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CARACTERIZACIÓN QUÍMICA Y BIOACTIVIDAD DE COMPUESTOS PRODUCIDOS POR LEVADURAS Y DERIVADOS DE LA VID

Memoria presentada por el licenciado EDWIN FERNÁNDEZ CRUZ para optar el Título de Doctor por la Universidad de Sevilla con la mención de "Doctor Internacional"

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A mis Padres

A Félix

"El mayor error que una persona puede cometer es tener miedo de cometer un error"

Elbert Hubbard

"The greatest mistake you can make in life is to be continually fearing you will make one"

Elbert Hubbard

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RESUMEN

En los últimos años, la melatonina ha sido identificada en diversas matrices alimentarias, principalmente de origen vegetal como frutas (uvas, fresas) y verduras (tomates, pimientos). Sin embargo, ha generado un notable interés el hecho de detectar melatonina en alimentos fermentados como el vino o la cerveza. Todo parece indicar que la presencia de esta molécula en alimentos fermentados está directamente relacionada con el metabolismo de las levaduras, especialmente del género *Saccharomyces cerevisiae*, durante los procesos fermentativos.

La melatonina es bien conocida en mamíferos como neurohormona sintetizada por la glándula pineal y su función principal es la de regular los ciclos circadianos de sueño/vigilia dependiendo de las condiciones de luz y oscuridad. Además, también ha mostrado otras propiedades bioactivas como molécula antioxidante, siendo una excelente captadora de especies reactivas de oxígeno. Por otro lado, ha sido también descrita en plantas y en microorganismos, aunque en este caso sus funciones no están completamente dilucidadas. La melatonina se forma a partir del aminóacido triptófano, que pertenece al grupo de aminoácidos aromáticos junto a la tirosina y la fenilalanina. Su proceso de formación en mamíferos contempla como intermediarios moléculas como el 5-hidroxitriptófano, la serotonina o la N-acetilserotonina. En plantas, la triptamina también puede formar parte de la ruta de formación de la melatonina en lugar del 5-hidroxitriptófano. Sin embargo, en microorganismos no está claramente definida su ruta metabólica de síntesis. En el caso de las levaduras, el metabolismo del triptófano produce asimismo el ácido indolacético y el triptofol, derivados de la ruta de Ehrlich.

Las técnicas analíticas para cuantificar melatonina en fluidos biológicos y alimentos abarcan desde el radioinmunoensayo, en el que destaca la técnica ELISA, hasta las técnicas de cromatografía líquida de alta resolución con detectores de masas de última generación. El principal reto es poder detectar y cuantificar concentraciones a nivel de traza. Las primeras han demostrado ser eficaces en fluidos biológicos como el plasma o la saliva. No obstante, cuando se han aplicado a matrices alimentarias, los resultados han sido dispares, hecho motivado por posibles interferentes de la compleja matriz alimentaria que puedan reaccionan con los anticuerpos utilizados en el ELISA. Por ello, la Cromatografía Líquida de Alta Resolución ha mostrado ser la mejor alternativa para el análisis de melatonina y de otros compuestos derivados del aminoácido triptófano.

En un principio, se desarrollaron métodos de cromatografía líquida con detectores de fluorescencia, pero debido a las bajas concentraciones de la melatonina en alimentos fermentados, los límites de detección y cuantificación de estos detectores fueron

insuficientes para la identificación inequívoca de esta molécula. En la actualidad, la cromatografía acoplada a espectrómetros de masas parece ser la más indicada para el estudio de la melatonina. Sin embargo, la mayor parte de métodos publicados solo contemplan el análisis de la melatonina de forma aislada, por lo que no permiten ofrecer una visión global sobre su posible vía de formación en alimentos fermentados.

La presente Tesis Doctoral tiene como uno de sus objetivos desarrollar un método cromatográfico acoplado a espectrometría de masas para identificar simultáneamente diferentes compuestos derivados del metabolismo del triptófano y relacionados con la ruta de síntesis de la melatonina. Además, se contempla el estudio de la estabilidad de estas moléculas frente al almacenamiento prolongado para comprobar el posible efecto matriz. Asimismo, se realizan diferentes ensayos de fermentación alcohólica tanto en mostos sintéticos como naturales a fin de seleccionar las levaduras con mayor capacidad de producción de compuestos indólicos derivados del triptófano. A su vez, se analizaron cervezas comerciales para dilucidar si en otras matrices distintas al vino se detectaba la presencia de compuestos indólicos.

Asimismo, se han realizado estudios de bioactividad de compuestos estilbenoides, derivados del aminoácido fenilalanina y presentes en raspones de la vid (*Vitis vinífera*). Estos ensayos tienen como objetivo evaluar la capacidad de estos compuestos frente a la inhibición del proceso de angiogénesis, que en adultos está involucrado en la patogenia del cáncer y las enfermedades cardiovasculares, consideradas como las principales causas de muerte en el mundo, según los últimos datos de la OMS. Debido a que el factor de crecimiento endotelial vascular (VEGF) es principal factor pro-angiogénico, la inhibición de VEGF es un mecanismo molecular plausible que demuestra una causa-efecto directa en la disminución del riesgo cardiovascular y de cáncer. Prueba del crucial rol de VEGF en la angiogénsis es el hecho de que ciertos fármacos frente al cáncer consisten en moléculas anti-VEGF, como el Avastin®. Sin embargo, debido al uso prolongado de las terapias anti-VEGF se han descrito serios efectos adversos, como hipertensión.

Ciertos compuestos polifenólicos presentes en los alimentos como el galato de epicatequina, quercetina, entre otros, han demostrado poseer potencial anti-VEGF a la vez que activan la enzima óxido nítrico sintetasa endotelial (eNOS), responsable de la vasodilatación. Por tanto, se evaluó el potencial anti-VEGF de 12 compuestos de la familia de los estilbenos en una línea de células endoteliales de vena de cordón umbilical humano (HUVEC). Además, se determinó su efecto en la cascada de señalización

intracelular, en concreto sobre las proteínas fosfolipasa gamma 1 (PLCγ1), responsable de la proliferación celular, la protein quinasa B (Akt) e eNOS.

Los resultados más notables derivan del desarrollo de un método analítico validado que por primera vez identificaba simultáneamente nueve derivados del metabolismo del triptófano. Dicho método ha sido de utilidad para evaluar el posible efecto matriz de muestras fermentadas, dando por resultado que la cerveza es la matriz que sufre menos alteraciones en cuanto a la composición de los compuestos indólicos, estableciendo como mejor temperatura de almacenamiento 4ºC. Las otras matrices probadas (vino, mosto sintético fermentado y extracto intracelular) fueron más inestables a lo largo del periodo de almacenamiento.

Por otro lado, se confirmó la producción de melatonina y otros compuestos derivados del metabolismo del triptófano como la serotonina, el ácido 3-indolacético, triptofol y un ester etílico del triptófano durante la fermentación alcohólica de mostos sintéticos, resultando la cepa de levaduras *Saccharomyces cerevisiae* Aroma White la mayor productora de melatonina. Los resultados, además, fueron reproducibles en mostos de uva natural de distintas variedades de uva (Corredera, Chardonnay, Moscatel, Palomino Fino, Sauvignon Blanc, Tempranillo, Vijiriega), donde la levadura Aroma White volvió a ser la mayor productora de melatonina. A su vez, destaca la descripción por primera vez de uno de los intermediarios de la ruta de la melatonina, la N-acetilserotonina, en fermentaciones llevadas a cabo en mostos de la variedad Tempranillo. Además, se ha analizado el contenido de melatonina y otros compuestos indólicos en diferentes cervezas comerciales, entre los que destaca la identificación por primera vez de 5-hidroxitriptófano, *N*-acetilserotonina y ácido 3-indolacético.

En cuanto a la bioactividad de estilbenos, se han seleccionado 5 compuestos (astringina, piceatanol, pallidol, ε- y ω-viniferina) capaces de inhibir VEGF (IC_{50} = 2,9-39,4 µM). Además, las dos viniferinas fueron capaces de inhibir PLCγ1 al mismo tiempo que activaron eNOS, por lo que cabría esperar que no presentaran el efecto hipertensivo asociado a los actuales fármacos anti-VEGF.

Los resultados son sin duda de interés a fin de mejorar los procesos de vinificación para que estos compuestos de la vid y los sintetizados por las levaduras durante la fermentación estén presentes en el vino, dándole un valor añadido al producto final, y trasladando las prácticas a la fabricación de otras bebidas fermentadas como la cerveza.

SUMMARY

In recent years, melatonin has been identified in different foods, mainly from plant origin such us fruits (grapes, strawberries) and vegetables (tomatoes and peppers). However, the identification of melatonin in fermented foods as beer and wine has attracted an increasing interest in research. Literature data indicate that the presence of this molecule in fermented food is due to the yeast metabolism, especially from Saccharomyces cerevisiae, during fermentative process.

Melatonin is a well-known neurohormone synthesized by the pineal gland of mammals. Its main function is related with circadian rhythms depending of light/darkness conditions. Besides, it has reported additional bioactive properties such as antioxidant, being an effective oxygen scavenger. On the other hand, it has also been described in plants and microorganism, although its role in both organism in not well elucidated. Melatonin is formed from the amino acid tryptophan that belongs to the aromatic amino acids groups. Mammal's metabolism of tryptophan encompasses different secondary metabolites such as 5-hydroxytryptophan, serotonin and N-acetylserotonin. In plants, tryptamine can be included on the melatonin pathway instead of 5-hydroxytryptophan. However, the metabolic pathway that produce melatonin in microorganisms is not completely defined. Nevertheless, amino acid catabolism by yeast known as the Ehrlich pathway mainly produces indole acetic acid and tryptophol.

Analytical techniques to quantify melatonin in biological fluids and foodstuff is quite diverse, covering from radioimmunoassay, being ELISA the most representative, to high resolution liquid chromatographic techniques coupled to a high resolution mass spectrometer. The main challenge of these techniques is to improve the detection and quantification limits of these compounds. ELISA technique is well performed when biological fluids are analysed. Nevertheless, its application to foodstuffs has been less repeatable, maybe due to the presence of interference in the food matrix that can react with the ELISA antibodies. For this reason, High Resolution Liquid Chromatography has proved to be the best technique for the melatonin and derived tryptophan compounds analysis.

At first, chromatographic methods with a fluorescence detector were used to determine melatonin in food. However, the low concentrations of melatonin presented in foods, and the limits of detection and quantitation of this detector make it ineffective to describe the presence of melatonin unequivocally. Nowadays, liquid chromatography coupled to mass spectrometers results the best option to study melatonin. However, most of the

reported methods only analysed melatonin. Thus, they cannot bring to light to a fully view of the likely formation pathways in fermented foods.

Therefore, one of the aims of the present doctoral thesis is to develop a chromatographic method couple to mass spectrometry that simultaneously identify different tryptophan derived compounds related with melatonin formation pathway. Additionally, the study of the compounds stability in different storage conditions was also included. Furthermore, fermentation experiments were performed in synthetic and natural must in order to find yeast with the best ability to synthesize indolic compounds derived from tryptophan. Eventually, commercial beers were analysed to dilucidate whether in other matrixes apart from wine indolic compounds are also produced.

Moreover, the bioactivity of stilbenoids compounds derived from the amino acid phenylalanine and present in grapevine stalks of *Vitis vinífera,* was also study. These experiments have the aim of evaluate the potential ability of these compounds to inhibit angiogenesis. This physiological process is involved in the development of cancer and cardiovascular diseases in adult population. Both diseases are consider the world's leading cause of death, according to the last data reported by the World Health Organisation. Since vascular endothelial growth factor (VEGF) is the main pro-angiogenic factor, its inhibition is a plausible molecular mechanism that shows a direct cause-effect for decreasing cardiovascular and cancer risk. One of the most remarkable proofs of VEGF role in angiogenesis is that some cancer treatments are based in an anti-VEGF drugs such as Avastin®, which is the most currently used. However, their lengthy treatment causes serious side effects, increasing the risk of hypertension.

Certain polyphenols that are present in foods such as epillogatechin gallate and quercetin, among others, have proved a potential anti-VEGF activity, allowing the activation at the same time of the endothelial nitric oxide synthase, responsible of vasodilation. Thus, anti-VEGF potential of twelve stilbenes was evaluated in human umbilical vein endothelial cells. In addition, the possible effect on the signalling intracellular downstream, particularly on phospholipase γ1 (PLC γ1, main responsible of cell proliferation), protein kinase B and eNOS was also studied.

Most remarkable results lead from the development and validation of an analytical method that identified simultaneously nine compounds derived from the amino acid tryptophan for the first time by UHPLC/HRMS technique in fermented synthetic must. The present method has been useful to evaluate the possible matrix effect of fermented samples, resulting beer as the matrix that caused less decrease of indolic compound

composition and establishing a storage temperature of 4ºC as the best alternative of conservation.

On the other hand, melatonin and related compounds derived from tryptophan metabolism such as serotonin, indole acetic acid, tryptophol and ethyl ester of tryptophan was confirm during the alcoholic fermentation of synthetic must, being the *S.cerevisiae* Aroma White the most melatonin producer. Additionally, it was proved the reproducibility of results in natural must of different grape varieties (Corredera, Chardonnay, Moscatel, Palomino Fino, Sauvignon Blanc, Tempranillo, Vijiriega), where Aroma White proved again to be the highest melatonin producer. Moreover, for the first time N-acetylserotonin was quantified during the alcoholic fermentation of Tempranillo must. The analysis of indolic compounds in beers pinpoints the identification of 5-hydroxytryptophan, N-acetylserotonin and indole acetic acid in beers as a novelty.

Regarding stilbenes bioactivity, astringin, piceatannol, pallidol, ε- and ω-viniferina inhibited VEGF activity (IC_{50} = 2.9-39.4 μM). In addition, both ε- and ω-viniferin were also able to inhibited PLCγ1, while activated eNOS, likely avoiding the side effects of hypertension, currently related with the anti-VEGF drugs treatment.

With no doubt, present results are remarkable to improve wine-making process by enhancing the presence of stilbenes from grapevine and the indolic compounds derived from the amino acid tryptophan by yeast metabolism in wines during the alcoholic fermentation, giving an added value to the final product and trying to emulate this practices to the production of other fermented beverages such as beer.

ABREVIATURAS

µg	Microgramos
µL	Microlitros
µM	Micromolar
3-IAA	Ácido 3-indolacético
3-IAD	Acetaldehido-3-indol
3-IPA	Ácido 3-indolpirúvico
5HT	Serotonina
5-HTRP	5-hidroxitriptófano
AC	Acetato de celulosa
ADY	Levaduras activas deshidratadas
AGC	Control automático de ganancia
Akt	Proteína quinasa B
ANOVA	Análisis de la varianza
AOAC	Asociación de Químicos Analíticos Oficiales
AW	Cepa de *S. cerevisiae* Aroma White
BCA	Ácido bicinconínico
BSA	Albumina de suero bovino
CABD	Centro Andaluz de Biología del Desarrollo
CT-QA23	Fermentación control con levadura QA23
CT-RF	Fermentación control con levadura Red Fruit
DMSO	Dimetil sulfóxido
DTT	Ditiotreitol (agente reductor de muestras)
ECV	Enfermedades cardiovasculares
EFSA	Autoridad Europea de Seguridad Alimentaria
EGCG	Epigalocatequin galato
EGM-2	Medio de crecimiento para células endoteliales 2
ELISA	Ensayo por inmunoabsorción ligado a enzimas
eNOS	Óxido nítrico sintasa endotelial
ESI	Ionización por electrospray
FA	Fermentación alcohólica
FPU	Formación de Profesorado Universitario

FULL MS^2	Espectro de masas con los principales fragmentos
FWHM	Anchura a media altura
HEPES	Ácido N-(2-hidroxietil) piperazina-N'-(2-etanosulfónico)
HESI	Ionización por electrospray calentada
HPLC	Cromatografía líquida de alta eficacia
HRMS	Espectrometría de masas de alta resolución
HSD	Tukey's Honest Significant Difference
HUVEC	Células endoteliales humanas de cordón umbilical
IATA	Instituto de Agroquímica y Tecnología Alimentaria
IC_{50}	Mitad de la concentración inhibitoria máxima
IFAPA	Instituto de investigación y Formación Agraria y Pesquera
IT	Tiempo de inyección
L	Litro
LC-DAD	Cromatografía líquida acoplada a un detector de diodos
LC-MS	Cromatografía líquida acoplada a un espectrómetro de masas
LDA	Análisis discriminante lineal
LDS	Dodecilsulfato de litio (tampón de muestra)
LOD	Límite de detección
LOQ	Límite de cuantificación
L-TRP EE	Etil ester de L-triptófano
L-TRP	L-triptófano
M. pulcherrima	*Metschnikowia pulcherrima*
m/z	Relación ente masa y carga
MEL	Melatonina
MeOH	Metanol
mg	Miligramo
Mha	Millones de hectáreas
MLT	Melatonina
mM	Milimolar
MS	Espectrometría de masas
MS-MS	Espectrometría de masas en tándem
n.d/nd	No detectable

n.q/nq	No cuantificable
NA-5HT	N-acetilserotonina
NACSERO	N-acetilserotonina
NCE	Energía de colisión normalizada
ng	Nanogramo
NL	Linea de ruido
nm	Nanómetro
NO	Óxido nítrico
NY	Nylon
OIV	Organización internacional del vino
pAkt	Proteína quinasa B fosforilada
PCA	Análisis de componentes principales
peNOS	Óxido nítrico sintasa endotelial fosforilada
PLCγ1	Fosfolipasa C gamma 1
pPLCγ1	Fosfolipasa C gamma 1 fosforilada
ppm	Partes por millón
PTFE	Politetrafluoroetileno
QTRAP	Trampa iónica de triple cuadrupolo
R^2	Coeficiente de determinación
RF	Cepa de *S. cerevisiae* Red Fruit
RIPA	Buffer de radioinmunoprecipitación
RHA	Rapontina, Raponticina
RP	Poder de resolución
rpm	Revoluciones por minuto
RSD	Desviación estándar relativa
R_T	Tiempo de retención
S. cerevisiae	*Saccharomyces cerevisiae*
SERO	Serotonina
SI-QA23	Fermentación secuencial de QA23 y *T. delbrueckii*
SI-RF	Fermentación secuencial de Red Fruit y *T. delbrueckii*
S-lens RF	Radio frecuencia de la lente S
SM	Mosto sintético

SO_2	Anhídrido sulfuroso/Dióxido de azufre
SP	Fermentación espontánea
SPE	Extracción en fase sólida
T. delbrueckii	*Torulaspora delbrueckii*
T^a	Temperatura
TBST	Buffer Tris-salino con Tween® 20
TOL	Triptofol
TRY	Triptamina
TRYPT	Triptamina
UHPLC	Cromatografía líquida de ultra-alta eficacia
UV/Vis	Luz ultravioleta/Luz visible
VEGF	Factor de crecimiento vascular endotelial
VEGFR-2	Receptor 2 de VEGF
YNB	Base nitrogenada para levaduras
YPD	Extracto de levadura con peptona y dextrosa

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INTRODUCCIÓN

BLOQUE I: COMPUESTOS INDÓLICOS

1. Triptófano

El triptófano, junto con la tirosina y la fenilalanina, conforman el grupo de los aminoácidos aromáticos (AAA), los cuales presentan anillos aromáticos en su estructura química **(Figura 1)**. Mientras que la fenilalanina y la tirosina se componen de anillos fenólicos, el triptófano posee un anillo indólico formado por un benceno (C6) y un anillo pirrólico (C5). Además, debido a esta estructura química, el triptófano es el aminoácido que presenta el mayor número de carbonos de todos los utilizados para la síntesis proteica, además de tener un fuerte carácter hidrofóbico (Palego et al. 2016).

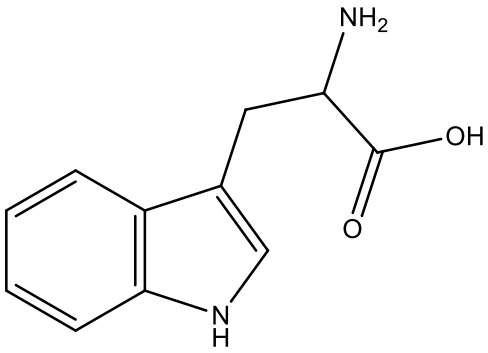

Figura 1. Estructura química del aminoácido L-triptófano

Hopkins & Cole describieron el triptófano por primera vez en 1901 (Hopkins and Cole 1901). Mientras que otros aminoácidos fueron aislados a través de una sencilla digestión ácida de proteínas, el triptófano precisó de un proceso más agresivo, con un aumento de la temperatura y el uso de tripsina, de donde cogió su nombre. Pocos años más tarde, en 1908, Ellinger y Flamand describieron la estructura por primera vez (Richard et al. 2009). El triptófano forma parte de los 9 aminoácidos esenciales para la nutrición humana, ya que se necesita de una fuente exógena para incorporarlo al organismo. Según un informe de la Organización Mundial de la Salud (OMS) sobre requerimientos de proteínas y aminoácidos en la nutrición humana, se estableció una cantidad diaria recomendada (CDR) de 250-425 mg/día, siendo la ingesta recomendada entre 3,6-6 mg/kg de peso al día de triptófano (WHO/FAO/UNU Expert Consultation 2007). Las principales fuentes de triptófano en la dieta proceden de alimentos de origen animal como la carne, pescado, marisco, huevo, leche y derivados lácteos, aunque también se encuentra en alimentos vegetales como frutos secos (anacardos, almendras, nueces), guisantes, patatas, cacao o uvas (Gutiérrez-Gamboa et al. 2018; Palego et al. 2016;

Richard et al. 2009). En la **Tabla 1** se observa el contenido aproximado de triptófano en diferentes fuentes alimentarias.

Tabla 1. Fuentes alimentarias de triptófano (adaptado de diferentes autores)

Alimento		Cantidad en mg por cada 100g/100 mL	Referencia
Atún en lata		1664,93	(Richard et al. 2009)
Queso cheddar		321	
Cacahuetes		229,28	
Leche entera		161,38	
Leche desnatada		121,47	
Pavo (carne magra)		90,39	
Copos de avena		73,5	
Pavo (carne grasa)		66,80	
Chocolate negro		56,43-63,49	
Pollo (carne grasa)		56,43	
Pollo (carne magra)		52,47	
Plátano		13,75	
Ciruelas pasas		11,76-14,28	
Pan blanco, integral		4,19-4,85	
Manzana		1,33	
Uva	Var. Kerner	0,2-8	(K. Hoenicke et al. 2001)
	Var. Riesling	0,4-3,5	(Linsenmeier, Löhnertz, and Schubert 2004)
	Var. Emir	10,9	(Ünal et al. 2015)
	Var. Narince	2,24	
	Var. Sultaniye	3,45	
	Var. Verdejo	0,25-0,4	(Ruiz-Rodríguez et al. 2017)
	Var. Treixadura	0,8-1,8	(Bouzas-Cid et al. 2018)
	Var. Chardonnay	0,5-4,5	(Vigentini et al. 2015)(Meng et al. 2018)
	Var. Croatina	0,52	(Vigentini et al. 2015)
	Var. Tempranillo	0,94-3	(Pérez-Álvarez et al. 2017) (Garde-Cerdán et al. 2017) (Gutiérrez-Gamboa et al. 2017) (González-Santamaría et al. 2018)
	Var. Monastrell	3,34	(Garde-Cerdán et al. 2017)
	Var. Carignan noir	0,62-1,55	(Gutiérrez-Gamboa et al. 2018)
	Var. Garnacha	1,31	(Gutiérrez-Gamboa et al. 2017)

1.1. Metabolismo del triptófano

Dentro del metabolismo del triptófano, existen numerosas rutas metabólicas implicadas que aparecen de manera detallada en la base de datos conocida como 'Enciclopedia de Kioto sobre genes y genomas' (KEGG) (Kanehisa et al. 2016, 2017; Ogata et al. 1999). Las rutas metabólicas en mamíferos, plantas o microorganismos se desarrollan en función de la presencia o ausencia de determinadas enzimas en su metabolismo. En humanos, el metabolismo del triptófano implica la formación de diferentes compuestos de gran interés biológico como la serotonina. Este metabolito se forma a través del triptófano por acción de la enzima triptófano hidroxilasa, dando lugar a 5-hidroxitriptófano, que posteriormente es descarboxilado a serotonina en una reacción dependiente de vitamina B6 (Le Floc'h, Otten, and Merlot 2011). Además, en la misma ruta, la serotonina puede acetilarse en N-acetilserotonina para la síntesis de melatonina, una neurohormona relacionada con los ciclos de sueño/vigilia y cuyo precursor también es el triptófano (de Almeida et al. 2011; Murch, KrishnaRaj, and Saxena 2000). Por otro lado, la formación de ácido antranílico y L-quinurenina, molécula necesaria para la síntesis de vitamina B3, también es dependiente del triptófano (Shibata and Fukuwatari 2012). aEn plantas destaca la formación de auxinas, principalmente ácido 3-indolacético, implicadas en el desarrollo y el crecimiento de la planta (Zhao 2010).En microorganismos como las levaduras,destaca su importancia en la producción de aromas en el vino (D. Chen, Chia, and Liu 2014) y en la síntesis de compuestos bioactivos derivados de su metabolismo en alimentos fermentados (Mas et al., 2014).

1.2. Fermentación alcohólica

La fermentación es un proceso catabólico en el que interviene diferentes microorganismos realizado en ausencia de oxígeno (condiciones anaerobias), dando lugar a diferentes productos orgánicos como el etanol, el ácido láctico o el ácido acético. Desde la Antigüedad se conoce la producción de productos fermentados como el vino y la cerveza en Oriente Medio y el Antiguo Egipto. Sin embargo, no es hasta el siglo XIX cuando se realizan los avances que permiten entender este proceso. Primero, Gay-Lussac, demostró la formación de gas y alcohol a partir de glucosa en 1810. Más tarde, Louis Pasteur verificó que los microorganismos (concretamente levaduras) eran las responsables de dicha conversión (Barnett 2000). Este proceso es esencial para la obtención de productos fermentados como el queso, el yogur, el pan, la cerveza o el vino.

Los productos fermentados se definen como aquellos alimentos o bebidas que resultan de una controlada acción de microorganismos que dan lugar a la conversión enzimática

de componentes mayoritarios y minoritarios (Marco et al. 2017). En estos casos, la presencia de microorganismos permite obtener un producto diferente a su materia prima de origen con características organolépticas propias (sabor, aroma, color), dándole un valor añadido al producto final.

Las levaduras del género *Saccharomyce*s son ampliamente utilizadas en la industria alimentaria, ya que son indispensables para la obtención pan, vino y cerveza (Hutkins 2006; Walker and Stewart 2016). Para que la fermentación alcohólica se dé correctamente, las levaduras necesitan una fuente de carbono para la obtención de energía (principalmente glucosa) y una fuente de nitrógeno para su crecimiento. Las fuentes de nitrógeno asimilable por las levaduras se conocen como YAN. En el caso de *S. cerevisiae*, las fuentes principales son el amonio y los aminoácidos. La primera es completamente asimilable mientras que de los aminoácidos, solo la glutamina y la asparragina se consideran altamente asimilables (Beltran et al. 2004), mientras que el resto son de asimilación lenta o más tardía (Crepin et al. 2012). Dentro de ellos se encontrarían los aminoácidos aromáticos como el triptófano.

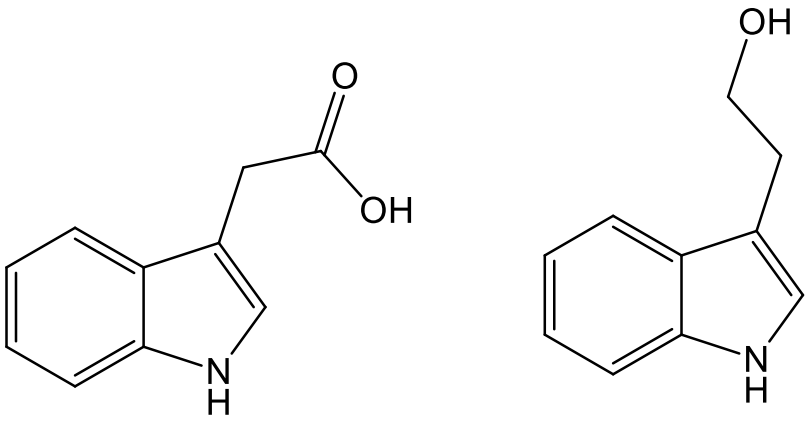

Figura 2. Estructuras químicas del ácido 3-indolacético (izquierda) y del triptofol (derecha).

En *S. cerevisiae*, la principal ruta durante la fermentación alcohólica es la ruta catabólica de Erhlich (Hazelwood et al. 2008). En ella, los aminoácidos sufren una reacción de transaminación, formando cetoácidos y posteriormente se descarboxilan para dar lugar a un aldehído superior. A partir de este compuesto, se generan tanto ácidos como alcoholes superiores por reacciones de oxidación o reducción (Dickinson 2003). En el caso del triptófano, el alcohol superior generado es el triptofol, mientras que el ácido superior es el 3-indolacético (Mas et al., 2014). Sus estructuras químicas aparecen reflejadas en la **Figura 2**. Sin embargo, *S.cerevisiae* también puede generar compuestos a través de otras rutas metabolicas. Entre ellas, destaca la formación de triptamina, formada a partir del triptófano en una sola reacción mediada por una

descarboxilasa y la formación de melatonina, aparentemente sintetizada a través del intermediario 5-hidroxitriptófano.

1.3. Melatonina

La melatonina (N-acetil-5-metoxitriptamina) es una neurohormona que fue descrita por primera vez en 1958 por Lerner (Lerner et al. 1958). Su estructura mantiene el anillo indólico del triptófano, con un grupo metoxi en posición 5 del anillo bencénico y una amida en posición 3 del anillo pirrólico **(Figura 3)**. Asociada principalmente a mamíferos, también se ha descrito ampliamente su síntesis en plantas (Arnao and Hernández-Ruiz 2015; Rüdiger Hardeland 2016; D. X. Tan et al. 2012) y en otros organismos no vertebrados como bacterias, levaduras y macroalgas (R Hardeland and Poeggeler 2003).

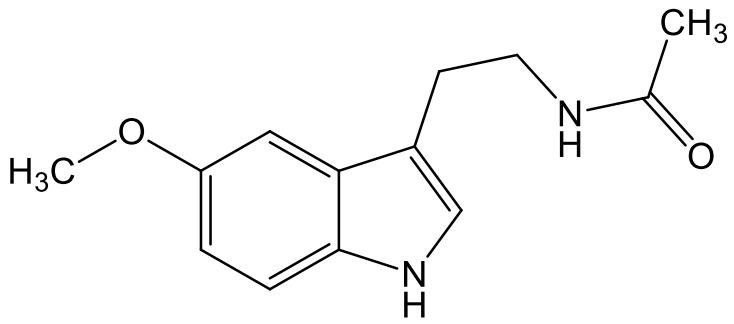

Figura 3. Estructura química de la melatonina

La melatonina se sintetiza principalmente por la glándula pineal en mamíferos (Reiter, 1991), aunque también se ha comprobado una síntesis extrapineal en otros tejidos como la retina, el bazo, el músculo esquelético o la piel entre otros (Acuña-Castroviejo et al. 2014; Slominski et al. 2017; Venegas et al. 2012).

Su función principal reside en el control de los ritmos circadianos dependientes de los ciclos de luz/oscuridad (Reiter, 1993). Sin embargo, la melatonina es un compuesto con numerosas propiedades bioactivas, destacando sobre todo su actividad antioxidante. La capacidad de la melatonina como captador de radicales libres o como molécula estimulante de enzimas antioxidantes ha sido ampliamente estudiada (Bonnefont-Rousselot & Collin, 2010; Reiter et al., 2016; Reiter, Tan, Manchester, & Qi, 2001). Metabolitos derivados del metabolismo de la melatonina como el N(1)-acetil-N(2)-formil-5 metoxikinuramina (AFMK) o el N1-acetil-5-metoxikinuramina (AMK) también han sido descritos como antioxidantes (Galano, Tan, and Reiter 2013).

Además, se ha descrito que la melatonina posee un efecto antiinflamatorio sobre el sistema nervioso central (Esposito and Cuzzocrea 2010), ejerce actividad ante la

aterosclerosis (Favero et al. 2014), mejora el sistema inmune (Lardone et al. 2014) e incluso podría ser de utilidad en tratamientos contra el cáncer gastrointestinal (Xin et al. 2015).

El Reglamento (CE) Nº 1924/2006 del Parlamento Europeo y del Consejo sobre declaraciones nutricionales y de propiedades saludables de los alimentos regula que debe haber una evidencia científica avalada por un panel de expertos para autorizar una declaración de propiedades saludables en cualquier alimento. Cabe destacar que la melatonina cuenta actualmente con dos declaraciones de propiedades saludables, según el Reglamento (UE) n ° 432/2012 de la Comisión, de 16 de mayo de 2012, por el que se establece una lista de declaraciones autorizadas de propiedades saludables de los alimentos distintas de las relativas a la reducción del riesgo de enfermedad y al desarrollo y la salud de los niños Texto pertinente a efectos del EEE (European Commission, 2012). El Panel de Productos Dietéticos, Nutrición y Alergias (NDA) de la EFSA (Autoridad Europea de Seguridad Alimentaria) fue el encargado de evaluar las posibles propiedades saludables de la melatonina. Dicho panel dictaminó en (EFSA (European Food Safety Authority, 2010) que la melatonina contribuye al alivio de los síntomas provocados por el jet lag. Esta declaración se utiliza cuando la concentración de melatonina en el alimento es de 0,5 mg por porción cuantificada de alimento, y debe suministrarse poco antes de dormir en el primer día de viaje y en los días posteriores a la llegada de destino. La segunda declaración autorizada establece que la melatonina contribuye a la reducción de la latencia de sueño, mejorando la calidad del sueño. Para ello, la concentración de melatonina en el alimento debe ser de al menos 1 mg por ración cuantificable.

1.4. Metabolismo de la melatonina

La ruta metabólica de la melatonina difiere entre mamíferos, plantas y microorganismos. En el caso de los mamíferos la ruta fue descrita por primera vez en 1960 por Axelrod y Weissbach (Axelrod and Weissbach 1960). Ellos postularon que el triptófano se convierte en 5-hidroxitriptófano por acción de la enzima triptófano hidroxilasa **(Figura 4)**. Posteriormente, esta molécula se descarboxila formando serotonina (5-hidroxitriptamina), la cual gracias a la acción de la enzima arilalquilamina-N-acetiltransferasa, se acetila en *N*-acetilserotonina (*N*-acetil-5-hidroxitriptamina). Por último, la hidroxiindol-O-metiltransferasa se encarga de metilar esta molécula para formar melatonina. En los mamíferos, la melatonina es metabolizada en el hígado donde se hidroxila y se transforma en 6-hidroximelatonina, el principal metabolito secundario de esta neurohormona que es excretado en la orina (Álvarez-Diduk et al. 2015).

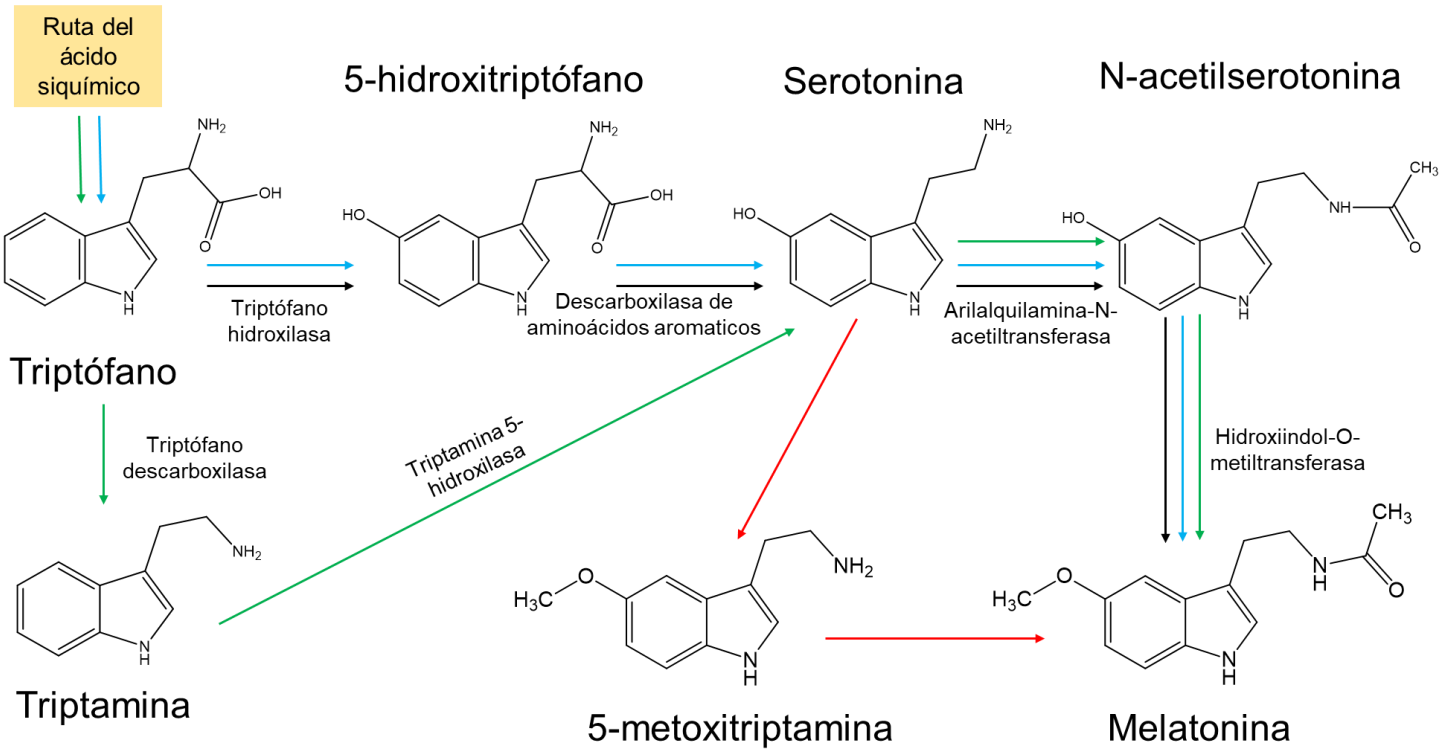

Figura 4. Metabolismo de la melatonina en mamíferos (negro), plantas (verde), levaduras (azul) y una ruta alternativa común a todos (rojo). Adaptado de Tan et al. (2016).

Las plantas también son capaces de sintetizar melatonina. Sin embargo, a diferencia de los mamíferos, el triptófano no es un aminoácido esencial, ya que pueden obtenerlo a través de la glucosa en un una ruta metabólica basada en la producción de ácido siquímico (Bochkov et al. 2012). Una vez obtenido el triptófano, éste es descarboxilado formando triptamina, que poteriormente es transformada en serotonina por la acción de la enzima triptamina 5-hidroxilasa. A partir de este compuesto, los pasos para la obtención de la melatonina se postulan similares a los del metabolismo en mamíferos, aunque las enzimas responsables no son homólogas a las del metabolismo animal, sino que tienen un origen distinto (Kang et al. 2013).

Sin embargo, en otros organismos como las levaduras, la síntesis no está completamente descrita. En 1999, Sprenger *et al.* demostraron que *Saccharomyces cerevisiae* es capaz de sintetizar melatonina y otros compuestos indólicos en un medio de cultivo simple, teniendo el triptófano como uno de los precursores principales (Sprenger et al. 1999). Poco después, en 2001, se realizaron estudios para comprobar qué enzima era la homóloga de la arilalquilamina-N-acetiltransferasa en la síntesis de melatonina por parte de *S. cerevisiae* (Ganguly et al. 2001). Hardeland y Poeggeler.,

concluyeron poco después que, aunque la melatonina se había detectado en diferentes organismos invertebrados (insectos, plantas, bacterias, levaduras) no estaba claro qué función poseía en sus respectivos metabolismos pero quizás no estuviese relacionada con los ritmos circadianos vistos en mamíferos (Hardeland & Poeggeler, 2003). Posteriormente, se estudió un posible efecto como antioxidante frente al estrés hipertónico en levaduras, pero los resultados mostraron que la melatonina no causaba ningún efecto positivo en el crecimiento de las mismas (Koziol et al. 2005).

En 2008, Park *et al.* estudiaron la conversión de 5-hidroxitriptófano en serotonina, y comprobaron que las levaduras del género *S. cerevisiae* poseían una actividad descarboxilasa limitada en cuanto a la velocidad de reacción (Park et al. 2008). Aunque parece ser que, a priori, el metabolismo de melatonina por parte de las levaduras es igual al de mamíferos, estudios más recientes discuten que posiblemente, la síntesis de melatonina no utilice en su ruta la conversión de serotonina en N-acetilserotonina, sino que el 5-hidroxitriptófano se metaboliza en 5-metoxitriptamina para dar lugar a la melatonina, extendiendo esta hipótesis no solo al metabolismo de las levaduras sino al metabolismo general de esta neurohormona (Tan et al., 2016). En la actualidad, los genes y enzimas involucrados en la síntesis de melatonina en levaduras no están completamente descritos.

1.5. Presencia de melatonina en alimentos

La melatonina se ha detectado y cuantificado en diversos alimentos, principalmente de origen vegetal. Uno de los primeros estudios se realizó en diversas frutas (taro, piña, fresa, manzana, tomate), verduras (espinacas, zanahoria, pepino, repollo, espárragos, cebolla) y cereales (arroz, cebada, maíz, avena), destacando el contenido de avena (1796,1 pg/g) y de maíz dulce (1366,1 pg/g) (Hattori et al. 1995). Posteriormente, otro estudio en las semillas de plantas comestibles destacó el contenido de melatonina en mostazas blancas y negras entre 129 y 189 ng/g de peso seco (Manchester et al. 2000) y un año más tarde, 2-16 pg/g en tomates (Van Tassel et al. 2001). Chen et al. realizaron un amplio estudio sobre melatonina en hierbas medicinales chinas, describiendo concentraciones entre 12 y 3771 ng/g (G. Chen et al. 2003). En 2006 se detectó por primera vez melatonina en ocho variedades de uva, destacando *Nebbiolo* 0,965 ng/g) y *Croatina* (0,870 ng/g) (Iriti, Rossoni, and Faoro 2006) y al año siguiente se describía por primera vez en diferentes aceites de oliva, entre 50 pg/mLde un aceite refinado de girasol y 119 pg/mLde un aceite con denominación de origen de Baena (de la Puerta et al. 2007).

Un año después, en 2008, se detectó melatonina por primera vez en vinos, a niveles entre 0,5 ng/mL en vinos de la variedad *Sangiovese* y 0,4 ng/mL en la variedad *Trebbiano* (Mercolini et al. 2008), siendo la primera vez que se cuantificaba en alimentos fermentados. Posteriormente, también se detectó melatonina en cervezas comerciales, en valores comprendidos entre 51.8 y 169.7 pg/mL (Maldonado, Moreno, and Calvo 2009a). Dado que la melatonina presente en estos alimentos no se presentaba de una manera inicial, se comenzó a pensar que las levaduras tenían un papel fundamental en la síntesis de este compuesto en alimentos fermentados. Poco tiempo después, en 2011 se confirmó por primera vez que las levaduras eran capaces de sintetizar melatonina durante la fermentación alcohólica de mostos de uva (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos-Villar, et al. 2011), determinando más tarde que en condiciones de fermentación controladas, la levadura requería la presencia de triptófano en el medio para sintetizar melatonina, pero la mayor presencia de este compuesto no implicaba una mayor producción de la misma (Rodríguez-Naranjo et al. 2012). A partir de entonces, se ha descrito la síntesis de melatonina en numerosas matrices alimentarias fermentadas por levaduras como el vino de granada (Mena et al. 2012), la cerveza artesanal (Garcia-Moreno, Calvo, and Maldonado 2013), el zumo de naranja (Fernández-Pachón et al. 2014) o el pan (Yılmaz, Kocadağlı, and Gökmen 2014).

No obstante, al observar las concentraciones de melatonina en las diferentes matrices, se puede comprobar que la mayor parte de las fuentes alimentarias de melatonina proceden de alimentos vegetales como frutas, verduras y cereales. Sin embargo, resulta remarcable el aporte de fuentes de melatonina derivadas del metabolismo de la *S.cerevisiae* en alimentos fermentados, aunque en todas ellas el aporte de este compuesto activo sea en pequeñas cantidades.

1.6. Otros derivados del triptófano en vino

Además de la melatonina, se han descrito en vinos otros compuestos pertenecientes a su ruta como la serotonina (Manfroi et al. 2009; Wang et al. 2014), aunque no suele ser habitual su cuantificación en vinos, dado las bajas concentraciones en las que se encuentra(ng/mL). Este intermediario desarrolla funciones vitales en el organismo, al ser una de las principales moléculas que influyen en el estado de ánimo. Además, ha mostrado tener propiedades neuroprotectoras frente a la formación de fibras de β-amiloide relacionadas con las enfermedades de Parkinson y Alzheimer (Hornedo-Ortega et al. 2018).

Por otro lado, el triptofol, alcohol superior derivado del triptófano por la ruta de Ehrlich, suele ser un metabolito habitual en el vino (Bordiga et al. 2016; Gil and Gómez-Cordovés

1986; Monagas, Bartolomé, and Gómez-Cordovés 2005). Su función parece estar relacionada como molécula *quorum sensing* durante la fermentación, es decir, compuestos que son expulsados en el medio y dan información a la población de levaduras para adaptar su metabolismo a las condiciones imperantes (Avbelj, Zupan, and Raspor 2016; Dickinson 2008; Dufour and Rao 2011).

El ácido 3-indolacético también se ha descrito en las fermentaciones llevadas a cabo por levaduras en vino (Maslov et al. 2011; Mihaljević Žulj et al. 2015), donde puede cuantificarse como compuesto libre o conjugado con otras moléculas (K. Hoenicke et al. 2001; Simat et al. 2004). El ácido 3-indolacético se considera un *off-flavour* durante la fermentación alcohólica (Katrin Hoenicke et al. 2002). En plantas si está reconocido su efecto en la elongación celular, crecimiento apical de las raíces o el tropismo (Westfall, Muehler, and Jez 2013). En líneas celulares de vena endotelial de cordón umbilical (HUVEC), ha mostrado bioactividad frente a la migración de líneas celulares en procesos relacionados con la angiogénesis (Cerezo et al. 2017).

En menor medida, la triptamina ha sido descrita durante la fermentación alcohólica en vinos (Beneduce et al. 2010; Rodriguez-Naranjo et al. 2013) y se suele aislar también en quesos fermentados (Chang, Ayres, and Sandine 1985; Mayer and Fiechter 2018) aunque no es una de las principales aminas biógenas derivadas del metabolismo de microorganismos. Es más, según las indicaciones de la EFSA sobre los límites máximos permitidos de aminas biógenas en alimentos, la triptamina no se contempla como de especial interés(EFSA Panel on Biological Hazards 2011).

1.7. Técnicas de análisis

La cuantificación de estos metabolitos en matrices alimentarias constituye un reto dentro del campo analítico debido a varios factores. En primer lugar, porque las concentraciones que se encuentran de melatonina y derivados en diversos alimentos es muy baja, del orden de trazas (ng/mL). En segundo lugar, el carácter anfipático de la melatonina hace difícil hallar un método de extracción fiable. Y en tercer lugar, debido a su gran capacidad como antioxidante, la melatonina reacciona rápidamente con otros constituyentes en las muestras por lo que hay que hacer un pretratamiento cuidadoso de la muestra (García-Parrilla, Cantos, and Troncoso 2009).

1.7.1. Técnicas inmunológicas

Estas técnicas fueron las primeras con las que se comenzó a describir la melatonina en diversas muestras alimentarias. Entre estas técnicas destacan el radioinmunonsayo

(RIA) y ELISA, diseñadas en un principio para el análisis de melatonina en fluidos biológicos.

En 1984 se desarrolló un método de RIA para analizar melatonina en plasma de rata, suero humano y orina (Vakkuri, Leppäluoto, and Vuolteenaho 1984) y en 1987 para estudiar los cambios en la concentración de melatonina en plasma y saliva humana (McIntyre et al. 1987). Sin embargo, su análisis en alimentos se utilizó una de las primeras veces en 1995, al cuantificar melatonina en plantas comestibles usando el radioinmunoensayo (Hattori et al. 1995).

Iriti et al, utilizó la técnica de ELISA para cuantificar melatonina en vinos, al igual que De la Puerta et al. para la identificación de este compuesto en aceites (Iriti, Rossoni, and Faoro 2006; de la Puerta et al. 2007). Más tarde, la determinación realizada de melatonina en cervezas se realizó usando un kit comercial disponible de ELISA (Maldonado, Moreno, and Calvo 2009b). En 2011, Rodríguez-Naranjo et al. probó que al usar el mismo kit en muestras de vino, el porcentaje de falsos positivos podía alcanzar hasta un 25%, descartando este método para el análisis en este tipo de matrices alimentarias, posiblemente provocados por interferentes que reaccionaban con los anticuerpos de la melatonina.(Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al. 2011).

Aunque los métodos inmunológicos ofrezcan unos límites que suelen situarse en el orden de pg/mL, nos encontramos ante una serie de desventajas al aplicarlos a matrices alimentarias. En primer lugar, los kits comerciales de ELISA para el análisis de la melatonina suelen alcanzar un precio elevado en el mercado, teniendo únicamente reactivo para procesar solo 96 muestras como máximo. En segundo lugar, al ser un análisis sumamente específico, solamente se puede realizar la determinación de un único compuesto, provocando la ausencia de información en cuanto a visión global de rutas metabólicas. Por último, la presencia de interferentes puede provocar una malinterpretación de los datos obtenidos, cuantificando otros compuestos que puedan reaccionar con el anticuerpo como si fueran melatonina. Por estos motivos, y a pesar de todos estos antecedentes, las técnicas cromatográficas fueron abriéndose paso en los análisis de compuestos indólicos, venciendo las dificultades que el ELISA no era capaz de solventar.

1.7.2. Técnicas cromatográficas

En los años 90, las técnicas cromatográficas empezaron a utilizarse con mayor frecuencia para la detección y cuantificación de la melatonina. Aunque Hattori et al. determinaron melatonina en plantas comestibles con radioimunoensayo, incorporaron

un análisis con cromatógrafo de líquidos y detector de fluorescencia para verificar los resultados (Hattori et al. 1995). Además, por aquel entonces se comprobó que moléculas como la serotonina y la triptamina se consideraban interferentes en los análisis de melatonina por HPLC.

Vitale et al. desarrollaron un método altamente sensible en fase normal con un detector de fluorescencia, permitiendo diferenciar con mayor exactitud estos compuestos de la melatonina (LOD = 3 pg/mL), ofreciendo mejores resultados que los detectores electroquímicos o la cromatografía en fase reversa. (Vitale et al. 1996). No obstante, en 1999 se utilizó la cromatografía líquida en fase reversa con detectores electroquímicos para determinar melatonina y otros compuestos indólicos relacionados derivados de la melatonina (Sprenger et al. 1999). Por aquel entonces, también se desarrolló un método más sensible con un HPLC de fase reversa y detector de fluorescencia pero añadiendo un paso de derivatización que parecía mejorar la sensibilidad (Iinuma et al. 1999). Sin embargo, los detectores electroquímicos coplados a un HPLC siguieron usándose en diferentes experimentos, como los realizadosen células de médula ósea o semillas de plantas comestibles, aunque obviando cualquier proceso de derivatización (Conti et al. 2000; Manchester et al. 2000).

Poco después, Cheng et al. analizó el contenido en hierbas tradicionales chinas usando un HPLC con fluorescencia acoplado a un espectrómetro de masas (G. Chen et al. 2003), siendo una de las primeras veces que se implementa esta técnica y obteniendo un LOD de 50 pg/mL. En 2006, Cao et al. realizaron mejoras en cuanto a la determinación de compuestos indólicos, al desarrollar un método que analizaba melatonina, serotonina y ácido 3-indolacético en extractos de plantas usando también un espectrómetro de masas en tándem (Cao et al. 2006). Los límites de detección que alcanzaron fueron de 5, 100 y 50 pg/mL para la melatonina, serotonina y ácido 3-indolacético respectivamente.

La determinación de melatonina en uvas por primera vez se realizó a través de un proceso de HPLC con fluorescencia, pero curiosamente, se utilizó el ELISA para verificar que no coeluían compuestos durante el análisis cromatográfico (Iriti, Rossoni, and Faoro 2006). Para cuantificar melatonina en vinos se usó el mismo método cromatográfico (HPLC-F), pero en esta ocasión, no se recurrió a la técnica ELISA como complemento analítico (Mercolini et al. 2008). Prácticamente a partir del año 2010, la mayor parte de métodos para la determinación de la melatonina y otros compuestos indólicos se realizó con espectrómetros de masas. Rodriguez-Naranjo et al. cuantificó melatonina en vinos usando un Qtrap (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al. 2011) con

un LOD de 51,72 ng/mL. Además, estas técnicas permitieron cuantificar melatonina en diversos alimentos, con un LOD entre 9,6 y 52,9 ng/mL (Kocadağlı, Yılmaz, and Gökmen 2014).

A día de hoy, las técnicas cromatográficas de UPLC acopladas a espectrómetros de alta resolución han probado ser la mejor alternativa para el estudio de la melatonina y otros compuestos indólicos derivados (Zieliński, Szawara-Nowak, and Wiczkowski 2016), ya que la sensibilidad alcanzada por estos equipos permite una cuantificación más fiable. que con otras técnicas.

BLOQUE II: Estilbenos

2. Estilbenos

Los estilbenos son un grupo de compuestos polifenólicos no flavonoides que se encuentran en diferentes familias de plantas, entre las que destaca la *Vitaceae*, siendo la *Vitis vinífera* uno de los géneros más representativos (Rivière, Pawlus, and Mérillon 2012). La uva y los derivados de la misma, como el vino, son las principales fuentes de estilbenos para nuestra dieta (Raúl F. Guerrero et al. 2009).

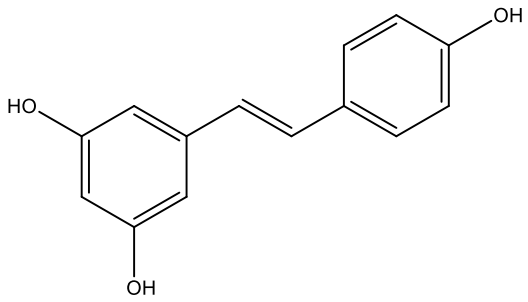

Figura 5. Estructura química del resveratrol.

