Tesis Doctoral

SEGUIMIENTO DE LA MELATONINA DURANTE LA ELABORACIÓN DE VINOS TINTOS Y BLANCOS:

ANÁLISIS, FORMACIÓN POR *SACCHAROMYCES* Y ACTIVIDAD ANTIOXIDANTE

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SEGUIMIENTO DE LA MELATONINA DURANTE LA ELABORACIÓN DE VINOS TINTOS Y BLANCOS

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UNIVERSIDAD DE SEVILLA

Facultad de Farmacia

Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal

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Memoria presentada por

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para optar al grado de Doctor.

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Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal

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INFORMA:

Que la Tesis Doctoral titulada "**Seguimiento de la melatonina durante la elaboración de vinos tintos y blancos: análisis, formación por *Saccharomyces* y actividad antioxidante"**, presentada por la Lda. Mª ISABEL RODRÍGUEZ NARANJO para optar al grado de Doctor por la Universidad de Sevilla, ha sido realizada en el Área de Nutrición y Bromatología de este Departamento bajo la dirección de las Dras. Mª CARMEN GARCÍA PARRILLA y EMMA CANTOS VILLAR, cumpliendo los requisitos exigidos.

Y para que así conste, firmo el presente en Sevilla,
Marzo de 2012.

Fdo. Ana María Cameán Fernández

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INFORMAN:

Que la Tesis Doctoral titulada **"Seguimiento de la melatonina durante la elaboración de vinos tintos y blancos: análisis, formación por *Saccharomyces* y actividad antioxidante",** ha sido realizada por la Lda. Mª ISABEL RODRÍGUEZ NARANJO en el Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Universidad de Sevilla, bajo su dirección y que reúne, a su juicio, las condiciones requeridas para optar al grado de Doctor por la Universidad de Sevilla.

Y para que así conste, firmamos en Sevilla,
Marzo de 2012.

Fdo. Mª Carmen García Parrilla

Fdo. Emma Cantos Villar

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PRESENTACIÓN DE LA TESIS

Esta tesis ha sido realizada en el Área de Nutrición y Bromatología de la Facultad de Farmacia de la Universidad de Sevilla bajo la dirección de las Dras. Mª Carmen García Parrilla y Emma Cantos Villar entre los años 2008 a 2012.

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La tesis se presenta con la siguiente estructura:

- Introducción general
- Justificación y objetivos del trabajo
- Diseño experimental
- Resultados: mostrados en cinco capítulos, cada uno formado por un artículo científico.
- Discusión general
- Conclusiones
- Bibliografía

INTRODUCCIÓN

DESCRIPCIÓN Y ESTRUCTURA DE LA MELATONINA

La melatonina es una hormona que se produce, principalmente, en la glándula pineal o epífisis a partir del aminoácido triptófano y está relacionada con el ciclo luz/oscuridad. Esta sustancia es muy conservada entre organismos distantes evolutivamente como son bacterias y protozoos, hongos, plantas, invertebrados y vertebrados (Hardeland y Poeggeler, 2003).

En 1917, MC. Cord y Allen observaron que si se añadía un extracto de la glándula pineal de la vaca sobre la piel de un sapo se producía su aclaración. A finales de los 50, Lerner y colaboradores, basándose en los estudios de Cord y Allen, aislaron la hormona pineal que producía este efecto a partir de las células de la glándula pineal bovina y describieron su estructura química: *N*-acetil-5-metoxitriptamina (Lerner et al., 1958).

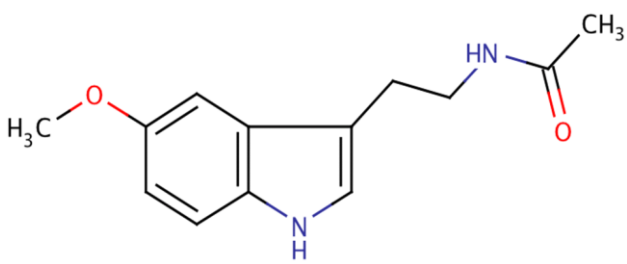

Figura 1. Estructura química de la Melatonina.

La *N*-acetil-5-metoxitriptamina presenta un anillo de tipo indólico y dos grupos funcionales principales, el grupo metoxi en posición 5 y el grupo amida en posición 3 (Posmyk y Janas, 2009). Estos grupos determinan su solubilidad tanto en medio acuoso como en orgánico, su carácter anfipático le permite traspasar las membranas celulares, ser transportada por fluidos biológicos y, además, le confieren especificidad para la unión a receptor. Estos grupos también son responsables de las propiedades antioxidantes de la melatonina (Hardeland et al., 2006).

SÍNTESIS Y METABOLISMO DE LA MELATONINA

La glándula pineal está localizada entre el mesencéfalo y el diencéfalo y forma parte del epitálamo. Esta glándula está formada por dos tipos de células: los pinealocitos, donde se sintetiza la melatonina y las células neuroglía (Brzezinski, 1997).

La producción de melatonina en esta glándula está controlada por el núcleo supraquiasmático (NSQ) que se encuentra sincronizado con el ciclo luz/oscuridad. Durante la noche, el NSQ envía señales neurales vía retino-hipotalámico-pineal e induce la liberación de noradrenalina (NA) nocturna desde el ganglio cervical superior (SCG). La unión de la noradrenalina a los receptores de membrana de los pinealocitos resulta en un incremento en el AMPc que conduce a la inducción de la *N*-acetiltransferasa y a la síntesis de melatonina (Figura 2).

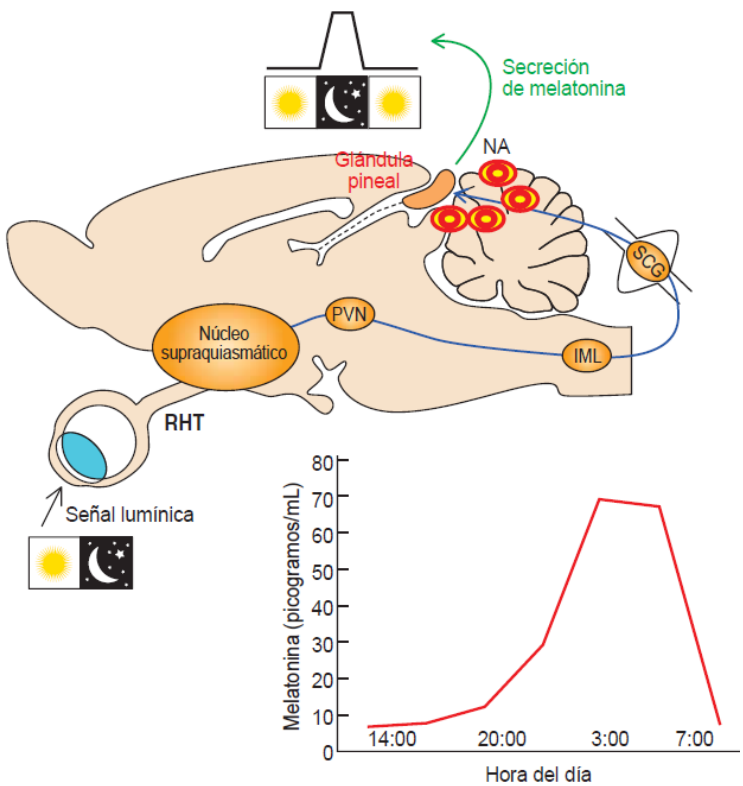

Figura 2. Esquema de la síntesis de melatonina en humanos (Tomado de Guerrero et al., 2007).

