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New insights into ethyl carbamate occurrence in fortified wines

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ABSTRACT

The occurrence of ethyl carbamate (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 arginine (Arg) being a direct precursor of EC in fortified wines, comparing it with known major precursors, urea and citrulline (Cit). Wine main sugars were appraised as possible catalysts, as previously pointed out. Model wines showed that: Arg can induce the EC formation under accelerated ageing, even without being metabolised by microorganisms; although much less reactive, Arg can play a significant role since high residual levels can occur in young fortified wines; glucose (Glc) and fructose (Fru) suppressed the EC formation from urea and Cit pathways, in about 11–26%.

Moreover, Madeira wine samples were investigated in order to appraise the eventual contribution of the alcoholic fortification. The results revealed that this step can promote a decrease of the amount of these EC precursors up to 46%.

Despite preliminary, additional information about the EC formation in fortified wines was obrained namely for designing new mitigation strategies, which can pass through the reduction of residual Arg.

1. Introduction

Fortified wines have unique organoleptic characteristics resulting from their specific winemaking processes, usually strongly dependent on ageing. All fortified wines hold a high alcohol content (150–220 mL/L) because of the addition of vinous alcohol or neutral grape spirit during the winemaking – alcoholic fortification. These wines are made in a wide range of styles with different sugar levels, from extra-dry to sweet (usually > 96 g/L). Information about the different production procedures of the world's best-known fortified wines, namely Sherry, Port and Madeira, is reported elsewhere (Pereira et al., 2019; Perestrelo et al., 2016). One potential hazard for fortified wines, like other fermented foods and beverages, is the natural occurrence of ethyl carbamate (EC) (JECFA, 2005), also known as urethane (CAS number 51-79-6). Based on EC toxicity studies Canadian authorities imposed, for the first time in 1985, legislation for EC concentration in alcoholic beverages. Regarding fortified wines, Canada imposed an EC limit of 100 μg/L and other countries did the same later (Weber et al., 2009). Also, the Food and Drug Administration (FDA) notified all countries exporting wines to the United States of America that they must meet the voluntary target for EC in wines (<60 μg/L in fortified wines) established by the American Industry (Jagerdeo et al., 2002). In 2007, the International Agency of Research on Cancer (IARC) reclassified EC as "probably carcinogenic to humans" (group 2A) (IARC, 2010).

In wines, EC is mainly formed after fermentation, by the reaction of carbamoyl compounds with ethanol (Jiao et al., 2014). Urea and citrulline (Cit) have been identified as the most relevant precursors of EC through reaction with ethanol (Azevedo et al., 2002; Jiao et al., 2014; Monteiro et al., 1989; Stevens et al., 1993). Temperature and maturation time are factors with marked influence on the final concentration of EC in wine, and in the concentration of its precursors (Azevedo et al., 2002; Kodama et al., 1994; Stevens et al., 1993). Hasnip et al. (2004) confirmed that the formation of EC is proportional to the concentration of urea, Cit and ethanol. Urea is mainly formed from arginine (Arg)

Abbreviations: EC, ethyl carbamate; Arg, arginine; Cit, citrulline; Fru, fructose; Glc, glucose; FW, fortified wine.

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metabolization inside of the yeast cells during the early and middle fermentation stages (Jiao et al., 2014). Cit is already present in grape juice, but it can also be generated through Arg anabolism, during fermentation by *Saccharomyces cerevisiae*, wherein ornithine and carbamoyl phosphate react to form Arg, with Cit acting as an intermediate product of this metabolism (Davis, 1986) and by the activity of lactic acid bacteria (LAB) through Arg degradation by the arginine deiminase pathway (Azevedo et al., 2002). Thus, Arg has being considered a preponderant metabolite for EC formation only after its metabolization by fermentative microorganisms (Butzke et al., 1997). Arg is commonly one of the most abundant amino acids in grape juice and its concentration is proportional to the increase of nitrogen fertilisation in the vineyard. An important decrease in the amino acid levels, including of Arg, during fortified wine's accelerated ageing was previously reported (Pereira et al., 2015), suggesting its transformation into new ageing-related metabolites.