La estructura química de los estilbenos se compone de un esqueleto carbonado formado por dos anillos aromáticos unidos entre sí por un puente etileno (1,2-difeniletileno) (Adrian et al. 2000). Las diferentes combinaciones de esta estructura dan lugar a los diferentes compuestos pertenecientes a los estilbenos. Las estructuras mas simples (monómeros) son aquellos compuestos con la estructura más sencilla, basada en un esqueleto C6-C2-C6 al que están unidos diferentes radicales, que pueden ser de distinta naturaleza (azúcares, grupos metilos, grupos metoxi, etc.). La posición en la que estos sustituyentes se encuentren en el plano dará lugar a diferentes configuraciones espaciales que serán características de cada molécula. El monómero de estilbenos más conocido es el resveratrol, cuya molécula presenta tres grupos hidroxilos, dos en uno

de los anillos aromáticos y otro en el restante **(Figure 5)**. El resveratrol se encuentra principalmente en la piel y en los raspones de la uva. Su metabolismo en plantas parte de la biosíntesis del amino ácido fenilalanina a través de la ruta del ácido siquímico. A partir de ese punto, el resveratrol sigue la ruta metabólica descrita en la **Figura 6**. La fenilalanina se transforma en ácido cinámico por acción de la fenilalanina amonio liasa. Posteriormente, la cinamato-4-hidroxilasa transforma el ácido cinámico en ácido p-cumárico, que por acción de la estilbeno sintetasa medida por la presencia de al menos 3 moléculas de malonilCoA da lugar al trans-resveratrol .

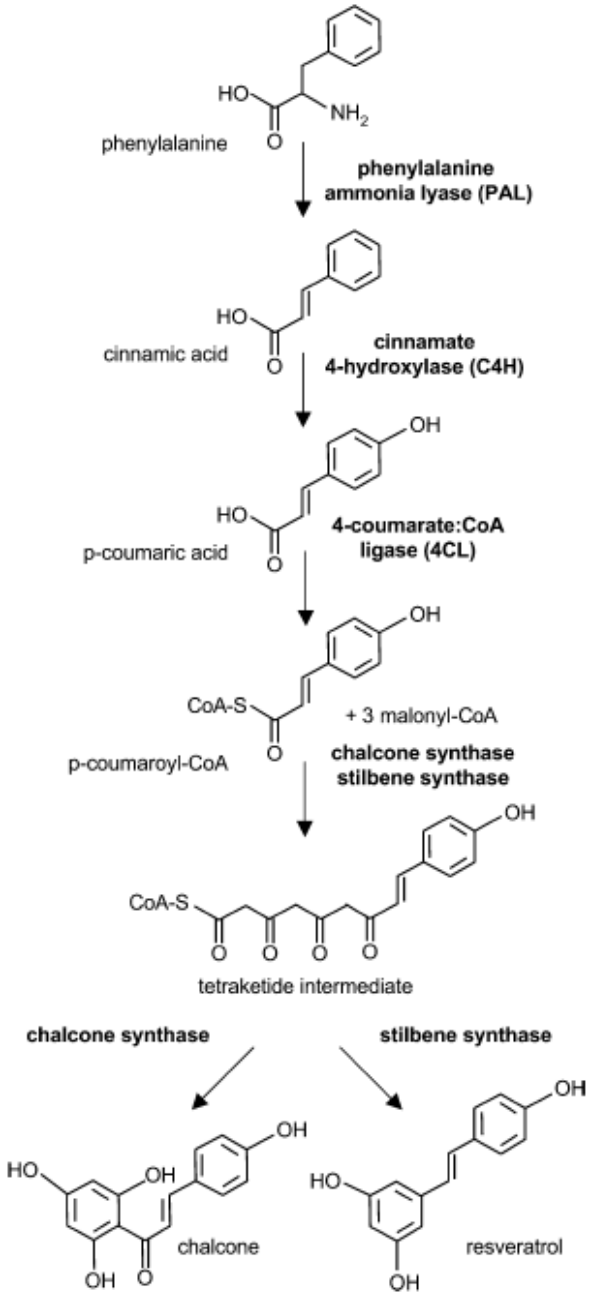

Figura 6. Metabolismo de formación del resveratrol a través de la fenilalanina (Chon et al., 2009)

2.1. Compuestos derivados

Además del resveratrol hay otros compuestos monoméricos que han cobrado importancia en los últimos años debido a su potencial como compuestos bioactivos como el piceatanol (Piotrowska, Kucinska, and Murias 2012), el piceido (Raúl F. Guerrero et al. 2009), el pterostilbeno (McCormack and McFadden 2013) o la rapontina (Tao et al. 2017), entre otros (Akinwumi, Bordun, and Anderson 2018). Al mismo tiempo, el resveratrol puede formar oligómeros con la unión de una o más moléculas de este compuesto, dando lugar a la formación de dímeros y tetrámeros (Liu et al. 2013; Mattivi et al. 2011).

Sin embargo, a diferencia del resveratrol o del piceido, no todos los compuestos derivados tienen una fuente alimentaria mayoritaria. Se han descrito algunos tetrámeros en vinos, como el hopeafenol y el isohopeafenol, (Decendit 2017; Guebailia et al. 2006), dímeros con las ε-viniferina (Vitrac et al. 2002) o el palidol (Flamini et al. 2013), aunque los más estudiados dado su potencial como compuestos bioactivos son el piceatanol y el pterostilbeno. No obstante, su presencia en alimentos no es tan estudiada, ya que sueln concentrarse en los raspones y en otras partes de la vid (Raúl F. Guerrero et al., 2016). Actualmente, se trabaja en el aprovechamiento de estos desechos para extraer compuestos bioactivos derivados del resveratrol (Biais et al. 2017; Rayne, Karacabey, and Mazza 2008; Sáez et al. 2018). Entre los usos potenciales de estos extractos, destacan la sustitución del anhídrido sulfuroso en el vino, dado las reacciones adversas que puede desencadenar en personas sensibles (Raúl F. Guerrero and Cantos-Villar 2015), presentando vinos con características organolépticas similares (Raposo et al. 2016, 2018).

3. Angiogenesis

La angiogénesis es un proceso fisiológico basado en la creación de nuevos capilares sanguíneos a través de otros preexistentes (Carmeliet and Jain 2000). Dicho proceso es vital durante la embriogénesis, las etapas de crecimiento y en la regeneración celular. Sin embargo, en adultos también se encuentra relacionado con procesos cancerosos y las enfermedades cardiovasculares derivadas por la formación de la placa de ateroma. también se encuentra activo en procesos tumorales y en el desarrollo de la placa de ateroma (Camaré et al. 2017). Para que las células puedan proliferar, es necesario sintetizar unos factores de crecimiento, entre los cuales destaca el factor de crecimiento vascular endotelial (VEGF) (Shibuya 2011). Dicho factor posee diferentes receptores de membrana, siendo el más activo el receptor 2 de VEGF (VEGFR-2) (Cébe-Suarez, Zehnder-Fjällman, and Ballmer-Hofer 2006).

En la actualidad, hay tratamientos contra el cáncer cuya diana es la inhibición de este proceso fisiológico (Glade-Bender, Kandel, and Yamashiro 2003; Ranieri et al. 2014). Para ello, inhiben la unión entre el VEGF y el VEGFR-2, evitando el desarrollo de una cascada de señalización intracelular mediada por diferentes proteínas. Entre ellas, destacan la fosfolipasa gamma 1 (PLCγ1), responsable de la proliferación celular (Shibuya 2011) que a su vez, también promueve la activación de la óxido nítrico sintasa endotelial (eNOS) mediada por la protein quinasa B (Akt) (Kolluru, Siamwala, and Chatterjee 2010). Sin embargo, la inhibición de esta cascada de señalización se traduce en una disminución de óxido nítrico, el cual es un potente vasodilatador. Los efectos adversos del tratamiento con fármacos anti-angiogénicos provoca a largo plazo hipertensión en los pacientes (Escalante and Zalpour 2011; Zhu et al. 2007). Por tanto, la búsqueda de compuestos capaces de inhibir el VEGF pero a su vez, mantener la producción de óxido nítrico cobra importancia a la hora de buscar tratamiento potenciales como futura alternativa.

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JUSTIFICACIÓN, HIPÓTESIS Y OBJETIVOS

Los productos fermentados se definen como aquellos alimentos o bebidas que resultan de la acción de microorganismos que dan lugar a la conversión enzimática de componentes mayoritarios y minoritarios (Marco et al. 2017). La presencia de microorganismos permite obtener un producto con valor añadido ya que mejora la conservación, el valor nutricional, la funcionalidad y las características organolépticas, entre otros aspectos; lo cual tiene un impacto económico positivo en su comercialización (Hutkins 2006).

Las levaduras del género *Saccharomyces*, son una de las principales encargadas de llevar a cabo el proceso de fermentación que da lugar a la obtención de productos como el pan, el vino o la cerveza. Durante este proceso, las levaduras precisan diversas fuentes de carbono, de nitrógeno asimilable (YAN) y de ausencia de oxígeno para su correcto desarrollo. De ellas, el nitrógeno es el factor más limitante para su crecimiento y actividad durante la fermentación alcohólica (Henschke & Jiranek 1993, Tesnière et al. 2015). Las principales fuentes de YAN son el amonio y los aminoácidos. El amonio es la preferida por las levaduras, debido a que es altamente asimilable. Entre los aminoácidos, la glutamina y la asparragina son los únicos que se consideran como preferidos para su metabolismo (Beltran et al. 2004). Sin embargo, los aminoácidos aromáticos (triptófano, fenilalanina y tirosina), si bien no son sus fuentes prioritarias de nitrógeno, cobran especial relevancia por los metabolitos que forman, ya que poseen propiedades bioactivas (antioxidantes, neuroprotectores, antiangiogénicos) e incluso algunos se han descrito como reguladores del crecimiento, afectando a la morfología de las levaduras (Dickinson 2008).

El metabolismo del triptófano conlleva la formación de otros compuestos como la melatonina, la serotonina o el ácido 3-indolacético durante la fermentación alcohólica (Maslov et al. 2011, Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos-Villar, et al. 2011, Mercolini et al. 2012). Dichos compuestos han demostrado tener propiedades bioactivas como antioxidantes, neuroprotectores y antiangiogénicos (Mas et al. 2014, Hornedo-Ortega et al. 2016, Cerezo et al. 2017). El triptofol, un alcohol superior derivado del metabolismo del triptófano, se ha demostrado que actúa como *quorum sensing* para las levaduras (Hazelwood et al. 2008, Avbelj et al. 2016). Sin embargo, todavía se desconocen tanto las vías de síntesis por parte de las levaduras como la función que estos compuestos ejercen durante la fermentación alcohólica.

Además, las concentraciones de algunos metabolitos como la melatonina han sido descritas en alimentos fermentados a niveles de traza (ng/mL) (Rodriguez-Naranjo, Gil-

Izquierdo, Troncoso, Cantos, et al. 2011, Kennaway 2017). Este hecho hace necesario la búsqueda de técnicas analíticas con la suficiente sensibilidad para poder cuantificar estos metabolitos en diferentes matrices. Al mismo tiempo, se requieren técnicas que permitan la detección simultánea de los distintos metabolitos intermediarios en la formación de compuestos bioactivos. Por ello, la cromatografía líquida de ultra-alta eficacia acoplada a un espectrómetro de masas de alta resolución (UHPLC/HRMS) es una técnica que cumple con estos requisitos. Para verificar la eficacia de la técnica, es preciso que los métodos utilizados se encuentren debidamente validados (Kruve et al. 2015).

Por tanto, la **hipótesis** de partida de la presente Tesis Doctoral supone que, disponer de métodos analíticos validados y sensibles que determinen simultáneamente diferentes metabolitos puede permitir mejorar el conocimiento de la síntesis de los compuestos derivados del triptófano durante la fermentación alcohólica, que ayude a establecer las condiciones para la formación de estos compuestos en productos fermentados.

La Tesis Doctoral tiene como **objetivo principal** la identificación y cuantificación de metabolitos derivados del aminoácido aromático triptófano en alimentos fermentados por la levadura *Saccharomyces cerevisiae* así como el estudio de la bioactividad de otros compuestos derivados de otro aminoácido aromático, la fenilalanina, en el metabolismo de la vid que da lugar a la formación de estilbenos.

Para la consecución de este objetivo, se definieron diferentes **objetivos específicos** a los que se llegaron después del planteamiento de una serie de cuestiones:

1. ¿Es posible cuantificar los compuestos derivados del metabolismo del triptófano simultáneamente y con suficiente sensibilidad?

En la bibliografía, existen diversos métodos para medir derivados del triptófano por separado, pero no hay ninguno que lleve a cabo un análisis simultáneo de diversos metabolitos. El primer objetivo requiere el desarrollo de un método analítico validado (Capítulo 1), que debe enfrentarse a los siguientes retos:

- Identificación simultánea de compuestos derivados del triptófano, principalmente los descritos en la ruta de síntesis de melatonina en plantas y mamíferos, que permita dilucidad si la levadura presenta dichos metabolitos en su ruta de síntesis (triptófano, 5-hidroxitriptófano, serotonina, *N*-acetilserotonina y melatonina). Asimismo, se contemplan otros compuestos de interés como el triptofol, el ácido 3-indolacético, la triptamina o el etil ester de triptófano.

- Validación del método analítico para poder cuantificar cantidades traza (ng/mL) de los compuestos minoritarios en muestras fermentadas por levaduras.

2. ¿Se ve afectada la estabilidad de los compuestos bioactivos derivados del triptófano por efecto de la matriz, temperatura y concentración?

La melatonina es una molécula muy inestable ante agentes externos como la luz y el oxígeno, debido a sus características antioxidantes (Friciu et al. 2016, Reiter et al. 2016). Además, otros compuestos derivados como el ácido 3-indolacético también presentan fotosensibilidad. Sin embargo, la composición de la matriz en la que se encuentran estos compuestos bioactivos puede afectar a su estabilidad a lo largo del periodo de almacenamiento de las muestras. Esta cuestión está recogida en el Capítulo 2 de la tesis y para llevarla a cabo se tuvieron en cuenta los siguientes objetivos:

- Evaluar el efecto de diferentes matrices (vino, cerveza, mosto sintético fermentado y extracto intracelular de levaduras *Saccharomyces*) en la estabilidad de los compuestos bioactivos derivados del triptófano.
- Determinar el impacto de distintas condiciones de temperatura (4ºC, -20ºC y -80ºC) y concentración (2, 200 ng/mL) a lo largo del periodo de almacenamiento (30 días) en los compuestos bioactivos mediante un método validado de UHPLC/HRMS.

3. ¿Cómo evolucionan los derivados del triptófano durante la fermentación alcohólica? ¿Presentan las cepas de los géneros *Saccharomyces* y no-*Saccharomyces* alguna diferencia en cuanto a su síntesis?

Previamente, se ha descrito que las levaduras de tipo *Saccharomyces* (QA23) sintetizan melatonina en condiciones de fermentación alcohólica. Sin embargo, no se ha estudiado la evolución de múltiples metabolitos derivados del triptófano en condiciones de fermentación con diferentes cepas comerciales (Capítulo 3). Por tanto, para la consecución de este objetivo fue necesario:

- Llevar a cabo fermentaciones alcohólicas con diferentes cepas de levaduras comerciales no estudiadas en este aspecto, de tipo *Saccharomyces* (Aroma White, Red Fruit, QA23, ES488, ICV GRE, UVAFERM BC) y no-*Saccharomyces* (*Torulaspora delbrueckii*, *Metschnikowia pulcherrima),* incluyendo la QA23 como control y utilizando mosto sintético estandarizado, con el objetivo de minimizar interferentes en la matriz.
- Analizar las muestras con el método analítico previamente validado para dilucidar qué cepas son las mayores productoras de compuestos bioactivos.

4. ¿Cómo evolucionan los compuestos derivados del triptófano al utilizar matrices más complejas?

El siguiente objetivo se centra en aquellas cepas de levaduras que producen mayores cantidades de compuestos derivados del triptófano con propiedades bioactivas halladas en el Capítulo 3. Se pretende evaluar la síntesis de estos compuestos durante la fermentación alcohólica en un medio más complejo (Capítulo 4). Para ello, fue necesario:

- Utilizar diferentes mostos naturales procedentes de variedades de uva blanca (Corredera, *Chardonnay*, Moscatel, Palomino Fino, *Sauvignon Blanc*, Vijiriega) y tinta (Tempranillo).
- El análisis de muestras tomadas durante la fermentación alcohólica para dilucidar la evolución de los compuestos derivados del triptófano en las diferentes cepas, con un método de UHPLC/HRMS previamente validado.

5. ¿Aparecen los compuestos bioactivos derivados del triptófano en otras bebidas fermentadas por las levaduras del género *Saccharomyces*?

Las levaduras del género *Saccharomyces* son capaces de fermentar otros sustratos como los cereales (trigo, cebada) para la obtención de bebidas alcohólicas de baja graduación como la cerveza. Hasta ahora, solo se han realizado análisis de melatonina en cervezas, a través de técnicas ELISA (Maldonado et al. 2009, Garcia-Moreno et al. 2013). Sin embargo, es necesario realizar estudios más completos sobre la presencia de otros compuestos derivados del triptófano con técnicas más sensibles. Este objetivo se abarca en el Capítulo 5 de la presente tesis, con los siguientes objetivos específicos:

- Optimización del tratamiento de muestra utilizado en anteriores análisis para permitir una mejor resolución de compuestos bioactivos en muestras de cerveza.
- Identificación y cuantificación de compuestos derivados del triptófano en extractos de cervezas comerciales para dilucidar aquellas marcas con mayor presencia de compuestos bioactivos en su composición.

6. Otros compuestos derivados del aminoácido aromático fenilalanina, ¿presentan actividad antiangiogénica?

Los estilbenos son compuestos que proceden del aminoácido fenilalanina, siendo el resveratrol el más estudiado. Su bioactividad ha sido extensamente descrita en la

bibliografía como compuesto antioxidante y como protector en enfermedades cardiovasculares (Smoliga et al. 2011, Bonnefont-Rousselot 2016). Entre ellas, la formación de la placa de ateroma en la aterosclerosis está influenciada por el proceso de angiogénesis, consistente en la creación de nuevos capilares sanguíneos a través de otros ya existentes. El factor de crecimiento vascular endotelial (VEGF) es uno de los principales mediadores de este proceso. Por tanto, la inhibición de VEGF es un mecanismo molecular plausible que demuestra una causa-efecto directa en la disminución del riesgo cardiovascular. Previamente, el resveratrol ha mostrado cierta actividad anti-VEGF (Cerezo et al. 2015). Sin embargo, no hay estudios sobre el efecto de otros estilbenos frente a esta inhibición. El capítulo 6 de la tesis incluye el desarrollo de los siguientes objetivos:

- Evaluación del potencial anti-VEGF de 12 compuestos derivados del estilbeno (ampelopsina A, astringina, ε-viniferina, hopeafenol, isohopeafenol, palidol, piceatanol, pterostilbeno, rapontin, r-viniferina, r2-viniferina y ω-viniferina) en células endoteliales de cordón umbilical humano (HUVEC). Uso de técnicas ELISA para hallar el valor de IC_{50} y establecer los compuestos más activos.
- Determinar el efecto de los estilbenos con mayor potencial anti-VEGF sobre la modulación de la cascada de señalización intracelular mediada por VEGF (PLCγ-1, Akt, eNOS). Evaluación de la fosforilación de dichas proteínas a través de técnicas de western-blot.

MATERIALES Y MÉTODOS

Para el desarrollo de la presente Tesis Doctoral, se han empleado los siguientes materiales:

1. MATERIALES

1.1. Patrones analíticos

Se utilizaron patrones analíticos comerciales con una pureza mayor al 97% en todos los casos. El nombre, proveedor y referencia los productos utilizados aparecen reflejados en la **Tabla 2**.

Tabla 2. Patrones utilizados para el análisis de compuestos indólicos

Compuesto	Proveedor	Referencia
5-hidroxi-L-triptófano	Sigma-Aldrich	H9772
5-hidroxitriptamina	Sigma-Aldrich	H9523
Ácido 3-indolacético	Panreac	57330
Etil ester de triptófano	Sigma-Aldrich	364517
L-triptófano	AppliChem	A3410
Melatonina	Sigma-Aldrich	M5250
N.acetil-5-hidroxitriptamina	Sigma-Aldrich	A1824
Triptamina	Sigma-Aldrich	193747
Triptofol	Sigma-Aldrich	T90301

1.2. Mosto sintético

Para la elaboración del mosto sintético se emplearon los reactivos que aparecen en la **Tabla 3**. La composición del mosto se basó en Riou et al. (1997) con algunas modificaciones en los elementos principales y el contenido de aminoácidos. El mosto contenía 300 mg/L de nitrógeno asimilable por las levaduras (YAN) de los cuales 120 mg/L procedían del cloruro amónico (NH_4Cl) y 180 mg/L de aminoácidos.

Tabla 3. Composición del mosto sintético (MS300), soluciones de aminoácidos, vitaminas y factores anaerobios.

	Compuesto	**Cantidad (g/L)**	**Proveedor**	**Referencia**
Elementos principales	Glucosa	100	Sigma-Aldrich	G5767
	Fructosa	100	VWR	24282.368
	Ácido cítrico	0,3	Merck	137002
	Ácido tartárico	3	VWR	20718.290
	Ácido málico	5	VWR	20365.290
	Fosfato monopotásico (KH_2PO_4)	0,75	Merck	104873
	Bisulfato potásico (KH_2SO_4)	0,5	VWR	26936.293
	Sulfato de magnesio heptahidratado ($MgSO_4 \cdot 7H_2O$)	0,25	Merck	105886
	Cloruro cálcico dihidratado ($CaCl_2 \cdot 2H_2O$)	0,155	Merck	102382
	Cloruro sódico (NaCl)	0,2	VWR	27810.295
	Cloruro amónico (NH_4Cl)	0,46	Sigma-Aldrich	254134
Aminoácidos	Bicarbonato de sodio	20	Panreac	131638
	Tirosina	1,5	Sigma-aldrich	93829
	Triptófano	13,4	Sigma-aldrich	93659
	Isoleucina	2,5	Sigma-aldrich	58879
	Ácido aspártico	3,4	Sigma-aldrich	11189

	Ácido glutámico	9,2	Sigma-aldrich	49449
	Arginina	28,3	Sigma-aldrich	A5006
	Leucina	3,7	Sigma-aldrich	61.819
	Treonina	5,8	Sigma-aldrich	89.179
	Glicina	1,4	Sigma-aldrich	50046
	Glutamina	38,4	Sigma-aldrich	G3126
	Alanina	11,2	Sigma-aldrich	5130
	Valina	3,4	Sigma-aldrich	94619
	Metionina	2,4	Sigma-aldrich	64319
	Fenilalanina	2,9	Sigma-aldrich	78019
	Serina	6,0	Sigma-aldrich	84959
	Histidina	2,6	Sigma-aldrich	53319
	Lisina	1,3	Sigma-aldrich	62840
	Cisteína	1,6	Sigma-aldrich	30089
	Prolina	46,1	Sigma-aldrich	P0380
Vitaminas	Mio-inositol	20	Alfa aesar	A13586
	Ácido nicotínico	0,2	Alfa aesar	A12683
	Pantotenato cálcico	1,5	Avantor	1443-03
	Clorhidrato de tiamina	0,025	Merck	1.08181.0025

	Clorhidrato de piridoxina	0,025	Applichem	A0957
	Biotina	0,003	Applichem	A0969
Oligoelementos	Sulfato de manganeso hidratado ($MnSO_4 \cdot H_2O$)	4	Prolabo	25303.233
	Sulfato de zinc hidratado ($ZnSO_4 \cdot H_2O$)	4	Panreac	141787
	Sulfato de cobre pentahidratado ($CuSO_4 \cdot 5H_2O$)	1	Prolabo	23174.233
	Yoduro de potasio (KI)	1	Sigma-Aldrich	207969
	Cloruro de cobalto hexahidratado ($CoCl_2 \cdot 6H_2O$)	0,4	Alfa aesar	B22031
	Ácido bórico (H_3BO_3)	1	Panreac	131015
	Molibdato de amonio $(NH_4)_6Mo_7O_{24}$	1	Sigma-aldrich	09878
Factores anaerobios*	Ergosterol	1,5/100mL	Sigma-Aldrich	E6510
	Ácido oleico	0,5mL/100mL	Prolabo	20447.293
	Tween 80	50 mL/100mL	Sigma-Aldrich	P1754
	Etanol	50 mL/100mL	Merck	K40430297

*Se prepara solución de 100 mL

1.3. Mostos de uva

El Instituto de Investigación y Formación Agraria y Pesquera (IFAPA) “Rancho de la Merced” de Jerez de la Frontera, España (Longitud 06:00:58 O, Latitud 36:45:29 N) se encargó de cultiva diferentes variedades de uva blanca

(Corredera, Chardonnay, Moscatel, Palomino Fino, Sauvignon Blanc, Vijiriega, Tempranillo) y tinta (Tempranillo).

1.4. Muestras de cerveza: *Capítulo 5*

El estudio sobre la presencia de compuestos indólicos se realizó en 19 tipos de cervezas comerciales **(Tabla 4)** ampliamente consumidas por la población española según el estudio *Brand Foodprint 2016* (Kantar Worldpanel, https://www.kantarworldpanel.com). Las cervezas fueron adquiridas en supermercados locales en formato de botella de vidrio, (200 y 500 mL). La mayoría de ellas fueron de tipo lager (baja fermentación), con la excepción de la cerveza Guinness (stout) y con un contenido alcohólico comprendido entre 4,6 y 7,2º.

Tabla 4. Cervezas seleccionadas para realizar el análisis de compuestos indólicos.

Nombre	*Tipo*	*Nombre*	*Tipo*
Alhambra tradicional	Lager tipo Pilsner	Estrella Damm	Lager tipo Pilsner
Ámbar	Lager tipo Pilsner	Estrella Galicia	Lager tipo Pilsner
Amstel	Lager tipo Pilsner	Guinness	Stout irlandesa seca
Buckler 0.0	Lager tipo Pilsner	Heineken	Lager tipo Pilsner
Budweiser	Lager americana	Mahou 5 estrellas	Lager tipo Pilsner
Carlsberg	Lager tipo Pilsner	Murphy´s Irish Red	Lager roja irlandesa
Corona	Lager americana	Paulaner	Lager blanca
Cruzcampo	Lager tipo Pilsner	San Miguel	Lager tipo Pilsner
Cruzcampo cruzial	Lager tipo Pilsner	Voll-Damm	Lager tipo Marzen
Desperados	Lager aromatizada		

1.5. Otros reactivos

El metanol grado LC-MS y el ácido clorhídrico al 37% fueron suministrados por Merck (Darmstadt, Alemania). El ácido fórmico (99%) de grado LC-MS y el etanol absoluto se adquirieron en Prolabo® (Obregón, México). El ácido N-(2-hidroxietil)

piperazina-N'-(2-etanosulfónico) (HEPES) fue proporcionado por Sigma-Aldrich (Barcelona, España).

1.6. Estilbenos

Se utilizaron 12 estilbenos derivados del resveratrol. Entre ellos, el picetanol y el pterostilbeno fueron adquiridos de Sigma-Aldrich (Barcelona, España). Debido a la falta de patrones comerciales, los restantes estilbenos (ampelopsina A, astringina, ε- y ω-viniferina,, hopeaphenol, isohopeaphenol, pallidol, rhapontin, r- y r-2-viniferina) fueron aislados de diferentes partes del pino (*Picea abies*) y de un extracto de vid (*Vitis vinífera*) suministrado por Actichem S.A. (Montauban, Francia) en el *Institut des Sciences de la Vigne et du Vin* de Burdeos, utilizando métodos previamente descritos (Biais et al., 2017; Gabaston et al., 2017).

1.7. Reactivos de biología molecular

Los reactivos utilizados (función, proveedor y referencia) para el caso de los experimentos de biología molecular se encuentran enumerados en la **Tabla 5**.

Tabla 5. Reactivos empleados para los experimentos de biología molecular.

Compuesto	Función	Proveedor	Referencia
Tris-HCl	Buffer NAPI	Applichem	A3452
Triton™ X-100	Buffer NAPI	Sigma-Aldrich	T8787
Ácido etilendiaminotetraacético (EDTA)	Buffer NAPI	Sigma Aldrich	E6758
Bifosfato de sodio	Buffer NAPI	AppliChem	A3902
Fosfato de sodio	Buffer NAPI	AppliChem	A3905
Inhibidores de proteasas	Buffer RIPA	Roche	05892970001
Benzonasa nuclease	Buffer RIPA	Novagen	70664
Inhibidores de fosfatasas	Buffer RIPA	Roche	4906837001
Solución de dodecil sulfato de sodio (SDS) al 10%	Buffer RIPA	Applichem	A0676
Desoxicolato de sodio (DOC)	Buffer RIPA	Sigma-Aldrich	D6750
Azul tripán (0.4%)	Colorante	Invitrogen	T10282

Suero bovino fetal (FBS)	Conservación de células	Biowest	S1810500
2-propanol	Conservación de células	AppliChem	A3465
Suplemento para medio de crecimiento (EGM-2)	Crecimiento de células	Lonza	H3CC-3162
Solución neutralizadora de tripsina (NTS)	Desarrollo de pases celulares	Lonza	CC5002
Tripsina/EDTA	Desarrollo de pases celulares	Lonza	CC5012
Dimetil sulfóxido	Disolución de patrones Conservación de células	Applichem	A3672
Kit de Sandwich ELISA PathScan® Phospho-VEGFR-2 (Tyr1175) (Cell Signalling)	ELISA	Cell Signalling	7335C
Ácido bicinconínico	Recuento de proteínas	Sigma-Aldrich	B9643
Sodio ortovanadato	Recuento de proteínas	Sigma-Aldrich	S6508
Solución de cobre (II)	Recuento de proteínas	Sigma-Aldrich	C2284
Albúmina de suero bovino	Recuento de proteínas	Sigma-Aldrich	A2153
Trizma®-base	TBST	Sigma-Aldrich	T6066
Tween ® 20	TBST	Sigma-Aldrich	P9416
Medio basal endotelial (EBM-2)	Tramiento con VEGF	Lonza	H3CC-3156
Buffer fosfato salino de Dulbecco (PBS) con cloruro de calcio y cloruro de magnesio (10x)	Tratamiento con VEGF	Sigma-Aldrich	D1283408
VEGF recombinante humano (165)	Tratamiento con VEGF	R&D systems	293-VE-010

Gentamicina	Tratamiento con VEGF	Lonza	H3CC-4083
Metanol (grado HPLC)	Western-blot	Merck	I525-0940
Anticuerpo PLCγ1 (D9H10, XPxp® Rabbit mAb)	Western-blot	Cell signalling	5690S
Anticuerpo fosforilado de PLCγ1 (Tyr783)	Western-blot	Cell signalling	2821S
Buffer de transferencia (20x)	Western-blot	NuPAGE	NP0006-1
Buffer de electrophoresis	Western-blot	NuPAGE	NP0001
Agente reductor para muestras (ditiotreitol) (10x)	Western-blot	NuPAGE	NP0009
Marcador de proteinas para electroforesis	Western-blot	NuPAGE	LC5602
Sustrato para quimioluminiscencia (Signal West Pico PLUS)	Western-blot	Pierce	34580
Buffer de separación (*stripping buffer*)	Western-blot	Pierce	21059
Anticuerpo fosforilado de eNOS (Ser1177) (C9C3, Rabbit mAb).	Western-blot	Cell signalling	9570S
Anticuerpo fosforilado de Akt (Ser473) (D9E, XP® Rabbit mAb)	Western-blot	Cell-signalling	4060S
Anticuerpo secundario (Rabbit IgG, HRP-Linked)	Western-blot	Cell signalling	7074S
Anticuerpo Akt	Western-blot	Cell Signalling	9272S
Anticuerpo eNOS	Western-blot	Cell Signalling	9572S
Solución de Ponceau S	Western-blot	Sigma-Aldrich	P3504
Antioxidante	Western-blot	NuPAGE	NP0005
Buffer de muestra (dodecilsulfato de litio) (4x)	Western-blot	NuPAGE	NP0007

1.8. Líneas celulares

Los experimentos realizados para comprobar la posible actividad anti-angiogénica de compuestos estilbenoides se hizo sobre la línea de células endoteliales de venas del cordón umbilical humano (HUVEC) suministradas por Lonza (Slough, Reino Unido).

1.9. Instrumentación

- Agitador magnético con calefacción, placa de aluminio (VWR, Radnor, Pensilvania, EE.UU).
- Agitador orbital MaxQ 4000 (Thermo Fisher Scientific, Bremen, Alemania).
- Agitador vórtex VV3 (VWR, Radnor, Pensilvania, EE.UU).
- Autoclave modelo AES 75L (Raypa, Barcelona, España).
- Balanza analítica AB204-S (Mettler Toledo, Columbus, Ohio, EE.UU).
- Baño de ultrasonidos J.P. Selecta UB-1538 (Abrera, Barcelona, España)
- Baño termostatizado BAE-2 (Raypa, Barcelona, España).
- Bomba de vacío MZ2CNT (Vacuubrand, Wetheim, Alemania).
- Cabina de seguridad biológica modelo Advantage (Thermo Fisher Scientific, Bremen, Alemania).
- Cabina de seguridad biológica modelo BIO II A Mini (Telstar).
- Centrífuga Allegra X-22R (Beckman Coulter, Pasadena, California, EE.UU).
- Centrífuga diferencial con rotor para tubos falcon de 15 ml modelo 3D-30 (Sigma-Aldrich, Barcelona, España).
- Centrífuga Heraeus Megafuge 16R (Thermo Fisher Scientific, Bremen, Alemania).
- Concentrador de muestras a vacío Hypervac-lite (Gyozen, Corea).
- Contador automático de células Countess®C10227 (Invitrogen, Carlsbad, California, EE.UU).
- Equipo de Agua Milli-Q modelo Nanopure Diamond (Barnstead, Alemania).
- Equipo de espectrometría de alta resolución (HRMS) consistente en un híbrido de cuadrupolo/Orbitrap ® Qexactive (Thermo Fisher Scientific, Bremen, Germany) con fuentes de ionización ESI, APCI y nanoESI

coplado a un cromatógrafo líquido de ultra-alta eficacia (UHPLC) (Thermo Fisher Scientific, Bremen, Germany).

- Equipo de quimioluminiscencia Amershan Imager 600 (GE Healthcare Life Sciences, Marlborough, MA, USA).
- Incubador de CO_2 modelo NU-4750E AutoFlow™ (Nuaire, Plymouth, UK).
- Incubadora de CO_2 para levaduras (INCO, Barcelona, España).
- Lector de placas multimodal para medidas de fluorescencia, luminiscencia y absorbancia modelo Synergy HT (BioTek ®, Izasa Scientific, Barcelona, España).
- Medidor pH Basic 20 (Crison, L'Hospitalet de Llobregat, Barcelona, España).
- Micropipeta de 10, 100 y 1000 µL Eppendorf Research plus (Hamburgo, Alemania).
- Micropipeta multicanal de 200 µL Eppendorf Research plus (Hamburgo, Alemania).
- Pipeta automatic FastPette™ V2 (Labnet International, Edison, NJ, EE.UU).
- Sistema de extracción en fase sólida al vacío (Supelco-Visiprep, Sigma-Aldrich, Barcelona, Spain).

1.10. Material fungible

- Agujas (0,8x120 mm) Sterican ® (Braun, Mensulgen, Alemania)
- Cámara contadora de células Countess™ (Invitrogen, Oregón, EE.UU)
- Cámara de Neubauer improved (Marienfeld, Alemania).
- Cartuchos desechables de SPE "*Phree™ phospholipid removal*" de 1 mL (Phenomenex ® Torrance, California, EE.UU).
- Cartuchos desechables de SPE Mega BE-C18, 1 gm, 6 mL (VARIAN, Agilent Technologies, Santa Clara, California, EE.UU).
- Columnas de UHPLC SB-C18 (2,1x5 mm, 1,8 µM) (Agilent, Technologies, Santa Clara, California, EE.UU).
- Eppendorff ámbar de poliprolileno de 1,5 mL (Brand, Wertjeim, Alemania)
- Film de sellado de poliéster para ELISA (146x79 mm) (VWR, Radnor, Pensilvania, EE.UU).

- Filtros de jeringa de 13 mm (0.2 µM) de membrana de nylon (Whatman, Buckinghamshire, UK).
- Filtros de jeringa de 13 mm (0.45 µM) con membrana de politetrafluoroetileno de (VWR, Radnor, Pensilvania, EE.UU).
- Filtros estériles de jeringa de 25 mm (0.2 µM) de membrana de acetato de celulosa (VWR, Radnor, Pensilvania, EE.UU).
- Filtros para botellas de 0.2 µM de Nalgene™ (Thermo Fisher Scientific, Bremen, Alemania)
- Geles de poliacrilamida (4-12% Bis-Tris), 1 mm, 12 pocillos (Thermo Fisher Scientific, Bremen, Alemania)
- Jeringas estériles sin aguja (1mL) Terumo ® (Leuven, Bélgica)
- Jeringas estériles sin aguja (5, 10, 20 mL) BD Discardit™ II (Huesca, España).
- Matraces aforados de 2 mL (Hirschmann, Eberstadt, Alemania)
- Matraces Erlenmeyer de 1 L (VWR, Radnor, Pensilvania, EE.UU).
- Membranas de nitrocelulosa de 0.2 µM, 7x8.4 cm (BioRad, Hercules, California, EE.UU).
- Microinsertos para viales de HPLC de 0,1 mL con muelle (VWR, Radnor, Pensilvania, EE.UU).
- Microinsertos para viales de HPLC de 0.2 mL (VWR, Radnor, Pensilvania, EE.UU).
- Pipetas de vidrio (Labbox Labware, S.L, Vilassar de Dalt, Barcelona, España)
- Pipetas estériles serológicas de 5, 10 y 25 mL (ALP, Chorges, Francia)
- Placas de 75 cm^2 para cultivo celular, en forma de U (Corning, Nueva York, EE.UU).
- Placas de 96 pocillos transparentes (VWR, Radnor, Pensilvania, EE.UU).
- Placas estériles de cultivo celular de 6 pocillos Falcon ® (Corning, Nueva York, EE.UU)
- Precolumnas para columna de UHPLC ZORBAX SB-C18 (2.1x5 mm, 1,8 µM) (Agilent, Technologies, Santa Clara, California, EE.UU).
- Puntas desechables para micropipetas de 10, 100 y 1000 µL (VWR, Radnor, Pensilvania, EE.UU).

- Rascadores estériles para cultivos celulares Falcon ® (Corning, Nueva York, EE.UU)
- Tapones de cauco natural perforados de 40 mm (diámetro superior) x 0,41 mm (diámetro inferior) x 40 mm (altura) (Saint-Gobain, Francia)
- Tubos de 30 mL estériles Sterilin San Martino - Trecate (NO) Italy
- Tubos de centrífuga estériles de 15 mL de polipropileno Falcon ® (Corning, Nueva York, EE.UU)
- Viales ámbar de HPLC de 1,5 mL (32x11.6 mm) con tapones de PTFE con septum rojo de 1 mm (VWR, Radnor, Pensilvania, EE.UU).

1.11. Software informático

- StatSoft, Inc. (2004). Statistica Software (Sistema software de análisis de datos), version 7.
- Thermo Fisher Scientific (2016) TraceFinder™ Software (Sistema de interpretacion de análisis por HRMS), version 3.1.
- Systat Software Inc. (2011) Sigmaplot Software (Sistema de representación gráfica), versión 12.
- Thermo Fisher Scientific (2013) Chromeleon™ Software (Sistema para programación de equipos de UHPLC) versión 7.1.
- Thermo Fisher Scientific (2013) Xcalibur™ Software (Sistema de interpretación de resultados tras análisis pro HRMS), version 3.0.63.
- CambridgeSoft Limited (2011) ChemDraw Software (Sistema de representación de estructuras químicas), versión 12.
- GraphPad Software, Inc. (2012) Grahpad Software (Sistema de estadística y representación de datos), versión 6.
- Microsoft Corporation (2013-2016) Paquete Office Excel® (Sistema de representación y organización de datos), versión 2013-2016.
- National Institute of Health (2016) ImageJ Software (Sistema de interpretación de bandas obtenidas por Western-Blot), versión 1.51.

1.12. Levaduras vínicas comerciales

Se seleccionaron un total de seis levaduras del género *Saccharomyces cerevisiae*, entre las cuales destacan dos cepas de la variante *S.cerevisiae bayanus* **(Tabla 6)**. Además, se incluyeron dos levaduras comerciales de

géneros no-*Saccharomyces*, *Metschnikowia pulcherrima* y *Torulaspora delbrueckii*. Todas las cepas fueron adquiridas liofilizadas.

Tabla 6. Cepas de levaduras comerciales utilizadas para el desarrollo de las fermentaciones

Género	**Cepa**	**Proveedor**
Metschnikowia pulcherrima	Flavia® MP346	Lallemand
Saccharomyces cerevisiae	Aroma White	Enartis
Saccharomyces cerevisiae	ES488	Enartis
Saccharomyces cerevisiae	Lalvin ICV GRE	Lallemand
Saccharomyces cerevisiae	Red Fruit	Enartis
Saccharomyces cerevisiae var. *bayanus*	Lalvin QA23	Lallemand
Saccharomyces cerevisiae var. *bayanus*	Uvaferm BC	Lallemand
Torulaspora delbrueckii	Biodiva™ TD291	Lallemand

2. MÉTODOS

2.1. Método de cromatografía líquida de ultra alta eficacia acoplado a espectrometría de masas (UHPLC/HRMS) para la determinación simultanea de compuestos indólicos: Validación *(Capítulo 1)*

Se utilizó un equipo de cromatografía líquida de ultra alta eficacia acoplada a un espectrómetro de masas de alta resolución (UHPLC/HRMS) consistente en un híbrido de cuadrupolo/Orbitrap ® Qexactive. El módulo de UHPLC Dionex™ 3000 constó de una bomba (HPG-3400RS), un cargador automático de muestras (WPS-3000RS) y un compartimento para la columna cromatográfica (TCC-3000RS). Se utilizó una column ZORBAX RRHD SB-C18 (2,1 × 100 mm, 1.8-µm tamaño de partícula) con una precolumna compatible con las mismas características que se mantuvo a 40º C durante el análisis.

El equipo constó de dos fases móviles, agua milliQ (A) y metanol (B), ambas con una concentración de 0.1% de ácido fórmico. Se programó un gradiente de elución durante 15 minutos con las siguientes características:

- 0-2 minutos: 95% A, 5% B
- 2-13 minutos: 0% A, 95% B
- 13.1-15 minutos: 95% A, 5% B.

El flujo seleccionado fue de 0.5 mL/min mientras que se fijó un volumen de inyección de 5 µL. El compartimento de muestras se mantuvo a 4ºC durante todo el análisis. El equipo se calibró semanalmente siguiendo las indicaciones del fabricante.

Con respecto a la espectrometría de masas, se utilizó una fuente de ionización en electrospray con temperatura (HESI) en modo positivo. Las características del método desarrollado se enumeran a continuación:

- Temperatura del horno: 440ºC
- Temperatura del capilar: 270ºC
- Voltaje en spray: 3.5 Kv
- Flujo de gas de envoltura (sheath gas): 53 unid. arbitrarias
- Flujo de gas auxiliar (auxiliary gas): 14 unid. arbitrarias
- Flujo de gas de barrido: 3 unid. Arbitrarias
- Energía de colisión normalizada (NCE): 20
- Poder de resolución: 35000 de anchura a media altura (FWHM).
- Margen de ventana: 1.0 m/z.
- Nivel de la radiofrecuencia de la lente (S-lens RF): 50%
- Tiempo máximo de inyección: 100 ms
- Control automático de ganancia (AGC) en modo dirigido: $1x10^6$ iones

Se eligió un método dirigido de fragmentación de masas (target MS^2) a través de las masas exactas de los diferentes compuestos indólicos. Los patrones de los nueve derivados del L-triptófano se infusionaron en el equipo utilizando una solución con una mezcla de todos los patrones (ver Tabla 2) a concentraciones entre 3 y 55 ng/mL. Tras ver los fragmentos mayoritarios y el tiempo de retención de cada compuesto, se decidió acotar los minutos del tiempo análisis de la siguiente forma:

- Serotonina/5-hidroxitriptófano: Minuto 0,5-2
- L-triptófano/Triptamina: Minuto 3,6-4,6
- N-acetilserotonina: Minuto 5-5,7

- Melatonina/Etil ester de triptófano: 6,1-7,6
- Triptofol/Ácido 3-indolacético: 6,6-7,6

La validación del método se llevó a cabo siguiendo los parámetros establecidos por la Asociación Oficial de Químicos Analíticos (AOAC). Se calcularon los parámetros de linealidad, repetividad, reproducibilidad y límites de detección y cuantificación.

2.2. Preparación del mosto sintético: *Capítulo 1, 2 y 3*

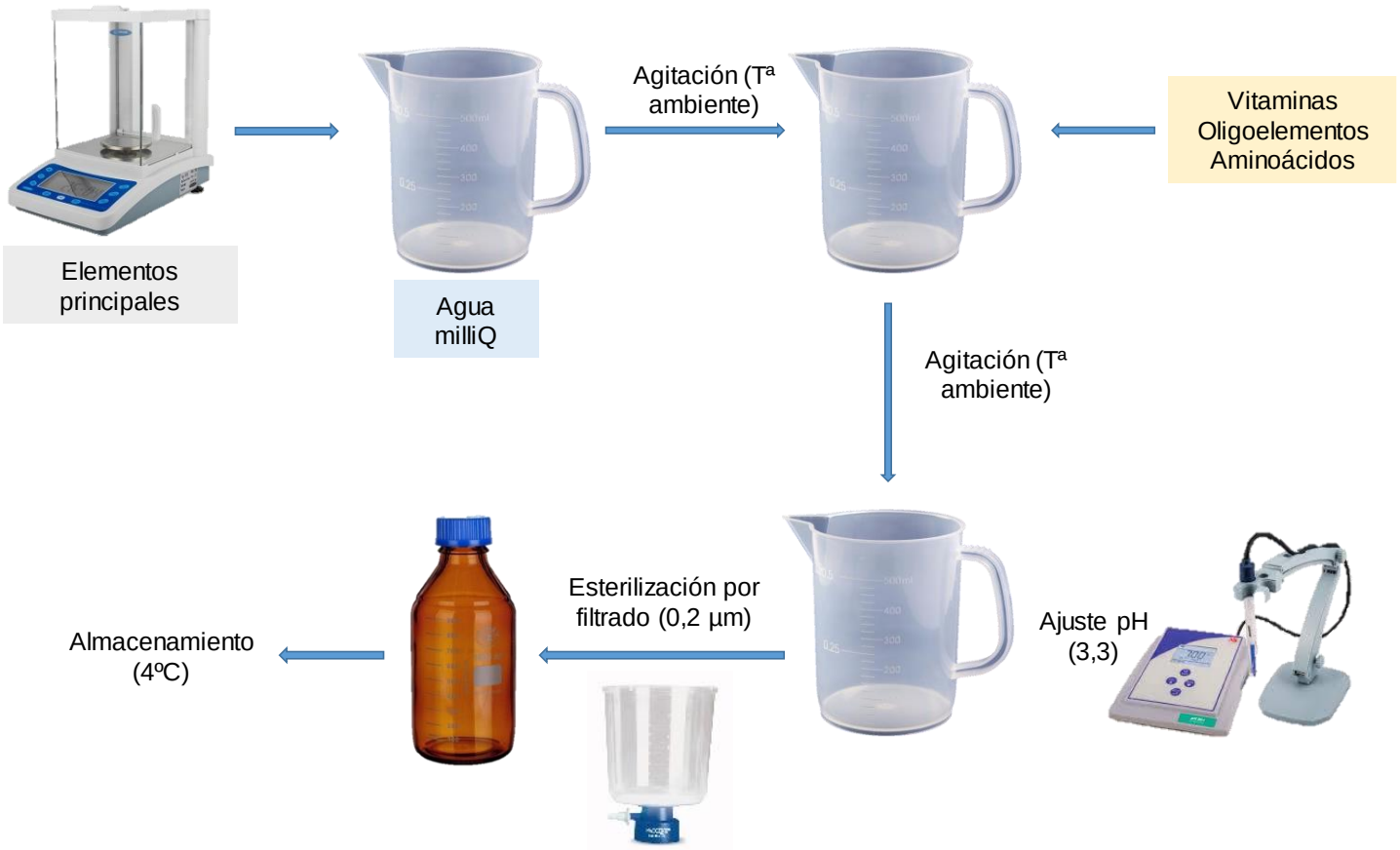

Figura 7. Procedimiento de preparación del mosto SM300

En primer lugar, se prepararon las soluciones de aminoácidos, vitaminas, oligoelementos y factores anaerobios. Los aminoácidos se disolvieron en una solución al 2% de bicarbonato de sodio, mientras que las soluciones de vitaminas y oligoelementos se prepararon en agua milliQ. Los factores anaerobios se disolvieron en una solución de etanol: Tween80 al 50% (v/v). Las soluciones de

vitaminas, aminoácidos y oligoelementos se esterilizaron por filtración (0.2 µM) y se tomaron las respectivas alicuotas. Posteriormente se añadieron al mosto 13,09 mL/L de la solución de aminoácidos, 10 mL/L de vitaminas y 1 mL/L de

oligoelementos y factores anaerobios. Los pasos para la preparación del mosto vienen indicados en la **Figura 7**.

2.2.1. Preinóculo para el mosto sintético

Antes de inocular el mosto sintético, las levaduras liofilizadas se rehidrataron (30 minutos, 37º C). Posteriormente, se sembraron en placas de medio sólido de peptona y dextrosa para levaduras (YPD, 2% peptona, 2% glucosa, 1% extracto de levadura y 2% agar) y se incubaron por un mínimo de 48 horas a 28ºC. Tras observar crecimiento en las placas, las levaduras se almacenaron a 4ºC hasta su uso. Todas las cepas se sembraron por duplicado.

Los preinóculos para las fermentaciones en mosto sintético se prepararon en medio YPD líquido esterilizado. Se utilizó un asa de siembra para coger colonias de las placas de YPD sólido y se introdujeron en un matraz Erlenmeyer con 150 mL de YPD. Seguidamente, el preinóculo se incubó en un agitador orbital a 28º C y 150 rpm durante 12-24 horas antes de la inoculación.

2.2.2. Inoculación en el mosto sintético

Se utilizaron 750 mL de mosto sintético en un matraz Erlenmeyer de 1 L de capacidad. Para asegurar las condiciones de esterilidad, los matraces se sellaron utilizando tapones de goma con dos salidas. En una de ellas, se introdujo un capilar de vidrio para permitir la liberación del dióxido de carbono; en la otra, se usó un tubo de silicona para la toma de muestra, cerrado en su extremo exterior por un tapón de plástico. Ttodos los materiales fueron previamente esterilizados.

Para la inoculación, se hizo el recuento del número de células del preinóculo en una cámara de Neubauer, por duplicado. Posteriormente, se utilizó un volumen del preinóculo suficiente para obtener una concentración inicial en el mosto sintético de 10^6 células/mL. Tras la inoculación, los matraces se incubaron a 28ºC con una agitación de 150 rpm para comenzar con la fermentación alcohólica. El seguimiento se realizó pesando diariamente los matraces y calculando la pérdida de dióxido de carbono hasta la estabilización del peso.

2.3. Elaboración del mosto de uva: *Capítulo 4*

Todas las uvas fueron de la cosecha de 2015 y se recolectaron en su estado óptimo de maduración. El procedimiento para la obtención del mosto se observa en la **Figura 8**. Los racimos de uvas se despalillaron, estrujaron y prensaron. Posteriormente, se adicionaron 40 mg/L de SO_2 y enzimas pectolíticas (2.5 mL/hL) previo al proceso de desfangado (24 h, 4ºC). El mosto resultante se introdujo en tanques de acero inoxidable de 100 L de capacidad. La variedad Tempranillo se utilizó para la preparación de un vino rosado.

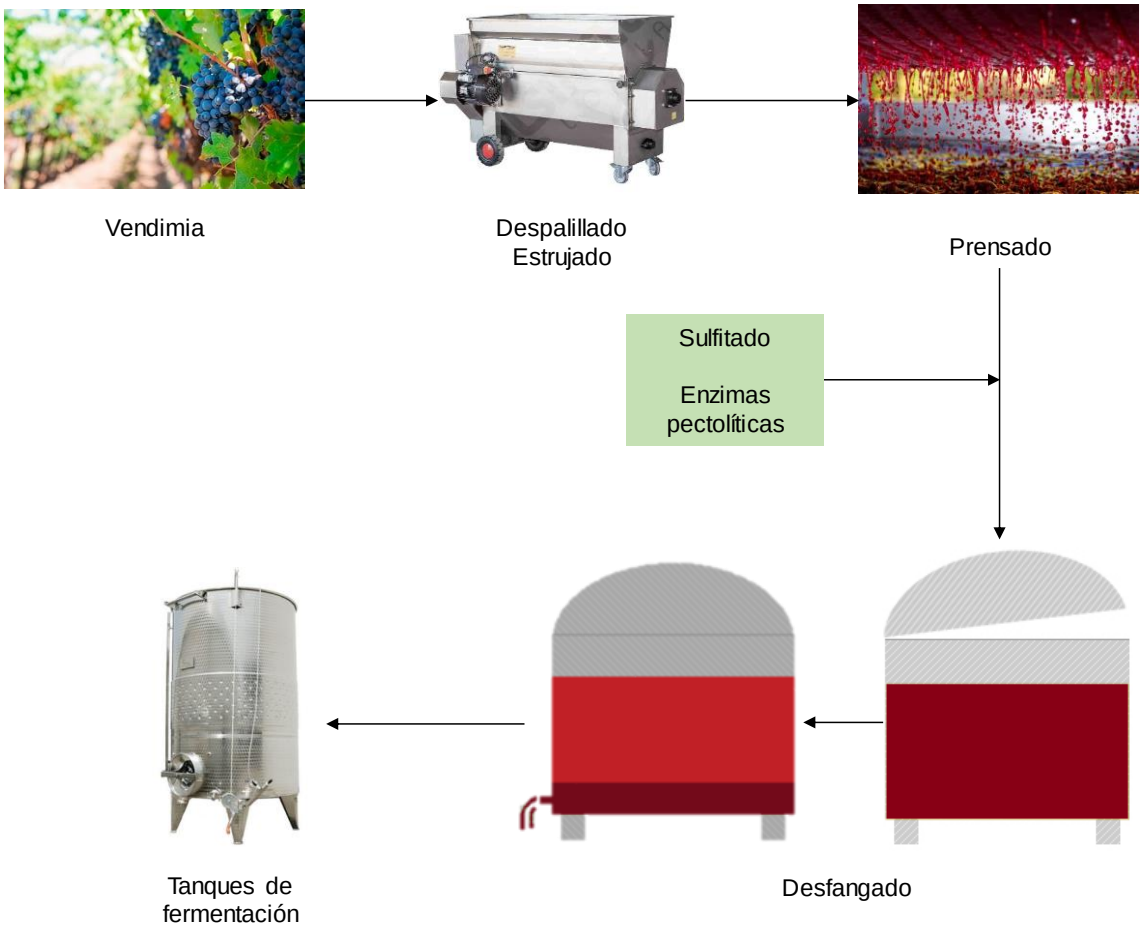

Figura 8. Proceso de obtención del mosto de uva para las fermentaciones del vino blanco y el rosado.