La formación de melatonina empieza con la captación del aminoácido triptófano (Trp) del torrente sanguíneo. El Trp se hidroxila en la mitocondria por la acción de la Trp-hidroxilasa. Gracias a la intervención de una enzima descarboxilasa, parte del 5-hidroxitritófano formado se convierte en serotonina en el citosol. Esta serotonina se acetila por la arilalquilamina-*N*-acetiltransferasa (AA-NAT) y forma *N*-acetilserotonina. Esta enzima, que constituye el paso limitante en la síntesis de melatonina, se encuentra bajo el control del NSQ y presenta un marcado ritmo circadiano. Finalmente, la hidroxiindol-*O*-metiltransferasa (HIOMT) forma melatonina por *O*-metilación de la *N*-acetilserotonina (Figura 3).

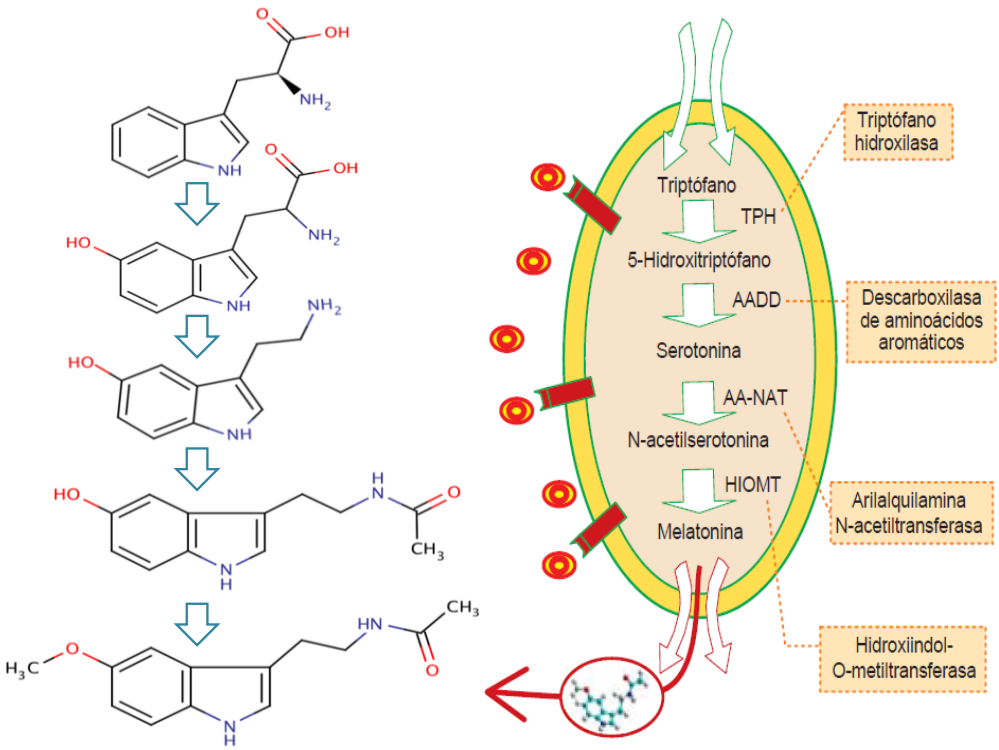

Figura 3. Esquema de la formación de melatonina en los pinealocitos a partir del aminoácido triptófano (Tomado y modificada de Guerrero et al., 2007).

La melatonina sintetizada se libera al sistema vascular desde donde accede al resto de los tejidos (Guerrero et al., 2007). Al contrario de lo que sucede con otras hormonas, la melatonina no se almacena en vesículas antes de su liberación (Reiter, 1981).

Además de la producción pineal, existen otros sitios extrapineales de síntesis de melatonina como son la glándula harderiana, el cerebelo, el tracto gastrointestinal, la retina, la piel y la médula ósea (Hardeland et al., 2006) y su concentración en algunos de estos órganos, donde ejerce una función antioxidante, puede ser del orden de miligramos.

En humanos, la melatonina se metaboliza en el hígado y en el riñón formando primero 6-hidroximelatonina (Yu et al., 1993), que después se conjuga con ácido sulfúrico o glucurónico, y se excreta fundamentalmente en forma de compuestos sulfatados o glucuronados en la orina. El 90% de la melatonina suele estar en forma de 6-sulfatoximelatonina en plasma y orina. La melatonina también se metaboliza en el cerebro en forma de derivados de kinuraminas, como *N*-acetil-5-metoxikinuramina (AMK) y N^1-Acetil-N^2-formil-5-metoxikinuramina (AFMK) (Hirata et al., 1974). Estas sustancias suponen un 15% del total de los metabolitos urinarios de la hormona.

BIODISPONIBILIDAD DE LA MELATONINA

La melatonina se elimina rápidamente (entre 0.5 y 5.6 minutos) después de suministrarla por vía intravenosa. La excreción urinaria del metabolito 6-sulfatoximelatonina evoluciona paralelamente a la concentración de melatonina en suero. Por vía oral, su biodisponibilidad es muy variable. Según Dollins et al., (1994), la concentración de melatonina en suero aumentó desde 15 a 410 ng L^{-1} después de 4 h de la administración de 1 mg de melatonina y desde 15 a 6825 ng L^{-1} después de 4 h de la administración de 10 mg de melatonina. Teniendo en cuenta que el nivel nocturno máximo de melatonina en sangre es de 200 ng L^{-1} y el nivel diurno de 10 ng L^{-1} aproximadamente, el aumento de melatonina en suero es muy significativo comparado con los valores fisiológicos. Un experimento realizado en 20 voluntarios donde se administraron 80 mg de melatonina en dosis única en 24 h dio como resultado un nivel de melatonina hasta 100 veces el nivel fisiológico nocturno y no se recuperó el nivel basal hasta 16 h después. No hubo evidencias de toxicidad aguda (ni accidentes graves, ni muertes) en los sujetos que tomaron estas dosis. Sin embargo, registraron efectos secundarios como trastornos gastrointestinales, hipotensión, dolor de cabeza y pesadillas vinculados

a la actividad farmacológica y al metabolismo de la melatonina (Waldhauser et al., 1990). Hasta el momento no se ha realizado la evaluación de riesgo de la melatonina pero debido a que es una neurohormona y que su concentración varía a lo largo del día, su administración con fines terapéuticos debería considerar las particularidades de esta molécula, y se ha de tener en cuanta tanto la dosis como el tiempo de administración y la duración del tratamiento.

