied to predict EC potential levels through the use of a linear correlation (34, 35).

2.1.3. Reaction between cyanic acid and ethanol

Chemically, the cyanic acid is a compound which is in the liquid or gaseous state at ambient temperature. Cyanic acid boiling point is 25.6 °C and its water solubility is high (36). There are several species which produce cyanogenic glycosides, secondary metabolites of plants, and the corresponding hydrolytic enzyme. Cyanogenic glycosides decompose in sugar and cyanohydrin by enzymatic or thermal cleavage. Hydrocyanic acid is formed after cyanohydrin decomposition and in the presence of oxygen, oxidizes to cyanic acid, a precursor of EC (Reaction 3 of Fig. 2) (37, 38). Cyanidric acid is more associated with stone fruit musts however, in grape must, only minor amounts were detected, resultant from grape seeds. Pressing increases the passage of cyanidric acid to must but the quantities released are considered reduced (37, 39).

There are several factors that have been mentioned as crucial in the formation of EC from cyanide, such as pH, light, ethanol content, temperature and presence of metallic species (19, 27, 40). Distillates beverages are those that normally present higher concentrations of EC, which is mainly produced during or after the distillation step. In this case, the volatile precursors of EC such as hydrocyanic acid and cyanic acid, present or formed during distillation, have a great impact in its production. Their low boiling points (approximately 30 ºC) make them likely to react in the gas phase with ethanol, producing EC. After obtaining the distillate, the presence of metallic salts (Cu(II) or Fe(III)) and light plays a crucial role in the kinetic of EC formation. It seems likely that distillation promotes the appearance of other natural EC precursors (19).

In 2007, the scientific panel opinion of the European Food Safety Authority (EFSA) gave emphasis to a particular issue, more associated to stone-fruit brandies, which brought up the relation between the presence of hydrocyanic acid in food and alcoholic beverages and the formation of EC during the maturation process. Long-term toxicity studies on hydrocyanic

acid and cyanides are still missing. Besides hydrocyanic acid did not reveal a genotoxicity potential its consumption at high concentrations, through fruit brandies, is considered undesirable (41).

2.1.4. Reaction between carbamoyl phosphate and ethanol

The carbamoyl phosphate can be formed, inside the yeasts or bacteria cells, by adenosine triphosphate (ATP), ammonia (NH_3) and carbon dioxide (CO_2) catalysed by the enzyme carbamoyl-phosphate synthase. Carbamoyl phosphate such as citrulline is an intermediate product of arginine anabolism during fermentation (fig. 4), principally during malolactic fermentation. The presence of this compound in the extracellular medium is rarely observed, having less attention of the scientific community in the control of EC occurrence. Carbamoyl phosphate ethanolysis leads to the formation of EC (Reaction 4 of Fig.2) (18, 24, 40).

2.2. Toxicity

The toxicity of EC was firstly recognized in 1943 in a work developed after the authors encountered a high incidence of lung cancer in laboratory animals anesthetized with EC (13). During the next two decades various experiences with animals were carried out in order to assess the risk associated with EC exposure. In general the results were consistent, taking into account that all species have developed different benign and malignant tumours (42). The EC shows a low acute toxicity, however its continued administration, at lower doses, promotes an increase of biological dysfunctions, carcinogenesis and mortality (16). The oxidation of EC to vinyl epoxide is the most worrying pathway in terms of genotoxicity (15). Other studies allowed to conclude that the metabolizing mechanism of EC occurs essentially in the liver and lung (14, 43, 44).

2.2.1. Carcinogenesis

In order to assess the toxicity of EC, several experiments were carried out with animals, being most of them rodents (15). In rats, orally treated with EC, it was verified an increase of carcinogenesis namely lung adenomas, lymphomas, tumours of the mammary

gland and others (14, 45). Topical application of EC also induced pulmonary adenomas and breast carcinomas (46). After administration of EC in hamsters, they experienced an increase incidence of cancers including melanoma, mammary gland tumours and carcinomas of the thyroid, ovary, liver and spleen, among others (47). The EC carcinogenesis was also verified in primates, with higher incidence in lung cancer and hepatocellular adenomas and carcinomas (48, 49).

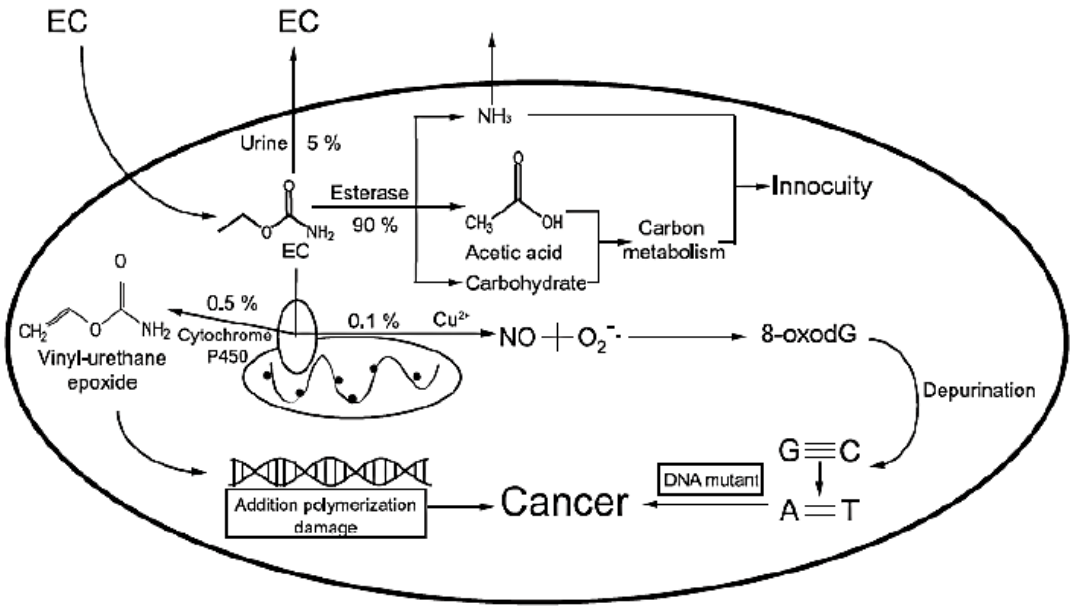


Fig. 5 – Carcinogenesis metabolism of EC (adapted from Hübner (50)).

More recently Hubner (50) described the EC mechanism of carcinogenesis, through a study of genotoxicity in salmonella, yeast and human lymphoblastic cells (Fig.5). The EC can be metabolized by two pathways. Over 90% of EC is hydrolysed to ethanol, ammonia and carbon dioxide by microsomal esterases and amidases (51, 52). The second metabolization pathway of EC occurs through its oxidation by cytochrome P450IIE1 (CYP2E1). This reaction originates 2-hydroxyethyl carbamate, N-hydroxyethyl carbamate and vinyl carbamate. The vinyl carbamate is then converted to the ultimate carcinogen, vinyl carbamate epoxide, by epoxidation (53-55). N-hydroxyethyl carbamate is a compound that generates nitric oxide, which can cause oxidative DNA damage and 2-hydroxyethylcarbamate can bind covalently to DNA, however they do not make a significant contribution to the carcinogenicity of EC (15,

56). It was also concluded that the metabolism of EC by CYP2E1 is necessary for its genotoxicity (43).

Major reviews were performed in 1989 by the California Department of Health Services, discussing the state of the art in the risk assessment for EC carcinogenicity (42). In 2005, the Codex Alimentarius commission of FAO and WHO considered the EC a relevant public health issue (16). More recently, in 2007, the EC was reclassified by the IARC as a "probably carcinogenic to humans" (Group 2A), the second most worrisome classification of IARC to carcinogenesis risk (15).

2.2.2. Influence of ethanol on ethyl carbamate metabolism

Besides the consumption of alcoholic beverages is the major exposure route to EC, the ethanol also interferes in its metabolization. Yamamoto (57) found that the administration of EC, together with ethanol, diminish the EC hydrolysis to carbon dioxide and its clearance (57). Chronic use of ethanol induces the formation of CYP2E1, the effect of ethanol in the decrease of EC clearance suggests that there is a competition for this oxidase enzymes (58). The neuropharmacological effects, like hypnotic and anaesthetic properties, of EC are increased wen the chemical is co-administered with ethanol (42). In a long-term study, during 2 years, of administration of EC in 5% of ethanol *vs.* drinking-water it was observed a decrease in body weight and an increase of biological dysfunctions and lethality.

2.3. Legislation

In 1985, an official report published by the Liquor Control Board of Ontario, in Canada, raised the attention to the topic of EC toxicity. The organization published the results of several alcoholic beverages with high levels of EC. In this report it was verified that fortified wines have the predisposition to form high levels of this compound (59). Canadian authorities, based on a study of EC toxicity developed by Schmahl (60), imposed the following limits: 30 µg/L in table wines, 100 µg/L in fortified wines, 150 µg/L in wine spirits and whiskies and 400 µg/L in fruit brandies and liquors, taking into account the consumption of each alcoholic beverage.

This legislation raised several problems at that time. There were no analytical methods with the required sensitivity nor accessible to most laboratories. Moreover, producers of alcoholic beverages did not know how to control EC levels in their products. However this imposition led to a great attention of the scientific community, resulting in a pronounced advance in the control of this toxic compound (15, 16, 19, 41, 59).

Public health options vary depending on the country and in the European Union does not exist a common legislation to EC maximum levels (41). Nowadays, some countries have established its own legislation (Table 1). The Czech Republic implemented an identical legislation to Canada. However, United States of America are the most restrictive in respect of table and fortified wines. Germany and Switzerland imposed limits values to fruit brandies and France, in addition to fruit brandies, also included distilled spirits in the legislation (19).