2.3.1. Inoculación del mosto de uva

Las variedades de uva blanca se inocularon con una sola cepa de levaduras (*S. cerevisiae* Aroma White) mientras que en la variedad tinta (Tempranillo) se realizaron 4 tipos de protocolos de inoculación **(Figura 9)**, los cuales se enumeran a continuación:

- Inoculación con cepa *S.cerevisiae* Ref Fruit (CT-RF).
- Inoculación con cepa *S.cerevisiae* var *bayanus* QA23 (CT-QA23).
- Inoculación con cepa *S.cerevisiae* Red Fruit con posterior inoculación secuencial de la cepa *T.delbrueckii* (SI-RF).
- Inoculación con cepa *S.cerevisiae* var *bayanus* QA23 con posterior inoculación secuencial de la cepa *T.delbrueckii* (SI-RF).

La levadura Aroma White liofilizada necesitó una hidratación previa. Para ello, se utilizó un protector (Go Ferm Protect®, Lallemand, Alemania) para disminuir el choque osmótico durante el proceso de rehidratación. Se mezclaron 30 g/hL de este compuesto en 1L de agua, calentando la solución (43ºC). Para añadir la levadura al vino, la diferencia de temperatura entre la levadura hidratada y el mosto no debe ser mayor a 10ºC. Por ello, tras homogeneizar la mezcla, se atempera por debajo de 40ºC para poder añadir la levadura a una concentración de 25 g/hL y se deja reposar 15 minutos. Posteriormente, se agita cuidadosamente la mezcla y se deja reposar nuevamente 30 minutos.

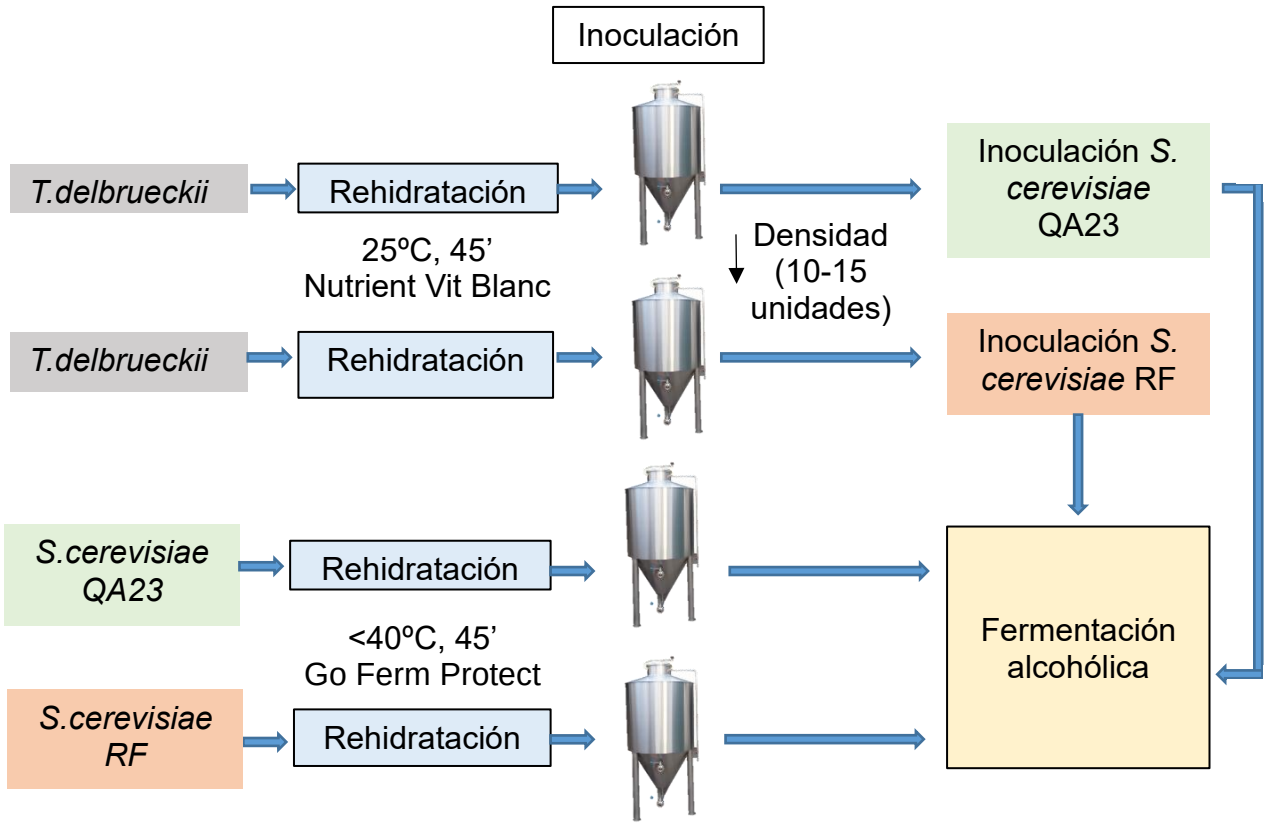

Figura 9. Esquema con los diferentes protocolos de inoculación en el mosto de la variedad Tempranillo.

En el caso de las inoculaciones secuenciales, primero se rehidrata la levadura *T. delbrueckii* (25 g/hL, 25ºC) durante 15 minutos, dejando reposar, agitando cuidadosamente y reposando de nuevo durante 30 minutos. Al igual que en la inoculación de la Aroma White, la diferencia de temperatura del mosto y de la levadura rehidratada no puede ser superior a 10 ºC. Para favorecer el metabolismo de las levaduras, se añaden al medio nutrientes (Nutrient Vit Blanc®, Lallemand, Alemania) a una concentración de 20 g/hL. Tras la inoculación de *T.delbrueckii*, la densidad del tanque de fermentación debe registrar una bajada de 10-15 puntos con respecto al valor original, antes de incorporar la levadura *S. cerevisiae* (ya sea QA23 o RF) junto con una nueva dosis de nutrientes.

El procedimiento de rehidratación para la QA23 o la RF es similar al descrito para la cepa Aroma White. Todas las fermentaciones se hicieron por triplicado manteniendo la temperatura a 16-18ºC hasta el final de la fermentación alcohólica (azucares residuales <3 g/L). Al mismo tiempo, se analizó un mosto fermentado espontáneamente sin la presencia de levaduras comerciales como control.

2.4. Ensayos para evaluar la estabilidad de compuestos derivados del metabolismo del L-triptófano en matrices fermentadas (*Capítulo 2)*

Tras la fermentación del mosto sintético llevado a cabo por la levadura del género *S. cerevisiae* Aroma White, se realizó un estudio de estabilidad de los compuestos derivados del aminoácido L-triptófano durante el almacenamiento, tras una fermentación de 15 días a 28ºC y 150 rpm. Asimismo, se analizaron extractos de medio intracelular para comparar la diferencia entre la matriz extracelular y la intracelular. Para comparar el estudio, se incluyeron muestras comerciales de vino y cerveza adquiridas en supermercados locales como producto final tras un proceso de fermentación. Se seleccionaron un vino blanco (variedad Palomino Fino) y una cerveza lager tipo *pilsner*, los cuales presentan unas matrices menos complejas para facilitar el posterior tratamiento de muestras. El tiempo de almacenamiento fue de 15 días para todas las muestras, y se realizaron alícuotas por separado para medir los experimentos por temperatura (4, -20 y -80ºC), y concentración (2 y 200 ng/mL, enriquecimiento)

2.5. Tratamiento de muestras: *Capítulo 1-5*

Todas las muestras de mosto (sintético o de uva), cervezas y vino fueron sometidas a un proceso de extracción en fase sólida con el objetivo de (I) limpiar la muestra de posibles interferentes que dificultaran el análisis posterior y (II) concentrar los analitos de interés. En la **Figura 10** se observa un cuadro resumen de los diferentes experimentos y sus puntos de muestreo, hasta llegar al tratamiento de extracción en fase sólida (SPE).

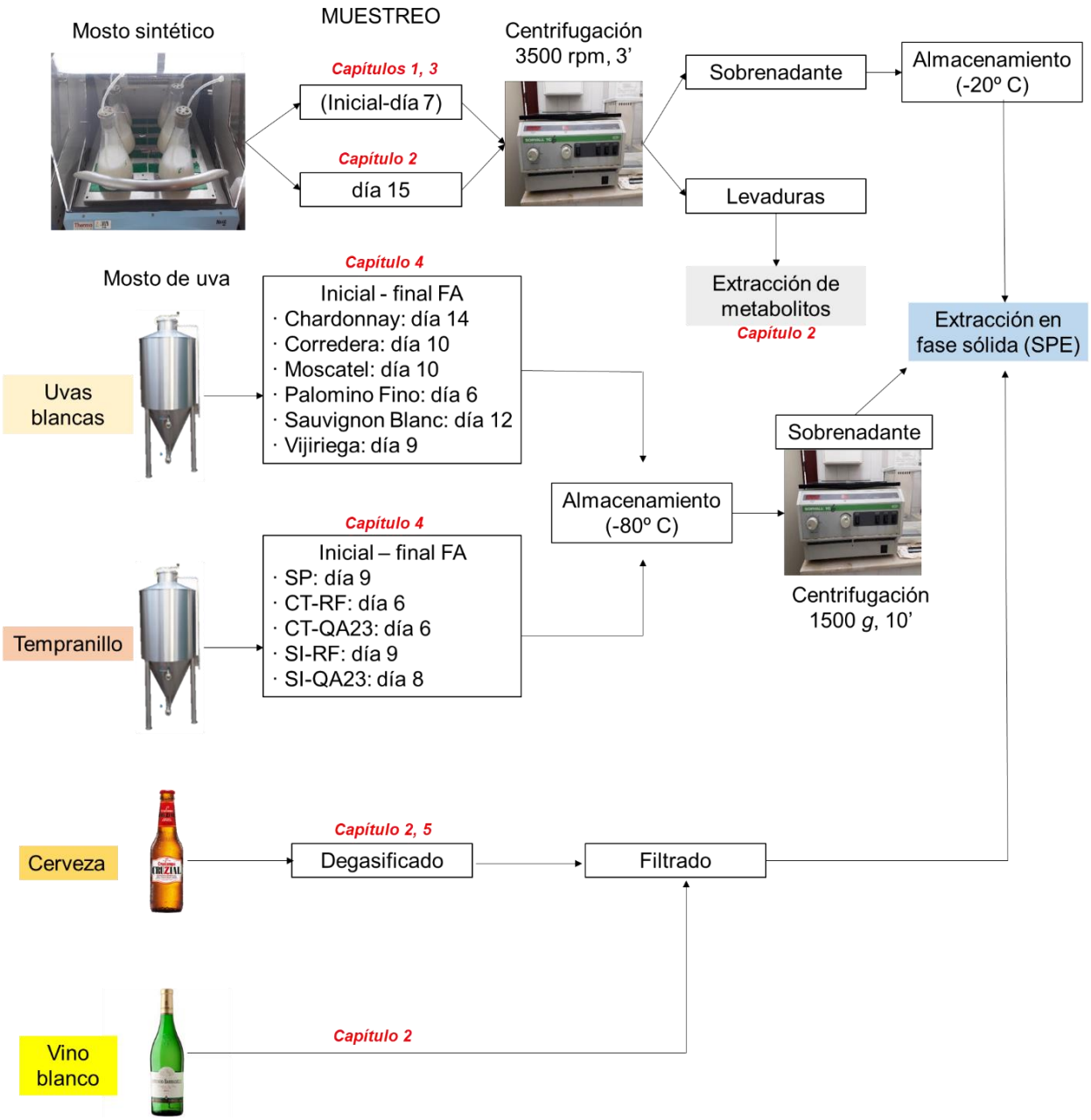

Figura 10. Muestreo realizado en las diferentes matrices alimentarias utilizadas en los capítulos 1-5.

2.5.1. Extracción en fase sólida

El procedimiento se realizó con cartuchos de extracción en fase sólida de tipo C18 (VARIAN, Agilent) caracterizados por ser altamente hidrofóbicos, con una capacidad de para procesar hasta 3 mL de muestra. Los cartuchos eran en primer lugar acondicionados (2 mL de metanol y 2 mL de agua MilliQ) antes de incorporar la muestra, cuyo volumen fue de 500 µL para los mostos (sintético y de uva), 1 mL para el vino y 1-2 mL para la cerveza. Los cartuchos conteniendo la muestra se limpiaron con 2 mL de una solución de agua:metanol al 10% (v/v) antes de extraer los analitos con metanol puro (1 mL).

Posteriormente, las muestras se introdujeron en un desecador a vacío hasta eliminar por completo el solvente. Una vez desecadas se resuspendieron en una solución de metanol:agua al 10% (v/v) con ácido fórmico (0,1%) a una concentración 3:1 con respecto al volumen inicial, con la excepción de las muestras utilizadas en el Capítulo 5 (cerveza) que fueron concentradas 10 veces.

2.5.2. Extracción de metabolitos intracelulares

Tras 15 días de fermentación en el mosto sintético, se realizó el recuento de levaduras en una cámara de Neubauer para poder extraer alícuotas de mosto final con una concentración de 10^9 cél/mL. Posteriormente, se separó la fracción celular del contenido extracelular por centrifugación. El pellet de levaduras se lavó dos veces para eliminar la presencia de analitos del medio extracelular.

La extracción de los metabolitos intracelulares se llevó a cabo utilizando un método con etanol caliente (Gonzalez, François, & Renaud, 1997). Este método utiliza una solución compuesta en un 75% por etanol y ácido N-(2-hidroxietil) piperazina-N'-(2-etanosulfónico) (HEPES) como buffer **(Figura 11)**. La solución se lleva a ebullición y posteriormente se añade al pellet de levaduras (3 mL). Inmediatamente después, se colocan en un baño termostatizado a 80º C por tres minutos. Transcurrido este tiempo, las muestras se dejan en hielo (al menos 3 minutos) antes de desecarlas a vacío. Luego, las células se resuspenden con agua MilliQ (3 mL) y se centrifugan (4ºC, 10 minutos, 5000 *g*). El sobrenadante

con los metabolitos intracelulares se almacena a -80ºC hasta el momento de su análisis.

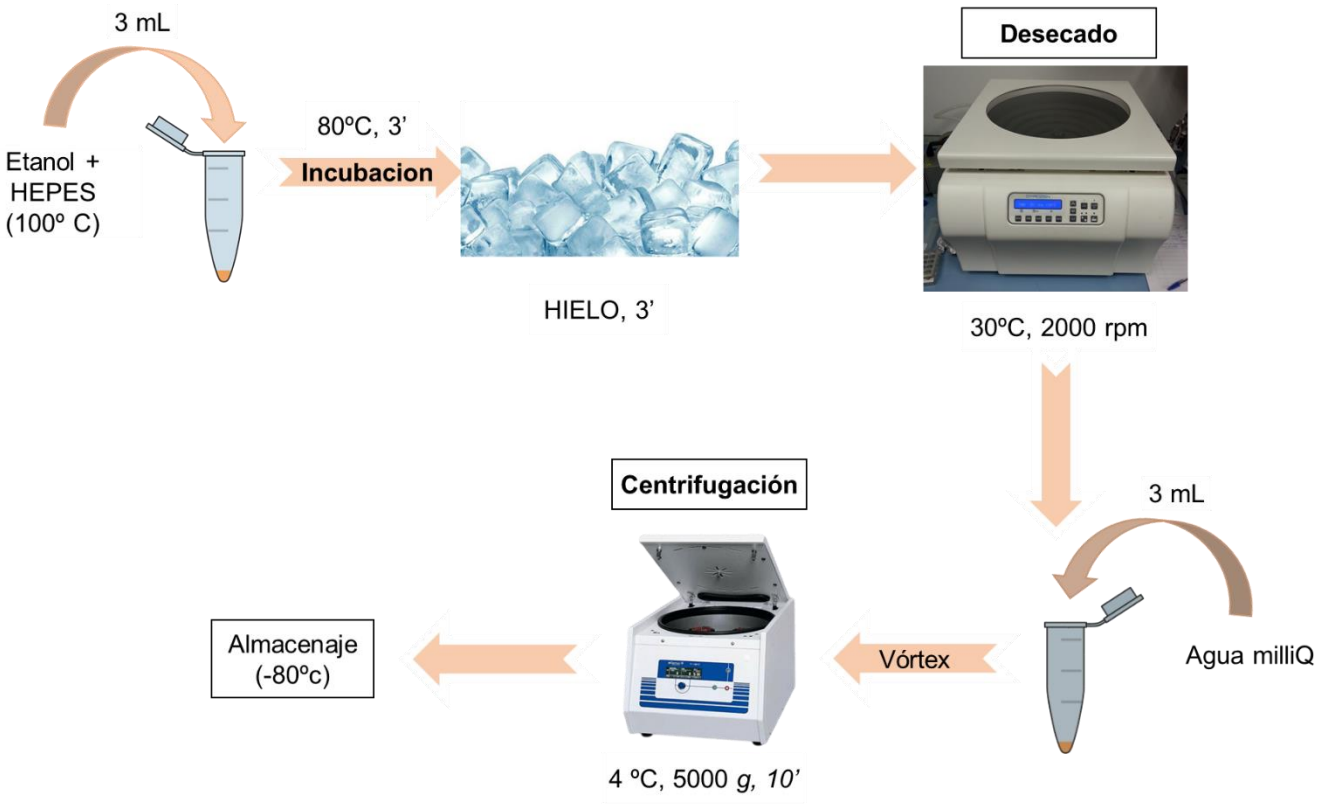

Figura 11. Esquema de la extracción intracelular por el método del etanol en caliente de González et al. 1997 para las muestras intracelulares utilizadas en el capítulo 2

2.5.2.1. Extracción en fase sólida para muestras intracelulares

Ya que las levaduras se someten a tratamientos agresivos para poder romper su pared celular, esta matriz suele presentar una alta concentración de fosfolípidos. Para evitar estas interferencias en el posterior análisis, las muestras deben tratarse con unos cartuchos de extracción en fase sólida para la eliminación de fosfolípidos (*Phree*, Phenomenex®). El adsorbente de estos cartuchos provoca la adhesión de fosfolípidos, previa precipitación de los mismos en la muestra. El procedimiento consiste en añadir a los cartuchos una solución de metanol y ácido fórmico (1%) y una pequeña cantidad de muestra (100 µL) respetando una proporción entre ambas soluciones de 1:4. Posteriormente, la mezcla se hace pasar por el cartucho con la ayuda de una bomba de vacío, estando en contacto con el material adsorbente al menos durante 5 minutos, para obtener el extracto

final. Los extractos resuspendidos se analizaron a través del equipo de UHPLC/HRMS con el método previamente validado.

2.6. Ensayos para la determinación de la actividad anti-angiogénica de los estilbenos (*Capítulo 6)*

2.6.1. Cultivo de células

Las células fueron utilizadas entre el pase 4 y 5. Se sembraron en placas de 6 pocillos estériles en un medio de crecimiento específico para HUVEC (*Endothelial Growth Medium-2*, EGM-2) suministrado por Lonza (Slough, UK) hasta la total confluencia de las mismas (5-6 días). Los cultivos se mantuvieron en incubadores a 37ºC a una atmósfera de un 5% de CO_2.

2.6.2. Tratamiento celular

Las placas con las células confluentes se sometieron a un tratamiento donde se utilizó un medio basal (*Endothelial Basal Medium, EBM*) adquirido por Lonza (Slough, UK) al que sólo se añadió antibiótico (15 mg de gentamicina + 7.5 µg de anfotericina B) para evitar la contaminación por microorganismos. El procedimiento se describe brevemente en la **Figura 12**.

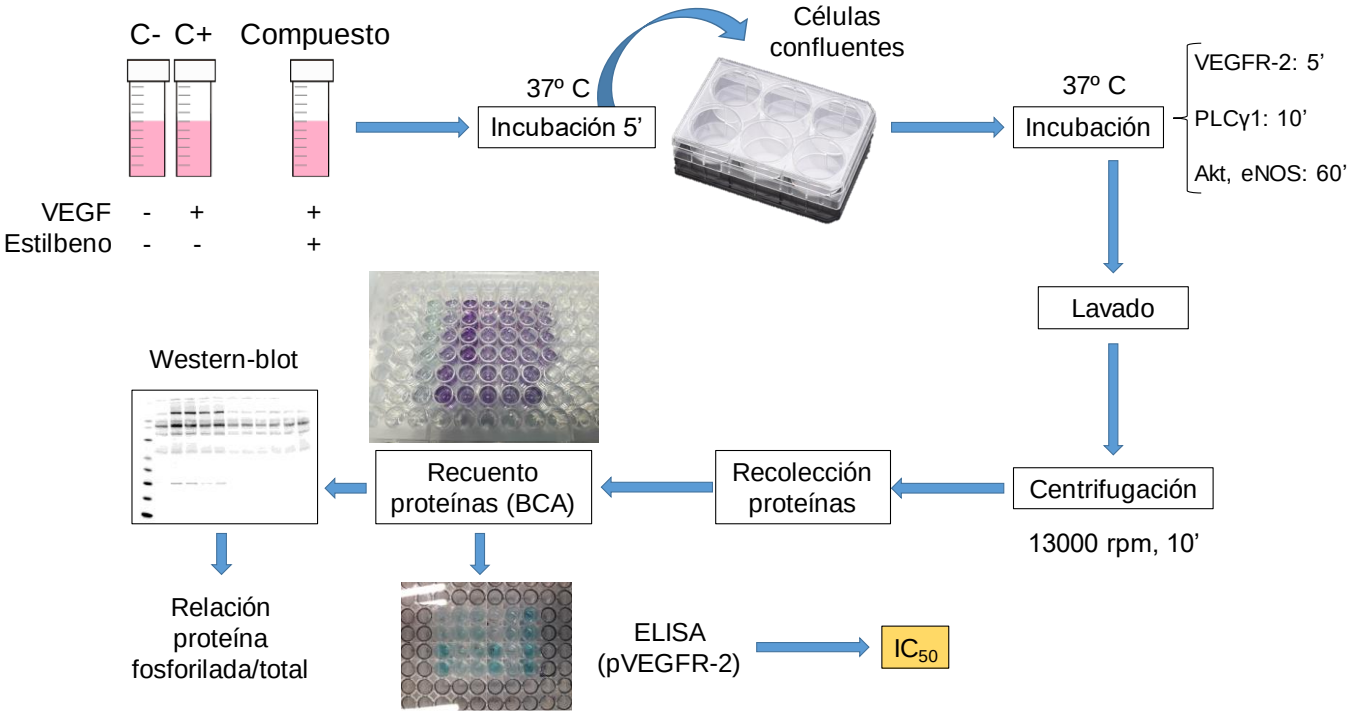

Figura 12. Procedimiento llevado a cabo sobre células HUVEC en presencia de estilbenos y VEGF

En primer lugar, se realiza un mix de VEGF (25 ng/mL) + estilbenos (0.5-50 µM) que se incuba durante 5 minutos. Posteriormente, las células se incuban con este mix a un tiempo variable, según la proteína a evaluar. En el caso de la evaluación de la inhibición del pVEGFR-2, se incuban 5 minutos, para PLCγ1 10 minutos y para Akt y eNOS 60 minutos (Moyle et al., 2015).

Tras la incubación, las células se lavan con un Buffer fosfato salino (PBS) y se lisan usando un buffer para radioinmunoprecipitación (RIPA) en cuya composición se encuentra un digestor de ADN (benzonasa nucleasa) y protectores de la proteína fosforilada (inhibidores de fosfatas y proteasas). Las proteínas son recolectadas tras utilizar raspadores desechables en cada pocillo y se centrifugan a 13000 rpm durante 10 minutos. Las muestras (el sobrenadante) se congelan a -80ºC.

2.6.3. Recuento de proteínas

Para el recuento de proteínas totales se utiliza el método espectrofotométrico del ácido bicinconínico (BCA). Este método utiliza la capacidad de algunos aminoácidos (cisteína, cistina, tirosina, triptófano) para convertir el ion de Cu^{+2} a Cu^{+1} en un medio salino a 37ºC. Posteriormente, ese ion de Cu^{+1} reacciona con el BCA dando lugar a un cromóforo, el cual se puede medir su absorbancia a 550 nm. La señal de las proteínas en las muestras se compara con una recta de calibrado realizada con albúmina de suero bovino (BSA). La determinación de las proteínas totales nos permite incluir en los ensayos de ELISA y Western blot la misma cantidad de proteínas de las distintas muestras de tal manera que se puedan comparar los resultados entre ellas.

2.6.4. Determinación del potencial anti-VEGF de los estilbenos por el método ELISA: Estimación del IC_{50}

Se utilizó un kit de ELISA específico que mide la fosforilación del VEGFR-2 en el residuo de Tirosina 1175 (Cell Signalling Technology, Danvers, MA, USA). El ensayo utiliza las muestras de proteínas a una concentración por pocillo normalizada, de manera que los resultados sean comparables entre sí. Las proteínas se incuban durante 1h a 37ºC con el anticuerpo específico para el

residuo de tirosina y posteriormente, se añade el anticuerpo secundario y se vuelve a incubar a (37ºC, 30 minutos). Transcurrido el tiempo, se agrega un sustrato a temperatura ambiente que aporta color al unirse con el anticuerpo secundario. Pasados 30 minutos, se añade una solución que interrumpe esta reacción, y se mide la absorbancia en un lector de placas a 450 y 550 nm. Los datos de absorbancia nos permiten realizar los cálculos de IC_{50} para cada estilbeno, utilizando el software GraphPad Prism.

2.6.5. Análisis de PLCγ1, Akt e eNOS por el ensayo de western-blot

El lisado de proteínas (la misma cantidad de proteínas para cada muestra) se mezcla con un tampón (dodecilsulfato de litio, LDS) y un agente reductor especial para la electroforesis (ditiotreitol, DTT). Tras mezclarlo, las proteínas se desnaturalizan en un termobloque (70ºC, 10 minutos) antes de colocarlas en un gel de poliacrilamida (4-12%) para la separación de las mismas (200 V, 45 minutos). Posteriormente, las proteínas se traspasan a una membrana de nitrocelulosa, la cual es tratada durante 1 h con una solución de albúmina de suero bovino (BSA) en TBST (5%). A continuación, se añaden los anticuerpos de las proteínas fosforiladas y se incuban a 4ºC al menos durante 12 h. Después, se incuba 1 h con el anticuerpo secundario y se mide la intensidad de las bandas por quimioluminiscencia con el kit de sustrato para quimioluminiscencia *Signal West Pico PLUS* (Thermo Fisher Scientific, Bremen, Germany). Tras esta media, las membranas vuelven a tratarse para separar los anticuerpos fosforilados y realizar el tratamiento de nuevo con BSA para inbucar las membranas con los anticuerpos totales.

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RESULTADOS

CAPÍTULO 1 / CHAPTER 1

Fernández-Cruz, E; Álvarez-Fernández, M. Antonia., Valero, E., Troncoso, A.M.,

García-Parrilla, M.C.

VALIDACIÓN DE UN MÉTODO ANALÍTICO PARA DETERMINAR MELATONINA Y OTROS COMPUESTOS DERIVADOS DEL METABOLISMO DEL L-TRIPTÓFANO POR UHPLC/HRMS

VALIDATION OF AN ANALYTICAL METHOD TO DETERMINE MELATONIN AND COMPOUNDS RELATED TO L-TRYPTOPHAN METABOLISM USING UHPLC/HRMS

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Validation of an analytical method to determine melatonin and compounds related to L-Tryptophan metabolism using UHPLC/HRMS

Fernández-Cruz, E.[a]; Álvarez-Fernández, M. Antonia.[a], Valero, E.[b,] Troncoso, A.M.[a,] García-Parrilla, M.C.[a]*

[a]*Department of Food Science and Nutrition, Faculty of Pharmacy, University of Seville, C/ Profesor García González 2, 41012, Sevilla, Spain.*

[b]*Department of Food Science and Nutrition, Faculty of Experimental Science, University Pablo de Olavide, Carretera de Utrera Km. 1, 41013, Sevilla, Spain.*

Corresponding author. Tel.: +34 95 4556759.

E-mail address: mcparrilla@us.es (MC. García-Parrilla).

Abstract

Melatonin is a bioactive compound that is present in wines because of the metabolism of L-Tryptophan by yeasts. Even though the complete pathway of synthesis is not well elucidated, certain related indolic compounds might be involved in it. Consequently, their determination is a matter of interest. On one hand, their formation during fermentation might be related to a specific role for yeast's metabolism, not known so far. On the other hand, the synthesis by yeasts of bioactive compounds with putative health benefits for consumers, such as melatonin or serotonin, is a relevant matter.

This paper aims to develop and validate an analytical method by Ultra High Performance Liquid Chromatography coupled to High Resolution Mass Spectrometry (UHPLC/HRMS) to monitor both melatonin and related indolic compounds, in order to decrease their detection limits and make it possible to assess their occurrence in culture medium and fermented products. In addition, the other objective is to evaluate the production of these compounds by a commercial *Saccharomyces cerevisiae* used to make white wines.

Diminishing the limit of detection below 0.5 ng mL^{-1} for all compounds under study is an achievement of this work. Furthermore, the strain under study (AROMA WHITE) has been found to synthetize melatonin and related compounds as serotonin. Additionally, the evolution of these compounds over time may contribute to understanding the role they play in yeast metabolism.

Keywords: indolic compounds, wine, yeast, *Saccharomyces*, synthetic must, exact mass.

Introduction

Melatonin (MEL) has previously been evidenced in wines (Mercolini et al. 2008, 2012, Stege et al. 2010, Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al. 2011, Vitalini et al. 2013, Vigentini et al. 2015). Despite it having been detected in wine after fermentation, revealing the role of *Saccharomyces cerevisia*e, little is known beyond this; specifically, its formation by yeasts under fermentation conditions needs to be unravelled. Nevertheless, L-tryptophan (L-TRP) has been pinpointed as its precursor (Sprenger et al. 1999, Murch et al. 2000). Additionally, serotonin (SERO), which happens to be an intermediary in the MEL pathway in plants (Iriti 2009, Feng et al. 2014), has been reported in wines (Manfroi et al. 2009, Wang et al. 2014). On the other hand, literature on the chemical composition of wines describes that some of these indolic compounds, such as Tryptophol (TOL) and 3-indolylacetic acid (3-IAA), are present in wines (Mattivi et al. 1999, Maslov et al. 2011, Favre et al. 2014) being the latest an off-flavour.

Other analytical techniques to determine L-TRP and derivative compounds in wines have been previously used (García-Parrilla et al. 2009). It is worth mentioning the ELISA assay, since it was used to identify MEL in serum (Welp et al. 2010). However, ELISA results were reported to be inaccurate for wine analysis due to matrix effects (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al. 2011). Indeed, the presence of a substance with a similar structure in wines could cause interference.

Originally, HPLC coupled with fluorescence detectors was used (Mattivi et al. 1999, Mercolini et al. 2008), but this technique is not suitable for unequivocal identification and therefore mass spectrometry techniques were preferred (Kocadağlı et al. 2014). At present, hybrid quadrupole-orbitrap offers a more accurate analysis of compounds based on the use of exact mass. Therefore, the optimization of an analytical method based on

high-resolution mass spectrometry (HRMS) techniques is required to achieve lower limit of detection (LOD) and limit of quantitation (LOQ) values, in addition to the subsequent validation of analytical parameters.

In order to gain more knowledge about L-TRP derivatives formation, a simultaneous analysis is necessary. The pathway in *Saccharomyces* has not been established yet. In animals and plants (Figure 1), the precursor is L-TRP, which is hydroxylated to form 5-hydroxytryptophan (5-HTRP). A decarboxylase transforms 5-HTRP into SERO and then into N-acetylserotonin (NACSERO). The last step in this pathway consists of MEL formation, through the action of a methyl transferase (Harumi & Matsushima 2000, Iriti & Varoni 2015). Plants also have an alternative pathway to synthesize MEL from L-TRP. A decarboxylase transforms it into Tryptamine (TRYPT) and then it is hydroxylated to SERO. The rest of the pathway is similar to animals (Tan et al. 2014).

Other compounds, such as TRYPT, 3-IAA and TOL are included in other pathways. In particular, TRYPT requires only one step to its formation, via L-TRP decarboxylase. Indeed, the literature reports these compounds in wines (Mattivi et al. 1999, Maslov et al. 2011, Rodriguez-Naranjo et al. 2013, Piasta et al. 2014, Mihaljević Žulj et al. 2015). On the other hand, L-Tryptophan ethyl ester (L-TRP EE), which has been recently reported to occur during alcoholic fermentation of must (Vigentini et al. 2015), presents the same exact mass as MEL. Due to a misinterpretation of the data, it was previously taken as a MEL isomer, but Iriti *et al*. elucidated its structure (Gardana et al. 2014). This compound is formed via esterification, although whether all *S.cerevisiae* strains are able to synthetize it is not clear, neither is the role it plays.

The aim of this work is to develop and validate an analytical method by Ultra High Performance Liquid Chromatography coupled to High Resolution Mass Spectrometry

(UHPLC/HRMS) techniques to simultaneously identify and quantify analytes related to L-TRP metabolism, in order to gain knowledge of yeast metabolism. To this end, optimization to achieve a low LOD is required, as MEL is in very low concentration and the same is expected for compounds involved in the pathway.

Materials and methods

Synthetic must

Synthetic must (SM) was prepared following the Riou *et al*. method (Riou et al. 1997), with a slight modification in sugar (100 g L^{-1} fructose and 100 g L^{-1} glucose) and organic acid content (tartaric acid 3 g L^{-1}, citric acid 0.3 g L^{-1} and malic acid 5 g L^{-1}). The other compounds were maintained without modifications: amino acids (purity ≥ 99%); mineral salts (KH_2PO_4 0.75 g L^{-1}, K_2SO_4 0.5 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.250 g L^{-1}, $CaCl_2$ 0.155 g L^{-1}, NaCl 0.200 g L^{-1}, NH_4Cl 0.460 g L^{-1}, $MnSO_4 \cdot H_2O$ 4 g L^{-1}, $ZnSO_4 \cdot H_2O$ 4 g L^{-1}, $CuSO_4 \cdot 5H_2O$ 1 g L^{-1}, KI 1g L^{-1}, $CoCl_2 \cdot 6H_2O$ 0.4 g L^{-1}, H_3Bo_3 1 g L^{-1} and $(NH_4)_6Mo_7O_{24}$ 1 g L^{-1}); vitamins (myo-inositol 2 g L^{-1}, calcium panthothenate 0.150 g L^{-1}, thiamine hydrochloride 0.025 g L^{-1}, nicotinic acid 0.200 g L^{-1}, pyridoxine 0.025 g L^{-1} and biotin 0,0003 g L^{-1}); anaerobic factors (ergosterol 1.5 g 100 mL^{-1}, oleic acid 0.5 g 100 mL^{-1} and Tween 80 0.5 ml L^{-1}). All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

L-TRP (cell culture grade) was supplied by Panreac (Darmstadt, Germany); standards of 5-HTRP, SERO (purity ≥ 98 %), NACSERO (TLC, purity ≥ 98 %), MEL (TLC, purity ≥ 98 %), TRYPT (analytical standard), TOL (purity 97 %), 3-IAA (T, purity > 98%) and L-TRP EE (AT, purity ≥ 99 %) were purchased from Sigma Aldrich (St. Louis, MO,

116 USA). Formic acid was provided by Prolabo® (Obregón, México) and methanol for
117 liquid chromatography was supplied by Merck (Darmstadt, Germany).

118

119 **Yeast strain**

120 A commercial strain of wine yeast was tested under fermentation conditions in SM. A
121 *S.cerevisiae* strain, currently selected for white wine making, was used (AROMA
122 WHITE). It was purchased from Enartis (Trecate (NO), Italy). The yeast was rehydrated
123 in a bath at 37 ºC for 30 minutes, following the manufacturer's instructions. Then, it was
124 plated on yeast extract peptone dextrose (YPD) agar and incubated at 28 ºC for 48 hours.
125 The pre-inoculum was prepared in 150 mL of YPD broth and left for 24 hours under
126 stirring (150 rpm) at 28° C, before inoculation in SM.

127

128 **SM inoculation**

129 SM was performed in 10 L of distilled water. Firstly, the amounts of sugars, acids and
130 salts described above were added. The resulting solution was stirred to homogenize the
131 medium and the pH was adjusted to 3.45 with NaOH. Subsequently, SM was autoclaved
132 at 121 ºC for 21 minutes. Then, amino acids, trace elements, vitamins and anaerobic
133 factors were added aseptically. This step prevents the degradation of thermolabile
134 compounds due to the high temperatures reached during the sterilization process. Finally,
135 just before inoculation, the SM was distributed in sterile Erlenmeyer flask of 1 L capacity
136 with 750 mL each. To end, they were inoculated with 10^5 cells mL^{-1}. Each flask was
137 capped with plugs with two gaps to maintain sterility. A Pasteur pipette was used in one
138 gap to allow the egress of carbon dioxide, while the other one was reserved for sampling.
139 Fermentation was carried out in triplicate.

Sampling

Sampling was performed daily for 9 days, taking 10 mL from each flask. Subsequently, their contents were divided into 2 aliquots: a 1 mL aliquot was diluted (1:100) for cell counting in a Neubauer chamber as a duplicate; the other 9 mL aliquot was centrifuged at 4,500 rpm for 3 minutes at room temperature. Following this, 2 mL of supernatant were taken, to measure the residual sugars in the medium, while the rest was stored at -20 ° C until analysis. Fermentations were considered to have finished when the residual sugars in the medium were below 2 g L^{-1}.

Sample preparation

Prior to UHPLC/HRMS analysis, sample compounds were extracted as previously described by Rodriguez-Naranjo *et al*. (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al. 2011). Briefly, C18 SPE cartridges (Variant, Agilent) were conditioned with 2 mL of methanol and 2 mL of milliQ water. Then, 500 µL of sample were loaded. Cartridges were washed with a 10% v/v methanol/water solution. Finally, analytes were eluted with 1 mL of methanol. Extracts were filtered (13 mm VWR Syringe Filter, 0.45 µm PTFE) to remove impurities before analysis.

UHPLC parameters

The analysis was carried out in an UHPLC Dionex Ultimate 3000 system (ThermoScientific, San Jose, USA) consisting of an RS Pump (HPG-3400RS), an RS Column Compartment (TCC-3000RS) and an RS Autosampler (WPS-3000RS). All devices were controlled by Chromeleon Express Software. The column used for the

163 analysis was a ZORBAX RRHD SB-C18 (2.1 x 100 mm, 1.8 µm particle size) with a
164 guard column (2.1 x 5 mm, 1.8 µm particle size). Both of them were purchased from
165 Agilent Technologies (Waldbronn, Germany). The analysis temperature was set at 40 ºC.

166 The chromatographic conditions consisted of two mobile phases, water (A) and methanol
167 (B), both with 0.1% formic acid with a gradient elution programmed as follows: 95% A,
168 5% B (0-2 min); 0% A, 100% B (2-13 min); 95% A, 5 % B (13.1-15 min). The flow
169 selected was 0.5 mL min^{-1} and the injection volume was 5 µL.

170

171 **HRMS parameters**

172 A Thermo Scientific Qexactive™ hybrid quadrupole-orbitrap mass spectrometer
173 (Bremen, Germany) was used coupled to the UHPLC system described earlier.
174 Compound ionization was performed through a heated electrospray ionization source
175 (HESI) in positive mode. The following parameters were optimized to carry out the
176 analysis: heater temperature was set at 440 ºC and capillary temperature at 270 ºC; spray
177 voltage was optimized at 3.5 kV; the flow rates of sheath gas, auxiliary gas and sweep
178 gas were stablished at 53, 14 and 3 arbitrary units, respectively. Once a week, the
179 Qexactive was calibrated according to the manufacturer's instructions for good practice.

180 A target-MS^2 mode was set to perform the analysis. The molecular formula and exact
181 mass of each compound were acquired from *PubChem* and *ChemSpider* public databases.
182 A mix of 9 standards at 3-55 ng mL^{-1} was infused into the system with a syringe at a low
183 flow rate (3 µL min^{-1}) in order to obtain the main fragments of every compound. A target
184 experiment using eight entries was used, since MEL and L-TRP EE possess the same
185 exact mass. The acquisition of every compound had a specific start-and end-time range.
186 SERO and 5-HTRP were analysed from 0.50 to 2 min; L-TRP and TRYPT from 3.60 to

4.60 min; NACSERO from 5 to 5.70 min; MEL and L-TRP EE from 6.10 to 7.60; and finally, TOL and 3-IAA from 6.60 to 7.60 min. The normalized collision energy used (NCE) was 20. In this mode, HRMS analysis had an RP of 35,000 FWHM, with an isolation window of 1.0 *m/z* and S-lens RF level of 50 %. For a maximum injection time (IT) of 100 ms, the automatic gain control (AGC) target analysed $1x10^6$ ions. Xcalibur Software (version 3.0.63) and TraceFinder™ Software (version 3.1) (Thermo Fisher Scientific, Waltham, MA) were used for the subsequent data analysis.

Method validation

This analytical method was validated following AOAC instructions (AOAC 2012). Linearity, LOD, LOQ, within-lab reproducibility and repeatability were the parameters analysed. Stock solutions of each compound were prepared with its proper solvent every time the analysis was carried out: milliQ water (L-TRP), ethanol (SERO, NACSERO, MEL, 3-IAA) or methanol (5-HTRP, TRYPT, TOL, L-TRP EE). They were maintained at -20ºC for a maximum of a month. A mixture of water:methanol (50:50) was used to prepare the suitable dilutions in order to build the calibration curves. For linearity, 10-point calibration curves were used in a range of 2300-0.01 ng mL^{-1} in triplicate for all compounds. LOD and LOQ were calculated through the calibration graph slope and its residual standard deviation. For LOD, a factor of 3.3 was used to calculate the value. On the other hand, LOQ was obtained by using a factor of 10.

A set of three samples spiked with different concentrations (50, 25 and 5 ng mL^{-1}) were employed to determine within-lab reproducibility and repeatability. The former was evaluated with a daily analysis of each spiked sample over 5 days. Repeatability was assessed in a single day-long work session, with 5 replicates of each concentration.

Statistical analysis

Differences between the indolic compounds during fermentation by *S.cerevisiae* AROMA WHITE were determined with one-way ANOVA analysis by the Statistica Software (StaSoft Inc 2005). The significance level was set at $p < 0.05$.

Results and discussion

UHPLC/HRMS method optimization

An analytical method by UHPLC/HRMS was optimized to determine L-TRP and derivative compounds. Figure 2 shows the chromatographic peaks obtained for each compound. A run time of less than 8 minutes was achieved. Good resolution was observed in the analysis due to the sensitivity of the Qexactive. The order of elution was as follows: SERO and 5-HTRP appeared first, with a retention time (R_T) of less than 2 minutes; subsequently, L-TRP and TRYPT eluted after 4 minutes. Even though there was a significant time space between the first two analytes and the following ones, the most suitable peak resolution was achieved this way. NACSERO had an R_T of 5.24 minutes, whereas L-TRP EE was eluted at 6.63 minutes. The rest of the compounds (TOL, 3-IAA, MEL) were obtained at an R_T of between 7.09 and 7.14 minutes. Exact mass allowed each peak to be identified, even though the R_T differs within just a few seconds. Table 1 displays the R_T, accurate mass of main fragment $[M+H]^+$, relative intensities found, chemical formula and calculated error (ppm) for the compounds included in this study. Error was below 3 ppm in all cases. SERO, TRYPT, 3-IAA acid and TOL had only one main fragment. SERO fragmentation *m/z* 160.07544 was the result of the loss of an amine group. This fact was common to most of the compounds, since its bond with the indolic ring was quite weak, as in the case of TRYPT (*m/z* 144.08038). TOL presented

dehydroxylation of the hydroxyl group (*m/z* 144.08053). 3-IAA obtained its main fragment *m/z* 130.06501 after the loss of the carboxyl group in the lateral chain. These functional groups were putatively the weakest, so they were easily breakable during the analysis. The 5-HTRP showed a main fragment *m/z* 204.06520 that was also due to a deamination. However, it also presented another fragment (*m/z* 162.05480), whose formation is not yet clear, but which was useful to identify the compound. NACSERO had the same identification fragment as SERO (*m/z* 160.07542). MEL and L-TRP EE shared a molecular formula and exact mass. Iriti *et al*. (Gardana et al. 2014) has previously described the pattern of fragmentation of each compound. Even though they had similar fragmentations determined with a QTRAP (*m/z* 233 → *m/z* 174 and *m/z* 233 → *m/z* 216), their different R_T and fragment intensities allowed them to be identified without any misinterpretation of the data obtained.

Method validation

Table 2 shows the validation parameters of the analytical method, including linearity, LOD, LOQ, within-lab reproducibility and repeatability for the 9 compounds analysed.

Linearity values were calculated through the correlation coefficient (R^2) of the curves obtained for each compound within the expected range of concentrations. All the values had an R^2 between 0.998-1.000, showing quite good linearity. The repeatability values obtained for all compounds were below 2% with the exception of SERO which highest value was near 21 % but within the AOAC recommendations. On the other hand, reproducibility reached values between 4 and 26 %. The AOAC recommends a reproducibility of 45-32 % for the low limit (5 ng mL^{-1}) and 32-16 % for the other concentrations analysed, so reproducibility was adequate.

Regarding LOD values, we could achieve to decrease MEL LOD to 0.0047 ng ml^{-1}. As far as MEL is concerned, other authors have reported LOD values for MEL determined by HPLC-MS/MS on different matrixes. Kocadağlı et al. analysed MEL in red wine and obtained a LOD of 0.034 ng mL^{-1} with a triple quadrupole (Kocadağlı et al. 2014). On the other hand, a LOD of 30.16 ng mL^{-1} was reported in a study of MEL during all the steps of the winemaking (Gomez et al. 2012). Rodriguez-Naranjo et al obtained a LOD of and 0.13 ng mL^{-1} by using a ion-trap in the study of MEL in wine (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al. 2011). Even though these values are quite low, they are higher than the one achieved in this work. Thus, working with a hybrid quadrupole-orbitrap mass spectrometer demonstrated a better election when it is necessary to low the LOD values in order to improve the analysis.

Analysis of indolic compounds during fermentation in SM

In order to test the suitability of the method to monitor the evolution of L-TRP derivatives during fermentation, an experiment with *S.cerevisiae* var. AROMA WHITE was performed. Figure 3 shows the evolution of cell density during alcoholic fermentation. Cell population underwent a significant growth rate reaching the maximum at day 2. Then, it decreased quickly at day 3 and it was remained constant until the end of alcoholic fermentation (day 7) in a SM. The indolic compounds under study were monitored over this period. The results are shown in Table 3. It is important to note that all of the compounds were determined at least once during the fermentation experiment, except for NACSERO. The concentration of L-TRP, which is the precursor of the pathway, decreased during fermentation, as expected. Yeasts need this amino acid to synthesize the rest of the compounds. Gil-Agustí *et al*. (Gil-Agustí et al. 2007) analysed L-TRP content

283 in wines and found low concentrations (0.20-1.98 ng mL^{-1}). In contrast, levels in must of
284 around 7000 ng mL^{-1} have been reported. Although its levels in the same wine after
285 alcoholic and malolactic fermentation were lower (3,660 ng mL^{-1} and $3.11x10^{6}$ ng mL^{-1},
286 respectively) (Wang et al. 2014) those values are higher than the ones obtained in this
287 experiment. On the other hand, the presence of 5-HTRP also decreased. To the best of
288 our knowledge, this is the first time it has been determined in SM fermented with
289 *S.cerevisiae*. At the beginning of fermentation, the highest concentrations were
290 quantified; these subsequently decreased during the process (19 to 3 ng mL^{-1}). Solutions
291 of amino acids, trace elements, vitamins and anaerobic factors added to the SM were
292 analysed and results showed that the initial amount of 5-HTRP that was observed in the
293 SM was originally present in the solution of amino acids added before the fermentation
294 process. Although yeasts did not synthetized this compound, they used it as a minority
295 nitrogen source.

296 SERO increased during fermentation from less than 1 ng mL^{-1} to more than 4.5 ng mL^{-1}.
297 Nevertheless, concentrations 1,000 times higher have been assessed in wines (nearly 3000
298 ng mL^{-1}) following malolactic fermentation (Wang et al. 2014). The maximum
299 concentration of MEL was achieved at day 2, coinciding with Rodríguez-Naranjo *et al*.
300 and Iriti et al. (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al. 2011,
301 Vigentini et al. 2015). It seems to be directly related with the cell growth at day 2, when
302 it also reached the maximum value. Then, MEL content also decreased until it completely
303 disappear at day 5. Other authors also detected MEL from day 2 but its concentration
304 remained constant during time (Gomez et al. 2012). TRYPT was also synthetized during
305 fermentation, but at lower levels (0.7 - 1.60 ng mL^{-1}) than have been reported before (Gil-
306 Agustí et al. 2007, Rodriguez-Naranjo et al. 2013). Even though yeasts played an

important role in its formation, the reached concentrations did not show this metabolite was a main one of the L-TRP metabolism.

Furthermore, the concentration of 3-IAA increased sharply in day 1 up to 84 ng mL^{-1} and then decreased, especially between day 1 and day 3 to 3 ng mL^{-1}. Maslov *et al*. found higher concentrations in final wines (25-30 ng mL^{-1}) (Maslov et al. 2011). This metabolite appeared in the solution of amino acids, so it was observed at day 0. The significant increase at day 1 brings to light that yeasts synthetized it during fermentation process. Sitbon *et al.* and Hoenicke *et al.* highlighted that 3-IAA can be conjugated with other compounds in order to protect the molecule from oxidation, showing a decrease at the following days (Sitbon et al. 1993, Hoenicke et al. 2001). This hypothesis is supported by our data.

On the other hand, TOL concentration was the highest of the analytes since it is a main metabolite of the L-TRP metabolism in yeasts (Mas et al. 2014). On day 1, the concentration obtained was 14,000 ng mL^{-1} and on day 3, it increased up to 19,000 ng mL^{-1}. Nevertheless, its levels had decreased to 16,500 ng mL^{-1} by day 7. Favre *et al*. found low quantities in Tannat wines (640-1,450 ng mL^{-1}), but the wine-making procedure influenced this data (Favre et al. 2014).

In relation to L-TRP EE, a continuous increase was noticed during fermentation: from 2.20 ng mL^{-1} on day 2, to 17.48 ng mL^{-1} on day 7. Iriti *et al*. also reported concentrations of this compound in the laboratory medium (Vigentini et al. 2015), but they found a tendency to decrease over time, which could be attributed either to the different strain used or medium composition. Unlike the SM used in this work, their medium compositionconsisted on buffered liquid mineral medium, containing glucose and yeast nitrogen base (YNB) with 2 different concentrations of L-TRP (20 and 100 mg L^{-1}).

Yeasts use L-TRP and transform it into metabolites by different pathways. In this study, the synthesis of MEL via 5-HTRP was studied. The obtained data supports that 5-HTRP is used during the first steps of the fermentation but is not synthetized by yeasts. Then, 5-HTRP decreases afterwards, while the other intermediates start to increase. Despite SERO being the precursor of MEL, it keeps increasing while MEL appears in a very tiny quantity and finally disappears. On the other hand, there are alternative pathways that were used by yeasts and were evidenced in the analysis. The formation of TOL and 3-IAA as main metabolites were the preferred pathways during fermentation process, according to other authors (Kradolfer et al. 1982, Mas et al. 2014). While TOL is clearly a waste product, which accumulates in the medium, 3-IAA have a maximum production peak and then starts to disappear until the end of fermentation. The formation of TRYPT occurred, but were not preferential by yeasts. Other pathways such as the kynurenic acid were not included on this study, even though other authors have evidenced it in the metabolism of *S. uvarum* (Mariko Shin, Tetsuro Shinguu, Keiji Sano 1991).

Conclusions

A validated method for the identification of derivative compounds of the L-TRP. metabolism was developed successfully and LOD and LOQ were lowered with the use of a hybrid quadrupole-orbitrap. It is clear that determined compounds (5-HTRP, SERO, MEL, 3-IAA, TRYPT, TOL, L-TRP EE) are involved in *Saccharomyces* metabolism and this is a point of departure for further studies, which are required to understand their role. In addition, 5-HTRP was identified in must for the first time. Nevertheless, there is still a need to study yeast metabolism of L-TRP derivatives in greater depth.

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Compliance with Ethical Standards

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Conflict of interest: Edwin Fernández-Cruz, M.Antonia Álvarez-Fernández, Eva Valero, A.M. Troncoso and M.C. García-Parrilla declare that they have no conflict of interest. This study does not contain any studies with human or animal subjects.

Ethical approval: This article does not contain any studies with human participants performed by any of the authors.

Informed consent: Not applicable.

