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Sotolon in Madeira Wine
New insights on the aroma impact and main formation pathways

MASTER DISSERTATION

João Marcelo Gontardo Gaspar
MASTER IN APPLIED BIOCHEMISTRY

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Resumo

O sotolon é um composto com aromas característicos a caramelo, ácer e caril. Depois de identificado pela primeira vez em vinhos franceses biologicamente envelhecidos, assim como no saqué, este tem sido identificado em muitos outros vinhos. Encontra-se, sobretudo, a altas concentrações em vinhos licorosos como o Vinho Madeira (MW), contribuindo para o seu *bouquet* único, e actuando também como possível marcador de idade. A parte experimental desta tese começou com o desenvolvimento de uma metodologia simples e rápida para sua quantificação. O método utiliza uma extracção líquido-líquido seguida de cromatografia líquida acoplada à espectrometria de massa em *tandem*, apresentando uma boa linearidade (R^2 = 0,9999), precisão (menor que 10% do desvio padrão relativo), recuperação (95%) e alta sensibilidade (limiar de quantificação de 0,04 μg/L). Pouco se sabe sobre a relevância do sotolon nos MW comercializados. Assim, o impacto odorífero da lactona foi estabelecido em Blends através dos valores de actividade odorífera (OAVs). Deste modo, o limiar de percepção olfactivo (OT) do sotolon foi estabelecido, pela primeira vez, no próprio MW obtendo-se um valor estimado (BET) de 112 μg/L. Após selecção de um painel, os BETs desceram até aos 23 μg/L. Os OAVs variaram entre 0,1–22 para os diferentes Blends e o sotolon revelou contribuir para o seu aroma. A formação da lactona em condições de envelhecimento semelhantes às do MW foi avaliada através de sistemas modelo submetidos a condições de envelhecimento acelerado (70 °C por um mês). Esta foi quantificada até 1,1 mg/L, tendo a presença de aminoácidos, de etanol, e de frutose contribuído para a sua formação. Pequenas quantidades do açúcar (1 g/L) foram suficientes para gerar sotolon a níveis superiores ao OT calculado. Embora mais estudos sejam necessários, este estudo sugere o papel de diferentes mecanismos na sua formação.

Palavras-chave: aroma do vinho, vinho fortificado, envelhecimento, processamento térmico, limiares olfactivos, análise sensorial

Abstract

Sotolon is a naturally occurring and strong aroma compound with characteristic caramel-, maple- and curry-like scents. Since its first identification in the biologically-aged *Vin Jaune* and in aged *sake*, sotolon has been identified in many other wines. Among these, it was identified at high concentrations in fortified wines such as Madeira wine (MW), contributing to their unique bouquet and acting as a possible age marker. The experimental part of this thesis started with the development of a simple, fast, and environmentally friendly methodology for its quantification. The method utilizes a single-step liquid-liquid extraction followed by liquid chromatography coupled with tandem mass spectrometry, showing good linearity (R^2 = 0.9999), intra-day and inter-day precision (lower than 10% of relative standard deviation), recovery of 95%, and high sensitivity (limit of quantification of 0.04 µg/L). Little is known about the flavour relevance of sotolon to commercialized MW wines. Thus, the odour impact of the lactone was established in MW Blends by the determination of odour activity values (OAVs). Thereby, the odour threshold (OT) of sotolon was established in the MW matrix for the first time. A preliminary best estimate threshold (BET) was estimated at 112 µg/L. Through further panel selection, the odour BETs were as low as 23 µg/L. OAVs varied between 0.1–22 among the different Blend styles and sotolon was revealed to contribute to their aroma. The formation of the lactone in MW-like ageing conditions was assessed by model systems submitted to accelerated ageing conditions (70 °C for one month). Sotolon was quantified at up to 1.1 mg/L with amino acid, ethanol, and fructose content contributing to its formation. Low fructose content (1 g/L) was enough to generate sotolon at levels higher than its OT. Although further studies are needed, this study suggests the role of different mechanisms in its formation.

Keywords: wine aroma, fortified wine, ageing, thermal processing, odour thresholds, sensory analysis

Declaration of authorship

Plagiarism consists of the presentation of ideas, opinions, phrases/texts, results, or conclusions from others as one's own work, even if there has been a translation. The practice of plagiarism constitutes a serious violation of academic ethics and may lead to disapproval or withdrawal from the academic degree, as well as to civil, criminal, and disciplinary liability.

Thus, I hereby declare that this thesis is the result of my own work, it is original, and I have acknowledged all outside material and sources used in its preparation and certify those were properly cited.

João Marcelo Gontardo Gaspar — Funchal, 15th December 2020

João Marcelo Gontardo Gaspar

Digitally signed by João Marcelo Gontardo Gaspar
Date: 2021.03.12 04:00:31 Z

When I heard the learn'd astronomer,
When the proofs, the figures, were ranged in columns before me,
When I was shown the charts and diagrams, to add, divide, and measure them,
When I sitting heard the astronomer where he lectured with much applause in the lecture-room,
How soon unaccountable I became tired and sick,
Till rising and gliding out I wander'd off by myself,
In the mystical moist night-air, and from time to time,
Look'd up in perfect silence at the stars.

—Walt Whitman

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List of abbreviations

2-KBA	2-ketobutyric acid
BET	best estimate threshold
FW	fortified wine
HIL	4-hydroxy-L-isoleucine
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
LLE	liquid-liquid extraction
MR	Maillard reaction
MSys	model system(s)
MW	Madeira wine
OAV	odour activity value
ORT	odour rejection threshold
OT	odour threshold
SFW	synthetic fortified wine
VDN	*Vins Doux Naturels*

Chapter 1

GENERAL INTRODUCTION

1.1 Introduction

Madeira wine (MW) is a well-known fortified wine (FW) mostly characterised by its richness and intensity of aromas acquired during its unusual ageing, but also by its surprising acidity, originated from the volcanic soils where the grapes are cultivated, well balanced with its sweetness. The resulting reactions that occur during the oxidative ageing conditions that these wines are exposed are important to the development of its typical and quite complex and persistent aroma, but also for its diverse colour range, varying from deep gold to deep brown. The result is one of the longest-lasting wines with spicy-, caramel-, coffee-, nutty-, to almond-like flavours; a bouquet especially sought-after and found in the finest and older Madeiras. These attributes result from the development and combination of many important aroma compounds formed during its ageing under heat [1]. One particular compound—***sotolon***—will be the focus of this thesis.

Unlike other well-known FWs, the relative production of MW is low. The yearly sales have stayed steadily around three million litre mark and the 2019 registered production was 3,162,937 litres according to IVBAM (the "Madeira Institute of Wine, Embroidery and Handicrafts") [2]. Most of it is produced and bottled as Blends and sold as either 3-, 5-, or 10-year-old wines. The average price for a 0.75 L bottle falls roughly between 5–10 € for a 3-year-old, 10–16 € for a 5-year-old, and 19–30 € for 10-year-old Blend [3,4]. There is quite a difference between these Blend wines' commercial value and those of the finest categories such as *frasqueira*, for example. The later can easily reach several hundred euros [4]. Although these Blends mostly represent the entry-level Madeiras, these are of economic importance as they constitute the bulk of MW sales, with producers establishing and maintaining their specific style and consistency among the various produced batches. Thus, it becomes interesting to study their composition, particularly its aromas.

The impact of sotolon has been well reported in FWs such as Port and Sherry. In the case of Madeira, for example, it has been proposed as an important and impactful compound to the aroma of this wine [5]. These studies show a strong correlation of the compound with ageing time, and sotolon is often associated with older premium Madeiras. While most of the research has been focused on the ageing methods and the compounds originated therefrom, little is known about their flavour relevance to the commercialized wines. Studies regarding the role of sotolon's flavour in the currently

commercialized Blends are particularly scarce. Contributing to this issue, Campo et al. [6] studied the aroma profile of four 10-year-old MW Blends and found sotolon as having a high odour impact on the aroma of these wines. However, almost 15 years have passed since its publication and it is expected that new winemaking procedures or strategies have been introduced in recent years. Thus, it seems important to study how these changes might be reflected in the impact of sotolon into the aroma of currently available wines.

One of the interesting peculiarities about sotolon is how it can act as an important compound in these niche wines while otherwise being associated with a deteriorating quality of dry white wines [7]. Understanding the mechanisms of formation of such a compound is then of great interest. Although sotolon has been quite studied, the mechanisms of formation in wines are still not so well elucidated. The probable pathways leading to its formation in biologically-aged wines seem well established [8]. On the other hand, in the case of oxidatively aged wines such as Madeira, the relationship with sugars, storage time, oxidation, and temperature has been pointed out but the formation mechanisms are still not fully understood [9]. The fact that sotolon is also present in a wide range of foodstuffs also makes it quite intriguing to keep studying and researching more about it.

Even though this thesis can be described mostly as an arrangement of preliminary experiments, the results may lead to new insights on the role and formation of sotolon, particularly in MWs.

1.2 General and specific objectives

The main aim of this study was to establish the odour impact of sotolon in currently available MW Blends and subsequently understand how this compound may be formed in this kind of beverage. To understand this, the following main objectives were put in place:

- Development of a fast and reliable method for the determination and quantification of sotolon in FW media;
- Choosing and preparing an appropriate sensory method for the determination of the odour threshold (OT) of sotolon in the MW matrix;
- Determination of the odour relevance of sotolon in MW Blends by means of calculating odour activity values (OAVs);
- Preparation of different combinations of model systems (MSys) submitted to accelerated ageing to better understand the formation of sotolon in MW-like ageing conditions.

1.3 Outline

This thesis is presented in four main parts; the first part (Chapter 2) gives general background information about the topic of this thesis, particularly on MW and sotolon. The second part (Chapter 3) is devoted to the determination and quantification of sotolon. The third part (Chapter 4)

is devoted to the sensorial impact of sotolon. And finally, the fourth part (Chapter 5) is related to the formation of sotolon in foodstuffs, particularly in wines.

A literature review on MW and sotolon is presented throughout Chapter 2. The background information on MW is particularly focused on the unusual and unique winemaking processes, namely the ageing methods of *estufagem* and *canteiro*. A brief summary of the last research done on this beverage is addressed, particularly regarding its composition and aroma key compounds acquired during its ageing. The compound sotolon is also briefly addressed in this chapter; from its first findings to its important role in many foodstuffs, as well as its impactful relationship with wines such as Madeira.

Chapter 3 is about the analytical methods employed for the determination and quantification of sotolon. Some of the most relevant research about this topic is firstly and briefly introduced. Then, the experimental part regarding the development of a fast and reliable method for its determination and quantification in FW media is addressed. This methodology is done by means of a miniaturized liquid-liquid extraction (LLE) and further analysis by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). This methodology is based and was published on the following paper:

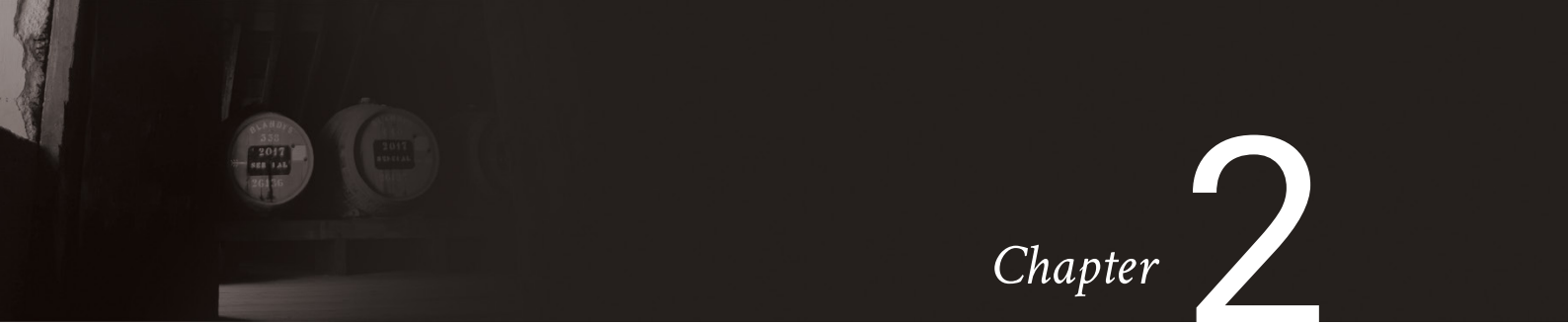
- *Rapid determination of sotolon in fortified wines using a miniaturized liquid-liquid extraction followed by LC-MS/MS analysis*
  Vanda Pereira, João M. Leça, João M. Gaspar, Ana C. Pereira, José C. Marques
  Journal of Analytical Methods in Chemistry, 2018; 1–7.

Chapter 4 introduces the sensorial characteristics of sotolon. The introduction briefly describes the topics of sensory analysis, the sense of smell, the concept of OT, and aroma research in foods. The flavour characteristics of sotolon and its impact in MW are also addressed. The experimental part is about the determination of the odour impact of sotolon in commercially available MW Blends. This is done by means of the determination of OTs and odour relevance by calculating OAVs in this beverage. Up to date, as far as we are aware, this is the first study reporting threshold data for sotolon in the MW matrix. These study findings are based and were published on the following papers:

- *Odor detection threshold (ODT) and odor rejection threshold (ORT) determination of sotolon in Madeira wine: a preliminary study*
  João M. Gaspar, Vanda Pereira, and José C. Marques
  AIMS Agriculture and Food, 2018; 172–180.

- *Is sotolon relevant to the aroma of Madeira wine Blends?*
  João M. Gaspar, Ana I. Freitas, Qianzhu Zhao, João M. Leça, Vanda Pereira, and José C. Marques
  Biomolecules, 2019.

Finally, in Chapter 5 an introduction on the scientific research about the formation of sotolon in foodstuffs is given. The experimental part then serves as an attempt at untangling the origins of sotolon in FWs. This preliminary study is based on MSys mimicking the MW-like ageing to understand its formation during those conditions.



Chapter 2

LITERATURE REVIEW: MADEIRA WINE AND SOTOLON

2.1 Madeira wine: what makes it unique?

MW is originated from the north Atlantic archipelago of Madeira, Portugal. It is an FW (17–22% alcohol by volume, ABV) of great local economic importance, well-renowned across the world. Its roots go almost as far as the discovery of the island, but its boom in fame and prestige is probably tied to the 18th century where the wine played an important role in the Atlantic trade with the Americas [10,11]. Madeira nowadays can be made in different styles depending on the grape variety and timing of fortification. The fortification with neutral grape spirit (96% vol.) inhibits the fermentative process which reflects on the level of residual sugars left in the wine and ultimately the MW style (Figure 2.1) [1].

Figure 2.1 The four typical Madeira wine styles according to the degree of sweetness (from dry to rich—or sweet).
Data from IVBAM [2].

The island's orography, the vineyards' volcanic soils, the grape varieties, and the resultant different styles with the characteristic acidity all contribute to the uniqueness of MW; but perhaps it is the ageing process that truly stands out. The peculiar processes employed during its ageing make this wine unique in the world. Contrary to most wines, Madeira is proposedly exposed to warm-oxidizing conditions, aiding not only to the appearance of the typical oxidized aromas (so-called *maderized*) but also to its well-renowned quality and longevity [1].

Many studies have been developed over the years and a wide amount of information is available in the literature. A simple research for "Madeira wine" in the Web of Science database, for example, results in more than 90 entries within chemistry-related fields. From those, a total of 54

papers were published in the last 10 years. This shows a crescent interest in acquiring more scientific knowledge about this beverage. It is, in fact, the ageing processes that gather the focus of most of the scientific publications on MW [1].

Most of MW suffers a "baking" step during its production. Young wines are exposed to an artificial heating process—*estufagem*—where wines are heated in steel vats (for at least three months according to the current legislation) with gradually increasing temperatures that can reach up to 55 °C. Then, wines can go through or, if made from white grape varieties, be exclusively aged in a traditional way in wooden casks—*canteiro*—where these are slowly aged in the warm attics of the wine cellars. More detailed information about these processes and the winemaking of MW can be found elsewhere [1,12]. While ageing, MWs are subjected to several changes resulting from the baking process and oxidative conditions [13]. The following highlighted research is mostly focused on the wine's major compound evolution and the typical flavour character acquired through the ageing processes.

With regards to compound evolution, Câmara et al. [14] firstly verified the influence of ageing on the level of 1,3-dioxane and 1,3-dioxolane isomers. Both dioxanes (*cis*- and *trans*-5-hydroxy-2-methyl-1,3-dioxane) and dioxolanes (*cis*- and *trans*-4-hydroxy-2-methyl-1,3-dioxolane) increased with age and showed a linear correlation, permitting their use as indicators of MW age. The *cis*-dioxane isomer was found at higher concentrations in all aged wines made from the four main white grape varieties under study (Sercial, Verdelho, Bual, and Malvasia). In another study involving MWs made from these same four white grape varieties, Câmara et al. [5,15] also observed an increase in the concentration of sotolon with ageing time. Sotolon content was higher in sweeter wines and a correlation with other sugar derivatives such as furfural, 5-methylfurfural, 5-hydroxymethylfurfural (HMF), and 5-ethoxymethylfurfural was established. Some of the major volatile changes during the ageing of MWs are related with the decrease in fatty acids ethyl esters and acetates, with the increase in ethyl esters from diprotic acids, such as ethyl lactate and diethyl succinate. These changes could explain the loss of the wine's freshness and fruity character [15]. Barrel ageing also brings an increase in oak lactones (*cis*- and *trans*-β-methyl-γ-octalactones) with ageing time. An increase is also observed for other γ-lactones, more importantly γ-heptalactone, γ-ethoxybutyrolactone, and pantolactone which are potential ageing markers [12,15]. Perestrelo et al. [16] identified 103 volatile compounds within the chemical groups of furans, lactones, volatile phenols, and acetals in monovarietal MWs. Independently of the variety, diethoxymethane, 1,1-diethoxyethane, 1,1-diethoxy-2-methyl-propane, 1-(1-ethoxyethoxy)-pentane, *trans*-dioxane, and 2-propyl-1,3-dioxane, 5-methylfurfural, and *cis*-oak lactone were identified as potential age markers. Specifically regarding the *estufagem* process, Pereira et al. [17,18] demonstrated that heating promotes the increase of the volatile fraction of both dry- and sweet-type Madeiras. Results have shown a particular increase in furans and esters and a slight decrease in alcohols, acetates, and fatty acids. After the *estufagem*, the increase in esters of organic acids such as diethyl succinate showed similar results as those obtained by Câmara et al. [15]. Like in oak-aged wines,

the decrease of most acetates could also contribute to the loss of fruitiness of these thermally processed wines. *Estufagem* was also shown to favour the development of the typical wine's tertiary aromas, particularly phenylacetaldehyde, β-damascenone, and 5-ethoxymethylfurfural. Later, Pereira et al. [19] further elucidated the process' contribution and the effect of the degradation of fructose and glucose to the acquired features of MW. Several volatile organic compounds were identified through the analysis of thermally processed sugar FW MSys. Most abounding compounds comprise furans, with HMF being the most abundant; 2(5*H*)-furanones were also identified, with sotolon being associated with the thermal degradation of fructose in acid medium. Freitas et al. [20] also demonstrated the *estufagem* process to accelerate the formation of the sotolon.

In summary, both ageing techniques lead to important and unique changes in the chemical composition of MW. As the ageing progresses, wine's fresh and fruity character is exchanged for a more complex aroma [13,21]. To better comprehend the aroma profile of MWs, dedicated sensorial studies were also applied. Campo et al. [6] characterized 10-year-old wines from the main four white grape varieties (Sercial, Verdelho, Bual, and Malvasia) with "candy", "nutty", "maderized", "toasty", "lacquer", and "dried fruit" descriptors. "Dried fruits" and "toasty" descriptors were characteristic of all four MWs studied, while "maderized", "candy", and "lacquer" were the most discriminative. The authors showed these oak-aged wines to have extremely complex aroma profiles (41 odorants) mainly rich in sotolon, phenylacetaldehyde, wood extractable aromas ((*Z*)-whiskylactone, for example), and other unknown odorants specific of MWs. Later, Oliveira e Silva et al. [22] evaluated the impact of forced-ageing on young Sercial and Malvasia MWs. Results showed the forced-ageing related compounds to have a higher impact on the wine's quality than those related to the primary and fermentative flavours. The wine's characteristic flavour was also shown to be due to the high levels of "aged marker compounds" such as sotolon, furfural, 5-methylfurfural, 5-ethoxymethylfurfural, methional, and phenylacetaldehyde. They also demonstrated similar descriptors of "dried fruit", "nutty", "musty", "baked", "oak", "mushroom", and "brown sugar" to best represent the volatile profile of MWs. The first two descriptors were attributed to sotolon, which was again demonstrated as being particularly impactful to the typicity of the MW bouquet. "Toasty", "dried fruits (nutty)", and "burnt sugar" were the common descriptors for both wines. Additionally, the forced-aged Malvasia was considered the most typical MW, evidencing the probable role of sugar in the aroma of these wines. Considering these findings, Figure 2.2 represents some of these main aroma descriptors and corresponding compounds related to the aged MW bouquet.

Figure 2.2 Main aroma descriptors and relevant odour compounds related with the aged Madeira bouquet. Based on the data from Campo et al. [6] and Oliveira e Silva et al. [22].

Although the previously cited research was focused on the volatile composition of MWs, it should be noted that the non-volatile fraction also plays an important role in the wine flavour [23]. The recently published reviews by Pereira et al. [1] and Perestrelo et al. [12] offer more detailed insights on both volatile and non-volatile composition of MWs, as well as additional details on applied chemometric approaches and other interesting topics.

2.2 Sotolon: a brief summary

2.2.1 Properties, nomenclature, and first findings

Sotolon (3-hydroxy-4,5-dimethyl-2(5*H*)-furanone; CAS # 28664-35-9) (Figure 2.3) is a naturally occurring and strong aroma compound with a characteristic caramel-, maple- and curry-like scent, depending on its concentration. It is also known as caramel furanone, sugar lactone, and/or fenugreek lactone [24]. At high concentrations, the characteristic fenugreek- and the curry-like smell is very apparent, while at lower concentrations the burnt sugary- and caramel-like nuances are noticeable [25]. Due to its intense sugary-sweet odour, sotolon is of great importance to the flavour and fragrance industry [26]. Its pungent flavour characteristics are a result of its low perceivable OTs (further discussed in Chapter 4). At room temperature sotolon appears as a viscous pale-yellow liquid and is particularly soluble and stable in alcoholic and acidic media [27,28]. Its density is 1.049 g/cm^3 and has a boiling point of 184 °C [29].

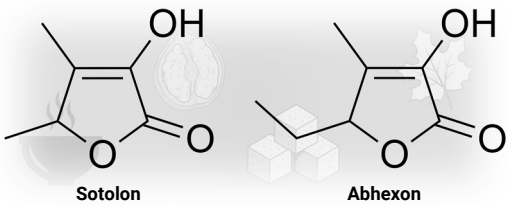


Figure 2.3 Chemical structures of sotolon and its ethyl analogue abhexon.

As it can be seen from its structure, sotolon is a heterocyclic organic compound with a four-carbon ring structure. It is a γ-lactone belonging to the family of compounds colloquially known as butenolides; these are dihydrofurans consisting of a 2-furanone skeleton [30]. Two butenolide isomers can be distinguished based on the double bond position: either $\Delta^{\alpha,\beta}$- or $\Delta^{\beta,\gamma}$-butenolides (Figure 2.4). In the past, butenolides were commonly known as crotonolactones and thus the isomers were also termed as "crotonolactone" and "isocrotonolactone", respectively [31–33]. Sotolon, in this case, belongs to the class of $\Delta^{\alpha,\beta}$-butenolides—the 2(5*H*)-furanones (Figure 2.5). Albeit the variation in literature for the order in which the substituent groups appear in the systematic name of sotolon, the 2(5*H*)-furanone nomenclature terminology is now predominantly used; the IUPAC name is defined as "3-hydroxy-4,5-dimethylfuran-2(5*H*)-one" although "3-hydroxy-4,5-dimethyl-2(5*H*)-furanone" is often used. The hydroxy group is sometimes given last (as "4,5-dimethyl-3-hydroxy-2(5*H*)-furanone") to emphasise the compound as a hydroxy-furanone [34]. The original term "butenolide" is also sometimes used (as "2-hydroxy-3,4-dimethyl-2-buten-1,4-olide") but is more common in earlier publications [35].

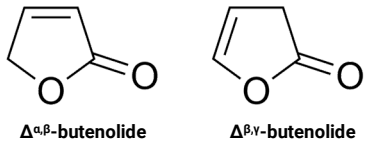
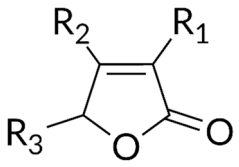


Figure 2.4 The two butenolide isomers.

The compound was identified for the first time in aged *sake* as a contributor to its burnt-like characteristic notes and *hine-ka* flavour [36]. *Hine-ka* roughly translates to "old stink" and is an unpleasant odour found in *sake* as a result of high amino-acid content or from storage at high temperatures [37]. Although initially associated with sotolon, recent research by Isogai et al. [38] showed polysulphides such as dimethyl trisulphide to highly correlate with the *hine-ka* overaged flavour, while sotolon did not. According to the authors, commercial aged *sake* stored for long periods, but without *hine-ka*, was characterized by high levels of sotolon, volatile aldehydes, furfural, and diethyl succinate. The resulting appealing honey- and soy-sauce-like aromas, which are distinguished from the *hine-ka* flavour, are thus associated to sotolon [37]. In the same year, in 1976, Dubois et al. [39] had also identified sotolon in the Jura *flor*-sherry wines—also known as *Vins Jaunes* (French for "yellow wines")—here being responsible for the nutty-like odour of the wine. The characteristic seasoning-like

and spicy-curry flavour notes of the fenugreek herb (*Trigonella foenum-graecum* L.) were also given to the presence of this aroma compound [40].