As aforementioned, urea is a major 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. However, Ough et al. (1988) verified that alcoholic fortification in the presence of yeast cells promoted 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 fortified without yeasts. They associated this result with the change of porosity of the yeast's membranes and the walls of its cells because of the high concentration of alcohol. Considering that alcoholic fortification may increase the urea excretion to the medium, the moment that alcoholic fortification is performed is important to the final concentration of urea in wine, since, if the fermentation is arrested at the point of maximum urea excretion, it will not be reabsorbed afterwards (An et al., 1993; Butzke et al., 1997; Jiao et al., 2014).

Alcoholic beverages industry has been committed to lower EC levels as far as possible. Thus, researchers have been motivated to develop strategies for the mitigation of EC in fermented foods and beverages (V. Gowd, Su, Karlovsky, & Chen, 2018a; 2018b). To minimize the EC levels in wine, the FDA suggests several strategies in a preventive action manual for winemakers (Butzke et al., 1997). These procedures are recommended to minimize EC content in table wines and most involve the reduction of storage time and temperature, the use of acidic ureases and genetic engineering tools focused on yeast genes responsible for urea metabolism. However, these strategies are not guaranteed to be viable solutions for the fortified wine sector without affecting product quality (Vemana Gowd et al., 2018a, 2018b; Hasnip et al., 2004; Zhao et al., 2013).

The present work aims to: investigate the possibility of Arg being a direct precursor of EC in fortified wines comparing it with known major precursors; assess if wine major sugars (Glc and Fru) have any influence in the EC occurrence; and ascertain the effect of alcoholic fortification in the transfer of EC precursors to the yeast extracellular medium, taking as a case study the production of Madeira wine, through the analysis of samples before and after fortification.

2. Material and methods

2.1. Model wine samples

Different model systems were prepared by adding individually each EC precursor (urea, Cit and Arg) at 100 mg/L to a synthetic wine solution (6 g/L of tartaric acid, 180 mL/L 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 was tested for the first time to find out its influence in the EC occurrence during wine ageing. 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). Experiment 1 intends to simulate the accelerated ageing process that is typical in the ageing used for the maturation of Madeira wines, which has been the focus of different studies (Pereira et al. 2013, 2014, 2017). Briefly, this fortified wine is produced on the volcanic soils of Madeira Islands (Portugal), either from red (Tinta Negra) or white (Sercial, Verdelho, Boal, and Malvasia) *Vitis vinifera* L. grapes, from manually cultivated vines, planted in small terraces. It holds an high acidity that imparts a characteristic freshness and a distinctive bouquet, acquired during a long-term ageing conducted at higher temperatures than those used in the maturation of still wines. Its maturation usually includes a heating step performed in stainless steel tanks up to 45 °C for at least 3 months, prior to the ageing in oak casks. A detailed description about its production can be found in literature (Pereira et al., 2019; Perestrelo et al., 2016).

Considering that temperature can be an important factor for the formation kinetics of EC 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 (Azevedo et al., 2002; Pereira et al. 2014, 2017). For a better understanding how samples were prepared, please check Table 1 of the "Results and discussion" section. All model wines were then analysed by LC-MS/MS to assess the EC formation.

2.2. Wine samples

Different trials of fortified wines were studied before and after the fortification process to assess the content of EC precursors. Twelve wines were obtained from *Tinta Negra* red grapes (*Vitis vinifera* L. cultivar) collected at 8 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 the carbon dioxide release during fermentation were used. Each microvinification started by the manual separation of grapes from stems and its crushing, adding about 60 mg/L of potassium metabisulfite, without adding commercial yeast. Pectinases and diammonium phosphate were also added. The grape skins, together with the free-run juice, followed 24 h of maceration before being separated. The alcoholic fermentation process was

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

Model Systems	Precursor	Sugar	EC (μg L)	
	(100 mg L)	(100 g L)	45 °C 4M	70 °C 1M
control - SW	–	–	n.d.	n.d.
Urea-wS	Urea	–	$2747 \pm 69^{a*}$	$10{,}554 \pm 280^{a+}$
Urea-Glc	Urea	glucose	$2173 \pm 14^{b*}$	$9389 \pm 70^{b+}$
Urea-Fru	Urea	fructose	$2036 \pm 8^{c*}$	$9336 \pm 718^{b+}$
Cit-wS	Citrulline	–	$388 \pm 14^{a**}$	$2538 \pm 259^{a++}$
Cit-Glc	Citrulline	glucose	$304 \pm 11^{b**}$	$2033 \pm 50^{b++}$
Cit-Fru	Citrulline	fructose	$286 \pm 8^{c**}$	$1879 \pm 48^{b++}$
Arg-wS	Arginine	–	$19 \pm 6^{a***}$	$41 \pm 8^{a+++}$
Arg-Glc	Arginine	glucose	$18 \pm 8^{a***}$	$40 \pm 10^{a+++}$
Arg-Fru	Arginine	fructose	$19 \pm 12^{a***}$	$43 \pm 5^{a+++}$