Por otra parte, para que la melatonina ingerida en la dieta sea beneficiosa para la salud debe absorberse en el tracto gastrointestinal, aumentar la concentración de la indolamina en sangre, y desde el torrente circulatorio debe acceder a las células. Su carácter anfipático favorece su absorción y le permite atravesar membranas biológicas, siendo identificada en distintas partes celulares: membranas, citoplasma, mitocondria y núcleo (Reiter et al., 2004). Un estudio llevado a cabo por Hattori et al., (1995) demostró que aumentaban los niveles de melatonina (de 19 a 33 ng L^{-1}) y la capacidad antioxidante en plasma después de 90 minutos de la ingesta de arroz que contenía 3.5 ng g^{-1} de melatonina. Otro estudio demostró que la concentración de melatonina en plasma de ratas aumentó de 11.5 $\pm$ 1.9 a 38.0 $\pm$ 4.3 ng L^{-1} tras la ingesta de 3 g de nueces de California (equivalente a 10.5 ng de melatonina) (Reiter et al., 2005) observándose paralelamente una elevación significativa de la capacidad antioxidante del plasma determinada mediante FRAP.

Aunque las concentraciones en alimentos son bajas, queda probado que la concentración plasmática de melatonina aumenta tras el consumo de cantidades razonables de alimentos. Teniendo en cuenta que el nivel diurno medio de melatonina en sangre es aproximadamente de 10 ng L^{-1}, el aumento por la ingesta de un alimento resulta significativo ya que puede llegar a triplicar la concentración de melatonina diurna en plasma, pero no llega al nivel nocturno de melatonina, aproximadamente 200 ng L^{-1}. Así, se puede considerar a la melatonina como un compuesto de interés por su actividad antioxidante y como constituyente natural de los alimentos.

Por tanto, la melatonina es un componente natural presente en alimentos con actividad biológica, debido principalmente a que puede actuar como un neutralizador de radicales libres y estimular la función de enzimas antioxidantes, así reducir el daño molecular en las células, disminuir la generación de productos

oxidantes y la destrucción oxidativa de lípidos y otras moléculas. Además, los metabolitos que se generan en las reacciones con antioxidantes (AFMK y AMK) también son neutralizadores de radicales libres (Reiter et al., 2007).

ACCIONES BIOLÓGICAS DE LA MELATONINA

Son numerosas las funciones asociadas a la melatonina que se han descrito en humanos. Así, se la relaciona con el desarrollo sexual, la regulación de los ritmos circadianos, presenta propiedades antioxidantes, antienvejecimiento y antiinflamatorias. La versatilidad funcional que se le atribuye a la melatonina se debe a que puede ejerce sus funciones a través de cuatro mecanismos de acción distintos:

1) Por unión a receptores de membrana: se une a receptores de membrana de 7 dominios transmembrana acoplados a proteínas G (GRCRs), como son los MT_1 y MT_2,

2) por interacción con proteínas de citoplasmáticas: puede interactuar con proteínas como la proteína quinasa C, la calmodulina o la proteína MT_3,

3) por interacción con receptores nucleares: puede unirse a receptores nucleares de la subfamilia de retinoicos (RZR/ROR),

4) por acción directa, neutralizando radicales libres: tiene la capacidad de atrapar y neutralizar radicales hidroxilo (Brzezinski, 1997).

Entre las funciones más significativas que se le atribuyen destacan:

- **Sincronizador del ritmo circadiano:** los ritmos circadianos (de latín *circa*, 'cerca' y *diez*, 'día') son oscilaciones de las variables biológicas en intervalos regulares de tiempo. Los humanos, al igual que el resto de animales, presentan una variación rítmica fisiológica (en procesos bioquímicos y de comportamiento) que se pone de manifiesto en la producción de ciertas moléculas como la melatonina (Guerrero et al., 2007).

La producción rítmica de melatonina en la glándula pineal, con valores mínimos diurnos y valores máximos nocturnos (del orden de picogramos) (Figura 2), es la

señal del reloj biológico, traduce la información del fotoperiodo al organismo, sincronizando los procesos neuroendocrinos con los cambios diarios y estacionales (Terrón, 2004). Por eso, la melatonina es considerada como la "hormona de la oscuridad". La liberación de la melatonina al torrente sanguíneo está estrechamente sincronizada con las horas habituales de sueño, la concentración en sangre varía de entre 10 a 200 ng L^{-1} del día a la noche. Alteraciones en esta sincronización se correlacionan normalmente con trastornos en el sueño (Brzezinski, 1997).

Además, la secreción de melatonina varía a lo largo de la vida según el siguiente modelo: durante los primeros seis meses de vida, los niveles nocturnos de melatonina son bajos, siendo entre los 1 a 3 años cuando se alcanzan los picos nocturnos más elevados y el ritmo circadiano. Entre los 15 y 20 años ocurre una caída en los niveles del 80 % debida al incremento de la talla del cuerpo, a pesar de la producción constante de melatonina después de la infancia. Durante las décadas siguientes disminuyen moderada y progresivamente hasta los 70-90 años, en que sus niveles son los más bajos (Teresa Galván et al., 2008).

- **Sueño:** el ritmo luz/oscuridad gracias a que la glándula pineal libera la de melatonina está estrechamente sincronizado con las horas habituales del sueño. Ciertas alteraciones en la sincronización debido a cambios de fase dia/noche o por ceguera, se correlacionan con alteraciones del sueño. La ingestión de melatonina afecta a la velocidad de concilio del sueño, así como a la duración y calidad del sueño (Brzezinski, 1997). Motivos por los cuales la Agencia Europea de Seguridad Alimentaria (EFSA) ha atribuido a la melatonina las alegaciones de propiedades saludables de ayudar a aliviar los síntomas de *jet lag* y ayudar a reducir el tiempo de concilio del sueño en estos dos últimos años (EFSA 2010, 2011).