R_1	R_2	R_3	
−OH	$-CH_3$	$-CH_3$	3-Hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolon)
−OH	$-CH_3$	$-C_2H_5$	5-Ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone (abhexon)
−OH	−OH	$-CHOH$ CH_2OH	5-(1,2-Dihydroxyethyl)-3,4-dihydroxy-2(5*H*)-furanone (ascorbic acid)
−OH	−OH	$-CH_2OH$	5-Hydroxymethyl-3,4-dihydroxy-2(5*H*)-furanone (erythroascorbic acid)
$-(CH_2)_3$, CH_3	-Br	=CHBr	3-Butyl-4-bromo-5-bromomethenyl-2(5*H*)-furanone
−Cl	$-CHCl_2$	−OH	3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (Mutagen X)

Figure 2.5 The naturally occurring 2(5*H*)-furanones.
The commonly used names are given in brackets. Adapted from Slaughter [34].

Its natural occurrence was first proposed and reported in 1975 by Rijkens and Boelens [41] in fenugreek as a "character-impact compound" of the plant. However, its presence was only later confirmed by Girardon et al. [42]. Interestingly, some sources report that sotolon was identified for the first time in 1967 in vegetable protein hydrolysates, citing the work by Sulser et al. [43]. In the original publication, Sulser and colleagues state that the aroma compound, which results from the degradation of threonine, is 5-ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone (also known as abhexon, emoxyfurone, or more commonly maple furanone)—the ethyl analogue of sotolon—one of the organic compounds having one of the lowest known OTs (Figure 2.3) [26]. Not only are the two compounds structurally similar, but they also possess very similar flavour properties and may share identical formation pathways, which might be the reason for some of these incoherent citations. Regardless, the work by Sulser and colleagues was of great importance as it further triggered new research findings of the formation pathways of sotolon (see Chapter 5).

The origin of the name dates to the late 1970s and early 1980s. During this time, a series of studies about the sugary flavour of raw cane sugar was published in the Proceedings of the Japan Academy, Series B journal. The group of authors were trying to isolate and identify the key substance responsible for the characteristic flavour of sugar cane molasses [44–46]. They had characterized over 40 compounds from cane molasses, most of them for the first time, but the synthetic mixture of these did not entirely reproduce the characteristic sugary flavour. The authors then postulated that the key flavour compound might be present at very low quantities and not among the main chromatogram peaks. Then, analysis of the minor peaks revealed 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone to be the

most probable flavour compound. Further isolation and sensory evaluation demonstrated that this lactone was indeed the key substance for the sugary flavour, having a very low OT value with aroma changing from caramel-like at low concentrations to curry-like at higher concentrations. The compound was then named as *sotolon* (sometimes referred to as sotolone) by the combination of the words *sotou* (Japanese for “raw sugar”) and "-olon" (from "enolic lactone") [46,47].

2.2.2 Chirality

Sotolon is also a chiral lactone and both enantiomers seem to occur naturally and in different ratios depending on the food matrix [48,49]. The first asymmetric synthesis was reported in 1983 by Okada et al. [50] from optically active tartaric acid. Another chiral synthesis was reported in 1992 by Monsandl et al. [49]. More recently, Nakahashi et al. [26] tried to determine the absolute configurations by a vibrational circular dichroism (VCD) approach. The authors found the absolute configurations of sotolon to be (*R*)-(–)-sotolon and (*S*)-(+)-sotolon (Figure 2.6).

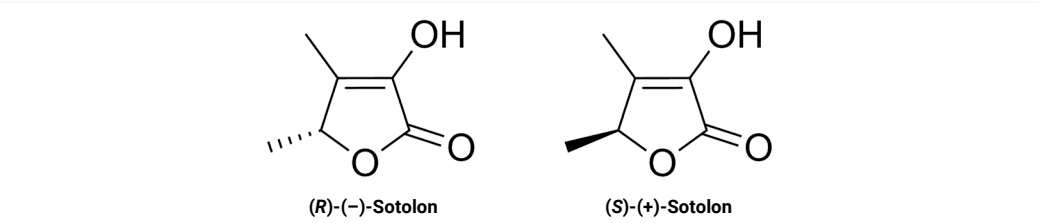


Figure 2.6 Absolute configurations of sotolon.

In fenugreek seeds, for example, isolated sotolon is optically active with (*S*)-sotolon being the dominant enantiomer (95%) [51]. Guichard et al. [49] also evaluated the enantiomeric ratios of sotolon in wines, including Sherry, *Vins Jaunes*, and Sauternes, and found the results to be too unspecific. The maximum enantiomeric excesses (%*ee*) in these wines were 58% and 44% for the (*R*)- and (*S*)-sotolon, respectively. Pons et al. [48] verified the distribution of both enantiomers among various French dry white wines of different vintages from the wine region of Bordeaux (Graves and Entre-Deux-Mers). Similarly to the reports of Guichard et al. [49], both forms were detected at different ratios: either as racemic, an excess of *R*, and an excess of *S*. The wine's vintage had no apparent correlation with the enantiomeric distribution pattern. The maximum %*ee* in these wines were 50% and 56% for the (*R*)- and (*S*)-sotolon, respectively. Although earlier studies reported that both enantiomers had the same aroma properties as of (±)-sotolon, these authors also demonstrated that the *S* form had a significantly lower odour perception threshold in model wine solution (further discussed in Chapter 4, see Table 4.5).

As addressed by Pons et al. [48], the different distribution patterns among these matrices can shed some light in the understanding of its formation. While the predominant presence of (*S*)-sotolon in fenugreek can be related to optically active precursors (further discussed in Chapter 5), in the case of wines it lacks better reasoning. Although Guichard et al. [49] suggested sotolon to not be affected

by partial racemization, Pons et al. [48] later postulated these different enantiomeric patterns in wines to occur, in part, as a result of the slow racemization of the lactone (over 20 months in model wine solution). The racemization was suggested to occur via a keto-enol tautomerism in a mildly acidic wine-like medium (pH 3–3.5) (Figure 2.7). Like in fenugreek, different enantiomeric proportions might result from enantiomerically pure precursors or intermediates, or from stereoselective reactions. Further racemization might then occur, which might explain the presence of low %*ee* and racemic forms of sotolon in wines submitted to relatively long ageing periods. On the other hand, the racemic form can result entirely from non-stereoselective mechanisms. Either way and as will be discussed in Chapter 5, the formation mechanisms of sotolon in wines still require further clarification.

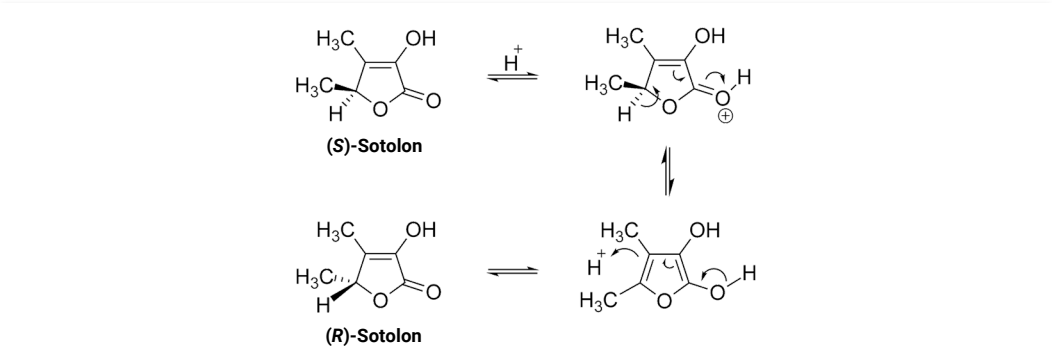


Figure 2.7 Sotolon racemization via enolization in mildly acidic medium.
Adapted from Pons et al. [48].

2.2.3 Environmental and health related effects

No known toxicologic effects are observed for sotolon; its content in foodstuffs does not raise any apparent concerns and no literature reports were found regarding serious health or environmental side effects. However, the lactone is linked with the characteristic odour of the maple syrup urine disease (MSUD; OMIM #: 248600) also known as the branched-chain ketoaciduria. This disease is a genetically inherited disorder that causes a deficiency in the branched-chain amino acid metabolism due to mutations in the branched-chain α-keto acid dehydrogenase complex, resulting in the blockage of the oxidative decarboxylation activity of the enzyme. Patients who suffer from the disease possess a characteristic sweet maple syrup-like aroma in their body fluids (particularly in urine) [52]. Interestingly, the "pseudo" MSUD is also commonly observed in Mediterranean individuals, correlated with the ingestion of fenugreek infusions [53].

More interestingly, sotolon was thought to be responsible for the maple syrupy smell that appeared across Manhattan in 2005. The pleasantly sweet smell remained a mystery up until 2009, when after a long investigation the responsible compound was isolated and narrowed to a fragrance factory nearby, Frutarom. The company often used fenugreek seeds to manufacture food flavours, releasing the pungent odour during the process. [54,55].

Recently, sotolon was also shown to act as a promising anti-pathogenic agent on infections caused by *Serratia marcescens* bacteria in humans [56].

2.2.4 Occurrence in foodstuffs

Sotolon occurs naturally in various foodstuffs. As stated before, it is particularly present as the key compound for the seasoning and savoury-like odour of fenugreek. Fenugreek is a leguminous herb widely cultivated in the Mediterranean, North African region, and India, often used in cooking as a traditional medicine against diabetes [57,58]. Fenugreek seeds can also be toasted and grind to be used as an essential ingredient in curry powders [51]. Reported concentrations show sotolon to range up to 25 mg/kg in fenugreek seeds, the highest amount found naturally [59]. Sotolon also plays a similar role to the seasoning-like flavour characteristic of the "love herb" lovage (*Levisticum officinale* W. D. J. Koch) [60]. In Table 2.1 some of the concentrations found for sotolon in natural plant-based sources are detailed. Additionally, sotolon can be found and contributes to the overall aroma of cane sugar [46], brewed coffee [61], condiments such as soy sauce [62], and many processed foods including meats [63], dairy [64], and alcoholic beverages [65,66].

Table 2.1 Concentrations found for sotolon in some natural plant-based sources.

natural source	sotolon (mg/kg)	reference
fenugreek (seeds)	3.3–25.12	[51,59]
Heracleum transcaucasicum Manden (dried shoots)	11.5	[67]
coffee beans (roasted)	0.63–1.87	[68,69]
lovage (dried leaves)	0.84	[59]
lovage (dried roots)	0.34	[59]
lovage (fresh leaves and stems)	0.03–0.05	[59]
cocoa beans (roasted)	0.013	[70]
coffee beans (raw)	< 0.01	[69]
cocoa beans (raw)	< 0.01	[70]
tomatoes	< 0.01	[71]

Studies regarding this compound are quite vast in the literature. Figure 2.8 shows the number of scientific publications related to each type of food-related matrix in which sotolon is reportedly studied. The collected data is based on publications retrieved from the Web of Science, Google Scholar, and Microsoft Academic databases using "sotolon" and "3-hydroxy-4,5-dimethyl-2(5*H*)-furanone" as search keywords. From the vast collection of publications, 184 were selected from food-related scientific fields regarding the detection/quantification by analytical means and/or being subject of food flavour analysis. The infographic thus represents a vast research that dates to its first findings in *sake*, in 1976, up to mid-2020.

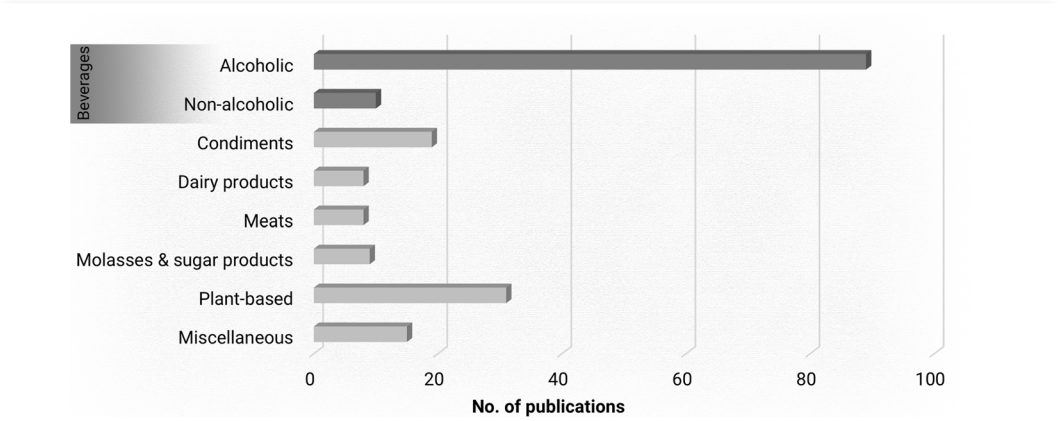


Figure 2.8 Number of scientific publications related to sotolon in foodstuffs.
Retrieved from the Web of Science, Google Scholar, and Microsoft Academic databases using "sotolon" and "3-hydroxy-4,5-dimethyl-2(5*H*)-furanone" as search keywords. Data corresponding from 1976–2020. Some papers may be considered within more than one category.

A vast part of the research surrounding sotolon in food matrices is focused on its presence and formation in alcoholic beverages, mostly in wines. From the 184 retrieved publications, 65 are within the wine category (Figure 2.9). Sotolon is showed to be quite stable in wine-like conditions [72]. Martin et al. [27] showed that sotolon is stable in dilute hydroalcoholic solution (14% vol. of ethanol) at acidic conditions (pH 3.10). On the contrary, sotolon was revealed to be unstable at alkaline pH [47]. Additionally, in aqueous solutions, only the enolic tautomer is observed [27].

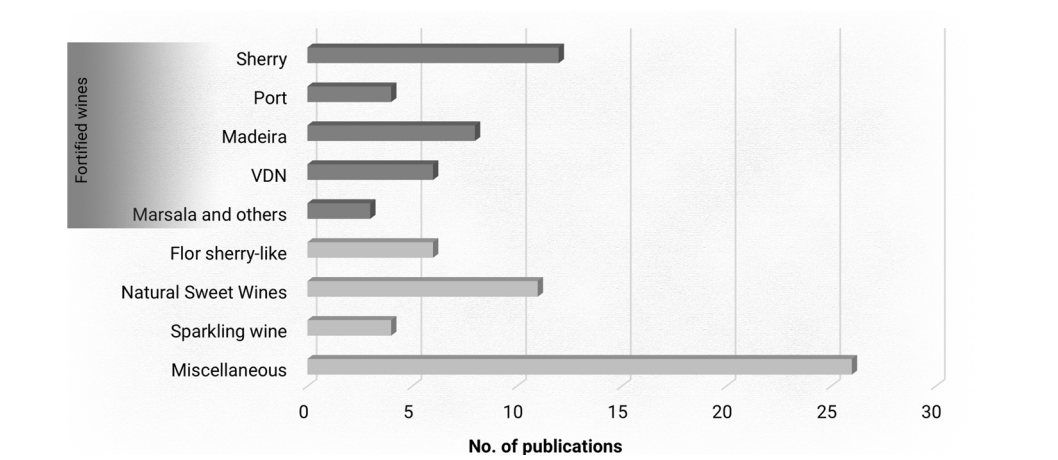


Figure 2.9 Number of scientific publications related to the presence of sotolon in wines.
Retrieved from the Web of Science, Google Scholar, and Microsoft Academic databases using "sotolon" and "3-hydroxy-4,5-dimethyl-2(5*H*)-furanone" as search keywords. Data corresponding from 1976–2020. Some papers may be considered within more than one category.

The occurrence of sotolon in wine was first reported by Dubois et al. [39] in 1976. The authors found this compound to be responsible for the "nutty" nuances of French *flor*-sherries. Since then, sotolon has been found to be an impactful compound to the aroma of botrytized wines [73], Sherry and Sherry-like biologically-aged wines [27,65,74,75], *Vins Doux Naturels* (VDN) [76], Port [66], and Madeira [5]. A brief overview of its occurrence and impact in some of these wines is followed.

2.2.4.1 Fortified wines

FWs are wines in which a distilled beverage or spirit has been added [77]. Thus, FWs can have an acquired alcoholic strength of 15–22% ABV. Some of the most well-known FWs come from the European countries of Spain, Portugal, France, and Italy, where the wine historically originates, although others are nowadays produced worldwide [1]. Sotolon is found to be particularly present in some wines from those regions, such as Sherry, Port and Madeira, VDN, and Marsala, respectively. A compilation of the quantification results for sotolon in FWs is presented in Table 2.2.

Table 2.2 Sotolon concentrations in well-known fortified wines.
Concentration values in µg/L unless otherwise specified.

wine	origin	sotolon	reference
Madeira	Portugal	< LOQ–2000	[5]
VDN	France	< LOQ–1378	[78]
Port	Portugal	< LOQ–958	[66,78]
Sherry	Spain	< LOQ–670	[65,79]
Marsala	Italy	0.02%[1]	[80]

VDN, *Vins Doux Naturels*; *LOQ*, limit of quantification; [1]relative peak area.

Sherry wines are FWs produced in the southern Spain region of Andalucía. These can notably be produced in different styles according to the employed vinification and ageing processes. The main fortified styles, however, are *Fino*, *Oloroso*, and *Amontillado* (DO Jerez-Xérès-Sherry) which correspond to the dry-styled (so-called *Generoso*) produced in the province of Cádiz. The same styles can also be produced in the towns of Montilla and Moriles (DO Montilla-Moriles) with a different designation. These styles are categorized according to the employed ageing method, as either: biological, oxidative, or a combination of both, respectively [81,82]. Additional information about the winemaking practices of Sherry can be found elsewhere [1]. Sotolon is showed to be an important odorant compound produced both during the biological ageing of *Fino* as well as during oxidative wine ageing [81]. For the sake of uniformity, the occurrence of sotolon in the biologically-aged Sherry will also be further mentioned in section 2.2.4.3 assigned to biologically-aged wines. Sotolon can range up to 670 µg/L in Sherry [79]. The first reported quantifications seem to be by Martin and Etiévant [83], showing a concentration of 36–143 µg/L in the case of *flor*-sherry. Zea and colleagues studied the aromatic characteristics of Sherry wines to a great extent [75,79,84–90]. With regards to *Oloroso* wines, which follow exclusively oxidative ageing, sotolon was found to be among the most odour active compound [89,90]. Its impact was higher in older wines, contributing to the walnut-, candyfloss-, and curry-like odour notes. Sotolon is also one of the main potent odorants in *Amontillado*, which results from both biological and oxidative ageing, sequentially. The same group determined the main odorant compounds in commercial *Amontillado* wines and compared them to exclusively biological and oxidatively aged Sherry to estimate the contribution of both processes [87]. The oxidative ageing was showed to influence the aroma of these wines to a greater extent than the biological ageing step. Sotolon was among the nutty and spicy odorant series and was found among all wine styles. It showed,

however, a much higher impact on the aroma of *Amontillado* (Table 2.3). Moyano et al. [79] also determined the main odorant compounds in wines still undergoing these ageing processes. Similarly, they found higher concentrations (up to 670 μg/L) in wines corresponding to the samples undergoing oxidative ageing. The odour perception of sotolon was also more intense in these wines (curry-like). Comparatively, lower concentrations (up to 310 μg/L) were found in wines during the biological ageing alone. Sotolon content also showed an increase with ageing time.

Table 2.3 Odorant impact of sotolon in dry-styled Sherry wines.
Data from Zea et al. [87].

Sherry wines					
Amontillado		*Fino*		*Oloroso*	
OAV	**rank[1]**	**OAV**	**rank[1]**	**OAV**	**rank[1]**
92 ± 8	1st	40 ± 4	4th	55 ± 4	1st

OAV, *odour activity value*; OAVs represent an average of nine values; [1]relative rank among the active odorant compounds (highest OAV).

The presence of sotolon also contributes to the aroma of the Portuguese Port and MWs. Detailed information about the winemaking of these FWs can be found elsewhere [1]. The lactone has been recognized as a key molecule for the "perceived age" of barrel-aged Port wine [91]. In Port, concentrations of sotolon can range up to about 1 mg/L and its formation is well correlated with storage time and sugar content [66,92]. Silva Ferreira et al. [66] firstly studied the aroma characteristics of Port wines aged for over 40 years in barrels. The authors found sotolon to be responsible for the "nutty" and "spicy-like" descriptors. Additionally, the OT of the compound was estimated at 19 μg/L in this wine. Likewise, sotolon was also found to be present in MWs with concentrations reaching higher values. Câmara et al. [5] reported values at up to 2 mg/L in wines aged between 1–25 years in oak casks. Higher concentrations were also found in wines with high residual sugar contents. The sotolon content in wines aged for 11 years was on average 258.7, 430.3, 540.6, and 825.8 μg/L for dry, medium dry, medium sweet, and sweet Madeiras, respectively. Its presence was also well correlated with ageing time, thus being suggested as a potential ageing marker of these wines [15]. Reports of Campo et al. [6] and Oliveira e Silva et al. [22] later demonstrated the role of sotolon to the wine's characteristic flavour.

Sotolon is present in the composition of VDN. The French term translates to "natural sweet wines" which is used to characterise the FWs made mainly in the regions of Rhône, Roussillon, and Languedoc in southern France [77]. The fortification is traditionally referred to as *mutage* and the winemaking processes can be very versatile with most wines undergoing oxidative ageing for several years. Most VDN is produced within the region of Roussillon with different appellations. VDN can be made from various red and white grape varieties, mostly: Grenache (noir, blanc, and gris), Maccabeu, Muscat, and Malvoisie du Roussillon [93,94]. Cutzach et al. [76,78,93,95,96] extensively studied the aromatic characteristics and role of sotolon in these wines. The authors firstly studied the volatile compounds responsible for the typical aroma of VDN from non-Muscat varieties: red wines made

mostly from Grenache noir (AOC Banyuls and AOC Rivesaltes), and white wines made from Grenache blanc, Grenache gris, and Macabeu (AOC Rivesaltes) of different ages. Sotolon was found for the first time in these types of wines with its concentration and sensory impact increasing quite regularly with wine ageing. In further studies, they found concentrations ranging up to about 1.4 mg/L with sotolon being more predominantly in white wine varieties. The "odour lability" of the compound was also verified. At relative lower concentrations (up to 300 μg/L) sotolon was responsible for the "prune" aroma of these wines; intermediate concentrations of about 300–600 μg/L resulted in "dried fig" aromas; above these values the typical "rancio" character was shown.

Another FW in which sotolon is found is Marsala produced in Sicily, Italy. Dugo et al. [80] characterized the volatile composition of different styles of Marsala wine. Sotolon was found to be present in *Superiore Riserva Dolce* Marsala, a sweet wine (above 100 g/L of residual sugars) with more than four years of ageing.

2.2.4.2 Natural sweet wines

Natural sweet wines are a type of sweet wine in which both alcoholic and sweetness degree derives exclusively from the grapes. Despite their classification and as the name implies, these have no relation with the French VDN as no fortification takes place during fermentation. The high sugar content of the final wine is obtained through late harvestings or other grape dehydration processes, which may include: frozen, overripe, dried, and/or raisined grapes [97,98].

Sotolon is a characteristic example of an impactful compound to the aroma of botrytised wines. These are sweet natural wines, also called noble rot wines, in which grape overripening occurs on the vine as a result of *Botrytis cinerea* infection. The French Sauternes and Hungarian *Tokaji Aszú* are well-known examples [98]. Masuda et al. [73], in 1984, were the first to find sotolon as a constituent of botrytized wine. The authors also evaluated the sensory characteristics of this compound in botrytised wines from France (Sauternes), Germany (*Trockenbeerenauslese*) and Japan. They reported sotolon as having a sugar-like and caramel-like aroma and concentrations ranging 5–20 μg/L. By spiking white wines with sotolon the authors verified the important role of the compound in the sweet and honey-like aroma of botrytized wine. While not necessarily directly related with the *B. cinerea* infection, sotolon is thought to be formed as a result of the optimal conditions during grape over-ripening [99]. More recently, Bailly et al. [100,101] and Sarrazin et al. [102] characterised the main odorants responsible for the typical aroma of botrytised wines from the Sauternes region and found sotolon to be among the most potent compounds. However, in the specific case of *Tokaji Aszú*, sotolon has not been identified as a volatile compound, probably as a result of its low concentration and analytical method choice not being appropriate to detect this lactone [103].

Some types of Sherry are also produced as sweet wines. This is the case of Pedro Ximénez (PX), which is produced from dehydrated Pedro Ximénez grapes. Although produced as Sherry, these sweet wines are mostly produced in the Montilla-Moriles region. Here, due to the favourable climatic

conditions, musts can reach high potential alcohol levels and fortification is not always required [104]. Thus, some of these can be considered a type of natural sweet wines. The aroma of PX was characterized by its richness in sotolon, which was among the most relevant aroma compounds. Campo et al. [74] quantified sotolon at 176 μg/L in a 1975 vintage Montilla-Moriles PX. This value was the highest among other analysed wines, which also included *Fino* Sherry and Sauternes. Similarly, *Vin de Paille* is a white natural sweet wine made from dehydrated grapes but produced in the Jura region of France. Guichard et al. [105] assessed the sotolon content in these wines and found its concentration to range 7–51 μg/L.