Mean value ($n = 6$) ± standard deviation; n.d. - not detected; wS - without sugar; SW - synthetic wine.
Different letters denote statistically significant differences ($P < 0.05$) by Holm-Sidak test. Different groups of data were analysed and these can be differentiated by the composition of special characters after the letters of each group.

conducted at about 20 ± 3 °C and stopped by the addition of neutral grape spirit (containing 95% (v/v) of ethanol) at different stages, from densities between 1040 and 1006 g/L, the extension of fermentation increases from the fortified wine FW1 to FW12 and the alcohol content was adjusted to about 170 mL/L. Each wine was obtained in duplicate and the densities were 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.

2.3. Ethyl carbamate analysis

The quantification of EC in the studied samples was achieved applying the method proposed by Leça et al. (2018), without any modification. EC present in model wines was concentrated by miniaturized liquid-liquid extraction with ethyl acetate as extraction solvent, separated from other extracted compounds by reversed-phase liquid chromatography (RP-HPLC), detected by electrospray tandem mass spectrometry (MS/MS) and quantified by internal standard calibration with butyl carbamate.

2.4. Urea analysis

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 fluorescence detection (FLD), with previous 9-xanthydrol derivatization of urea in the HPLC loop. Three replicates of samples and standards were analysed in duplicate.

2.4.1. 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).

Urea stock solution (50 mg/L) was prepared in synthetic wine. Six calibration solutions (0.19; 0.25; 0.5; 1; 5 and 10 mg/L) were obtained by successive dilutions of this solution also in synthetic wine. Each calibration point was prepared in triplicate and twice analysed, within the range of 0.19–10.0 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).

2.4.2. 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 °C. 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 8.60 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.

2.4.3. Derivatization

The derivatization reaction was performed into 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 column. All solutions used for derivatization were filtered through Chromafil PTFE 0.2 μm syringe filters.

2.5. Arginine and citrulline analysis

The analysis of Arg and Cit in the sample set of wines in fortification 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.

2.5.1. Standards and reagents

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

Arg and Cit (both with a purity grade > 97%) were obtained from Sigma–Aldrich (St. Louis, MO, USA). 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 at 300 mg/L, which was used to prepare the calibration points (1, 2.5, 5, 20, 50, 100 and 300 mg/L), by successive dilutions. All samples and calibration points were filtered through Chromafil PTFE 0.2 μm syringe filters.

2.5.2. Apparatus 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 °C. A gradient elution was used 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.

2.5.3. Derivatization

Before derivatization, 100 μL of each sample 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. 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 into the loop for 2 min and then loaded into the column.

2.6. Data processing

Significant differences were evaluated by the analysis of variance (one-way ANOVA with Holm-Sidak method) using the statistical software SigmaPlot 12.0.

3. Results and discussion

3.1. Formation of ethyl carbamate during the accelerated ageing of fortified wine model systems

Table 1 summarizes the EC formation levels in the model systems mimicking fortified wine conditions, studied under accelerated ageing conditions (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.

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 pointed out by previous studies (Butzke et al., 2013; Jiao et al., 2014; Moreno-Arribas et al., 2009; Weber et al., 2009; Zhao et al., 2013). A model system with 100 mg/L of Arg lead to the formation of EC levels varying between 18 and 43 μg/L. These results suggest that the conversion percentage of this precursor into EC is low (<1% mole/mole). However, considering that Pereira et al. (2015) showed that Arg content in young fortified wines was found up to levels of 356 mg/L, EC formed through this via during the ageing of fortified wines must be considered. According to rough estimates, the occurrence of Arg at these concentrations can account with up to 64 μg/L of EC at standard ageing conditions.