Table 1 - Maximum levels for ethyl carbamate in alcoholic beverages

Country	Wine (µg/L)	Fortified wine (µg/L)	Distilled spirit (µg/L)	Sake (µg/L)	Fruit brandy (µg/L)
Canada	30	100	150	200	400
USA	15	60	n.r.	n.r.	n.r.
Czech Republic	30	100	150	200	400
Switzerland	n.r.	n.r.	n.r.	n.r.	1000
France	n.r.	n.r.	150	n.r.	1000
Germany	n.r.	n.r.	n.r.	n.r.	800

n.r. - no regulation

2.4. Determination

The EC was quantified for the first time in alcoholic beverages by Lofroth (17), demonstrating its formation from the degradation of diethyl pyrocarbonate. Walker (61) developed the first methodology to quantify EC by gas chromatography (GC). The method allowed to quantify the compound in the order to 100 µg/L. For each analysis it was necessary 250 mL of wine and the extraction process was highly complex and time-consuming. With slight changes on the same methodology, Ough (18) detected for the first time levels of 10 µg/L of the compound.

Since then, the GC has been considered the preferential technique to quantify EC in alcoholic beverages. The emergence of new detectors, like mass spectrometer detector (MS), and new techniques of extraction have motivated the development of new methodologies, with simplified extraction processes and higher sensitivities (15, 19, 42). The legislation imposed by Canada authorities, in 1985, also forced the scientific community to develop new analytical methods. The analysis of this compound in complex matrixes, such as alcoholic beverages, is hard to achieve because of the low concentration levels (in the order of µg/L) and due to the high interference caused by other matrix components, even when extensive clean-up procedures are applied (15).

Nowadays, the GC coupled with MS has become the excellence technique for the quantification of EC. Usually the MS detector is operating in selected-ion monitoring mode and in general the ions m/z 89, 74, and 62 are used to identify the compound and the ion m/z 62 is used for quantification. The propyl carbamate (PC) or butyl carbamate (BC) have been used as internal standard, however there has been an increase tendency to use EC labelled with deuterium, 13C or 15N isotopes, in mass spectrometric detection (15, 19).

The Association of Official Analytical Chemists International adopted as their official methods, to quantify EC in different matrixes, two GC methods, one with MS detection and other by thermal energy analyser detection (TEA) (62, 63). The International Organisation of Vine and Wine (OIV) official method, for determination of EC in beverages, requires a GC/MS equipment, using selected ion monitoring mode, and PC as internal standard. This method involves a complex and time-consuming extraction procedure, using a diatomaceous earth solid phase extraction column (64).

The advanced chromatographic separation system, by multidimensional GC, with a previous solid-phase extraction (SPE) simplifies the extraction procedure to determine EC, however it is necessary to remove the ethanol from the sample by a time-consuming technique (65). Another advanced approach is the tandem MS (MS-MS) detection that improves the sensitivity and specificity of the methods (66, 67). Liquid chromatography is also used to quantify the EC using derivatization with 9-xanthydrol and fluorescence detection (HPLC-FLD) (68-70).

The solid-phase microextraction (SPME) is a solvent-free technique characterized by simple extraction procedures and be easy to apply, which can be automatize. In this sense, the

head space solid-phase microextraction (HS-SPME) has been successfully applied to quantify EC in wine and spirits. The HS-SPME followed by GC-MS-MS analysis to determine this compound in alcoholic beverages have been widely used by the scientific community, generating several articles (15, 66, 71). Emergent techniques, like MEPS, may provide simpler and more robust methods for the EC determination in alcoholic beverages, which contributes to its prevention and control.

2.5. Prevention and control

Identifying the causes of human cancer is the priority on its prevention and today the high incidence of this disease continues to increase. The EC raises some concerns in terms of public health as a "probably carcinogenic to humans" being a problem more associated with alcoholic beverages consumption. The prevention and control of EC levels, used in the beverage industries, have obtained good results, however it seems to be important to keep implementing these effective preventive and control actions. There has been a clear reduction of this compound in commercial products over the past 20 years. These results are due to the efforts made in the identification of the main precursors, the understanding of its formation mechanisms, as well as the impact of external factors such as light, temperature and time of storage or ageing (15, 16).

Nowadays, there are still no general methods for EC prevention in all foods and beverages but some different strategies have been developed and applied in industrial scale. Based on recent research, the mitigation of EC in alcoholic beverages can be achieved by the modification of raw materials and by the optimization of the fermentation parameters, like using commercial yeasts that excretes low concentrations of urea and by the addition of acid urease that degrades urea or even by the modification of the fermentation yeast cells (19, 20).

Multiple factors can affect the EC formation, depending on the considered fermented or distilled beverage. According to the Food and Drug Administration (FDA), urea, citrulline, and arginine participate in the formation of EC in most fermented beverages. The reaction between urea and ethanol seems to be the key reaction in its formation in wine. Vineyard fertilization with urea, ammonia and other N-fertilizers has a direct influence in the nitrogen content of musts that potentiate the occurrence of EC. N-fertilizers should only be used to

provide sufficient nutrients for yeast cell growth. FDA also indicates the enzymatic hydrolysis of urea, with urease enzyme, as a way to mitigate EC.

The fortified wine producers have to take into account that the fortification step increases the urea excretion by yeast cells. Urea is usually formed during the early and middle stages of fermentation and laboratory tests should be performed in order to minimize its concentration in solution (72).

In general, some techniques can support the prevention of EC occurrence, such us lowering the pH, lowering the ethanol content and lowering the temperature during the fermentation and storage. Supplementing adjustments at the beginning of the fermentation with diammonium phosphate is also a preventive technique. These techniques are not effectiveness and cannot be applied to all fermentative practices. Additionally, factors such as oxygen and light irradiation play an important role in EC formation (20). Another solution proposed in wine production was the use of commercial yeast with low urea excretion. The solution to reduce EC that appears to be more effective and that is inclusively recommended by FDA is the application of acid urease. Acid urease has an optimum pH compatible with wine, and its feasibility to remove urea has been demonstrated. The industrial application of acid urease in wineries still limited because of longstanding and expensive procedures. The perspectives about acid urease utilisation are good and a lot of research has been accomplished (20, 72).

Methods involving metabolic engineering, through the modification of fermentative yeasts, have been successfully applied. In fact, 89% of EC reduction can be achieved. These methods are based on the repression of urea by inhibiting the expression of arginase (CAR1) or by controlling the amount of urea in medium, through the enhancing of the genes that regulate its metabolism and the transport (DUR1,2 and DUR3) (Fig. 6). Consequently, these methods implicate changes on the beverages flavour (20, 73).

Regarding distilled spirits, the precursor that raises major concerns is the cyanic acid. The high-quality of raw materials, high hygienic standards, the optimization of the fermentation conditions and the conditions and time of storage play an important role in EC mitigation. Some procedures have been tested namely the addition of potassium metabisulfite or an oxygen absorber, the addition of enzymes to decompose cyanide, the destoning of the fruit prior to mashing, the addition of copper salts or copper catalysts in the distillation

process, the application of steam, low temperature, high reflux rate distillation and also metabolic engineering. These methods reduce the EC levels, but none is completely effective and most of them promotes accentuated changes on the flavour and a set of other problems (20).

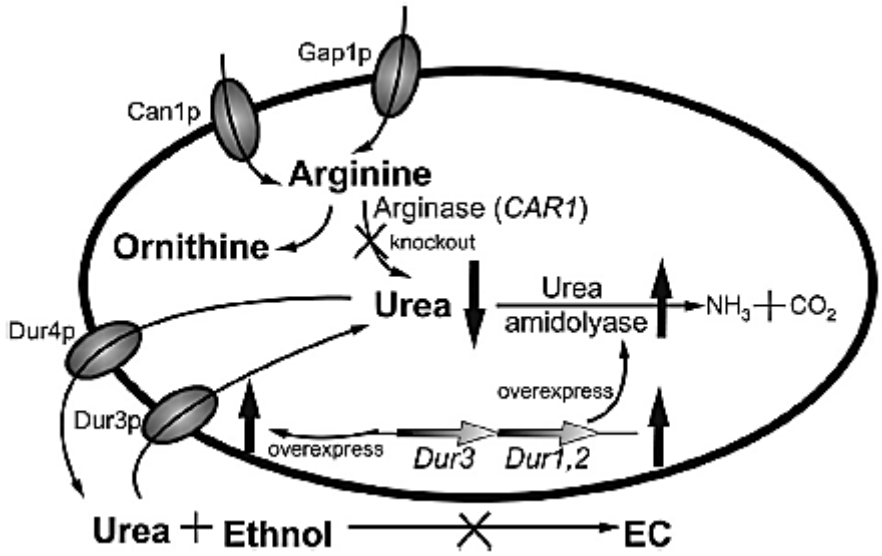


Fig. 6 – Diagram of the two approaches used in metabolic engineering methods for EC elimination. Repression of the gene encoding for arginase (CAR 1) and the enhancing of genes that regulate the metabolism and transport of urea (DUR1,2 and DUR3) (adapted from Butzke **(**73)).

3. MADEIRA WINE

Madeira wine is a fortified wine with an alcoholic content between 17 to 22%. Still nowadays, Madeira wine is an internationally well-recognized product, not only by its History but also by its peculiar characteristics, representing a product with significant value to Madeira Island's economy (Portugal). It is characterized by a marked and intense flavour and it is usually served as an aperitif or dessert wine, being also recognized for its high longevity even after opening. Madeira wine unique features are, in part, due to the characteristics of the island, namely from the soil of volcanic origin, the proximity to the sea and the moderate climatic conditions. The traditional white varieties are Sercial, Verdelho, Boal and Malvasia and the red variety is Tinta Negra, all *Vitis Vinifera* L. species.

Madeira wine has a unique winemaking process and the extension of fermentation is defined according to the grape variety and the style desired (dry, medium-dry, medium-sweet and sweet wines may be produced). The fermentation is stopped by the addition of grape spirit containing 95% v/v of ethanol. After the post-fermentation treatments, the obtained wine undergoes a maturation process that can go directly into wood casks, in an ageing process named *canteiro,* or can be previously submitted to a unique accelerated ageing process, the *estufagem* process. In *canteiro*, the wine remains in casks for a minimum period of 3 years. On the other hand, in *estufagem* the fortified wine is usually heated at about 45 °C for a minimum period of 3 months. In the past, this process emerged to reproduce the wine ageing during those long sea voyages. This process is another particularity that makes Madeira wine a distinctive fortified wine (74-78).