Maslov et al. [106] performed an aroma characterization on "predicate" wines from Croatia. These are another type of natural sweet wines obtained either through dried, frozen and/or noble rot infected grapes. The authors had also found sotolon to be among the highest contributing wine compounds to the overall aroma composition. The quantified values were also much higher than any other natural sweet wines, reaching MW-like levels. Results also showed an increase in sotolon concentration during ageing ranging 238–1509 μg/L.

2.2.4.3 Biologically-aged wines

The biological ageing of wines is characterized by the development of yeast strains on the wine's surface after alcoholic fermentation (Figure 2.10). Some of these wines are produced in Andalucía (Spain), Sardinia (Italy), Tokaj (Hungary), and Jura (France) regions [107]. The biofilm is formed as a result of the yeast's metabolism changing from fermentation to aerobic respiration (oxidative metabolism) when exposed to air due to the allowed headspace in casks [108]. This yeast film naturally prevents the wine from any further significant oxidation [81]. The yeast's metabolism also promotes peculiar organoleptic and compositional changes in these wines, such as the formation of sotolon [109].

The occurrence of sotolon in wine was first reported in the biologically-aged *Vin Jaune* [39]. This is a wine produced in the French Jura region (AOC Château-Chalon, AOC Arbois, AOC Côtes du Jura, and AOC L'Etoile) exclusively from Savagnin grapes [110]. Due to a peculiar sherry-like process of ageing, these wines are also known as French *flor*-sherries. As described before, Sherry wines can be made in different ways with some being subjected to this peculiar biological ageing. This is the case of the dry-styled *Fino* and *Amontillado* Sherry, their similar Montilla-Moriles counterparts, and *Manzanilla* (DO Manzanilla-Sanlúcar de Barrameda), which are aged under this film of yeasts known as *flor*. *Vins Jaunes* are thus also marked by the presence of this biofilm (here called *voile*) during their ageing process, which occurs undisrupted for at least six years and three months [110]. Earlier studies have found sotolon to contribute to the aroma of *Vin Jaune* [39]. Later, Martin et al. [27] and Guichard et al. [105] quantified sotolon ranging 75–268 μg/L in these wines, and a good correlation between its concentration and the wine's typicity was also observed. Pham et al. [111] verified that the amount of sotolon increased with time during the six years of biological ageing of *Vin Jaune*. Also, the concentration followed a gradient with higher concentrations found at the bottom of the barrels. More

recently, Collin et al. [112] confirmed the key role of sotolon to the typical curry notes found in these wines. With regards to Sherry, Martin and Etiévant [83] were the first to quantify sotolon in these wines. Sotolon concentration ranged from 36–143 μg/L in *flor*-sherry. Sotolon was also showed to be more prevalent in *Vin Jaune* rather than in *Fino* Sherry [65]. With a concentration ranging up to 500 μg/L, the same group also showed the role of the lactone to the typical aroma of these biologically-aged wines. Moreno et al. [75] assessed the evolution of aroma compounds during the biological ageing of *Fino* Sherry. Sotolon was shown to increase linearly (up to 191 μg/L) after five years. The lactone was thus among the compounds suggested as best markers of the changes in biologically-aged Sherry wines. Zea et al. [86] later demonstrated the role of sotolon's spicy-like notes to the aroma of *Fino*.

Figure 2.10 *Flor* yeast growing at the surface of a Sherry wine ageing in barrel.
Image credit: Deb Harkness via Flickr https://flic.kr/p/4TGVuE.

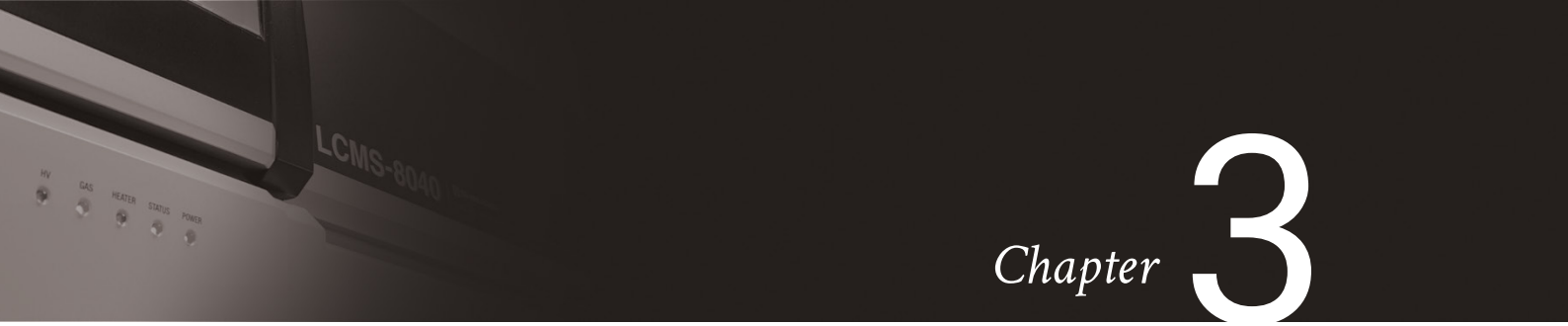
Although Tokaj is mostly known for its noble rot *Tokaji Aszú*, some wines such as the dry *Tokaji Szamorodni* are also aged under a *flor* velum, making them quite reminiscent of Sherry [113]. Guichard et al. [105] quantified sotolon ranging 84–142 μg/L in *Tokaji* partially aged under a yeast film.

2.2.4.4 Negative role in dry white wines

The characterisation of the key compounds responsible for the oxidised aroma of spoiled wines has been vastly studied [114–116]. While the positive contribution of sotolon to oxidatively aged wines is well evident, in the case of wines traditionally made under reductive conditions its presence is considered to result in off-flavours [117]. Some authors have focused on the contribution of sotolon to the oxidation off-flavour arising during the atypical ageing of dry white wines. It is responsible for the so-called "premox"—the premature oxidative wine ageing, usually associated with the loss of freshness and varietal qualities [114] Due to its organoleptic properties, relative minimal concentrations are sufficient to spoil these wines [48,118].

Escudero et al. [114] monitored the changes in the flavour profiles of white wines during oxidation. Sotolon was found to be among the highest impact odorants. The similar contribution of sotolon was also reported by Lavigne and Dubourdieu [119]. Silva Ferreira et al. [120] then verified the development of off-flavours characterized as "honey-like", "farm-feed", "hay", and "woody-like"

during the storage of bottled dry white wines at extreme conditions (45 °C and O_2 saturation). The same group later identified sotolon to be among the most important contributors to the oxidation defect. The authors also employed a forced ageing experiment to promote the oxidation-spoiled aroma. A relation was established between the concentration of these oxidative-related compounds and sample sensorial scores ("most spoiled") [115]. Pons et al. [7,48,118] contributed with further insights on the role and formation of sotolon in these wines. Major findings were related with the utmost flavour contribution of the *S* enantiomeric form in oxidized white wines as well as the involvement of 2-ketobutyric acid (2-KBA) as the potential precursor for its formation (further discussed in Chapter 5).



Chapter 3

SOTOLON IDENTIFICATION AND QUANTIFICATION IN FORTIFIED WINE

DEVELOPMENT OF A METHODOLOGY FOR THE DETERMINATION AND QUANTIFICATION OF SOTOLON IN FORTIFIED WINE MEDIA BY A MINIATURIZED LIQUID-LIQUID EXTRACTION AND LC-MS/MS ANALYSIS

3.1 Introduction

Sotolon's identification and quantification in food matrices have been diversely reported in the literature (see Chapter 2). The first reported identifications of the compound date back to 1976 when Takahashi et al. [36] identified it as the burnt flavouring compound of aged *sake*. Although sotolon had been synthesized before [35,121,122], this was the first time that the compound was isolated from a natural product. After isolation by Diaion HP-20, silicic acid and Dowex 1-X8 column chromatography, following extraction with chloroform, the authors identified and quantified sotolon in aged *sake* with the aid of thin-layer and gas chromatography (GC), and detection by mass spectrometry (MS) and ultra-violet spectroscopy (UV).

The occurrence of sotolon in a wine matrix was first reported in the Jura *Vin Jaune* by Dubois et al. [39] in 1976. Due to the unavailability of the publication at the time of writing, quantification data could not be confirmed. Also, no clear cross-references regarding quantitative results were found. Thus, according to the available literature, the first reported quantifications in wine media seem to be by Masuda et al. [73] in 1984. The authors found sotolon to be the key flavour substance of botrytised wine. Sotolon was here reportedly separated by column chromatography with DEAE-Sephadex A-25 (OH^-) and silica gel. The separation was preceded by LLEs of botrytised wine (1.2 litres of wine) with a solvent mixture of ether–*n*-pentane–dichloromethane. The quantification was done by gas chromatography-mass spectroscopy (GC-MS) (Finnigan 4000 GC-MS system). Ions at 128 *m*/*z* were used for quantification. Sotolon content in the botrytised wines was found in the range 5–20 μg/L.

Since then, sotolon has been identified and quantified in many other wines—particularly in well-known FWs such as Port [66], Sherry [65] and Madeira [5]—at concentrations that can reach up to 2 mg/L, as in the case of MW [5]. The occurrence of the lactone in FWs was previously discussed in Chapter 2 (see Table 2.2 for a summary of the quantification results for sotolon in some of these wines).

As a potent odorant, sotolon has been extensively quantified in some wine matrices by GC techniques. Two-dimensional GC (2D-GC) and capillary 2D-GC were employed in *flor*-sherries [65,83]. High-resolution gas chromatography-mass spectrometry-olfactometry (GC-MS-O) was used to quantify the compound in Spanish white wines undergoing oxidation [114]. Other GC-MS methods were used in the quantification in different wine types [5,7,91,112,123,124]. More recently, gas chromatography coupled with tandem mass spectroscopy (GC-MS/MS) was used for quantification in white wines [125,126]. Some authors also proposed liquid chromatography (LC) techniques to quantify sotolon. The use of high-performance liquid chromatography-UV (HPLC-UV) [105,127] and ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) [127,128] has been reported.

Sotolon is a chiral lactone (see Figure 2.6) and thus enantiomeric studies are of interest. Some methodologies have been used for chiral separation and identification. Chiral GC-MS has been used for the separation and study of sotolon enantiomers in wine. Pons et al. [48] evaluated the enantiomeric ratios (ERs) of both (*S*)-sotolon and (*R*)-sotolon in several dry white wines using a β-cyclodextrin chiral main column (HP Chiral-20β; J&W, France) connected to a fused silica precolumn (BP20; SGE, France). The separation of sotolon enantiomers showed the presence of nonracemic forms. Maximum enantiomeric excess was 50% and 56% for (*R*)-sotolon and (*S*)-sotolon, respectively. Following a similar methodology, Guichard et al. [49] had also studied the ERs of sotolon in *Vin Jaune*, Sherry and botrytized wines by 2D-GC coupled with a flame ionisation detector. The three types of distribution patterns were also revealed: the racemic form and both an excess of the *R*-and *S*-form. Much like the study by Pons et al. [48], the presence of the enantiomers in the wines was not systematic, although Pons et al. [48] attribute the racemization of sotolon as a possible explanation for the enantiomeric distributions observed. More recently, Xie et al. [129] studied the resolution of racemic sotolon by packed column supercritical fluid chromatography, developing a method for the isolation of optically active sotolon from the synthetic racemic molecule. Furthermore, VCD spectroscopy was used to confirm the absolute configurations of sotolon as (*R*)-(+)-sotolon and (*S*)-(–)-sotolon [26,130].

The low quantities usually found in wines (typically in the micro-range) imposes a challenge for the sotolon quantification in these complex matrices. Due to the very low OTs (further discussed in Chapter 3), sotolon might be sensory detectable in many matrices but not always by quantitative analytical means [52]. In the case of FWs, the problem is exacerbated as a result of the higher ethanol content which is known to affect the isolation of volatile compounds and having an effect on the extraction abilities of different solvent and adsorbent systems [131]. LLE is often carried-out before chromatographic separation [9]. Solid-phase extraction (SPE) can also be employed [123,131]. The extraction step is then followed by a preconcentration step [9]. The chemical and physical characteristics of sotolon can also negatively affect its quantification by some analytical methods. Due to the high boiling point (184 °C) headspace sampling such as dynamic headspace (DHS) and solid-phase microextraction (SPME) are not so favourable [127].

Due to these needed extra-steps, some of the applied chromatographic methods for the quantification of sotolon in wines are often laborious, relying upon a relatively high amount of solvent volumes and long-lasting sample extractions. Recently, Gabrielli et al. [127] addressed these issues and proposed two novel HPLC-UV and UHPLC-MS methods for the determination of sotolon in wines. The proposed methods were based on LLEs with relatively faster extraction times and lower volumes of wine (30 mL) and solvents (40 mL), which provided a faster and easier-to-apply analysis procedure when compared to other proposed procedures [7,114,124,132,133]. Briefly, for the UHPLC-MS separation, an Acquity H-Class UPLC system connected to a Xevo triple quadrupole mass spectrometer (Waters, Milford, MA, USA) was used. The separation was performed on a reversed-phase Acquity UPLC BEH C18, 2.1 × 100 mm, 1.7 μm column (Waters, Milford, MA, USA), mobile phases consisting of (A) 1% formic acid in water and (B) methanol:acetonitrile:isopropanol (49:49:2). The MS detection was carried out in electrospray ionisation (ESI) in the positive ionization mode. Sotolon analysis was in multiple reaction monitoring (MRM) mode using the precursor ion at 129 m/z and the product ions at 55 and 83 m/z for quantification. For the HPLC-UV method, the separation was performed in an Agilent 1260 Series system fitted with a diode array detector (DAD) (Agilent, Palo Alto, CA, USA). Chromatographic separation through a reversed-phase Kinetex C18 100 × 3 mm × 2.6 μm column (Phenomenex, Torrance, CA, USA), mobile phases (A) water and (B) methanol. Sotolon was detected and quantified at 235 nm. The analysis time was 6.5 and 20 min for the UHPLC-MS and HPLC-UV techniques, respectively. Although relatively rapid analytical methods, the sample preparation and extraction procedures are still quite demanding: the use of NaCl is needed; the extraction step is done twice with dichloromethane for a total of 20 min; anhydrous Na_2SO_4 is used as a drying agent; and further purification of the dry material is applied with PVPP resin before filtration. Besides, these methodologies were only employed and validated for South African white wines and might not be feasible in complex FWs.

More recently, a microextraction by packed sorbent (MEPS) was proposed by Freitas et al. [134]. Sotolon analysis was then carried out by UHPLC in an Acquity H-Class UPLC system (Waters, Milford, MA, USA) combined with a 2996 photodiode array (PDA) detector. Identification was made by comparison of both retention time and spectral characteristics at 233 nm. Even though the method allowed a minimum sample and solvent volume usage, in addition to being successfully applied to both table white and MWs, without automatization it still is a quite laborious procedure. Although the focus of these currently trending environment-friendly techniques toward low sample and reagent consumption, which is greatly appreciated, these techniques involve a demanding work that may not be compatible in an oenology laboratory setting.

The positive contribution of sotolon to the overall quality of FWs such as older Madeiras [22], Port [66], *Fino* Sherry [75] and French FWs [76] is well known. Its positively influence on non-FWs such as *Vins Jaunes* and botrytised wines has also been reported [65,73]. The negative impact, however, and its role in the off-flavour character of prematurely aged dry white wines has been observed [115].

Thus, the determination of the sotolon content in these types of wines can be of great interest when monitoring its formation, whether its presence is desirable or not.

The aim of this part of the study was the proposal of a simple and rapid quantification method for sotolon in FWs—a balance between both sensitivity and readiness of the method was chased. Thus, validation parameters such as precision, repeatability, recovery, linearity, limit of detection (LOD) and limit of quantification (LOQ) were assessed. Further implementation of the method was done in multiple MWs.

3.2 Materials and methods

3.2.1 Chemicals

All chemicals used had a purity grade higher than 97%. Food-grade sotolon standard was purchased from Sigma-Aldrich Fine Chemicals (SAFC) (St. Louis, MA). Absolute ethanol was from Sigma-Aldrich (Steinheim, Germany). Ethyl acetate was from Fisher Scientific (Leicestershire, UK). Formic acid, L-(+)-tartaric acid, sodium hydroxide and UPLC grade methanol were obtained from Panreac (Barcelona, Spain). Ultra-pure water with a resistivity of >18 MΩ.cm (type 1) was obtained from a Millipore Simplicity® UV apparatus (Milford, MA).

3.2.2 Synthetic fortified wine and sotolon standard solutions

Synthetic fortified wine (SFW) was prepared in ultra-pure water and consisted of a solution containing 6 g/L of tartaric acid, 18% vol. of ethanol/water, and pH adjusted to 3.5 with a 1 M sodium hydroxide solution. Standard (400 mg/L) and working (200 mg/L) solutions of sotolon were rigorously prepared in ethanol and water, respectively. These solutions were used for the preparation of seven calibration points by spiking the SFW and FW, within the validation range 1–2000 μg/L.

3.2.3 Wine samples

Sotolon content was assessed in a set of 44 FWs with different ageing times, sweetness degrees, and ethanol content using the developed methodology. The wines' age ranged up to 115 years old and alcoholic strength was 18–20% ABV.

3.2.4 Sample preparation

The low quantities usually found in wines imposes a challenge for the sotolon quantification in these complex matrices. To overcome this problem, an LLE procedure is needed.

A QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) experimental procedure was first tested to extract sotolon. QuEChERS is a widely used extraction and clean-up technique characterized by high flexibility that can be adapted to different applications to various classes of compounds in several food matrices [135]. To do so, 10 mL of wine were placed into 50-mL PTFE

centrifuge tubes with 1 g of sodium citrate tribasic dehydrate, 500 mg sodium citrate dibasic sesquihydrate, 1 g of sodium chloride, and 4 g of anhydrous magnesium sulphate. Both acetonitrile and ethyl acetate were tested as extractant solvents. Four mL of each solvent was added into the centrifuge tubes, each tested separately. The tubes were vortexed for 5 min and then centrifuged for 5 min at 4400 rpm (Eppendorf 5702, NY, USA). After centrifugation and separation of the liquid phases, the organic layer was evaporated under a moderate nitrogen stream.

A second procedure was then tested by removing the salts and buffers from the QuEChERS sample preparation. The addition of the salts and buffers decreased the extraction yield of sotolon, and its removal further simplified the extraction procedure. Thus, the developed extraction protocol was nominated as a miniaturized LLE.

Full factorial design was then used to perform the optimization of the extraction procedure. The sample and extractant volume were chosen as variables. For the sample, 8, 10, and 15 mL volumes were tested; while for the extractant, 4, 5, and 8 mL volumes were chosen. The combinations of two experimental variables at these three volume levels were randomly tested using an FW. Data analysis was performed with Matlab software (version R2016b) to estimate the best combination of sample/extractant volumes.

3.2.5 Apparatus and chromatographic conditions

The HPLC separation was performed in an LC-MS/MS system from Shimadzu (Kyoto, Japan). The system is composed by a Nexera X2 UHPLC system with binary LC-30AD pumps, a DGU-20 A5 degassing unit, a CTO-20A column oven, a SIL-30AC autosampler, and an LCMS-8040 triple quadrupole mass spectrometer, equipped with an electron spray ionization (ESI) module. Purified nitrogen (Genius 1050; Peak Scientific, Inchinnan, Scotland, UK) was used as the drying gas.

The column used was a Kinetex C18, 150 × 2.1 mm, 2.6 μm particle size, 100 Å pore size, from Phenomenex (Torrance, CA, USA). The chromatographic separation of the sample extracts was carried out in reversed-phase with a linear gradient with solutions (A) methanol and (B) acidified water (0.1% formic acid) and the flow rate was set to 0.4 mL/min (Table 3.1). The injection volume was 5 μL, with each sample being injected twice, while standard extracts were analysed three times. All eluents were filtered through hydrophilic polypropylene 0.2 μm pore size membrane filters (Pall Corporation, Ann Arbor, MI, USA), while the sample and wine extracts were filtered with Chromafil PTFE 0.2 μm pore size syringe filters (Macherey-Nagel, Düren, Germany) before injection and analysis.

The MS detection was carried out using ESI in the positive ionization mode, and the optimized conditions were as follows: the desolvation line temperature was maintained at 250 °C and the block heater at 400 °C, while the nebulizing gas flow was set to 2.5 L/min and the drying gas flow to 17.5 L/min. Sotolon was analysed in the multiple reaction-monitoring (MRM) mode, using the following ion transitions: 129.1 $m/z \longrightarrow$ 55.1 m/z (for quantification) and 129.1 $m/z \longrightarrow$ 83.0 m/z (for identification). The optimal collision energy (−18 eV) was optimized by the direct injection of a

standard solution of sotolon (10 mg/L) performing various automatically programmed tests by the Labsolutions 5.7 software from Shimadzu (Kyoto, Japan). The data acquisition and peak integration processing were both performed with Labsolutions 5.7 software.

Table 3.1 Linear gradient elution used for the chromatographic separation.
Eluent A is methanol, and B is acidified water (0.1% formic acid).

time (min.)	flow (mL/min.)	eluent A (%)	eluent B (%)
0.0	0.4	5	95
4.0	0.4	5	95
6.0	0.4	30	70
7.0	0.4	100	0
10.0	0.4	5	95
15.0	0.4	5	95

3.2.6 Method validation

3.2.6.1 Selectivity

Selectivity was firstly appraised by the analysis of FW to ensure the chromatograms were free of interferences originating from the wine matrix. The selectivity of the method was further confirmed through the additional analysis of 44 FWs with different ageing times, sweetness degrees, and alcoholic strength.

3.2.6.2 Matrix effect

The matrix effect was assessed through the slope comparison method [136,137], using equation 3.1. Curves were obtained by plotting the sotolon peak areas to the corresponding concentrations, between 25 and 200 μg/L.

$$\%\ \mathrm{ME} = \left[\frac{(\mathrm{sFW} - \mathrm{sSFW})}{\mathrm{sSFW}}\right] \times 100 \qquad (3.1)$$

sFW, slope of the fortified wine curve; *sSFW, slope of the synthetic wine curve.*

3.2.6.3 Linearity

The external standard calibration method was adopted. A seven-point concentration scale was prepared with sotolon standard at 1, 10, 25, 50, 125, 1000, and 2000 μg/L in SFW. Each point was extracted and injected in triplicate, and the calibration curve was plotted.

3.2.6.4 Sensitivity

Sensitivity was evaluated by the determination of the limit of detection (LOD) and limit of quantification (LOQ). These parameters were estimated based on the Signal-to-Noise (S/N) approach. Thus, S/N = 3 and S/N = 10 were considered for LOD and LOQ, respectively.

3.2.6.5 Precision

Intra-day and inter-day tests with two standard solutions and one FW were used to assess the repeatability and reproducibility, respectively. These two parameters were expressed as relative standard deviation (%RSD). Intra-day %RSD was evaluated through the response of 10 successive analysis, while 5 analyses of the same samples were tested in three different days to assess the inter-day %RSD.

3.2.6.6 Accuracy

The accuracy was assessed through recovery tests. An FW was spiked with known amounts of sotolon at two representative concentration levels, within the calibration range (250 and 1000 μg/L). Recovery was calculated by comparing the mean values of three replicates with the theoretical concentrations.

3.3 Results and discussion

3.3.1 Analytical method development

To obtain reliable analytical results, a robust methodology should be applied. Sample preparation procedures can often be quite laborious and time-consuming, especially when the analytes are found at such low concentrations, as is usually the case for the occurrence of sotolon in complex matrices such as FWs. Thus, a simple and fast miniaturized LLE methodology was employed. Then, LC-MS/MS in MRM mode was selected for the analysis in FWs. LC-MS/MS is a technique with high selectivity and high sensitivity, thus being a method of choice [138]. LC, and in particular LC-MS has previously been used to quantify sotolon in wines [127]. Although this previously proposed method showed a good performance, and efforts were made to obtain a less extensive sample preparation and clean-up when in comparison to previous methodologies, the extraction procedure was still quite demanding.

3.3.1.1 Optimized extraction procedure

As aforementioned, the extraction of sotolon from FW was initially based on a QuEChERS experimental procedure. Since the addition of salts and buffers decreased the extraction yield, these were therefore removed from the extraction procedure. This further simplified the extraction procedure. Our findings evidenced ethyl acetate as revealing the best performance, thus, it was chosen as the optimal solvent for the extraction of sotolon in FWs. When in comparison with acetonitrile, interference-free chromatograms with less background noise were obtained. Full factorial design was used to perform the optimization of the extraction procedure [139], thus, eight millilitres of ethyl acetate were added to 15 mL of wine in 50-mL PTFE centrifuge tubes. This mixture was vortexed for five minutes and then centrifuged for 10 min at 4400 rpm. After the separation of both phases, the upper organic phase was collected and evaporated under a moderate nitrogen flow. The dried residue was then dissolved in 0.1% formic acid up to a final volume of 1 mL and filtered through Chromafil Xtra PTFE 0.20 μm syringe filters (Macherey-Nagel, Düren, Germany) into 2-mL HPLC

vials. Each solution was extracted in duplicate and 5 μL of the extract was injected into the LC-MS/MS system for further analysis.