The EC concentration in urea and Cit systems at 100 mg/L varied between 2036 and 10,554 μg/L and 286 to 2538 μg/L, respectively (Table 1). Thus, at the same conditions, urea was the precursor with the higher EC formation rate, being on average 5-fold higher than Cit and 201-fold higher than Arg (Table 1). 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. The urea content was found to vary between 0.005 and 1 mg/L (Francis, 2006) in different types of wine, including in fortified ones, which is considerably lower than the amounts herein tested. Cit occurrence is usually more associated with the occurrence of malolactic fermentation and in white table wines, levels ranging between 9 and 31 mg/L can be found (Terrade et al., 2006). Despite malolactic fermentation not being usually induced in the vinification of the fortified wines, this kind of fermentation can occur spontaneously by the bacteria naturally present and increasing the content of Cit, eventually contributing for EC development during wine ageing.

Regarding the effect of wine major sugars, the results herein obtained are not in agreement with the conclusions driven by an academic study developed by Martins (2011), wherein Glc was pointed out as being 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 the EC formation (Table 1). In the systems containing urea, the depletion varied between 11 and 26%, while in Cit systems it varied between 20 and 26%. No significant differences were detected between Glc or Fru, although lower EC values were in general obtained in Fru model systems. Thus, there must be other factors that have a higher contribution for the EC formation in sweet wines than sugars, which indeed showed a suppression effect in the current study.

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

3.2. Impact of fortification on the passage of EC precursors from intercellular yeast to the wine medium

The effect of the fortification step on passaging the precursors from the intercellular to the extracellular yeast medium was evaluated, with the addition of about 170 mL/L 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 red *Vitis vinifera* L. grape variety *Tinta Negra*. In general, the concentration of precursors considerably differed among wines (Table 2), not being verified a trend according to the extension of fermentation, which increases from the fortified wine FW1 to FW12. This variation can be justified by the 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 the parcels are located. Thus, the grapes sampling was made to obtain wines with the greatest variability as much as possible. Thus, this heterogeneity 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 and 4.85 mg/L in the sample set analysed, which, according to rough estimates, could originate up to about 500 μg/L of EC with long-term ageing of fortified wines. The fortification step generally decreased the most reactive precursor to form EC in the wine medium, between 5 and 33% (Table 2). This was observed in 8 of the 12 fortified wines under study. The decrease can be justified by the dilution promoted by the ethanol added. Another possibility is related to the reduced solubility of urea with the increase of ethanol concentration as previously suggested by (Lee et al., 1972) and demonstrated by (Capuci et al., 2016). An increase of 22% was only observed in 1 of the 12 wines analysed. The increase can be justified by the porosity change of yeast cells by the addition of ethanol (Ough et al., 1988). In 3 other wines, the change in the urea concentration was not statistically significant (Table 2).

Regarding Cit, it was only quantifiable in 7 of the 12 wines analysed, between 0.21 and 15.1 mg/L (Table 2) which, according to rough estimates, could originate up to about 380 μg/L of EC with long-term ageing. Cit occurrence is more closely related with the activity of LAB, since in the alcoholic fermentation it only occurs as an intermediate of Arg anabolism (Arena et al., 1999; Davis, 1986; Jiao et al., 2014). Thus, the eventual presence of LAB in the medium can justify the higher Cit content in some wines, since malolactic fermentation can naturally occur in wines (Henríquez-Aedo et al., 2016; Izquierdo Cañas et al., 2008; H. M.; Li et al., 2019). In wines where Cit was quantifiable, its concentration decreased with the fortification, between 11 and 46%. This decrease can also be related to the dilution that naturally occurs on the fortification step and by the reduced solubility of Cit with increased ethanol content (S. Li et al., 2016).

Arg, the less reactive EC precursor, was also quantified in the wines under fortification and the results are depicted on Table 2. Arg levels varied between 1.6 and 901 mg/L. The fortification step promoted variations in the Arg contents and trends. When the Arg content in wines before the fortification was between 1.6 mg/L and 20.3 mg/L (5 out of the 12 wines), there was an increase up to 2.9-fold times on its concentration after alcoholic fortification. The increase of Arg in the medium can be due to the effect of the ethanol addition on the porosity of yeast membranes (Ough et al., 1988) or even by yeast autolysis (Hernawan et al., 1995). When higher Arg contents were found, namely between 115 mg/L and 217 mg/L (2 out of 12 wines), no statistically significant differences were observed with the adding vinous alcohol. At levels higher than 687 mg/L, a decrease of Arg up 34% was detected, which can be explained by the evidence recently found by Bowden et al. (2018) that ethanol ascending concentration lower the Arg solubility.