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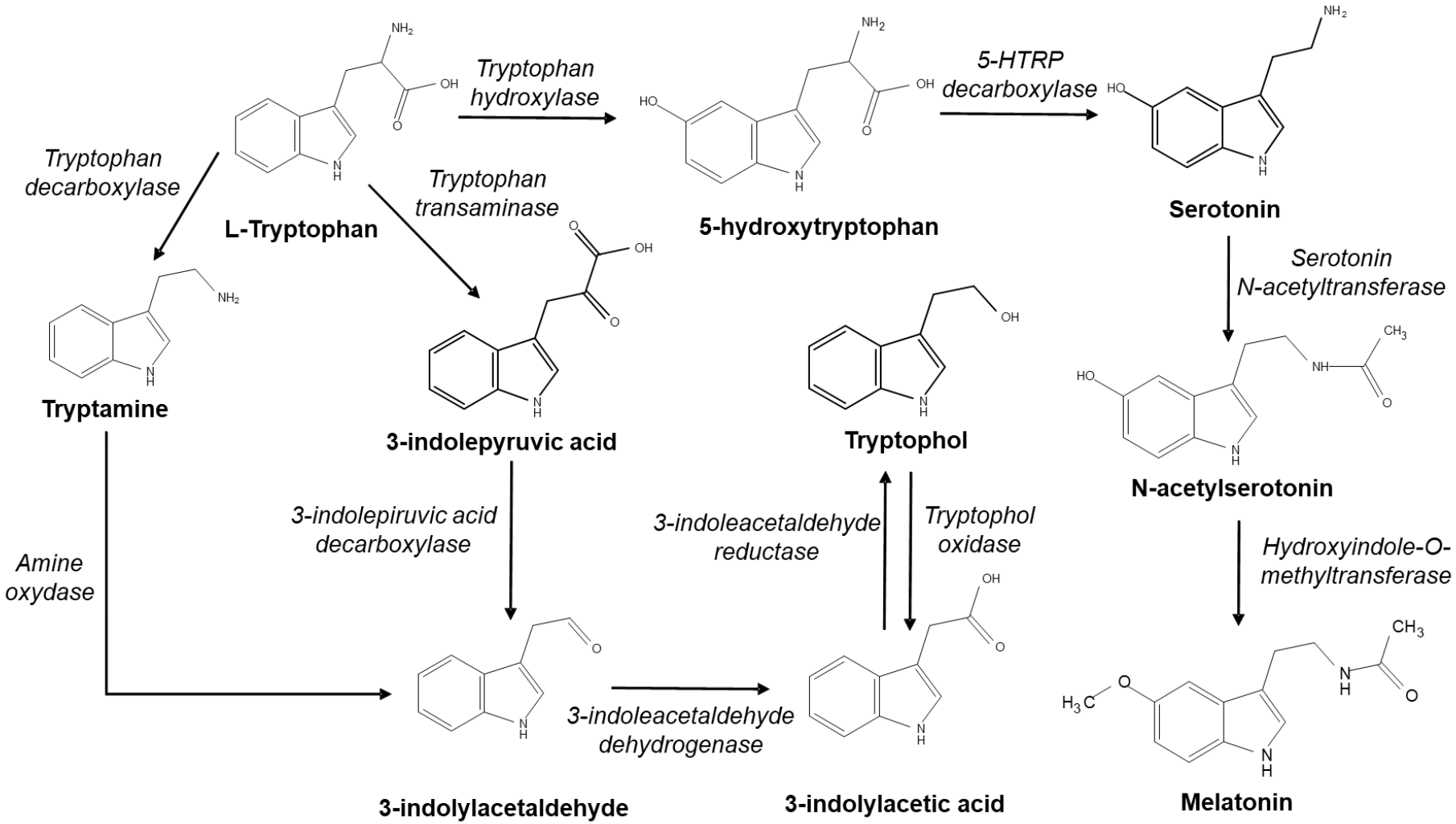

Figure 1. Synthesis of compounds derived from L-Tryptophan in animals and plants

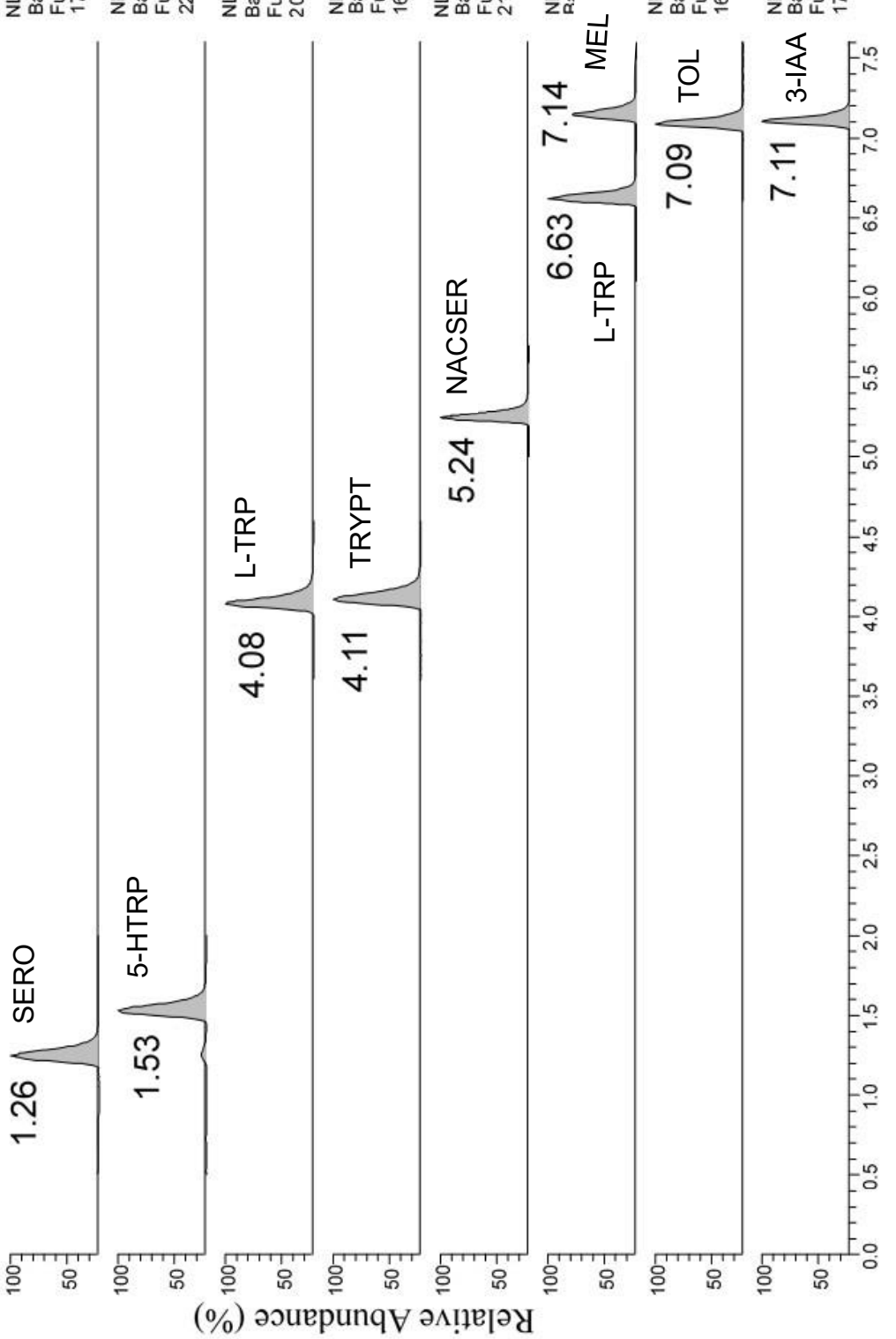

Figure 2. Target MS^2 mode results with R_T and relative abundance of each indolic compound

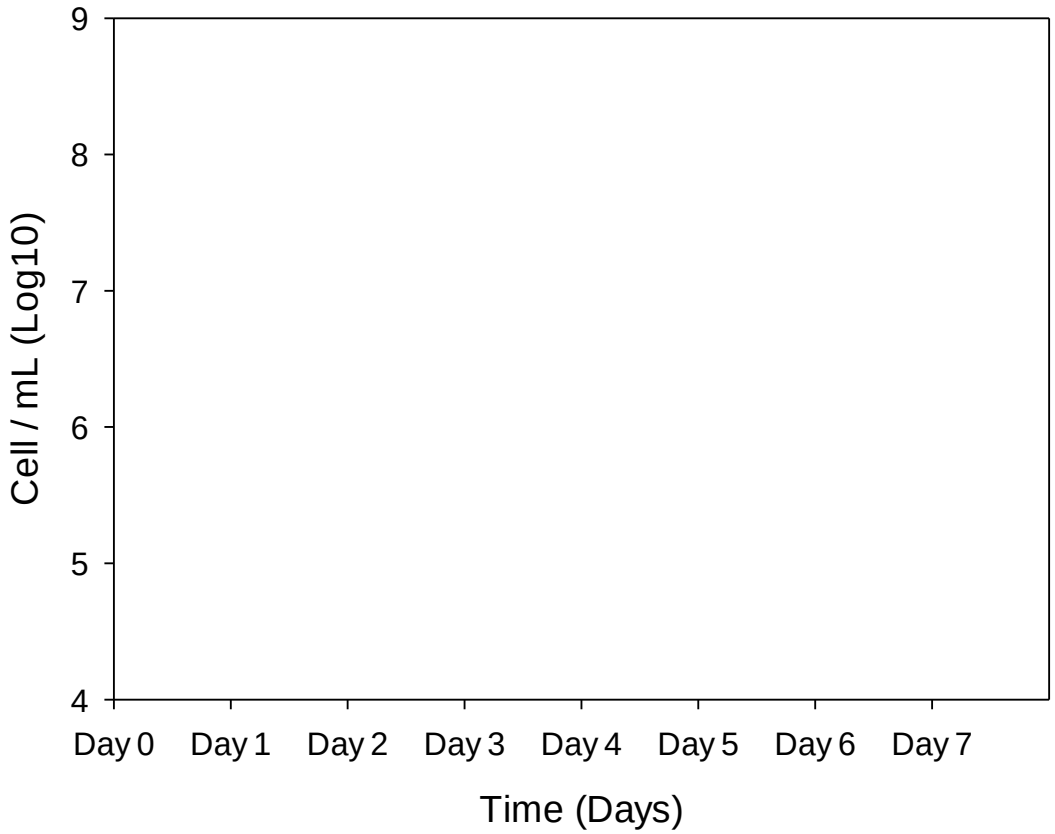

Figure 3. Evolution of cell population during 7 days of the fermentation process by *S.cerevisiae* (AROMA WHITE)

Compound	RT (min)	$[M+H]^+$	Main fragment	Theorical fragment	Relative intensities	Formula	Error (ppm)
SERO	1.26	177.10224	160.07544	160.07569	100	$C_{10}H_{10}ON$	1.57
5-HTRP	1.53	221.09207	204.06520	204.06552	100	$C_{11}H_{10}O_3N$	1.54
			162.05480	162.05496	15	$C_9H_8O_2N$	0.96
L-TRP	4.08	205.09715	188.07030	188.07061	100	$C_{11}H_{10}O_2N$	1.61
			146.05992	146.06004	23	C_9H_8ON	0.85
			159.09143	159.09167	4	$C_{10}H_{11}N_2$	1.53
TRYPT	4.12	161.10732	144.08038	144.08078	100	$C_{10}H_{10}N$	2.72
NACSERO	5.24	219.11280	160.07542	160.07569	100	$C_{10}H_{10}ON$	1.73
			202.08595	202.08626	6	$C_{12}H_{12}O_2N$	1.50
			219.11241	219.11280	5	$C_{12}H_{15}O_2N_2$	1.81
L-TRP EE	6.63	233.12845	216.10153	216.10191	100	$C_{13}H_{14}O_2N$	1.73
			174.09118	174.09134	20	$C_{11}H_{12}ON$	0.89
			159.09149	159.09167	12	$C_{10}H_{11}N_2$	1.15
			132.08069	132.08078	9	$C_9H_{10}N$	0.70
TOL	7.09	162.09134	144.08053	144.08078	100	$C_{10}H_{10}N$	1.70
3-IAA	7.11	176.07061	130.06501	130.06513	100	C_9H_8N	0.87
			176.07046	176.07061	15	$C_{10}H_{10}O_2N$	0.85
MEL	7.14	233.12845	174.09100	174.09134	100	$C_{11}H_{12}ON$	1.94
			216.10153	216.10191	7	$C_{13}H_{14}O_2N$	1.72
			233.12795	233.12845	5	$C_{13}H_{17}O_2N_2$	2.18

Table 1. Main fragments of each indolic compound derived from L-TRP using UHPLC/HRMS.

Compound	R^2	Range	LOD	LOQ	Reproducibility			Repeatability		
					Low level	Medium level	High level	Low level	Medium level	High level
SERO	0.9999	0.5-70	0.0067	0.0204	17.89	26.16	13.42	0.01	21.13	15.38
5-HTRP	0.9999	20-1	0.0066	0.0200	7.84	6.18	5.30	0.00	0.30	0.32
L-TRP	1.0000	10-2300	0.0013	0.0039	9.72	9.85	10.26	0.01	0.53	0.44
TRYPT	0.9999	0.5-70	0.0018	0.0056	12.25	10.74	9.82	0.00	0.54	0.76
NACSERO	1.0000	0.5-70	0.0038	0.0114	20.46	16.64	14.18	0.04	0.87	0.72
L-TRP EE	0.9999	0.5-25	0.0054	0.0163	12.61	12.56	12.77	0.00	1.78	0.63
TOL	1.0000	20-2300	0.0030	0.0090	6.18	4.64	4.32	0.00	0.66	0.96
3-IAA	0.9998	0.5-100	0.0029	0.0089	5.94	5.05	4.16	0.02	0.09	0.19
MEL	0.9999	0.01-8	0.0047	0.0144	22.53	22.52	21.11	0.00	1.11	0.90

Table 2. Validation parameters of analytical method: linearity (R^2), range of calibration, LOD, LOQ (ng mL^{-1}), within-lab reproducibility and repeatability in three levels each (5, 25 and 50 ng mL^{-1}) represented by RSD (%).

Compound	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
SERO	nq	0.67 ± 0.31^{cdefgh}	2.41 ± 0.46^{befgh}	2.50 ± 0.64^{bgh}	3.35 ± 0.17^{bcgh}	3.44 ± 0.23^{bcgh}	4.61 ± 0.55^{bcdef}	4.70 ± 0.63^{bcdef}
5-HTRP	23.28 ± 0.02^{bcdefgh}	18.51 ± 0.19^{acdefgh}	10.90 ± 0.03^{abdefgh}	8.70 ± 0.18^{abcefgh}	5.96 ± 0.06^{abcdfgh}	4.93 ± 0.03^{abcdegh}	4.04 ± 0.11^{abcdefh}	3.12 ± 0.03^{abcdefg}
L-TRP	$27914.49 \pm 58.03^{bcdefgh}$	$5346.33 \pm 68.51^{acdefgh}$	$836.20 \pm 33.24^{abdefgh}$	26.81 ± 1.10^{abcgh}	26.60 ± 0.43^{abcfgh}	25.40 ± 0.45^{abcegh}	34.83 ± 1.02^{abcdefh}	46.50 ± 0.61^{abcdefg}
TRYPT	2.45 ± 0.02^{bcdegh}	0.79 ± 0.28^{agh}	0.88 ± 0.12^{adegh}	1.25 ± 0.11^{ac}	1.35 ± 0.26^{ac}	1.47 ± 0.55	1.68 ± 0.28^{abc}	1.53 ± 0.27^{abc}
NACSERO	nq	nd	nd	nd	nd	nd	nd	nd
L-TRP EE	0.94 ± 0.01^{cdefgh}	nq	2.20 ± 0.01^{adefgh}	3.43 ± 0.10^{acefgh}	5.40 ± 0.06^{acdfgh}	10.02 ± 0.05^{acdegh}	12.30 ± 0.08^{acdefh}	17.48 ± 0.13^{acdefg}
TOL	$174.97 \pm 0.74^{bcdefgh}$	$14157.97 \pm 222.12^{acdefgh}$	$16245.06 \pm 288.86^{abdefgh}$	$19206.29 \pm 473.57^{abch}$	$19277.6 \pm 40.95^{abcfgh}$	$18603.58 \pm 58.36^{abce}$	$18231.62 \pm 383.12^{abce}$	$18255.62 \pm 220.04^{abcde}$
3-IAA	0.75 ± 0.04^{bcdefg}	83.81 ± 1.86^{acdefg}	12.58 ± 0.40^{abdefg}	3.63 ± 0.18^{abcefg}	1.66 ± 0.01^{abcdg}	1.66 ± 0.05^{abcdg}	0.88 ± 0.03^{abcdef}	nq
MEL	nq	0.06 ± 0.01^{ce}	0.14 ± 0.00^{bdef}	0.07 ± 0.01^{ce}	0.04 ± 0.01^{bcdf}	0.06 ± 0.00^{ce}	nq	nq

Main values and standard deviation are represented (ng mL^{-1})

[a]Significant differences with day 0

[b]Significant differences with day 1

[c]Significant differences with day 2

[d]Significant differences with day 3

[e]Significant differences with day 4

[f]Significant differences with day 5

[g]Significant differences with day 6

[h]Significant differences with day 7

Table 3. Evolution of indolic compounds related to L-TRP during fermentation in SM (day 1-7). Main values and standard deviation are represented (ng mL^{-1}).

CAPÍTULO 2 / CHAPTER 2

Fernández-Cruz, E., Carrasco-Galán, F., Cerezo-López, A.B., Troncoso, A. M., García-Parrilla, M.C.

EFECTO MATRIZ Y SU INFLUENCIA EN LA ESTABILIDAD DE MELATONINA Y COMPUESTOS DERIVADOS DEL TRIPTÓFANO EN DIFERENTES CONDICIONES DE ALMACENAMIENTO

MATRIX EFFECT ON THE STABILITY OF MELATONIN AND OTHER TRYPTOPHAN DERIVED COMPOUNDS AT DIFFERENT STORAGE CONDITIONS

FIRST DRAFT

Matrix effect on the stability of melatonin and other tryptophan derived compounds at different storage conditions

Fernández-Cruz, E., Carrasco-Galán, F., Cerezo-López, A.B., Troncoso, A. M., García-Parrilla, M.C*.

Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal. Facultad de Farmacia. Universidad de Sevilla. C/ Profesor García González 2, 41012, Sevilla, Spain

*Corresponding author:

E-mail address: mcparrilla@us.es (M.C. García-Parrilla)

Abstract

Melatonin is a bioactive compound that is synthesised by yeast during alcoholic fermentation, using the amino acid tryptophan as a precursor. Its occurrence has been described in fermented products such as wine, beer, synthetic medium and so others at trace levels. Due to the low concentrations found in these fermented products, melatonin and other derived compounds of the amino acid L-tryptophan are suitable to be affected by the composition of different matrices. In the present work, effects of temperature (4º, -20º, -80ºC) and concentration (low and high) were analised in four matrixes (white wine, pilsner beer, fermented synthetic must, intracellular *Sacharomyces cerevisiae* extract) during storage. Results show that beer is the matrix with less changes during storage in low and high presence of indolic compounds, being 4ºC for 1 week the best condition to maintain them unalterable.

Introduction

Melatonin (MLT) is a neurohormone that is synthesised from the amino acid tryptophan (L-TRP) in mammals, plants and microorganisms (Murch et al., 2000; Slominski et al., 2002; Sprenger et al., 1999). In mammals, the metabolic pathway encompasses different intermediates such as 5-hydroxytryptophan (5-HTRP), serotonin (5-HT) and N-acetylserotonin (NA5-HT) in mammals (Slominski et al., 2002). Plants can also follow this pathway, but they use tryptamine (TRY) instead of 5-HTRP to form serotonin (5-HT). Microorganism such as yeast have proved to synthesized MLT in presence of L-TRP (Rodríguez-Naranjo et al., 2012; Sprenger et al., 1999), although the conversion of 5-HTRP to 5HT is quite rate-limiting in S. cerevisiae. However, MLT has been reported in fermented products by the presence of this yeast such as wine (Rodriguez-Naranjo et al., 2011), beer (Garcia-Moreno et al., 2013), orange juice (Fernández-Pachón et al., 2014) and bread (Yılmaz et al., 2014).

Melatonin concentration in fermented beverages is usually at trace levels (ng/mL). Wine is the alcoholic beverage where MLT has been quantified the most (Meng et al., 2017) However, its amount depends on the analytical technique. In Sangiovese and Trebbiano wines, between 0.4-0.5 ng/mL of MLT were reported using a HPLC-F. Even lower concentrations (0.16-0.32 ng/mL) were found in Cabernet Sauvignon, Malbec and Chardonnay wines using a capillary electrochromatography (Stege et al., 2010). However, higher levels) were found in Merlot and Groppello (4-8 ng/mL wines using a triple quadrupole mass spectrometer (Vitalini et al., 2011). Rodriguez et al. analysed MLT with an ion trap mass spectrometer in different wines and found lower concentrations (5.1-129.5 ng/mL). Melatonin content in beer has been scarcely reported in literature. Concentrations between 51.8 y 169.7 pg/mL were found in commercial beers. However, ELISA assay was performed instead of chromatographic techniques (Maldonado et al.,

2009). Other compounds of the MEL pathway such serotonin have been described in beers (Kirschbaum et al., 1999). However, other L-TRP derived compounds from the catabolic Ehrlich pathway by yeast (Hazelwood et al., 2008) have been described in beers such as tryptophol (Bartolomé et al., 2000), which is also present in wines (Favre et al., 2014; Gil and Gómez-Cordovés, 1986), and 3-indolacetic acid (3-IAA) (Maslov et al., 2011).

These compounds are formed during the alcoholic fermentation by yeast (Rodríguez-Naranjo et al., 2012). It has been reported the synthesis of MLT in pomegranate wines (Mena et al., 2012), orange juice (Fernández-Pachón et al., 2014) and synthetic must (Fernández-Cruz et al., 2017). However, fermented must is an intermediate product and changes should be consider to take place.

Due to the complexity of food matrices and the low amounts of MEL and L-TRP-derived compounds reported on literature is surprising to find no works studying the matrix effect on stability of these compounds during storage conditions. The aim of this work is to study different conditions of temperature and indolic compound concentration during storage time in order to gain more knowledge about how the occurrence of these compounds is suitable to be affected.

Material and methods

Reageants

Standards of 5-HTRP, SERO, NACSERO, MEL, TRYPT, 3-IAA, TOL and L-TRP EE and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were supplied by Sigma Aldrick (St. Louis, MO, USA). L-tryptophan standard was purchased from Panreac (Darmstadt, Germany). Methanol LC/MS grade was supplied from Merck

(Darmstadt, Germany) and formic acid (99%) LC/MS grade was provided from Prolabo® (Obregon, Mexico).

Commercial beverages

Both pilsner beer and white wine bottle were purchased in a local market at glass-bottle format. Pilsner beer has an alcoholic content of 4.6º while white wine had 12.5º.

Synthetic must and fermentations

Synthetic medium (SM) was based on previous works (Fernández-Cruz et al., 2016). Basically, SM presents 200 g/Lof sugars (fructose and glucose 1:1) and nitrogen sources provided by ammonium and amino acids, allowing a yeast assimilable nitrogen (YAN) of 300 mg/L. Synthetic must was prepared under sterile conditions and was fermented with a *Saccharomyces cerevisiae* strain (Aroma White). The inoculum was prepared to have an initial population of 10^6 cell/mL. Fermentation was carried out for 15 days in 1 L flasks with 750 mL of SM at 28ºC and 150 rpm. Flasks were daily weighed to ensure the correct development of fermentations that were performed by triplicate.

Sampling

After 15 days of fermentations, cells were counted with a Neubauer chamber in order to take SM aliquots containing 10^9 cell/mL. Then, samples were centrifuged and the supernatant was collected in dark-flasks. On the other hand, cell pellet was washed twice with milliQ water to remove the extracellular metabolites. Intracelullar compounds were extracted following the method of González et al. (Gonzalez et al., 1997). Briefly, a solution of ethanol (75%) with HEPES (50 µM) was heat until boiling and added to the pellet. Then, samples were placed in a heated bath (80ºC) for 3 minutes and then, were place on ice. Subsequently, they were dried with a vacuum concentrator (HyperVACLITE, Gyrozen, Seoul, Korea) and the pellet was suspended in milliQ water.

Samples were centrifuge (5000 *g*, 10 minutes, 4ºC) and the supernatant was collected in a dark-glass vial.

Aliquot preparation

Three clusters of the four matrices were prepared as follows: solutions with no standard addition; solutions enriched with 2 ng/mL of each indolic compound; solution with 200 ng/mL of indolic compound. The three clusters were repeated twice more in order to storage the same samples at 4º, -20ºC and -80ºC. Samples were initially treated and subsequently at 7 and 15 days.

Solid phase extraction

Commercial beverages and synthetic must were loaded in a solid phase extraction (SPE) cartridge (VALIAN, Agilent) following the method described for Fernandez-Cruz et al. (Fernández-Cruz et al., 2017). Extracts were dried in a vaccum concentrator (HyperVACLITE, Gyrozen, Seoul, Korea) and suspended in methanol:milliQ water solution (1:1) with formic acid (0.1%) previously to the UHPLC/HRMS analysis.

UHPLC/HRMS

An UHPLC Dionex Ultimate 3000 system (ThermoScientific, San Jose, USA), coupled to a Thermo Scientific Q-Exactive™ hybrid quadrupole-orbitrap mass spectrometer (Bremen, Germany), was used**.** The system was controlled with Chromeleon Express software. UHPLC conditions were set according to a previously validated method (Fernández-Cruz et al., 2016). The programs used for the subsequent data analysis comprised Xcalibur Software (version 3.0.63), to confirm compound identification, and TraceFinder™ Software (version 3.1), in order to quantify the different analytes.

RESULTS & DISCUSSION

Eight compounds of the L-TRP metabolism and L-TRP itself were storage for 15 days at different conditions of temperature (4ºC, -20ºC and .80ºC). Results are shown in Figures 1-7. As can be seen, most of the compounds of MEL pathway were not identified in solution with no standard added, although they were quantifies in the solutions enriched with 2 and 200 ng/mL.

Beer is the matrix where tryptophan (Figure 1) is more stable at the different temperatures, with no significant changes until day 15. Initial L-TRP values of beer were the highest of the analysed matrices (750 ng/mL) since the rest only reached a level of 50 ng/mL. Other authors reported similar values for final fermented synthetic must (Fernández-Cruz et al., 2017). At day 15 L-TRP started to decrease in the four matrices, although the more enriched was able to maintain the original concentration.

5-hydroxytryptophan (Figure 2)was also analysed. Is was only quantified in the 200 ng/mL cluster. It was increased in beer and intracellular content, being more unstable in synthetic must and wine. These compound was previously described during alcoholic fermentation and it disappeared at the end of fermentative process (Fernández-Cruz et al., 2016).

Serotonin (Figure 3) increased its concentration during the storage time from day 0 to day 15. N-acetilserotonin have the same trend, but it was significantly from day 7 to day 15 in beer and synthetic must.

Melatonin (Figure 4) showed different trend depending on the matrix. At first, it was not quantified neither no-enriched nor 2 ng/mL solution. The 200 ng/mL cluster shows that beer maintained MEL content unalterable. However, while fermented synthetic must and intracellular media increased their MEL content, in wine melatonin disappear at the 15.

3-indolacetic acid was practically stable in all the matrices with the exception of wine, where it has a pronounced drop, disappearing at day 15.

Tryptophol was completely stable in beer and synthetic must, but was almost non-identified in wines.This contrast with other authors, since they reported than tryptophol trend is to be accumulated during fermentation (Fernández-Cruz et al., 2017).

Conclusions

Beer was the matrix were most of the indolic compounds were more stable at different concentrations and temperatures, being the most suitable for the conservation of indolic compounds. However, at day 15 some of them decreased. Better results were obtained within the first 7 days.

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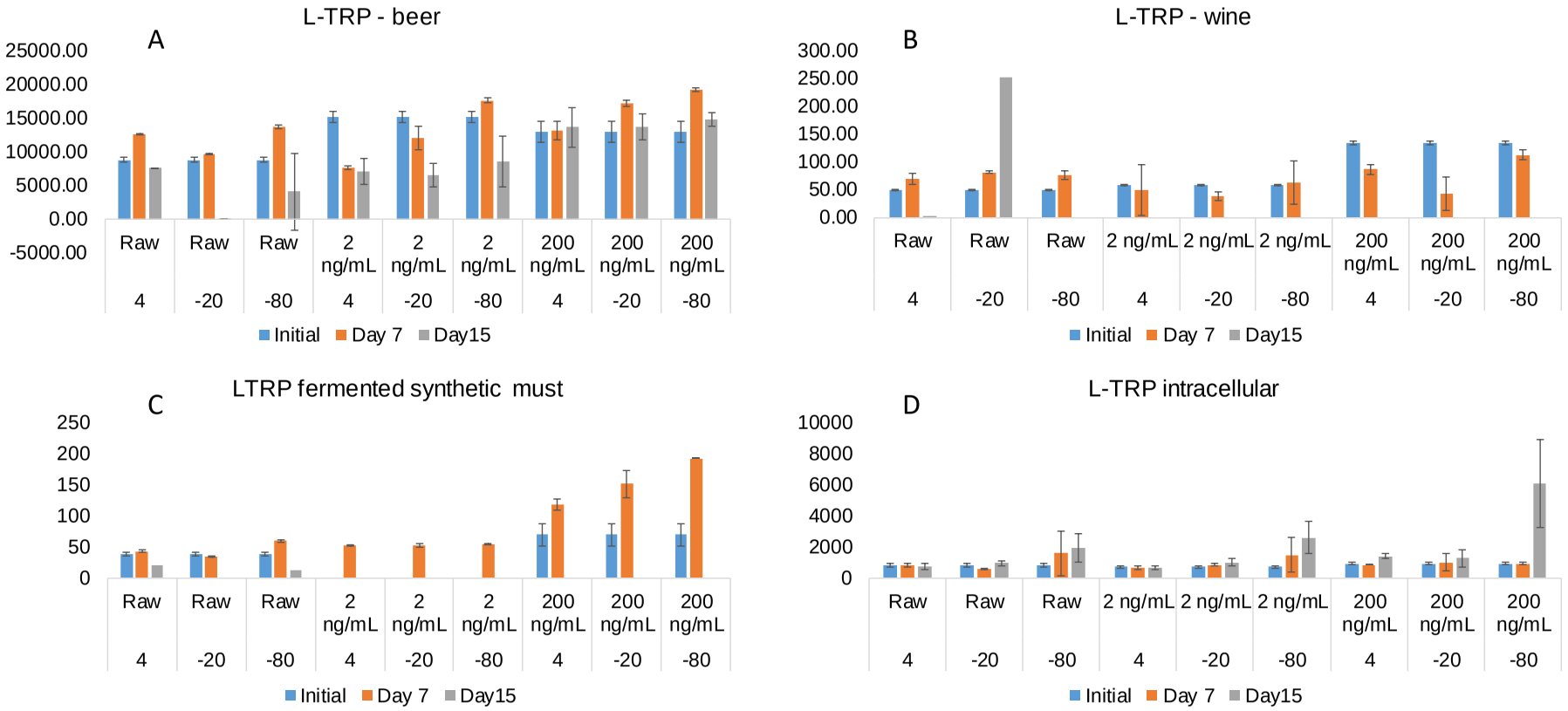

Figure 1. L-tryptophan content in beer (A), wine (B), synthetic fermented must (C) and intracellular extract (D) during storage conditions of 4ºC, -20ºC, -80ºC

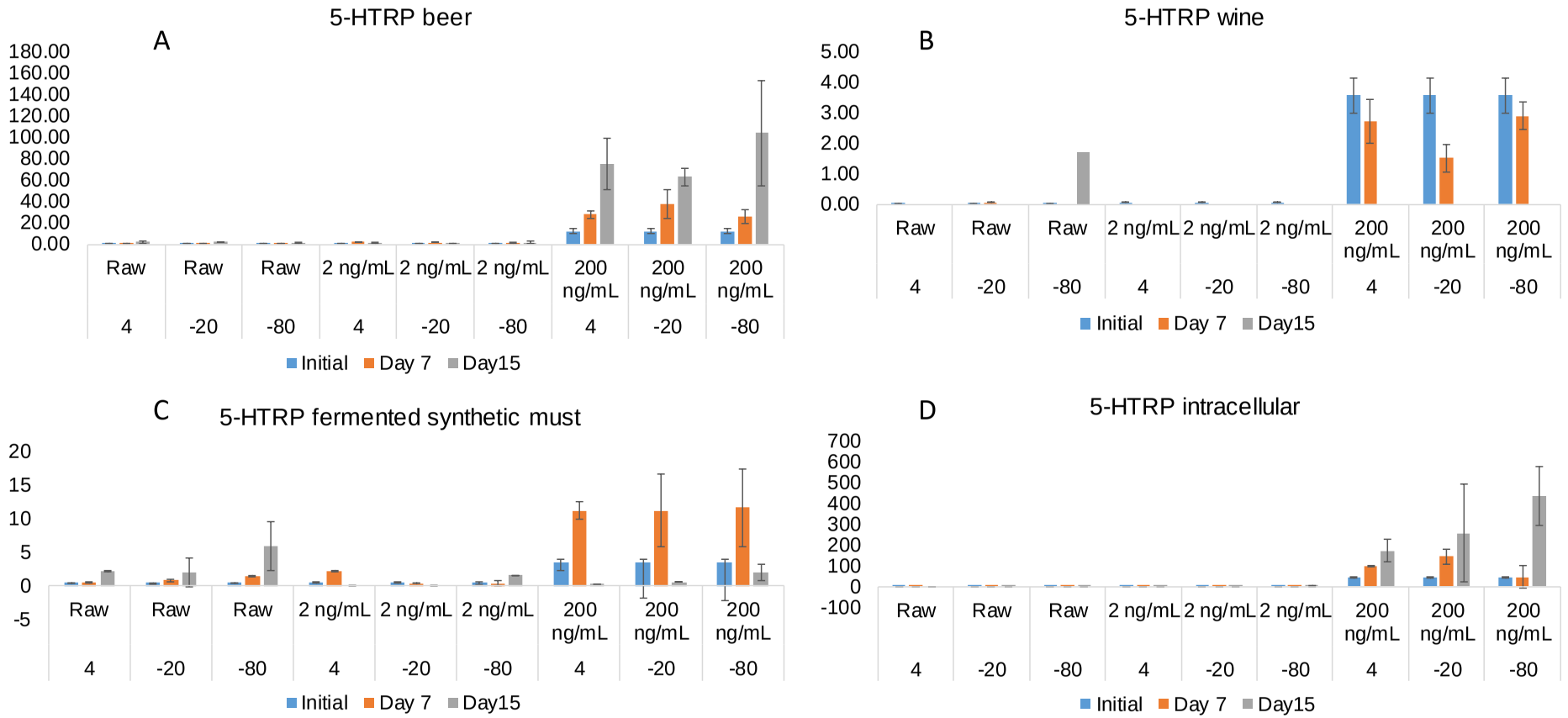

Figure 2. 5-hidrotryptophan content in beer (A), wine (B), synthetic fermented must (C) and intracellular extract (D) during storage conditions of 4ºC, -20ºC, -80ºC

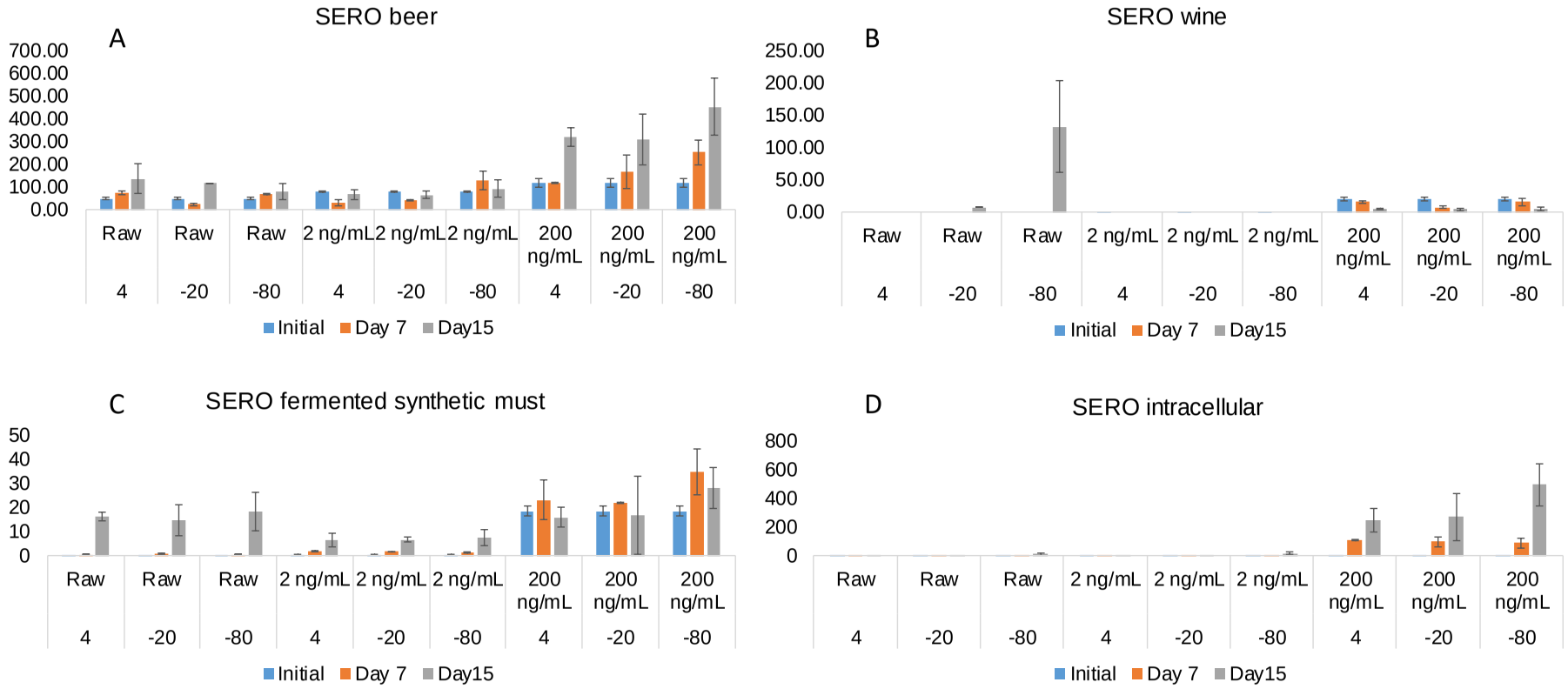

Figure 3. Serotonin content in beer (A), wine (B), synthetic fermented must (C) and intracellular extract (D) during storage conditions of 4ºC, -20ºC, -80ºC

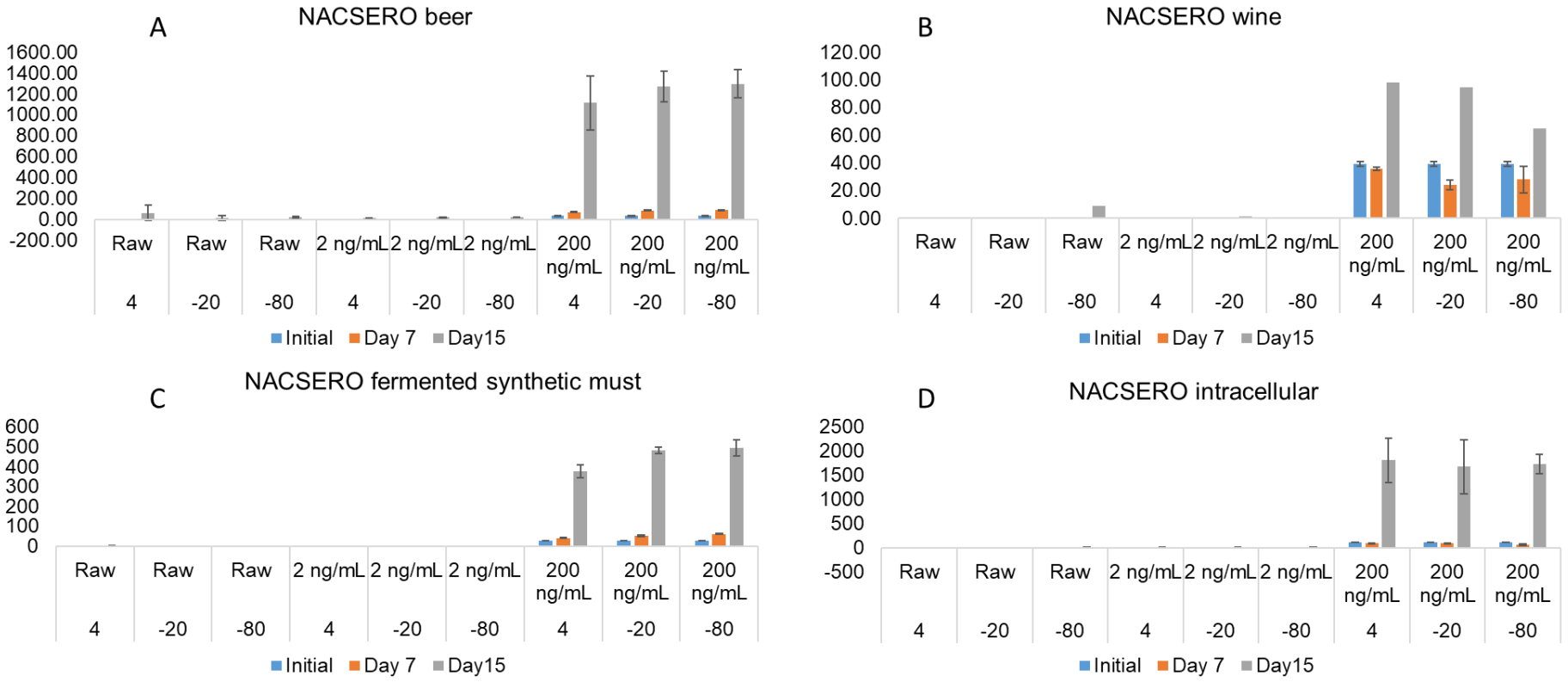

Figure 4. N-acetylserotonin content in beer (A), wine (B), synthetic fermented must (C) and intracellular extract (D) during storage conditions of 4ºC, -20ºC, -80ºC

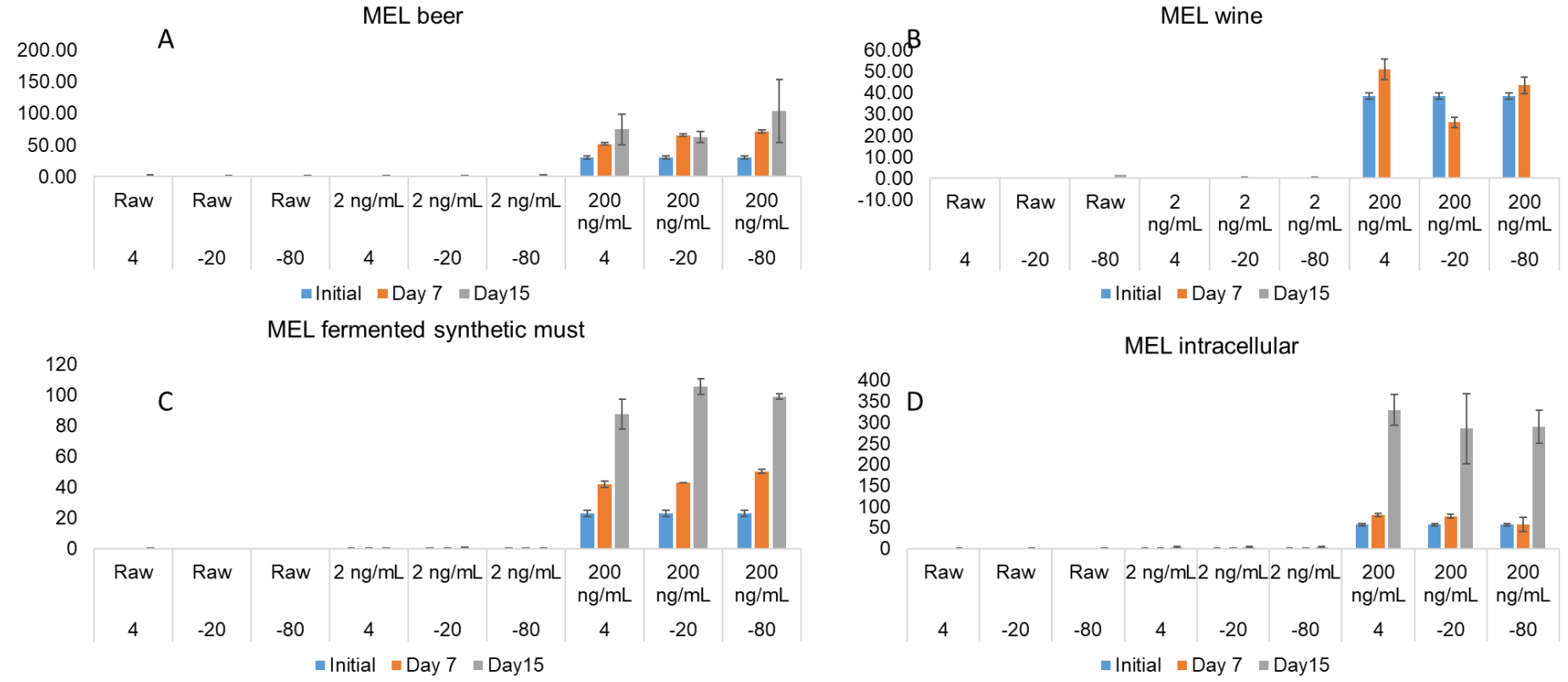

Figure 5. Melatonin content in beer (A), wine (B), synthetic fermented must (C) and intracellular extract (D) during storage conditions of 4ºC, -20ºC, -80ºC

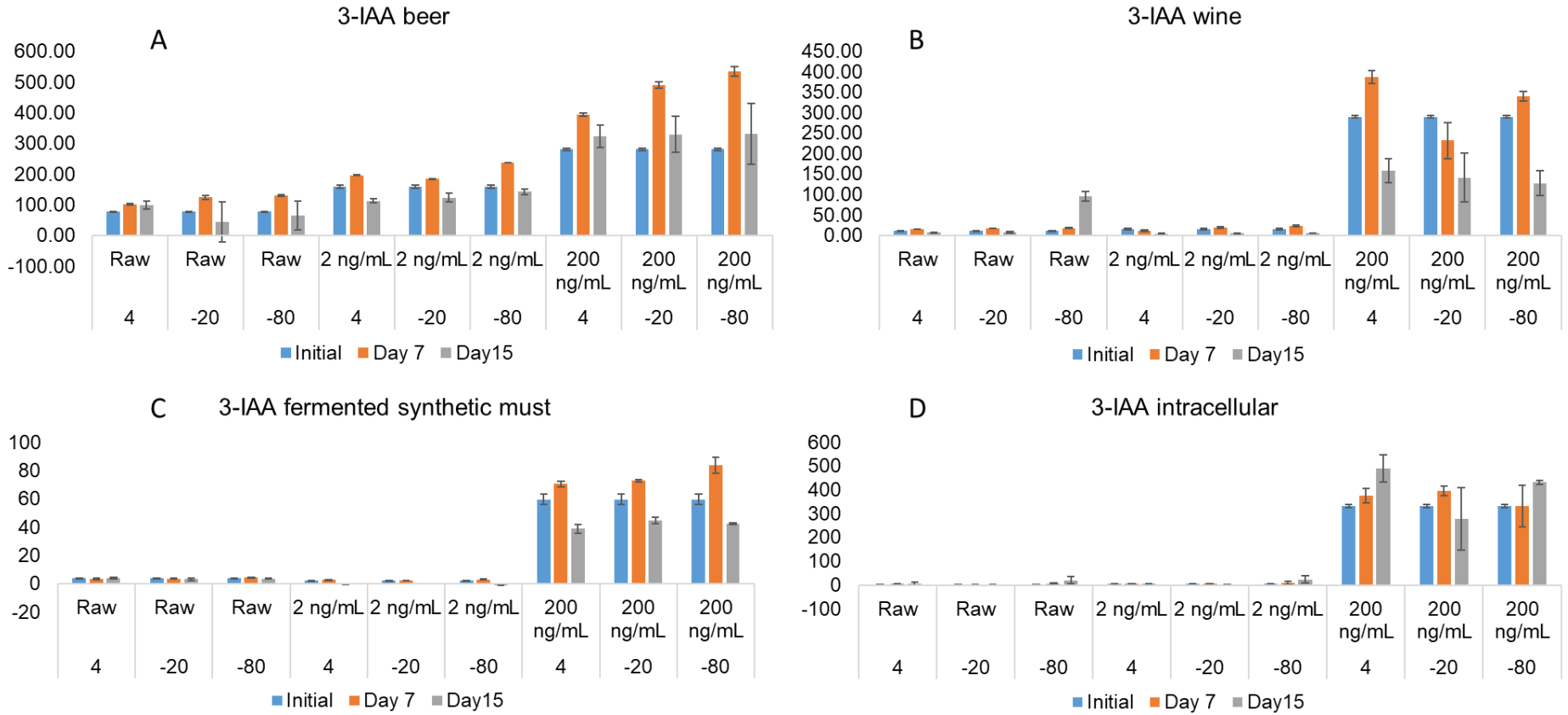

Figure 6. 3-Indoleacetic acid content in beer (A), wine (B), synthetic fermented must (C) and intracellular extract (D) during storage conditions of 4ºC, -20ºC, -80ºC

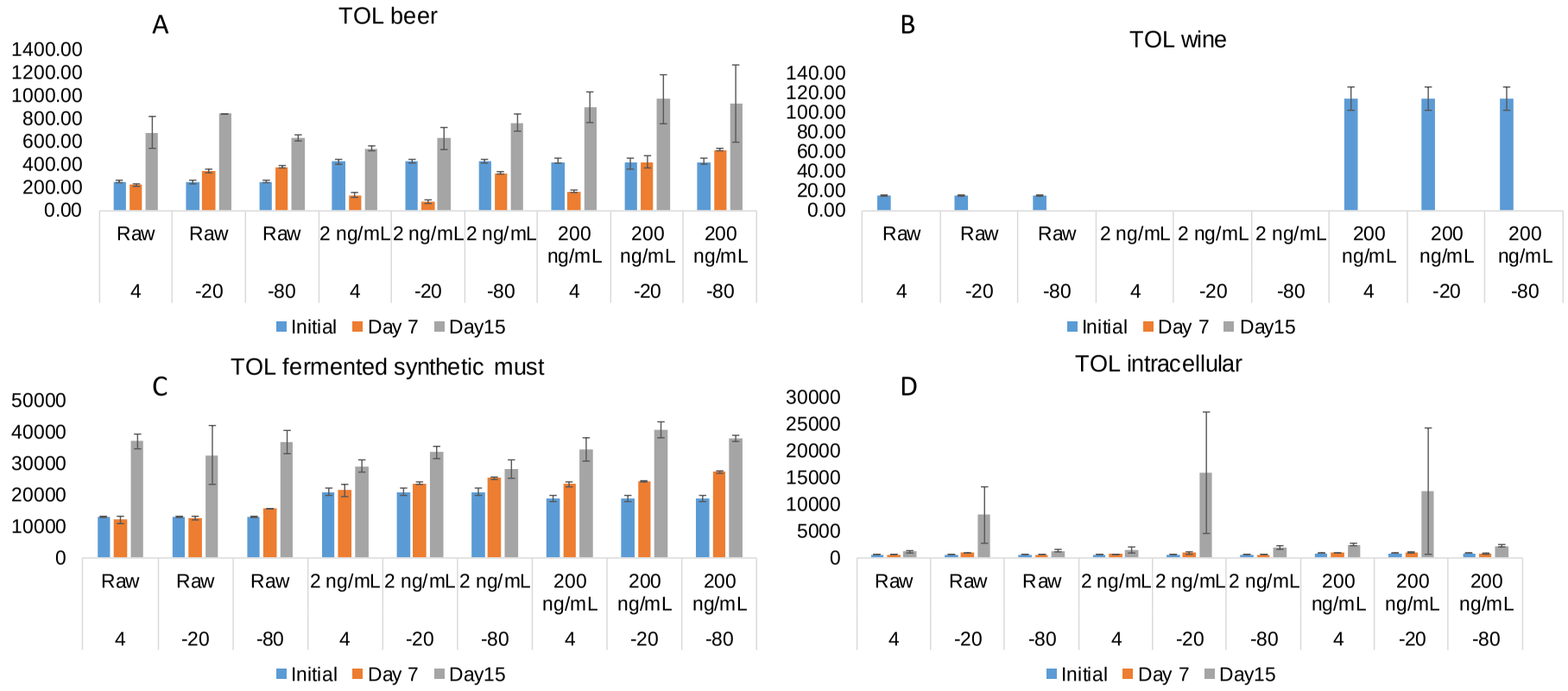

Figure 7. 3-Indoleacetic acid content in beer (A), wine (B), synthetic fermented must (C) and intracellular extract (D) during storage conditions of 4ºC, -20ºC, -80ºC

CAPÍTULO 3/ CHAPTER 3

Fernández-Cruz, E., Álvarez Fernández, M. A., Valero, E., Troncoso, A. M., García-Parrilla, M.C.

MELATONINA Y COMPUESTOS DERIVADOS DEL METABOLISMO DEL TRIPTÓFANO PRODUCIDOS DURANTE LA FERMENTACIÓN ALCOHÓLICA POR DIFERENTES LEVADURAS VÍNICAS

MELATONIN AND DERIVED L-TRYPTOPHAN METABOLITES PRODUCED DURING ALCOHOLIC FERMENTATION BY DIFFERENT WINE YEAST STRAINS

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Melatonin and derived L-tryptophan metabolites produced during alcoholic fermentation by different wine yeast strains

Fernández-Cruz, E;[a]; Álvarez-Fernández, M.A;[a] Valero, E.;[b] Troncoso, A.M.;[a] García-Parrilla, M.C.[a]*

[a]*Department of Food Science and Nutrition, Faculty of Pharmacy, University of Seville, C/ Profesor García González 2, 41012, Sevilla, Spain.*

[b]*Department of Food Science and Nutrition, Faculty of Experimental Science, University Pablo de Olavide, Carretera de Utrera Km. 1, 41013, Sevilla, Spain.*

*Corresponding author. Tel.: +34 95 4556759.

*Email address: mcparrilla@us.es (M.C. García-Parrilla).

ABSTRACT

Melatonin is a neurohormone involved in the regulation of circadian rhythms in humans. Evidence has recently been found of its occurrence in wines and its role in the winemaking process. The yeast Saccharomyces cerevisiae is consequently thought to be important in Melatonin synthesis, but limited data and reference texts are available on this synthetic pathway.

This paper aims to elucidate whether the synthetic pathway of Melatonin in Saccharomyces and non-Saccharomyces strains involves these intermediates. To this end, seven commercial strains comprising Saccharomyces cerevisiae (Red Fruit, ES488, Lalvin QA23, Uvaferm BC, and Lalvin ICV GRE) and non-Saccharomyces (Torulaspora

delbrueckii and Metschnikowia pulcherrima) were monitored, under controlled fermentation conditions, in synthetic must, for seven days. Samples were analysed using a UHPLC—HRMS system (Qexactive).

Five out of the seven strains formed melatonin during the fermentation process: three S.cerevisiae strains and the two non-Saccharomyces. Additionally, other compounds derived from L-tryptophan occurred during fermentation.

Keywords: *Saccharomyces cerevisiae*, synthetic must, bioactive, indolic compounds, HRMS

Chemical compounds studied in this article:

L-tryptophan (PubChem CID: 6305); 5-hydroxy-L-tryptophan (PubChem CID: 439280); 5-hydroxytryptamine (PubChem CID: 5202); N-Acetyl-5-hydroxytryptamine (PubChem CID: 903); Melatonin (PubChem CID: 896); Indole-3-acetic acid (PubChem CID: 802); Tryptamine (PubChem CID: 1150); Tryptophol (PubChem CID: 10685); L-Tryptophan ethyl ester hydrochloride (PubChem CID: 134519).