3.3.1.2 Method's performance evaluation

The method's selectivity, matrix effect, linearity, sensitivity, precision, and accuracy were appraised. Table 3.2 shows the performance evaluation results obtained for the proposed extraction method. The miniaturized LLE method showed good selectivity, as the chromatograms of both SFW and FWs were free of interferences at the retention time of sotolon (Figure 3.1). Sensitivity was evaluated by the determination of the lowest concentration of sotolon that could be measured. The determined limit of detection (LOD) and limit of quantification (LOQ) were 0.011 and 0.037 μg/L, respectively. Both values are well below the OTs found in wines and comparable to some other recent reported methods [125–128,134]. For the matrix effect evaluation, sotolon peak area was plotted against the corresponding concentration, and no matrix effect was encountered (ME = 13%). The linearity of the method was confirmed by the good correlation coefficient (R^2 = 0.9999) obtained. Both repeatability and reproducibility never exceeded 10% of RSD, showing good precision. Finally, for the accuracy of the method, recovery experiments showed results higher than 92%, demonstrating that the method is accurate.

Table 3.2 Performance results obtained for the proposed method.

parameter	result
linear regression	$A_{sotolon}$ = 46437 [sotolon] + 43722
linear concentration range (μg/L)	1.0–2000
R^2	0.9999
LOD (μg/L)	0.011
LOQ (μg/L)	0.037
repeatability (% RSD)	3.4–6.4
reproducibility (% RSD)	5.4–10.0
recovery (%)	
FW + sotolon (250 μg/L)	92
FW + sotolon (1000 μg/L)	98

$A_{sotolon}$, sotolon peak area; [sotolon], sotolon concentration (μg/L); LOD, limit of detection; LOQ, limit of quantification; FW, fortified wine.

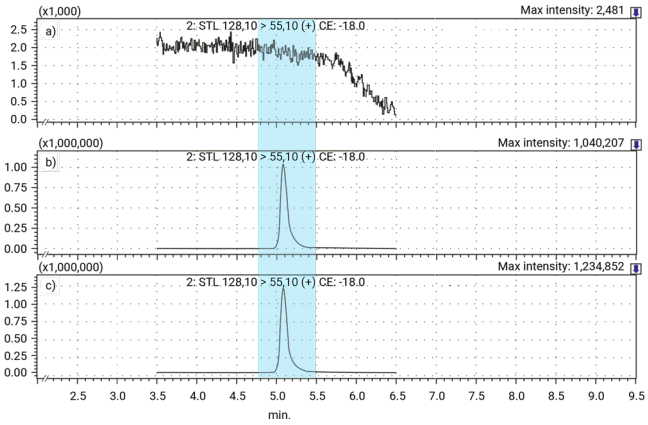


Figure 3.1 Obtained chromatograms using the proposed extraction procedure.
Chromatograms from a (a) synthetic wine solution, (b) standard sotolon calibration solution (250 µg/L), and (c) fortified wine.

3.3.2 Sotolon quantification in Madeira wines

The proposed LC-MS/MS methodology was then applied to the determination and quantification of sotolon in 44 MWs. The wines were of different ages and varied in sweetness levels (from dry to sweet) covering usual ageing periods and styles of this kind of wines. Sotolon was detected and quantified in all wine samples at concentrations ranging between 6.3 ± 0.4 and 810 ± 20 µg/L (Table 3.3) revealing that the method covers the concentration range usually observed for the compound in this kind of FWs. The quantification data of the 44 wines is shown in Table A-1 of Appendix A, adapted from the original publication.

These results confirm the applicability of the method in FWs. Also, the developed method proofed itself useful in the quantification of sotolon in several MSys mimicking FW submitted to accelerated ageing, as will be further described in Chapter 5. Furthermore, the method was also implemented in the quantification of sotolon in various commercially available MW Blends. These were used for the preparation of the sensory analysis tests, as well as the determination of the respective OAVs for sotolon in this kind of beverage, as will be discussed in Chapter 4.

Table 3.3 Application of the method to the quantification of sotolon in a range of fortified wines with different ages and styles.
Sotolon values expressed as mean value ± standard deviation.

no. of samples	wine style	age range (years)	sotolon (µg/L)
5	dry	und–38	113–427
6	medium-dry	und–40	142.0–494
10	medium-sweet	und–115	137–810
23	sweet	und–21	6.3–697

und, undisclosed age.

Chapter 4

SENSORIAL IMPACT OF SOTOLON

DETERMINATION OF THE ODOUR THRESHOLD AND ODOUR RELEVANCE OF SOTOLON IN COMMERCIALLY AVAILABLE MADEIRA WINE BLENDS

4.1 Introduction

As it was discussed in Chapter 2, sotolon is a powerful odour compound present in many foodstuffs. It contributes to the characteristic sensorial impression of burnt nuances in cane sugar [46] and aged *sake* [36], to the curry odour of fenugreek seeds [42] and is particularly significant to the aroma of wines. Although considered to be a key off-flavour compound in table dry white wines [48,118], responsible for "premox", in the case of the well-known FWs it is seen as having a positive contribution to the wine aroma [1]. In this type of wines, sotolon can reach relatively high concentrations (Table 2.2) when in comparison to wines with theoretical perceivable "premox" spoilage [115,124].

Wine is a complex mixture, more so is an FW such as Madeira, which is purposely exposed to oxidizing conditions and unusual ageing methods resulting in the formation of many odorant compounds. Pondering that such compound may be the key to the overall wine aroma makes it interesting to study its flavour properties. This part of this thesis is focused on the study of the sensorial impact of sotolon in FWs, wherein MW is the case-study. Hence, the present study seeks to determine the orthonasal threshold and relative odour significance of sotolon in commercially available MW Blends.

4.1.1 Sensory analysis

Sensory analysis—or sensory evaluation—is a scientific field which studies and considers the five human senses of sight, smell, taste, touch, and hearing and applies it to the study of consumer products. It is an interdisciplinary science which encompasses principles and methods from fields such as chemistry, statistics, psychology, physiology, and others [140]. As it is summarised by Stone et al. [141], it is a field that comprises a set of techniques to evoke, measure, analyse and interpret human responses to certain stimuli perceived through these five senses. Sensory evaluation has been in continuous growth since it emerged as a unique discipline, and despite the new developments in methodologies, certainly, it will always rely on human subjects as the instruments of analysis.

There are many ways of evaluating a response to a stimulus, and the method of approach is dependent on the purpose of the test. Sensorial test methods can be categorized in many ways, usually classified according to their main purpose. Nevertheless, the classical proposed classification is to divide the test methods into three classes: discrimination, descriptive, and affective testing. However, the classification can be shortened to two main types—analytical and affective—thus considering discrimination and descriptive testing as two types of analytical methods (Table 4.1) [141].

Table 4.1 Classification of test methodologies in sensory evaluation.
Adapted from Lawless and Heymann [142].

class	question of interest	type	test types
discrimination	is Product A different from Product B?	analytical	difference tests: triangle test; duo-trio test.
descriptive	how does Product A and B differ from each other?	analytical	descriptive analysis: paired-comparison tests; *n*-alternative forced choice tests (*n*-AFC).
affective	how well are Product A and B liked?	affective (hedonic)	hedonic tests: paired-comparison tests.

Lawless and Heymann [142], and Stone et al. [141] described in great detail these types of methodologies in their reviews. In summary, discrimination tests have the purpose of verifying if a difference between products exists. This difference can be evaluated through a variety of methods known as difference tests. Examples of the most employed difference tests are the triangle, the duo-trio, and the paired comparison; Table 4.2 briefly explains each one. Although more difficult to implement, descriptive tests provide much more (qualitative and/or quantitative) information about a product's perceived sensory characteristics. The Quantitative Descriptive Analysis® (or QDA®) is one example of a descriptive analysis method. Briefly, in QDA® analysis, participants are subjected to a wide range of products related with the product category of interest. The characteristics and descriptions of the products are determined as a group. Duplicates and redundant terms are then excluded, and after the necessary refinements, a specific vocabulary is obtained. Then, the assessment of the product of interest is made using the standard attribute descriptors. The intensities (from "weak" to "strong") of each agreed characteristic are individually assessed in a specific order, and the final results are often reported as a spider web diagram. The classical Flavour Profile and the Texture Profile are other examples that rely on these same principles of identifying and quantifying product attributes. QDA®, however, was introduced to overcome some shortcomings of these older methodologies and is frequently the method of choice. Finally, the affective (or hedonic) tests involve consumer preference and acceptance to attempt to quantify the degree of liking or disliking of a product. The 9-point hedonic scale is often used as a capable test of evaluating the magnitude of product acceptance. It is important to keep in mind that some newer methods of analysis may not fit within this categorization [141].

Table 4.2 Examples of the most employed difference tests in sensory analysis.
Adapted from Cowey and Travis [143].

difference test	presentation	test mechanic
triangle	three coded samples are presented. One sample (A) is different from the other two equal products (B).	to evaluate each sample, choosing which one is most different from the other two. Possible serving orders: AAB, ABA, BAA, BBA, BAB, ABB.
duo-trio	like the triangle test, three samples are presented but the first sample is a reference (R) and the other two (A, B) are coded.	to evaluate each sample, the first one as a reference, indicating which is the most similar to the control sample. Possible serving orders: R AB, R BA.
paired comparison	two coded samples are presented.	to evaluate each sample, choosing the product which has more of a designated characteristic (sweetness, for example). Possible serving orders: AB, BA.

Regarding the instrument of analysis—the panellists—we can also sub-divide them in three categories: non-trained, trained, and consumer panellist. A panel consisting of non-trained panellists is formed by participants that are usually new to sensory analysis and will generally have less sensitivity to the stimuli in question. This type of panel is used in discriminative evaluation, although in some cases the panel should be trained. This panel can then undergo periodical training sessions to get familiarized with the sensory methodologies and to develop a good ability to recall, recognize and describe a range of sensory stimuli related with the products under study, thus being called a trained panel. A trained panel is mainly used in descriptive analysis or anytime product attribute evaluation is pretended. Lastly, the term consumer panel is used to describe a group of panellists that are representative of the product's consumer population [140,142]. Aside from the panel expertise, sensory measurements can be subjected to variability and bias due to physiological (adaptation to the stimulus due to continued exposure, for example) and psychological factors (expectation error; error of habituation; order of presentation of the samples; or lack of motivation, for example). [140]. Additionally, age and gender may influence sensory sensitivity and responses [144,145].

In summary, we can consider sensory analysis as a tool that enables the understanding of the properties of foods, providing helpful information to the food product developers [142]. It is particularly useful and essential in the winemaking industry as wine quality is often a result of a good control of the winemaking and ageing processes through both analytical and sensorial evaluations. Nevertheless, sensory evaluation is not only limited to the study of food matrices but also encompasses other materials [141].

4.1.1.1 The sense of smell

From the five senses, the sense of smell is probably the most complex. It is particularly important in food product development as it allows the assessment of the attribute of flavour (a combination of odour, taste, and overall mouthfeel) [146].

The human nose can distinguish thousands of different odours [147]. This is due to the relatively high number of olfactory receptors and mode of action. Volatile molecules can enter the nasal cavity region directly through the nostrils (orthonasal) or from the back of the oral cavity through the

nasopharynx (retronasal) after food mastication or swallowing [148]. Although both ways bring odour molecules to the olfactory epithelium, the process of odour perception is thought to be different [149–151]. As is described by Trimmer and Mainland [152], once the volatile compounds reach the main olfactory epithelium, dissolved in the nasal mucus layer, they will interact and be detected by the receptors on the cilia of the olfactory sensory neurons. These are bipolar neurons and are the only ones that can directly contact both the external environment and the central nervous system [152]. This interaction is dependent on the nature and properties of the compound. The neuron's axon extends and merges with other axons forming the olfactory fila which converge to the olfactory bulb. Through neurological response, signals reach the olfactory cortex, wherein all the information is processed, and the odorant is perceived. Odours are thought to be decoded across receptors through a combination mechanism, although the rules influencing this combination are still not known. Besides, receptors are also susceptible to the odorant concentration. The authors state that a given receptor has different response profiles to different odorant concentrations but the action mechanisms for the encoding of both odour identity and concentration is also unclear.

4.1.1.2 The concept of threshold and odour threshold determinations

A sensory threshold is defined as the specific amount of a stimulus required to produce an effect [153]. By definition, absolute thresholds are the lowest stimulus level or intensity that can be detected [154,155]. However, there can be defined four types of thresholds: detection (absolute), recognition, difference, and terminal threshold. These are shortly described in Table 4.3. As is explained by Bi and Ennis [155], absolute thresholds should mark a sharp point between sensation and no sensation. In theory, the concept of threshold assumes that this transition point is independent of the testing conditions. However, given that sensory responses are affected by many factors, the threshold is treated as a statistical concept. For a group, it is defined as the concentration for which the probability of detection is 0.5 under the conditions of the test [156].

Apart from the four main types of thresholds, a fifth new type was recently proposed. Prescott et al. [157] studied the effect of 2,4,6-trichloroanisole (TCA), responsible for the cork taint defect, on the preference responses of wine consumers. The authors combined the paired preference test (a type of test based on the paired comparison test [158]) with the method of constant stimuli threshold procedure. The objective was to determine the point at which the concentration level of TCA in Chardonnay wine would become unpleasant to the panel and was termed as the consumer rejection threshold. This rejection threshold concept has then been applied to 1,8-cineole in red wine [159], sweetness acceptance in Semillon [160], ethyl phenylacetate in red wine [161], and 1,1,6-trimethyl-1,2-dihydronaphthalene in Riesling wines [162].

OTs are determined by a series of trials. First, the individual thresholds are assessed, and then a group threshold is calculated given the individual responses [153]. The ascending forced-choice method of limits is one of the procedures used for determining OTs. It is based on the classical psychophysical method of limits, where the stimulus is presented in an ascending order of intensity

until the substance is detected [142]. This is the method that is described in the widely used ASTM E679 standard procedure [163]. The ASTM E679 prescribes a rapid and reliable methodology for the determination of threshold values from only 50 to 100 three alternative forced-choice (3-AFC) presentations. The 3-AFC test is a type of discrimination test like the triangular test. It consists of the panellist choosing the different sample from a set of three (two "blanks" and one "target") with the concentration of the target sample constantly increasing. The test implies a forced-choice, so the panellists must make a selection even if it is only a guess. A best estimate threshold (BET) is then obtained based on the response pattern of the panel rather than the conventional group threshold (the concentration level for which the probability of detection is 0.5 by 50% of the panel) [156,164]. Although straightforward, attention is necessary when planning the test to minimize some of its flaws [165]. The procedure's threshold determination relies on the correct and incorrect response pattern of the panel. When the responses change from incorrect to consistently correct, an individual threshold is obtained. Because of this, it may be important to define stopping rules and understand how many correct responses mean being "consistently correct", thus avoiding bias by correct guessing. Preliminary testing is thus often employed to set up and estimate the proper concentration range, the number of trials, dilution scale-steps and panel selection, for example [142].

As with every other sensory evaluation, these tests should be carried out in distract- and odour-free rooms. Panellists assess the product individually, preferably in individual booths, and are told to abstain from smoking, eating, and/or using fragranced cosmetics prior to the tests. Variations among panellists represent the main error source when estimating group thresholds [155]. Some degree of variability is even expected within a single panellist [142]. Thus, it is important to set up the sensory test accordingly.

Table 4.3 Types of sensory thresholds.
Adapted from Lawless and Heymann [142].

threshold type	description
detection (or absolute)	the lowest amount of a substance that can be perceived and differentiated from the background.
recognition	the lowest amount of a substance that can be perceived and correctly recognized.
difference[1]	the smallest difference in the amount of a substance that can be perceived.
terminal	the highest amount of a substance above which no increase in intensity is perceived.
consumer rejection threshold	the amount at which a consumer preference occurs for a sample not containing the substance

[1]Difference thresholds are also related and sometimes referred to as "just noticeable difference" (or JND) which is associated with the Weber and Fechner's laws.

One important point to keep in mind is that by definition detection (absolute) thresholds require a 0-level background media (blank). This means that typically detection threshold determinations should be performed in pure water or pure air [142]. Thus, performing a detection threshold in a food matrix seems theoretically impossible and what ends up being determined is a difference threshold instead. However, the distinction between detection and difference threshold is

not always clear. Thresholds reported in the literature are often inconsistent, especially in the case of food matrices and media that includes endogenous levels of the stimulus in study [166,167]. Because of the 0-level requirement, and in the case of a background media situation such as beer or wine, some authors consider the difference threshold and detection threshold to be the same entity [168]. Others consider that a detection threshold is obtained even if the reference sample contains the stimulus as long as it is at sub-threshold level, but the responses may be affected both by absolute and difference thresholds [169].

Although these brief notions and information on sensory analysis and OT determinations are enough for the purpose of this study, there is much more interesting information available in the literature. A read of the review by Amerine et al. [170] is suggested. The more recent literature reviews by Meilgaard et al. [140], and Lawless and Heymann [142,171] on sensory evaluation and threshold concepts are suggested as well and were the basis of writing most of this introduction.

4.1.2 Aroma in foods

Food matrices contain a vast number of volatile compounds. A volatile screening may give a list of compounds with relatively high concentrations, but not all would necessarily contribute to the overall aroma profile of the product. Sensory analysis tackles this problem and measures the odour perception of the product's constituents. With the combination of both analytical and sensory testing, it is possible to obtain a good understanding of the aromatic characteristics of the food product [172].

Parker [172] summarises some of the methods typically employed in aroma research which are the key elements of the so-called sensomics approach in food flavour science. As described, aroma extract dilution analysis (AEDA) is one of the most frequently used techniques to assesses an aroma extract through GC-olfactometry (GC-O). By serial dilutions of the original extract, it is possible to find the compounds that potentially contribute to the aroma of the food product. The number of times an extract is diluted before a specific aroma is lost is defined as the relative flavour dilution (FD). To accurately quantify the most important compounds, quantification through stable isotope dilution analysis is often employed. OTs, as were described before, are determined for specific compounds of interest, and are used to OAVs. An OAV is defined as the ratio of the concentration of an aroma compound to its OT. OAVs > 1 indicate that the compound is present above its threshold value, likely contributing to the aroma of the food. To completely address the role of the individual compounds, recombinates are prepared by reconstructing the aroma of the food. Aroma models are prepared by mixing the pure aroma compounds in the respective proportions originally found in the product. Omission tests, which study the effect of the elimination of compounds from the aroma model, are then used to determine and decode the food flavour [173].

4.1.3 Sotolon's flavour characteristics

Sotolon is a well-known pungent compound with a very high odour strength. At room temperature, the pure compound appears as a viscous and pale-yellow liquid, possessing an extremely strong caramel-, maple-, burnt sugary-, and curry-like smell [28]. The familiar and interesting flavour characteristics of sotolon are a result of its low perceivable OT. It is reported to have an extremely low odour perception threshold (0.00001–0.001 ppb) when diffused in air [47,61]. Low OTs (≤ 20 μg/L) are also reported when sotolon is diluted in water, with most reports showing a ≤ 1 μg/L detection threshold. Table 4.4 presents a summary of some of the available sensory thresholds reported for sotolon in different liquid matrices, including some alcoholic beverages.

Table 4.4 Summary of the available sensory threshold determinations found in literature for sotolon in different matrices.
Reported values are for orthonasal threshold determinations unless otherwise specified. Threshold values in μg/L unless otherwise specified.

matrix	details	detection threshold	recognition threshold	difference threshold	reference
diffused in air	n/a	0.001[1]	n/a	n/a	[47]
diffused in air	n/a	0.00001–0.00002	n/a	n/a	[61]
water	n/a	0.5–1[2]	n/a	n/a	[35]
water	n/a	0.3	n/a	n/a	[174]
water	n/a	0.49	1.1	n/a	[175]
water	n/a	20[3]	n/a	n/a	[68]
water	n/a	0.01[1]	n/a	n/a	[46]
water	n/a	0.3[3]	n/a	n/a	[176]
water	n/a	1.7[3]	n/a	n/a	[177]
water	n/a	1.7[3]	n/a	n/a	[178]
water	n/a	1.7[3]	n/a	n/a	[71]
water	n/a	0.08[2,3].	n/a	n/a	[179]
oil	n/a	0.2[3]	n/a	n/a	[174]
acetic acid solution	7% acetic acid/water (*w*/*v*).	16	n/a	n/a	[180,181]
hydroalcoholic solution	12% ethanol/water (*v*/*v*); 5 g/L tartaric acid; pH 3.5.	6.3	n/a	n/a	[182]
hydroalcoholic solution	40% ethanol/water (*v*/*v*).	24.2	n/a	n/a	[183]
hydroalcoholic solution	0.04% ethanol/water (*v*/*v*); 7.2 g/L glucose; 2.1 g/L fructose; 0.6 g/L sucrose; 26.9 g/L maltose; 3.6 g/L maltotriose; pH 4.50.	8.68–28.3; 1.24–5.80[2]	n/a	n/a	[184]

(*continued*)					
hydroalcoholic solution	10% ethanol/water (*v*/*v*)	5	n/a	n/a	[185]
hydroalcoholic solution	n/a	2	n/a	n/a	[119]
hydroalcoholic solution	10% ethanol/water (*v*/*v*); 5 g/L tartaric acid; pH 3.2.	9	n/a	n/a	[6]
hydroalcoholic solution	12% ethanol/water (*v*/*v*).	2.5[1]	n/a	n/a	[73]
hydroalcoholic solution	11% ethanol/water (*v*/*v*); 4 g tartaric acid; pH 3.5	0.7	n/a	n/a	[186]
hydroalcoholic solution	18% ethanol/water (*v*/*v*); 100 g/L sugar; pH 3.5	10	n/a	n/a	[78]
beer	Belgian blond beer: 6.3% ABV; pH 4.2.	n/a	n/a	8	[187]
wine	white wine	n/a	n/a	15	[65]
wine	white wine	n/a	n/a	8	[119]
wine	Port wine	n/a	n/a	19	[66]

ABV, alcohol by volume; *n/a, not applicable or not available*; [1]value in ppb; [2]retronasal threshold; [3]value in µg/kg.

Due to its intense sugary-sweet odour, sotolon is of great importance for the flavour and fragrance industry [26]. It is widely used as a seasoning in food products like curry, pickles, chutneys, vanilla extracts, and artificial maple syrup, as well as in tobacco flavourings [51,129]. Interestingly, a recently published research demonstrates the potential use of sotolon, along with benzaldehyde and vanillin, in the creation and acceptability of a tawny Port-like fragrance [188]. Besides its impactful contribution to foodstuffs, sotolon's characteristic sweet aroma is also reported as an olfactory clinical index for the maple syrup urine disease (MSUD) [52].

Sotolon is also a chiral compound (see Figure 2.6) and both enantiomers seem to occur naturally at different ratios depending on the matrix [48,49]. Considering the possibility that each enantiomer could have distinct odour properties, Okada et al. [50] were the first to synthesize both sotolon's enantiomers from the enantiomers of tartaric acid. The authors reported that both enantiomers exhibited the same sugary flavour and insect attractancy as of the racemic compound mixture. However, more recently, Pons et al. [48] studied the distribution and organoleptic impact of sotolon enantiomers in French dry white wines from the wine regions of Bordeaux (Graves and Entre-Deux-Mers). The authors found the perception threshold of the (*S*)-sotolon to be 100-fold lower than that of the (*R*) enantiomer in both wine and model wine solution (5 g/L L-tartaric acid; 12% vol. of ethanol; pH 3.5) (Table 4.5). With a perception threshold of 0.8 and 5 µg/L in model and white wine matrices, respectively, the study suggests (*S*)-sotolon to be the key-contributor to the characteristic prematurely aged aroma in dry white wines. More importantly, the study also demonstrated that the

impact of sotolon in dry white wines is dependent on the distribution of its enantiomeric forms. Both enantiomers' aroma nuances are indeed quite similar. The (*S*)-sotolon has a curry, walnut and strong caramel-like odour while the (*R*) form has a walnut, rancid odour [129].

Table 4.5 Odour perception thresholds and descriptors for sotolon enantiomers.
Data from Pons et al. [48].

enantiomeric form	threshold (µg/L)		descriptors
	wine model solution[1]	wine[2]	
(*R*)-sotolon	89	121	walnut, rancid
(*S*)-sotolon	0.8	5	curry, walnut
racemic mixture	2[4]	8[4]	curry, walnut

[1]12% ethanol/water (*v*/*v*); 5 g/L L-(+)-tartaric acid, pH 3.5; [2]French dry white wines from Bordeaux region; [3]assessment in wine model solution; [4]value from Lavigne and Dubourdieu [119].

4.1.4 Odour impact on Madeira wine

Sotolon can be found at relatively high concentrations in MW. This fact combined with the reported low OTs makes sotolon a potential key flavour compound of this beverage. The first findings started with the research developed by Câmara et al. [5] where the authors, for the first time, studied the levels of sotolon and its relationship to sugar content. They found a strong correlation between sotolon content, sugars, and ageing time. Flavour profile studies were later employed by Campo et al. [6] which found sotolon to have a high impact in the odour of 10-year-old MW Blends made with the white grape varieties Sercial, Verdelho, Bual, and Malvasia. The authors found sotolon's OAVs ranging between 1.6–2.1, although the OTs used to calculate these were obtained in 10% vol. ethanol/water mixture which may not properly represent the MW matrix. Later, Oliveira e Silva et al. [22] identified sotolon to be responsible for the "nutty"/"dried fruits" aroma descriptor common to both Sercial and Malvasia reference wines. This was the highest impact flavour descriptor obtained for both wines through GC-O and AEDA (FD of 256). The authors also demonstrated the importance of sotolon in the typicity of MWs. Typicity scores were positively correlated with sotolon, sugar, and baking time, while negatively correlated with fermentation length.