Madeira wine has been raising interest in the scientific community, resulting in a considerable number of scientific studies. Its volatile profile is the aspect that has been receiving great attention and, up to the moment, has originated more scientific articles (79-91). Chemometric studies in Madeira wine, using advanced multivariate statistical methodologies, have also been applied in order to characterize the ageing process, defining patterns of ageing and predicting wine age (92-96), as well as allowing the quantification compounds of interest, in a quick and easy way (96). The *estufagem* process was deeply studied by Pereira (97), that evaluated the effect of this ageing process in the main characteristics and constituents of Madeira wine, resulting several scientific papers (88, 98-101).

Perestrelo (67) developed a Head Space - Solid Phase Microextraction (HS-SPME) method combined with comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC × GC-ToFMS) to quantify EC in fortified wines. Their results revealed that 50% of the analysed wines exceeded the 100 µg/L in a sampling of only 20 monovarietal Madeira wines. GC × GC-ToFMS is one of the most advanced technologies to identify and quantify vestigial compounds, though Madeira wine sector as well as most laboratories does not have access to this kind of expensive equipment. In this sense arises the necessity to develop an analytical methodology to quantify EC in Madeira wine without using sophisticated and expensive equipment. Moreover, it was also verified the necessity to study the EC evolution during the ageing process in order to further develop strategies to mitigate its formation.

PART II - DEVELOPED METHODOLOGY

This Part of the work is based on the following publication:

Rapid and sensitive methodology for determination of ethyl carbamate in fortified wines using microextraction by packed sorbent and gas chromatography with mass spectrometric detection

J. M. Leça, V. Pereira, A. C. Pereira, J. C. Marques

Analytica Chimica Acta, 2014;811:29-35

4. METHODOLOGY FOR DETERMINATION OF ETHYL CARBAMATE IN FORTIFIED WINES USING MEPS EXTRACCION WITH A GC-MS EQUIPMENT

Abstract:

This section presents a new methodology to quantify EC in fortified wines. The presented approach combines the MEPS, using a hand-held automated analytical syringe, with one-dimensional GC coupled with mass spectrometry detection (GC–MS). The performance of different MEPS sorbent materials was tested, namely SIL, C2, C8, C18 and M1. Also, several extraction solvents and the matrix effect were evaluated. Experimental data showed that C8 and dichloromethane were the best sorbent/solvent pair to extract EC. Concerning solvent and sample volumes optimization used in MEPS extraction an experimental design (DoE) was carried out. The best extraction yield was achieved passing 300 µL of sample and 100 µL of dichloromethane. The method validation was performed using a matrix-matched calibration using both sweet and dry fortified wines, to minimize the matrix effect. The proposed methodology presented good linearity (R^2 = 0.999) and high sensitivity, with quite low limits of detection (LOD) and quantification (LOQ), 1.5 and 4.5 µg/L, respectively. The recoveries varied between 97 and 106%, while the method precision (repeatability and reproducibility) was lower than 7%. The applicability of the methodology was confirmed through the analysis of 16 fortified wines, with values ranging between 7.3 and 206 µg/L. All chromatograms showed good peak resolution, confirming its selectivity. The developed MEPS/GC-MS methodology arises as an important tool to quantify EC in fortified wines, combining efficiency and effectiveness, with simpler, faster and affordable analytical procedures that provide great sensitivity without using sophisticated and expensive equipment.

4.1. Introduction

The concerns raised by the toxicological aspects of EC together with the implemented legislations and low concentration levels found in wines (µg/L), as well as the occurrence of interferences on detection, has motivated several researchers to develop new methods to determine EC in wines. The ability to quantify the EC and the simplification of its

quantification, with the advancement of technology, has played an important role in EC control and prevention. Nonetheless nowadays the quantification of the EC stills not accessible to most wine producers and usually involves expensive equipment, such as GC-MS, and complex and extensive extraction procedures or simple extraction procedures associated with variants of the GC-MS, much more expensive, such as multidimensional gas chromatography (GC-GC) or triple quadrupole mass spectrometry detection (MS/MS) (15, 19).

Several extraction and chromatographic techniques have been used, including continuous liquid-liquid extraction (LLE) with Soxhlet apparatus (102), derivatization with 9-xanthydrol followed by high performance liquid chromatography (HPLC) with fluorescence detection (69) and even LLE after derivatization, followed by GC–MS (103). Other methods make use of solid phase extraction (SPE), but using multidimensional gas chromatography with mass spectrometry (GC-GC/MS) (65) or liquid chromatography with tandem mass spectrometry (LC-MS/MS) for detection (70). Several efforts have been done to develop new methodologies to determine EC without using long procedures and hard-working analyses, combining precision to high sensitivity. In this regard, headspace solid phase microextraction (HS-SPME) has been gaining great highlighting using gas chromatography with tandem mass spectrometry detection (GC-MS/MS) (104) and two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC–ToFMS) (67).

Recently, MEPS has also becoming emergent, arising as a feasible and easy-to-use extraction technique. MEPS derives from the miniaturization of the conventional SPE, but with additional advantages namely uses small sample and solvent volumes (microliters) and consequently reduces the environmental impact, increases the analysis sensitivity and enables the direct injection into the LC or GC instruments. The small cartridge can be packed or coated with different silica-based polymers: SIL (unmodified silica), C2 (ethyl), C8 (octyl), C18 (octadecyl) and M1 (80% C8 and 20% SCX - strong cation exchanger using sulfonic acid bonded silica), providing selective and suitable sampling conditions (105). The MEPS technique has been used to determine other compounds of interest for the alcoholic beverages industry (106-108), however, as far as we know, it has never been applied for the analytical determination of EC.

The aim of this part of the study was to develop a fast, simple and sensitive methodology to quantify EC in fortified wines using MEPS extraction combined with one-dimensional GC-MS equipment.

4.2. Materials and methods

4.2.1. Chemicals and samples

EC was purchased from Acros Organics (Geel, Belgium), while butyl carbamate (BC), used as internal standard (IS), was obtained from Sigma–Aldrich (Steinheim, Germany). All standards had a purity grade of more than 97%. Absolute ethanol, > 99.8% (GC), was purchased from Sigma–Aldrich (Steinheim, Germany), tartaric acid and methanol from Panreac (Barcelona, Spain) while acetonitrile, ethyl acetate and dichloromethane were from Fisher Scientific (Leicestershire, UK). Ultra-pure water (18 MΩ) was prepared by the Simplicity®UV ultrapure water (type 1) apparatus from Millipore (Milford, MA, USA).

EC and BC stock solutions of 1 g/L were prepared by dissolving appropriate amounts of each compound in ultra-pure water. In order to obtain the matrix-matched calibration solutions, suitable dilutions of the stock solutions were prepared with ultra-pure water, to obtain the intermediate solutions of 50 mg/L in EC and 10 mg/L in BC, which were then used to spike dry and sweet fortified young wines. Each calibration point was extracted in triplicate, within the validation range 5-400 µg/L.

The sweet and dry fortified wines used to perform the matrix-matched calibrations were obtained from *Vitis vinifera* L. white varieties and were absent of quantifiable amounts of EC and BC. Regarding the application sample set, 16 fortified wines, aged up to 36 years old and with ethanol contents between 18 to 20% were analysed using the developed methodology.

4.2.2. Apparatus and chromatographic conditions

eVol® MEPS™ hand-held automated analytical syringe (SGE Analytical Science, Australia) of 500 µL was used and MEPS barrel insert needles (BINs, 8 µL, 45 µm particle

size and 60 Å pore size), containing 4 mg of different packing polymers (SIL, C2, C8, C18 and M1) were tested to optimize the extraction.

All analyses were carried out using a GC-MS system, the TRACE GC Ultra gas chromatograph equipped with the ISQ single quadrupole and the TriPlus autosampler (liquid mode) from Thermo Scientific (Hudson, NH, USA). The column was a DB-WAX 60 m × 0.250 mm with 0.50 μm film thickness from Agilent J&W (Folsom, CA, USA). The carrier gas was helium at a constant flow rate of 1 mL/min. The injector port that was kept at 230 °C, in splitless mode, while the transfer line and the ion source were maintained at 230 and 240 °C, respectively. The oven temperature program started at 40 °C, hold 1 min, increased to 180 °C at 20 °C/min and hold for 15 min, with a total GC run time of 23 min.

The mass spectrometer was operated in electron impact (EI) mode at 70 eV. Initially, some tests with standards and samples were performed with chromatograms obtained in total ion count (TIC), in the range m/z 30–400, to ensure the retention time of EC and BC. Then, selective ion monitoring (SIM) of the three characteristic ions m/z 62, 74 and 89 of both compounds was tested in order to ensure good resolution. Also, to increase the sensitivity and to meet quantification purposes, further analyses were performed using the ion m/z 62.

4.2.3. MEPS optimization

As aforementioned, retention times of EC and BC were previously determined using individual standards dissolved in dichloromethane, with chromatograms recorded in TIC. Several solvents were individually analysed in order to check the absence of EC, specifically ethanol, methanol, acetone, ethyl acetate, acetonitrile and dichloromethane, through direct injection into GC-MS. Additionally, several commercially available sorbent materials (SIL, C2, C8, C18 and M1) were tested and the extraction was performed with all EC free solvents. Meantime, the best extraction solvent was also chosen. The standard solution used for these tests was set to 100 μg/L of EC (limit imposed by Canada) spiked with 24 μg/L of BC (internal standard). After choosing the ideal BIN and extraction solvent, the MEPS procedure was then optimized performing an DoE. This is an experimental strategy in which factors (experimental variables that can affect the response) are varied together, instead of one at a time. The experiments carried out are designed economically and efficiently, while individual and combined factors are evaluated (109). In this study, the analysed factors were the sample

and solvent volumes to be used in the extraction procedure. As response variable, the GC-MS data was used, namely to evaluate the factors-levels combination that ensure its maximization. For each factor, three levels were examined, varying from 200 to 1000 µL and 100 to 350 µL to sample and solvent volumes, respectively. The plan to carry out the experiments as well as the data analysis was computed using Matlab software (version 7.6, the Mathworks Inc.).