Table 2
– Concentration of urea, citrulline and arginine in the fortified wine medium before and after the alcoholic fortification step.

Sample	Urea				Citrulline				Arginine			
	Before Fortification		After Fortification		Before Fortification		After Fortification		Before Fortification		After Fortification	
	CC (mg L)	SD	CC (mg L)	SD	CC (mg L)	SD	CC (mg L)	SD	CC (mg L)	SD	CC (mg L)	SD
FW1	2.10[a]	0.09	1.7[a]	0.2	10[a]	1	5.9[b]	0.8	764[a]	47	505[b]	73
FW2	2.5[a]	0.1	1.6[a]	0.2	12[a]	1	6.5[b]	1.0	749[a]	12	539[b]	33
FW3	1.87[a]	0.05	1.2[a]	0.1	11.1[a]	0.6	6.7[b]	0.8	787[a]	32	632[b]	43
FW4	4.8[a]	0.6	3.6[a]	0.1	0.21	0.01	n.q.		1.6[a]	0.2	4.3[b]	1.0
FW5	4.5[a]	0.7	4.0[b]	0.5	n.q.		n.d.		2.51[a]	0.09	7.3[b]	0.9
FW6	0.43[a]	0.04	0.41[b]	0.02	1.31[a]	0.03	1.17[b]	0.02	15[a]	2	27.7[b]	0.7
FW7	0.66[a]	0.04	0.81[b]	0.04	12.7[a]	0.2	7.4[b]	0.6	687[a]	9	491[b]	25
FW8	0.98[a]	0.09	0.75[b]	0.09	15.1[a]	0.8	8.3[b]	0.7	901[a]	46	666[b]	65
FW9	0.37[a]	0.05	0.21[b]	0.02	n.q.		n.d.		2.8[a]	0.3	4.1[b]	0.2
FW10	0.40[a]	0.03	0.35[b]	0.05	0.65[a]	0.01	0.54[b]	0.01	217[a]	54	184[a]	55
FW11	0.55[a]	0.07	0.42[b]	0.06	n.d.		n.d.		20.3[a]	0.1	32[b]	3
FW12	1.1[a]	0.1	0.96[a]	0.08	n.d.		n.d.		115[a]	41	80[a]	35

CC - concentration; SD - standard deviation; n.d. - not detected; n.q. - not quantifiable.
Mean value (n = 6); different letters within the same wine denote statistically significant differences ($P < 0.05$) by Holm-Sidak test.

4. Conclusions

This study allows to conclude that Arg can induce the formation of EC, without being previously metabolised by fermentative microorganisms. Despite not being an important EC precursor, when compared with urea and Cit, as significant levels of Arg can be found in young fortified wines, before ageing, it should be considered as a possible contributor for the occurrence of EC with ageing. Thus, the need for the industrial control of the nitrogen sources during the fermentation becomes reinforced to avoid an excessive amount of residual Arg. It can also be concluded that wine main sugars, Glc and Fru, can mitigate the EC formation by the urea and pathways during wine ageing (in about 11–26%), but not the Arg pathway. The kinetic formation of EC is differently affected by the temperature rise (long-term ageing): the Cit pathway seems to be more favoured, increasing approximately 7-fold, followed by the pathways of urea (4-fold) and Arg (2-fold). Alcoholic fortification is not a critical process in terms of transferring the two most reactive EC precursors, urea and Cit, to the medium of wine. This vinification step affects the transfer of the less reactive precursor, Arg, only when it occurs in low concentrations in the medium before alcoholic fortification.

CRediT authorship contribution statement

João M. Leça: Formal analysis, Investigation, Writing – original draft. **Vanda Pereira:** Methodology, Resources, Writing – original draft. **Andreia Miranda:** Investigation, on fermentation. **José Luis Vilchez:** Supervision, Writing – review & editing. **José C. Marques:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