- **Maduración sexual y reproducción:** la melatonina actúa como un vínculo entre el cuerpo y el ritmo luz/oscuridad del medio ambiente. Una prolongación en las horas de oscuridad afecta a la secreción de melatonina y desempeña un papel clave en el comportamiento sexual y reproductivo (Arendt, 1989). Como se señaló anteriormente, hay un descenso de las concentraciones nocturnas de melatonina

en suero progresivamente a lo largo de la infancia y la adolescencia. No se conoce si esta reducción se relaciona con cambios en la tasa de secreción o en el aumento del tamaño del cuerpo. No hay datos disponibles en humanos para conocer los posibles mecanismos que pueden relacionar la disminución de la secreción de la melatonina que se produce cuando los niños crecen. Son necesarios más estudios para determinar la relación entre la disminución de las concentraciones de melatonina en suero y el momento en que ocurre la pubertad y su tasa de progresión (Brzezinski, 1997).

- **Antioxidante:** existe un extenso número de publicaciones que avalan la capacidad antioxidante que tiene la melatonina. Ejerce su actividad antioxidante como neutralizador directo de radicales libres (Hardeland et al., 1993; 1995; Allegra et al., 2003), aumentando la eficacia de otros antioxidantes (Gitto et al., 2001), estimulando la acción o la síntesis de enzimas antioxidantes (Reiter et al., 2000; Rodríguez et al., 2004; Mayo et al., 2002) o protegiéndolas del daño oxidativo (Mayo et al., 2003a; 2003b), o incrementando la eficacia de la cadena de transporte de electrones mitocondrial (Okatani et al., 2003).

Además, la oxidación de la melatonina produce otros metabolitos biológicamente activos como la N^1-acetil-N^2-formil-5-metoxikynuramina (AFMK) y la N^1-acetil-5-metoxikynuramina (AMK) (Hardeland y Pandi-Perumal, 2005) (Figura 4).

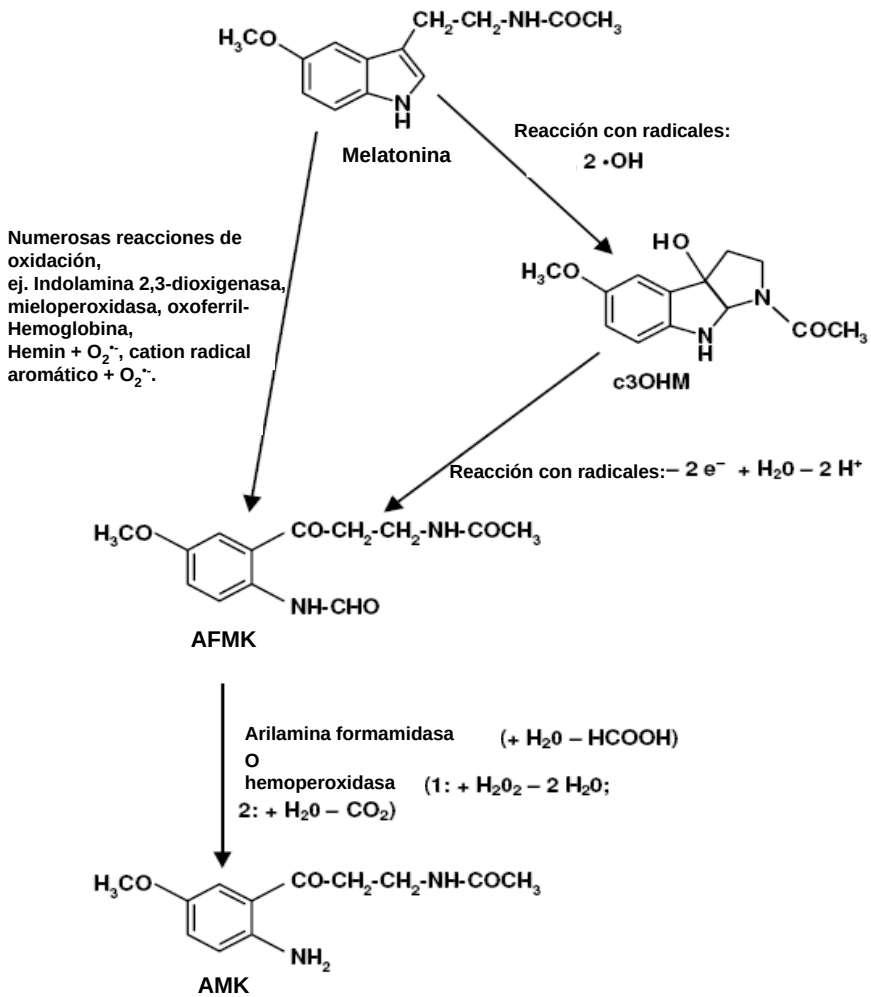

Figura 4. Reacciones de oxidación de melatonina (AFMK: N^1-acetil-N^2-formil-5-metoxikinuramina y AMK: N^1-acetil-5-metoxikinuramina). (Hardeland y Pandi-Perumal, 2005).

La concentración en fluidos como la bilis u órganos como la retina o el intestino, donde la melatonina ejerce una función puramente antioxidante frente al estrés oxidativo (Reiter et al., 2003), puede llegar a ser del orden de miligramo. Y, debido a su característica anfifílica, esta molécula puede atravesar fácilmente las barreras fisiológicas y reducir el daño oxidativo tanto en el medio acuoso como en las membranas lipídicas de las células (Reiter et al., 2004).

- **Antienvejecimiento:** a la melatonina se la relaciona con el envejecimiento debido a que se produce una disminución de la síntesis de esta molécula con la edad (Reiter et al., 1995). Sin embargo, esta reducción con la edad puede ser una consecuencia del proceso de envejecimiento en vez de su causa. Lo que está claro es que esta indolamina puede ejercer efecto protector contra el daño oxidativo a las células, pero esto no es suficiente para apoyar la idea de su efecto antiedad.

- **Antitumoral:** un estudio realizado en ratas demostró que la melatonina influía en el crecimiento de tumores. Después de realizarles una pinealectomía se produjo el aumento de tumores en las ratas y la administración de melatonina revirtió este efecto, inhibiendo los tumores (Tamarkin et al., 1981). Este efecto antitumoral puede deberse también a la actividad antioxidante, pero sobre todo tiene que ver con la capacidad inmunomoduladora de la melatonina, que es capaz de influir en la respuesta inmunitaria tanto innata como adaptativa (Guerrero et al., 2007).

MELATONINA EN PLANTAS

El descubrimiento de melatonina en una especie distinta a los vertebrados fue en el alga dinoflagelada *Gonyaulax* (*Lingulodinium polyedrum*) (Poeggeler et al., 1991). En 1995, dos grupos de investigadores, Dubbels et al., (1995) y Hattori et al., (1995) publicaron la presencia de melatonina en plantas.