4.2.4. MEPS optimized procedure

Firstly, 5 mL of sample/standard solution, previously spiked with 12 µL of internal standard (BC solution of 10 mg/L), were filtered through 0.45 µm syringe Acrodisc GHP filters (Pall Gelman Sciences, Ann Arbor, MI, USA). Following this step, samples were then extracted using the C8 sorbent, which was selected to extract EC, after being performed the optimization tests. Before each extraction, the sorbent was washed and conditioned twice with 500 µL of methanol, dichloromethane and ultra-pure water, at about 33 µL/s. Then, 300 µL of sample were passed through the sorbent at a flow rate of about 5 µL/s. Thereafter, a drying step was performed passing, five-fold, 500 µL of air at 250 µL/s. EC was then eluted with 100 µL of dichloromethane, aspirating at 1.7 µL/s and dispensing at 33 µL/s, approximately. Each sample and standard solution was extracted in triplicate and 3 µL of extract were injected twice into the GC-MS port. Each BIN was used for about 120 extractions. The DoE optimized MEPS extraction procedure is schematized in Fig. 7.

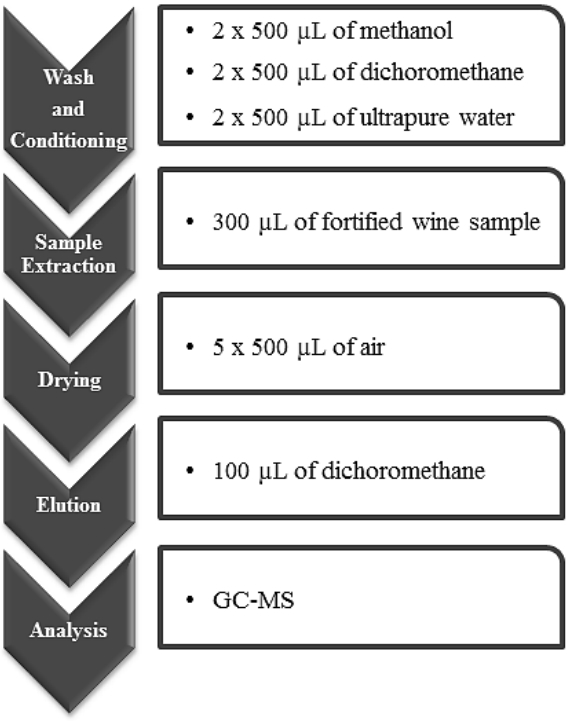


Fig. 7 - DoE optimized MEPS procedure for determination of EC in fortified wines.

4.2.5. Method validation

The described MEPS/GC-MS methodology for determination of EC in fortified wines was validated in terms of linearity, sensitivity, matrix effect, selectivity, precision and accuracy.

The working standard solutions were prepared by spiking both dry and sweet fortified wines at six different concentration levels: 5, 10, 50, 100, 200 and 400 µg/L of EC with 24 µg/L of BC as internal standard. Calibration curves were obtained by plotting the analyte peak area ratio (EC area/IS area) from the six increasing standard solutions against the corresponding EC concentration. The linearity was determined based on the linear regression results.

Sensitivity was evaluated determining the limit of detection (LOD) and limit of quantification (LOQ) as follow: LOD=3.3 σ/b and LOQ=10 σ/b, with σ as the intercept standard deviation and b the slope.

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 (6 g/L of tartaric acid, 18% of ethanol and pH 3.50) and those obtained by spiking dry and sweet fortified wines with known amounts (matrix-matched calibration), by the following equation (110):

% ME = [((slope of matrix-matched calibration - slope of synthetic wine calibration))/(slope of synthetic wine calibration)]×100

Selectivity was appraised by the analysis of several fortified wines, among which were chosen those that were used for the matrix-matched calibration, to ensure the absence of chromatographic interferences, at the retention times of EC and BC (SIM at m/z 62), which could compromise EC quantification. Synthetic wine blanks were also evaluated.

Precision was estimated from inter- and intra-day analysis of the standard solutions and fortified wines. Intra-day repeatability was assessed by 10 successive replicate determinations of 2 samples and a working standard solution, while inter-day reproducibility was assessed by the analyses of the same samples in 3 different days. These two parameters were expressed as relative standard deviation (%RSD).

The accuracy of the method was assessed through a recovery study, spiking a fortified wine in triplicate, with known amounts of EC at three representative concentrations levels, within the calibration range. Average recovery was calculated by comparing mean values of the 3 replicates with theoretical concentrations of each one. Carry-over was also investigated by running a blank sample after extracting the working standard solutions with the highest content of EC.

4.3. Results and discussion

Firstly, a concentrated solution of EC, diluted in dichloromethane, was directly injected into the GC-MS and recorded at full scan mode (total ion count) to identify and determine its retention time. Then, several ramp temperatures were tested in order to optimize the GC-MS analysis of EC. At the same time, to ensure the absence of interfering substances at EC retention time (14.1 min), some non-optimized MEPS extracts of fortified wine samples were analysed with both TIC and SIM modes. At SIM mode, the analyses were performed

recording the sum of the three major ions m/z 62, 74 and 89 and also, only the characteristic ion m/z 62. It was found interferences at the EC retention time when the recording was done with the sum of the ions m/z 62, 74 and 89. Indeed, the TIC mode analysis confirmed that the matrix of some fortified wines was very complex and concentrated, compromising the sensitivity. In this sense, it was chosen to perform SIM analysis only at m/z 62, which assured enough sensitivity to analyse EC with an excellent performance. Similar strategy has already been adopted by other authors (65, 111, 112).

4.3.1. Extraction solvent survey

Taking into account the objective of developing an extraction method with MEPS, the potential extraction solvents were analysed looking for the presence of EC, with the SIM mode at m/z 62. The obtained results showed that only acetonitrile, ethyl acetate and dichloromethane were EC free solvents. Methanol, ethanol and acetone solvents had measurable amounts of EC, mainly ethanol, which presented the peak with the greatest area of EC (Fig. 8).

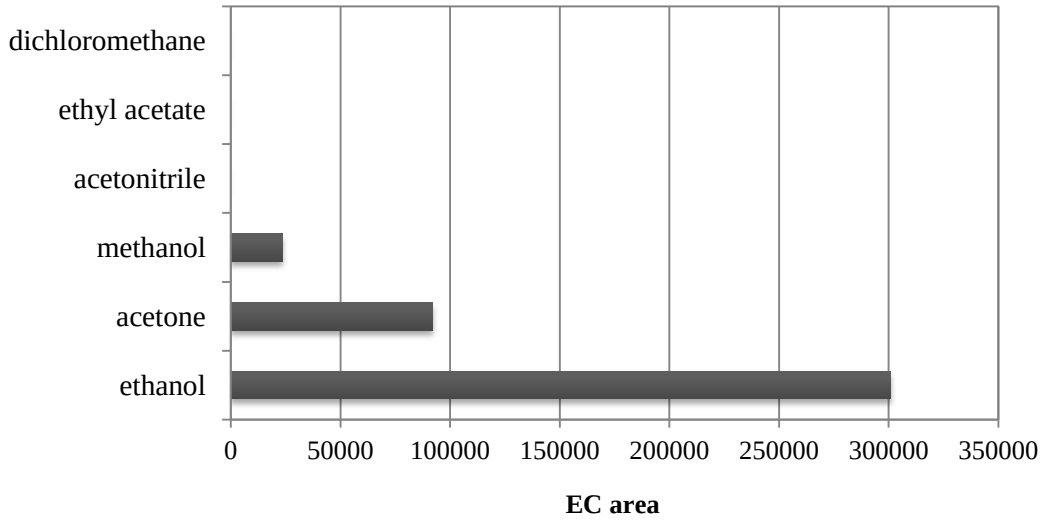


Fig. 8 - EC checking of the possible extraction solvents: ethanol, methanol, acetone, acetonitrile, ethyl acetate and dichloromethane.

This result led us to avoid the use of this solvent for calibration purposes, considering that EC presence in ethanol could affect its quantification. Actually, the EC determination in alcoholic beverages presupposes its use to simulate the matrix, since ethanol has direct

influence on the EC extraction. Thus, this fact must be taken into account on the development of analytical methods, once standards solutions are currently prepared with a certain percentage of ethanol.

Actually, we have tried to find an ethanol source that was absent of EC. In this sense, 3 bottles of ethanol > 99.8% (GC) of the same brand, available in the laboratory, were tracked through GC-MS direct injection, in order to quantify EC (Table 2). In this case, the calibration was prepared based on standards diluted in dichloromethane. The resulting calibration showed good linearity and sensitivity results (R^2 = 0.999 and LOQ = 15.21 μg/L). The results revealed that ethanol bottles presented concentrations ranging from 25.0 to 27.9 μg/L.

Table 2 - Concentration of EC in the ethanol available in the laboratory (> 99.8% (GC)), Sigma - Aldrich)

Ethanol	Concentration (μg/L)	SD
Batch 1	25.0	0.5
Batch 2	27.9	0.3
Batch 3	26.3	0.4

SD - standard deviation

Therefore, the synthetic wine, usually used for the preparation of standards, can have an additional EC concentration of about 4.5 μg/L derived from the added ethanol (18%).

4.3.2. Selection of the MEPS sorbent and extraction solvent

After solvents survey, several tests were conducted in order to select the best solvent/sorbent pair. To perform this task, a non-optimized MEPS procedure was carried out using a 500 μL syringe coupled with a hand-held automatic system. This syringe was fitted with a removable BIN containing 4 mg of sorbent material. The performance of the sorbent materials SIL, C2, C8, C18 and M1 were tested with the 3 extraction solvents free of EC. Very recently, new sorbents became commercially available, which could be further tested.