While the OT was estimated for Port wine, no current studies report threshold values for the case of Madeira with information on sotolon's flavour role in this wine being particularly scarce. Also, considering that some advances in the MW production have naturally been adopted along the years, it becomes interesting to determine the relevance of sotolon and its reflection in the aroma of currently produced MW Blends.

4.2 Material and methods

4.2.1 Chemicals

All chemicals had a purity grade higher than 97%. Food-grade sotolon standard and absolute ethanol were purchased from Sigma-Aldrich Fine Chemicals (SAFC, Sigma-Aldrich) (St. Louis, MA) and

Sigma-Aldrich (Steinheim, Germany), respectively. L-(+)-Tartaric acid and sodium hydroxide were from Panreac (Barcelona, Spain). Ultra-pure water with a resistivity of >18 MΩ.cm (type 1) was obtained from a Millipore Simplicity® UV apparatus (Milford, MA).

4.2.2 Overall sensory procedure

The first step in the determination of the sotolon's odour impact followed a sensory analysis for the determination of the OT of this compound in MW Blends. This sensory analysis was divided into two main parts. Additionally, some initial trials were performed to better understand how the testing methodology could be employed and to estimate the proper concentration range to be used.

The selection of the appropriate matrix solution for OT determinations is sometimes a difficult task. In this case, sotolon is a characteristic compound that is naturally found in MWs. So, it made sense that one should choose a wine that, ideally, has the lowest amount of sotolon. Silva Ferreira et al. [66] encountered similar challenges when determining the OT of sotolon in Port. As a compromise solution, the authors selected a 3-year-old wine to perform the threshold evaluation. It is known that difference thresholds values tend to increase in proportion to the concentration of the target compound [142]. Thus, here 3-year-old MW Blends were chosen for the analysis, as these seemed to be a good compromise between the endogenous sotolon content and the most accurate OT to answer the experimental question. Although not comparable, this threshold value would be the most approximate to the hypothetical absolute threshold.

The first part consisted of a preliminary test carried out with a non-trained panel. Here a preliminary OT was determined for sweet-type 3-year-old MW. The odour rejection threshold (ORT) was also attempted using a sweet-type 5-year-old wine. Although no training was involved, this first part served as a way to introduce the panellists to the kinetics of the sensory test and to familiarize them with the sotolon odorant stimulus. Then, the second part of the study was employed with six panellists selected from both the previous study and additional trials. The aim was to re-evaluate the OT of sotolon in MW by re-submitting the selected panel to repeated analysis with four other wines. The found OTs permitted the evaluation of the OAVs in different MW Blends.

All sensory evaluation tests took part in a temperature-controlled room free of odours, noise, and other major distractions, at the University of Madeira. Wines were poured in ISO tasting glasses and these were coded with three-digit random numbers, covered with plastic petri-dishes, and randomly arranged for each evaluation test. Each tasting session was prepared one hour prior to the sensory evaluations. The sensory studies were composed of repetitive sessions using the same panel participants whenever possible. Panellists were also asked not to eat, drink or smoke during the 30 minutes prior to the testing sessions.

4.2.3 Preliminary sensory study

4.2.3.1 Panellists

For the first procedure, panellists were recruited from the University of Madeira via online form. To characterize the panel, individuals who chose to participate in the sensory tests were also asked to fill a short screening questionnaire indicating their familiarization with MW and general sensory analysis knowledge. A total of 22 panellists, 13 females and 9 males, aged 21–62 years-old accepted to participate in the study. From the 16 responses of the screening questionnaire, 69% were familiarized with discrimination tests, had previously participated in similar tests, although the vast majority (69%) rarely consume FW (Figure 4.1).

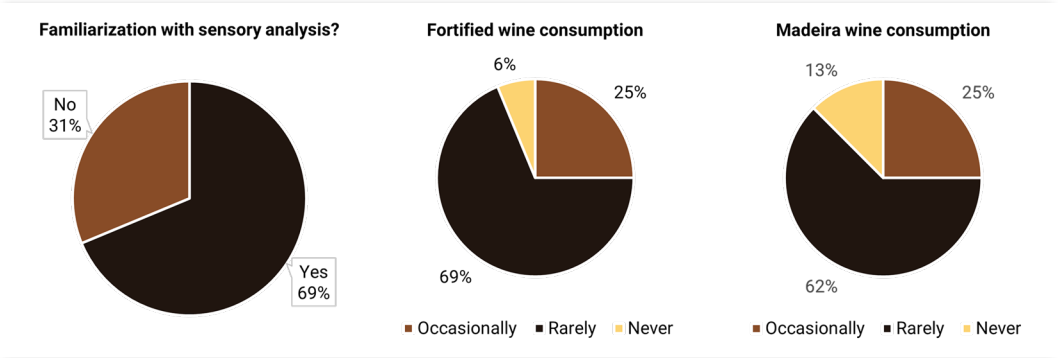


Figure 4.1 Results from the screening questionnaire.

4.2.3.2 Wine samples

Two commercially available wines were used. A 3-year-old sweet MW with relatively low sotolon content (6.3 ± 0.4 µg/L) was used for the OT evaluation. A sweet 5-year-old wine (174 ± 6 µg/L of sotolon) was used for the ORT assessment. The sotolon content of the non-spiked wines was determined by the LC-MS/MS method described in Chapter 3.

4.2.3.3 Sample preparation

A 100 mg/L working solution of sotolon was prepared in SFW. The SFW was prepared as described in Chapter 3 (18% vol. of ethanol; 6 g/L of tartaric acid; and pH adjusted to 3.5 with 1 M sodium hydroxide solution). This solution was used to spike the wine samples with known concentrations of the target compound.

For the OT determination, the sample preparation involved spiking the 3-year-old wine with increasing concentration of sotolon: 4, 14, 34, 74, 154 and 314 µg/L. This 6-step concentration series was established according to the initial trials and based on the previously reported OT for sotolon in Port wine [66]. The same procedure was employed for the concentration series of the solutions used to determine the ORT. The 5-year-old wine was spiked with increasingly 2-fold concentrations ranging 253–3464 µg/L of spiked sotolon.

4.2.3.4 Best estimate threshold determination

The ascending forced-choice method of limits described before by the ASTM E679 standard practice was followed [163]. Each concentration scale-step was represented by a 3-AFC presentation which comprised a triad. One glass was filled with 20 mL of spiked wine (target sample) and two others were filled with 20 mL of the base wine (blanks) at each concentration scale-step. From the set of 22 volunteers, only 19 (11 females and 8 males), aged 21–62 years old, performed the sensory evaluation. The panel was instructed to smell each sample and choose the one that was different from the other two, starting from the less concentrated scale-step. The responses were registered in a paper ballot where the corresponding three-digit code was circled (Figure B-1). As the method employed a forced choice, it was required that each panellist would make a selection even if it were to be a guess. Repetitive testing was performed to obtain enough 3-AFC presentations. The sensory evaluation took place in four sessions: two sessions per day (one in the middle of the morning and the other at the middle of the afternoon) for two consecutive days. Most of the panellists (90%) attended at least two sessions and a total of 318 3-AFC presentations were acquired.

Data analysis was also employed as described by the ASTM E679 procedure. Each individual response was collected, and the panel judgements were "translated" to correct or incorrect guesses. Each time the panellist incorrectly discriminated a sample, an incorrect response was reported at that specific concentration scale-step and was marked with a "0". Otherwise, when a sample was correctly discriminated, a correct response was reported with a "+" sign. The orthonasal BET for each trial was calculated by taking the geometric mean at these concentration scale-points where the panellist's response changed from incorrect to consistently correct. This meant taking the geometric mean of the last miss (0) and the next correct concentration (+). For the purpose of this test, an assumption is made that if the concentration range were extended and a lower concentration scale-step were to be tested the panel would miss it; contrariwise, if a higher concentration scale-step would be tested the panel would correctly discriminate it. Thus, in case of an incorrect response at the highest concentration available, the individual BET was obtained by taking the geometric mean of that concentration and the next higher hypothetical scale-step. If in the case of a complete run of correct judgements, the BET was calculated by taking the geometric mean between the lowest concentration and the next lower hypothetical scale-step. The arithmetic mean of each individual BET was then calculated, and the group BET was obtained.

4.2.3.5 Odour rejection threshold determination

The ORT determination followed the procedure of Prescott et al. [157]. This procedure is based on the paired preference test procedure described by the ISO 5495 standard [158]. Only 19 volunteers were able to participate in this study: 12 females and 7 males aged between 21–62 years old. The procedure was similar to that of the odour BET determination, but only two samples were presented per each of the five scale-steps used: one glass filled with 20 mL of the spiked wine (target sample) and

one with the base wine (blank). The panel was instructed to smell each sample and to choose the sample that was overall preferred, starting from the less concentrated scale-step. The responses were registered in a paper ballot where the preferred corresponding three-digit code was circled. A forced-choice was also required, and the panel had to make a selection even if there was no noticeable preference. Repetitive testing was performed to obtain a sufficient number of evaluations. The sensory evaluation took place in four sessions: two sessions per day (one in the middle of the morning and the other at the middle of the afternoon) for two consecutive days.

Data was collected and analysed, and the proportion of judges preferring the control sample was plotted against the sotolon concentration. The minimum responses necessary to establish a significative preference was determined accordingly [157].

4.2.4 Sensory evaluation with a selected panel

4.2.4.1 Panel selection

Following the preliminary test, the panel selection was based on individual performance and availability for further repeated testing. Individual performance was established by the percentage of correct responses (at least 50%) and individual BETs. From the initial pool, a total of 6 panellists were selected, 1 male and 5 females, aged between 30–46 years old.

4.2.4.2 Wine samples

Four MW Blends were used during the sensory tests. These were 3-year-old commercially available wines of two different styles (dry and sweet) from two local producers (Table 4.6). Some basic oenological parameters were determined by a TDI Bacchus 3 Multispec analyser (Barcelona, Spain). The calibrations for the determination of alcoholic strength, density, volatile acidity, titratable acidity, and pH were previously employed following the OIV reference methods OIV-MA-AS312-01A:R2016, OIV-MA-AS2-01A:R2012, OIV-MA-AS313-02:R2015, OIV-MA-AS313-01:R2015, and OIV-MA-AS313-15:R2011, respectively [189]. Residual sugar determination was calibrated based on the Lane-Eynon method [190]. Significant differences between these parameters were evaluated by the analysis of variance (one-way ANOVA with the Holm-Sidak method) using the Minitab, LLC Minitab® 17 statistical software (State College, PA).

Table 4.6 Three-year-old Madeira wine Blends selected for the sensory evaluation.
Oenological parameters obtained in triplicate with values expressed as mean concentration ± standard deviation. Different letters represent statistically significant differences ($p < 0.05$) across rows by Holm-Sidak test.

parameter	producer A		producer B	
	dry-styled	sweet-styled	dry-styled	sweet-styled
alcoholic strength (% ABV)	18.03 ± 0.01^{a}	18.53 ± 0.02^{b}	19.23 ± 0.03^{c}	19.28 ± 0.03^{c}
density (g/mL)	1.0033 ± 0.0001^{a}	1.0263 ± 0.0002^{b}	1.0049 ± 0.0002^{c}	1.0274 ± 0.0003^{d}
volatile acidity (g/L)	0.39 ± 0.01^{a}	0.40 ± 0.00^{a}	0.44 ± 0.03^{a,b}	0.49 ± 0.04^{b}
titratable acidity (g/L)	4.46 ± 0.06^{a}	4.92 ± 0.01^{b}	5.1 ± 0.1^{b}	4.95 ± 0.06^{b}
pH	3.52 ± 0.01^{a,b}	3.51 ± 0.01^{a}	3.51 ± 0.01^{a}	3.54 ± 0.02^{b}
residual sugars (g/L)	52.1 ± 0.8^{a}	112.9 ± 0.5^{b}	63 ± 1^{c}	120 ± 1^{d}

ABV, alcohol by volume.

A different set of 89 MW Blends (sampled in 2017) was used to determine the odour impact of sotolon. These wines were from four local producers and comprised the four sweetness styles of Madeira (dry, medium dry, medium sweet, and sweet) (Table 4.7). The sotolon content of these wines was determined by the LC-MS/MS method described in Chapter 3 which allowed the calculation of the OAVs.

Table 4.7 Sampled Madeira wine Blends for the evaluation of the sotolon's odour impact.

blend age	blend style	no. sampled
3-year-old	dry	11
	medium dry	10
	medium sweet	6
	sweet	14
5-year-old	dry	5
	medium dry	7
	medium sweet	9
	sweet	8
10-year-old	dry	4
	medium dry	4
	medium sweet	5
	sweet	6

4.2.4.3 Sample preparation

A standard stock (3.96 g/L) and working (100 mg/L) solution of sotolon was prepared in both ethanol and SFW (18% vol. of ethanol; 6 g/L of tartaric acid; and pH adjusted to 3.5 with 1 M sodium hydroxide solution), respectively. The working solution was used to spike the wine samples with known concentrations of the target compound. Each 3-year-old blend was spiked to 625 μg/L and by serial dilutions, a five scale-step 2.5-fold concentration series was obtained: 16, 40, 100, 250, and 625 μg/L.

4.2.4.4 Best estimate threshold determination

The ASTM E679 standard practice was followed to assess the odour BET for each of the four wine Blends. Each concentration scale-step was represented by a 3-AFC presentation which comprised a triad. One glass was filled with 30 mL of spiked wine (target sample) and two others were filled with 30 mL of the base wine (blanks). Repetitive testing was performed to obtain enough 3-AFC presentations. For each wine, the sensory evaluation consisted of duplicate sessions per day (one in the middle of the morning and the other at the middle of the afternoon). A total of 55 3-AFC presentations were acquired per wine. The individual threshold values were treated as independent in-between sessions. The rest of the procedure and the analysis of data followed the same procedure as of the preliminary sensory study described before.

4.2.4.5 Odour activity values

The odour impact of sotolon into the aroma of 3-, 5-, and 10-year-old Blends was appraised by the calculation of the respective OAVs. These values were obtained by dividing the quantified concentration of sotolon in each wine by the lowest estimated OT found, as described before.

4.3 Results and discussion

4.3.1 Preliminary evaluation

An initial preliminary sensory study was carried out to determine the OT and ORT for sotolon in sweet-styled 3-year-old MW. This first study also served to familiarize the panellists with the stimulus and the discrimination sensory testing. After 318 3-AFC presentations, the odour BET was estimated at 112 μg/L by the 22 panellists. This threshold was evaluated in a wine containing 6.3 ± 0.4 μg/L of endogenous sotolon. Individual BETs are depicted in Figure 4.2. Refer to Appendix B where the individual BETs obtained for each trial are given in Table B-1. Prior to this study, no reports were found for threshold evaluation of sotolon in the MW matrix. Preliminary tests evidence an estimated OT of about 6-fold higher than the obtained by Silva Ferreira et al. [66] for Port wine (19 μg/L). Although both FWs have comparable organoleptic characteristics [1], the different complexities of both Port and Madeira may have implications in the different perceptions of sotolon.

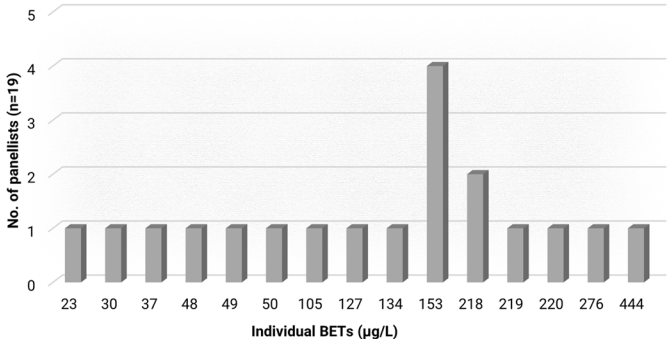


Figure 4.2 Distribution of the individual odour best estimate thresholds (BETs) for sotolon in sweet-styled 3-year-old Madeira wine.

The acceptability of sotolon was appraised through an ORT evaluation. The methodology of Prescott et al. [157] was adopted using a sweet-styled 5-year-old MW, as described before. After repeated testing, a total of 48 responses were obtained. The minimum number of responses necessary to establish a significative preference for one of the samples, at a 5% significance level, was 32 (67%) [191]. Figure 4.3 illustrates the results of the ORT determination. The number of responses preferring the non-spiked wine never exceeded this limen at any of the concentration scale-steps (max 46%). Since the evaluation test did not meet the requirement for the rejection of the null hypothesis (which establishes that a distinction cannot be made between the samples in terms of preference) no ORT was found for sotolon in these testing conditions. Higher concentration scale-steps should be further evaluated.

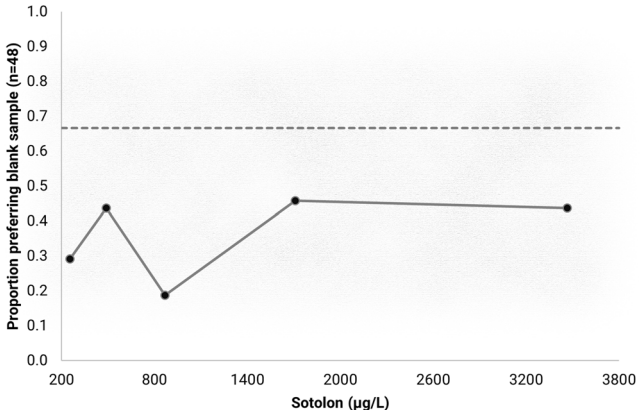


Figure 4.3 Proportion of the panellists' preference responses towards the blank wine samples during the odour rejection threshold sensory tests.

4.3.2 Sensory analysis with a selected panel

The preliminary study allowed the first sight about the odour perception of sotolon in a sweet-styled 3-year-old MW blend. However, and expecting this value was lower, the evaluation was re-tested with different 3-year-old Blends, this time using a selected panel (Table 4.8). The apparent variation of the preliminary responses prompted this new evaluation, as the non-familiarization with the sotolon stimulus and the design of the first experiment might have caused it. A new optimized concentration

range with less 3-AFC presentations was also adopted, thus reducing the possible effect of fatigue and adaptation. The OT obtained would then be used to evaluate the sotolon odour impact in MW Blends.

Table 4.8 Performance of the 22 panellists during the preliminary sensory trials.
Panellists highlighted in bold were selected.

panellist	correct responses (%)	no. of 3-AFC presentations	BET (µg/L)
S1	30	10	170
S2	30	10	170
S3	60	10	226
S4	**52**	**36**	**125**
S5	**58**	**22**	**71**
S6	38	11	170
S7	**67**	**36**	**93**
S8	62	35	141
S9	56	36	202
S10	38	16	264
S11	**77**	**27**	**80**
S12	35	36	921
S13	48	30	543
S14	**70**	**41**	**67**
S15	33	17	170
S16	42	12	283
S17[1]	**67**	**6**	**57**
S18	67	6	453
S19	59	19	937
S20	43	7	1358
S21	**86**	**7**	**85**
S22	71	7	170

3-AFC, three alternative forced-choice; BET, best estimate threshold; [1]panellist S17 was selected but was not available to perform during the following tests.

4.3.2.1 Odour threshold determinations

A total of 55 3-AFC presentations were acquired per MW blend. Table 4.9 shows the results obtained for each wine. The complete dataset and each individual BET are presented in Tables B-2 to B-5 of Appendix B. The odour BETs ranged from 23.3 to 68.7 µg/L, much lower than those of the preliminary study (112 µg/L). This decrease may be a result of the higher acuity of the selected panel and the overall familiarization gained from repeated testing [142,192]. The odour BET obtained for the sweet-styled wine from producer A (35.3 µg/L) was higher than of the corresponding dry-style (23.3 µg/L). The opposite was observed for the other producer's wines (41.7 and 68.7 µg/L, respectively). The variability of the threshold values may be explained by the matrix itself, as flavour release or retention is affected by the intrinsic chemical properties [193]. Matrix effects are known to influence the odour perceptiveness of specific stimuli, particularly the ethanol content in alcoholic beverages [194–196]. These results reinforce the idea of measuring OTs using in the beverage matrix itself. Here, the differences in BETs seem to be more relevant between wine producers than between wine styles. The results also suggest that the sugar content (which is reflected by the wine style) was not a key-

influencing factor on sotolon perceptibility. It is, however, important to note that the natural variability of the sensory test may account for such observed differences [142,163]. Although still higher, the BETs here determined are now closer to the previously estimated OT in Port wine [66]. The effect of the matrix in OT evaluation could also be observed when comparing these values with those reported by Campo et al. [6] in a model wine solution (9 μg/L).

Table 4.9 Selected panel's odour best estimate thresholds (BETs) for the orthonasal evaluation of sotolon in 3-year-old Madeira wine Blends.
Panel BET calculated as the geometric mean of the individual BETs from six panellists during duplicate sessions (total of 55 three alternative forced-choice presentations).

	producer A		**producer B**	
	dry-styled	sweet-styled	dry-styled	sweet-styled
panel BET (μg/L)	23.3	35.3	68.7	41.7
$\log_{10}$	1.4	1.5	1.8	1.6
$\log_{10}$ standard deviation	0.4	0.6	0.6	0.7

4.3.2.2 Odour activity values

The odour impact of sotolon was assessed in 89 wine samples. These were Blends of different styles and ages. The sotolon content in these wines ranged between 2.0 ± 0.9 to 516 ± 17 μg/L. The OAVs were then calculated using the lowest odour BET value previously obtained in the wine matrix (23.3 μg/L). The results are depicted in Figure 4.4. Sotolon might contribute to the overall aroma in wine Blends where OAV is higher than one. In this case, OAVs within the ranged 0.1–22 averaged 2.8, 6.3, and 9.8 for 3-, 5-, and 10-year-old Blends, respectively. The quantification data and OAV values are reported in more detail in Table B-6 of Appendix B as well. The results show that sotolon was perceptible and contributed to the overall flavour of most wines (94%). Ten-year-old Blends mostly had OAVs ≥ 10. As was expected, sotolon has a higher odour relevance in older Blends which confirms the known correlation with wine age [5]. The correlation between sotolon and sugar content in MW is known, with sugar degradation mechanisms potentially playing an important role in its formation [5,19]. Although this relationship was not as clear in terms of OAVs, higher values were found for sweet-styled 5- and 10-year-old Blends. The OAVs calculated for the 10-year-old Blends are significantly higher than those observed by Campo et al. [6] (ranging 1.6–2.1). Overall, the results suggest that sotolon has a relevant and important odour contribution to the aroma of current commercial MW Blends.

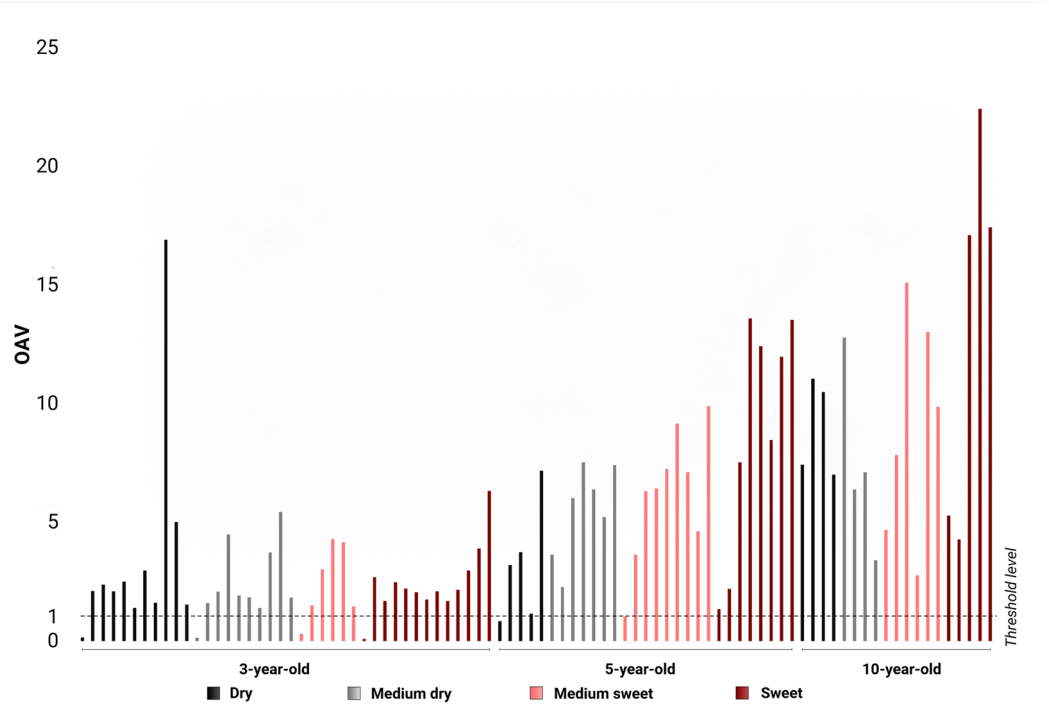


Figure 4.4 Odour activity values (OAVs) determined in the 3-, 5-, and 10-year-old Madeira wine Blends.

Chapter 5

PRELIMINARY STUDIES ON THE FORMATION OF SOTOLON IN FORTIFIED WINE

ROLE OF THE ACCELERATED AGEING IN THE FORMATION OF SOTOLON IN MADEIRA WINE

5.1 Introduction

Potent odorant compounds are of great interest in the flavour and food industry, as they can greatly affect the food quality and consumer acceptance [197,198]. The exceptional contribution of sotolon to the aroma of different foodstuffs naturally prompted numerous studies to focus on the identification of its formation pathways [25]. These studies are of great interest to better understand how sotolon is formed, especially wherein it may play a positive role in the perceivable quality of the food product. This knowledge may potentiate the development or adjustment of the production procedures to benefit its formation. Oppositely, in such cases where the presence of sotolon is seen as unfavourable, managing its formation could also be crucial.