Desde entonces, numerosos estudios han confirmado la existencia de la indolamina en raíces, hojas, frutas y semillas de gran variedad de especies vegetales (Dubbels et al., 1995; Hattori et al., 1995, Manchester et al., 2000; Burkhardt et al., 2001; Iriti et al., 2006) (Tabla1).

Nombre común	Nombre científico	Órgano	Melatonina (ng g^{-1})	Referencia
Banana	*Musa paradisiaca* (L.)	Fruto	0.002	Dubbels et al. 1995
Fresa Silvestre	*Fragaria ananassa* (Duch.)	Fruto	0.01	Hattori et al. 1995
Cebolla	*Allium cepa* (L.)	Bulbo	0.03	Hattori et al. 1995
Pepino	*Cucumis sativus* (L.)	Fruto	0.03	Hattori et al. 1995
Piña	*Ananas comosus* (Stickm.) Merill.	Fruto	0.04	Hattori et al. 1995
Manzana	*Malus domestica* (Borkh)	Fruto	0.05	Hattori et al. 1995
Zanahoria	*Daucus carota*	Raíz	0.06	Hattori et al. 1995
Repollo	*Brassica oleracea* (L.)	Hoja	0.1	Hattori et al. 1995
Cebada	*Hordeum vulgare* (L.)	Semilla	0.4	Hattori et al. 1995
Tomate	*Lycopersicon esculentum* (Mill.)	Fruto	0.5	Dubbels et al. 1995
Rábano	*Raphanus sativus* (L.)	Raíz	0.7	Hattori et al. 1995
Arroz	*Oryza sativa japonica* (L.)	Semilla	1	Hattori et al. 1995
Avena	*Avena sativa* (L.)	Semilla	2	Hattori et al. 1995
Hierba de San Juan	*Hypericum perforatum* (L.)	Hoja	2	Murch et al. 1997
Maíz	*Zea mays* (L.)	Semilla	2	Hattori et al. 1995
Apio	*Apium graveolens* (L.)	Semilla	7	Manchester et al. 2000
Alfalfa	*Medicago sativum* (L.)	Semilla	16	Manchester et al. 2000
Cereza	*Prunus cerasus* (L.)	Fruto	18	Burkhardt et al. 2001
Girasol	*Foeniculum vulgare* (Gilib.)	Semilla	29	Manchester et al. 2000
Almendra	*Prunus amygdalus* (Batsch)	Semilla	39	Manchester et al. 2000
Uva	*Vitis vinifera*	Fruto	96	Iriti et al. 2006
Mostaza negra	*Brassica nigra* (L.)	Semilla	129	Manchester et al. 2000

Tabla 1. Melatonina en algunas plantas.

En plantas, el precursor de la melatonina es el aminoácido L-triptófano al igual que en vertebrados. Sin embargo, la síntesis de serotonina se realiza por una vía diferente que en animales. En primer lugar, el aminoácido precursor es descarboxilado por la triptófano descarboxilasa (TrpDC) para formar triptamina, que con la triptamina 5-hidroxilasa forma serotonina. La melatonina puede sintetizarse por dos vías a partir de la serotonina en plantas: vía 5-

metoxitriptamina como en mamíferos o vía *N*-acetilserotonina, proceso catalizado por varias enzimas (Posmyk y Janas, 2009).

En la figura 4 se observa que el aminoácido L-triptófano, precursor de la melatonina, es utilizado para la síntesis de ácido 3-indolacético (IAA) y otras auxinas. Las auxinas son un grupo de fitohormonas que se producen en distintas partes de las plantas cuando se encuentran en fase de crecimiento activo y regulan muchos aspectos del desarrollo vegetal, influyen en el crecimiento de los órganos estimulando la elongación o alargamiento de ciertas células e inhibiendo el crecimiento de otras, en función de la cantidad de auxina en el tejido vegetal y su distribución.

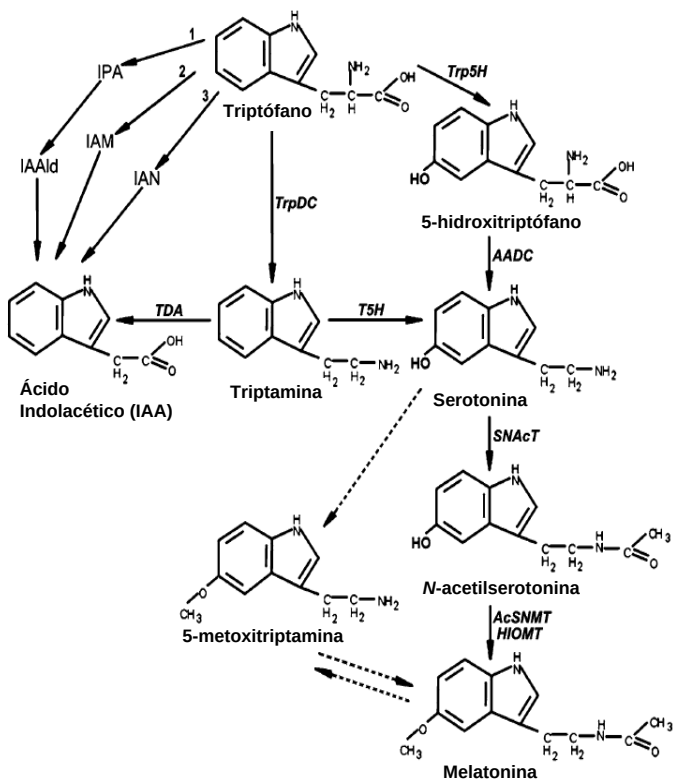

Figura 5. Síntesis de Melatonina en Plantas. Enzimas implicadas: Trp5H triptófano 5-hidroxilasa, AADC descarboxilasa de aminoácido aromático, TrpDC triptófano descarboxilasa, T5H triptamina 5-hidroxilasa, TDA triptamina desaminasa, SNAcT serotonina N-acetiltransferasa, AcSNMT acetiloserotonina N-metiltransferasa, HIOMT hidroxiindoli-O-metiltransferasa. Intermedios en la síntesis de ácido indolacético: (1) ácido indolpirúvico (IPA) y indolacetaldehido (IAAld), (2) indolacetamida (IAM), y (3) indolacetonitrilo (IAN) (Tomada de Posmyk y Janas, 2009).