An, D., & Ough, C. S. (1993). Urea excretion and uptake by wine yeasts as affected by various factors. *American Journal of Enology and Viticulture, 44*(1), 35.
Arena, M. E., Saguir, F. M., & Manca de Nadra, M. C. (1999). Arginine, citrulline and ornithine metabolism by lactic acid bacteria from wine. *International Journal of Food Microbiology, 52*(3), 155–161.
Azevedo, Z., Couto, J. A., & Hogg, T. (2002). Citrulline as the main precursor of ethyl carbamate in model fortified wines inoculated with Lactobacillus hilgardii: A marker of the levels in a spoiled fortified wine. *Letters in Applied Microbiology, 34*, 32–36.
Bowden, N. A., Sanders, J. P. M., & Bruins, M. E. (2018). Solubility of the Proteinogenic α-amino acids in water, ethanol, and ethanol–water mixtures. *Journal of Chemical & Engineering Data, 63*(3), 488–497.
Butzke, C. E., & Bisson, L. F. (1997). *Ethyl Carbamate preventative action manual, US Food and Drug Administration, Center for food safety and Applied nutrition Ethyl carbamate preventative action manual.* Washington: US, Food and Drug Administration.
Butzke, C. E., & Bisson, L. F. (2013). *Ethyl Carbamate preventative action manual, US Food and Drug Administration, Center for food safety and Applied nutrition Ethyl carbamate preventative action manual. US.* Washington: Food and Drug Administration.
Capuci, A. P. S., Carvalho, N. D.d., Jr, M. R. F., & Malagoni, R. A. (2016). Solubility of Urea in ethanol-water mixtures and pure ethanol from 278.1K to 333.1K. *Revista ION, 29*(2), 125–133.
Davis, R. H. (1986). Compartmental and regulatory mechanisms in the arginine pathways of Neurospora crassa and Saccharomyces cerevisiae. *Microbiological Reviews, 50*, 280–313.
Francis, P. S. (2006). The determination of urea in wine – a review. *Australian Journal of Grape and Wine Research, 12*(2), 97–106.
Gowd, V., Su, H., Karlovsky, P., & Chen, W. (2018a). Ethyl carbamate: An emerging food and environmental toxicant. *Food Chemistry, 248*, 312–321.
Gowd, V., Su, H., Karlovsky, P., & Chen, W. (2018b). Ethyl carbamate: An emerging food and environmental toxicant. *Food Chemistry, 248*, 312–321.
Hasnip, S., Caputi, A., Crews, C., & Brereton, P. (2004). Effects of storage time and temperature on the concentration of ethyl carbamate and its precursors in wine. *Food Additives & Contaminants, 21*, 1155–1161.
Henríquez-Aedo, K., Durán, D., Garcia, A., Hengst, M. B., & Aranda, M. (2016). Identification of biogenic amines-producing lactic acid bacteria isolated from spontaneous malolactic fermentation of chilean red wines. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology, 68*, 183–189.
Hernawan, T., & Fleet, G. (1995). Chemical and cytological changes during the autolysis of yeasts. *Journal of Industrial Microbiology, 14*(6), 440–450.
IARC. (2010). Alcohol consumption and ethyl carbamate. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 96*, 3–1383.
Izquierdo Cañas, P. M., García Romero, E., Gómez Alonso, S., Fernández González, M., & Palop Herreros, M. L. L. (2008). Amino acids and biogenic amines during spontaneous malolactic fermentation in Tempranillo red wines. *Journal of Food Composition and Analysis, 21*(8), 731–735.
Jagerdeo, E., Dugar, S., Foster, G. D., & Schenck, H. (2002). Analysis of ethyl carbamate in wines using solid-phase extraction and multidimensional gas chromatography/mass spectrometry. *Journal of Agricultural and Food Chemistry, 50*(21), 5797–5802.
JECFA. (2005). *Summary and conclusions of the sixty-fourth meeting of the joint FAO/WHO expert committee on food additives codex alimentarius*.
Jiao, Z., Dong, Y., & Chen, Q. (2014). Ethyl carbamate in fermented beverages: Presence, analytical chemistry, formation mechanism, and mitigation Proposals. *Comprehensive Reviews in Food Science and Food Safety, 13*, 611–626.
Kodama, S., Suzuki, T., Fujinawa, S., Teja, P., & Yotsuzuka, F. (1994). Urea contribution to ethyl carbamate formation in commercial wines during storage. *American Journal of Enology and Viticulture, 45*, 17–24.