1. INTRODUCTION

Melatonin (MEL) is a bioactive compound with antioxidant properties, in addition to its well-known effect of regulating circadian rhythms (Reiter, 1993; Bonnefont-Rousselot and Collin, 2010). Its occurrence has been evidenced in alcoholic beverages such as beer and wine (Maldonado, Moreno and Calvo, 2009; M. Isabel Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, and Garcia-Parrilla, 2011a). Neither of these groups of authors were able to determine it in grapes (pulp, seed or skins). However, the latter group monitored the whole winemaking process, and highlighted that MEL is formed during the fermentation stage (M. Isabel Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos-Villar, and Garcia-Parrilla, 2011b). This fact pinpoints the role of yeasts, in particular *Saccharomyces cerevisiae*, in the occurrence of MEL in wine.

The MEL synthesis pathway in vertebrates is shown in Figure 1; it includes the following compounds: L-tryptophan (L-TRP), 5-hydroxytryptophan (5-HTRP), Serotonin (SERO), N-acetylserotonin (NACSERO) and MEL (Iriti, 2009; Feng, Wang, Zhao, Han, and Dai, 2014). Moreover, other compounds with an indolic ring are present in wine, including 3-indolylacetic acid (3-IAA) (Maslov et al. 2011), Tryptamine (TRYPT) (Gil-Agustí, Carda-Broch, Monferrer-Pons, and Esteve-Romero, 2007), Tryptophol (TOL) (Favre et al. 2014) and L-Tryptophan ethyl ester (L-TRP EE) (Vigentini et al. 2015), among others. As far as our knowledge is concerned, their formation and significance are not well understood.

The study of L-TRP metabolism during the fermentation process is of interest because it is the precursor of the bioactive compounds that can be synthesised from it, such as MEL and SERO. Additionally, other compounds with an indolic structure can have an impact on sensorial properties, as is the case with 3-IAA. Consequently, the significance of the

MEL molecule for yeast needs to be explored, and this paper aims to contribute to unravelling its role.

It is well known that *S.cerevisiae* uses NH_4 as its preferred nitrogen source. However, in conditions where this compound is restricted, it can use other sources, such as amino acids. When this occurs, yeasts follow the Ehrlich pathway. In the specific case of the amino acid L-TRP, the main metabolites of this pathway are TOL, as the most synthesised higher alcohol, and 3-IAA as the most present higher acid (Mas et al. 2014).

In this context, it would be interesting to broaden knowledge about how the different strains use L-TRP, and specifically: the day of fermentation when it is used, the molecules with an indolic structure that are formed during fermentation, and if the strains under study synthesise these compounds in similar concentrations. Concerning *S.cerevisiae* strains, it is known that they consume L-TRP in the early stages of alcoholic fermentation (Bisson, 1991), as well as other amino acids present at low concentrations.

Nowadays, non-*Saccharomyces* yeasts are being investigated in depth (Comitini et al., 2011; Gonzalez, Quirós, and Morales, 2013; Suárez-Lepe and Morata, 2012). One of the main reasons for this interest is that these strains can be used to produce wines with lower alcohol content, frequently through sequential inoculation of *Saccharomyces* strains (Contreras et al., 2014; Quirós, Rojas, Gonzalez, and Morales, 2014). In addition, they can be used to improve wine quality in terms of producing flavour compounds, stabilizing wine colour, and promoting enzymatic activity, to decrease protein and polysaccharide levels in the end product, among other uses (Jolly, Varela, and Pretorius, 2014; Suárez-Lepe and Morata, 2012). However, their ability to form the bioactive MEL has not been explored so far, which is why we have included them in our study.

This paper aims to: (i) explore whether the strains under study can produce certain indolic compounds derived from L-TRP, as vertebrates do; (ii) select the strain that can synthesise bioactive compounds in the highest quantities; and (iii) ascertain when bioactive compounds are formed during alcoholic fermentation. To this end, seven commercial wine yeast strains were studied during alcoholic fermentation in a synthetic medium, by means of a previously validated method involving Ultra High Performance Liquid Chromatography coupled to High Resolution Mass Spectrometry, to allow the simultaneous determination of nine compounds with an indolic-derived structure (Fernández-Cruz, Álvarez-Fernández, Valero, Troncoso, and García-Parrilla, 2016).

2. MATERIALS AND METHODS

2.1. Reagents and Materials

L-TRP standard was supplied by Panreac (Darmstadt, Germany). The rest of the standards (5-HTRP, SERO, NACSERO, MEL, TRYPT, 3-IAA, TOL and L-TRP EE) were purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol for liquid chromatography was supplied from Merck (Darmstadt, Germany) and formic acid was provided by Prolabo® (Obregon, Mexico).

2.2. Wine yeast strains

Seven commercial wine yeast strains were selected, to study their ability to synthesise bioactive compounds under fermentation conditions in a controlled medium. Five of them were *S.cerevisiae* and two were non-*Saccharomyces*. Yeast species, strains and providers are shown in Table 1. Since all the strains were provided as active dried yeast, they were rehydrated for 30 min. at 37 ºC and plated on yeast extract peptone dextrose (YPD) agar (2 % peptone, 2 % glucose, 1 % yeast extract and 2 % agar). Then, they were incubated

at 28 ºC in an oven, for 2 days. Afterwards, the pre-culture was prepared in 150 mL of YPD broth (2 % peptone, 2 % glucose and 1 % yeast extract) and shaken at 150 rpm, 28 ºC, overnight, before inoculation.

2.3. Alcoholic fermentation conditions

Synthetic must (SM) was prepared based on Riou et al. (Riou, Nicaud, Barre, and Gaillardin, 1997), with some modifications. Glucose and fructose (100 g L^{-1} each) were used as a carbon source. The amounts of malic and citric acid were changed to 5 and 0.5 g L^{-1}, respectively. In addition, 3 g L^{-1} of tartaric acid were included. Uracil was not added to the SM. All the compounds were supplied by Sigma Aldrich (St. Louis, MO, USA). Mineral salts ($CaCl_2$ 0.155 g L^{-1}, KH_2PO_4 0.75 g L^{-1}, K_2SO_4 0.5 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.250 g L^{-1}, NaCl 0.200 g L^{-1}, NH_4Cl 0.460 g L^{-1}), sugars and organic acids were the first to be added. Subsequently, the solution was shaken and the pH of the medium was adjusted to 3.5, with NaOH. Then, SM was autoclaved at 121 ºC for 21 minutes. Thermolabile compounds—anaerobic factors (oleic acid 0.5 g 100 mL^{-1}**,** ergosterol 1.5 g 100 mL^{-1}, and Tween 80 0.5 ml L^{-1}), amino acids, and vitamins (biotin 0.003 mg L^{-1}, calcium pantothenate 1.5 mg L^{-1}, chlorohydrate pyridoxine 0.25 mg L^{-1}, chlorohydrate thiamine 0.25 mg L^{-1}, myo-inositol 20 mg L^{-1}, nicotinic acid 2 mg L^{-1}) and trace elements ($CoCl_2 \cdot 6H_2O$ 0.4 g L^{-1}, $CuSO_4 \cdot 5H_2O$ 1 g L^{-1}, H_3Bo_3 1 g L^{-1}, , KI 1 g L^{-1}, $MnSO_4 \cdot H_2O$ 4 g L^{-1}, $(NH_4)_6Mo_7O_{24}$ 1 g L^{-1}, $ZnSO_4 \cdot H_2O$ 4 g L^{-1})—were later added, in aseptic conditions, in order to prevent degradation through the sterilization process. Erlenmeyer flasks with 750 mL of SM were inoculated with 10^5 cells mL^{-1} and capped with plugs equipped with a capillary, to allow the egress of carbon dioxide. To monitor fermentation, each flask was weighed daily, before and after sampling, in order to calculate the carbon dioxide released. Fermentations were performed in triplicate.

391 2.4. UHPLC/HRMS analysis

392 An UHPLC Dionex Ultimate 3000 system (ThermoScientific, San Jose, USA), coupled
393 to a Thermo Scientific Q-Exactive^{TM} hybrid quadrupole-orbitrap mass spectrometer
394 (Bremen, Germany), was used**.** The UHPLC system consisted of a pump (HPG-3400RS),
395 a column compartment (TCC-3000RS) and an autosampler (WPS-3000RS). The UHPLC
396 system was controlled using Chromeleon Express software. The UHPLC/HRMS
397 conditions were based on the Fernández-Cruz et al. method (Fernández-Cruz et al. 2016).
398 A ZORBAX RRHD SB-C18 column (2.1 x 100 mm, 1.8 µm particle size) with a guard
399 column (2.1 x 5 mm, 1.8 µm particle size) was used. Both of them were purchased from
400 Agilent Technologies (Waldbronn, Germany). Water (A) and methanol (B), both with 0.1
401 % formic acid, were selected as mobile phases. A gradient elution was programmed as
402 follows: 0–2 min 95 % A (5 % B); 2–13 min 0 % A (100 % B); 13.1–15 min 95 % A (5
403 % B). In order to identify the indolic compounds, solutions containing the nine analytes
404 were prepared in a range of 2300–0.01 ng mL^{-1}. Injection volume was 5 µL and the flow
405 was set at 0.5 mL min^{-1}. The temperature of the analysis was maintained at 40 ºC.

406 A heated electrospray ionization source (HESI) was used in positive mode to develop the
407 analysis. A target-MS^2 mode was selected using the molecular formula and exact mass of
408 the indolic compounds, both of which are available in public databases, such as *PubChem*
409 and *ChemSpider*. The main HRMS parameters were optimized: heater and capillary
410 temperature (440 ºC and 270 ºC, respectively), spray voltage (3.5 kV), and flow rates of
411 sheat gas, sweep gas and auxiliary gas (53, 3 and 14 arbitrary units respectively).
412 Additional mass spectrometry parameters were also established. Normalized collision
413 energy (NCE) had a value of between 15 and 25. S-lens RF had a level of 50 %, and mass
414 resolving power (RP), expressed by full width at half maximum (FWHM), was set at
415 35,000, with an isolation window of 1.0 m/z. The AGC target value was $1x10^6$ ions, and

416 the maximum injection time (IT) 100 ms. The programs used for the subsequent data
417 analysis comprised Xcalibur Software (version 3.0.63), to confirm compound
418 identification, and TraceFinder™ Software (version 3.1), in order to quantify the different
419 analytes.

420

421 2.5. Statistical analysis

422 Differences between commercial strains and their synthesis of indolic compounds were
423 tested by one-way ANOVA analysis, using Statistica Software (StaSoft Inc., 2004).
424 Differences were considered significant when $p < 0.05$.

425

426 **3. RESULTS AND DISCUSSION**

427 Eight compounds deriving from L-TRP (5-HTRP, SERO, NACSERO, MEL, 3-IAA,
428 TRYPT, TOL and L-TRP EE) an L-TRP itself were assessed during alcoholic
429 fermentation (for 7 days) and unequivocally identified through their retention time, exact
430 mass and matching their mass spectra with that of the commercial standard. Quantitation
431 was performed using external calibration curves with 10 points, which were freshly
432 prepared at every analytical session. The evolution of each compound included in this
433 study that was formed by the 7 yeast strains during the fermentation process is shown in
434 Figure 2.

435 As can be observed, some indolic compounds were present in the SM at day 0. To try to
436 clarify the source of these compounds, the different solutions (amino acids, vitamins,
437 trace elements and anaerobic factors) added to the SM after the sterilization process were
438 analysed. The results are displayed in Table 2. Surprisingly, amino acid solutions showed
439 a significant presence of 5-HTRP, NACSERO, TOL and L-TRP EE. On the other hand,

solutions of trace elements and vitamins presented low concentrations of 5-HTRP, SERO and L-TRP EE, below 1 ng mL^{-1}. The solution of anaerobic factors presented a concentration of NACSERO of 2.02 ng mL^{-1}. Finally, TOL was detected in all solutions, between 23 and 36 ng mL^{-1}. Although the concentrations reported seem to be significant, it has to be taken into account that the volume of each solution added to the SM was 10 mL (trace elements and anaerobic factors), 100 mL (vitamins) and 130.9 mL (amino acids), and the final volume of SM was 10 L. Therefore, the initial concentration diminished when the solutions were added, which explains the scarce occurrence of some of the indolic compounds at day 0.

L-TRP is the starting point of the synthesis of MEL and the other related compounds (College, 2012; Mas et al., 2014; Sprenger, Hardeland, Fuhrberg, and Han, 1999). Yeasts tend to consume either ammonium or certain amino acids as a nitrogen source (Bell and Henschke, 2005; Jiranek, Langridge, and Henschke, 1995; Ter Schure, Van Riel, and Verrips, 2000). The amount of nitrogen in the SM aimed to imitate that of grape must composition, so the yeasts would use the available nitrogen source in the exponential phase, in order to grow. As expected under these conditions, a high consumption of L-TRP was observed. Its concentration then diminished significantly during the fermentation process (Figure 2A). *Saccharomyces* and non-*Saccharomyces* strains consumed L-TRP in very different quantities. While *Saccharomyces* diminished the amount of L-TRP to below 25 ng mL^{-1}, the minimum level reached by *T. delbrueckii* was much higher (2,733 ng mL^{-1}). *M. pulcherrima* achieved a concentration of 301 ng mL^{-1} of L-TRP, but took longer than the rest of the strains to reach that value.

5-HTRP was also identified and quantified during the fermentation process (Figure 2B). Figure 3 shows the mass spectra fragmentation pattern obtained. It was present from preparation of the SM, and the yeasts consumed it during the first stages of fermentation.

Apparently, yeasts can use this compound as a nitrogen source in a similar way to L-TRP. Non-*Saccharomyces* strains use this compound to a lesser extent than *Saccharomyces* ones. This compound has scarcely been studied in yeast metabolism (Fernández-Cruz et al., 2016; Park, Kang, Park, and Back, 2008; Sprenger et al., 1999), although it has been reported in human plasma, as a 5-hydroxyindolic compound, and is apparently related to the control and regulation of the functions of the central and peripheral nervous systems (Sakaguchi et al., 2015).

Conversely, the other intermediates of MEL metabolism (SERO and NACSERO) were not quantified during the fermentation process, with the exception of the QA23 strain, which showed SERO at day 7 (result not shown in Figure 2). NACSERO was not quantified either, except for the *M. pulcherrima* value at day 1 (0.29 ng mL^{-1}) (Figure 2C). This fact is likely due to L-TRP consumption of this strain. Its metabolism is the slowest of the different commercial strains, so it is possible to quantify this metabolite in the early stages of alcoholic fermentation.

MEL was evidenced during fermentation in 5 out of the 7 strains (Figure 2D). Its occurrence was scattered, since it tended to appear and disappear in the SM as time progressed. In the case of *Saccharomyces* strains, Red Fruit and ES488 were the ones that synthesised it. On the other hand, with regard to *S.cerevisiae* var. *bayanus*, only QA23 formed MEL. It has been reported that this strain is capable of forming MEL (María Isabel Rodriguez-Naranjo, Torija, Mas, Cantos-Villar, and Garcia-Parrilla, 2012). Synthesis of MEL took place at the end of the exponential growth phase. Red Fruit reached a maximum level of 2.24 ng mL^{-1} at day 2, after most of the L-TRP had been consumed. This also confirms what other authors have reported (Rodríguez-Naranjo et al. 2012, Vigentini et al. 2015, Fernández-Cruz et al. 2016). The quantity of MEL progressively decreased until day 4, in accordance with a lower consumption of L-TRP; for the

following 2 days, L-TRP was not consumed and MEL was not detected. However, at day 7, MEL was observed. Figure 4 shows sugar concentration in the SM during the fermentation process. As can be observed, *S. cerevisiae* strains had consumed the entire sugar content by day 3. Red Fruit, ES488 and QA23 strains showed a similar pattern. However, ES488 and QA23 had not synthesised MEL by day 3. There are no prior MEL studies involving Red Fruit and ES488 strains, making this a new contribution of this paper.

As far as the Uvaferm BC and ICV GRE strains are concerned, neither of them synthesised any MEL at all. In a previous study, a related *S. cerevisiae* wine strain, Aroma White, was found to form MEL from day 1 to day 5, but at a low concentration (Fernández-Cruz et al. 2016), with a maximum level of 0.14 ng mL^{-1} at day 2. Unlike these strains, *T. delbrueckii* and *M. pulcherrima* did not form MEL until day 4, which coincided with low levels of L-TRP in the medium. Once again, MEL was observed at day 7. This formation is located at the midpoint of the stationary phase, so these strains took longer to synthesise MEL than the *Saccharomyces* strains. At the same time, non-*Saccharomyces* strains did not reach a sugar level below 2 g L^{-1} at the end of alcoholic fermentation (Figure 4). This occurrence is an original contribution because, so far, there have been no studies that address the metabolism of MEL in non-*Saccharomyces* yeasts.

3-IAA and TOL are the most abundant indolic compounds analysed during alcoholic fermentation. Both of them are products derived from L-TRP, via the Ehrlich pathway. While 3-IAA is the higher acid derivative of L-TRP, TOL is the higher alcohol (Mas et al. 2014). The strains included in this study reached the maximum concentration of 3-IAA at day 1 (Figure 2E); this is linked to the decrease of L-TRP in the medium. Each yeast consumed this compound differently. The lowest values were obtained from QA23 and *M. pulcherrima* strains (56 and 103 ng mL^{-1}, respectively). Red Fruit, Uvaferm BC

515 and ICV GRE strains formed 3-IAA in a concentration range of 200–300 ng mL^{-1}. On the
516 other hand, ES488 and *T. delbrueckii* were the most productive yeasts, with values above
517 400 ng mL^{-1}. Its decrease during fermentation may be caused by conjugation of 3-IAA
518 with other structures (Hoenicke, Simat, Steinhart, Köhler, and Schwab, 2001; Sitbon et
519 al., 1993) and the failure of yeasts to use L-TRP in the end stages of fermentation.
520 Regarding TOL, its concentration increased sharply at day 2, in the case of
521 *Saccharomyces* strains, and at day 3, in the case of non-*Saccharomyces* (Figure 2G). In
522 every case, once it reached its maximum, it remained almost unaltered in the medium.
523 This might suggest that yeasts did not need TOL for their metabolism at all. It seems to
524 be an end product of L-TRP yeast metabolism.
525 TRYPT (Figure 2F) is a biogenic amine (BA), which is considered an undesirable
526 compound due to its potential health concerns. The European Food Safety Authority
527 (EFSA) scientific opinion about biogenic amines and their risks showed that TRYPT was
528 present in alcoholic beverages, mostly in beer (EFSA Panel on Biological Hazards 2011).
529 However, TRYPT is not a main BA typically present in wine, and current European Law
530 does not regulate its safety levels.
531 Finally, L-TRP EE is a newly identified compound, recently described in wines, involved
532 in *S. cerevisiae* metabolism during alcoholic fermentation (Vigentini et al. 2015). It is
533 closely related to MEL, since their exact mass is identical. The results show that all strains
534 were able to synthesise this compound (Figure 2H). Nevertheless, each strain reached
535 different levels of production. *Saccharomyces* strains Red Fruit and ES488 had an
536 ascending formation of L-TRP EE from day 3 to day 5, with a sharp drop at day 6.
537 Surprisingly, at day 7, the concentration increased again. The other *Saccharomyces* strain
538 (ICV GRE) had irregular concentration values, with high values at day 4, 6 and 7 (5.87,
539 8.23 and 5.62 ng mL^{-1}, respectively). *Saccharomyces cerevisiae* var. *bayanus* Uvaferm

BC and QA23 did not share a similar pattern of synthesis. While the Uvaferm BC strain hardly formed L-TRP EE, the QA23 one reached two peaks, at day 2 (5.93 ng mL^{-1}) and day 7 (7.03 ng mL^{-1}).

On the other hand, non-*Saccharomyces* strains synthesised this compound throughout the fermentation process. *T. delbrueckii* produced the highest concentration, at day 2 (8.41 ng mL^{-1}), and although this strain also experienced a sharp drop at day 3, its concentration from day 5 was fairly constant. The values of *M. pulcherrima* strain L-TRP EE oscillated during the alcoholic fermentation, with maximum values at day 1 (6.07 ng mL^{-1}) and day 3 (6.67 ng mL^{-1}). Therefore, the non-*Saccharomyces* strains synthesised L-TRP EE more quickly than the others.

4. CONCLUSION

The compounds studied in relation to L-TRP metabolism developed by yeasts show significant differences between *Saccharomyces* and non-*Saccharomyces* wine strains. Under alcoholic fermentation conditions, all the strains were capable of synthesising different indolic compounds from L-TRP. 5-HTRP can be used by yeasts to form intermediates of MEL metabolism. Even though SERO and NACSERO are precursors of MEL, they were barely quantified during alcoholic fermentation. 3-IAA and TOL were the most synthesised compounds. All the strains synthesised TRYPT, but at safe levels. L-TRP EE was also formed by all the strains. It is clear that all these analytes are involved in *Saccharomyces* metabolism, and this is a point of departure for further studies, which are required to fully understand their roles.

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Compliance with Ethical Standards

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Conflicts of interest: Edwin Fernández-Cruz, M.Antonia Álvarez-Fernández, Eva Valero, A.M. Troncoso and M.C. García-Parrilla declare that they have no conflicts of interest. This study does not contain any studies involving human or animal subjects.

Ethical approval: This article does not contain any studies performed by any of the authors with human participants.

Informed consent: Not applicable.

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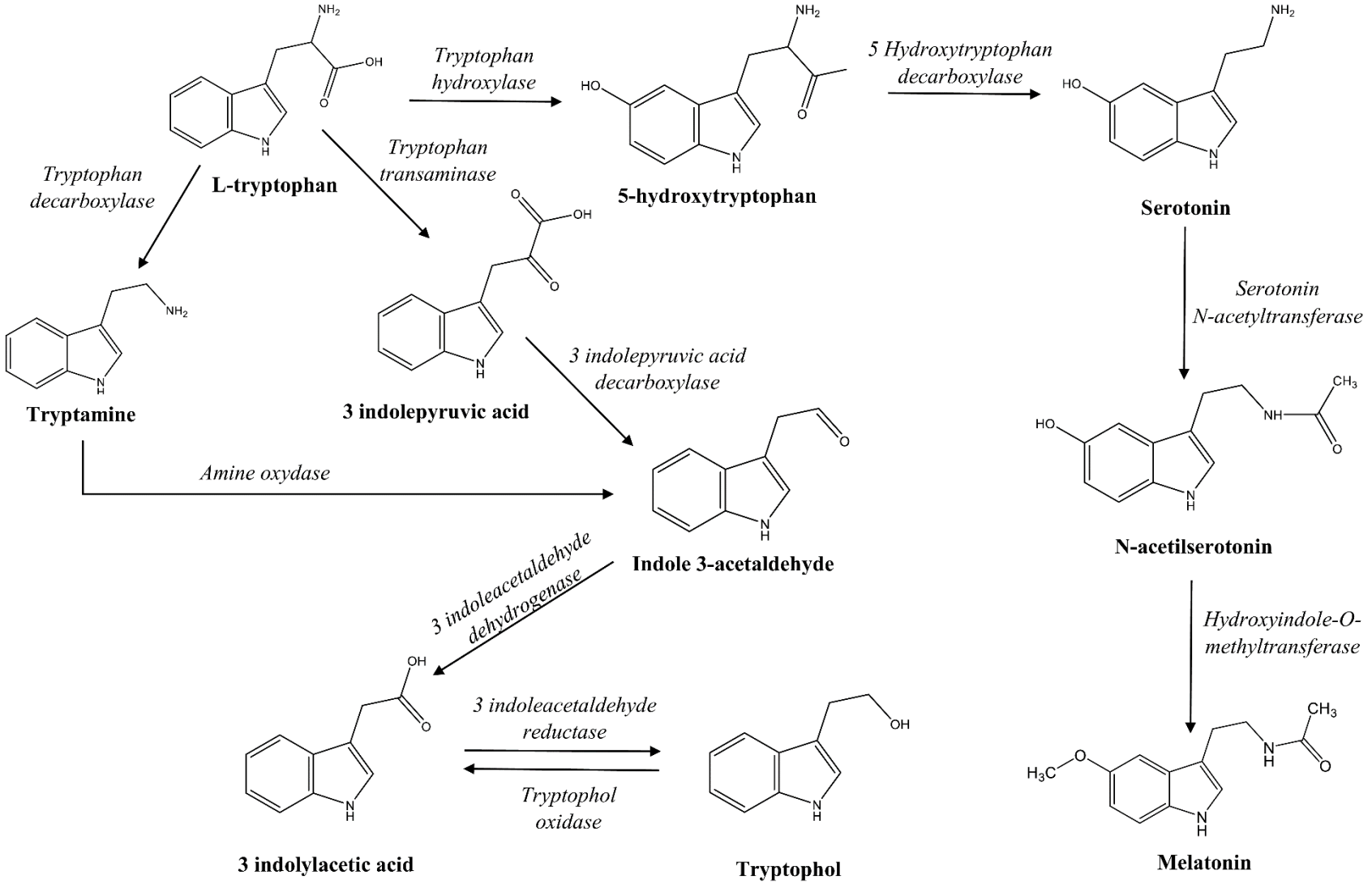
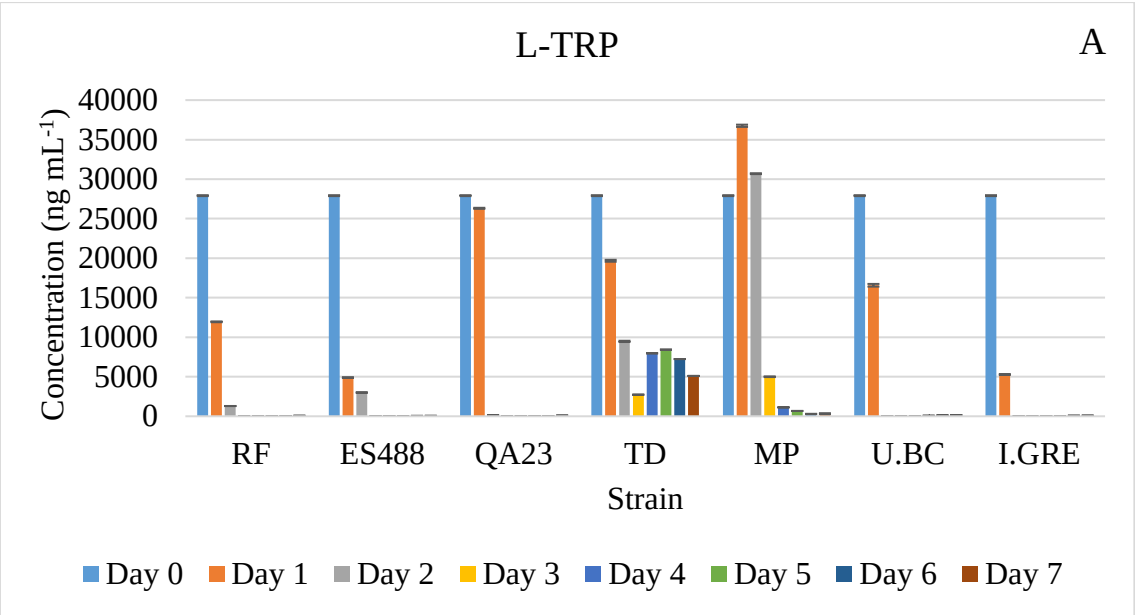


Figure 1. Metabolism of indolic compounds derived from L-TRP



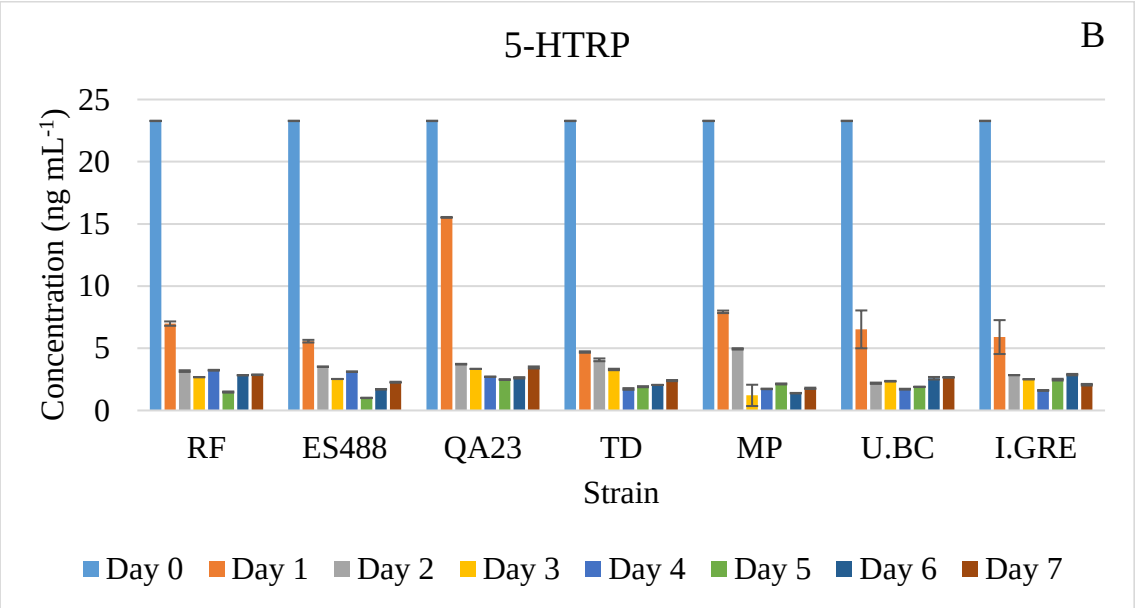
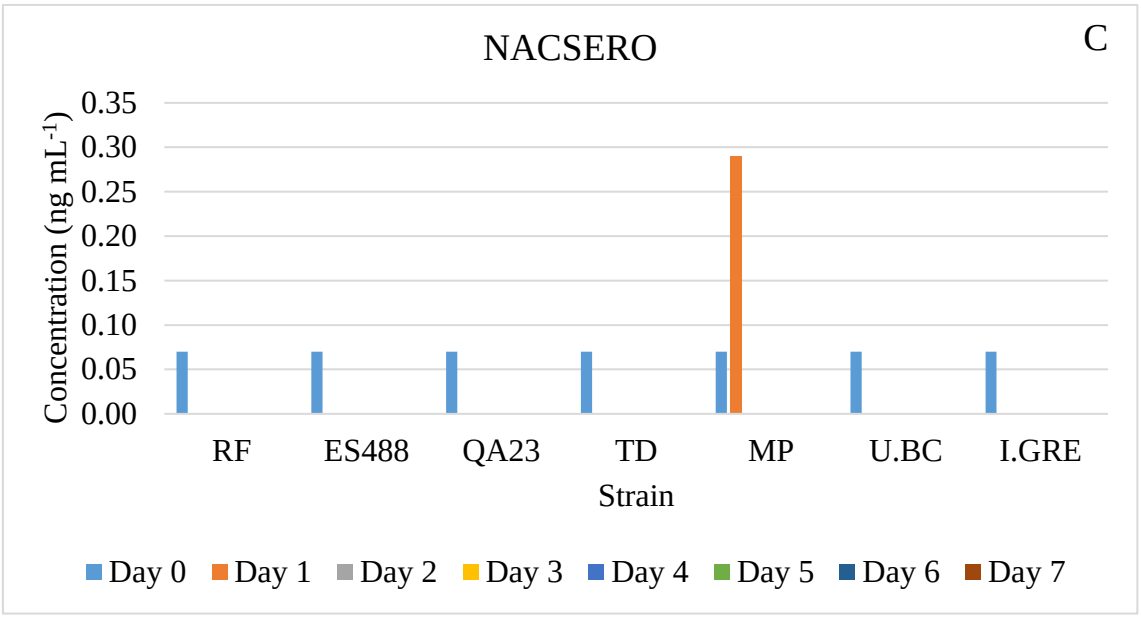


Figure 2 (A-H). Evolution of the indolic compounds concentration

L-TRP: L-tryptophan; 5-HTRP: 5-hydroxytryptophan; NACSERO: N-acetilserotonin; MEL: Melatonin; 3-IAA: 3-indolylacetic acid; TRYPT: Tryptamine; TOL: Tryptophol; L-TRP EE: L-tryptophan ethyl ester

RF: Red Fruit; TD: *Torulaspora delbrueckii*; MP: *Metschnikowia pulcherrima*; U.BC: Uvaferm BC; I.GRE: ICV-GRE.



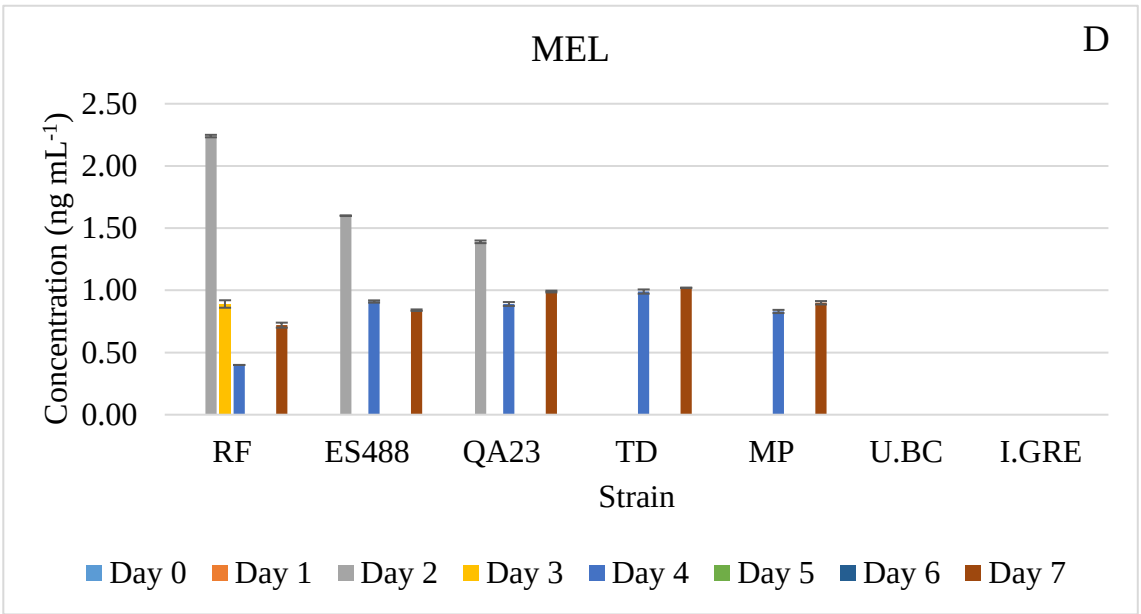
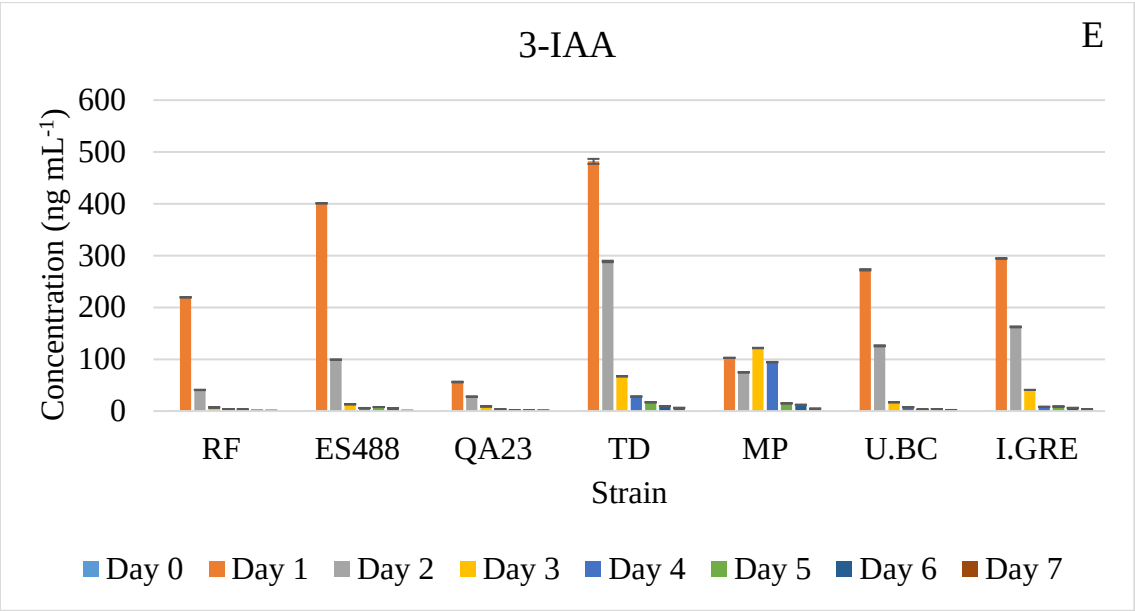


Figure 2 (A-H). Evolution of the indolic compounds concentration (continued)



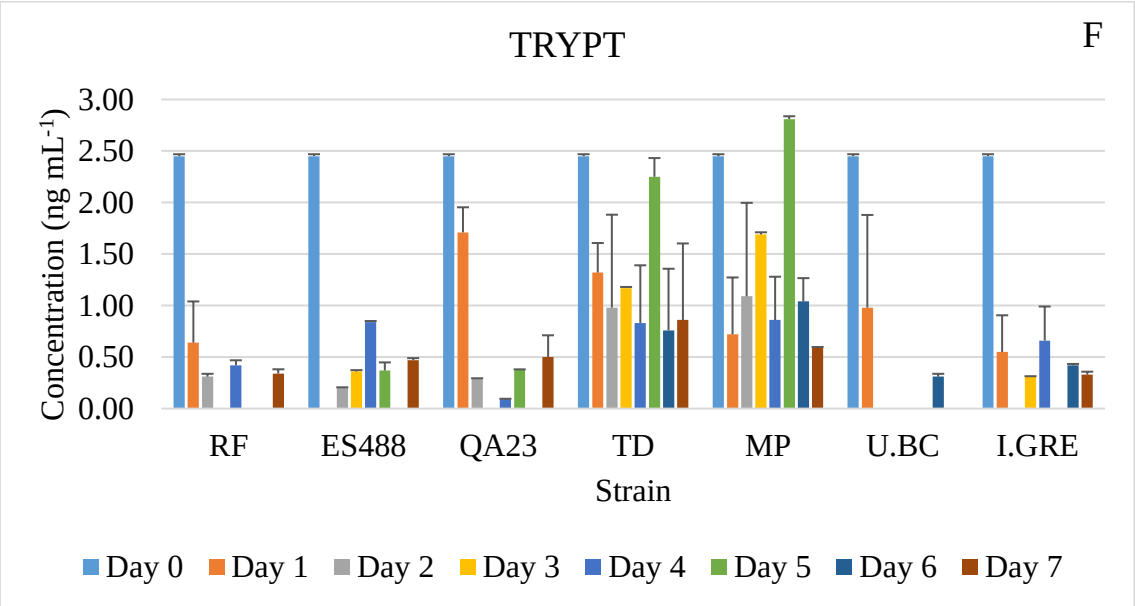
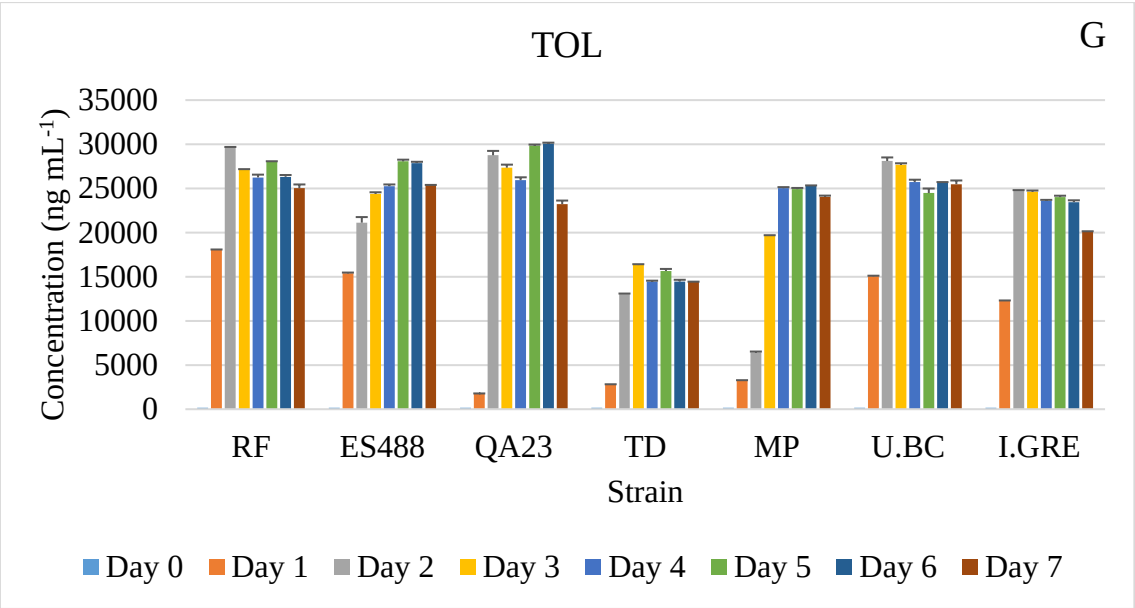


Figure 2 (A-H). Evolution of the indolic compounds concentration (continued)



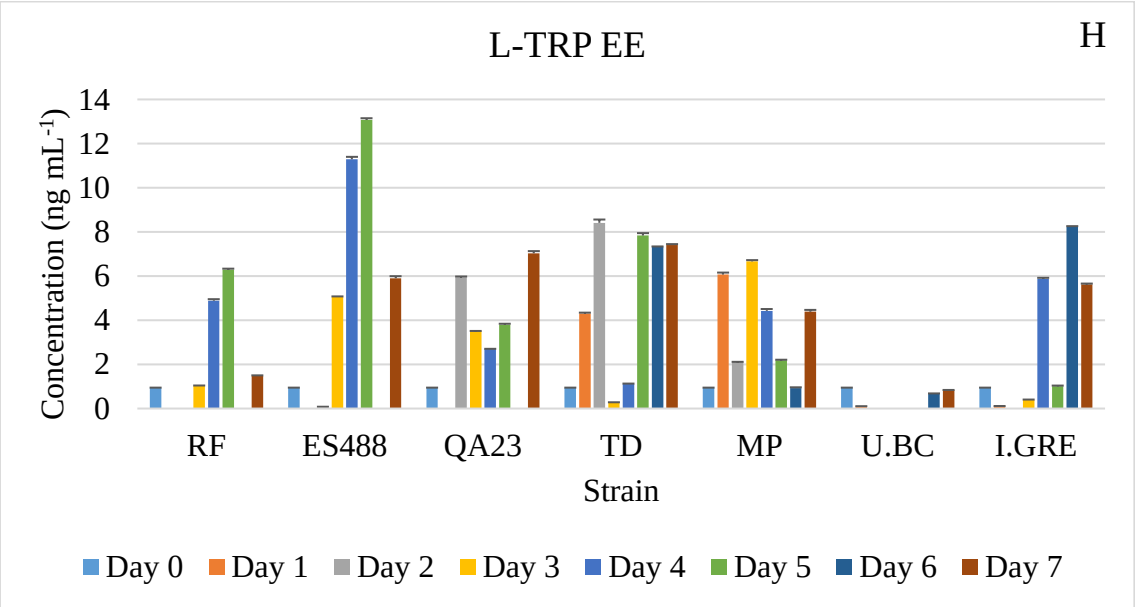


Figure 2 (A-H). Evolution of the indolic compounds concentration (continued)

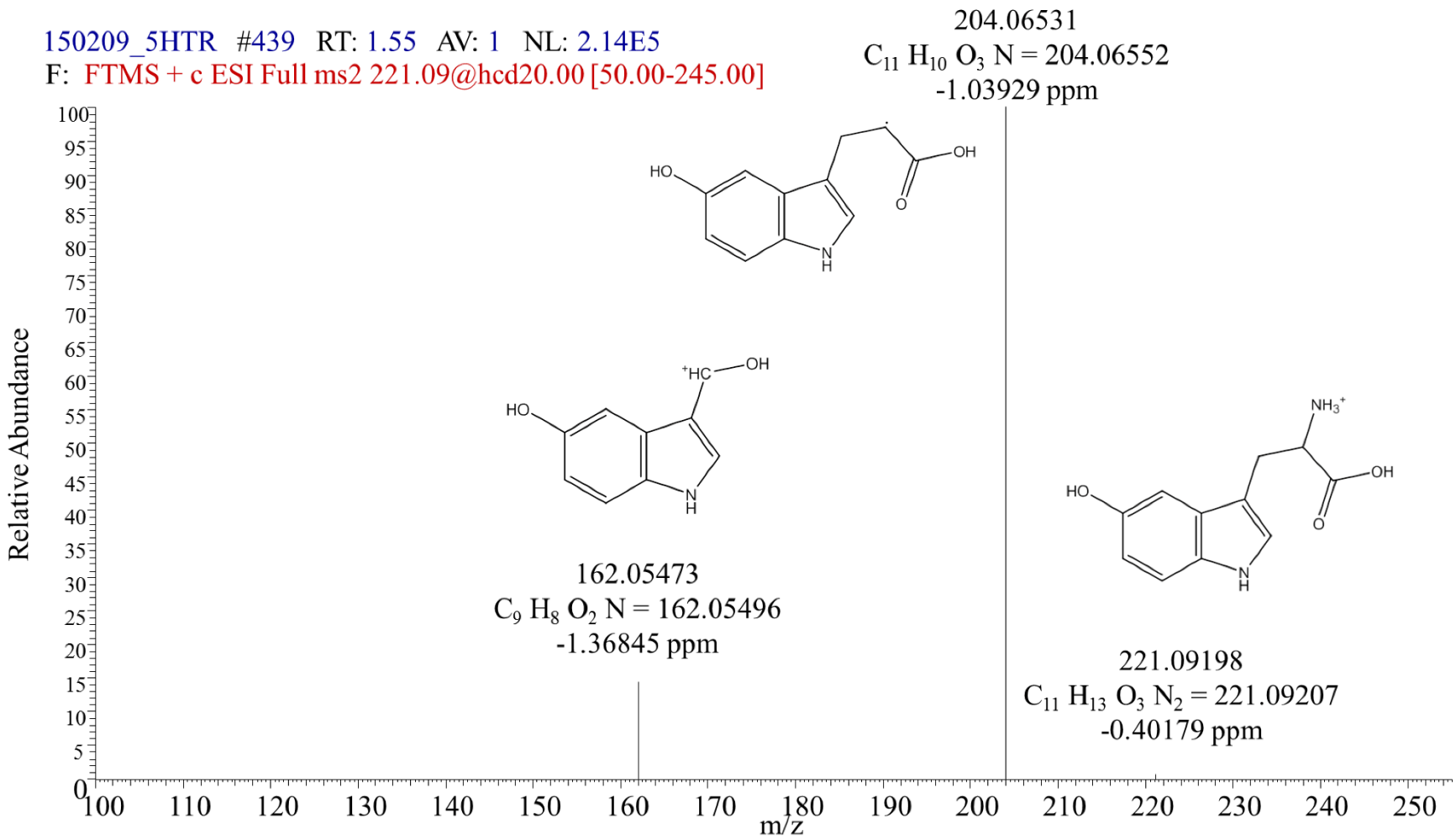


Figure 3. Growth curves of the commercial wine yeast strains

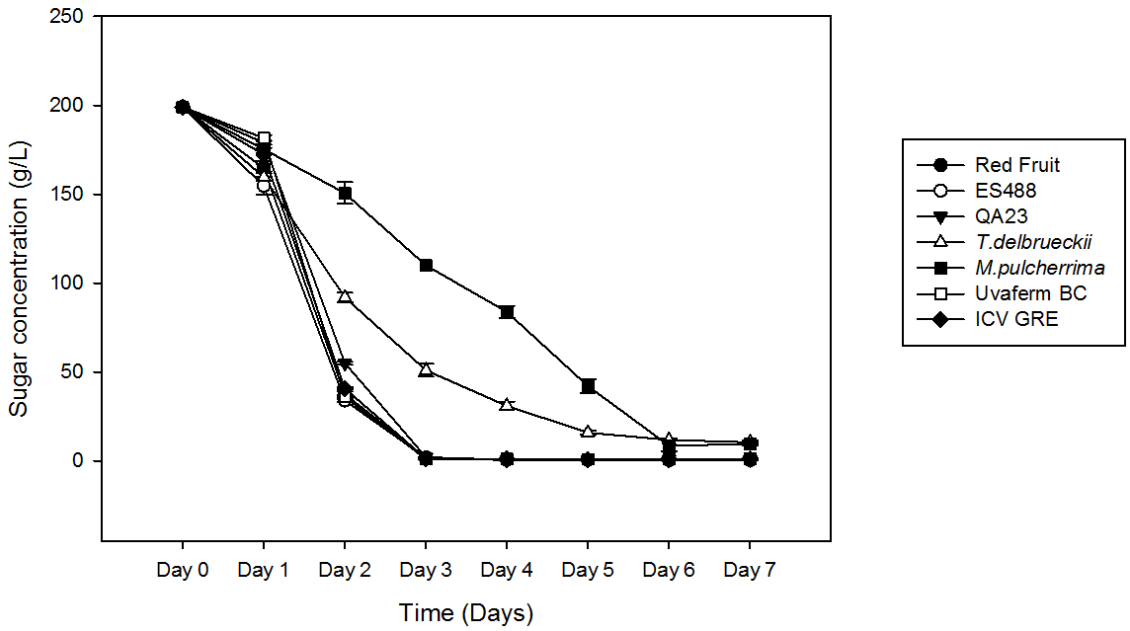


Figure 4. Sugar concentration during fermentation process

CAPÍTULO 4 / CHAPTER 4

Fernández-Cruz, E., Cerezo-López, A.B., Cantos-Villar, E., Troncoso, A. M., García-Parrilla, M.C.

SEGUIMIENTO DE METABOLITOS DEL L-TRIPTÓFANO DURANTE FERMENTACIONES EN MOSTO DE UVA NATURAL: EFECTO DEL TIPO DE INOCULACIÓN Y LA VARIEDAD DE UVA EN LA SÍNTESIS DE MELATONINA Y COMPUESTOS INDÓLICOS RELACIONADOS

TIME COURSE OF L-TRYPTOPHAN METABOLITES WHEN FERMENTING NATURAL GRAPE MUSTS: EFFECT OF INOCULATION TREATMENTS AND CULTIVAR IN MELATONIN AND RELATED INDOLIC COMPOUNDS OCCURRENCE

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Time course of L-tryptophan metabolites when fermenting natural grape musts: Effect of inoculation treatments and cultivar in melatonin and related indolic compounds occurrence

E. FERNÁNDEZ-CRUZ[1], A.B. CEREZO[1], E. CANTOS-VILLAR[2], A. M. TRONCOSO[1] and M.C. GARCÍA-PARRILLA[1]

[1] Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal. Facultad de Farmacia. Universidad de Sevilla, 41012, Sevilla, Spain; [2] Instituto de Investigación y Formación Agraria y Pesquera (IFAPA), Centro Rancho de la Merced, Consejería de Agricultura, Pesca y Desarrollo Rural (CAPDA), Junta de Andalucía, Ctra,Trebujena, Km 2.1, 11471, Jerez de la Frontera, Spain.

Corresponding author: Dr.María del Carmen García-Parrilla, mcparrilla@us.es.

Short title: Occurrence of L-tryptophan derivatives compounds in different grape cultivars

Abstract

Background and Aims: Tryptophan is involved in the formation of bioactive compounds such as melatonin and 3-indoleacetic acid, by yeast. Melatonin is a neurohormone whose occurrence in wine has been widely reported in recent years. However, occurrence of melatonin and other indolic compounds related to tryptophan metabolism by wine yeast strains has been sacarcely reported on grape musts. This work examined the occurrence of these compounds during the alcoholic fermentation of musts from seven grape cultivars (Corredera, Chardonnay, Moscatel, Palomino Fino, Sauvignon Blanc, Tempranillo and Vijiriega).

Methods and Results: Must was fermented with three *Saccharomyces cerevisiae* strains and with sequential inoculation with the non-*Saccharomyces* yeast *Torulaspora delbrueckii*. Fermented must samples were analysed by UHPLC/HRMS to determine the concentration of: L-tryptophan, 5-hydroxytryptophan, 5-hydroxytryptamine, *N*-acetyl-5-hydroxytryptamine, melatonin, 3-indoleacetic acid, tryptamine, tryptophol and L-tryptophan ethyl ester. Fermentation with Aroma White gave different profile of indolic compound occurrence during alcoholic fermentation depending on the cultivar. The yeast strain used did not influence the occurrence of indolic compounds. Instead, fermentation time was found to be a more influential factor.

Significance of the study: This is the first study that quantifies 5-hydroxytryptophan and *N*-acetyl-5-hydroxytryptamine during the alcoholic fermentation of grape must. The occurrence of compounds with bioactive potential, for example 3-indoleacetic acid and melatonin, during fermentation with commercial yeast strains is also described.

Keywords: *3-indoleacetic acid, melatonin, qexactive,* Saccharomyces cerevisiae, *wine*

Introduction

The amino acid L-tryptophan (L-TRP) contains an indolic ring which confers a strong hydrophobic character (Palego et al. 2016). In addition to taking part in the biosynthesis of proteins, it is involved in the metabolism of several compounds of interest, such as 5-hydroxytryptamine (5-HT) and melatonin (MEL), their principal precursor being L-TRP (Murch et al. 2000). The occurrence of MEL has been reported in many plant-based foods and beverages, including grapes (Iriti 2009, Mercolini et al. 2012), strawberries (Stürtz et al. 2011), pomegranate wines (Mena et al. 2012), sweet cherries (Zhao et al. 2013), and peppers and tomatoes (Riga et al. 2014), amongst others (Feng et al. 2014).