This final part of this thesis is thus focused on the formation of sotolon in FW media. Since it was shown that sotolon can be found at high concentrations in these types of wines and its particular impact to the aroma is known, the study of its formation is of interest. The effect of the accelerated ageing in the formation of sotolon was herein studied through MSys simulating the accelerated ageing of MW.

5.1.1 Sotolon formation pathways: role of 2-ketobutyric acid

Interestingly, some sources describe sotolon as being a breakdown product of threonine, specifically citing the work of Sulser et al. [43]. However, according to the original publication, the formation via its precursor 2-KBA was proposed to explain the occurrence of abhexon in hydrolysed vegetable protein, and not sotolon. Later, the same authors characterized abhexon as the flavouring principle of seasonings from plant protein hydrolysates [35]. Nonetheless, recent research showed that a similar mechanism might be involved in the formation of sotolon in the French *flor*-sherry *Vin Jaune* [8]. Although the compound is known to be originated in several ways, and despite its significant role in wine, its natural formation is still not well elucidated [19]. Some of the findings on its formation are

briefly discussed below and a summary diagram of the proposed formation pathways in beverages is depicted in Figure 5.1.

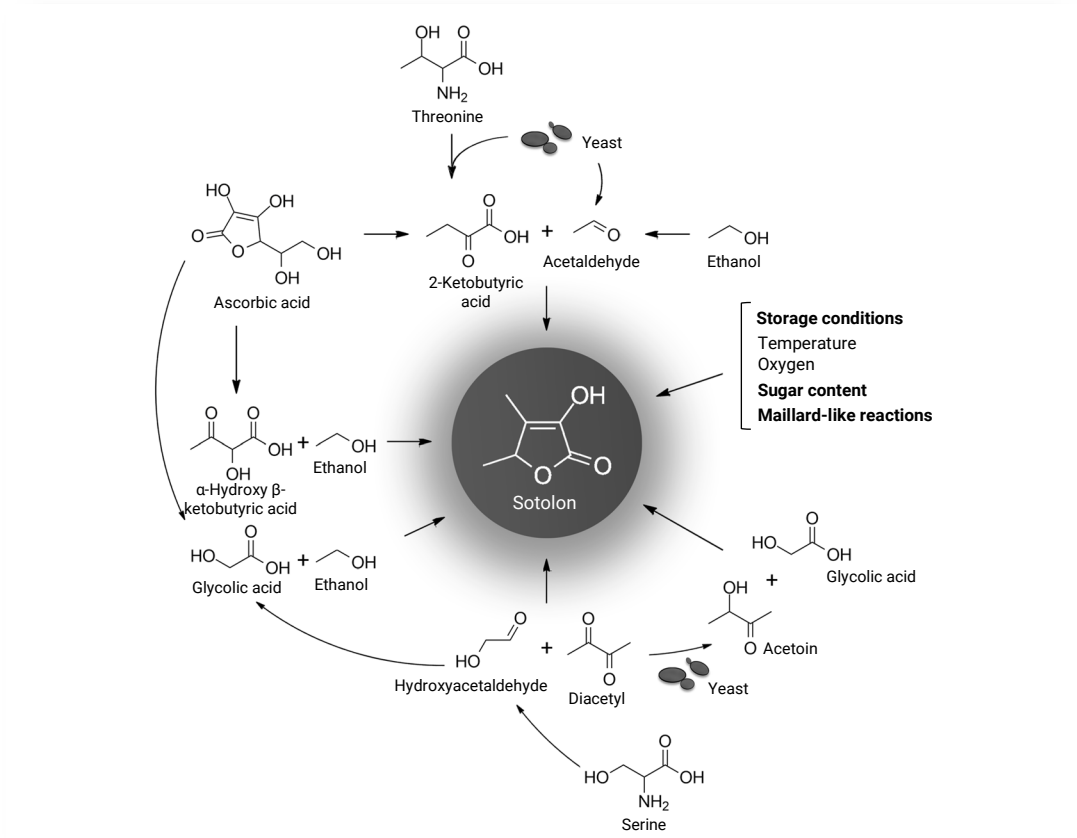


Figure 5.1 Summary of the proposed formation pathways for sotolon in beverages.
Based on Darriet and Pons [117], and Scholtes et al. [187].

In 1967, Sulser et al. [43] firstly proposed abhexon to be formed via the acid-hydrolysis of 2-KBA in protein hydrolysates. The compound 2-ketobutyric (or α-ketobutyric) acid is a short-chained keto acid known to occur due to the degradation of threonine in hydrolysed vegetable protein [199]. Years later, the formation of sotolon has been suggested to occur through similar mechanisms [91]. In 1976, Dubois et al. [39] firstly proposed sotolon to occur due to an aldol condensation between 2-KBA and pyruvic acid after heating (100 °C for 24 hours), a mechanism which could be involved in the formation of sotolon in *Vin Jaune*. Takahashi et al. [36] studied its formation in aged *sake* and suggested the condensation between 2-KBA and acetaldehyde to form the compound. A synthetic route from 2-KBA and aldehydes had also been previously reported by Rödel and Hempel [122]. Years later, Pham et al. [8] suggested the same precursors to be involved when studying its formation in synthetic media resembling *Vin Jaune*. In a 2010 report, Pons et al. [7] showed the same aldol condensation to be responsible for the low concentrations of sotolon found in prematurely aged white wines. Under mild conditions (40 °C for 30 days) experiments with model wine systems (12% vol. of ethanol, 5 g/L of tartaric acid, pH 3.5, 8 mg/L dissolved oxygen) containing 2-KBA (10 mg/L) and variable amounts of

acetaldehyde showed that sotolon was formed when the acetaldehyde content exceeded 500 μg/L (Table 5.1).

According to several studies, amino acids such as threonine are a potential source of 2-KBA in wine media [117]. However, and although other possible pathways have been proposed, the factors affecting the presence of 2-KBA still seem debatable. Pons et al. [7], for example, showed it to be formed as a result of the oxidative degradation of ascorbic acid in dry white wines. Ascorbic acid is an antioxidant and exogenous compound sometimes added to wine in combination with sulfur dioxide (SO_2) to protect against oxidation [200]. Nevertheless, the authors found the α-keto acid to be present even in wines not supplemented with ascorbic acid, which suggested the involvement of other compounds and/or pathways in its formation. Some of the potential precursors and pathways regarding the formation of this seemingly important keto acid, which can directly affect the formation of sotolon in wine media, are shortly described below.

Table 5.1 Sotolon formation with increasing acetaldehyde concentration in model wine solution containing 2-ketobutyric acid (10 mg/L).
The model wine solution consisted of 12% vol ethanol, 5 g/L tartaric acid, and pH 3.5. Solutions were kept at 40 °C for 30 days and dissolved oxygen was adjusted to 8 mg/L by air bubbling before sealing. Data from Pons et al. [7].

	acetaldehyde (μg/L)				
	0	100	200	500	1000
sotolon (μg/L)	nd	nd	nd	tr	1.1[1]

nd, *not detected*; *tr*, *trace amounts*; [1]average of triplicates.

5.1.1.1 Formation as a result of yeast metabolism

Sotolon was shown to be present in the well-known Sherry, an FW mainly produced in southern Spain. Some types of Sherry wines are subjected to a peculiar biological ageing under *flor* [1]. This is the case of the dried styles *Fino*, *Amontillado* and *Manzanilla*, as well as the similar sherry-like styles produced in Montilla-Moriles which grow aerobically at 15–15.5% vol. of ethanol content [74]. Although not an FW, the sherry-like *Vin Jaune* from the French region of Jura is also marked by the presence of this biofilm—here called *voile*—during its ageing process [110]. Other known biologically-aged wines are also produced in Italy (*Vernaccia di Oristano*) and Hungary (*Tokaji Szamorodni*) [107]. A reduction in the content of amino acids is observed during this biological ageing, as these provide the main source of nitrogen for the yeasts [201]. The enzymatic deamination of L-threonine by *flor* yeasts is thought to originate 2-KBA which, followed by an aldol condensation with acetaldehyde, leads to the formation of sotolon [7]. This mechanism was originally proposed to explain the formation of the lactone in *Vin Jaune* (Figure 5.2) [8].

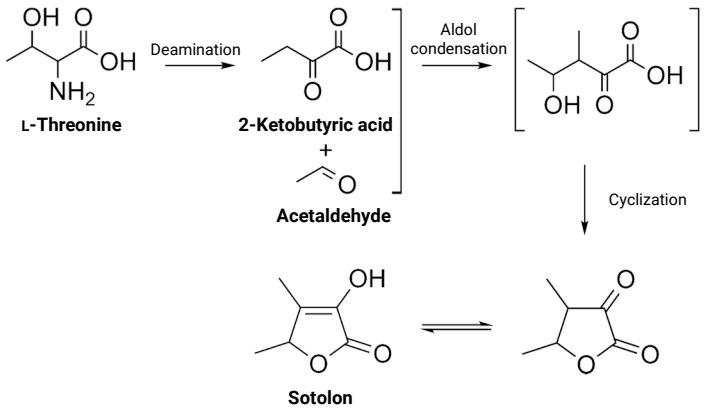


Figure 5.2 Proposed mechanism for the formation of sotolon in *Vin Jaune*.
Adapted from Pham et al. [8].

The formation of 2-KBA can also occur during the primary fermentative process. In their study, Pons et al. [7] found 2-KBA to be produced by the yeasts during the alcoholic fermentation. The authors evaluated the role of several *Saccharomyces cerevisiae* strains in the formation of 2-KBA during fermentation. The α-keto acid content varied within the range 0.9–7.1 mg/L at the end of the fermentation and the yeast strain had a high impact on its formation. As explained by the authors, this variation may be due to the threonine deaminase activity, an enzyme responsible for the deamination of threonine through the Ehrlich pathway in which 2-KBA is a known intermediate.

5.1.1.2 Non-enzymatic formation from threonine

Takahashi et al. [36] studied the effect of accelerated ageing in the formation of sotolon in *sake*. After one month of ageing at about 60 °C, the authors detected some known degradation by-products of threonine, including 2-KBA. The presence of these by-products was suggested as a result of the acid-degradation of threonine, which is found at high concentration in fresh *sake*. Acetaldehyde was also found as an acid-degradation by-product of threonine and was suggested to contribute to the formation of sotolon via an aldol condensation with 2-KBA.

5.1.1.3 Formation from acetaldehyde

Acetaldehyde (also referred to as ethanal) is the major aldehyde found in wines, with concentrations sometimes reaching up to 1000 mg/L [17]. It is known to result either through the course of fermentation or due to the oxidation of ethanol [202]. In FWs concentrations were shown to range within 12–800 mg/L [203]. Higher concentrations are usually found in *Fino* Sherry, normally at the range 230–550 mg/L, allowing the differentiation of this style from other Sherry [202]. In the case of MWs relative lower concentrations were observed (18–117 mg/L) [15,204]. The aldol condensation between 2-KBA and acetaldehyde has been extensively linked to sotolon, suggesting the role of acetaldehyde as key for the formation of the lactone in those beverages [91,187]. Although 2-KBA was shown to be formed by either enzymatic or chemical deamination of threonine, it can also be derived from acetaldehyde itself. Pisarnitskiĭ et al. [205] found 2-KBA to occur as a result of the condensation

of two molecules of acetaldehyde under oxidative conditions. The authors also propose the higher levels of sotolon found on Sherry and MWs as a result of this strict oxidative mechanism.

5.1.1.4 Formation from ascorbic acid

Burnt- and spicy-like off-flavours associated with the presence of sotolon can result during the storage of citrus soft drinks [206]. König et al. [133] proposed the sotolon formation to be linked to ethanol and ascorbic acid in model solution simulating the storage conditions of these beverages. MSys consisting of 250 μL of ethanol, 83 mg of ascorbic acid, and 42 ml of water were stored at 70 °C for two weeks. The authors studied the formation mechanisms with labelled isotopic precursors and identified carbons from both ascorbic acid and ethanol in the carbon skeleton of sotolon. It was suggested that the formation may be due to two different pathways either involving one or two molecules of ethanol (Figure 5.3). Additionally, the storage of the same MSys under nitrogen inhibited the occurrence of sotolon, indicating the importance of oxygen in its formation under those conditions.

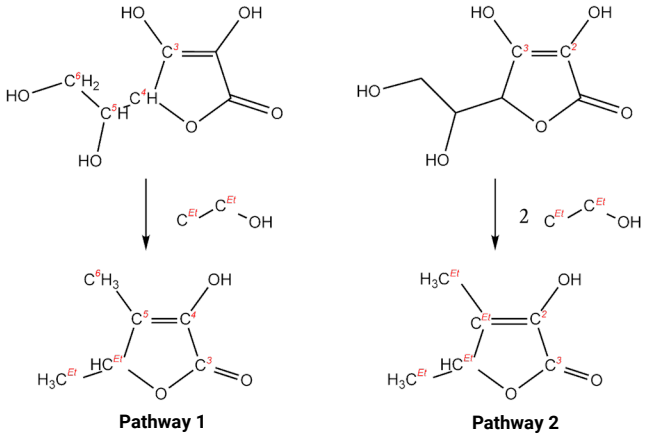


Figure 5.3 Formation of sotolon from ascorbic acid and ethanol.
The red superscript numbered carbons represent those originating from ascorbic acid; C_{Et} represents those originating from ethanol. Adapted from König et al. [133].

Inspired by these findings, Pons et al. [7] tried to find 2-KBA among the degradation by-products of ascorbic acid. Initial results showed 2-KBA to be a minor degradation product of ascorbic acid in model wine solution (12% vol. of ethanol, pH 3.5, 8 mg/L of dissolved oxygen). Further aqueous and dilute alcohol acid model solutions containing ascorbic acid were prepared and heated for two days at 70 ºC. Significant amounts of both sotolon and 2-KBA were only detected in the alcoholic solutions (Table 5.2). Isotopic labelling using deuterated ethanol resulted in unlabelled 2-KBA, which suggested it was formed via the oxidative degradation of ascorbic acid.

Scholtes et al. [187] recently suggested α-hydroxy-β-ketobutyric acid (or 2-hydroxy-3-oxobutanoic acid) to result from the oxidation of ascorbic acid, generating sotolon through the reaction with one molecule of ethanol. The second pathway proposed by König et al. [133], involving two ethanol molecules, may rely on the formation of glycolic acid as an intermediate.

Table 5.2 Formation of sotolon and 2-ketobutyric acid (2-KBA) in model solutions containing ascorbic acid (3 g/L) and ethanol (12% vol.).
Solutions were kept at 70 °C for two days and dissolved oxygen was adjusted to 8 mg/L by air bubbling before sealing. Adapted from Pons et al. [7].

	model solution		
	control	control + ascorbic acid	control + ascorbic acid + ethanol
sotolon (µg/L)	nd	nd	30[1]
2-KBA (mg/L)	nd	nd	0.8[1]

nd, *not detected*; [1]average of triplicates.

5.1.2 Formation through Maillard-type reactions

It is well accepted that Maillard-type reactions are responsible for the generation of sotolon, particularly during the thermal processing of foods [207]. Sotolon shares a similar sugary aroma with other Maillard-derived compounds such as furaneol, maltol or cyclotene. The branched carbon skeleton of sotolon, in contrast to the straight carbon chain structure of these other three compounds, however, suggests a different formation pathway during the Maillard reaction (MR) [47]. Some Maillard-type reactions were assessed for the formation of sotolon in thermally processed MSys. Sotolon was shown to be formed through MR intermediates such as pyruvic and ketoglutaric acids [208]. Kobayashi [47] started with an aqueous mixture of both glutamic and pyruvic acids at pH 8 and, after boiling for four hours, identified sotolon in an ether extract. It was suggested that 2-ketoglutaric acid originated from the oxidation of glutamic acid, which then yielded sotolon through a somewhat complex amino-carbonyl reaction dependent on the pH and temperature conditions (Figure 5.4). Hofmann and Schieberle [209,210] applied aroma extract dilution analysis to understand the role of cysteine on the generation of volatiles linked to the MR. They have identified sotolon in solvent extracts from thermally treated binary mixtures of cysteine (3.3 mmol) and sugars (10 mmol): L-cysteine/D-ribose, L-cysteine/D-glucose, L-cysteine/L-rhamnose. Although sotolon was not among the most odour-active compounds, the FD factor was highest within the acidic volatile fraction of L-cysteine/L-rhamnose MSys. The same authors also found the binary mixture of hydroxyacetaldehyde/butane-2,3-dione (diacetyl) to generate sotolon within the same heating conditions [207]. The temperature was raised from 20 to 145 °C within 20 minutes and the amounts of sotolon were particularly high at pH 5.0. The authors, however, reported much lower yields when the pH was lowered to 3.0. Dry-heating of the precursor mixture also yielded sotolon, but again at much lower amounts. Due to the high levels of diacetyl found in Port (up to 10 mg/L), Silva Ferreira et al. had also suggested the role of this mechanism in these wines, although the presence of hydroxyacetaldehyde was still to be confirmed [91]. More recently, Scholtes et al. [187] found hydroxyacetaldehyde together with acetoin (the reduced form of diacetyl) to generate sotolon in beer MSys (5% vol. of ethanol, pH 4.5) after 30 days at 60 °C. The authors also suggested a potential combination of acetoin and serine-derived compounds, such as glycolic acid, to be precursors in the formation of sotolon. Glycolic acid can either be obtained enzymatically by the oxidation of

hydroxyacetaldehyde or through the Strecker degradation of serine to hydroxyacetaldehyde and further oxidation in heat-treated media [211].

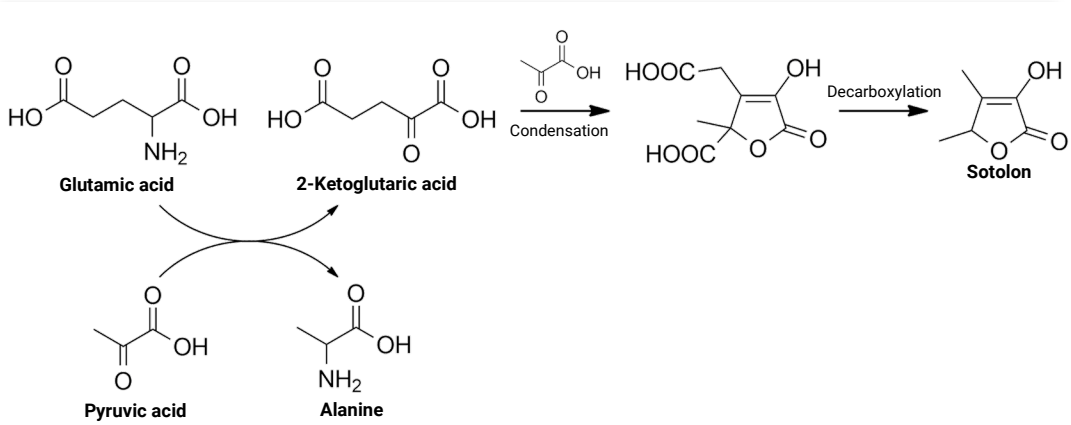


Figure 5.4 Formation of sotolon during an amino-carbonyl reaction involving 2-ketoglutaric acid.
Adapted from Kobayashi [47].

3-Amino-4,5-dimethyl-2(5*H*)-furanone has been found to be present in cultures of *Lactarius helvus*, a mushroom with a characteristic fenugreek-like smell attributed to the presence of sotolon [212]. Because of the apparent "ease of conversion" of 3-amino-4,5-dimethyl-2(5*H*)-furanone into sotolon, Guerra et al. [208] further investigated its formation. These authors studied the effect of amino acids and α-keto acids on the thermal generation of this amino lactone. The precursor was found to be thermally generated for the first time from both glycine/pyruvic acid and alanine/glyoxylic acid MSys. Interestingly, pyruvic acid is a yeast metabolic by-product while glyoxylic acid is a known oxidative degradation product of tartaric acid in wine media [213,214]. Isotope labelling experiments also indicated the formation of 3-amino-4,5-dimethyl-2(5*H*)-furanone to occur via different pathways. Guerra and colleagues then proposed a two-step formation pathway involving the compound 4,5-dimethyl-2,3-furandione which in turn can be formed by two different pathways (Figure 5.5).

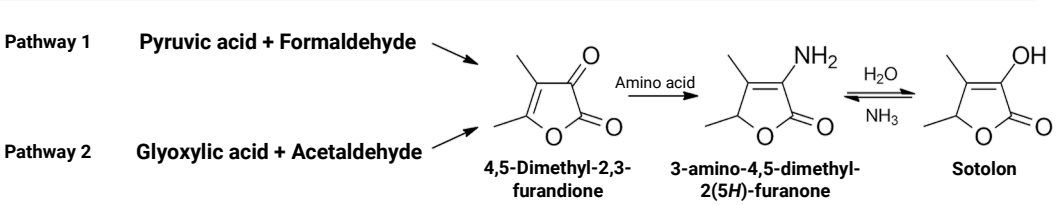


Figure 5.5 Proposed pathways for the formation of the sotolon precursor 3-amino-4,5-dimethyl-2(5*H*)-furanone.
Adapted from Guerra et al. [208].

In the first study reporting sotolon in MW, Câmara et al. [5] demonstrated a strong correlation between sotolon and other well-known furanic sugar derivates (furfural, 5-methylfurfural, and HMF). Previously, Silva Ferreira et al. [66] had also encountered similar results when studying the role of sotolon in Port wine. These results suggest that MRs could take part in the formation of sotolon during the ageing of these alcoholic beverages.

5.1.3 Formation from 4-hydroxyisoleucine

4-Hydroxy-L-isoleucine (HIL) is the most abundant free amino-acid in fenugreek and it was first isolated and identified in the plant seeds by Fowden et al. [215]. The natural occurrence of sotolon was first proposed in fenugreek by Rijkens and Boelens [41] and later confirmed by Girardon et al. [42]. Here it is thought to be formed by the thermal degradation of HIL. The similarities between sotolon and HIL, as pointed out by Girardon et al. [42], prompted Blank et al. [51,216–218] to study its formation in the plant. The authors assessed this formation by reacting HIL with different carbonyl compounds in a phosphate-buffered MSys (pH 5.0) subjected to high temperatures (100 °C) for 60 minutes. The acid-catalysed cyclization of HIL leads to the formation of the corresponding amino acid lactone form (3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone) which when reacting with an α-dicarbonyl formed a Schiff base. The Schiff base is then rearranged and hydrolysed to finally form sotolon (Figure 5.6). With temperatures above 70°°C for up to 10 hours and using methylglyoxal as the carbonyl source provided a better yield in sotolon (Figure 5.7). The authors also found the HIL lactonization to be a rate-limiting step of the reaction, as the intermediate lactone produced sotolon with a better yield than from HIL directly. This lactonization is thought to be favoured under acidic conditions. This sotolon formation hypothesis is further supported by the fact that (*S*)-sotolon is the predominant enantiomer in fenugreek which is in agreement with the stereochemistry of the predominant HIL enantiomeric form (2*S*,3*R*,4*S*) isolated from the plant [51]. As explained by Slaughter [34] position 4 of (2*S*,3*R*,4*S*)-HIL becomes position 5 in (*S*)-sotolon and asymmetry is lost at positions 2 and 3 as a result of double-bonding (Figure 5.8).

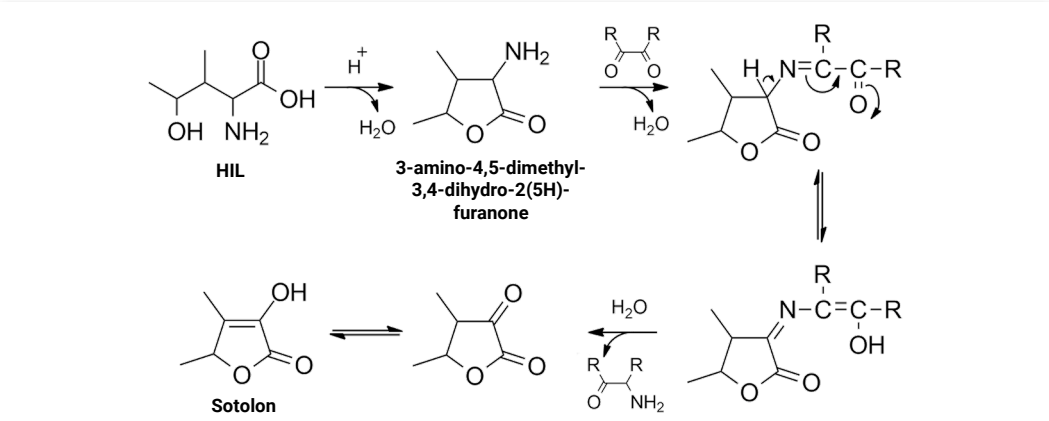
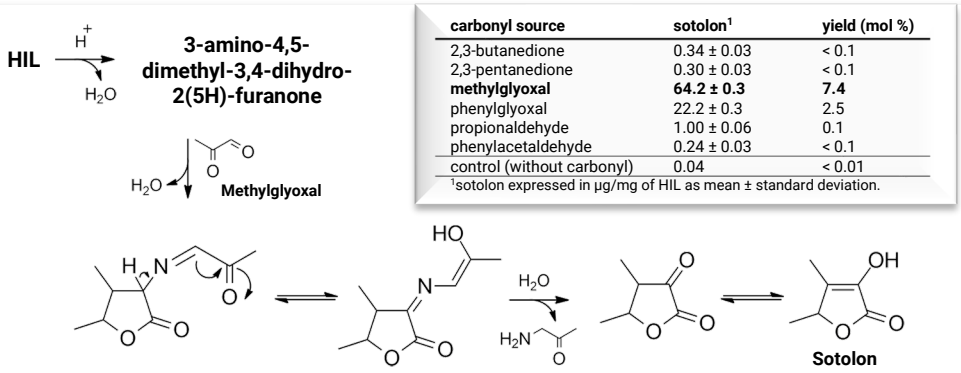


Figure 5.6 Proposed generic formation mechanism for sotolon from 4-hydroxy-L-isoleucine (HIL) in fenugreek. Adapted from Blank et al. [216,217].



carbonyl source	sotolon[1]	yield (mol %)
2,3-butanedione	0.34 ± 0.03	< 0.1
2,3-pentanedione	0.30 ± 0.03	< 0.1
methylglyoxal	**64.2 ± 0.3**	**7.4**
phenylglyoxal	22.2 ± 0.3	2.5
propionaldehyde	1.00 ± 0.06	0.1
phenylacetaldehyde	0.24 ± 0.03	< 0.1
control (without carbonyl)	0.04	< 0.01

[1]sotolon expressed in µg/mg of HIL as mean ± standard deviation.