Las funciones fisiológicas que la melatonina desempeña en plantas no son muy conocidas. Al igual que en el Reino Animal, parece estar implicada en la regulación del ciclo circadiano, relacionando el ciclo día/noche con los procesos biológicos de las plantas. Por otra parte, la melatonina es una indolamina con estructura relacionada con el triptófano (un aminoácido esencial en humanos), la serotonina (un neurotransmisor) y el ácido 3-indolacético (IAA) que pertenecen a la familia de las auxinas que actúan regulando el crecimiento y el desarrollo de las plantas, por tanto la melatonina puede desempeñar la función de fitohormona (Arnao y Hernández-Ruiz, 2006).

Otra de las funciones de la melatonina es proteger las plantas del estrés oxidativo producido por los factores medioambientales como el ozono, la radiación ultravioleta, la temperatura, etc. reduciendo el daño oxidativo de moléculas como los ácidos nucleicos, proteínas y lípidos. La melatonina tiene una gran capacidad antioxidante debido a que es soluble tanto en agua como en lípidos a baja concentración y actúa de forma directa o a través de otros mecanismos disminuyendo el daño oxidativo (Posmyk y Janas, 2009). La oxidación de la melatonina produce otros metabolitos biológicamente activos como la N^1-acetil-N^2-formil-5-metoxikynuramina (AFMK) y la N^1-acetil-5-metoxikynuramina (AMK) (Hardeland y Pandi-Perumal, 2005) (Figura 4).

PRESENCIA EN ALIMENTOS Y SUPLEMENTOS DE MELATONINA

Recientemente se ha descrito la presencia de melatonina en alimentos que forman parte de la dieta diaria. Frutas como uva, manzana, fresa y banana constituyen una fuente de melatonina. Además, esta indolamina ha sido identificada en productos de origen vegetal como el aceite de oliva (119 ng L^{-1}) (De la Puerta et al., 2007) y en bebidas fermentadas como la cerveza (170 ng L^{-1}) (Maldonado et al., 2009) y el vino (500 ng L^{-1}) (Mercolini et al., 2008). Otra fuente importante de melatonina dietética es la leche de vaca, en la que se han encontrado niveles de hasta 10.5 ng L^{-1}.

El análisis de melatonina en alimentos presenta ciertas dificultades. En primer lugar, su concentración en algunos alimentos de origen vegetal suele ser muy pequeña (ng g^{-1} a µg g^{-1}), y por tanto el método analítico debe ser sensible. En segundo lugar, por el carácter anfipático de la molécula, es muy difícil elegir un método de extracción que dé lugar a la recuperación completa y al contenido exacto. Y, por último, la melatonina es un antioxidante y reacciona rápidamente con especies reactivas de oxígeno y nitrógeno (Tan et al. 2007).

Los métodos de extracción deben ser los adecuados para conseguir la suficiente selectividad y sensibilidad en la detección de la indolamina, teniendo en cuenta que los vegetales presentan una matriz compleja. Las técnicas analíticas utilizadas para determinar melatonina en plantas y alimentos son: el radioinmunoensayo (RIA) (Van Tassel et al. 2001a), la inmunoprecipitación (Pape y Luening, 2006), la inmunoabsorción ligando-enzima (ELISA) (Iriti es al. 2006; Kollmann et al. 2008; Maldonado et al. 2006), cromatografía de gases con espectometría de masas (GC-MS) (Van Tassel et al. 2001b; Dubbels et al. 1995), cromatografía líquida con detector de fluorescencia (LC-FL) (Arnao y Hernández-Ruiz, 2007; Mercolini et al. 2008), cromatografía líquida con espectrometría de masas (LC-MS) (Yang et al. 2002).

Además de un componente natural de la dieta, la melatonina se consume en muchos países del mundo en suplementos dietéticos, sola o en combinación con otros ingredientes como vitaminas, extractos de hierbas, etc. Normalmente, se comercializan en comprimidos de 1, 3 y 5 mg. Estos comprimidos se absorben entre las encías y la mejilla o bajo la lengua. También hay líquidos y cremas que se aplican sobre la piel como protección para las quemaduras de sol o como agente contra el envejecimiento.

El principal uso de estas fórmulas es el alivio de los síntomas del *jet lag*, con el fin de ajustar los ciclos de sueño/vigilia en personas cuyos turnos de trabajo cambian diariamente y para ayudar a las personas ciegas a establecer un ciclo de día y de noche. También para el tratamiento del insomnio y como ayuda para dormir después del uso de los medicamentos llamados benzodiazepinas. Sin embargo, otros usos son el tratamiento de la enfermedad de Alzheimer, la depresión, el síndrome de fatiga crónica, la fibromialgia, las migrañas y otros dolores de cabeza,

para el síndrome del intestino irritable, la pérdida de masa ósea y la epilepsia, entre otras, para los cuales no se ha demostrado que sea efectiva.

La dosis típica consumida suele ser de entre 0.3-5 mg al acostarse, pero puede llegar a ser de 10 a 50 mg diarios en tratamientos de cefaleas de racimo, cáncer de próstata y tumores (Portal MedlinePlus).

ALEGACIONES DE LAS PROPIEDADES SALUDABLES DE LA MELATONINA.

El Reglamento (CE) Nº 1924/2006 del Parlamento Europeo y del Consejo es una normativa que regula la declaración de alegaciones nutricionales y propiedades saludables de los alimentos en la Unión Europea desde el 1 de julio de 2007. Tras su entrada en vigor, la evidencia científica debe ser el soporte que sustente las declaraciones que realice un producto. La Agencia Europea de Seguridad Alimentaria (EFSA) realiza su evaluación y un dictamen científico que se considera para la autorización de la comercialización del producto por la Comisión Europea. El artículo 13 de dicho reglamento es el que regula las declaraciones de propiedades saludables distintas de las relativas a la reducción del riesgo de enfermedad y al desarrollo y salud de los niños.

La Comisión Europea solicitó al Panel de Productos Dietéticos, Nutrición y Alergias (NDA) que realizase una evaluación científica a cerca de las propiedades saludables de la melatonina en relación con el alivio de los síntomas del *jet lag*, la reducción de la latencia del sueño y su contribución a la calidad del sueño. Para la realización del informe solicitado, el panel de NDA tuvo en cuenta la lista de alegaciones de la salud propuesta en el artículo 13 del reglamento suministrada por los Estados miembros y algunas referencias recibidas en la EFSA. Los datos recibidos por el panel en referencia a la lista de alegaciones de la salud de la melatonina fueron las siguientes (Tabla 2):

ID	Alimento o ingrediente	Relación con la salud	Redacción de propuestas
1953	Melatonina	Regulación del ciclo sueño-vigilia	Ayuda a reducir los efectos del *jet lag*. Ayuda a reducir la fase adormecimiento. Ayuda a regular el ritmo circadiano. Mejora el ciclo sueño-vigilia Contribuye a la mejora de la calidad del sueño. Ayuda a reconciliar el sueño de forma natural.
Condiciones de Uso: Hasta 5 mg al dia			

Tabla 2. Principales propuestas de alegaciones sobre la salud para la melatonina.