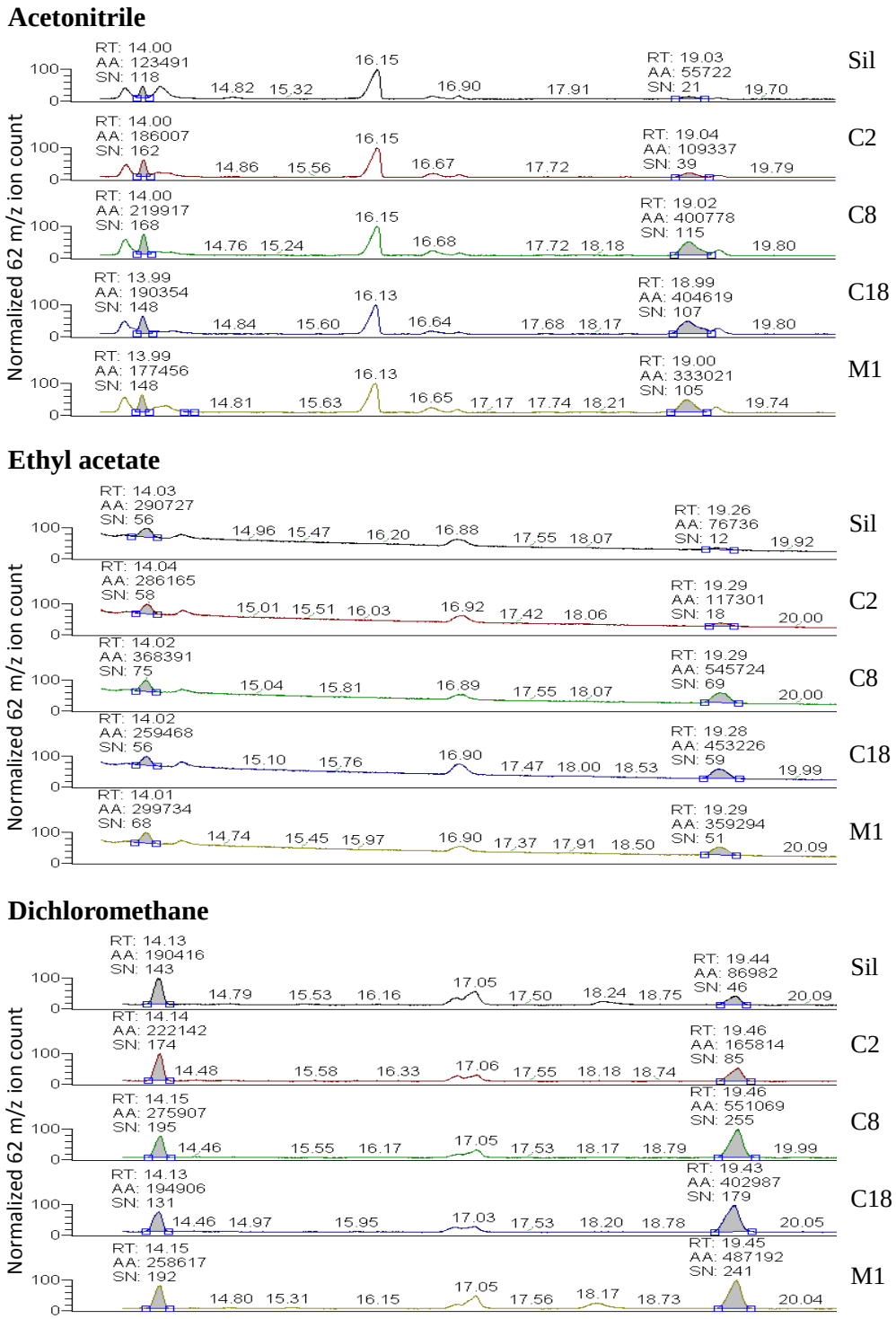


Fig. 9 - Typical chromatograms of the sorbent materials SIL, C2, C8, C18 and M1 using the extraction solvents acetonitrile, ethyl acetate and dichloromethane. EC RT ≈ 14 min, BC RT ≈ 19 min. RT - retention time; AA – peak area; SN - signal to noise ratio.

The obtained results were compared to select the best BIN and solvent to extract and quantify EC in fortified wines. Regarding the extraction solvent, it was verified that acetonitrile extracts less EC compared to the other two extraction solvents, regardless the BIN used. Moreover, acetonitrile extracted some interfering substances that co-eluted with the EC and BC peaks (Fig. 9). In turn, ethyl acetate and dichloromethane were the solvents with higher efficiency in the extraction of EC and BC. Actually, ethyl acetate extracts more EC than dichloromethane, however, causes a change in the baseline, reducing the signal to noise ratio (S/N) of both EC and BC peaks (Fig. 9). Furthermore, the EC peak of the ethyl acetate extracts presented an inferior resolution, as depicted in Fig. 9. Considering these results, dichloromethane was chosen as extraction solvent.

The BIN with C8 sorbent material presented the best efficiency to extract EC (Fig. 9), using dichloromethane as extraction solvent. Thus, C8 BIN and dichloromethane were chosen to perform the MEPS/GC-MS methodology for the determination of EC in fortified wines.

4.3.3. MEPS extraction optimization

After choosing the C8/dichloromethane pair to extract EC, an DoE was carried out to optimize the extraction in order to obtain the best response in the GC-MS equipment. The sample and extraction solvent volumes were the chosen variables. The sample volumes analysed were 200, 500 and 1000 µL, while the tested solvent volumes were 100, 200 and 350 µL. Fig. 10 depicts the result of the statistical DoE approach. The two factors analysed were plotted against the response variable in order to visualize the combination that maximizes the GC-MS response. Moreover, the response of other interferences was also analysed in order to ensure that the chosen factors combination maximize the S/N of the methodology used.

The optimum conditions were achieved by maximizing the second order function, which has sample and solvent volume as dependent variables and GC response as independent variable. As illustrated by Fig. 10, the maximum EC peak area can be achieved by using 100 µL of dichloromethane and 300 µL of wine sample. Other conditions that also affect the MEPS extraction, such as aspiration/dispense rates and conditioning/equilibration steps, were adjusted (section 2.4) taking into account the tips reported by previous methods, ensuring efficiency and effectiveness (113, 114).

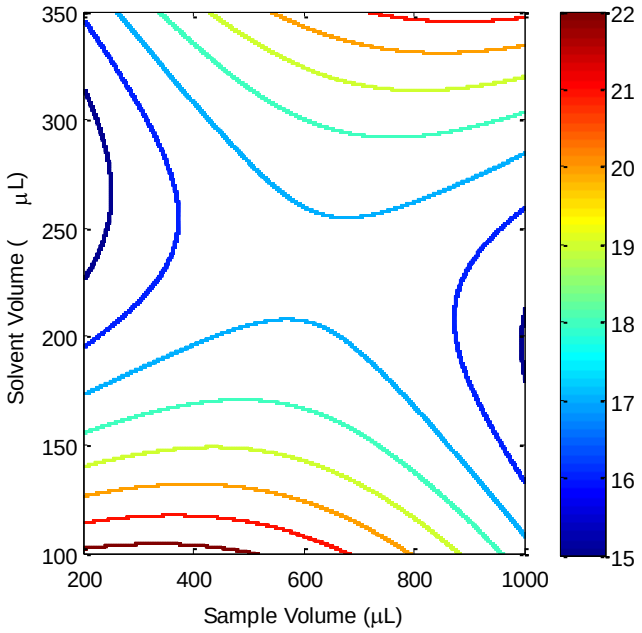


Fig. 10 - DoE to optimize the MEPS extraction with C8 BIN with sample volume, extraction solvent volume and the response in GC-MS equipment as variables. The colormap illustrates the variation of GC-MS response, where the maximum is delimited by the dark red line.

4.3.4. Matrix effects

The matrix effect can compromise the results generated by an analytical method, especially when it is intended to analyse samples of high complexity, such as fortified wines. Thus, the variation percentages of the slopes of three calibration curves, accessed with synthetic, dry and sweet fortified wines as samples matrix and using the optimized extraction, were compared to evaluate the matrix influence on the extraction procedure and analysis (Fig. 11).

Although there is no limit values established for matrix effect, it can be considered that up to 15% of matrix suppression or enhancement is acceptable. In the present study a value of 17% was obtained between synthetic and the both fortified wines. A negligible difference was found (about 0.3%) between the two types of wines, not having a considerable matrix effect between these two types of fortified wines.

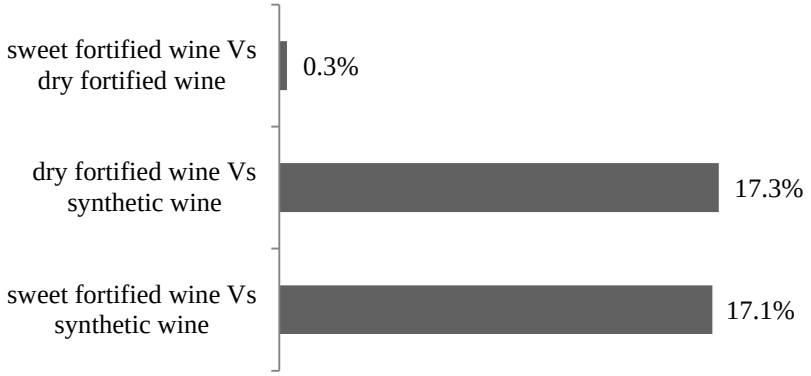


Fig. 11 - Percentage of the matrix effect between the slopes of the three calibration curves: synthetic wine and sweet and dry fortified wine.

4.3.5. Method validation

Faced with the lack of an ethanol completely free of EC, together with the fact that was observed matrix effect between synthetic and fortified wines, it was decided to adopt the matrix-matched calibration approach to overcome these drawbacks. To accomplish this calibration, the selectivity of the proposed methodology was firstly assessed by the analysis of the sweet and dry fortified wines specially selected, which were further used to generate the matrix-matched calibration. The results revealed that there were no interferences at EC and BC retention times, 14.1 and 19.4 min, respectively, as demonstrated in Fig. 12.

A single calibration curve was then obtained by the average response of the six concentration levels prepared with both sweet and dry fortified wine standard solutions. Each one was extracted in triplicate and injected twice. A good correlation coefficient (R^2 = 0.999) was observed, confirming the linearity of the method. Table 3 depicts some of the validation results.