Yeast such as *Saccharomyces cerevisiae* are capable of metabolising L-TRP to produce MEL. This was first reported for yeast grown in two different media (Sprenger et al. 1999) and then in fermented products including beer (Maldonado et al. 2009), wine (Stege et al. 2010, Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al. 2011, Mercolini et al. 2012) and bread (Yılmaz et al. 2014). Different metabolic pathways have been proposed for the production of MEL from L-TRP. The synthesis of MEL by yeast was initially proposed to follow the same steps as those in animals, that is via 5-hydroxytryptophan (5-HTRP), 5-HT and *N*-acetyl-5-hydroxytryptamine (NA5-HT) (Figure 1). A subsequent study, however, reported that the capacity of *S. cerevisiae* to synthesise 5-HT via 5-HTRP was limited (Park et al. 2008). This suggested that MEL formation via 5-HTRP was not the only viable pathway. An alternative pathway for synthesising MEL from L-TRP via 5-methoxytryptamine (Figure 1) has recently been proposed (Tan et al. 2016). Different *Saccharomyces* and non-*Saccharomyces* yeast have been reported to produce MEL during alcoholic fermentation (AF), for example in synthetic must (SM) by *S. cerevisiae* (Aroma White, Red Fruit, QA23, ES488, IGV-GRE and Uvaferm strains), *Torulaspora*

delbrueckii or *Metschnikowia pulcherrima* (Rodríguez-Naranjo et al. 2012, Fernández-Cruz et al. 2017), and in orange juice by *Pichia kluyveri* (Fernández-Pachón et al. 2014). Several previous studies have investigated the formation of L-TRP derivatives by the QA23 yeast strain, due to its ability to rapidly complete fermentation and its wide use in white, rosé and red winemaking (Rodríguez-Naranjo et al. 2012, Blanco et al. 2013, Fernández-Cruz et al. 2017, González et al. 2018). By comparison, *S. cerevisiae* strains, such as Aroma White (AW) and Red Fruit (RF), have been used only sporadically in laboratories, but have recently been studied in SM (Fernández-Cruz et al. 2016, 2017, Muñíz-Calvo et al. 2017). Whereas AW is widely used in white winemaking, RF imparts appealing sensory properties to rosé and red wines. Moreover, the use of *non-Saccharomyces* yeasts in winemaking, such as *T. delbrueckii*, is considered an emerging biotechnological tool of great commercial interest (Puertas et al. 2017). *Torulaspora. delbrueckii is* characterised by the production of its low acetaldehyde, acetic acid, hydrogen sulfide and volatile phenol (Renault et al. 2009) As such, its sequential inoculation with *S. cerevisiae* is considered to improve wine quality (Bely et al. 2008, van Breda et al. 2013, Azzolini et al. 2014, González-Royo et al. 2015, Renault et al. 2015). It is therefore attracting the interest of winemakers to improve the fermentation process, which in turn will produce wines with improved characteristics such as color, aroma and better technological properties (Suárez-Lepe and Morata 2012).

In addition to 5-HT and MEL, L-TRP metabolism encompasses other metabolites as shown in Figure 1. Different pathways have been proposed for the synthesis of indolic compounds such as 3-indoleacetic acid (3-IAA) (Maslov et al. 2011). This compound is a phytohormone that has been exhaustively described in both plants and micro-organisms such as bacteria (Epstein and Ludwig-Muller 1993, Spaepen et al. 2007, Yu et al. 2014,

Cook et al. 2016). Together with L-TRP metabolites, such as tryptophol (TOL), tryptamine (TRY) and L-tryptophan ethyl ester (L-TRP EE) (Gil-Agustí et al. 2007, Favre et al. 2014, Gardana et al. 2014, Tudela et al. 2016), these compounds are of interest as bioactives due to their potential as antioxidants (Bonnefont-Rousselot and Collin 2010), endothelial function protectors (Jadhav et al. 2012), antiangiogenic molecules (Cerezo et al. 2017) and neuroprotective agents (Hornedo-Ortega et al. 2017).

The present study aimed to test whether different grape must substrates, yeast strains and inoculation procedures influence the formation of L-TRP metabolites in semi-industrial fermentations. In addition, the study aimed to ascertain the conditions which most efficiently produce L-TRP metabolites, some of which have bioactive potential.

Materials and methods

Reagents

The compounds, 5-HTRP, TOL, 3-IAA, 5-HT, NA5-HT, MEL, L-TRP EE, and TRY of high-grade purity (>97%) were purchased from Sigma Aldrich (St Louis, MO, USA); L-Tryptophan from Panreac (Darmstadt, Germany); analytical grade methanol for UHPLC analysis from Merck (Darmstadt, Germany); and formic acid from Prolabo (Obregón, México).

Grapes

Grapes from six white cultivars, Corredera, Chardonnay, Moscatel, Palomino Fino, Sauvignon Blanc and Vijiriega, commonly grown in the south of Spain, and one red cultivar, Tempranillo, were harvested from vineyards located at the Instituto de Investigación y Formación Agraria y Pesquera (IFAPA) in Jerez de la Frontera, Spain (SW Spain, long. 06:00:58W, lat. 36:45:29N).

117 *Yeast strains*

118 Yeasts were selected according to their efficiency in producing MEL and other L-TRP
119 derived compounds reported in previous work (Fernández-Cruz et al. 2016, 2017): *S.*
120 *cerevisiae* Aroma White AW (Enartis, Trecate, Italy), *S. cerevisiae* Lalvin YSEO QA23®
121 (Lallemand, Bayern, Germany), *S. cerevisiae* Red Fruit RF (Enartis) and *T. delbrueckii*
122 TD291 Biodiva (Lallemand). The yeasts were supplied as commercial active dehydrated
123 yeasts (ADY).

124 *Winemaking procedure*

125 **White winemaking.** Grapes from the six white cultivars were harvested, destemmed,
126 crushed and pressed. A pectolytic enzyme preparation (2.5 mL/hL, Enartis ZYM) and 40
127 mg/L of SO_2 (Sulfosol, Sepsa-Enartis) were added to the must which was held for 24 h
128 at 4ºC . Subsequently, the clarified must was placed in a 100 L stainless steel vessel. The
129 AF was induced by inoculation with the Aroma White AW yeast strain (Enartis) and
130 monitored at 18ºC. Fermentations were in triplicate (n = 3) for each cultivar. Fermentation
131 was considered finished when the concentration of residual sugar was less than 3 g/L.

132 **Rosé winemaking.** Tempranillo must was produced with a pneumatic press. A pectolytic
133 enzyme preparation (3 mL/hL, Enartis ZYM) and 40 mg/L of SO_2 were added. The must
134 was then placed into 15 stainless steel 10 L vats.

135 The Tempranillo must was subjected to five inoculation treatments: (i) inoculation with
136 *S. cerevisiae* QA23 yeast (CT-QA23); (ii) inoculation with *S. cerevisiae* RF yeast (CT-
137 RF); (iii) sequential inoculation (SI-QA23) with *T. delbrueckii* TD291 and then with *S.*
138 *cerevisiae* QA23, i.e. when density had decreased by 15 points after the start of AF; (iv)
139 sequential inoculation (SI-RF) with *T. delbrueckii* TD291 and then with *S. cerevisiae* RF,
140 again when density had decreased by 15 points; and (v) spontaneous fermentation (SP),

that is without inoculation with commercial yeast. All fermentations were maintained at 16–18 ºC and were in triplicate (*n*=3).

Sampling

Samples (15 mL) of white and rosé must were taken after the dejuicing process. For white fermentations, additional samples (15 mL) were taken daily from inoculation until the end of AF, i.e. when the residual sugar level were less than 3 g/L. For Tempranillo fermentations, CT-QA23 and CT-RF, were sampled daily until day 6, while SP, SI-QA23 and SI-RF were sampled daily until day 9. Samples were also taken on day 13–14. The duration of AF and final residual sugar level for wines of each cultivar are provided as supporting information (Table S1, Figure S1). Samples were stored at -80 ºC prior to analysis.

Sample preparation

Samples were thawed and centrifuged for 10 min at 1500 *g* (Sorvall TC Dupont centrifuge, Thermo Fisher Scientific, Barcelona, Spain)) to remove lees. The resulting supernatant was collected in 15 mL falcon tubes and an aliquot (500 µL) extracted as described by Fernández-Cruz et al. (2017). Extracts were placed in a vacuum concentrator (HyperVACLITE, GYROZEN, Seoul, Korea) until total dryness was achieved (34ºC, 2000 rpm). Samples were subsequently reconstituted in aqueous methanol (1:1, 167 µL) to a 3:1 final concentration. Prior to UHPLC/HRMS analysis, samples were filtered (13 mm VWR syringe filters, 0.45 µm PTFE) and placed in dark coloured glass vials.

UHPLC/HRMS analysis

Extracts were analysed with a UHPLC system (Dionex Ultimate 3000, Thermo Fisher, San Jose, CA, USA) comprising an autosampler (WPS-3000RS), pump (HPG-3400RS)

and column compartment (TCC-3000RS). Separation was achieved using a 2.1 x 100 mm SB-C18 column (Zorbax RRHD, 1.8 µm particle size) fitted with a guard column (2.1 x 5 mm, 1.8 µm particle size). UHPLC system was coupled to a hybrid Quadrupole-Orbitrap Qexactive equipment (Thermo Scientific, Bremen, Germany). UHPLC and HRMS conditions were set according to a previously validated method (Fernández-Cruz et al. 2016). Each sample replicate was injected in duplicate ($\underline{n}$ = 6). More information about the UHPLC/HRMS data is provided in Table S2. The system was controlled with Chromeleon Express software. Xcalibur software (v.3.0.63) and TraceFinder software (v. 3.1) were used for data analysis. All software was provided by Thermo Scientific (Brenen, Germany).

Statistical analysis

The data were subjected to Multiple linear regression, ANOVA and Tukey's Honest Significant Difference (HSD) to determine any significant differences ($p < 0.05$). In addition, Linear discriminant analysis (LDA) was also applied. Statistica software (StatSoft, 2005, Oklahoma, USA) v.7.0) was used to perform statistical analyses.

Results and discussion

Composition of must from white grape cultivars during fermentation with S. cerevisiae *Aroma White*

Must from six white grape cultivars, (Corredera, Chardonnay, Moscatel, Palomino Fino, Sauvignon Blanc, and Vijiriega, were analysed during AF with the AW *S. cerevisiae* strain to determine the occurrence of indolic compounds derived from L-TRP metabolism. It must be noted that, in all cases, AW *S. cerevisiae* was the main strain implanted during AF (data not shown).

189 Figure 2 shows changes in the concentration of the precursor amino acid L-TRP, together
190 with metabolites of the MEL pathway (5-HTRP, 5-HT, NA5-HT and MEL), the higher
191 alcohol TOL and other compounds of interest (3-IAA and L-TRP EE), during AF. The
192 initial L-TRP must concentration ranged from 0.4 to 2.3 mg/L (416–2313 ng/mL), with
193 Corredera and Chardonnay containing the lowest and highest concentration, respectively
194 (Figure 2a). The amino acid profile is influenced by several parameters, such as the soil
195 composition of the area where the grapes were cultivated, the cultivar employed (Garde-
196 Cerdán et al. 2009), irrigation (Bouzas-Cid et al. 2018), leaf treatments (Ruiz-Rodríguez
197 et al. 2017) and terroir (Moreno et al. 2015, Uriarte et al. 2016). Despite it being one of
198 the main amino acids, however, little attention has been paid to L-TRP and few studies
199 report the L-TRP concentration of grapes and must in different cultivars. The
200 concentration measured in the current study is much lower than that previously reported
201 (Table 1). At the end of AF, L-TRP values had decreased significantly, ranging from 4.2
202 ng/mL for Vijiriega to 1902 ng/mL for Moscatel.
203 The first intermediate metabolite in the MEL pathway, described previously for animals,
204 plants and yeasts, is 5-HTRP (Figure 1). This study is the first to report the occurrence of
205 5-HTRP in grape must during AF. As shown in Figure 2b, 5-HRTP was initially present
206 at a low concentration (less than 2.5 ng/mL) in must prior to AF for all cultivars;
207 significant quantities were then consumed by the end of AF. The progressive decrease in
208 5-HTRP concentration ranged from 55% for Corredera to 99% for Sauvignon Blanc. It
209 appears that yeast are capable of consuming 5-HRTP quickly during the early stages of
210 AF (i.e. during days 1–3). A similar trend was previously observed during the AF of SM
211 (Fernández-Cruz et al. 2016).

The subsequent MEL pathway intermediates, 5-HT and NA5-HT, were not detected in the white musts, however, the final metabolite, MEL, was quantified during AF of all samples, except Palomino Fino. Melatonin was measured at a concentration ranging from 0.10–0.45 ng/mL in the initial must samples (i.e. prior to AF) (Figure 2c). Accumulation of MEL followed two trends during AF, despite the use of the same yeast strain. A zigzag plot trend was observed for the MEL concentration in Corredera and Moscatel, with significant change observed during AF. In contrast, MEL concentration remained constant during AF of Chardonnay, Sauvignon Blanc, and Vijiriega (Figure 2c). A previous study reported the maximum production of MEL on day 2 for a SM fermentation with the *S. cerevisiae* AW strain (Fernández-Cruz et al. 2016). In this study, however, more than one peak in MEL production was found during AF (Figure 2c). There was a maximum peak for MEL at the end of AF (i.e. on day 9) for Chardonnay, while other cultivars, e.g. Corredera and Moscatel, gave multiple peaks, especially during the first stages of AF (i.e. between days 1–6). These results show a remarkable difference amongst the substrates studied, resulting in each cultivar displaying different trends in MEL production.

The occurrence of L-TRP EE during AF of white grape must was highly variable (Figure 2d). . In five of the six initial samples, L-TRP EE was present. The exception, Corredera, L-TRP EE did not appear until day 3. Moreover, Corredera, Palomino Fino and Vijiriega gave relatively constant L-TRP EE values, with little change during AF (ca. 0.1 ng/mL). In Chardonnay and White Muscat, L-TRP-EE concentration was found to slightly increase up until the end of AF (0.5 ng per 10^6 CFU), when it then diminished (Vigentini et al. 2015). In an earlier study, we reported an increase in L-TRP EE concentration in SM during AF using the *S. cerevisiae* AW strain under laboratory conditions, with a final

concentration of 17.5 ng/mL at the end of AF (Fernández-Cruz et al. 2016). Other researchers have also reported the formation of L-TRP EE in growth medium and grape musts, under laboratory conditions. In growth medium, L-TRP EE increased in concentration 24 h after inoculation (12.0–208.1 ng/mL) with different strains of *S. cerevisiae* (UMY255, EC1118 and IOC18–2007), but it decreased to negligible levels (0.3–1.6 ng/mL) 72 h after inoculation (Vigentini et al. 2015). In the current study, the maximum concentration of L-TRP EE found in white grape must (0.1 ng/mL) was lower than that reported in SM (17.5 ng/mL). Apparently, yeast produce ethyl esters from amino acids part way through AF, when the concentration of alcohol in the fermenting medium is high (Lambrechts and Pretorius 2000). The L-TRP EE appears to be formed from L-TRP (Figure 1) in a one-step reaction (Arapitsas et al. 2018).

The initial concentration of 3-IAA observed in the six white grape musts was negligible (<1 ng/mL), in agreement with that previously observed in the initial must obtained from Kerner and Malvasia grapes, i.e. <5 ng/mL (Simat et al. 2004, Maslov et al. 2011). Our results show a remarkable increase in 3-IAA during the early stages of AF for all must samples (Figure 2e), indicating production due to yeast metabolism. In yeast, the occurrence of 3-IAA is reported to be dependent on L-TRP (Contreras, Curtin, et al. 2014, Nutaratat et al. 2016). This relationship was not found in the current study, even though two different accumulation trends were observed during AF. Chardonnay, Moscatel, Sauvignon Blanc and Vijiriega showed a significant decrease in 3-IAA at day 3, relative to their initial 3-IAA concentrations. A similar trend was previously reported for Müller-Thurgau grape must fermented with *S. cerevisiae* Uvaferm CM, where the highest concentration of 3-IAA (being 70 ng/mL) occurred during turbulent fermentation, with

the concentration diminishing at the end of AF (Simat et al. 2004). In the case of SM fermented by the *S. cerevisiae* AW strain, the main trend observed was an initial increase, and then a decrease in 3-IAA concentration, from 83.8 ng/mL at day 1 to 0.9 ng/mL by the end of AF (Fernández-Cruz et al. 2016). In contrast, Corredera and Palomino Fino gave a relatively consistent 3-IAA concentration, being 16.5 and 14.8 ng/mL, respectively, at the end of AF, that is the concentration was significantly higher than that prior to AF (Figure 2e). Similarly, a study involving fermentation of Riesling grape must by four commercial *S. cerevisiae* strains (Uvaferm CEG, Lalvin Cross, Anchor VIN and Anchor Exotics SPH) reported an increase in 3-IAA during AF of 10–35 ng/mL, but concentration did not diminish at the end of fermentation (Mihaljević Žulj et al. 2015). These data suggest that the combination of yeast strain and grape cultivar play a decisive role in the accumulation of 3-IAA.

Tryptophol was the compound with the highest concentration during the AF of must from the six white grape cultivars (Figure 2f). Tryptophol was present in the initial must at concentration of 3.3 and 31.3 ng/mL for Moscatel and Palomino Fino, respectively. . Two different accumulation patterns were then observed. Corredera, Chardonnay, Palomino Fino and Sauvignon Blanc mildly accumulated TOL during AF with a final concentrations ranging from 365 to 666 ng/mL. Whereas for Moscatel and Vijiriega, a significant sharp increase (1170–1217 ng/mL) was observed after 1–2 days of AF and was constant at the end of AF, giving a significantly higher concentration of TOL compared with that observed for other cultivars. The same trend was reported for AF of must from Müller-Thurgau grapes, with TOL being synthesised during the first stages of the fermentation process and increased until the end of AF (Simat et al. 2004). In SM

fermented with *S. cerevisiae* AW strain, TOL also increased in concentration during the first 3 days of AF (Fernández-Cruz et al. 2016). As is known, TOL is the fusel alcohol of the amino acid L-TRP and it is produced by yeast during AF via the Ehrlich pathway (Hazelwood et al. 2008, Mas et al. 2014). Synthesis of TOL takes places after the formation of two intermediate metabolites, 3-indolpyruvic acid (3-IPA) and 2-indolacetaldehyde (3-IAD), which derive from L-TRP (Dickinson 2003) (Figure 1). In our samples, this pathway appears to be the one that yeast preferred during AF, since most of the L-TRP present in the initial must was transformed into TOL.

Linear discriminant analysis was performed with variety selected as the independent variable. Forward stepwise analysis included the following variables: 5-HTRP, MEL, 3IAA, TRYPT, TOL, L-TRP EE in the model. 5-Hydroxytryptamine and NA5-HT could not be included, as they were not detected in a sufficient number of samples. Classification rates of 100% were achieved. Figure 3 shows that samples were grouped according to cultivar using Factors 1 and 3. This indicates the production of indolic compounds depends largely on the grape cultivar being fermented, in addition to the yeast strain.

Effect of yeast strain on the composition of Tempranillo must during fermentation

Tempranillo must was inoculated with several yeast strains after the dejuicing process and they were implanted during AF of each yeasting procedure (data not shown). Figure 4 shows the compositional changes observed during the five different fermentations carried out with Tempranillo must. Figure 4a shows an initial L-TRP concentration of 3492 ng/mL, which was considerably lower than the concentration previously reported for must of different red grape cultivars, being 9410 to 30 000 ng/mL (Table 1). This may

reflect differences in soil type and climate, as previously suggested (Fernández-Marín et al. 2013). However, compared with results obtained for the white grape cultivars (Figure 2a), the initial concentration of L-TRP was highest in Tempranillo must. The accumulation of L-TRP remained similar to that observed for the white must, i.e. significant consumption of L-TRP was observed between days 1 and 4, irrespective of the inoculation treatment; with negligible concentrations detected from day 5. The same trend was previously observed in SM (Fernández-Cruz et al. 2017).

For 5-HTRP (Figure 4b), both the initial concentration (being 2.3 ng/mL) and the accumulation trend were similar to those observed for white grape must (Figure 2b). All of the fermentations followed the same pattern, regardless of which yeast strain was inoculated, even the spontaneous fermentation. Like L-TRP, 5-HTRP was exhausted during the early stages of AF, but it was not produced in either the white or Tempranillo grape must. We reported the same trend for 5-HTRP occurrence in SM fermented with *S. saccharomyces* RF, QA23 and *T. delbrueckii* under laboratory conditions (Fernández-Cruz et al. 2017). These results show that use of the same yeast strains under either semi-industrial or laboratory conditions gave similar 5-HTRP consumption patterns. Thus, it appears that the inoculation method does not significantly affect 5-HTRP consumption during AF, regardless of fermentation scale.

As far as we are concerned, this is the first time NA5-HT has been quantified during AF of Tempranillo musts. NA5-HT was present in the initial must at 0.25 ng/mL (Figure 4c). During AF, NA5-HT levels peaked between days 3–4 and then decreased substantially until the end of AF, to give final concentrations of 0.3 ng/mL. In an earlier study, we reported NA5-HT at negligible concentrations (0.1 ng/mL) at the start of fermentations

328 in SM, with NA5-HT disappearing during AF (Fernández-Cruz et al. 2017). NA5-HT has
329 been quantified in commercial sparkling wines such as Cava, Reserva, and Gran Reserva,
330 at higher concentrations, i.e. 0.3 to 2.0 ng/mL (Tudela et al. 2016).

331 Following the corresponding pathway (Figure 1) MEL is one of the final L-TRP
332 metabolites. In this study, MEL was present in Tempranillo must at a low concentration,
333 i.e. 0.07 ng/mL (Figure 4d). A considerably higher MEL concentration has been reported
334 in red grape must after pressing, that is 74.1, 77.7, 241.2 and 322.7 ng/L for Cabernet
335 Sauvignon, Tempranillo, Merlot and Tintilla de Rota, respectively (Rodriguez-Naranjo et
336 al. 2011). During AF, considerable variation in MEL was observed, similar to that
337 described above during AF of white grape must (Figure 2c).

338 The concentration of 3-IAA in Tempranillo must (Figure 4e) also followed the trend
339 described during AF of white grape must (Figure 2e). 3-Indole acetic acid was initially
340 present at a concentration of 2.8 ng/mL but the concentration subsequently increased
341 during the first few days of fermentation, reaching a maximum (irrespective of
342 inoculation treatment) between days 2–4. The concentration of 3-IAA then decreased
343 significantly until the end of AF, with the final concentration ranging from 7.2 to 10.8
344 ng/mL, for CT-RF and SP samples, respectively. SM fermentations performed with RF
345 and QA23 yeast strains showed similar trends (Fernández-Cruz et al. 2017).

346 The initial concentration of TOL in Tempranillo must (Figure 4f) was low, at just 4.9
347 ng/mL, compared with the level (123 ng/mL) reported for Monastrell grape must (Bordiga
348 et al. 2016). The concentration of TOL then increased significantly during AF, reaching
349 a final concentration of between 1979 for CT-QA23 and 2668 ng/mL for SP.

L-Tryptophan ethyl ester was not detected in the initial Tempranillo must, but it accumulated in all fermentations, irrespective of inoculation treatment, from day 1 at quite a constant concentration (data not shown), with no change in concentration observed during AF (0.032–0.042 ng/mL).

Linear discriminant analysis was performed to compare the occurrence of indolic compounds as a function of both inoculation treatment and the duration of fermentation, that is from the initial must (i.e. at day 0) to day 6. All of the quantified compounds where included in the analysis. Figure 5 shows the plots obtained with a forward stepwise method in which 35 samples were analysed. Four groups, representing the initial must, and the must after 1, 5 and 6 days of fermentation, were clearly differentiated (Figure 5a). Samples collected on days 2, 3 and 4, representing the midpoint of AF, formed one uniform group, with few noticeable differences. The greatest changes were therefore observed during the early stages of AF, where L-TRP metabolism by yeast was more noticeable. In contrast, the production of indolic compounds during AF was not strongly influenced by the inoculation treatment (Figure 5b); that is separation of samples was achieved only when the duration of fermentation was the variable.

Conclusion

Must from different cultivars gave different profiles of the indolic compounds derived from the amino acid L-TRP. Although the presence of L-TRP in the initial must is essential, the occurrence of individual indolic compounds was not always related to the initial concentration of L-TRP. Changes in the concentration of 5-HTRP and NA5-HT, intermediates of the MEL pathway in yeast metabolism, were described for the first time during the AF of grape must fermented by different yeast strains. The accumulation of

MEL and 3-IAA, compounds considered to have bioactivity potential, was also reported during AF, with an especially high concentration observed for Corredera. The higher alcohol, TOL, was the L-TRP metabolite found to be present at the highest final concentrations in all white fermentations, with L-TRP being the pathway preferred by yeast. In a common substrate such as Tempranillo must, inoculation treatments involving different yeast strains did not affect the occurrence of indolic compounds during AF. Their occurrence was, however, influenced by the duration of fermentation. Further studies are required to ascertain the combination of grape cultivar and inoculation strategy that optimises the concentration of indolic compounds so as to realise their potential bioactivity in wine.

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Table 1. L-Tryptophan concentration reported in white and red grape cultivars.

Type of grape	Grape variety	Amount (ng mL^{-1})	Reference
White	*Kerner*	2,000-80,000	(Hoenicke et al. 2001)
	Riesling	4,000-35,000	(Linsenmeier et al. 2004)
	White grapes from different countries	200-11,000	(Bell & Henschke 2005)
	White grapes from Alentego region (Portugal)	5,500-17,800	(Herbert et al. 2006)
	Albana	70-117	(Mercolini et al. 2012)
	Emir	108,760	(Ünal et al. 2015)
	Narince	22,410	(Ünal et al. 2015)
	Sultaniye	34,520	(Ünal et al. 2015)
	Verdejo	2,500-4000	(Ruiz-Rodríguez et al. 2017)
	Treixadura	8,000-18,000	(Bouzas-Cid et al. 2018)
	Chardonnay	5,000-45,000	(Meng et al. 2018)
		3,400	(Vigentini et al. 2015)
Red	*Sangiovese*	70-133	(Mercolini et al. 2012)
	Croatina	5,200	(Vigentini et al. 2015)
	Tempranillo	9,410-17,630	(Pérez-Álvarez et al. 2017)
		23,380	(Garde-Cerdán et al. 2017)
		25,810	(Gutiérrez-Gamboa et al. 2017)
		30,000	(González-Santamaría et al. 2018)
	Monastrell	33,420	(Garde-Cerdán et al. 2017)
	Carignan noir	6,240-15,540	(Gutiérrez-Gamboa, Carrasco-Quiroz, et al. 2018)
	Garnacha	13,100	(Gutiérrez-Gamboa, Portu, et al. 2018)

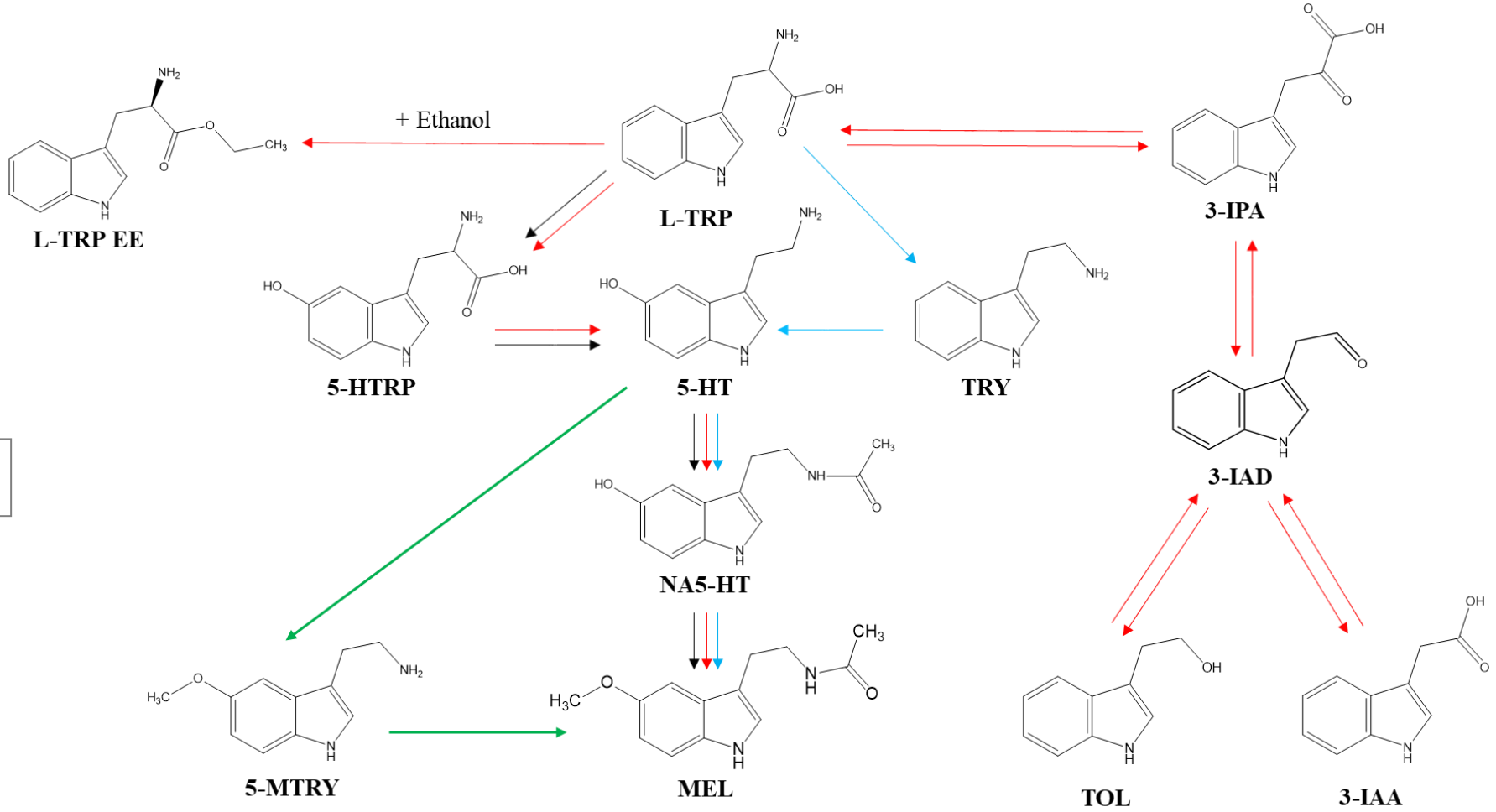

Figure 1. L-Tryptophan pathways showing formation of different indolic compounds by animals (——), plants (——) and yeast (——) and by alternative common pathways (——). Adapted from Dickinson et al. 2003, Simat et al. 2004 and Tan et al. 2016. L-TRP, L-tryptophan; 5-HTRP, 5-hydroxytryptophan; 5-HT, 5-hydroxytryptamine; NA5-HT:,*N*-acetyl-5-hydroxytryptamine; MEL: melatonin; TRY: tryptamine; 5-MTRY: 5-methoxytryptamine; 3-IPA, 3-indolepyruvic acid; 3-IAD, 3-indole acetaldehyde; TOL, Tryptophol; 3-IAA, 3-indoleacetic acid

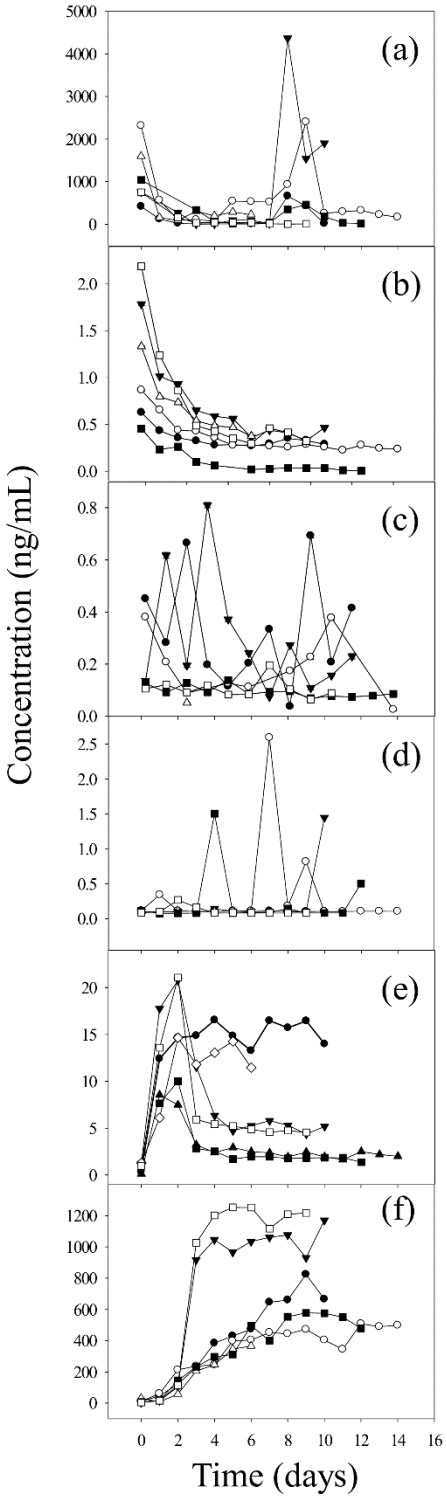

Figure 2. Effect of *Saccharomyces cerevisiae* Aroma White strain on the concentration of (a) L-tryptophan, (b) 5-hydroxytryptophan, (c) melatonin, (d) L-tryptophan ethyl ester, € 3-indoleacetic acid and (f) tryptophol during the fermentation of musts of the white grape cultivars, Corredera (●); Chardonnay (▲); Moscatel (▼); Palomino Fino (◊); Sauvignon Blanc (■) and Vijiriega (□).Values are means of six replicates (n = 6) ± standard deviation.

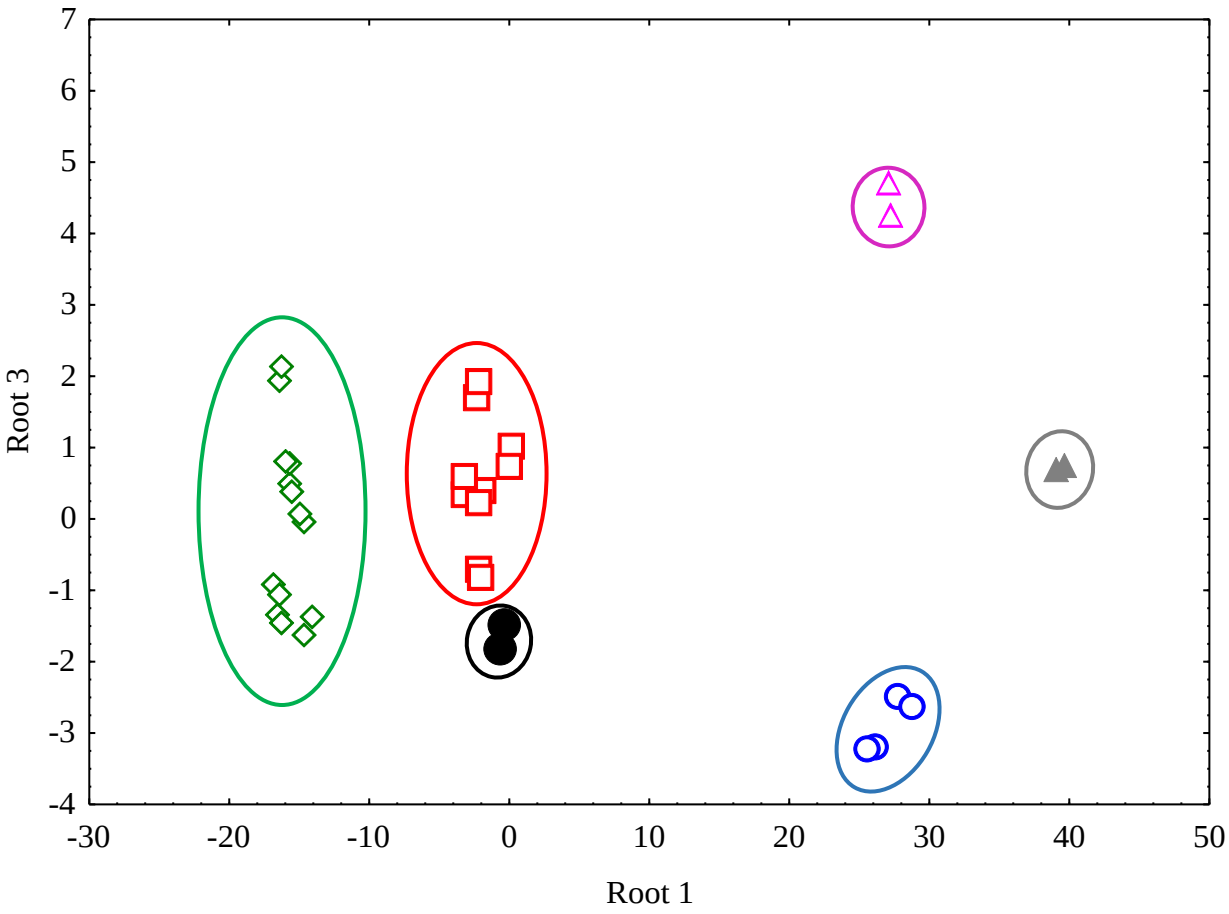

Figure 3. Linear discriminant analysis performed on must of the white grape cultivars Corredera (○), Chardonnay (□), Moscatel (◊), Palomino Fino (△), Sauvignon Blanc (●) and Vijiriega (■) at different points during alcoholic fermentation by *Saccharomyces cerevisiae* Aroma White.

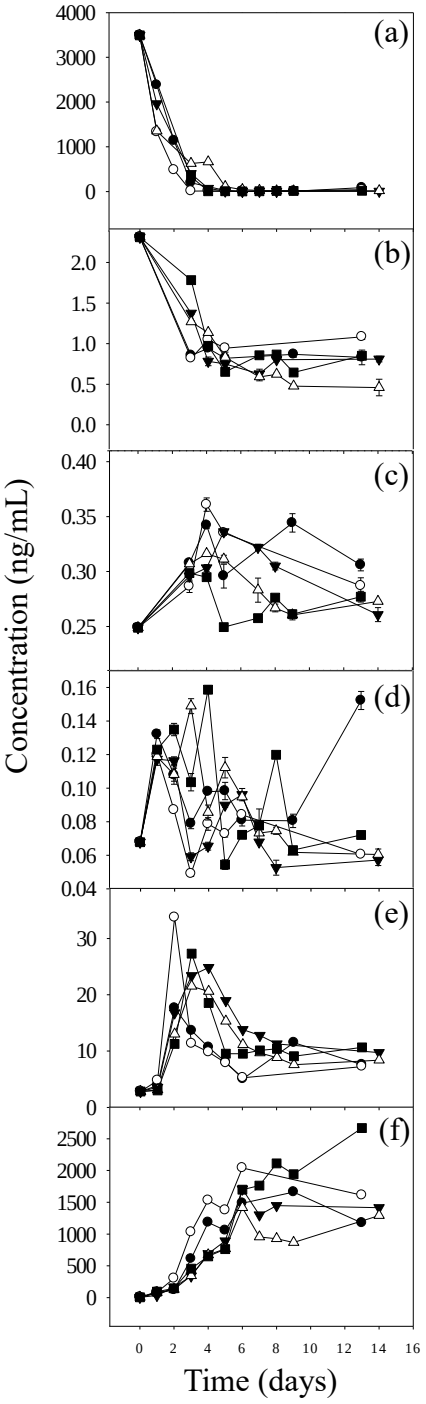

Figure 4. Effect of yeast on the concentration of (a) L-tryptophan, (b) 5-hydroxytryptophan, (c) melatonin, (d) L-tryptophan ethyl ester, (e) 3-indoleacetic acid and (f) tryptophol in Tempranillo grape must fermented with: *Saccharomyces cerevisiae* QA23 (●);*S. cerevisiae* Red Fruit (○);sequential inoculation with *T. delbrueckii* and *S. cerevisiae* QA23 (▼);sequential inoculation with *T. delbrueckii* and *S. cerevisiae* Red Fruit (Δ); spontaneous fermentation (■). Mean ± SD, Values are means of six replicates (n = 6) ± SD.

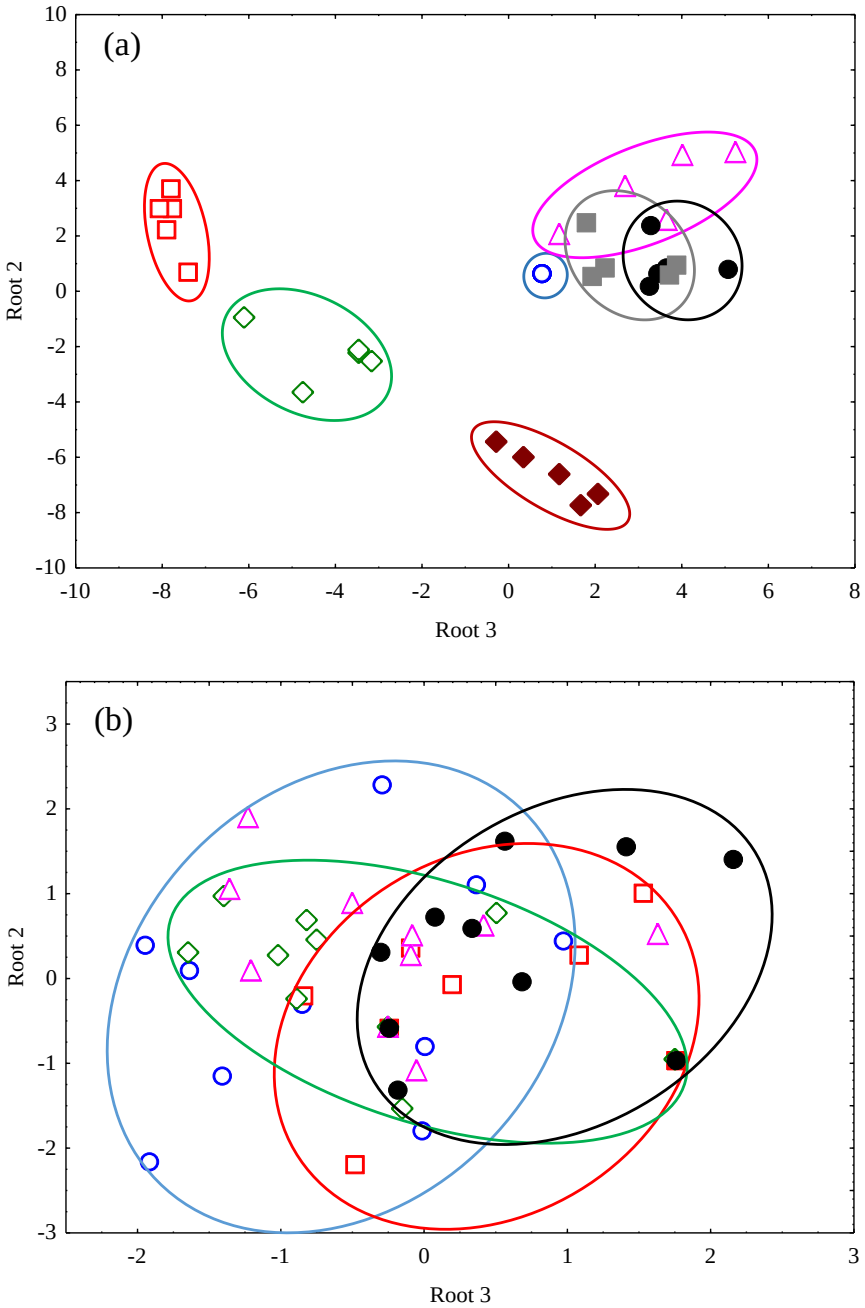

Figure 5. Linear discriminant analysis performed on must of the red grape cultivar Tempranillo fermented (a) with *Saccharomyces cerevisiae* QA23/RF at day 0 (○), day 1 (□), day 2 (◊), day 3 (Δ), day 4 (●), day 5 (■) and day 6 (♦) and (b) with inoculation of ESP (○), RF (□), QA (◊), ISRF (Δ) and ISQA (●)

Supplementary material

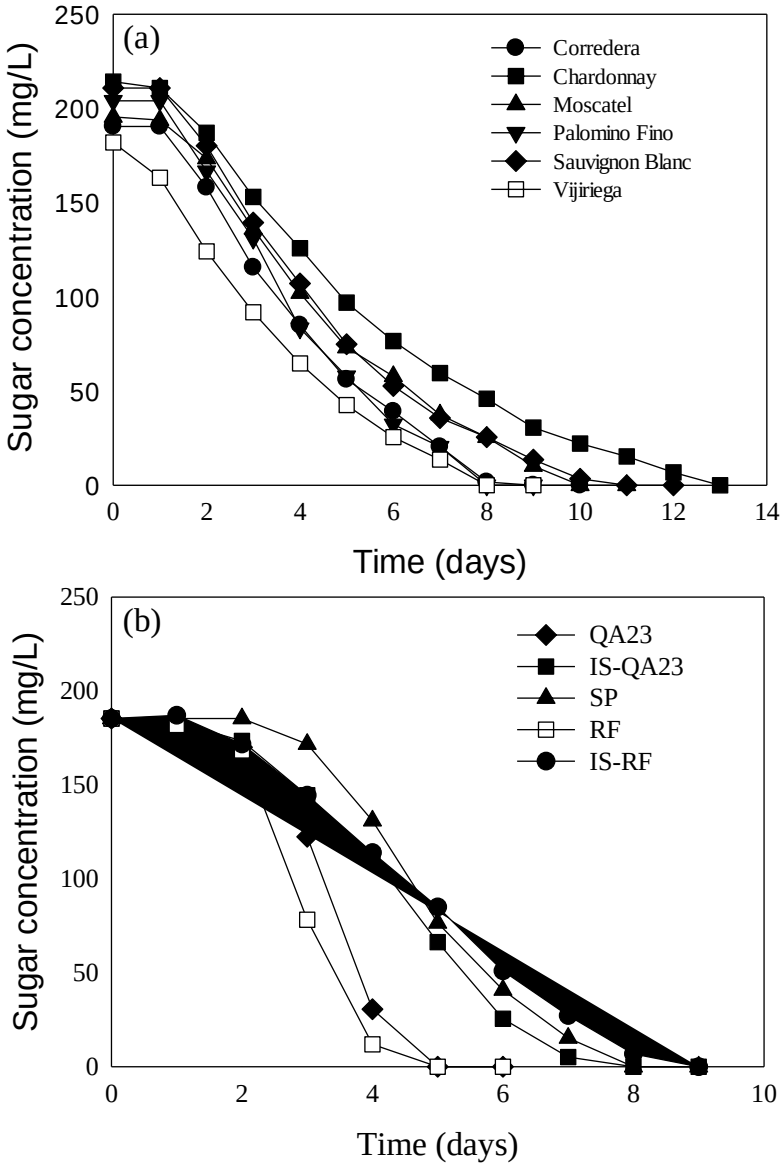

Figure S1. Sugar consumption during alcoholic fermentation of must from (a) the white grape cultivars Corredera (●), Chardonnay (■), Moscatel (▲), Palomino Fino (▼), Sauvignon Blanc (♦) and Vijiriega (□) and from (b) Tempranillo following inoculation with *Saccharomyces cerevisiae* QA23 (♦) and *S. cerevisiae* RF (□), sequential inoculation with *Torulaspora delbrueckii* and *S. cerevisiae* QA23 (■) and with *Torulaspora delbrueckii* and *S. cerevisiae* RF (●), and spontaneous fermentation (▲).

Table S1. Effect of grape cultivar on the duration of alcoholic fermentation (AF) and residual sugar concentration at the end of fermentation.

Grape variety	End of AF	Residual sugars (g L^{-1})
Chardonnay (white)	14 days	1.95
Corredera (white)	10 days	2.33
Moscatel (white)	10 days	1.47
Palomino fino (white)	6 days	2.00
Sauvignon Blanc (white)	12 days	1.55
Vijiriega (white)	9 days	1.33
Tempranillo (rosé)	13 days	0.56-1.2

Table S2. Retention time, protonated ion, main fragment, theorical fragment, relative intensity, formula and error of the different indolic compounds analysed during the alcoholic fermentation.

Compound	RT (min)	$[M+H]^+$	Main fragment	Theorical fragment	Relative intensities	Formula	Error (ppm)
5-HT	1.05	177.10224	160.07567	160.07569	100	$C_{10}H_{10}ON$	0.14
5-HTRP	1.27	221.09207	204.06540	204.06552	100	$C_{11}H_{10}O_3N$	0.59
TRY	3.65	161.10732	144.08066	144.08078	100	$C_{10}H_{10}N$	0.82
L-TRP	3.85	205.09715	188.07043	188.07061	100	$C_{11}H_{10}O_2N$	0.91
			146.06004	146.06004	21	C_9H_8ON	0.02
			159.09152	159.09167	4	$C_{10}H_{11}N_2$	0.96
NA5-HT	5.26	219.11280	160.07568	160.07569	100	$C_{10}H_{10}ON$	0.04
			202.08618	202.08626	6	$C_{12}H_{12}O_2N$	0.36
			219.11272	219.11280	5	$C_{12}H_{15}O_2N_2$	0.40
L-TRP EE	6.40	233.12845	216.10185	216.10191	100	$C_{13}H_{14}O_2N$	0.24
			174.09135	174.09134	18	$C_{11}H_{12}ON$	0.08
			159.09164	159.09167	12	$C_{10}H_{11}N_2$	0.19
			132.08072	132.08078	8	$C_9H_{10}N$	0.43
TOL	7.15	162.09134	144.08061	144.08078	100	$C_{10}H_{10}N$	1.14
			162.09128	162.09134	17	$C_{10}H_{12}ON$	
3-IAA	7.16	176.07061	130.06523	130.06513	100	C_9H_8N	0.81
			176.07060	176.07061	16	$C_{10}H_{10}O_2N$	0.02
MEL	7.21	233.12845	174.09151	174.09134	100	$C_{11}H_{12}ON$	0.17
			216.10204	216.10191	7	$C_{13}H_{14}O_2N$	0.13
			233.12851	233.12845	5	$C_{13}H_{17}O_2N_2$	0.06

CAPÍTULO 5 / CHAPTER 5

Fernández-Cruz, E., Carrasco-Galán, F., Cerezo-López, A.B., Cantos-Villar, E., Troncoso, A. M., García-Parrilla, M.C.

DETERMINACIÓN DE COMPUESTOS INDÓLICOS DERIVADOS DEL METABOLISMO DEL L-TRIPTÓFANO EN DIFERENTES CERVEZAS COMERCIALES

DETERMINATION OF INDOLIC COMPOUNDS DERIVED FROM L-TRYPTOPHAN METABOLISM IN DIFFERENT COMMERCIAL BEERS

IN PREPARATION TO BE SUBMITTED TO FOOD CHEMISTRY

Determination of indolic compounds derived from L-tryptophan metabolism in different commercial beers

Fernández-Cruz, E.[1], Carrasco-Galán, F.[1], Cerezo-López, A.B.[1], Troncoso, A. M.[1], García-Parrilla, M.C[1]*.

[1]Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal. Facultad de Farmacia. Universidad de Sevilla. C/ Profesor García González 2, 41012, Sevilla, Spain

*Corresponding author:

E-mail address: mcparrilla@us.es (M.C. García-Parrilla)

Abstract

Beer is the most consumed alcoholic beverage around the world. Despite of the alcohol content, it presents some bioactive compounds derived from hops such as polyphenols. However, there is other compounds of interest derived from the amino acid *L*-tryptophan than can be present in beer. A group of 19 beer commercial samples were chosen to study different metabolites, optimizing sample treatment and analyzing by UHPLC/HRMS technique. Results show that PTFE filters is the best option to avoid loss derived from sample treatment. Additionaly, for the first time has been reported the occurrence of 5-hydroxytryptophan, *N*-acetyl-5-hydroxytryptamine, 3-indolacetic acid and *L*-tryptophan ethyl ester. Although concentrations found are low to outcome a remarkable effect of bioactive compound after the dietary intake, they could be useful as supporting sources on daily intake.

Keywords: melatonin, 3-indolacetic acid, beer, bioactive, HRMS

INTRODUCTION

Beer is the most consumed alcoholic beer around the world, with a consumption of 200 billlion liters per year (Colen & Swinnen, 2016; Stack, Gartland, & Keane, 2016). Beer contains, apart of alcohol, amino acids, carbohydrates, vitamins and also bioactive compounds such as polyphenols and melanoidins, mostly from hops and malt (Arranz, Chiva-Blanch, Valderas-Martinez, Casas, & Estruch, 2014; González-SanJosé, Rodríguez, & Valls-Bellés, 2016). Focusing on the nitrogen compounds, the amount of amino acids in beer is highlyinfluenced by the raw material used. In Europe the most common cereals to produce beer are barley and corn, although rice, wheat, oat, sorghum, rye and triticale can be also used as an alternative (Bogdan & Kordialik-Bogacka, 2017). Additionally, during brewing, the germination and mashing procedures usually involve proteolytic processes that increase the amount of amino acids in malt and wort (Hellwig, Beer, Witte, & Henle, 2018). Amino acids are not the nitrogen source most preferred by brewing yeasts. Despite that, thay can be assimilatedandaccording to the following assimilation pattern fast, intermediate, slow and no absorbednitrogen source (Kabelová, Dvořáková, Čížková, Dostálek, & Melzoch, 2008).

The amino acid L-tryptophan (L-TRP) is considered as a slow absorption nitrogen source for yeast (Beltran, Novo, Rozès, Mas, & Guillamón, 2004), therefore its presence in wort and beer usually goes unnoticed since it is not crucial for the fermentation step. Nevertheless,, L-TRP is the precursor of some bioactive compounds such as melatonin (MLT), serotonin (5-HT) or 3-indolacetic acid (3-IAA) which have been been reported in fermented beverages such as wine due to the yeast metabolism (Li, 2008; Mihaljević Žulj et al., 2015; Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, & García-Parrilla,

2011). Among these, MLT and 5-HT as well as some higher alcohols such as tryptophol (TOL) have been previously described in beer (Bartolomé, Peña-Neira, & Gómez-Cordovés, 2000; Kirschbaum, Meier, & Brückner, 1999; Kocadağlı, Yılmaz, & Gökmen, 2014; Maldonado, Moreno, & Calvo, 2009a).

However, little is known about the occurrence of L-TRP derived compounds in beer. The role of the yeast has been recently highlighted as relevant for the occurrence of MLT in fermented products such as wine (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos-Villar, & García-Parrilla, 2011) and it was also reported in orange juice fermented with *Saccharomyces* strains (Fernández-Pachón et al., 2014). Consequently, it is plausible to suppose that MLT and other related L-TRP metabolites might be present in other fermented products as beers. However, literature about them is almost non-exsisting right now. As, other compounds related with L-TRP such as 5-hydroxytryptophan (5-HTRP), *N*-acetyl-5-hydroxytryptamine (NA-5HT) and ethyl ester of tryptophan (L-TRP EE) have not been reported yet in beers.

Since Garcia-Moreno et al. reported the determination of MLT in beers by means of ELISA technique, the advances in the field mostly consist on….On the other hand, the issues concerning the analytical challenge as…..have not beed addressed as far as we know. Indeed, The low concentrations reached by MLT in beers requires a quite sensitive identification technique to quantify it. Hence, it seems that ELISA analysis to determine the concentration of MLT in beer is the most accurate (Garcia-Moreno et al., 2013; Maldonado, Moreno, & Calvo, 2009b). However, it has been reported that ELISA has a huge variability when is tested on complex matrices (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al., 2011). Currently, the UHPLC/HRMS technique is able to

identify and quantify trace concentration metabolites unequivocally (Fernández-Cruz, Álvarez-Fernández, Valero, Troncoso, & García-Parrilla, 2016; Kocadağlı et al., 2014; Vivian, Aoyagui, de Oliveira, & Catharino, 2016)

The aim of this work is to elucidate the occurrence of different L-TRP derivates compounds in commercial beers, widely consumed in Spain territory using a validated UHPLC/HRMS and optimizing the sample treatment in order to improve the identification of bioactive compounds in beers and provide data to be included in food composition tables.