En la evaluación científica se tuvo en cuenta la caracterización de la melatonina, la relevancia de los efectos sobre la salud humana, así como la base científica de los efectos propuestos. A la vista de estos resultados, el Panel concluyó que la relación causa-efecto entre el consumo de melatonina y el alivio de los síntomas del *jet lag* estaban suficientemente probados en estudios científicos y que se deben a trastornos del sueño como consecuencia del estilo de vida, horarios de trabajo, etc. de la población en general. Y, por tanto, motivo de la consideración como alegación sobre la salud.

Teniendo en cuenta todos los datos, el Panel de expertos NDA emitió el informe científico publicado en la revista de la EFSA en 2010 (EFSA journal, 2010) con las siguientes conclusiones:

- La melatonina esta suficientemente caracterizada como alimento/ingrediente.

- El alivio de los síntomas del *jet lag* podría ser un efecto fisiológico beneficioso para la población, y queda establecida una relación causa-efecto entre el consumo de melatonina y el alivio del *jet lag*. La dosis de melatonina debe estar entre 0.5 y 5 mg, que deben tomarse el primer día y algunos días posteriores a la llegada al destino. Siendo la población objetivo, la población en general.

- La reducción de la fase de adormecimiento y la mejora de la calidad del sueño podrían ser efectos fisiológicos beneficiosos, pero no se ha establecido una relación causa-efecto entre el consumo de melatonina y estos efectos.

En 2011, el Panel de expertos NDA publicó (EFSA journal, 2011a), después de haber analizado diferentes meta-análisis, que existía una relación causa-efecto entre el consumo de melatonina y la reducción de la fase de adormecimiento. La melatonina ayudaba a reducir el tiempo de conciliación del sueño y la población diana era la población en general. Para este efecto, debía consumirse 1 mg de melatonina cerca de la hora de dormir.

En los dos últimos años, la EFSA ha considerado a la melatonina como un constituyente de los alimentos, y le ha atribuido las alegaciones de propiedades saludables por ayudar a aliviar los síntomas de *jet lag* y por ayudar a reducir el tiempo de concilio del sueño.

AMINAS BIÓGENAS: RELACIÓN CON LA MELATONINA

Las aminas biógenas son bases nitrogenadas de bajo peso molecular, su estructura química puede ser alifática (agmatina, cadaverina, espermita, espermidina, metilamina, putrescina), aromática (tiramina, feniletilamina) o heterocíclica (histamina, triptamina) (Anexo I).

Estos compuestos son generados durante el metabolismo de vegetales, microorganismos y animales, y se forman principalmente por descarboxilación de un aminoácido o por aminación y transaminación de aldehidos y cetonas (Ascar y Treptow, 1986). Se encuentran frecuentemente en alimentos fermentados como queso, salchichas, vino y cerveza (Silla Santos, 1996). La formación de aminas biógenas durante la fermentación tiene lugar si en el alimento se encuentra el aminoácido precursor y si los microorganismos encargados de la síntesis poseen las enzimas amino-descarboxilasas capaces de descarboxilar a los aminoácidos. La aparición de aminas biógenas se debe a la acción de levaduras (Goñi y Azpilicueta, 2001) y bacterias (Arena y Manca de Narda, 2001; Lonvaud-Funel, 2001; Constantini et al., 2009) encargadas de los procesos de fermentación. Además, otros factores como las condiciones en las que se produce el crecimiento de los microorganismos, las condiciones de pH, temperatura, oxigenación, etc. pueden influir en su contenido (Ancín-Azpilicueta et al., 2008).

La técnica más empleada hoy en día para el análisis y cuantificación de aminas biógenas en vinos es el HPLC acoplada a un detector espectrofotométrico (DAD), fluorimétrico (Fl) o a uno de espectrometría de masas (MS) (Anlı y Bayram, 2008). Pero la dificultad que implica la detección de estos compuestos a baja concentración en matrices complejas como los alimentos (García-Marino et al., 2010), ha hecho que se plantee el uso del método de derivatización pre o postcolumna. Los reactivos derivatizantes se utilizan para incrementar la sensibilidad de la detección y evitar interferencias (Önal, 2007; García-Marino et al., 2010).

Las funciones biológicas de las aminas biógenas son diversas, por ejemplo, las aminas putrescina, cadaverina, espermidina y espermina son indispensables para el crecimiento celular, la triptamina es un neurotransmisor y la histamina y la tiramina son mediadores de la inflamación. Sin embargo, dependiendo de la concentración, muchas de ellas pueden ejercer beneficios o perjudicar la salud. Las aminas biógenas más importantes por su efecto tóxico son la histamina y la tiramina. La ingesta de altas concentraciones pueden causar efectos adversos como dolor de cabeza, hipo- o hipertensión, nauseas, palpitaciones, intoxicación renal, hemorragia cerebral y hasta la muerte, dependiendo de la sensibilidad del individuo y de su capacidad de desintoxicación (Silla Santos, 1996; Shalaby, 1996).

La presencia de aminas biógenas en alimentos tiene un gran interés debido a los efectos negativos sobre la salud de los consumidores. En 2011, la Autoridad Europea de Seguridad Alimentaria (EFSA) publicó la opinión del Panel de Expertos de Riesgos Biológicos (BIOHAZ) sobre la evaluación del riesgo de la formación de aminas biógenas en alimentos fermentados. Este estudio reveló que la histamina y la tiramina son las aminas biógenas más tóxicas presentes en los alimentos y que para estimar la evaluación de riesgo son necesarios más estudios de estos compuestos (EFSA Journal, 2011b). El Reglamento (CE) 2073/2005 establece límites legales del contenido de histamina en los productos de la pesca para especies de pescados asociados a un alto contenido en histidina entre 100 – 200 mg kg^{-1} y para productos pesqueros procedentes de especies de pescados asociados a un alto contenido de histidina y madurados enzimáticamente en

salmuera entre 200 – 400 mg kg^{-1}. Sin embargo, hasta el momento, no se han establecido límites legales, dentro de la Unión Europea, para otros alimentos.

La melatonina es una indolamina muy relacionada estructuralmente con la triptamina, y con la que comparte precursor principal, el aminoácido triptófano. Al igual que las aminas biógenas, la melatonina se forma a partir del metabolismo de plantas y de microorganismos. Sin embargo, existe una gran diferencia entre la melatonina y el resto de aminas biógenas, y es que no se han descrito ningún efecto adverso debido a la presencia de melatonina en ningún alimento.