Lee, F.-M., & Lahti, L. E. (1972). Solubility of urea in water-alcohol mixtures. *Journal of Chemical & Engineering Data, 17*(3), 304–306.
Leça, J. M., Pereira, V., Pereira, A. C., & Marques, J. C. (2018). A sensitive method for the rapid determination of underivatized ethyl carbamate in fortified wine by liquid chromatography-electrospray tandem mass spectrometry. [journal article]. *Food Analytical Methods, 11*(2), 327–333.
Li, H. M., Jiang, D. Q., Dai, Z. G., Zhang, Y. S., Zhang, Y., Sun, S. Y., & Zhao, Y. P. (2019). Aromatic property of cherry wine produced by malolactic fermentation of controlled and spontaneous on the bacterial evolution. *International Journal of Food Properties, 22*(1), 1270–1282.
Li, S., Jiang, L., Qiu, J., & Wang, P. (2016). Solubility and solution thermodynamics of the δ form of l-citrulline in water + ethanol binary solvent mixtures. *Journal of Chemical & Engineering Data, 61*(1), 264–271.
Martins, C. S. F. (2011). *Estratégias para a redução de carbamato de etilo em vinhos Madeira.* Universidade de Aveiro.
Monteiro, F. F., Trousdale, E. K., & Bisson, L. F. (1989). Ethyl carbamate formation in wine: Use of radioactively Labeled precursors to demonstrate the involvement of urea. *American Journal of Enology and Viticulture, 40*(1), 1–8.
Moreno-Arribas, M. V., & Polo, M. C. (2009). *Nitrogen compounds* (1 ed.). New York: Springer.
Ough, C. S., Crowell, E. A., & Mooney, L. A. (1988). formation of ethyl carbamate precursors during grape juice (chardonnay) fermentation. I. Addition of amino acids, urea, and ammonia: Effects of fortification on intracellular and extracellular precursors. *American Journal of Enology and Viticulture, 39*, 243–249.
Pereira, V., Albuquerque, F., Cacho, J., & Marques, J. C. (2013). Polyphenols, antioxidant potential and color of fortified wines during accelerated ageing: The Madeira wine case study. *Molecules, 18*(3), 2997–3017.
Pereira, V., Cacho, J., & Marques, J. C. (2014). Volatile profile of Madeira wines submitted to traditional accelerated ageing. *Food Chemistry, 162*, 122–134.
Pereira, V., Pereira, A. C., & Marques, J. C. (2019). 13 - emerging trends in fortified wines: A scientific Perspective. In A. M. Grumezescu, & A. M. Holban (Eds.), *Alcoholic beverages* (pp. 419–470). Woodhead Publishing.
Pereira, V., Pereira, A. C., xe9, #, Perez Trujillo, J. P., Cacho, J., & Marques, J. C. (2015). Amino acids and biogenic amines evolution during the estufagem of fortified wines. *Journal of Chemistry, 9*, 2015.
Pereira, V., Santos, M., Cacho, J., & Marques, J. C. (2017). Assessment of the development of browning, antioxidant activity and volatile organic compounds in thermally processed sugar model wines. *Lebensmittel-Wissenschaft & Technologie, 75*, 719–726.
Perestrelo, R., Silva, C., Pereira, J., & Câmara, J. S. (2016). Wines: Madeira, Port and Sherry fortified wines – the sui generis and notable Peculiarities. Major differences and chemical Patterns. In B. Caballero, P. M. Finglas, & F. Toldrá (Eds.), *Encyclopedia of food and health* (pp. 534–555). Oxford: Academic Press.
Stevens, D. F., & Ough, C. S. (1993). Ethyl carbamate formation: Reaction of urea and citrulline with ethanol in wine under low to normal temperature conditions. *American Journal of Enology and Viticulture, 44*, 309–312.
Terrade, N., & Mira de Orduña, R. (2006). Impact of winemaking practices on arginine and citrulline metabolism during and after malolactic fermentation. *Journal of Applied Microbiology, 101*(2), 406–411.
Weber, J. V., & Sharypov, V. I. (2009). Ethyl carbamate in foods and beverages: A review. *Environmental Chemistry Letters, 7*, 233–247.
Zhang, J., Liu, G., Zhang, Y., Gao, Q., Wang, D., & Liu, H. (2014). Simultaneous determination of ethyl carbamate and urea in alcoholic beverages by high-Performance liquid chromatography coupled with fluorescence detection. *Journal of Agricultural and Food Chemistry, 62*(13), 2797–2802.
Zhao, X., Du, G., Zou, H., Fu, J., Zhou, J., & Chen, J. (2013). Progress in preventing the accumulation of ethyl carbamate in alcoholic beverages. *Trends in Food Science & Technology, 32*, 97–107.