MATERIAL AND METHODS

Reagents

Standards of indolic compounds (3-IAA, 5-HTRP, L-TRP, L-TRP EE, MEL, NACSERO, SERO, TRYP, TOL) were supplied by Sigma Aldrich (Barcelona, Spain). Methanol of LC/MS grade was provided by Merck (Darmstadt, Germany). Formic acid for LC/MS with a 99% purity was supplied by Prolabo ® (Obregon, Mexico).

Beer samples

Beer brands were selected to be representative of beer consumption in Spain. The Brand Foodprint 2016 study (*Kantar Worldpanel*, https://www.kantarworldpanel.com) about the main food brands sold was used to extract the most consumed beers in Spain by autonomous region. As a result, nineteen beer brands were purchased from different local supermarkets in the glass-bottle format (Table 1). Most beers belonged to the lager type

with the exception of Guinness (stout) and have an alcoholic degree between 0.0 and 7.2º. Voll-dam was not widely consumed, but it was included since it was previously reported to have a high level of MEL (Maldonado et al., 2009b).

Filtration step optimization

In order to test the effect that filtration step caused on the concentration of indolic compounds, three filters with different membrane materials were selected according to those previously referenced in literature (citas). Table 2 shows the literature that reports the used filters to analyse indolic compounds and the filters used prior to the determination of L-TRP related compounds, being nylon (NY) the most common for UHPLC/HRMS analysis. Moreover, polytetrafluoroethylene (PTFE) and cellulose acetate (CA) filters have been also used (Smart, Aggio, Van Houtte, & Villas-Bôas, 2010). To test them and for the sake of comparison, three different stock solutions of nine indolic compounds standards were prepared, based on the limits of quantification (LOQ) previously reported by Fernández-Cruz (Fernández-Cruz et al., 2016). Concentrations of LOQ, LOQ + 50% and 3x LOQ were filtered with the different membranes and were analysed by the above mentioned UHPLC/HRMS validated method. All samples were dried in a vaccum concentrator (HyperVACLITE, GYOZEN, Korea) at 30ºC, 2000 rpm and subsequently rediluted in a dark HPLC vial with 500 µL solution of 10% methanol:water with formic acid (0.1 %) prior to UHPLC/HRMS analysis. Additionally, same stock solutions without filtration step were analysed to compare the signal given by the filtered ones.

Solid phase extraction optimization

Solid phase extraction (SPE) procedure was also tested with different SPE cartridges as shown in Table 2. . To test whether SPE procedure affects the final concentration of the indolic compounds, four types of cartridges were tested: C18 Bond Elut (10112102118, Agilent), C18 Strata^{TM}-X (S100-HCH, Phenomenex), Strata® C18-E (S001-HCH, Phenomenex©) and C8 Clean Screen® Dau (CSDAU503, UCT). SPE was performed according to Fernandez-Cruz et al. (Fernández-Cruz, Álvarez-Fernández, Valero, Troncoso, & García-Parrilla, 2017). All cartridges were conditioned with 2 mL methanol and after 2 mL milliQ water. Then, 500 µL of indolic standard solutions at different concentrations (LOQ, 3x LOQ and 50x LOQ) were loaded. Cartridges were subsequently washed with 2 ml of a 10% methanol:water solution. Indolic compounds were eluted with 1 mL of methanol. Extracts were placed in dark brown eppendorfs and dried and rediluted as described in filters step optimization section. Stock solutions of LOQ, 3x LOQ and 50x LOQ solutions were also analysed without loading them in the SPE cartridges.

Sample treatment

Aliquots of 5 mL of each beer sample were degassed for 30 minutes in an UB-1488 ultrasonic bath (J.P.Selecta, Barcelona, Spain). Then, samples were filtered before the SPE procedure. SPE was performed as described in SPE optimization section, using a sample volume of 2 mL. Then, extracts were dried (30 ºC, 2000 rpm) and rediluted in dark HPLC vials up to a 10:1 concentration prior to UHPLC/HRMS analysis.

UHPLC/HRMS analysis

All the reconstituted solutions were analysed by a UHPLC/HRMS system with a previously validated method with the conditions set according to Fernández-Cruz et al. (Fernández-Cruz et al., 2016, 2017).

Statistical analysis

Statistical differences through the Student´s t-test were performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla California USA, www.graphpad.com). Principal Components Analysis was performedwith the StatSoft, Inc. (2004). STATISTICA (data analysis software system), version 7.0 (www.statsoft.com).

RESULTS AND DISCUSSION

Optimization of filtration and SPE procedure

Three types of filter membranes (NY, CA and PTFE) were tested to study the effect on the concentration of indolic compounds derived from the amino acid L-TRP. Results show that CA filters retained most of the indolic compounds severely with the exception of 5-HTRP (Figure 1). Concerning to NY membrane, L-TRP, NA-5HT, MEL, 3-IAA and TOL concentrations were significantly affected. In particular, it is worth to mention that N-acetylserotonin, Melatonin… can be retained as much as a …However, PTFE filters retained …proved to be the best option to filter samples in order to study the occurrence of indolic compounds, since it only affected the concentration of two compounds (L-TRP and L-TRP EE). Figure 1 displays the percentage of the two concentrations tested of the indolic compounds, being LOQ the lowest and 3x LOQ the highest.

164 The precursor L-TRP had significant losses when NY, CA and PTFE filters are used
165 (29%, 46% and 38% respectively) at LOQ concentration (Figure 1A). On the other hand,
166 no changes were observed when the 3x LOQ concentration was tested on the different
167 filters. This fact is important, since the L-TRP concentrations found in fermented
168 beverages are usually much higher (Fernández-Cruz et al., 2017; Jia, Kang, Park, Lee, &
169 Kwon, 2011; Kabelová et al., 2008; Zhu, Zhang, & Gong, 2017).

170 However, compounds from the MEL synthesis pathway (5-HTRP, SERO, NACSERO
171 and MEL itself) have been reported at quite low concentrations (ng/mL, pg/mL), closely
172 to LOQ (Kocadağlı et al., 2014; Mena, Gil-Izquierdo, Moreno, Martí, & García-Viguera,
173 2012; Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al., 2011). 5-HTRP was
174 not affected by any filter type (Figure 1B), there was a strong loss of SERO (99% and
175 69% for LOQ and 3x LOQ, respectively) with CA filters (Figure 1C). The intermediate
176 NACSERO and MEL were the compounds of the MEL pathway more affected by the
177 filter membrane (Figure 1D-E). CA filters retained between 61 and 48% of NACSERO
178 initial concentration while NY filters affected between 46 and 75% for LOQ and 3x LOQ
179 respectively.

180 Other compounds such as 3-IAA, TOL, TRY and L-TRP EE were severely affected by
181 CA filters (Figure 1 F-I), while NY was only effective with TRY and L-TRP EE. PTFE
182 filters show a good recovery for these compounds. In fermented samples, TOL is the main
183 L-TRP derived compound found, with a concentration expressed at mg/L (Bordiga et al.,
184 2016; Fernández-Cruz et al., 2016) while 3-IAA is quantified in a lesser concentration
185 (ng/mL) (Fernández-Cruz et al., 2017; Hoenicke, Simat, Steinhart, Köhler, & Schwab,
186 2001). However, the occurrence of TRY and L-TRP EE is scarce (Tudela et al., 2016;

187 Vigentini et al., 2015) and it is important to asses that NY and PTFE filters can be used
188 indistinctly. Consequently the selection of the filters is crucial for the results to be
189 obtained.

190 Thus, PTFE filters are the best option to use in the sample treatment prior to
191 UHPLC/HRMS analysis since it do not interfere the concentration of these compounds
192 in fermented samples.

193

194 *Indolic compounds content in commercial beers*

195 Nineteen commercial beers were analysed by UHPLC/HRMS in order to study the
196 occurrence of different indolic compounds related with the aromatic amino acid L-TRP
197 such as 5-HTRP, SERO, NACSERO, MEL, 3-IAA, TRYP, TOL and L-TRP EE. Results
198 are displayed in Table 3. To the best of our knowledge, this paper reports for the first time
199 the occurrence of some of L-TRP derived compounds such as 5-HTRP, NACSERO, 3-
200 IAA and L-TRP EE on beer samples. That were unequivocally identified by HRMS

201 All beers show a remarkable content of L-TRP (Table 3A), ranging from the 348.08
202 ng/mL of San Miguel to 6508.83 ng/mL of the Voll-Damm. Beer presents in its
203 composition different content of amino acids. In the case of L-TRP, it is usually classified
204 as a secondary nitrogen source that is consumed when the primary sources are depleted
205 (Bogdan & Kordialik-Bogacka, 2017). However, the raw material to obtain wort also
206 provides a different profile of amino acids initial concentration, especially when sources
207 different from malt such as wheat, barley, corn, sorghum or rice among others (Bogdan
208 & Kordialik-Bogacka, 2017). Toh et al. (2018) reported a concentration of 7.92 mg/L of

tryptophan using the *S. cerevisiae* strain S-04 after 14 days of fermentation. This value was higher than the value described for Voll-Damm which reached the highest L-TRP concentration of the beers analysed.

5-HTRP (Table 3A) is the first intermediate of the MEL pathway. It was found in all commercial beers, ranging from 0.15 ng/mL from San Miguel to 1.05 ng/mL of Cruzcampo cruzial. Previously, it has been reported low concentrations (below 5 ng/mL) in synthetic media fermented by commercial *S. cerevisiae* strains after 7 days (Fernández-Cruz et al., 2016, 2017). In final beers with subsequent treatments after alcoholic fermentation process, it was expected to be found even at lower concentrations.

Following the pathway, 5-HT was also found in all beer samples from 0.99 to 22.35 ng/mL (Guinness and Buckler 0.0 respectively). Serotonin was described in different beers samples from 3.5 to 24.2 mg/L (Kirschbaum et al., 1999). This high 5-HT concentration values were reported using a derivatization process with para-Nitrobenzyloxycarbonyl Chloride and a HPLC/DAD analysis. Beers included on these study were not submitted to a derivatization process and an UHPLC/HRMS was used instead. This could explain the huge difference between the 5HT found in the literature and the values reported in this work. On the other hand, *Saccharomyces cerevisiae* proved to produce 5-HT during alcoholic fermentation, reaching values at day 7 of 4.7 ng/mL (Fernández-Cruz et al., 2016). Likely, the strain used in each brewer industry may caused the differences observed among these commercial beers.

The immediately intermediate prior to MLT synthesis pathway is NA-5HT (Table 3A). As far as we are concerned, this is the first time that is quantified on commercial beer samples. Although it is not a main compound of beer, it appears in all commercial brands

in a range from 0.02 ng/mL of San Miguel to 0.39 ng/mL of Paulaner. Melatonin content is even lower than that found on NA-5HT, ranging from 29.3 pg/mL of Alhambra tradicional to 9.95 of Voll-Damm. It seems than beers are able to synthesize 5-HT but cannot form higher amounts of NA-5HT and MLT. However, MLT pathways of *S. cerevisiae* is still completely undescribed. Melatonin was reported in beers previously, also with commercial beers at concentrations between 51.8 to 169.7 ng/mL using the ELISA technique (Maldonado et al., 2009a). Our results from UHPLC/HRMS show lower levels, which explain the differences of MLT content. Moreover, when brewing process were performed in laboratory conditions with no final treatments, it was found a MLT concentration of 333 pg/mL. Likely, the treatment performed on beer in brewer industries may affect the final content of MLT, being craft beer procedures the best option to maintain possible bioactive compounds on beer.

TOL and 3-IAA are well known as the main higher alcohol and acid respectively on yeast metabolism, following the Ehrlich pathway (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008; Mas et al., 2014). The 3-IAA appears in all the beer samples, ranging from 7.68 ng/mL of Guinness to 143.42 ng/mL in Desperados (Table 3B). Respecting TOL, concentration range was higher (51.72 ng/mL from Buckler to 1892.61 ng/mL of San Miguel). While TOL is usually reported on beer, this is the first time that 3-IAA is quantified in commercial beers. Previously, both TOL and 3-IAA has been reported on synthetic must and wines (Fernández-Cruz et al., 2017; Mihaljević Žulj et al., 2015) at similar concentrations, being TOL the main metabolite derived from L-TRP during alcoholic fermentation. While TOL has proved to be a quorum sensing molecule (Avbelj, Zupan, & Raspor, 2016; Dickinson, 2008) it is not clear the role of 3-IAA on yeast.

255 Other minoritarian metabolites were also quantified on commercial beers. Tryptamine is
256 usually included when a study of biogenic amines on beer is performed. Concentrations
257 in beer were found at 100 ng/mL (Gómez-Alonso, Hermosín-Gutiérrez, & García-
258 Romero, 2007) and 137-4239 ng/mL (Nalazek-Rudnicka & Wasik, 2017). However, in
259 mant ocassions is has been not detected on beer samples (Jia et al., 2011; Kirschbaum et
260 al., 1999; Redruello et al., 2017). Our samples described TRY content from 2.64 ng/mL
261 of Buckler 0.0 to 38.41 ng/mL of Voll-Damm. These values are lower than the previously
262 reported, but higher than the TRY concentrations found in wines (Fernández-Cruz et al.,
263 2017; Rodriguez-Naranjo, Ordóñez, Callejón, Cantos-Villar, & García-Parrilla, 2013).
264 Thus, tryptamine formation in beer seem to be a not preferred pathway. There is an
265 additional, the L-TRP EE that has also described for the first time in beers, ranging 0.05-
266 2.57 ng/mL (Buckler 0.0, San Miguel respectively). L-TRP EE is not present on L-TRP
267 yeast metabolism, but it is formed due to the ethanol presence during alcoholic
268 fermentation (Arapitsas, Guella, & Mattivi, 2018). The concentration of L-TRP EE has
269 been reported to increase along the alcoholic fermentation by *S. cerevisiae* (Fernández-
270 Cruz et al., 2017). However, other studies show that L-TRP EE increase its concentration
271 on the early stages of alcoholic fermentation in grape must, but disappear quickly after 3
272 days from the inoculation day (Vigentini et al., 2015).

273

274 *Impact of beer consumption on L-tryptophan derivatives compounds occurrence in diet*

275 The World Health Organisation estimate the average alcohol consumption in 20-40 g
276 alcohol/day for women and 40-60 g alcohol/day for men. However, other authors report
277 lower values of 10-12 g alcohol/day for women and 20-24 g alcohol/day for men.

278 Depending on the alcohol content of beer, the daily amount allowed to avoid an excessive
279 alcohol consumption changes. Beers included in this study have an average alcohol
280 content of 5.2º per bottle of 200 mL (Table 1) with the exception of Voll-Damm (7.2º).
281 Under OMS recommendations, the consumption of 1 beer per day would not exceed the
282 average alcohol consumption neither woman nor men. In other cases, with 10-12 g
283 alcohol/day, 2 bottles will be enough to reach the average alcohol consumption for
284 women, and 4 bottles for men.

285 Bioactive compounds such as 3-IAA, 5-HT and MLT have different bioactive properties.
286 The 3-IAA was effective against the phosphorylation of vascular endothelial growth
287 factor (VEGF) mediated by receptor 2 (VEGFR-2) at 0.9074 mM (A.B. Cerezo, Hornedo-
288 Ortega, Álvarez-Fernández, Troncoso, & García-Parrilla, 2017); 5-HT was reported with
289 high inhibition of amyloid β-peptide aggregation evidenced its power as neuroprotective
290 at 100 µM (Hornedo-Ortega et al., 2018); MLT has proved to be an incredible antioxidant
291 (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012). Although beer
292 content of these metabolite is not enough to fulfil a benefitial intake, it could be an
293 additional source to daily intake.

294 **Conclusions**

295 Beer sample treatment is an important step to analyse bioactive compounds of L-TRP
296 metabolism using a UHPLC/HRMS technique. Filters of PTFE are shown to be the most
297 proper equipment to diminish possible loss of minoritarian bioactive compounds such as
298 MLT, 5-HT and 3-IAA. Moreover, commercial beer is also a source of bioactive
299 compounds although concentrations found are not at high levels but it could be reinforce
300 the presence on diet with other foods and beverages.

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Table 1. Names, alcoholic degree and fermentation type of the different beers commercialised in Spain beers

Name	*Alcoholic degree*	*Beer type*
Alhambra tradicional	4.6	Pilsner lager
Ámbar	5.9	Pilsner lager
Amstel	5.0	Pilsner lager
Buckler 0.0	0.0	Pilsner lager
Budweiser	5.0	American lager
Carlsberg	5.0	Pilsner lager
Corona	4.6	American lager
Cruzcampo	4.8	Lager pilsner
Cruzcampo cruzial	5.9	Lager pilsner
Desperados	5.9	Flavoured Lager
Estrella Damm	5.4	Pilsner lager
Estrella Galicia	5.5	Pilsner lager
Guinness	5.0	Irish dry stout
Heineken	5.0	Pilsner lager
Mahou 5 estrellas	5.5	Pilsner lager
Murphy´s Irish Red	5.0	Irish red ale lager
Paulaner	5.5	Weissbier lager
San Miguel	5.4	Pilsner lager
Voll-Damm	7.2	Märzenbier lager

Table 2. Sample treatment of different indolic compounds using solid phase extraction (SPE) cardridges and filters.

Compound	*Sample*	*Cartridge*	*Eluyent*	*Technique*	*Concentration*	*Filter*	*Reference*
Melatonin	Grape skin	C18 Sep-Pak silica column	MeOH	HPLC-FD ELISA	0.031-0.965 ng/g 2.4-428.3 pg/mL	Nylon	Iriti et al. (2006)
	Grape skin Wine	---	MeOH	Capillary electrochromatography	0.6-1.2 ng/g 0.16-0.32 ng/mL	Nylon (0.45 µm)	Stege et al (2010)
	Wine	---	---	UPLC-MS/MS	4.1-8.1 ng/mL	---	Vitalini et al. (2011)
	Wine	HLB Oasys	MeOH	UPLC-MS/MS	0.05-0.62 ng/mL	---	Vitalini et al. (2013)
	Grape skin	---	---	HPLC-ESI-MS/MS	8.9-158.9 ng/g	---	Boccalandro et al. (2011)
	Grape skin	---	MeOH	UPLC-MS/MS	120-160 ng/g	0.45 µm	Gomez et al. (2012)
	Grape skin	Strata C8 (500 mg/6 mL)	MeOH	LC MS/MS. UHPLC MS/MS	440 ng/g	PTFE (0.22 µm)	Gomez et al. (2013)
	Grape		1% aqueous formic acid	UPLC-(ToF) MS	100.000 - 150.000 ng/g	Ultrafree MC filtered centrifuge tubes 0.2 µm	Murch et al. (2010)
	Grape		MeOH	HPLC-FD	1.2-1.5 ng/g	0.45 µm syringe membrane	Mercolini et al. (2012)

						Sartorious	
	Red and white wine	VARIAN BondElut C18 100 mg, 1 mL	MeOH	HPLC-FD	0.4 y 0.5 ng/mL	0.2 µm syringe membrane Sartorious	Mercolini et al. (2008)
	Red and white wine			Capillary electrochromatography	0.16, 0.24, 0.32 ng/mL	Nylon 0.45 µm	Stege et al. (2010)
	Red wine			UPLC-MS/MS	4.1 y 8.1 ng/mL		Vitalini et al. (2011)
	Pressed wines			LC-MS/MS	74-322 ng/mL	0.45 µm	Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos-Villar, et al. (2011)
	Racked wines	Oasis MCX 30 mg	1.2 mL of the 100 mM NaOH:MeOH (65:35) solution	LC-MS/MS	250-423 ng/mL	0.45 µm PTFE	Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al. (2011)
	Monovarietal and polyvarietal red wines,white and dessert wines, modena	HLB Oasys 100 mg SPE cartridge	MeOH	UPLC-HR/MS	0.14-0.62 ng/mL 0.05-0.31 ng/mL 0.18 ng/mL		Vitalini et al. (2013)

	balsamic vinegars				0-0.31 ng/mL 0.11-0.13 ng/mL		
	Albana grappa, grape juice		MeOH	HPLC-FD	0.3 ng/mL 0.5 ng/mL	0.45 µm syringe membrane Sartorious	Mercolini et al. (2012)
	Cherry	Strata X Oasis HLB	2 mL MeOH 1 mL 2 % formic acid in MeOH	HPLC-Electronic detection	11-28 ng/g	Whatman de 0.2 µm	Rosado et al. (2017)
	Food (including beer and wine)			LC–MS/MS	0.05-20 ng/mL	Syringe filter 0.45 µm	Kocadağlı et al. (2014)
	Fruit juice			Vortex assisted dispersive liquid-liquid microextraction HPLC	0.23 ng/mL	Nylon syringe filter 0.22 µm	Afzali et al. (2016)
	Sparkling wines			UHPLC-MS/MS	0.17 µg/L	Nylon membrane filter 0.2 µm	Tudela et al. (2016)
Serotonine	Urine and plasma	Oasis® WCX (weak cation exchange) 10 by 1 mm SPE cartridges		XLC-MS/MS	0.03 µg/L y 0.9 µg/L		Wilhelmina et al. (2010)
	Blood	Oasis MAX cartridges (30 mg, 1 mL)	1.5 mL of MeOH containing 50 µL of 1N, pH 3 citrate buffer	HPLC	0.05 ng/mL (LOD)	Varian nylon filters (47 mm diameter, 0.2 µm	Addolorata Saracino et al. (2010)

						pore size)	
	Plasma, cerebrospinal fluid and tissue	C 18 reversed-phase extraction columns	1.0 mol / l acetic acid that contained ascorbic acid	HPLC-F	Signal-to-noise ratio of 3 for serotonin at concentrations of 67 nmol/L.		Kema et al. (2001)
	Sparkling wines			UHPLC-MS/MS	65.34 µg/L	Nylon membrane filter 0.2 µm	Tudela et al. (2016)
Tryptophan	Plasma, cerebrospinal fluid and tissue	C 18 reversed-phase extraction columns	1.0 mol / l acetic acid that contained ascorbic acid	HPLC-FD	Signal-to-noise ratio of 3 for tryptophan at concentrations of 19 nmol/L.		Kema et al. (2001)
	Plasma	Isolute® PRS (propylsulphonic acid based strong cation exchange) 10mm×1 mm SPE		XLC–MS/MS	30 nmol/L (LOD)		Wilhelmina et al. (2009)
	Wheat, barley, corn and rice flour	Column packed with Ni–Al(NO3−) LDH nano-sorbent	3 mL of 2 mol L−1 NaOH solution	SPE-SD	0.01 µg/L (LOD)		Zadeh et al. (2015)
	Sparkling wines			UHPLC-MS/MS	0.43 µg/L	Nylon membrane	Tudela et al. (2016)

						filter 0.2 µm	
Tryptophan etil éster	Wine	StrataX SPE column	3 mL MeOH	UPLC-MS/MS			Gardana et al. (2014)
	Sparkling wines			UHPLC-MS/MS	0.03 µg/L	Nylon membrane filter 0.2 µm	Tudela et al. (2016)
N-acetil Serotonine	Neuronal tissues and cell culture media	LC-MS-MS quantification of NACSERO in neuronal tissues and cell culture media	Pajkovic et al.				
	Sparkling wines			UHPLC-MS/MS	0.17 µg/L	Nylon membrane filter 0.2 µm	Tudela et al. (2016)
5-hidroxi-Triptophan	Amniotic fluid	Cleanert PEP–2 (6 mL/200 mg) SPE column	1 mL0.5% acidifiedMeOH followed by 1 mL MeOH	GC–MS	0.36 µg/L (LOD)		Shi et al. (2017)
	Vinos espumosos			UHPLC-MS/MS	0.36 µg/L	Nylon membrane filter 0.2 µm	Tudela et al. (2016)
	Plasma, cerebrospinal fluid and tissue		1.0 mol / l acetic acid that contained ascorbic acid	HPLC-FD	Signal-to-noise ratio of 3 for 5-HTP at concentrati		Kema et al. (2001)

					ons of 17 nmol/L.		
3-IAA	Wheat extracts	C18 SPE cartridges (3 mL, 500 mg) from Varian	1 mL 80% MeOH	Solid-phase extraction and liquid chromatography–electrospray tandem mass spectrometry	0.005 µg/mL		Hou et al. (2008)
Tryptamine	Cerebroespinal fluid	Polypropylene 1 mL SPE columns secured by glass-fiber frits	5% acetic acid in MeOH	HPLC-FD	60.3 nmol/L	Membrane filters	Lulinski et al. (2017)
	Tuna			UHPLC-HILIC	0.114-0.115 ppm	Syringe filters Titan-2 17 mm, 0.45 µm	Randy et al. (2011)
	Fish	STRATA X cartridge	2 + 2 mL of a mixture MeOH/acetic acid (99:1, v/v)	LC- MS/MS-FD	0.15 mg/kg	13 mm/ 0.45 µm (Whatman No. 1 paper)	Sagranti et al. (2012)
Triptofol	Cerveza		ACN/water/formic acid	RP-HPLC			Li et al. (2008)
	Miniferm entation in Wine Yeast			HPLC-FD	11 ng/mL	0.2 µm filters	Zupan et al. (2012)

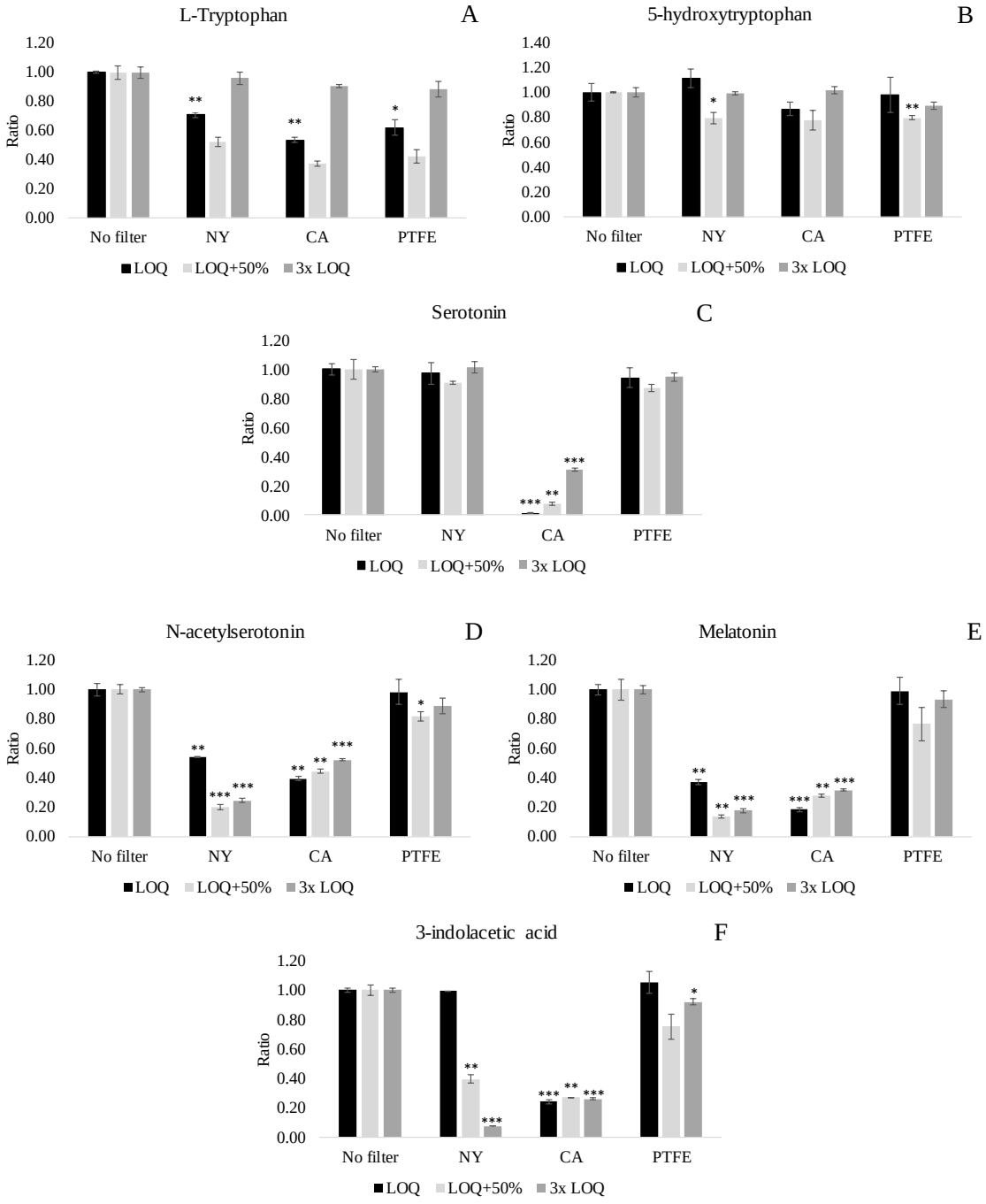
Table 3A. Indolic compounds related with MEL pathway found in commercial beers. Concentrations are expressed in ng/mL (*n*=3)

Beer	L-tryptophan	5-hydroxytryptophan	Serotonin	N-acetylserotonin	Melatonin*
Alhambra tradicional	1890.85 ± 20.34	0.28 ± 0.00	6.77 ± 0.10	0.06 ± 0.00	29.2 ± 1.27
Ámbar	3648.60 ± 36.70	0.20 ± 0.00	3.97 ± 0.10	0.14 ± 0.00	13.5 ± 0.00
Amstel	3510.67 ± 80.38	0.42 ± 0.02	8.38 ± 0.10	0.26 ± 0.00	23.7 ± 0.57
Buckler 0.0	4163.76 ± 66.63	0.46 ± 0.02	22.35 ± 0.32	0.36 ± 0.00	21.7 ± 0.99
Budweiser	3832.94 ± 83.81	0.27 ± 0.01	6.01 ± 0.12	0.15 ± 0.00	15.7 ± 0.71
Carlsberg	3621.13 ± 25.86	0.32 ± 0.01	5.43 ± 0.23	0.28 ± 0.01	18.4 ± 2.12
Corona	4535.70 ± 67.84	0.30 ± 0.01	1.32 ± 0.01	0.19 ± 0.00	13.5 ± 0.42
Cruzcampo	6078.06 ± 36.93	0.35 ± 0.03	11.48 ± 0.22	0.29 ± 0.00	27.15 ± 0.21
Cruzcampo cruzial	4108.09 ± 9.42	1.05 ± 0.02	7.14 ± 0.12	0.31 ± 0.01	18.8 ± 1.13
Desperados	4432.98 ± 398.26	0.33 ± 0.03	1.63 ± 0.17	0.25 ± 0.02	25.6 ± 2.83
Estrella Damm	3744.20 ± 161.19	0.27 ± 0.01	1.81 ± 0.06	0.11 ± 0.00	10.85 ± 0.78
Estrella Galicia	4559.18 ± 75.47	0.22 ± 0.00	7.15 ± 0.07	0.21 ± 0.00	16.3 ± 0.57
Guinness	4767.53 ± 31.78	0.36 ± 0.00	0.99 ± 0.01	0.22 ± 0.00	17.7 ± 0.71
Heineken	3938.52 ± 10.78	0.83 ± 0.00	8.85 ± 0.02	0.35 ± 0.00	21.15 ± 0.21
Mahou 5 estrellas	1102.37 ± 29.52	0.17 ± 0.01	10.64 ± 0.20	0.06 ± 0.00	20.2 ± 0.99
Murphy´s Irish Red	5935.79 ± 10.56	0.73 ± 0.01	16.23 ± 0.90	0.21 ± 0.01	19.1 ± 1.84
Paulaner	1624.19 ± 26.50	0.48 ± 0.02	2.09 ± 0.05	0.39 ± 0.00	10.8 ± 0.28
San Miguel	348.08 ± 5.51	0.15 ± 0.01	7.60 ± 0.04	0.02 ± 0.00	27.85 ± 1.34
Voll-Damm	6534.82 ± 378.16	0.34 ± 0.03	2.35 ± 0.00	0.21 ± 0.01	9.95 ± 1.06

Table 3B. L-tryptophan (L-TRP) derived compounds belonging to different pathways no related with melatonin (MEL). Concentrations are expressed in ng/mL (*n*=3).

Beer	3-IAA	TRYP	TOL	L-TRP EE
Alhambra tradicional	82.31 ± 0.09	18.47 ± 0.07	1511.33 ± 23.95	0.32 ± 0.00
Ámbar	65.99 ± 0.95	12.57 ± 0.16	293.48 ± 5.98	1.02 ± 0.01
Amstel	7.93 ± 0.03	24.23 ± 0.29	116.78 ± 1.05	0.73 ± 0.01
Buckler 0.0	11.72 ± 0.00	2.64 ± 0.06	51.72 ± 1.22	0.05 ± 0.00
Budweiser	25.94 ± 0.62	31.14 ± 0.75	104.12 ± 2.44	1.42 ± 0.01
Carlsberg	20.09 ± 0.58	23.62 ± 1.26	366.26 ± 15.71	0.49 ± 0.03
Corona	25.05 ± 0.61	9.15 ± 0.15	82.17 ± 1.44	0.44 ± 0.01
Cruzcampo	66.55 ± 0.16	29.77 ± 0.05	163.04 ± 0.38	0.74 ± 0.00
Cruzcampo cruzial	15.45 ± 0.48	36.59 ± 0.49	149.14 ± 5.26	0.81 ± 0.03
Desperados	143.42 ± 3.11	20.51 ± 1.71	139.01 ± 8.43	0.49 ± 0.04
Estrella Damm	35.62 ± 0.06	14.81 ± 0.58	58.81 ± 0.99	0.89 ± 0.03
Estrella Galicia	63.13 ± 0.22	16.90 ± 0.23	106.42 ± 1.09	0.47 ± 0.01
Guinness	7.68 ± 0.06	38.09 ± 0.19	81.23 ± 0.01	1.52 ± 0.00
Heineken	7.79 ± 0.01	19.26 ± 0.24	97.26 ± 1.23	0.31 ± 0.00
Mahou 5 estrellas	100.81 ± 0.34	16.54 ± 0.64	1354.27 ± 22.22	0.61 ± 0.02
Murphy´s Irish Red	18.13 ± 0.53	34.62 ± 1.68	182.15 ± 2.61	0.58 ± 0.03
Paulaner	11.82 ± 0.20	31.86 ± 0.08	819.16 ± 1.58	1.48 ± 0.04
San Miguel	109.06 ± 0.31	25.75 ± 0.29	1892.61 ± 20.60	2.57 ± 0.01
Voll-Damm	40.73 ± 1.24	38.41 ± 2.79	61.42 ± 3.39	2.04 ± 0.16

Figure 1. Filters results for the indolic compounds at different concentration (LOQ, LOQ+50%, 3x LOQ).



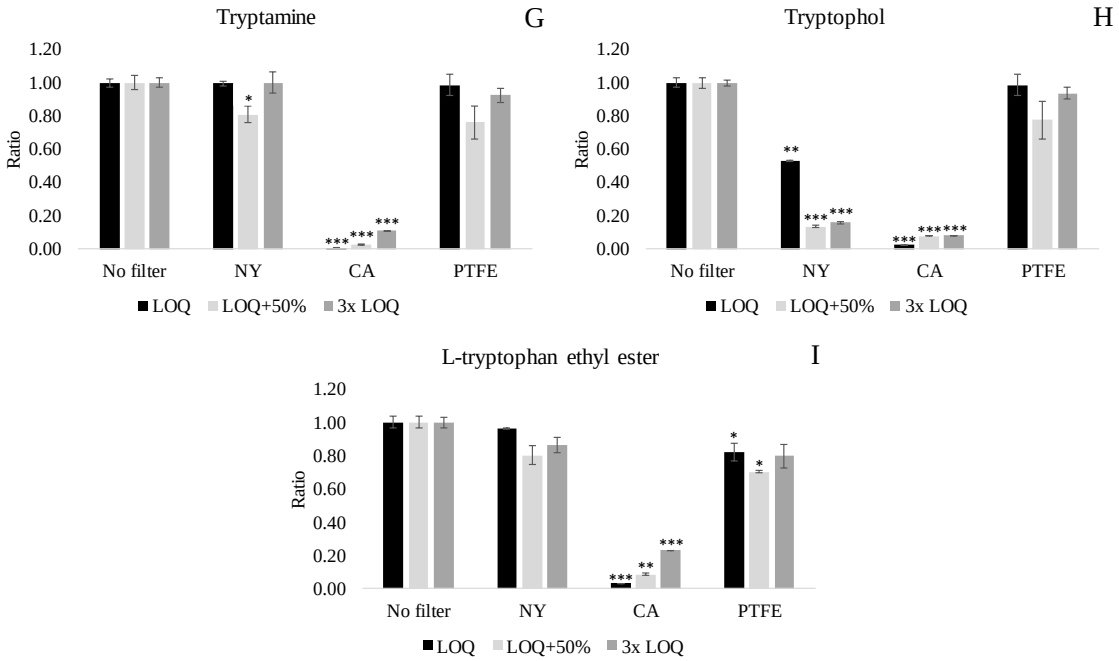
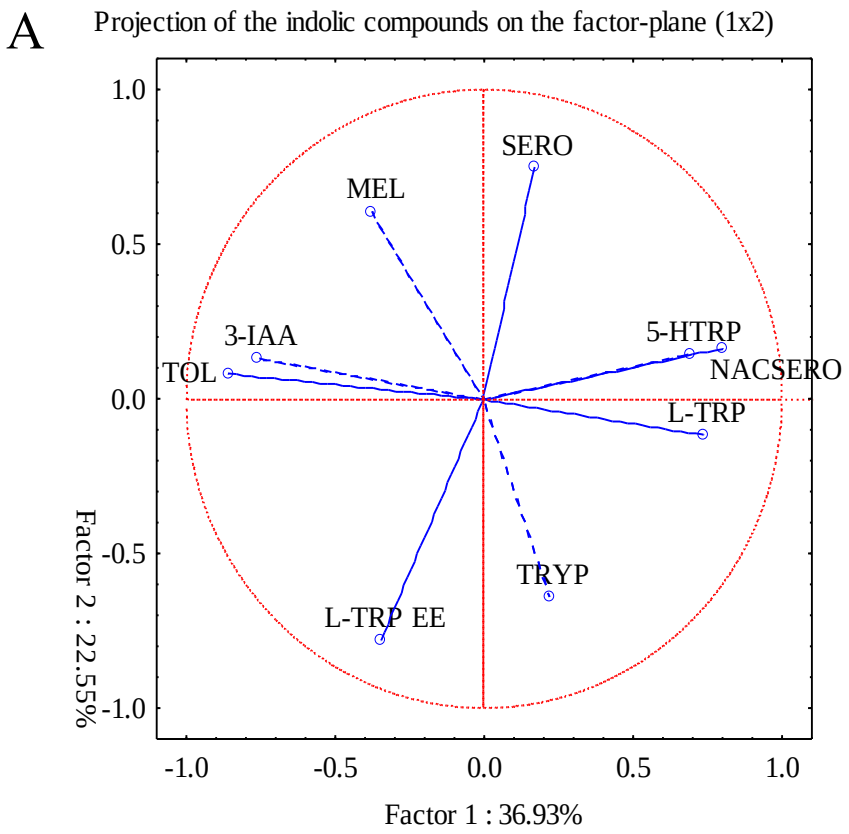
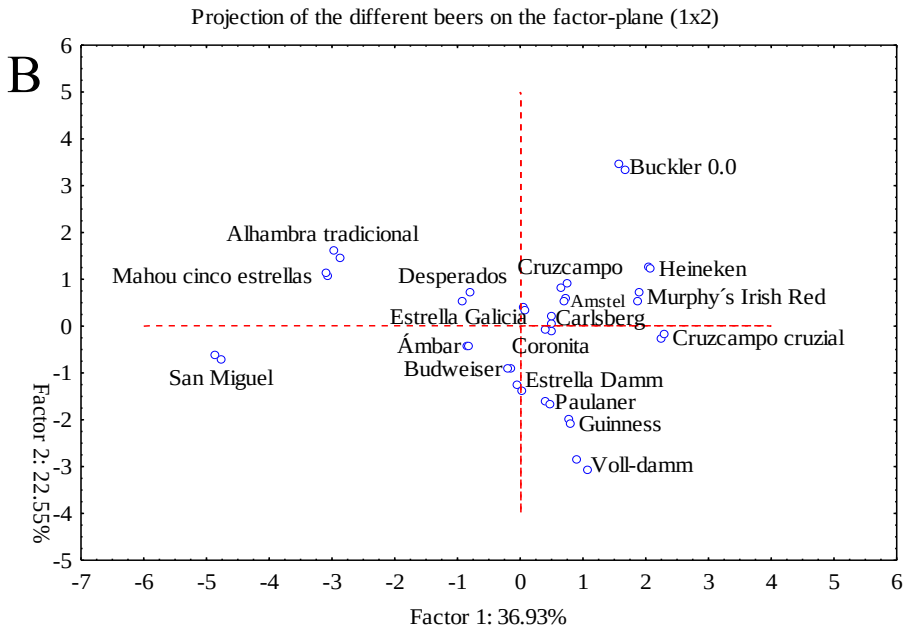

Tryptamine
G
Tryptophol
H
Ratio
1.20
1.00
0.80
0.60
0.40
0.20
0.00
No filter
NY
CA
PTFE
LOQ
LOQ+50%
3x LOQ
L-tryptophan ethyl ester
I

Figure 2. Principal Component Analysis (PCA) performed on indolic compounds (A) and commercial beers (B).





CAPÍTULO 6 / CHAPTER 6

Fernández-Cruz, E., Cerezo López, A.B., Cantos-Villar, E., Richard, T., Troncoso, A. M., García-Parrilla, M.C.

EFECTO DE DIFERENTES ESTILBENOS PROCEDENTES DE LA VID (*Vitis vinífera*) EN LA INHIBICIÓN DE LA FOSFORILACIÓN DEL VEGFR-2

EFFECT OF STILBENES DERIVED FROM *VITIS SPP* ON THE INHIBITION OF VEGFR-2 PHOSPHORYLATION

SUBMITTED TO MOLECULAR NUTRITION & FOOD RESEARCH

Effect of stilbenes derived from *Vitis spp* on the inhibition of VEGFR-2 phosphorylation

Fernández-Cruz, E.[1], Cerezo, A.B.[1], Cantos-Villar, E.[2], Richard, T[3], Troncoso, A.M[1], García-Parrilla, M.C[1]*.

[1]Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal. Facultad de Farmacia. Universidad de Sevilla. C/ Profesor García González 2, 41012, Sevilla, Spain

[2]Instituto de Investigación y Formación Agraria y Pesquera (IFAPA), Centro Rancho de la Merced, Consejería de Agricultura, Pesca y Desarrollo Rural (CAPDA), Junta de Andalucía. Ctra. Trebujena, Km 2.1, 11471 Jerez de la Frontera, Spain.

[3]University of Bordeaux, ISVV Bordeaux-Aquitaine, 71 Avenue Edouard Bourleaux, 33883 Villenave d'Ornon Cedex, France.

*Corresponding author. Tel.: +34 95 4556759.

•E-mail address: mcparrilla@us.es (M.C. García-Parrilla)

Abbreviations:

Akt, protein kinase B; BCA, bicinchoninic acid; DMSO, dimethyl sulfoxide; EGM-2, endothelial cell growth medium-2; eNOS, endothelial nitric oxide synthase; EGCG, epigallocatechin gallate; HUVEC, human umbilical vein endothelial cells; NO, nitric oxide; PLCγ1, phospholipase gamma 1; RIPA, radioinmunoprecipitation assay buffer; TBST, Tris-buffered saline with Tween® 20; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor 2.

Keywords: Antiangiogenic, Astringin, HUVEC, Stilbenes, VEGF

ABSTRACT

Scope: Stilbenes are phenolic compounds present in different higher plant families which have shown different biological activities such as antioxidant properties, anti-tumoral and anti-atherosclerotic effect, among others. Angiogenesis is a key process involved in both cancer and cardiovascular diseases, being the vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 the main triggers. Certain polyphenol compounds such as flavonoids have shown to potently inhibit VEGF and consequently, angiogenesis. Therefore, the present work aims to evaluate the potential effect of stilbenes on the inhibition of VEGF and their subsequent effect on the downstream signalling pathway (PLCγ1, Akt and eNOS).

Methods and Results. The activation of VEGFR-2 was study through ELISA assay while the phosphorylation of intracellular downstream proteins PLCγ1, Akt and eNOS was tested by western-blot. On one hand, astringin, pallidol and ω-viniferin showed the lowest IC_{50} values (2.90, 4.42 and 6.10 μM, respectively) against VEGFR-2 activation. On the other hand, PLCγ1 phosphorylation was inhibited by ε-viniferin, astringin and ω-viniferin, while Akt and eNOS activation was enhanced only by ε-viniferin and ω-viniferin.

Conclusion: These data demonstrate for the first time that stilbenes such as astringin, pallidol, ω-viniferin and ε-viniferin show anti-angiogenic properties, being potential anti-VEGF ingredients in food and beverages. In addition, ε-viniferin and ω-viniferin possess potential anti-angiogenic effect likely preventing the side effects caused by anti-VEGF drugs on hypertension.

1. INTRODUCTION

Stilbenes are secondary metabolites classified as non-flavonoid polyphenols, with a monomeric structure comprised by two aromatic rings joined by an ethylene ring [1]. These compounds derive from the amino acid phenylalanine via phenylpropanoid pathway, through the enzyme stilbene synthase [2] and are synthesized by different higher plant families such as *Leguminoseae*, *Pinaceae* and *Vitaceae* among others [3]. The most representative compound of stilbenes family is the resveratrol, which is found in food such as cranberries, peanuts, pistachios or chocolate, but table grapes and red wine are the main source on its dietary intake [4, 5]. Besides resveratrol, other stilbenes, such as astringin, piceatannol, pallidol, ε-viniferin and hopeaphenol, have also been described in grapes and wines (Table 1) [6-19].

Moreover, ε-viniferin is not only present in diet sources but also in grape cane, grapevine and root extracts of *Vitis vinifera* as well as other stilbenoids compounds such as ω-viniferins, *r*-viniferin, *r2*-viniferin and ampelopsin A, which are currently being studied for their promising biological effects [20–24] and currently investigated as an alternative to SO_2 in winemaking [25, 26].

Resveratrol has shown antioxidant, anti-inflammatory, antidiabetic, neuroprotective, antiaging, anti-cancer and cardioprotective effect [5, 27–30]. Regarding its cardioprotective effect, resveratrol has proved to reduce the formation of atherosclerotic plaques and restores flow-mediated dilation in rabbits fed on high-cholesterol diet [31]. Resveratrol supplementation in mice also delayed spontaneous mammary tumour development and reduced metastasis [32]. Additionally, pterostilbene, a resveratrol methoxylated monomer, has also shown antioxidant properties [33], reducing blood pressure [34], improving cardiac function [35] and inhibiting effect on aortic vascular smooth muscle cells growth [36]. Besides, piceatannol has demonstrated an inhibitory effect on the proliferation and migration of human aortic smooth muscle cells [37].

Angiogenesis, which involves the formation of new capillary vessels from pre-existing ones [38], plays a crucial role on both cancer and cardiovascular diseases. It drives tumor cell growth as well as the development and destabilization of atherosclerotic plaques [39, 40]. The most pro-angiogenic factor involved in this process is the vascular endothelial growth factor (VEGF) [41] by binding to its receptor VEGFR-2, which is the main mediator of the proliferation, migration, survival and permeability process on endothelial cells [42]. In fact, the inhibition of VEGF is an objective for current pharmacological therapies against cancer, being recently developed and approved the use of antibodies anti-VEGF such us Avastin®, among others, for the treatment of colon, lung, breast, kidney and liver cancer [43, 44]. However, their lengthy treatment causes serious side effects, increasing the risk of hypertension, since anti-VEGF drugs inhibit also nitric oxide (NO) production (a potent vasodilator) as a consequence of VEGF signaling inhibition [45, 46].

Recently, it has been reported that VEGF is the key molecular target for certain polyphenol compounds, such as epigallocatechin gallate (EGCG), procyanidin oligomers, quercetin, among others, which potently inhibit VEGF signaling and angiogenesis at physiological concentrations (IC_{50}: 0.08-1 μM) [47, 48]. Additionally, EGCG and tetrameric procyanidin showed that while inhibited phospholipase gamma 1 (PLCγ1), which is the principal regulator of the cell proliferation, increased activation (phosphorylation) of the endothelial nitric oxide synthase (eNOS), via protein kinase B (Akt), which might still induce NO bioavailability [47]. These data show that these bioactive compounds might be a promising alternative for the prevention of cancer and cardiovascular diseases, reducing angiogenesis induced by VEGF while avoid the inhibition of eNOS, and the subsequent hypertension risk caused by the drugs treatment [49].

Resveratrol has been the unique stilbene compound evaluated for the inhibition of VEGF-induced VEGFR-2 activation, which showed a weak inhibitory effect (24% of inhibition at 50 μM) [48]. However, it is necessary to explore the inhibitory effect of the different compounds of this family

of polyphenols, since different substituents on the phenolic rings (total OH, catechol, methyl and glycosyl groups) affect to their potential inhibitory effect [48]. Therefore, the aim of this work is to evaluate the potential anti-VEGF effect of twelve different stilbene compounds present in food, including monomers, dimers and tetramers, and whether they are also able to active eNOS on endothelial cells.

2. MATERIAL AND METHODS

2.1. Cell culture

Human Umbilical Vein Endothelial Cells (HUVEC) and the Endothelial Cell Growth Medium-2 (EGM-2) was provided by Lonza (Slough, UK). HUVEC were cultured at 37 ºC in an atmosphere at 5% CO_2 between passages 4 and 5 in 6-well plates until be completely confluent.

2.2. Stilbenes extraction and purification

Figure 1 shows the chemical structure of the stilbenes included in the present work: resveratrol, piceid, ampelopsin A, astringin, ε-viniferin, hopeaphenol, isohopeaphenol, pallidol, piceatannol, pterostilbene, rhapontin, *r*-viniferin (vitisin B), *r2*-viniferin (vitisin A) and ω-viniferin. Piceatannol and pterostilbene standards were purchased by Sigma-Aldrich (Steinheim, Germany). The rest of stilbenes were extracted and purified from a grapevine raw shoot following the method described by Biais et al. [24], except astringin and rhapontin that were extracted based on Gabaston et al. method [50]. Each compound was diluted with dimethyl sulfoxide (DMSO) and storage at -20ºC until HUVEC treatment.

2.3. Determination of VEGFR-2 phosphorylation by ELISA

Vehicle control (≤ 0.1% DMSO) and VEGF ($VEGF_{165}$, R&D Systems, Minneapolis, MN, USA) at 25 ng/mL concentration (≤ 0.1% DMSO) were used as negative and positive controls, respectively. Following the previously described plausible molecular mechanism for polyphenols

inhibition of VEGF [47], stilbenes were incubated for 5 minutes with VEGF in Endothelial Basal Medium (EBM) at different concentrations (1 µM - 50 µM, DMSO final concentration < 0.1 %). Subsequently, confluent HUVECs were treated with the mix stilbene-VEGF for 5 min by duplicate. HUVECs were then lysed with radioinmunoprecipitation assay (RIPA) buffer and subsequently centrifuged at 4 ºC, 13000 rpm for 10 minutes. Finally, a bicinchoninic acid assay (BCA) was performed to determine protein content in the supernatant.

A PathScan Phospho-VEGFR-2 (Tyr1175) sandwich ELISA kit (Cell Signalling Technology, Danvers, MA, USA) was used to measure the phosphorylated VEGFR-2 levels in the lysates, following the manufacturer´s instruction. Afterwards, half maximal inhibitory concentration (IC_{50}) with confidence intervals for each stilbene was determined with the GraphPad Prism software v. 6.00 (GraphPad Software, La Jolla California USA). Each sample was also analysed by duplicate.

2.4. Western Blot analysis for PLCγ1, Akt and eNOS

Compounds with the highest anti-VEGF effect were evaluated for their potential effect on the modulation of the activity of the dowmstream signalling proteins PLCγ1, Akt and eNOS. The experimental conditions were the same that the above explained (section 2.3), including in addition the evaluation of the compounds effect alone. All compounds were tested by duplicate. The protein lysates were mixed with a sample buffer (LDS, NuPAGE) and a reducing agent (DTT, NuPAGE) before heating them for 10 minutes at 70ºC to denature proteins. Subsequently, electrophoresis was performed in a 4-12% Bis-Tris gels (NuPAGE) and then, proteins were transferred into nitrocellulose membranes of 0.2 µM (Bio-Rad). Tris-buffered saline with Tween® 20 (TBST) was mix with bovine serum albumin (BSA) to a final concentration of 5% for blotting the membranes before the addition of antibodies directed against phospho-PLCγ1 (Tyr783), PLC γ1, phospho-Akt (Ser 473), Akt and phospho-eNOS (Ser 1177) and eNOS (Cell Signaling Technology). Membranes were incubated overnight at 4ºC and then, anti-rabbit IgG-

HRP antibody (Cell signalling Technology) in TBST+BSA (5%) was added to incubate membranes for 1h at room temperature. Image analysis of bands was performed on an Amershan Imager 600 station (GE Healthcare live sciences, Marlborough, MA, USA) after treating membranes with SuperSignal West Pico chemiluminescent substrate (Therno Scientific, Hitchin, UK). Samples were analysed by triplicate.

2.6. Statistical analysis

GraphPad Prism software V.6.00 (GraphPad Software, La Jolla California USA) was used to determine significant differences between samples through Student´s t-test.

3. RESULTS & DISCUSSION

3.1. Inhibition of VEGFR-2 activation by stilbenes

It has been already demonstrated that certain polyphenols inhibit VEGFR-2 activation by tightly binding to VEGF and avoiding, as a consequence, its binding to the receptor [47]. More than fifty polyphenols compounds from different families have been tested for its anti-VEGF effect, however, resveratrol was the only stilbene reported, showing a weak inhibition of VEGFR-2 phosphorylation (24% at 50 μM). Since different structural features affect the potential inhibition of VEGF, in the present work, twelve different stilbenes, including monomers (astringin, piceatannol, pterostilbene and rhapontin), dimers (ampelopsin A, ε-viniferin, pallidol and ω-viniferin) and tetramers (hopeaphenol, isohopeaphenol, r-viniferin and r2-viniferin) were assessed for the first time on the potential anti-angiogenic effect of this family of polyphenols (Table 2).

3.1.1. Monomers

Piceatannol has a quite similar structure as resveratrol with an additional OH group forming a catechol group, which enhance the inhibition of the VEGFR-2 phosphorylation, showing a 3.5-

folds higher inhibitory effect than resveratrol (IC_{50} = 37.70 µM). Similarly, the presence of a catechol group on flavonoids such as luteolin, orobol or quercetin showed an enhanced inhibitory effect against VEGFR-2 activation [51]. Piceatannol has been previously reported to reduce the VEGF expression from cancer cells, indirectly preventing the angiogenesis [52]. However, this is the first time that piceatannol prove to directly inhibit angiogenesis on endothelial cells.