The method sensitivity was evaluated by LOD and LOQ determinations, calculated based on the obtained linear regression. The LOD and LOQ were low (1.5 and 4.5 μg/L, respectively), being close or even lower to those found in literature (65, 67, 70, 71, 102-104, 111, 112, 115-117), conferring to the developed methodology a great sensitivity to analyse EC in fortified wines.

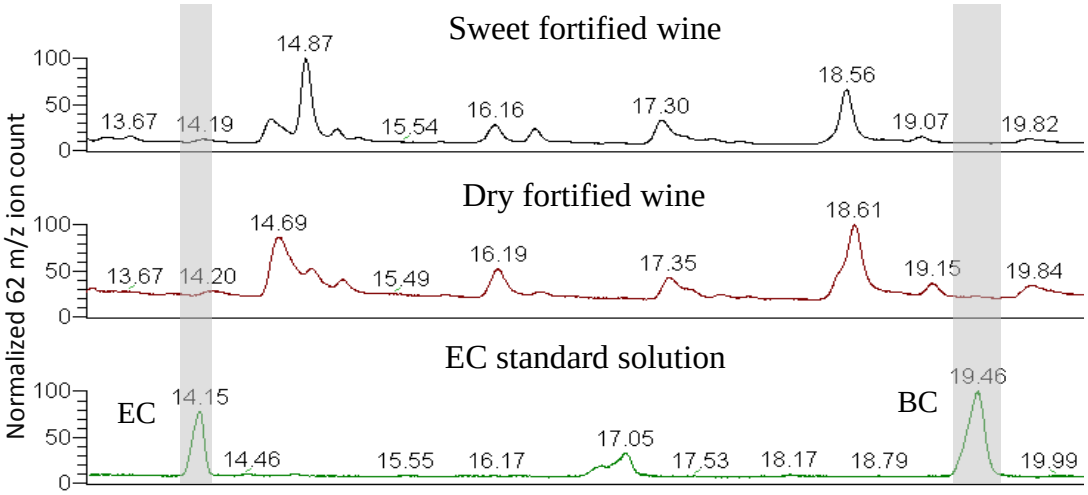


Fig. 12 - Chromatograms of the fortified wine samples used to generate the matrix-matched calibration and a 100 µg/L standard solution of EC with 24 µg/L of BC. EC – ethyl carbamate; BC – butyl carbamate.

Recovery study was carried out to determine the accuracy of the method, by spiking a fortified wine with known amounts of EC, at three concentration levels representative of the calibration range. The wine sample was analysed before and after the addition of 3 different amounts of EC. The recoveries ranged between 97 and 106%, demonstrating the good accuracy of the developed methodology (Table 3).

The method precision (repeatability and reproducibility) was evaluated by the variation of intra- and inter-day (three different days with an interval of 5 days between them) repetition of the method. Repeatability was accessed by 5 successive extractions injected twice of 100 µg/L standard solution and 2 fortified wines, with different concentrations. The reproducibility was estimated by the variation between the intra-day results and those obtained in inter-day analyses, through the extraction (triplicate) and injection (duplicate) of the same 3 samples. The results revealed a good repeatability (5 – 7%) and reproducibility (4 – 7%) of the methodology, since all RSD values were lower than 7%.

Additionally, the analysis of blanks after extracting the standard solutions with the highest content of EC, confirmed the absence of carry-over between extractions.

Table 3 - Validation results obtained for the proposed MEPS/GC-MS methodology

Parameter	Result	
linear regression ($y=mx+b$)	0.01045x + 0.13741	
Linear concentration range	5-400 μg/L	
R^2	0.9999	
LOD (μg/L)	1.5	
LOQ (μg/L)	4.5	
Recovery	Cc ± SD (μg/L)	%
FW	26 ± 2	-
FW + EC 50 μg/L	78 ± 4	106
FW + EC 100 μg/L	123 ± 6	97
FW + EC 200 μg/L	228 ± 10	101

LOD - limit of detection; LOQ- limit of quantification; Cc - Concentration; FW - fortified wine; SD - standard deviation

4.3.6. Analysis of fortified wine samples

To evaluate the applicability of the proposed MEPS/GC-MS methodology for determination of EC in fortified wines a set of fortified wines, aged up to 36 years old, were analysed. All samples were extracted in triplicate and injected twice. The results are shown in Table 4. The older wines were analysed in order to check the adopted linear range, as EC content is expected to increase with age (118).

The obtained chromatograms showed that the applicability of the MEPS/GC-MS methodology to quantify EC in fortified wines was achieved, since they showed a good peak resolution, confirming its selectivity. Additionally, the quantified concentrations varied from 7.3 to 206 μg/L, showing that the developed methodology covers the range interest of the compound (Table 4). Actually, the fact that wines with higher content of EC were in general associated with higher ageing periods was also demonstrated.

Table 4 - Application of the proposed methodology for the EC quantification of 16 fortified wines.

	Wine age (years)	Concentration (μg/L)	SD (n=6)
FW_1	5	28	3
FW_2	5	31	3
FW_3	5	22	4
FW_4	3	18	2
FW_5	5	38	2
FW_6	3	50	2
FW_7	5	13	2
FW_8	unk	7.6	0.1
FW_9	17	76.1	0.7
FW_{10}	16	85.5	0.9
FW_{11}	36	132	5
FW_{12}	18	138	5
FW_{13}	18	107	3
FW_{14}	17	93	3
FW_{15}	25	206	7
FW_{16}	unk	7.3	0.3

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

4.4. Conclusion remarks

A fast, simple and sensitive methodology was developed and optimized to quantify EC in fortified wines using MEPS extraction, through a hand-held automated analytical syringe, with GC–MS detection. The best solvent/sorbent pair was selected after testing several sorbent materials and EC free extraction solvents. C8 BIN and dichloromethane were the most efficient pair to extract EC. MEPS extraction was optimized performing an DoE, varying sample and extraction solvent volumes. The best response could be achieved with the passage of 300 μL of sample and 100 μL of dichloromethane.

The matrix effect study revealed that a noticeable effect of both sweet and dry fortified wines exists relative to synthetic wine. In turn, and together with the fact that it was not found an ethanol completely free of EC commercially available, a matrix-matched calibration was

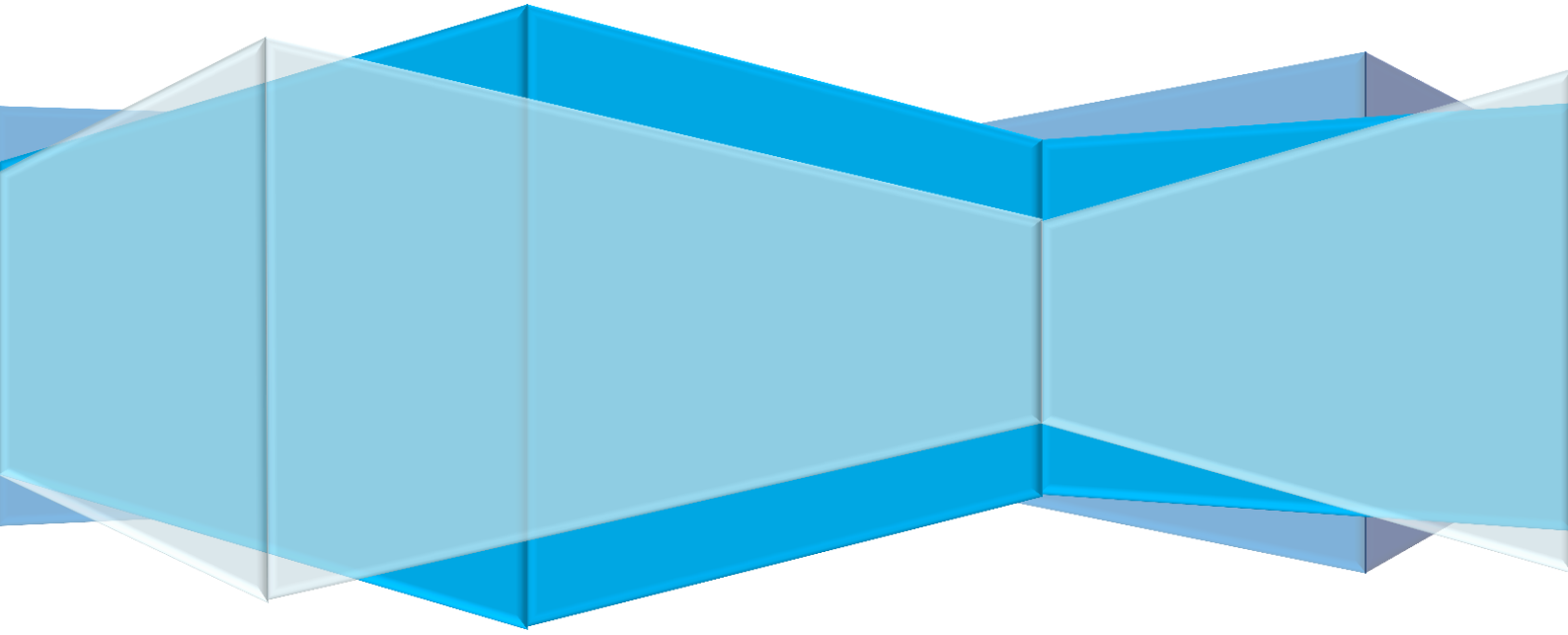
performed using both sweet and dry fortified wines. The analytical methodology was then validated, showing good results in terms of linearity, sensitivity, selectivity, precision and accuracy. The applicability of the methodology was demonstrated by the analysis of a set of 16 fortified wines, with values ranging between 7.3 and 206 μg/L. The corresponding chromatograms showed good precision and resolution.

Finally, it can be concluded that the presented MEPS/GC-MS methodology is an excellent tool to quantify EC in fortified wines, gathering efficiency and effectiveness, without using long and hard-working procedures, like the conventional methodology adopted by the OIV.