Figure 5.7 Formation of sotolon from 4-hydroxy-L-isoleucine (HIL) and methylglyoxal.
Adapted from Blank et al. [218].

Apart from fenugreek, HIL is also present in *L. helvus* where sotolon is thought to be the key compound responsible for its odour, as discussed earlier [212]. Unlike Blank and colleagues, Peraza-Luna et al. [219] did not find 3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone among the compounds in hairy root cultures of fenugreek. Contrariwise, they have found 3-amino-4,5-dimethyl-2(5*H*)-furanone, which also found in *L. helvus* and a potential precursor of sotolon, as discussed earlier. As HIL is also present in *L. helvus* [212], the authors conclude that the formation of sotolon in both the plant and mushroom might share several intermediary compounds.

The oxidative deamination of HIL through an enzymatic route has also been suggested [220]. More recently, Lanfermann et al. [221] using isotope labelling experiments have shown the existence of an oxygenase activity in the pathway of sotolon from L-isoleucine in cultures of *Laetiporus sulphureus*, an edible mushroom grown on tree trunks and branches [24]. As proposed, these findings provide a new insight into the "cold formation" of sotolon.

5.1.4 Other factors affecting its formation in wines

Although the main formation pathways for sotolon are still not well elucidated it is accepted that sugar concentration, storage time, oxidation, and temperature are known factors associated with sotolon development in wines [9].

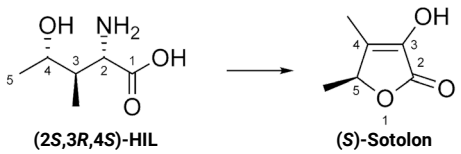


Figure 5.8 Stereochemistry of (2*S*,3*R*,4*S*)-4-hydroxy-L-isoleucine (HIL) and (*S*)-sotolon.
Adapted from Blank et al. [51].

5.1.4.1 Sugar content and storage time

Sotolon formation in FWs is suggested to be favoured by high sugar content. Silva Ferreira et al. [66] found a high correlation between its formation and sugar derivatives such as furfural and HMF in barrel-aged Port wines aged up to 60 years-old ($r = 0.9015$ for HMF). Câmara et al. [5] later

demonstrated the same apparent relationship in 86 MWs of different styles aged up to 25 years-old ($r = 0.9291$ and 0.9458 for furfural and HMF, respectively). A correlation was also found for 5-methylfurfural and 5-ethoxymethyfurfural ($r = 0.9442$ and 0.9045, respectively). In both studies, sotolon showed a linear increase in formation with ageing time ($r > 0.95$ and $r = 0.917$ for the port and MW study, respectively). The correlated furanic sugar derivatives are also known to be linked with wine ageing [132]. Thus, this lactone has been suggested as an important key marker of wine oxidative ageing.

Pereira et al. [19] evaluated the contribution of the thermal degradation of fructose and glucose to the overall MW features. Sotolon was identified only in fructose MSys (125 g/L of fructose dissolved in model wine consisting of 18% vol. of ethanol, 6 g/L of tartaric acid, pH 3.5) after storage at 70 °C for one month. This study suggested the thermal degradation of fructose in acidic medium as the main responsible for the formation of sotolon in sweet FWs.

5.1.4.2 Oxygen and oxidative conditions

The mechanisms responsible for the formation of sotolon are thought to involve oxygen and oxidative conditions [124]. The oxidative conditions may explain its presence in oxidatively aged wines such as Port [66], Madeira [5], and VDN [76].

Cutzach et al. [95] investigated the ageing of sweet FWs under various accelerated ageing conditions (stored at 37 °C with and without the presence of oxygen) and found the concentration of sotolon to increase linearly over time. The sotolon content in an oxygenated environment was higher than that observed in the absence of air. The authors also noticed a higher increase factor in sweet fortified white wine when in comparison to the increase observed in sweet fortified red wine after six months. This was suggested as due to the antioxidant activity of polyphenolic compounds in red wines acting on acetaldehyde, which probably acts as the limiting factor for the sotolon formation.

Escudero et al. [114] studied the odorants generated during wine oxidation through GC-O/AEDA. White wines were submitted to a saturated oxygen environment and stored at 20 °C for up to five weeks. Sotolon was shown to be an impact odorant of oxidized wine.

A forced ageing experiment regarding the presence of sotolon in both *Colheita* and *Vintage* Port wines demonstrated the formation of the lactone to be dependent on the amount of dissolved oxygen [91]. The study involved a 2-year-old red Port wine submitted to an oxygen saturated environment for up to 59 days of storage at different temperatures. After 59 days at 60 °C, sotolon concentration reached 300 μg/L, for a total of 22 mg/L of consumed oxygen. The same wine was also aged in a non-saturated environment and a third portion was also supplemented with SO_2 (free SO_2 = 74 mg/L) and submitted to the same conditions. The authors reported the rates of formation to be about ten-fold higher for the wine samples submitted to the oxygen saturated environment when in comparison to the non-saturated samples. The treatment with SO_2 also inhibited the sotolon formation. The authors suggest this as a result of the high reactivity of oxygen with sulphur dioxide

and/or the combination of SO_2 with the carbonyl group of sotolon precursors, consequently preventing the aldol reaction involved in its formation.

Lavigne et al. [124] assayed the content of sotolon in dry white wines during eight months of barrel ageing either with and without the presence of lees. Sotolon showed a higher content in wines aged in new barrels without lees. The authors suggested the capacity of lees to combine oxygen to explain the prevention of sotolon formation. During bottle ageing, sotolon concentration was also correlated with the amount of dissolved oxygen (R^2 = 0.938). Sensory evaluation indicated a correlation between the sotolon content and the perception of oxidation aroma (R^2 > 0.7), which suggested the lactone to be a good marker of defective ageing in white wines during bottle ageing.

The role of antioxidant compounds, such as glutathione, in the formation of sotolon during the storage of bottled dry white wines had also been evaluated [119]. Dubourdieu and Lavigne [222] showed the addition of GSH (10 mg/L) to prevent sotolon formation in dry white wines after three years of storage. The concentration of sotolon was about three-fold less than that of the non-supplemented control bottle.

5.1.4.3 Storage temperature

Cutzach et al. [96] investigated the ageing of white sweet FWs under various conditions, including storage temperature. The wines were stored in air-conditioned (16–18 °C) and unconditioned environments (8–33 °C). Although not by much, the development of sotolon was higher in the unconditioned environment (Table 5.3). The authors also proposed new oak barrels to promote the alcohol oxidation reactions required for the formation of sotolon.

A forced ageing experiment regarding the presence of sotolon in Port wines also demonstrated the formation of the lactone to be dependent on temperature, even at a higher extent than oxygen [91]. A 2-year-old red Port wine was stored at 15, 45, and 60 °C for up to 59 days. At 15 °C no significant changes were observed in the concentration of sotolon. Both at 45 and 60 °C the concentrations increased with time, reaching 300 μg/L at 60ºC. More recent research has also confirmed sotolon formation to be highly dependent upon oxygen and temperature conditions [92]. Its concentration in Port wines may be due to a synergistic effect between these two parameters.

Pereira et al. [19] demonstrated the contribution of temperature to the formation of sotolon as a result of the thermal degradation of fructose in MSys (125 g/L fructose, 18% vol. of ethanol, 6 g/L of tartaric acid, pH 3.5) submitted to accelerated ageing conditions (stored at 70 °C for one month).

Table 5.3 Evolution of sotolon in white sweet fortified wines under different storage conditions.
Sotolon was assessed at two points: 6th and 30th month of ageing in each respective container. The concentration range (in μg/L) represents the evolution between these two periods. Data from Cutzach et al. [96].

air-conditioned winery (16–18 °C)			**non-air-conditioned winery (8–33 °C)**		
concrete vats	2-year-old barrels	new barrels	concrete vat	2-year-old barrels	new barrels
0–3	3–6	2–94	2–13	5–11	7–105

5.2 Materials and methods

5.2.1 Chemicals

All chemicals used had a purity grade higher than 98% except for hydrochloric acid (37%). D-(–)-Fructose was from Merck Co. (Darmstadt, Germany). L-Amino acids arginine, cysteine, threonine and aspartic acid, and γ-aminobutyric acid (GABA) were purchased from Fluka Analytica (Sigma-Aldrich) (Steinheim, Germany). L-(+)-Tartaric acid and sodium hydroxide were from Panreac (Barcelona, Spain). Hydrochloric acid (37%) and absolute ethanol were from Sigma-Aldrich (Steinheim, Germany). Ultra-pure water with a resistivity >18 MΩ.cm (type 1) was obtained from a Millipore Simplicity® UV apparatus (Milford, MA).

5.2.2 Preparation of the model systems

Fructose and amino acids (arginine, cysteine, GABA, aspartic acid, and threonine) were used in different combinations for the preparation of the MSys in both acidified water and SFW media. The selection of each amino acids and fructose was based on previous studies reporting the most important amino acids present in the MW matrix and the role of fructose in the formation of sotolon, respectively [19,223,224]. Seeing how threonine is showed to have an important role in sotolon formation, this amino acid was also evaluated to compare with the other amino acid MSys in the sweet SFW media. Duplicates of all MSys were prepared (Table 5.4). Most MSys were prepared in SFW in the same manner as described in Chapter 3 (18% vol. of ethanol; 6 g/L of tartaric acid; and pH adjusted to 3.5 with 1 M sodium hydroxide solution). Amino acids (100 mg/L) were also added to evaluate the possible role of the MR. Water MSys were acidified to pH 3.5 with a dilute hydrochloric acid solution. The fructose content was set at 1 and 100 g/L to simulate extra-dry and sweet wines, respectively.

Each MSys solution was poured into 125 mL amber bottles. The bottles were filled to about half their volume (60 mL of MSys) and stored at 70 ± 0.5 °C in a Memmert UFE 400 oven (Schwabach, Germany) for one month to simulate long-term ageing. This is assuming that these conditions could mimic the long-term ageing of MW during *canteiro*—or yet mimic the reactions that could possibly occur during the *estufagem* process. After the storage period, the sotolon content of each MSys was assessed in duplicate by the LC-MS/MS method previously described in Chapter 3.

Table 5.4 Prepared model systems (MSys) submitted to accelerated ageing (n = 4).

MSys code	pH	ethanol (% vol.)	L-(+)-tartaric acid (g/L)	D-(−)-Fructose (g/L)	L-amino acid (100 mg/L)
EtOH	3.5[1]	18	n/a	n/a	n/a
Fru1	3.5[1]	n/a	n/a	1	n/a
Fru100	3.5[1]	n/a	n/a	100	n/a
Fru1EtOH	3.5[1]	18	n/a	1	n/a
Fru100EtOH	3.5[1]	18	n/a	100	n/a
SFW	3.5	18	6	n/a	n/a
SFW_Fru1	3.5	18	6	1	n/a
SFW_Fru1Arg	3.5	18	6	1	arginine
SFW_Fru1Cys	3.5	18	6	1	cysteine
SFW_Fru1GABA	3.5	18	6	1	GABA
SFW_Fru1Asp	3.5	18	6	1	aspartic acid
SFW_Fru100	3.5	18	6	100	n/a
SFW_Fru100 Arg	3.5	18	6	100	arginine
SFW_Fru100 Cys	3.5	18	6	100	cysteine
SFW_Fru100 GABA	3.5	18	6	100	GABA
SFW_Fru100 Asp	3.5	18	6	100	aspartic acid
SFW_Fru100Thr	3.5	18	6	100	threonine

SFW, *synthetic fortified wine*; *n/a*, *not applicable*; *GABA*, *γ-aminobutyric acid*, [1]acidified with dilute hydrochloric acid solution.

5.3 Results and discussion

5.3.1 Quantification results

The quantification of sotolon in the model solutions was assessed after one month of storage at 70 °C. The results are presented in a graphical form in Figure 5.9; they are also presented in more detail in Table C-1 of Appendix C. The sotolon content was shown to range from not detected up to 1142 ± 70 μg/L. These results are in accordance with what is normally found for oxidatively aged wines such as Madeira. Câmara et al. [5] quantified up to 2 mg/L of sotolon in MWs aged up to 25 years following *estufagem* and/or maturation in oak casks. Sotolon content was shown to be favoured by high sugar content. While the sotolon content in wines aged for 11 years was on average 825.8 μg/L for sweet-styled Madeiras, the values for dry-styled wines were only 258.7 μg/L. Freitas et al. [20] demonstrated the *estufagem* process to accelerate the formation of the lactone. Wines submitted to heating conditions (45 °C for 120 days), prior to *canteiro* ageing for 1080 days, increased the sotolon content at up to 6-fold (up to about 405 μg/L). Again, higher concentrations were mostly observed in the aged sweet wines. Thus, the accelerated ageing conditions here used can probably be used to better understand the long-term ageing of MW and other similar FWs, particularly for the case of sweet-styled wines. The average sotolon content also falls right in the range observed for other FWs as well, namely Port, VDN and Sherry (see Table 2.2).

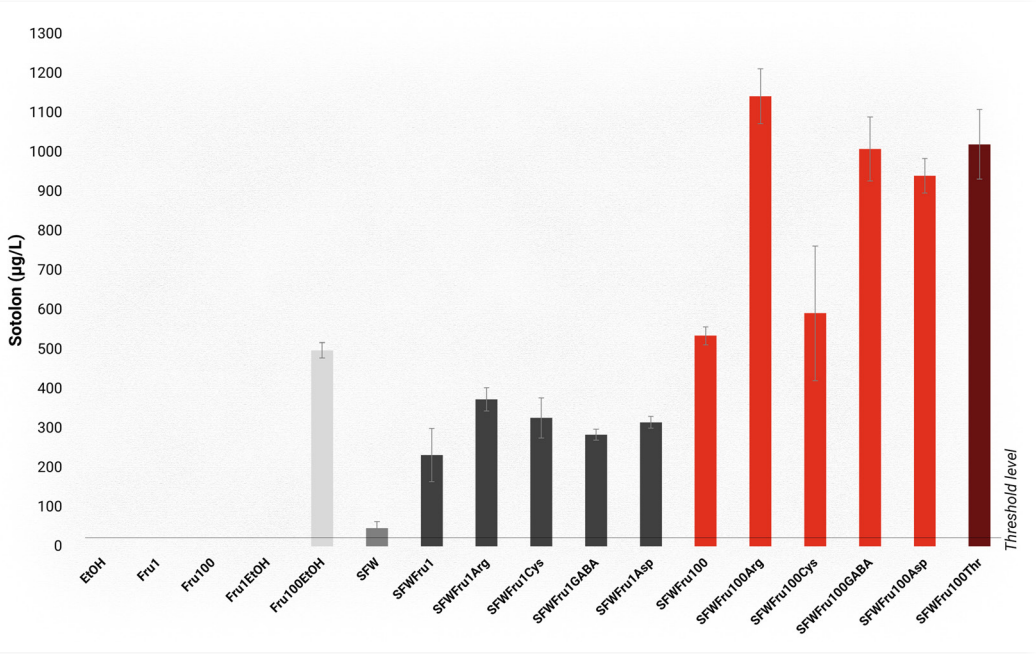


Figure 5.9 Quantification of sotolon in the model systems (MSys) submitted to accelerated ageing conditions (70 °C for one month).

At a first glance, it is clear that the formation of sotolon was particularly higher in MSys simulating a sweet FW (100 g/L of fructose) when in comparison to the extra-dry MSys (1 g/L of fructose). The "SFWFru100" MSys was quantified at 534 ± 23 µg/L of sotolon. Pereira et al. [19] assessed the development of aroma volatiles in thermally processed sweet MSys (125 g/L of sugar). These were stored in the same conditions as of those of this current study and sotolon was also found to be formed. This current study expands on those results and introduces extra-dry MSys. In this case, even in such MSys with a low amount of sugar, sotolon content reached up to 373 ± 30 µg/L ("SFWFru1Arg"). This is quite a remarkable result; while sotolon is often associated and strongly correlated with sweet wines, the quantified amount in this MSys is still quite high and falls within the range of sotolon usually found for these types of wines, aged for long periods of time. These results are in accordance with what was previously reported by Cutzach et al. [76] in real samples. For the first time, the authors had found high amounts of sotolon in dry wines (< 5 g/L of sugars) kept at oxidative ageing conditions: up to 572 µg/L in a dry white wine with more than seven years of barrel ageing. Even though sweet wines (VDN) reached a far higher sotolon content—up to 1399 µg/L in a sweet white wine with 29 years of both barrel and bottle ageing—the authors were still surprised with the amount generated in the dry wines. Although the correlation of sotolon with sugar content is evident, and if sugars do in fact play a role in the sotolon formation in FWs, one could still expect relatively high amounts of the lactone to be generated even in almost extra-dry wines where fortification is employed at the end of the primary fermentation. This means that if the winemaker decides to make an extra-dry wine, aged in similar conditions, sotolon would possible still contribute to the overall aroma.

The "SFW" MSys devoid of either sugar or amino acid is of particular interest. This MSys had the least quantified amount of sotolon (46 ± 17 μg/L) after the ageing period. Although a relatively lower value, this concentration is still quite above the value of the OT determined for sotolon in MW (23.3 μg/L). This result might imply a different route of formation for sotolon without the involvement of sugars, and further contribute to the hypothesis of sotolon being impactful to the aroma of extra-dry wines aged in similar conditions.

The addition of the selected amino acids (100 mg/L) had an increased effect on the generation of sotolon, particularly in sweeter MSys. The highest sotolon content (1142 ± 70 μg/L) was found for the MSys containing arginine in sweet SFW ("SFWFru100Arg"). When comparing it with the "SFWFru100", the content in sotolon more than doubled (113% increase). The other MSys prepared with amino acids and sugars, namely "SFWFru100GABA", "SFWFru100Asp" and "SFWFru100Thr" also revealed an increase in sotolon, respectively, except "SFWFru100Cys". Interestingly, the amount of sotolon in "SFWFru100Cys" was 591 ± 23 μg/L, which was not significantly different from the amount found in the "SFWFru100" MSys. In the case of the extra-dry MSys, a slight increase is also observed. This increase, however, is only significant in "SFWFru1Arg" MSys (61% increase) when compared to "SFWFru1". Also, within these amino acid MSys, no statistically significant differences were observed.

A preliminary attempt at deconstructing these MSys was made by either removing ethanol or sugar, without the presence of tartaric acid. These MSys were still acidic (pH 3.5) by acidifying with a dilute hydrochloric acid solution. None of these MSys generated sotolon at detectable amounts except for "Fru100EtOH". This MSys is essentially the same as "SFWFru100" but without tartaric acid in its composition. The quantified amount of the lactone was quite high (497 ± 19.2 μg/L). No statistically significant difference was observed between this value and the quantified amount in the "SFWFru100" MSys (534 ± 23 μg/L).

5.3.2 Insights on the probable formation pathways

The aim of the study was not to pinpoint a detailed formation route for sotolon, but rather analyse the quantified MSys and withdraw some preliminary conclusions about its formation in MW-like conditions. While not well understood, the formation of sotolon is thought to differ among foodstuffs. Even among wines, there seems to be a consensus that the formation in wines exposed to oxidative conditions is different than that for wines involved in biological ageing, for example. It is then clear that some of the proposed formation mechanisms for sotolon might not hold in the conditions here tested. MW is proposedly exposed to oxidative conditions and its ageing during *canteiro* does not involve the Sherry-like *flor* yeasts. Thus, the mechanisms proposed for *Vin Jaune*, involving the condensation of 2-KBA with acetaldehyde, might not be involved in this case. On the other hand, considering the similarities between Madeira, *Oloroso* Sherry, Port and some VDN, the formation mechanisms might be concurrent among them.

Following, the present results will be discussed according to previous reports regarding the formation of sotolon in other foodstuffs. The aim is to draw a parallel between the here obtained quantification data and the probable mechanisms reported in other studies, particularly in those that involve MW-like conditions.

5.3.2.1 Role of fructose, amino acids, and the Maillard reaction

First, and without taking the deconstructed MSys into account, clearly the higher content of fructose resulted in a greater amount of sotolon. It is also evidenced that the introduction of amino acids contributed to a higher yield. These results, along with the storage conditions employed during the study, permit to consider the MR. The MR is a quite complex process, and its development requires the presence of amino compounds, sugars, and high temperatures. A temperature of 70 °C is technically never achieved under normal circumstances during the baking process neither during the *estufagem* nor *canteiro* ageing of MW. However, and as was previously discussed, it is fair to assume that one month in these conditions could perhaps simulate the long-term ageing of MW during *canteiro*—or yet mimic the reactions that could occur during the *estufagem* process.

These results expand on those from Pereira et al [19]. The authors assessed the effect of fructose and glucose thermal degradation for the MW features in similar storage conditions as this current study, namely during accelerated ageing at 70 °C for one month. They have found sotolon to be only generated in fructose-containing MSys, which suggested a reduced contribution from the MR in its formation. However, the addition of amino acids to these MSys had a positive effect on the formation of sotolon, but as all MSys were simulating sweet FWs (125 g/L of sugar), the role of MR could not be assured. During this present study, extra-dry MSys were assessed, and a slight increase was also observed with the addition of amino acids to these MSys. However, this increase seems to be only significant in the "SFWFru1Arg". The increase is thus much higher within the sweet MSys, except for "SFWFru100Cys". Apart from the role of MR, these results seem to also corroborate the hypothesis by Pereira et al [19], suggesting the fructose degradation in acidic medium, favoured by high temperatures, to play a greater role in the formation of sotolon. Even a low amount of fructose was enough to solely contribute to significant amounts of sotolon. Unfortunately, the role of glucose could not be assessed during this study.

Sotolon quantification studies in oxidatively aged wines are often accompanied by strong correlations with furan-based compounds arising from the MR. As stated before, Silva Ferreira et al. [66] and Câmara et al. [5] found a high correlation between the formation of sotolon and sugar derivatives such as furfural and HMF in barrel-aged port and MWs, respectively. More recently, Maslov et al. [106] also found high amounts of stolon in Croatian predicate natural sweet wines (up to about 1.5 mg/L) of different vintages. Both sotolon and furfural were also showed to increase with the wine's age. Even though the quantification of these furan compounds was not employed in this present study, these previous reports along with the results obtained by the amino acid MSys can imply the potential role of Maillard-type reactions as well.

Although the incorporation of the amino acids increased sotolon concentration, albeit not significant in every respective MSys, the results of both threonine and cysteine can be highlighted.

Role of threonine

The role of threonine was assessed in a sweet FW MSys ("SFWFru100Thr"). As was discussed before, this amino acid is thought to be a potential source of 2-KBA, a well-known intermediary for the formation of sotolon in biologically-aged wines and/or *sake* [8,36]. Here, the non-enzymatic formation of 2-KBA through the acid-degradation of threonine could be tested. If somehow the "SFWFru100Thr" MSys generated a significantly higher amount of sotolon when in comparison to the addition of the other sweet and amino acid MSys, one could attribute its formation to the potential role of this mechanism. The results, however, were very similar among the tested amino acids. In fact, arginine contributed more to the sotolon formation than any other amino acid. Actually, in the specific case of Port wine, Silva Ferreira et al. [66] did not observe any correlation of 2-KBA with sotolon nor with ageing time. Although high quantities were found (up to 2 mg/L) in wines older than 10 years these values were always lower than 0.5 mg/L. The possibility that 2-KBA might play a role in the formation of the lactone is still plausible, but its presence might to not be exclusively related to the acid-degradation of threonine. 2-KBA was also shown to be derived from acetaldehyde in earlier reports. The aldol condensation between 2-KBA and acetaldehyde was thought to be one of the more probable routes of formation involved in wines subjected to oxidative conditions [205]. Acetaldehyde is particularly present in oxidatively and biologically-aged wines as a result of either ethanol oxidation or *flor* yeast activity, respectively. High values are normally found in *Fino* Sherry wines (230–550 mg/L) [202]. In the case of MW, relative lower concentrations were previously reported (18–117 mg/L) [15,204]. However, this mechanism cannot be verified within these studied conditions. Composition studies on the aged MSys could then be of great interest to better understand the formation of the lactone through these mechanisms.