Astringin, which is the piceatannol glucoside (Figure 1), was the stilbene tested that exhibited the highest inhibitory activity against VEGFR-2 phosphorylation (IC_{50} = 2.90 µM), showing a 14-folds more effectiveness against the phosphorylation of VEGFR-2 than its aglycone piceatannol (IC_{50} = 37.70 µM). In contrast, piceid, which is the main natural resveratrol glucoside form, did not showed significant differences on the inhibition of VEGFR-2 phosphorylation compared with resveratrol (28% and 24 % respectively, at 50 µM) [51]. Additionally, Cerezo et al. (2015) previously showed that different glycosylated forms of quercetin were completely ineffective in inhibiting the VEGFR-2 phosphorylation [51] compared with quercetin (IC_{50} = 0.754 µM). In the case of stilbenes, the presence of the catechol group in one of the rings of the astringin and a 3-*O*-glucoside group seems to be the structural combination that higher enhance the anti-VEGF activity. Therefore, our present results prove for the first time that a glycoside polyphenol can be also a good candidate for anti-VEGF activity.

Considering the highest astringin concentration reported in red wine (38.1 mg/L, Table 1) and the bioavailability data for related stilbenoids (piceatannol, pterostilbene and resveratrol 50%, 80% and 30% of bioavailability, respectively) [53, 54], since there is no available data about astringin bioavailability, between 2 and 4 glasses of red wine (medium glass of wine = 150 mL) would be enough to reach an active astringin concentration in plasma (3.4-4.5 µM). Since alcohol content limits wine consumption for a healthy diet, food supplements would be a good option to reach an astringin active concentration in plasma without the alcohol limitation.

The other two monomers tested, pterostilbene and rhapontin, present methyl groups on their structures. Pterostilbene (3',5'-dimethoxy-resveratrol) is a result of a double methylation of the resveratrol in positions 3' and 5'. Although pterostilbene caused a low inhibition activity against VEGR-2 phosphorylation at 50 μM (40 %), was more effective than resveratrol (24 %). In flavonoids, methyl groups on the B-ring strongly diminished the bioactivity against VEGF phosphorylation [51]. In stilbenes, it seems that the presence of methyl groups allows certain bioactivity against the phosphorylation of VEGFR-2. On the other hand, rhapontin was completely ineffective at 50 μM. It presents a methyl group on 4' position and a 3-O-glucosyde group. In this case, it seems that the presence of both glycoside and methyl groups strongly diminished the effect against the phosphorylation of VEGFR-2.

3.1.2. Dimers

The order of anti-VEGF potency for the dimers of stilbenes were as follows: pallidol (IC_{50} = 4.42 μM) > ω-viniferin (IC_{50} = 6.10 μM) > ε-viniferin (IC_{50} = 18.84 μM). Ampelopsin A was completely ineffective at 50 μM. ε-viniferin has previously showed anti-proliferative effect (at 10 μM) on vascular smooth muscle cells [55]. However, this is the first time that ε-viniferin and ω-viniferin have shown anti-angiogenic activity by inhibiting VEGF. Comparing the anti-VEGF activity of stilbenoids dimers with procyanidins dimers previously reported [51], the former present higher effectiveness (IC_{50} = 4.42 – 18.84 μM, IC_{50} = 52.58 μM, respectively). The reactivity differences between stilbene dimers could be related to their three-dimensional structures. For example, ω- and ε-viniferin have the same plane structure and differ only by the orientation of their phenol rings. Cerezo et al. (2015) have demonstrated that flavonoids with near-planarity on their structure such as quercetin or myricetin displayed potent anti-VEGF activity [51], since near-planar structures have been shown to more easily enter hydrophobic pockets in proteins [56, 57]. In addition, the complexity of the ampelopsin A structure might not

enter in the pocket of the VEGF molecule, making it completely ineffective [47]. Structure-activity relationship studies are needed to better understand the properties of these compounds.

3.1.3. Tetramers

High molecular weight stilbenes (tetramers) caused a low inhibition effect (<25%) on VEGFR-2 phosphorylation at 1 μM, (Table 2). These results contrast with that obtained in procyanidins, where the more polymeryzed structures confers the greatest activity against the VEGFR-2 phosphorylation, reaching IC_{50} values of 0.28 μM for the procyanidin tetramer [51].

3.2. Modulation of downstream signalling proteins (PLCγ1, Akt and eNOS) in HUVEC by stilbenes

To check whether the inhibition of the VEGFR-2-activating activity of VEGF by stilbenes also regulates signalling events downstream of pVEGFR-2, the inhibition of PLCγ1 phosphorylation, the first protein activated in response to VEGFR-2 activation and responsible of cell proliferation, by the most potent stilbenes (piceatannol, astringin, pallidol, ε -viniferin and ω-viniferin) was evaluated. In addition, phosphorylation of Akt and eNOS, which are activated later in the VEGF signalling cascade and accountable for NO production were also tested. Two sets of concentrations were assessed depending on the IC_{50} values of stilbenes, to ensure that VEGFR-2 phosphorylation was completely inhibited (10 μM for astringin, pallidol and ω-viniferin; and 50 μM for piceatannol and ε-viniferin).

3.2.1. Phosphorylation of PLCγ1

Pallidol presented IC_{50} values for VEGF inhibition below to 10 μM (Table 2) but had no effect against the phosphorylation of PLCγ1 in presence of VEGF (Figure 2A). Astringin, ε- and ω-viniferins showed a significant inhibition of PLCγ1 phosphorylation mediated by VEGF (86, 55 and 68 %, respectively) being astringin the most effective (Figure 2 C-F). Astringin treatment in presence of VEGF presented a pPLCγ1/PLCγ1 ratio 7-folds lower than VEGF alone (Figure 2C),

while ε-viniferin reached a ratio 3 folds lower (Figure 2E). These data are in agreement with Moyle et al. (2015) [47], who showed that certain polyphenols such as a procyanidin tetramer isolated from apple (dp4) and EGCG from green tea inhibited VEGFR-2 phosphorylation and as a consequence they totally inactivated PLCγ1 phosphorylation at 1 μM. As far as we are concerned, our data show for the first time the potential of certain stilbenes for the inhibition of PLCγ1 (at 10 and 50 μM) and, as a likely consequence, the cell proliferation of endothelial cells.

3.2.2. Phosphorylation of Akt and eNOS

The importance of activating eNOS lies on the fact that anti-VEGF drugs currently used in the cancer treatment of colon, lung, breast, kidney and liver cancer [43, 44] have demonstrated to cause serious side effects, increasing the risk of hypertension, by inhibiting the NO production (a potent vasodilator) as a consequence of VEGF signalling inhibition [45, 46]. Akt is a protein prior activated in the signalling cascade by VEGF that has proved to activate eNOS phosphorylation, among different alternative pathways.

Although ε- and ω-viniferins inhibited VEGFR-2 and PLCγ1 phosphorylation (Table 2 and Figure 2), VEGF-induced phosphorylation of Akt was not inhibited but significantly enhanced by these compounds, both in presence and absence of VEGF (more than twenty-folds higher for ε-viniferin and more than two-folds higher for ω-viniferin) regarding negative control (Figure 3A-D). However, astringin, which inhibited VEGFR-2 and PLCγ1 phosphorylation, did not enhanced the Akt activation (Figure 3A).

Since pAkt is known to activate the eNOS enzyme (peNOS), we evaluated the effects of the stilbenes on eNOS activation. The present data show that VEGF alone increase the peNOS/eNOS ratio (Figure 4). The ε-viniferin increased the peNOS/eNOS ratio 3.2 and 3-folds higher in presence and absence of VEGF, respectively (Figure 4A). Additionally, ω-viniferin, in presence of VEGF, slightly increased the peNOS/eNOS ratio regarding negative control, (Figure 4B).

These data are in agreement with those previously reported for EGCG and dp4 also at 10 μM, which although inhibited VEGFR-2 and PLCγ1 were able to increase or retain the activation of pAkt and peNOS both in presence and absence of VEGF [47]. The hypothesis postulated was that polyphenol compounds were able to activate Akt and eNOS by means of other surface receptors or by directly generating ROS in a receptor-independent fashion since Kim et al. (2007) demonstrated that EGCG activated Akt, eNOS and NO production in BAECs by these possible ways [58]. Similarly, resveratrol has been reported to activate eNOS via AMP-activated protein kinase, estrogen receptors and sirtuin 1 on endothelial cells [49]. A similar unexplored mechanism might be involved in the activation of Akt and eNOS by ε- and ω-viniferins.

The present results reinforce the notion that certain polyphenols are potent VEGF inhibitors but still may induce NO production by increasing phosphorylation of Akt and also eNOS, revealing for the first time that ε- and ω-viniferins show the same trend. Although, ε-viniferin and ω-viniferin are mainly present in grapevine shoot, root and grape cane extracts [25], they are gaining importance due to their promising use in winemaking as an alternative to SO_2 to avoid its adverse effects {Formatting Citation}. Therefore, further research should be conducted to focus on the determination of this mechanism and to evaluate the final concentration of these compounds in the final product within the use of those extracts, and the different winemaking conditions to improve their content in wines.

The others investigated compounds, such as astringin or piceatannol did not showed a significant stimulation of either Akt and eNOS (Figures 3 and 4), although they have demonstrated to be potential inhibitors of VEGF (Table 2). These data demonstrate that not all the polyphenols that inhibit VEGF are able to show the beneficial effect of activating eNOS and likely induce NO production.

4. CONCLUDING REMARKS

This is the first time that stilbenes such as astringin, pallidol, ω-viniferin and ε-viniferin show potential anti-VEGF effect in endothelial cells (most IC_{50} < 10 μM) and subsequent angiogenesis. Additionally, both ε- and ω-viniferins proved to inhibit the downstream phosphorylation of PLCγ1, responsible for cell proliferation, while stimulate the phosphorylation of Akt and eNOS. The present data reveal for the first time that stilbenes such as ω-viniferin and ε-viniferin possess potential anti-angiogenic effect likely preventing the side effect caused by anti-VEGF drugs on NO bioavailability. Therefore, these compounds present good potential for their future exploitation as anti-VEGF ingredients in foods and beverages.

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Declarations

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Conflicts of interest: E. Fernandez-Cruz, A. B. Cerezo, E. Cantos-Villar, T. Richard, A.M. Troncoso and M.C. García-Parrilla declare that they have no conflicts of interest.

Ethical approval: This study does not contain any studies performed by any of the authors with human participants.

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Table 1. Dietary sources of stilbenes derived from resveratrol.

Compound	*Source*	*Amount*	*Reference*
E-Astringin	*Raboso Piave* grape	106.0 ± 5.8 µg/Kg	Flamini et al. (2013)
Trans-astringin	French red wines	N.D.–38.1 mg/L	Vitrac et al. (2002)
	French white wines	N.D.–8.5 mg/L	
	Brazilian red wines	4.35-25.72 mg/L	Vitrac et al. (2005)
	Canadian red wines	0.04-0.35 mg /L	Naugler et al. (2007)
	Italian red wines	N.D.-1.83 mg /L	Buiarelli et al. (2007)
	Italian white wines	N.D.-0.72 mg /L	
	Portuguese red wines	N.D.-35.9 mg /L	Ribeiro de Lima et al. (1999)
	Portuguese white wines	N.D.-15.6 mg/L	
	French red wines	2.5-26.1 mg/L	
Cis-astringin	Italian red wines	N.D.-1.59 mg/L	Buiarelli et al. (2007)
	Italian white wines	N.D.-1.32 mg/L	
	Primitivo grapes	884.2 ± 3.4 µg/Kg	Flamini et al. (2013)
Z-astringin	*Raboso Piave* grapes	101.7 ± 3.3 µg/Kg	
	Primitivo grapes	121.4 ± 1.0 µg/Kg	
Piceatannol	Raboso Piave grapes	41.8 ± 0.5 µg/Kg	
	Primitivo grapes	281.5 ± 10.2 µg/Kg	
Trans-piceatannol	Italian red wines	N.D.-5.22 mg/L	Buiarelli et al. (2007)
	Italian white wines	N.D.-0.59 mg/L	

	Cabernet Sauvignon berries (without seeds)	0.052 mg/kg fw	Bavaresco et al. (2002)
	Monastrell grapes	0.78 ± 0.1 mg/kg fw	Cantos et al. (2003)
	Monastrell wines	208.4 ± 3.6 µg/L	
Pallidol	Raboso Piave grapes	21.7 ± 0.2 µg/Kg	Flamini et al. (2013)
	French red wines	0.5–4.8 mg/L	Vitrac et al. (2002)
	Canadian red wines	0.06-0.40 mg /L	Naugler et al. (2007)
	South African red wines	0.20-9.20 mg /L	Guebailia et al. (2006)
	Primitivo grapes	356.2 ± 2.6 µg/Kg	Flamini et al. (2013)
	French red wines	1.33-2.22 mg /L	Landrault et al. (2002)
	French rosé wines	0.38 mg /L	
Z-ε-viniferin	Raboso Piave grapes	214.6 ± 5.7 µg/Kg	Flamini et al. (2013)
	Primitivo grapes	380.4 ± 7.0 µg/Kg	
E-ε-viniferin	Raboso Piave grapes	592.5 ± 11.6 µg/Kg	
	Primitivo grapes	702.1 ± 3.4 µg/Kg	
Trans- ε-viniferin	North African red wines	0.2-1.2 mg/L	Guebailia et al. (2006)
	Merlot wine	1.20 ± 0.05 mg/L	Amira-Guebailia et al. (2009)
	Cabernet Sauvignon wine	0.69 ± 0.08 mg/L	
	Ksar wine	0.49 ± 0.08 mg/L	
	Amjad wine	0.20 ± 0.04 mg/L	

ε-viniferin	Red wine	0.014 mg/L	Hurtado-Gaitán et al. (2017)
	Brazilian red wines	0.19-4.35 mg/L	Vitrac et al. (2005)
	French red wines	0.10-1.63 mg/L	Landrault et al. (2002)
	French botrytized sweet white wines	0.08-0.17 mg/L	
Cis- ε-viniferin	Merlot	0.30 ± 0.03 mg/L	Amira-Guebailia et al. (2009)
	Cabernet Sauvignon wine	0.35 ± 0.04 mg/L	
	Cuvée du Président wine	0.22 ± 0.06 mg/L	
	Gris d'Algérie wine	0.10 ± 0.02 mg/L	
	Muscat wine	0.38 ± 0.08 mg/L	
	Ksar wine	0.38 ± 0.06 mg/L	
	Amjad wine	0.20 ± 0.05 mg/L	
	Guerrouane wine	1.12 ± 0.09 mg/L	
	Terrale wine	0.13 ± 0.05 mg/L	
Hopeaphenol	South African red wines	0.30-3.80 mg/L	Guebailia et al. (2006)

Footnote: fw: fresh weight; dw: dry weight

Table 2. Table 2. Inhibition of 12 different stilbenes on VEGF phosphorylation induced via VEGFR-2

Compound	Size	Total OH (Position)	Others (Position)	IC50 (μM)
***E*-Astringin**	Monomer	7	Glucoside (3)	2.90 (2.655-3.169)
Pallidol	Dimer	6	---	4.42 (3.839-5.089)
***E*-ω-viniferin**	Dimer	5	---	6.102 (5.039-7.389)
R2-viniferin (Vitisin A)	Tetramer	10	---	8.672 (8.117-9.265)
***E*-ε-viniferin**	Dimer	5	---	18.84 (17.400-20.390)
***E*-Piceatannol**	Monomer	4 (3,5,3',4')	---	39.70 (37.610-41.890)
				Inhibition (%)
Pterostilbene	Monomer	1 (4')	OCH_3 (3, 5)	40.7 (50 μM)
R-viniferin (Vitisin B)	Tetramer	9	---	23.4 (1 μM)
Isohopeaphenol	Tetramer	10	---	16.2 (1 μM)
Rhapontin	Monomer	6 (5, 5')	OCH_3 (4'), Glucoside (3)	Ineffective (50 μM)
(+)-Ampelopsin A	Dimer	6	---	Ineffective (50 μM)
Hopeaphenol	Tetramer	10	---	Ineffective (1 μM)

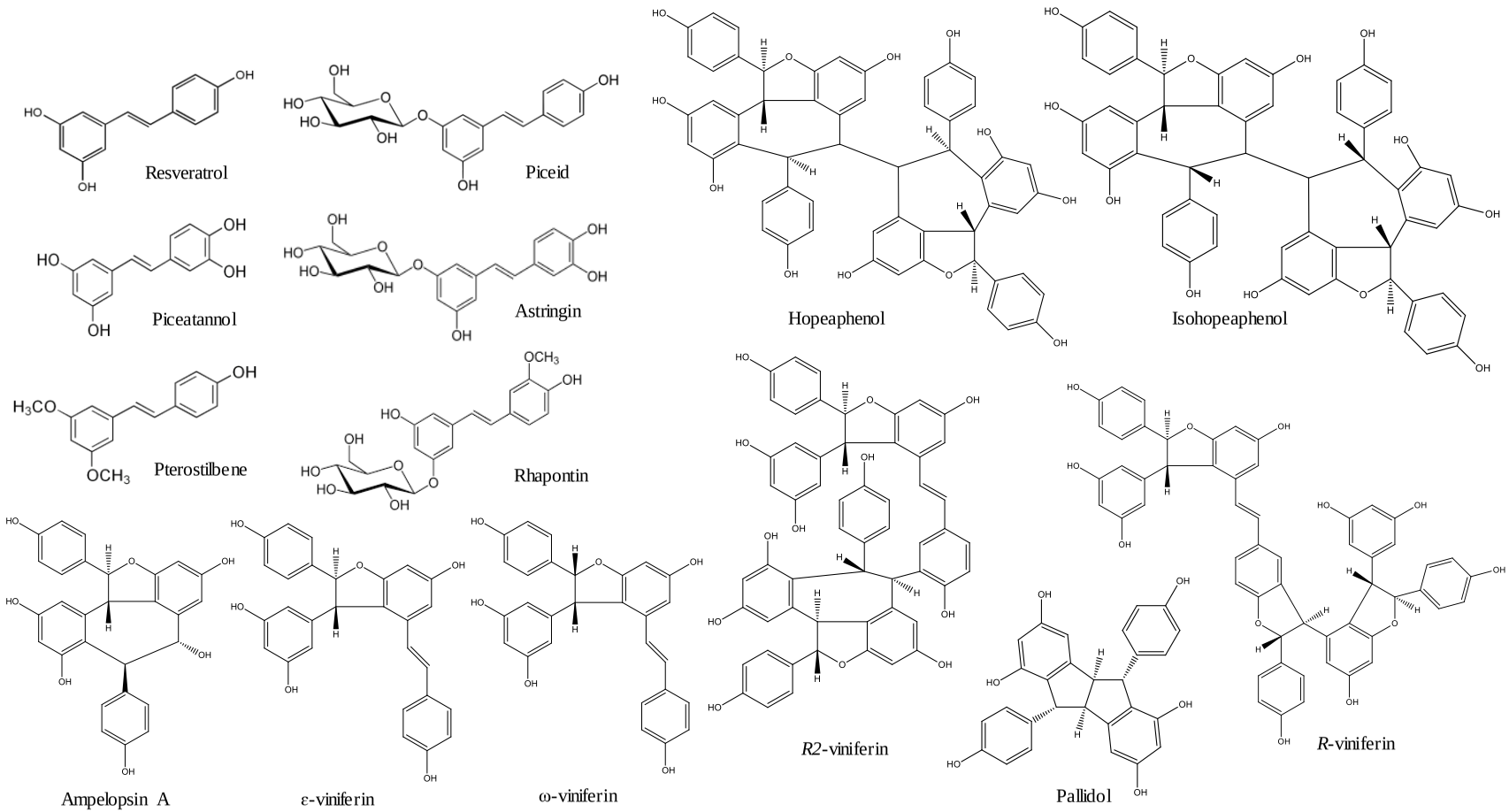

Figure 1. Stilbenes tested on the inhibition of VEGFR-2 phosphorylation in HUVEC.

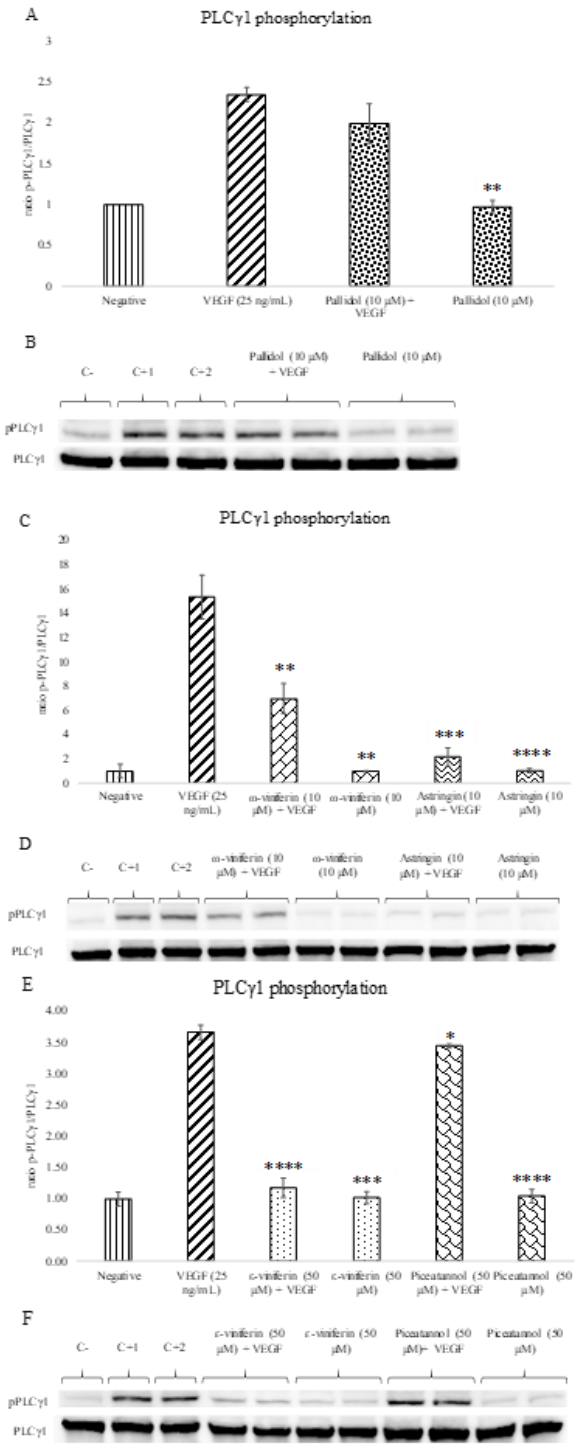

Figure 2. Pallidol caused no inhibition on PLCγ1 phosphorylation (A). Astringin, ω-viniferin (C) and ε-viniferin (E) showed a significant inhibition of PLCγ1 phosphorylation (86, 68 and 55% respectively). Basal medium containing VEGF (25 ng/mL) was pre-incubated with astringin, ω-viniferin, pallidol (10 µM), ε-viniferin and piceatannol (50 µM) separately. HUVECs were subsequently incubated for 10 minutes with the pre-incubated solution. Cell were lysed and proteins were separated on an SDS-PAGE gel and probed with the corresponding PLCγ1 antibodies. Generated bands (B, D, F) and ratios for pPLCγ1/PLCγ1 (A, C, E) are displayed, *$p < 0.05$, **$p < 0.01$, *** p<0.001, ****p<0.0001 versus VEGF positive control. Western blot analysis was performed by triplicate (n=3).

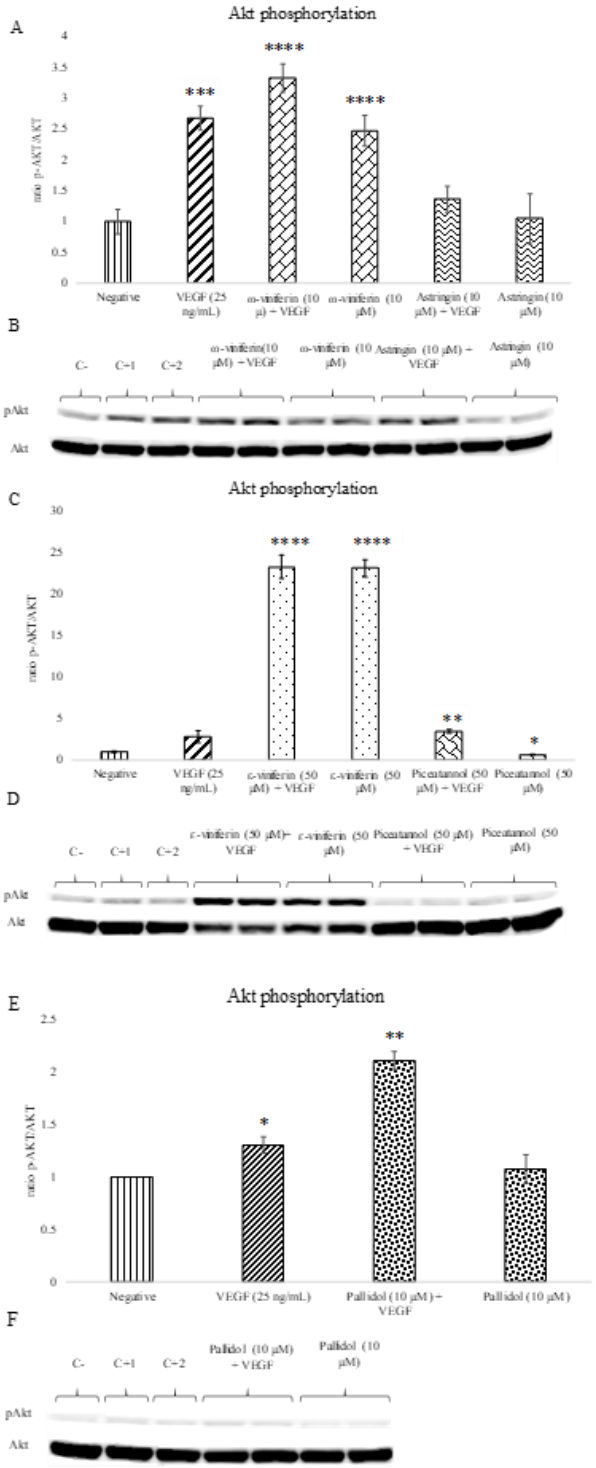

Figure 3. Both ω-viniferin (A) and ε-viniferin (C) did not inhibited, and even enhanced the phosphorylation of Akt. Astringin (A), piceatanol (C) and pallidol (E) caused inhibition of Akt. HUVEC were treated incubated 60 minutes in basal medium containing preincubated VEGF (25 ng/mL) with astringin, pallidol, ω-viniferin (10 µM), ε-viniferin and piceatannol (50 µM) separately. Then, HUVEC were lysed and proteins were separated on and SDS-PAGE gel to be treated with specific Akt antibodies. Western blot bands (B, D, F) and ratios for pAkt/Akt are presented, *$p < 0.05$, **$p < 0.01$, *** p<0.001, ****p<0.0001 versus negative control. Analysis was performed by triplicate (n=3).

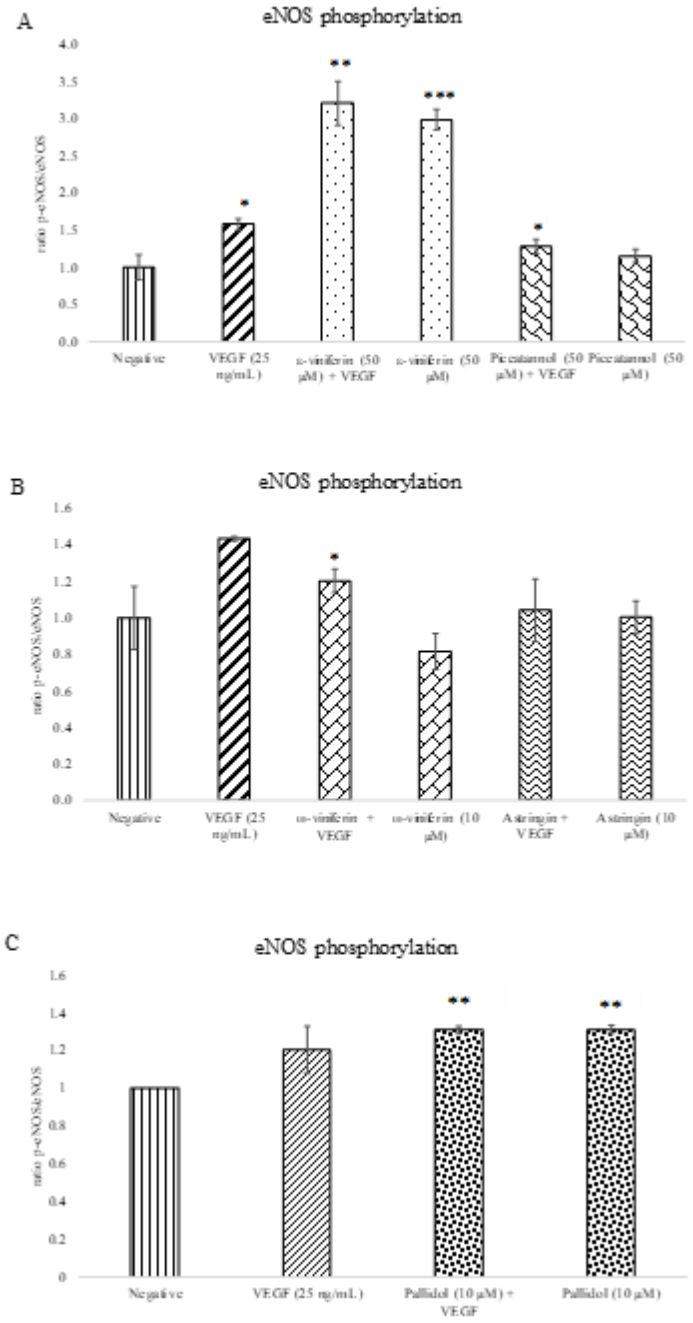

Figure 4. ε-viniferin (A) significantly enhances the phosphorylation of eNOS, while ω-viniferin (B) slightly allows the activation of eNOS. Piceatannol (A), astringin (B) and pallidol (C) inhibited eNOS phosphorylation. VEGF (25 ng/mL) was incubated in basal medium with astringin, pallidol, ω-viniferin (10 µM), ε-viniferin and piceatannol (50 µM) separately. HUVEC were treated 60 min with the mix VEGF/stilbene and the subsequent lysed proteins were separated on an gel (SDS-PAGE) and treated with eNOS antibodies. Ratios for peNOS/eNOS are displayed, *$p < 0.05$, **$p < 0.01$, *** $p<0.001$ versus VEGF positive control. Western blot analysis was performed by triplicate (n=3).

DISCUSIÓN GENERAL

La presente Tesis doctoral se ha centrado fundamentalmente en la identificación y cuantificación de metabolitos derivados del metabolismo del aminoácido L-triptófano durante la fermentación alcohólica (mosto sintético y mosto de uva) y en bebidas comerciales (cerveza). Además, se ha llevado a cabo la determinación de la bioactividad, concretamente en la inhibición frente a la angiogénesis de los estilbenos, que son compuestos derivados del aminoácido aromático fenilalanina y que se producen en el metabolismo de la vid

Esta Tesis doctoral se plantea como el estudio del metabolismo del aminoácido triptófano por parte de las levaduras vínicas con la consiguiente producción de compuestos bioactivos de interés en bebidas fermentadas. Respecto al metabolismo de las levaduras, ya se había descrito la formación de ácido 3 indolacético y del triptofol como metabolitos del triptófano por la ruta de Ehrlich en la fermentación alcohólica (Gil & Gómez-Cordovés, 1986; Hoenicke, Simat, Steinhart, Köhler, & Schwab, 2001). Sin embargo, la presencia de melatonina en vinos se ha puesto en evidencia recientemente (Mercolini et al., 2008), así como su síntesis por parte de las levaduras durante la fermentación alcohólica (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos-Villar, & García-Parrilla, 2011). Esta molécula, de gran interés como compuesto bioactivo, desempeña en mamíferos funciones relacionadas con el ciclo del sueño/vigilia en el ritmo circadiano (Reiter, 1993).

El análisis de la melatonina en matrices alimentarias resulta complejo, debido a sus características anfipáticas, su bajo contenido en alimentos a niveles traza (ng/mL) y su rápida reacción con las especies reactivas de oxígeno por su carácter antioxidante (Mercolini, Mandrioli, & Raggi, 2012; Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, & García-Parrilla, 2011; Tan, Zanghi, Manchester, & Reiter, 2014). Por ello, la melatonina precisa de técnicas lo suficientemente sensibles y fiables para su determinación.

Lás técnicas de radioinmunoensayo (ELISA) para cuantificar melatonina han sido ampliamente utilizadas en matrices alimentarias (de la Puerta et al., 2007; Garcia-Moreno, Calvo, & Maldonado, 2013b; Iriti, Rossoni, & Faoro, 2006), pero el porcentaje de falsos positivos por interferentes que reaccionen con los anticuerpo del ELISA hace que sean poco fiables (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al.,

2011). Por otro lado, las técnicas cromatográficas acopladas a espectrómetros de masas se presentan como la opción más adecuada para el estudio de la melatonina, ya que se han descrito en la bibliografía límites de detección de 0,034 ng/mL, 0,13 ng/mL y 30,16 ng/mL usando un triple cuadrupolo (Gomez, Raba, Cerutti, & Silva, 2012; Kocadağli, Yilmaz, & Gökmen, 2014; Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al., 2011).

Hasta ahora, la mayor parte de los métodos cromatográficos publicados contemplaban el análisis de la melatonina de manera aislada (García-Parrilla, Cantos, & Troncoso, 2009; Gomez, Hernández, Martinez, Silva, & Cerutti, 2013; Russel J Reiter, Tan, Manchester, & Qi, 2001). Sin embargo, este abordaje analítico no permite abarcar el estudio de las posibles vías por las cuales las levaduras sintetizan este compuesto bioactivo. Nuestra hipótesis de partida indicaba que, para obtener información sobre la síntesis de melatonina, es necesario una metodología que nos permita una visión global sobre el metabolismo.

En esta tesis, se desarrolló una metodología capaz de abarcar el estudio de metabolitos relacionados con la melatonina (Capitulo 1), identificando nueve compuestos del metabolismo del triptófano (5-hidroxitriptófano, serotonina, N-acetilserotonina, melatonina, triptamina, triptofol, ácido 3-indolacético, etil ester de triptófano) de manera simultánea por primera vez en un tiempo de análisis corto (15 minutos), con unos límites de detección comprendidos entre 1,3 y 6,7 pg/mL. Estos valores representan un hito analítico, ya que los métodos cromatográficos que comprenden un número elevado de compuestos suelen disminuir la sensibilidad. La validación del método se realizó en un equipo de cromatografía líquida de ultra-alta eficacia acoplada a un espectrómetro de masas de alta resolución (Qexactive). Los valores de linealidad (R^2) fueron de 0.998-1 y los de repetitividad y reproducibilidad inferiores al 2% para todos los compuestos, salvo la serotonina (21%), todos ellos dentro de los valores establecidos por la AOAC.

Además, con el método validado se identificó y cuantificó un compuesto con la misma masa molecular que la melatonina, el etil ester de triptófano. El espectro de masas de este compuesto, fue descrito detalladamente por Gardana et al. (Gardana, Iriti, Stuknytė, De Noni, & Simonetti, 2014). Su m/z (233) coincide con el de la melatonina. Sin embargo, con el método validado se comprobó que la intensidad de uno de los fragmentos de la melatonina (m/z 216) era mucho más intensa en el etil ester de triptófano, siendo este su ion confirmatorio en su análisis. Debido a este hecho, es posible que la detección de

isómeros de melatonina en otros equipos menos sensibles al Qexactive y reportados previamente en la bibliografía, diera lugar a errores de interpretación. En el método validado también es un hito el poder diferenciar inequívocamente ambas moléculas en una misma muestra, ya que ambas aparecen durante la fermentación alcohólica (Vigentini et al., 2015).

Tras validar un método capaz de detectar hasta niveles trazas (ng/mL) diferentes compuestos derivados del triptófano, se realizó un estudio para comprobar la influencia del posible efecto matriz en la cuantificación de estos compuestos durante diferentes periodos de almacenamiento (Capítulo 2). Además, el tratamiento de la muestra también es importante para evitar pérdidas de metabolitos antes de realizar el análisis definitivo. Se conoce que el triptófano, aunque es estable, puede disminuir su concentración en una solución con otros aminoácidos (Friedman & Cuq, 1988). Por otro lado, la melatonina reacciona rápidamente con los radicales de oxígeno por lo que suele ser una molécula muy reactiva Reiter et al., 2001), y el ácido 3-indolacético se analiza en condiciones de oscuridad para que no se vea afectado por la luz..

Los resultados mostraron que los compuestos son más estables en la cerveza que en cualquiera de las otras matrices (vino, mosto sintético fermentado y extracto intracelular). Este hecho está causado posiblemente porque la cerveza presentó una concentración inicial de compuestos mayor que en las otras matrices. Al mismo tiempo, la temperatura de 4ºC parece ser la más indicada para mantener estos compuestos sin cambios significativos durante 1 semana de almacenamiento. Posteriormente, todos los derivados del triptófano sufren cambios que son significativos tras 15 días de almacenamiento, en todas las temperaturas analizadas. Por tanto, se establecería un tiempo de almacenamiento óptimo de 7 días para que los compuestos indólicos con propiedades bioactivas, como el ácido 3-indolacético o la melatonina, se mantuviesen inalterables en el medio.

Una vez validado el método para diferentes matrices, cabe preguntarse: ¿Cuál es el impacto de las levaduras en el metabolismo del triptófano? En primer lugar, se midió la concentración de estos metabolitos en un medio simple (mosto sintético) para evitar posibles interferencias de la matriz (Capítulo 3). Se inocularon cinco cepas de levadura *S.cerevisiae* y dos cepas no-*Saccharomyces (T.delbrueckii* y *M.pulcherrima)*. El análisis a través del método validado permitió identificar por primera vez 5-hidroxitriptófano durante la fermentación alcohólica en todas las cepas de levaduras. La disminución de

este compuesto durante el proceso fermentativo muestra que las levaduras lo consumen, probablemente para formar los derivados de la ruta de la melatonina, como la serotonina. De todas las cepas utilizadas, solamente la Aroma White produjo serotonina durante la fermentación alcohólica, ya que su concentración aumentó a medida que avanzaba la fermentación alcohólica (de 0,67 a 4,60 ng/mL). Este compuesto sí había sido descrito previamente en vinos, a concentraciones superiores en torno a 3 mg/L (Shi et al., 2017). Asimismo la presencia de serotonina es mayor tras el desarrollo de la fermentación maloláctica (Wang, Ye, Zhu, Wu, & Duan, 2014).

Aunque la melatonina pudo ser cuantificada para la mayor parte de cepas, la Red Fruit fue la mayor productora. Además, la cuantificación de melatonina en cepas no-*Saccharomyces* como *T.delbrueckii* y *M. pulcherrima*, resultó de sumo interés pues aún no se había descrito. Tras su análisis, se pudo establecer que la síntesis de melatonina tiene lugar al final de la fase exponencial. Las cepas no-Saccharomyces sintetizan melatonina más tarde debido a la menor velocidad de crecimiento durante la fermentación(Gobert et al., 2017). Al mismo tiempo, se observó que la melatonina no tenía un patrón de síntesis determinado, sino que aparecía y desaparecía del medio a medida que avanzaba la fermentación.

Por otro lado, el triptofol y el ácido 3-indolacético, como se esperaba, se pudieron cuantificar en todas las muestras. Mientras que el triptofol se acumuló en el medio, el ácido 3-indolacético disminuyó su concentración después de una notable producción al principio de la fermentación en todas las cepas de levadura. Mientras que el triptofol sí tiene una función descrita como molécula *quorum sensing* (Chen & Fink, 2006), se desconoce si el ácido 3-indolacético tiene una función en las levaduras, aunque el descenso de su concentración puede estar también provocado por su unión a otras moléculas (Hoenicke et al., 2001).

Tras comprobar el metabolismo del triptófano por parte de las levaduras en un medio sintético, cabía enfrentarse al siguiente reto. ¿Es reproducible en medios complejos? Para dar respuesta a esta pregunta, se seleccionaron aquellas cepas con mejores resultados en la producción de compuestos indólicos bioactivos como melatonina y ácido3-indolacético(*S. cerevisiae* Aroma White, Red Fruit y *T.delbrueckii*) así como una cepa testigo (QA23) cuya síntesis de melatonina se ha descrito previamente (Rodríguez-Naranjo, Torija, Mas, Cantos-Villar, & García-Parrilla, 2012). Además, se realizaron fermentaciones de mostos de uva procedente de diferentes variedades (Corredera,

Chardonnay, Moscatel, Palomino Fino, *Sauvignon Blanc*, Vijiriega y Tempranillo) (Capítulo 4).

Ante todo, se descubrió que el patrón de síntesis de la mayoría de compuestos observado en el mosto sintético se mantenía en los mostos de uva: consumo de triptófano e hidroxitriptófano al principio de la fermentación; acumulación de triptofol en el medio; producción de ácido 3-indolacético con su posterior descenso. Sin embargo, la aparición y desaparición de la melatonina a lo largo de la fermentación es un patrón que no pudo ser reproducido en todas las fermentaciones. La combinación de la cepa Aroma White y el mosto de uva de la variedad Corredera fue la que produjo mayor cantidad de melatonina.

Por otro lado, este experimento permitió por primera vez la cuantificación de N-acetilserotonina en mostos de uva de la variedad Tempranillo, siendo un hito del presente trabajo. Aunque su síntesis fue irregular, permitió verificar que la ruta de la melatonina en levaduras se realiza a través de este intermediario. Pero, ¿por qué estas diferencias entre el mosto sintético y el mosto de uva? Además de la complejidad que este último presenta, las fermentaciones no se realizaron a la misma temperatura. Mientras que la levadura se desarrolla de manera óptima a 28ºC, las fermentaciones en bodegas oscilan entre los 16 y 18ºC. Por tanto, aunque se ha comprobado que la temperatura y el medio no afectan a gran parte de los compuestos, parece ser que en lo concerniente a la ruta de la melatonina pueden verse afectadas por estas diferencias.

El ácido 3-indolacético y el triptofol se sintetizaron en las primeras etapas de la fermentación alcohólica, pero a diferencia del mosto sintético, no alcanzaron concentraciones tan elevadas. Este hecho puede estar relacionado con la menor concentración de triptófano en el mosto de uva (70-80000 ng/mL en variedades blancas), el cual depende de diversos factores como el clima, el suelo o la fertilización (Bouzas-Cid, Falqué, Orriols, & Mirás-Avalos, 2018; Linsenmeier, Löhnertz, & Schubert, 2004; Ruiz-Rodríguez et al., 2017). No obstante, cabe destacar que en dos variedades de uva blanca (Corredera y Palomino Fino) el contenido de ácido 3-indolacético alcanzado en los primeros días de la fermentación alcohólica se mantuvo constante, a diferencia de la disminución significativa observada en otras variedades. Por tanto, no solo la concentración de triptófano afecta a la síntesis de compuestos derivados, sino que la complejidad del medio, en este caso mosto de uva debe, afecta a la síntesis del ácido 3-indolacético.

Para completar el estudio se realizó la determinación de compuestos indólicos en 19 cervezas comerciales producidas por una fermentación llevada a cabo por *S. cerevisiae* (**Capítulo 5**). Debido a que los compuestos indólicos de la ruta de la melatonina se encontraron en concentraciones muy bajas en mostos, especialmente serotonina, N-acetilserotonina y melatonina, las muestras se concentraron 10 veces. Además, tras el tratamiento de muestras se comprobó que los filtros de politetrafluoroetileno mostraron ser los que menos compuestos retenían, mientras que los de nylon y acetato de celulosa afectaban negativamente al contenido de compuestos indólicos en la muestra. Aunque en la bibliografía se han utilizado en alguna ocasión filtros de para el procesado de muestras con melatonina (Gomez et al., 2013), la mayoría de artículos en los que se ha descrito este compuesto han utilizado filtros de nylon (Iriti et al., 2006; Stege, Sombra, Messina, Martinez, & Silva, 2010). Por tanto, sería conveniente que a la hora de determinar compuestos indólicos en cervezas se utilizaran los filtros de politetrafluoroetileno de manera preferente.

Hasta ahora, los estudios acerca del contenido de melatonina en se habían centrado exclusivamente en la melatonina. Por un lado, se demostró que el consumo de cerveza aumentaba el nivel de melatonina en plasma al poco tiempo de haberla consumido, constituyendo una fuente dietética de la misma (Maldonado, Moreno, & Calvo, 2009). Por otro lado, se comprobó que la melatonina estaba presente desde el mosto de cebada, y que además, su síntesis tenía lugar en el mosto tras añadir azúcar al final de la primera fermentación (Garcia-Moreno, Calvo, & Maldonado, 2013a). Sin embargo, en ambos experimentos, la melatonina se cuantificó por el método ELISA,con las limitaciones que est determinación comporta..

Una gran diferencia con respecto al vino es la mayor concentración de triptófano detectada en el producto final. En un principio, los niveles de triptófano vienen marcados por la materia prima de origen, que en el caso de la cerveza se compone de diferentes cereales como la cebada, el trigo, maíz, sorgo o arroz entre otros cereales (Bogdan & Kordialik-Bogacka, 2017). Estas cantidades nos podrían llevar a dos hipótesis: que el triptófano presente en las materias primas de la cerveza supera al de la uva o que las levaduras durante la fermentación alcohólica de cerveza no consumen tanto triptófano como se esperaba. No obstante, queda esclarecido que en la fermentación de las cervezas, las levaduras también siguen la ruta de la melatonina. Además, la cerveza

puede aportar triptófano en la dieta como precursor para la síntesis de los otros derivados.

Por otro lado, tanto el 5-hidroxitriptófano como la *N*-acetil serotonina se cuantificaron por primera vez en cervezas comerciales, aunque a concentraciones muy bajas (<1 ng/mL). La melatonina también se cuantificó en todas las muestras de cerveza, aunque el rango fue incluso inferior al descrito por los otros dos precursores, del orden de 9-30 pg/mL. Previamente, los trabajos donde se analizó melatonina en cervezas describieron concentraciones ligeramente superiores, entre 52 y 169 pg/mL (Kocadağli et al., 2014; Maldonado et al., 2009). Las variaciones que se observan posiblemente estén relacionadas por la técnica de análisis empleada. En el trabajo de Maldonado et al. se usó ELISA para cuantificar las muestras, siendo un método cuyos falsos positivos y negativos pueden alterar los resultados finales (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, et al., 2011). Kocadağli sí utilizó un método de LC/MS-MS en el que solo cuantificó melatonina, lo cual aumentó la sensibilidad.

Asimismo se realizó una investigación complementaria centrada en la bioactividad de compuestos relacionados con otro aminoácido aromático, la fenilalanina. Los estilbenos son metabolitos polifenólicos derivados del aminoácido fenilalanina a través del metabolismo del ácido fenilpropanoico en plantas. El **capítulo 6**, estudió la bioactividad de 12 estilbenos (ampelopsina A, astringina, ε-viniferina, hopeafenol, isohopeapfenol, palidol, picetanol, pterostilbeno, rhapontina, r-viniferina, r2-viniferina y ω-viniferina) presentes en su gran mayoría en los raspones de la vid frente a la activación del VEGFR-2 para inhibir el proceso de angiogénesis.

En la actualidad se están realizando estudios sobre el aislamiento de estos estilbenos en raspones y otras partes de la vid con el objeto de sustituirlo por el anhidro sulfuroso como gente antimicrobiano y antioxidante, para evitar los efectos adversos que éste provoca en personas sensibles (Biais et al., 2017; Chaher et al., 2014). (Guerrero & Cantos-Villar, 2015). El uso de estos compuestos permitiría obtener vinos con un mayor contenido en compuestos bioactivos r (Raposo et al., 2016, 2018).

Hasta ahora, solo se habían realizado experimentos con el resveratrol y piceido frente a la inhibición de la fosforilación del VEGFR-2 (Cerezo, Winterbone, Moyle, Needs, & Kroon, 2015) dando como resultado una inhibición tan solo del 24% y 28%, respectivamente (50 μM). Los estilbenos con mayor potencial anti-VEGF fueron

astringina, (2,90 µM), el pallidol (4.42 µM), la ω-viniferina (6,10 µM), la ε-viniferina (18,84 µM) y el piceatannol (39.70 µM). Las diferencias entre los valores de cada estilbenos tienen su relación en su estructura química. Al igual que en los polifenoles, es posible que la conformación espacial de los estilbenos y su ajuste en el *pocket* de la estructura del VEGF determinen su efecto (Atrahimovich, Vaya, & Khatib, 2013; Xiao et al., 2011).

Con el objeto de comprobar que los estilbenos al inhibir VEGF además inhiben la proliferación celular que es una de las fases iniciales del proceso de angiogénesis, se evaluó el efecto de estos compuestos sobre la inhibición de la principal proteína intracelular implicada en la proliferación celular, como es PLCγ1. Esta proteína fue inhibida significativamente por las viniferinas ε y ω y por la astringina, mientras que el piceatanol, aunque la inhibía, no significativamente como en los otros compuestos.

Se han desarrollado fármacos anti-VEGF que actualmente se utilizan en las terapias frente al cancer, como son el bevacizumab, sorafenib o sunitinib (Luvero et al., 2017). Sin embargo, al inhibir la fosforilación de VEGFR-2, también inhiben la activación de eNOS provocando a largo plazo hipertensión en los pacientes (Escalante & Zalpour, 2011; Li & Kroetz, 2018; Zhu, Wu, Dahut, & Parikh, 2007). Esto se produce porque al inhibir toda la cascada de reacción intracelular, se inhibe la producción de óxido nítrico via eNOS, el cual es un potente vasodilatador. Por tanto, es importante encontrar moléculas que puedan inhibir el receptor de VEGFR-2 y PLCγ1, pero que promuevan la activación de eNOS. Las viniferinas ε- y ω-, demostraron promover significativamente la fosforilación de eNOS, vía Akt, al mismo tiempo que inhibían la activación de PLCγ1 y VEGFR-2. Sin embargo, solamente la ε-viniferina ha sido cuantificada en vinos tintos (Amira-Guebailia et al., 2009; Hurtado-Gaitán, Sellés-Marchart, Martínez-Márquez, Samper-Herrero, & Bru-Martínez, 2017; Landrault et al., 2002), puesto que la ω-viniferin se ha identificado en los raspones de la vid (Biais et al., 2017) Además, el pallidol demostró mantener la activación de eNOS, aunque debe producirse por otras vías distintas de las de Akt.

Con estos resultados, se abre una puerta a investigaciones futuras para que los compuestos más activos puedan aparecer en los productos finales (vino) a una concentración activa, o incluso, su implementación en posibles terapias alternativas para inhibir el proceso angiogénico.

CONCLUSIONES

1. Se ha desarrollado un método validado para el análisis simultáneo de 9 compuestos indólicos en un equipo de UHPLC/HRMS (Qexactive), con unos valores de LOD inferiores a los que había publicados en la bibliografía

2. Los filtros de politetrafluoroetileno demostraron afectar mínimamente a la concentración de compuestos indólicos, siendo por tanto los más indicados para su uso en el tratamiento de muestras fermentadas.

3. Se ha identificado y cuantificado por primera vez el contenido de 5-hidroxitriptófano en muestras de cerveza, vino y mosto sintético.

4. La estabilidad de los compuestos indólicos está muy ligada a la matriz y a la concentración en las muestras, siendo más estables a temperaturas de refrigeración (4º C) durante al menos 7 días, permitiendo el consumo doméstico de compuestos bioactivos dentro de un periodo razonable tras una apertura.

5. Las levaduras no-Saccharomyces son capaces de sintetizar melatonina en diferentes condiciones de fermentación, tanto en mosto sintético como en mosto de uva, a concentraciones del orden de pg/mL. Sin embargo, fue imposible verificar un patrón de síntesis de melatonina reproducible en diferentes medios.

6. La síntesis de compuestos indólicos depende en mayor medida del tiempo de fermentación, más que de la cepa de levadura utilizada, así como de la velocidad con la que llegan al final de la fase exponencial.

7. La mejor combinación para la producción de melatonina en mostos de uva fue el uso de la levadura Aroma White en mostos de la variedad Corredera.

8. Las cervezas contienen todos los compuestos de la ruta de la melatonina, aunque a concentraciones traza, cuantificándose por primera vez el ácido 3-indolacético y la *N*-acetilserotonina en cervezas comerciales.

9. Los compuestos estilbenoides derivados del resveratrol como la astringina, el palidol, el piceatannol y la ε- y ω-viniferina presentan potencial anti-VEGF en células endoteliales de vena de cordón umbilical humano.
10. Las viniferinas ε- y ω-viniferina, además, son capaces de inhibir la principal proteína involucrada en la proliferación celular (PLCγ1) a la vez que activan la fosforilación de eNOS via Akt, abriendo un campo de investigación para combatir los efectos adversos hipertensivos debidos al tratamiento prolongado con medicamentos anti-VEGF.

CONCLUSIONS

1. A validated method for the simultaneous analysis of nine compounds derived from the L-tryptophan metabolism was developed in a UHPLC/HRMS (Qexactive), lowering the LOD of these indolic compounds respecting the reported ones in literature

2. Politetrafluoroetylene filters showed be the most effective for sample treatment to study the indolic compounds in fermented beverages.

3. For the first time, 5-hydroxytryptophan was quantified in different samples after a alcoholis fermentation carried out by yeast.

4. Indolic compounds stability was suitable to be affected because of both matrix and concentration range, being more stable at 4ºC and maintaining the concentration until a maximum period of 7 days, being reasonable for a domestic storage time of opened beverages.

5. Non-*Saccharomyces* strains are able to synthesise melatonin at different fermentation conditions in both synthetic and natural grape must at trace levels (ng/mL). However, we cannot establish a repetitive trend of synthesis for melatonin in the different sample tested.

6. Indolic compounds synthesis leads on the fermentation time more than yeast used, being influenced by the total exponential phase.

7. Best combination for melatonin production in natural grape must resulted on Aroma White and Corredera grape variety.

8. Beers contain all the melatonin pathway intermediates at trace levels. For the first time it was quantified 3-indoleacetic acid, 5-hydroxytryptophan and N-acetylserotonin in commercial beers.

9. Stilbenes derived from resveratrol such as astringin, pallidol, Piceatannol, ε- and ω-viniferin have proved to be potent anti-angiogenic compounds in HUVEC.

10. Additionally, ε- and ω-viniferin were able to inhibit cellular proliferation via PLCγ1 and, at the same time, allowing the phosphorylation of eNOS via Akt, allowing new fields for future researches in alternative treatments to anti-VEGF drugs.