PART III - MONITORING OF ETHYL CARBAMATE DURING THE AGEING PROCESS

This part of the study is being prepared to be submitted in Food Chemistry, entitled as:

Evolution of ethyl carbamate in fortified wines during ageing process



5. EVOLUTION OF ETHYL CARBAMATE IN MADEIRA WINE: *CANTEIRO VS. ESTUFAGEM*.

Abstract:

This section presents the study of the EC formation in Madeira wines under *estufagem vs. canteiro* ageing during 18 months. Sweet wines made from Malvasia and Tinta Negra grape varieties were chosen in order to evaluate the EC formation kinetic, using the previously developed method. According to the results, Tinta Negra variety has more propensity to form EC comparatively to Malvasia. This might be explained by the fact that Tinta Negra has a higher content in amino acids, namely arginine. In the other hand, it was verified that *estufagem* process (45 ºC for 4 months) clearly increases the EC formation kinetic. Furthermore, during this process the EC concentration has a linear increase ($R^2 \geq 0.977$), proportional to the ageing time. Despite this, when the wines are submitted to *canteiro* the EC values remain almost constant during the following 14 months. In Malvasia wine aged by *canteiro* the EC concentration did not suffer significant changes. Contrarily, the Tinta Negra wine submitted to *canteiro* presented an exponential tendency ($R^2 = 0.939$) to increase the EC concentration, between the 1st and the 4th month, and then a linear tendency ($R^2 = 0.999$) up to the end of the ageing period, never exceeding the values obtained for the same wine when submitted firstly to *estufagem*. The results also suggest that Tinta Negra wines either aged including the *estufagem* step or aged only by *canteiro* might maintain the same trend, that is, at about 2.5 years of maturation the concentrations of EC will be identical, not surpassing the 70 µg/L, which is within the limit imposed by most countries. In general, according to the obtained results, it might be concluded that *estufagem* step does not seem to be the most critical factor for the EC development, unlike the quantity of EC precursors in medium associated with grape variety and growth factors.

5.1. Introduction

Besides being formed during fermentation, EC can be further formed during wine maturation and storage, depending on maturation and storage conditions, such as duration and temperature, therefore great variations in EC concentration may occur at the final consumption

point. As aforementioned, the EC formation increases with temperature and storage time. Citrulline and urea seems to have an important role on EC occurrence during ageing and storage, despite these two precursors undergo faster decline reactions. Several authors have been demonstrated that the rate of EC formation, in wine, decreases with the decline of citrulline and urea (24, 118, 119), however, others argue that there are other relevant compounds contributing to the EC formation (26).

Kodama (120) concluded that the EC occurrence during wine storage time is related to the urea content. Their results verified that the EC formation is represented by a linear correlation relative to urea concentration, at different temperatures. In the same study, the obtained EC/urea equations were mathematically evaluated and the authors concluded that urea should be less than 2 mg/L to maintain the EC values within the established legislation, in table and fortified wines. Hasnip (118) also confirmed that the rate of EC formation in wine is proportional to the concentration of urea. The same correlation was similarly observed relative to citrulline. Moreover, it was also concluded that the concentration of ethanol and temperature increases the kinetics of the EC formation. According to Stevens (26) the reaction of urea and ethanol at room temperature has a moderate kinetic. Additionally, the exposure to UV light it has pointed out as a factor that promotes a significant increase of the EC concentration in stone-fruit spirits (121). However, we cannot affirm that the same occurs in wines taking into account that the main formation pathways are different.

Madeira wine as a fortified wine has a high alcoholic content (17 to 22%) and, as mentioned before, there are two traditional maturation processes to age these wines: *estufagem* and *canteiro*. Most wines, especially those produced from Tinta Negra, are submitted to *estufagem.* Pereira (122), developed a model to predict Madeira wine age, based on *Malvasia* wine samples, and concluded that 3 months of *estufagem* at 45 °C are equivalent to 4.6 years of maturation in *canteiro*.

The aim of this part of the study was the monitoring of EC formation during both ageing processes, *estufagem* and *canteiro,* of two sweet wines made from Malvasia and Tinta Negra grape varieties, in order to appraise the formation kinetic in each type of ageing.

5.2. Materials and methods

The methodology used to quantify EC in the studied fortified wines was the proposed by Leça (123), previously described in section 4.2.4. The chemicals, equipment and the chromatographic conditions used are also specified in de sections 4.2.1 and 4.2.2, respectively.

5.2.1. Samples

It was used two traditional *Vitis vinifera* L. varieties, Malvasia and Tinta Negra. The grapes were collected in 2011 from different locations of the Madeira Island and the wines were produced in a local winery, according to their practices. Pectins and diammonium phosphate were added to the musts. A solution of 10% of sulfite was also added to obtain a concentration of about 60 mg/L. Commercial yeasts were not added and the grape skins together with the grape juice followed 24 hours of maceration. After the separation of the skins, the fermentative process was controlled below 25 ºC, in stainless steel tanks. The fermentations of Malvasia and Tinta Negra sweet wines were stopped when the must specific gravity reached about 1046 g/L, by the addition of natural grape spirit (containing 95% (v/v) of ethanol), raising the alcohol content up to about 17% (v/v). After fortification, the wines were clarified and stabilized through bentonite clays and albuminocol gelatines. Each wine, Malvasia and Tinta Negra, was divided in two fractions of 200 L. One fraction was submitted to *estufagem* at 45 °C, in a pilot scale system equipped with 200 L stainless steel vats, for 4 months (120 days) before being transferred to *canteiro* ageing. The other fraction was directly placed into oak casks to age only through the *canteiro* process. These wines were followed during 18 months and the sampling was collected in triplicate at different stages of the ageing process. The samples were stored at -26 °C before analysis.

5.3. Results and discussion

The applicability of the developed MEPS/GC-MS methodology (123) was previously demonstrated, revealing to be robust in the quantification of EC in fortified wine samples. Moreover, the low limits of detection and quantification obtained, indicated that this methodology can be used in routine analysis or in the EC monitoring, since the moment of its

formation. The method was then applied to study the EC formation kinetic using the sample set previously described.

According to Fig. 13, it can be verified that in general, the wines obtained from Tinta Negra have more tendency to form EC comparatively to Malvasia variety, when comparing the two maturation processes, *estufagem* and *canteiro*. Taking into account that the winemaking process was very similar, this fact may be explained by several reasons such as the different locations, soil conditions and treatments applied to the vineyards, especially the amount of fertilizers added (124). Consequently, the amino acid composition can be different. Indeed, Pereira (97) demonstrated that Tinta Negra sweet wine presented a higher content in amino acids, namely arginine, than Malvasia wine. Another reason, can be associated to the fact that yeasts with more proneness to ferment each grape variety may be different and different yeast strains excrete different concentrations of EC precursors to the medium (72). Furthermore, taking into account that the initial specific gravity of the must of these two varieties was different (1067 g/L and 1070 g/L for Malvasia and Tinta Negra, respectively), to reach the density of 1046 g/L, these wines are submitted to different extensions of fermentation before being added the natural grape spirit (fortification step). This difference can also account the effect that were endogenous yeast that conducted fermentation and that the membranes porosity of yeast cells are sensitive to fortification, especially to the moment that it is performed, since influences the extraction of EC precursors to the medium (125).

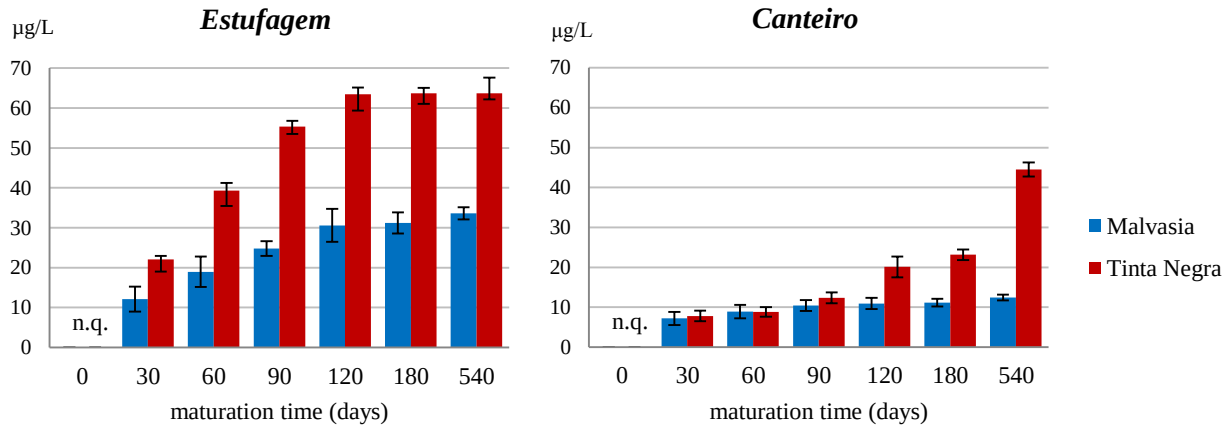


Fig. 13 - Evolution of ethyl carbamate in sweet wines made from two *Vitis vinifera* L .varieties, Malvasi and Tinta Negra, aged by two different maturation processes, e*stufagem* and *canteiro*.

It could also be verified that the *estufagem* process clearly increases the formation kinetic of EC, confirming some results already obtained in other studies (26, 118). In addition, this study reveals that the wine heating at 45 ºC for 4 months (*estufagem*) promotes linear increase of EC (R^2 = 0.998 and 0.977 for Malvasia and Tinta Negra, respectively) proportional to the ageing time (Fig. 14). It is interesting to notice that it was found that the slop of the Tinta Negra trend line (m = 0.467) was 2-fold higher than the slope of the Malvasia regression line (m = 0.204) (Fig. 14), once again demonstrating that has greater predisposition to form EC.