Potential role of cysteine on the inhibition of sotolon

The MSys were all stored with a considerable amount of headspace and the role of oxygen and/or oxidative conditions can be considered. The results obtained from the "SFWFru100Cys" MSys are of particular interest and might be related to the oxidative mechanisms in the formation of sotolon. The amount of sotolon in this MSys was only 591 ± 23 μg/L. This quantified value was quite low when compared to the amounts generated among the other amino acid sweet MSys. Cysteine is known to be involved in the formation of strong odorants during the MR [225]. Hofmann and Schieberle [209] found sotolon to be formed in thermally processed binary mixtures of cysteine and sugars, although sotolon contributed with lower FD factors to the overall odour.

Sulphur dioxide (SO_2) is a substance with antiseptic, antioxidant, and antioxidasic properties that has been vastly used in winemaking [226]. Although considered to be toxic and allergenic, sulfites are still considered the most effective additives at controlling wine oxidation [227]. Cysteine is a sulfur-

containing amino acid and, along with glutamic acid and glycine, is part of the composition of a well-known tripeptide naturally found in wine—glutathione (γ-glutamyl-cysteinyl-glycine). The reductive property of the free sulfhydryl group supported by cysteine contributes to some beneficial antioxidant effects of glutathione during the wine ageing [228]. Some studies have focused on the possibility of glutathione replacing SO_2 in winemaking, acting as a natural antioxidant [229]. Glutathione can exist in either reduced (commonly known as GSH) or oxidised form (glutathione disulfide or GSSG). Dubourdieu and Lavigne [222] have shown the addition of 10 mg/L of GSH to prevent sotolon formation in dry white wines after three years of storage. In some way, a similar mechanism through the free amino acid could have occurred in the "SFWFru100Cys" MSys, causing the observed decrease which might be related to the oxidative formation of sotolon. Free cysteine is known to readily oxidise to cystine (its corresponding disulfide) forming the cysteine/cystine redox couple. This antioxidant activity would be similar to the GSH/GSSG redox couple [230]. This result is interesting as it could confirm that an oxidative mechanism does indeed take part in the formation of sotolon in these studied conditions. However, chemical oxidation requires transition metal ions [231] and to the best of our knowledge these were not among the MSys composition. Still, the quantified amount of sotolon found for this MSys can be related with a lower yield caused by the activity of cysteine or indicative of a different mechanism taking place. It would seem as if the presence of cysteine supressed a major formation mechanism and the relative lower amount of sotolon was related with "left-over" mechanisms. Perhaps fructose degradation mechanisms are persistent, as the quantified amount is similar and not significantly different from the amount in "SFWFru100" MSys.

5.3.2.2 The deconstructed model systems

A preliminary attempt at further deconstructing these MSys was also made to better elucidate the main formation pathways of sotolon in such FWs. Among these MSys, no sotolon was detected in all but the "Fru100EtOH" MSys, which generated sotolon in moderate amounts (497 ± 19.2 μg/L).

These results imply that sotolon is formed from fructose only when ethanol (18% vol.) is present in the composition of the MSys. The "Fru1EtOH" MSys, however, did not generate detectable amounts of sotolon. The only difference between this MSys and "SFWFru1" is the acid used to acidify them: hydrochloric acid and tartaric acid, respectively. In this case, it seems like the presence of tartaric acid might have played a role in the generation of sotolon. On the other hand, no statistical difference was observed for the amounts formed between "Fru100EtOH" and "SFWFru100" MSys. Thus, the role of tartaric acid is not clearly demonstrated, and further tests should be employed.

The results from the "Fru1" and "Fru100" MSys also further elucidate the potential role of ethanol in the formation of sotolon. While the degradation of fructose in acidic media was suggested as a probable mechanism, it seems like it could not solely contribute to the formation of sotolon in the absence of ethanol. Given the high concentrations of sotolon found in some FWs, it is not at all farfetched to expect ethanol to have a major contribution to its formation. The higher levels of the lactone in FWs was actually suggested to be a result of a strict oxidative mechanism involving

acetaldehyde [205]. Acetaldehyde is known to result, in part, due to the oxidation of ethanol [232]. Interestingly, it would be plausible that acetaldehyde could then originate 2-KBA and further condensation led to the formation of sotolon, as previously proposed by Pisarnitskiĭ et al. [205] in some foodstuffs. In this case, however, and similarly to fructose, ethanol by itself ("EtOH") did not lead to the formation of the lactone. This was an interesting result and can be explained by the mechanics of ethanol oxidation in wines. The oxidation of ethanol to acetaldehyde is metal-catalysed and particularly dependent on the activity of the Fe^{3+}/Fe^{2+} redox couple in the reduction of hydrogen peroxide (H_2O_2) [232]. Such transition metals were not added to the composition of these MSys. Actually, the production of this aldehyde by the direct oxidation of ethanol is thought to be quite scarce [202]. Thus, this mechanism could not be verified within these studied conditions. This could suggest that 2-KBA did not have a major role, if any at all, in the formation of sotolon among these studied MSys. While its presence as a result of either ethanol oxidation or through oxidation of threonine is still plausible in a real case scenario, it possibly does not constitute the only source of sotolon in these wines. This is in part in agreement with Silva Ferreira et al. [66], which did not observe any apparent correlation between sotolon content and 2-KBA in Port wine.

In summary, sotolon could be formed due to different mechanisms in these wines. As proposed by Silva Ferreira et al [66] for Port wine, the "hybrid-formation" of the compound is also here suggested. These pathways most likely involve sugars, either through Maillard-like reactions and/or sugar degradation mechanisms due to the high temperatures and acidic media. Furthermore, these mechanisms seem to be dependent on ethanol content. The results obtained from "SFWFru100Cys" are of particular interest as they could imply the major role of oxidative reactions in its formation, which would be in accordance to the reported importance of oxygen in its formation [92]. Further studies are still necessary to better elucidate these mechanisms.

Chapter 6

GENERAL CONCLUSIONS

6.1 Main remarks

The main aim of this study was to establish the odour impact of sotolon in currently available MW Blends and subsequently understand how this compound may be formed in this kind of beverage. The experimental part of this thesis, however, started with the development of a simple, fast, and environmentally-friendly methodology for the quantification of sotolon in these FW matrices. The proposed methodology was validated, showing good performance results in terms of linearity, sensitivity, selectivity, precision, and accuracy. The method then proofed itself useful in the following experiments related to the main goals of this thesis. The following results stand out.

Regarding the sensorial impact of sotolon in MW Blends:

- The OT of sotolon was determined in MW for the first time. Sensorial tests followed the ASTM E679 practice with 22 panellists. From a total of 318 3-AFC presentations, the OT was estimated at 112 μg/L in a sweet 3-year-old MW Blend. This value was about 6-fold higher than the previously reported value for Port wine.
- The ORT of sotolon was also attempted in MW for the first time. Within the concentration range of 253–3464 μg/L no rejection was observed for a sweet 5-year-old MW Blend.
- Further evaluations of the OT of sotolon in MW were reassessed with a selected panel and an improved sensorial design. This time, BETs ranged from 23 up to 69 μg/L for four 3-year-old MW Blends from a total of 55 3-AFC presentations for each wine.
- The lowest determined OT was used to study the impact of sotolon in MW. The odour relevance of sotolon was appraised in the aroma of 3-, 5-, and 10-year-old MW Blends. By calculating the OAVs, results showed a distribution ranging 0.1–22, increasing with Blend age. Sotolon was found to be a key contributor to the overall aroma of these wines, although more impactful in sweet-styled and 10-year-old Blends.

Regarding the sotolon formation:

- The analysis of the 17 MSys submitted to accelerated ageing revealed concentrations of sotolon ranging from not detected up to about 1.1 mg/L. These values are in accordance with what is normally found for oxidatively-aged wines, particularly sweet MW aged for a long

period. Thus, these accelerated ageing conditions can probably be used to simulate the long-term ageing of Madeira-like wines.

- For the first time, fructose levels as low as 1 g/L were shown to generate sotolon at levels higher than its OT in MWs. However, the presence of ethanol (18% vol.) was required for this formation.
- The addition of amino acids to the composition of the sweet MSys significantly increased sotolon (up to about 113%)—except for the case of cysteine—thus allowing for the consideration of the role of the MR.
- The acid-catalysed fructose degradation mechanism, as previously hypothesised by Pereira et al [19], was corroborated to play a major role in the formation of sotolon.
- Results from cysteine-containing MSys suggest the potential role of an oxidative mechanism taking part in the formation of sotolon. However, due to the nature of chemical oxidation mechanisms in wines, the role of these mechanisms raises some doubts in its formation within the studied conditions.

6.2 What to look forward

The present thesis results allowed a better understanding of the odour impact of sotolon in currently available MW Blends. The accelerated ageing experiments with the MSys also revealed some insights on the formation of the lactone in this beverage. However, the following topics are of great interest and can be further explored.

6.2.1 The effect of sotolon on the wine's expected value and aromatic complexity

Earlier studies have demonstrated the importance of sotolon to the aroma of many wines. As discussed, the determination of OTs and calculation of OAVs serve as a good tool to estimate the relative odour significance of odorant compounds in complex matrices. Here the focus was only on one compound—sotolon—as its impact had already been demonstrated among other compounds in MW by the works of Campo et al. [6] and Oliveira e Silva et al. [22]. Mainly based on that premise, the results here demonstrated and confirmed the impact of sotolon in currently available MW Blends. Although sensorial data may not be easily comparable—but considering that OAVs are directly related with the compound concentration—it was possible to verify that sotolon is found at higher levels in current MW Blends when in comparison to the MW Blends used in the earlier studies. As a potential key compound, it is thus of great interest to understand if sotolon content is correlated with the expected value and aromatic complexity of these wines. Further sensorial analysis could confirm a crescent market interest in more complex MW, which is then directly reflected in the sotolon content of these wines.

6.2.2 Further investigation into the formation pathways of sotolon

The results obtained from the quantification of sotolon in the MSys submitted to accelerated ageing probably raised more questions than answers. Although the role of fructose, ethanol and amino acid addition was somewhat clear, some of these results need to be further elucidated and re-assessed. From the role of tartaric acid to a better assessment of the role of cysteine, and the preparation of other MSys with different combinations, the possibilities are quite overwhelming. Foremost, it seems important to firstly re-evaluate these same MSys in an *estufagem*-like setting (45 °C for three months, for example). Although these results might correctly simulate long ageing periods, it would be of great importance to assess the mechanisms of formation during the *estufagem* process and its occurrence in younger MWs. Apart from the focus on MSys, studies could also make use of enantiomeric analysis in MWs, further verifying the contribution of stereoselective mechanisms to the sotolon's formation.

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Appendix A. Supplementary material regarding the quantification of sotolon in fortified wines through the developed method

Table A-1 Detailed results of the quantification of sotolon in the 44 fortified wines.
Sotolon values expressed as mean value ± standard deviation.

sample	age (years)	wine style	sotolon (µg/L)
1	und	sweet	8.3 ± 0.9
2	3	sweet	6.3 ± 0.4
3	3	sweet	9.6 ± 0.4
4	3	sweet	83 ± 1
5	3	sweet	66.2 ± 0.6
6	3	sweet	62 ± 3
7	3	sweet	65 ± 3
8	3	sweet	63.1 ± 0.5
9	3	sweet	17 ± 1
10	3	sweet	18.0 ± 0.9
11	5	sweet	145 ± 6
12	5	sweet	173 ± 6
13	5	sweet	254 ± 7
14	5	sweet	320 ± 4
15	5	sweet	111 ± 3
16	6	sweet	264 ± 16
17	7	sweet	214 ± 2
18	9	sweet	268 ± 13
19	13	sweet	493 ± 5
20	17	sweet	509 ± 24
21	18	sweet	645 ± 8
22	19	sweet	461 ± 15
23	21	sweet	697 ± 20
24	und	medium sweet	550 ± 13
25	und	medium sweet	398 ± 28
26	11	medium sweet	137 ± 5
27	20	medium sweet	283 ± 30
28	22	medium sweet	346 ± 20
29	51	medium sweet	623 ± 27
30	55	medium sweet	487 ± 75
31	87	medium sweet	739 ± 49
32	97	medium sweet	393 ± 25
33	115	medium sweet	810 ± 20
34	und	medium dry	494 ± 87
35	und	medium dry	274 ± 10
36	5	medium dry	176 ± 19
37	20	medium dry	283 ± 13
38	22	medium dry	142.0 ± 0.4
39	40	medium dry	417 ± 9
40	und	dry	427 ± 15
41	und	dry	186 ± 1
42	12	dry	113 ± 8
43	27	dry	242 ± 12
44	38	dry	346 ± 3

und, undisclosed age.

Appendix B. Supplementary material regarding the sensorial impact of sotolon in Madeira wine

Tese de Mestrado em Bioquímica Aplicada

Aromas-chave do vinho Madeira: impacto sensorial e principais precursores

João Marcelo Gontardo Gaspar

Orientador: Prof. Dr. José Carlos Marques

Co-orientador: Dra. Vanda Pereira

3-AFC TEST Código: ODTXX.X

Nome: _____ Data: _____

Idade: _____

INSTRUÇÕES:

Em cada grupo de 3 amostras, duas são idênticas. **Cheire as 3 amostras e indique, com um círculo, a amostra diferente para cada um dos 5 grupos**. Caso não consiga distinguir deve seleccionar uma amostra ao acaso.

Grupo 1	311	944	717
Grupo 2	613	883	121
Grupo 3	574	157	179
Grupo 4	921	513	594
Grupo 5	783	615	991

Figure B-1 Specimen of the paper ballot given to each panellist for the determination of the odour threshold of sotolon in Madeira wine through the three-alternative forced-choice (3-AFC) test.

Table B-1 Individual best estimate thresholds (BETs) obtained for each trial for the determination of the odour threshold of sotolon in sweet-styled 3-year-old Madeira wine.
Individual BETs calculated as the geometric mean of the last missed and the next correct concentration. Panel threshold calculated as the arithmetic mean of each individual BET.

	individual log_{10} BETs within each trial						
panellist	1st	2nd	3rd	4th	other[1]	avg	SD
P1	2.03	2.34	nt	nt	nt	2.19	0.22
P2	2.34	2.03	nt	nt	nt	2.19	0.22
P3	2.34	2.34	nt	nt	nt	2.34	0.00
P4	2.34	2.34	1.70	2.03	nt	2.10	0.31
P5	2.03	1.34	2.03	1.34	nt	1.68	0.40
P6	2.03	nt	2.34	nt.	nt	2.19	0.22
P7	1.70	2.03	0.87	0.87	nt	1.37	0.59
P8	2.03	1.34	1.70	nt.	nt	1.69	0.34
P9	2.34	2.03	2.65	2.34	nt	2.34	0.25
P10	2.34	2.03	nt	2.65	nt	2.34	0.31
P11	2.03	1.70	0.45	2.34	1.34	1.57	0.73
P12	2.03	nt	2.65	2.65	nt	2.44	0.36
P13	2.34	nt	1.70	nt	nt	2.02	0.45
P14	1.70	1.70	2.03	0.45	nt	1.47	0.70
P15	nt	2.34	1.70	2.34	nt	2.13	0.37
P16	nt	2.03	2.34	nt	nt	2.19	0.22
P17	nt	nt	2.65	2.03	nt	2.34	0.44
P18	nt	nt	1.70	nt	nt	1.70	n/a
P19	nt	nt	2.65	nt	nt	2.65	n/a
						log_{10} total	38.93
						log_{10} avg	2.05
						log_{10} SD	0.36
						BET	112

nt, *not tested*; a*vg*, *average (arithmetic mean)*; *SD*, *standard deviation*; *n/a*, *not applicable*;
[1]panellist P11 performed a repetition of the second trial.

Table B-2 Individual odour best estimate thresholds (BETs) and selected panel BET found for sotolon in dry-styled 3-year-old Madeira wine from producer A.
Individual BETs calculated as the geometric mean of the last missed and the next correct concentration. Panel threshold calculated as the arithmetic mean of each individual BET.

	concentration scale-steps (µg/L)					BET	
panellist	16	40	100	250	625	value	log_{10}
S4	+	+	+	+	+	10	1.01
S5	0	+	+	+	+	25	1.40
S7	+	+	+	+	+	10	1.01
S11	+	0	0	+	+	158	2.20
S14	+	+	+	+	+	10	1.01
S21	0	0	+	+	+	63	1.80
S4	+	+	+	+	+	10	1.01
S7	0	+	+	+	+	25	1.40
S11	+	+	+	+	+	10	1.01
S14	0	+	+	+	+	25	1.40
S21	0	0	+	+	+	63	1.80
						log_{10} total	15.04
						log_{10} avg	1.37
						log_{10} SD	0.42
						BET	23.3

+, correct judgement at corresponding scale-step; 0, incorrect judgement at corresponding scale-step; avg, average (arithmetic mean); SD, standard deviation.

Table B-3 Individual odour best estimate thresholds (BETs) and selected panel BET found for sotolon in sweet-styled 3-year-old Madeira wine from producer A.
Individual BETs calculated as the geometric mean of the last missed and the next correct concentration. Panel threshold calculated as the arithmetic mean of each individual BET.

	concentration scale-steps (µg/L)					BET	
panellist	16	40	100	250	625	value	log_{10}
S4	+	+	+	+	+	10	1.01
S5	0	+	+	+	+	25	1.40
S7	+	+	+	+	+	10	1.01
S11	+	+	+	+	+	10	1.01
S14	0	0	+	+	+	63	1.80
S21	0	0	+	+	+	63	1.80
S4	+	+	+	+	+	10	1.01
S7	0	+	+	+	+	25	1.40
S11	0	0	0	+	+	158	2.20
S14	0	+	+	+	+	25	1.40
S21	+	0	0	+	0	988	2.99
						log_{10} total	17.03
						log_{10} avg	1.55
						log_{10} SD	0.62
						BET	35.3

+, correct judgement at corresponding scale-step; 0, incorrect judgement at corresponding scale-step; avg, average (arithmetic mean); SD, standard deviation.

Table B-4 Individual odour best estimate thresholds (BETs) and selected panel BET found for sotolon in dry-styled 3-year-old Madeira wine from producer B.
Individual BETs calculated as the geometric mean of the last missed and the next correct concentration. Panel threshold calculated as the arithmetic mean of each individual BET.

	concentration scale-steps (µg/L)					**BET**	
panellist	16	40	100	250	625	value	log_{10}
S4	+	0	+	+	+	63	1.80
S5	0	0	0	+	+	158	2.20
S7	+	0	+	+	+	63	1.80
S11	0	0	+	+	+	63	1.80
S14	0	+	+	+	+	25	1.40
S21	0	+	0	0	+	395	2.60
S4	+	+	+	+	+	10	1.01
S7	+	0	+	+	+	63	1.80
S11	0	0	0	+	+	158	2.20
S14	0	+	+	+	+	25	1.40
S21	0	0	+	0	0	988	2.99
						log_{10} total	20.21
						log_{10} avg	1.84
						log_{10} SD	0.63
						BET	68.7

+, correct judgement at corresponding scale-step; 0, incorrect judgement at corresponding scale-step; avg, average (arithmetic mean); SD, standard deviation.

Table B-5 Individual odour best estimate thresholds (BETs) and selected panel BET found for sotolon in sweet-styled 3-year-old Madeira wine from producer B.
Individual BETs calculated as the geometric mean of the last missed and the next correct concentration. Panel threshold calculated as the arithmetic mean of each individual BET.

	concentration scale-steps (µg/L)					**BET**	
panellist	16	40	100	250	625	value	log_{10}
S4	+	+	+	+	+	10	1.01
S5	0	+	+	+	0	988	2.99
S7	+	+	+	+	+	10	1.01
S11	0	+	+	+	+	25	1.40
S14	0	0	+	+	+	63	1.80
S21	0	0	+	+	+	63	1.80
S4	0	+	+	+	+	25	1.40
S7	0	+	+	+	+	25	1.40
S11	+	+	+	+	+	10	1.01
S14	+	+	+	+	+	10	1.01
S21	0	0	0	+	0	988	2.99
						log_{10} total	17.82
						log_{10} avg	1.62
						log_{10} SD	0.74
						BET	41.7

+, correct judgement at corresponding scale-step; 0, incorrect judgement at corresponding scale-step; avg, average (arithmetic mean); SD, standard deviation.

Table B-6 Concentration and odour activity values (OAVs) for sotolon in the studied Madeira wine Blends.
Sotolon values expressed as mean value ± standard deviation.

sample	age (years)	wine style	sotolon (µg/L)	OAV
1	3-year-old	dry	82.2 ± 4.0	3.6
2			3.6 ± 1.0	< 1
3			48.6 ± 2.3	2.1
4			54.8 ± 2.5	2.4
5			48.4 ± 2.7	2.1
6			57.5 ± 2.3	2.5
7			32.1 ± 4.4	1.4
8			68.3 ± 1.2	3.0
9			37.0 ± 0.4	1.6
10			389.2 ± 12.3	16.9
11			115.5 ± 9.8	5.0
12	3-year-old	medium dry	35.5 ± 4.7	1.5
13			3.3 ± 0.3	< 1
14			36.8 ± 0.7	1.6
15			48.0 ± 1.4	2.1
16			103.5 ± 4.1	4.5
17			44.2 ± 4.7	1.9
18			42.3 ± 1.7	1.8
19			32.2 ± 3.0	1.4
20			86.0 ± 0.5	3.7
21			125.2 ± 3.9	5.4
22	3-year-old	medium sweet	42.1 ± 6.8	1.8
23			6.9 ± 0.6	< 1
24			34.5 ± 1.6	1.5
25			69.7 ± 0.9	3.0
26			98.8 ± 1.4	4.3
27			96.1 ± 0.7	4.2
28	3-year-old	sweet	33.6 ± 9.2	1.5
29			2.0 ± 0.9	< 1
30			61.9 ± 10.1	2.7
31			38.9 ± 0.7	1.7
32			57.1 ± 5.1	2.5
33			50.8 ± 3.8	2.2
34			47.3 ± 2.3	2.1
35			40.4 ± 2.9	1.8
36			48.4 ± 2.6	2.1
37			38.8 ± 1.3	1.7
38			49.8 ± 3.2	2.2
39			68.3 ± 1.4	3.0
40			89.9 ± 7.1	3.9
41			145.5 ± 7.6	6.3
42	5-year-old	dry	19.4 ± 2.8	< 1
43			73.6 ± 7.2	3.2
44			86.2 ± 10.3	3.7
45			26.7 ± 2.0	1.2
46			165.3 ± 7.1	7.2
47	5-year-old	medium dry	84.0 ± 1.7	3.7
48			52.4 ± 4.6	2.3
49			138.6 ± 4.5	6.0
50			173.5 ± 8.0	7.5
51			147.2 ± 1.6	6.4
52			120.2 ± 4.0	5.2
53			170.7 ± 4.4	7.4
54	5-year-old	medium sweet	23.9 ± 0.4	1.0
55			84.0 ± 7.8	3.7

(*continued*)				
56	5-year-old	medium sweet	145.4 ± 8.8	6.3
57			147.9 ± 9.6	6.4
58			167.0 ± 11.0	7.3
59			210.9 ± 4.3	9.2
60			163.7 ± 2.1	7.1
61			106.5 ± 14.2	4.6
62			227.7 ± 14.2	9.9
63	5-year-old	sweet	31.1 ± 3.6	1.4
64			50.7 ± 3.5	2.2
65			173.4 ± 11.7	7.5
66			313.0 ± 4.4	13.6
67			286.0 ± 6.0	12.4
68			195.1 ± 14.8	8.5
69			275.6 ± 5.7	12.0
70			311.4 ± 9.1	13.5
71	10-year-old	dry	171.0 ± 16.0	7.4
72			254.4 ± 11.0	11.1
73			241.6 ± 15.8	10.5
74			161.3 ± 5.1	7.0
75	10-year-old	medium dry	294.2 ± 1.7	12.8
76			147.1 ± 3.2	6.4
77			163.7 ± 19.0	7.1
78			78.5 ± 1.5	3.4
79	10-year-old	medium sweet	108.0 ± 1.7	4.7
80			180.5 ± 3.2	7.8
81			347.5 ± 19.0	15.1
82			63.8 ± 2.1	2.8
83			299.6 ± 0.6	13.0
84	10-year-old	sweet	227.3 ± 20.7	9.9
85			121.6 ± 7.5	5.3
86			98.4 ± 1.8	4.3
87			393.6 ± 24.5	
88			516.1 ± 16.7	
89			401.0 ± 21.0	

Appendix C. Supplementary material regarding the quantification of sotolon in model systems submitted to accelerated ageing

Table C-1 Detailed quantification results for sotolon in the model systems (MSys) submitted to accelerated ageing conditions.
Results expressed as mean value ± standard deviation.

MSys code	sotolon (µg/L)
EtOH	nd
Fru1	nd
Fru100	nd
Fru1EtOH	nd
Fru100EtOH	497 ± 19.2
SFW	46 ± 17
SFW_Fru1	232 ± 68
SFW_Fru1Arg	373 ± 30
SFW_Fru1Cys	326 ± 51
SFW_Fru1GABA	284 ± 14
SFW_Fru1Asp	315 ± 15
SFW_Fru100	534 ± 23
SFW_Fru100 Arg	1142 ± 70
SFW_Fru100 Cys	591 ± 171
SFW_Fru100 GABA	1008 ± 81
SFW_Fru100 Asp	940 ± 44
SFW_Fru100Thr	1020 ± 88

nd, not detected.