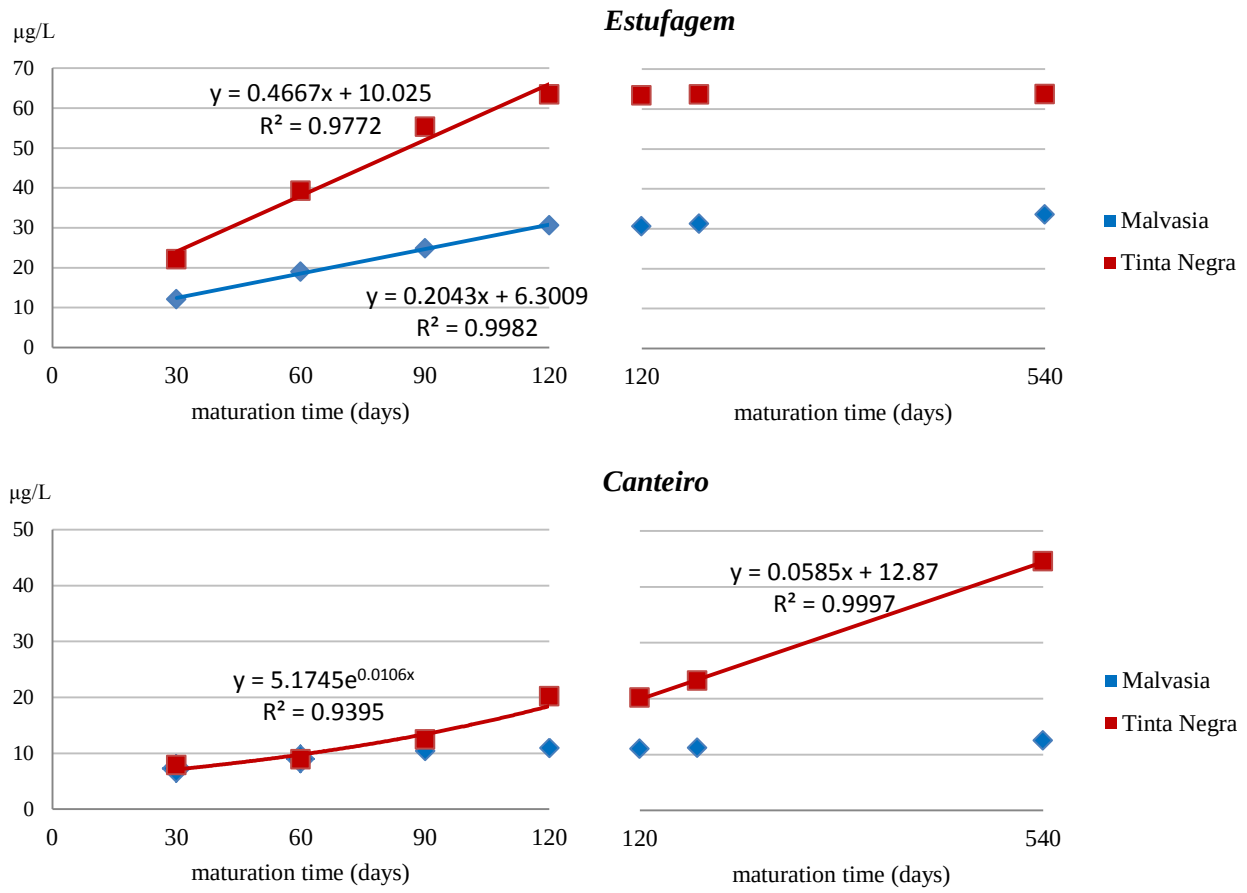


Fig. 14 – Trends of EC evolution during Madeira wine maturation, *estufagem* and *canteiro* of two wine made by Malvasia and Tinta Negra grape varieties.

Under the *canteiro* system (Fig. 14), both wines presented different patterns for the EC evolution, as well as did not presented a linear evolution trend as observed in the *estufagem* process. The formation kinetic of EC in Malvasia wine under *canteiro* was not favoured since the concentration values did not suffer significant changes up to the 540 days. On the other

hand, the Tinta Negra wine submitted only to *canteiro* ageing presented an exponential tendency to increase the EC concentration (R^2 = 0.939) between the 30 and 120 days and thenceforth a linear tendency was observed (R^2 = 0.999) (Fig. 14).

In Tinta Negra wines (Fig. 13 and 14) we can verify that *estufagem* increase the formation kinetic of EC, however when the wine returns to *canteiro* the EC values remain almost constant during the following 14 months. The same result was observed in Malvasia never surpassing the 40 µg/L, which is well above the most restricted limit imposed by the USA. These results may be related to an accentuated decrease of EC precursors in the wine medium during *estufagem* (118). Nonetheless, in regard to Tinta Negra wine exclusively aged under the *canteiro* process, despite the exponential growth of the EC concentration up to the 120 days, the maximum concentration attained was much lower than for the same wine submitted to *estufagem*, even before 540 days (18 months). These results suggest that Tinta Negra wines either aged including the *estufagem* step or aged only by *canteiro* might maintain the same trend, i.e., at about 2.5 years of maturation the concentrations of EC will be identical, not surpassing the 70 µg/L, which is within the limit imposed by most countries.

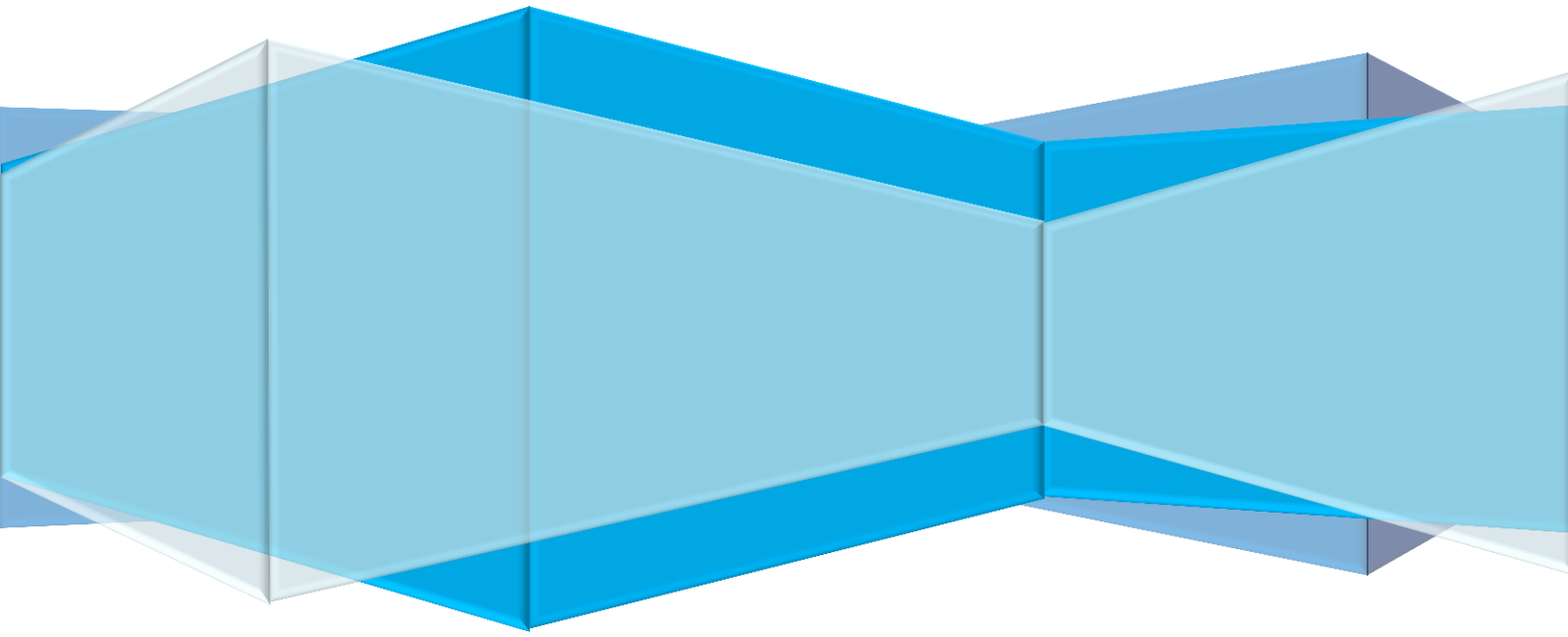
From the obtained results, it can be highlight that the quantity of EC precursors in Madeira wine medium appears to be the most important factor, which depends on the grape variety, soil composition, treatments applied to vineyards, yeasts strains and fermentation conditions (72).

5.4. Concluding remarks

From this study it might be concluded that *estufagem* step does not seem to be the most critical factor for EC development, unlike the amount of precursors. Thus, in order to control the EC occurrence in Madeira wines its precursors must be monitored before the maturation process. Taking into consideration that the amount of precursors might be dependent from several factors, from grape variety to fermentation conditions it is reasonable to think that this study should be extended, taking into consideration the dry wines, in order to compare their behaviour relative to sweet wines, as well as the monitoring of EC precursors during a similar experiment. Additionally, from the EC monitoring of a representative number of Madeira

wines during these two ageing processes, it could be developed a prediction model, based on chemometrics approaches.

PART IV - CONCLUSIONS AND PERSPECTIVES



6. CONCLUSIONS

From the overall work it can be concluded that the MEPS/GC-MS methodology developed is an excellent tool to quantify EC in fortified wines, as demonstrated by the validation parameters. This methodology uses an emergent extraction technique and combines a simple, faster and affordable extraction procedure with great sensitivity and effectiveness, without using sophisticated and expensive equipment. The method revealed to be robust and also useful for routine analysis as well as for the EC monitoring since the moment of its formation.

From the study of EC formation during Madeira wine ageing, for 18 months, it was concluded that the *estufagem* process increases the EC formation, nonetheless, after its application, the EC concentration remain almost constant during the following ageing time. It also became clear that *estufagem* step does not seem to be the key factor for the EC development, but instead the amount of precursors in the Madeira wine medium.

7. FUTURE PERSPECTIVES

As future work, we consider important monitoring a representative number of Madeira wines, including dry wines, during three years of both ageing processes and identify patterns of EC evolution, in order to develop a prediction model, based on chemometric approaches. It would also be relevant to quantify this problematic and to develop or apply methodologies to quantify the EC precursors at earlier stages of vinification. Moreover, it should be developed strategies to mitigate and control the EC formation in fortified wines.

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