JUSTIFICACIÓN Y OBJETIVOS

En la relación alimentación-salud, la presencia de compuestos con actividad biológica en alimentos cobra especial relevancia. Cada vez más la evidencia científica avala los beneficios del consumo de alimentos con compuestos con actividad biológica. Más allá del valor calórico y del contenido en nutrientes, los alimentos proporcionan una gran variedad de sustancias con propiedades potencialmente saludables. Aumenta así el interés científico por conocer la identidad de estos compuestos bioactivos y la necesidad de disponer de métodos analíticos que permitan obtener datos fiables en cuanto al contenido en los diferentes alimentos. Además, la comprensión de su origen y formación permite establecer estrategias conducentes a obtener alimentos más saludables de gran interés para el elaborador de productos alimenticios.

Existen datos de que un consumo elevado de frutas y verduras aumenta la excreción urinaria de 6-sulfatoximelatonina, metabolito de la melatonina (Nagata et al., 2005). La melatonina es una molécula que ha dado lugar a una extensa bibliografía sobre sus propiedades biológicas por ser una hormona, presentar propiedades antioxidantes y tener una serie de efectos beneficiosos. Se ha verificado la capacidad de la melatonina de atrapar especies reactivas de oxígeno ($ONOO^-$; $NO^\cdot$; H_2O_2) y además, el metabolito que se forma tras su oxidación la N^1-acetil-N^2-formil-5-metosikinuramina (AFMK) neutraliza los radicales hidroxilo y se ha aislado en diversos tejidos (Hardeland y Pandi-Perumal, 2005). Su administración crónica a ratones reduce el estrés oxidativo asociado al envejecimiento (Nogues et al., 2006).

A diferencia de los compuestos polifenólicos, bioactivos ampliamente estudiados, las características de solubilidad de la melatonina permiten que atraviese bien las barreras fisiológicas. En la célula se ha encontrado en el citosol, núcleo, membranas celulares y mitocondria, es decir, está presente en aquellos lugares donde se producen las especies reactivas de oxígeno y el efecto de un antioxidante tiene mayor trascendencia (Hardeland et al., 2006).

Esta Tesis Doctoral es el resultado de la realización del Proyecto de Excelencia financiado por la Junta de Andalucía titulado "*Determinación de melatonina en uvas, vinos y otros alimentos de Andalucía*" (P07-AGR-02480). Al inicio del

presente trabajo, la presencia de melatonina en estos alimentos era una cuestión prácticamente inédita. Y por tanto, unos de los principales retos a afrontar fue disponer de métodos analíticos suficientemente sensibles para las concentraciones a analizar, específicos y reproducibles. La mayoría de los estudios se habían basado en el test ELISA, validado para fluídos biológicos (plasma y suero). No obstante, los alimentos presentan una gran complejidad y es preciso validar los métodos de análisis en estas matrices.

La presente Tesis Doctoral tiene como **principal objetivo** la determinación de melatonina en uvas y vinos de Andalucía y realizar su seguimiento a lo largo del proceso de vinificación. Se pretende evaluar posibles efectos sobre la salud determinando sus propiedades antioxidantes y el contenido de otras aminas que pudieran estar presentes en estos vinos.

Para alcanzar estos propósitos se plantaron las siguientes cuestiones, cuyas respuestas dieron lugar a los objetivos específicos y a las tareas a realizar de este trabajo de investigación:

1. ¿Cómo medir el contenido de melatonina en uvas y vinos?

El primer objetivo que se planteó es disponer de métodos analíticos validados para el análisis de melatonina en uvas y vinos. Por tanto, se necesita la puesta a punto del proceso de extracción y de métodos analíticos, sensibles y específicos. Se necesitan técnicas fiables para determinar el contenido en melatonina en uvas y vinos. Además, debe evitarse la alteración de la melatonina presente en la muestra y obtener una buena recuperación.

Se evaluarán diferentes métodos y se determinará la sensibilidad y especificidad para determinar melatonina en alimentos. El método Enzyme-Linked ImmunoSorbent Assay (ELISA) utilizado para fluidos biológicos será validado para muestras de vino. Pero, dado que el precio del kit es elevado, se utilizarán otros métodos como la cromatografía líquida con detector de fluorescencia (HPLC-FL), más comunes en los laboratorios de análisis de alimentos. Para garantizar la inequívoca identificación de la melatonina se empleará la técnica de espectrometría de masas (LC-MS/MS). En el Capítulo 1 se llevan a cabo todos los hitos planteados y, además, se analizan las muestras de vino.

2. ¿Dónde se encuentra la melatonina presente en las uvas? Y ¿Se modifica este contenido a lo largo del proceso de vinificación?

El segundo objetivo es estudiar en qué parte de la uva se encuentra la melatonina (piel, pulpa o semilla) y proponer técnicas de vinificación encaminadas a salvaguardar la concentración de melatonina en el producto final. Asimismo, se pretende evaluar cómo inciden las distintas etapas del proceso de vinificación sobre la concentración de melatonina desde el mosto al vino.

Se partirá de uvas cultivadas en el centro IFAPA *Rancho de la Merced* en condiciones perfectamente controladas. Se elegirán variedades de mesa y de vinificación (blancas y tintas), considerando aquellas más extendidas y de mayor importancia para Andalucía como las variedades autóctonas andaluzas, *Palomino Fino* y *Tintilla de Rota*. Se evaluará qué variedad es más productora de melatonina.

A partir de ellas se ensayarán vinificaciones en condiciones controladas siguiendo el proceso en etapas clave. En el caso de vinificación en tinto las etapas clave son: el mosto inicial, el prensado y el deslío. En el caso de vinificación en blanco: el mosto inicial, el desfangado y el deslío, al objeto de conocer la evolución de la melatonina durante dichas etapas. Este estudio nos podría dar idea de cómo mejorar el contenido de melatonina en los vinos para darles un valor añadido como producto. Además, se incluye el estudio del contenido de melatonina en vinos elaborados con crianza biológica (bajo velo de flor), por su relevancia en Andalucía (Capítulo 2).

3. ¿Podría *Saccharomyces* producir melatonina en las condiciones de fermentación? ¿Se comportan de la misma manera las diferentes cepas comerciales de uso frecuente en enología?

Según un artículo publicado en 1999, *S. cerevisiae* fue capaz de sintetizar melatonina durante su crecimiento en medio de cultivo y en presencia de precursores como triptófano y serotonina (Sprenger et al., 1999). Por tanto, el tercer objetivo se centra en comprobar si ciertas levaduras pueden sintetizar

melatonina durante la fermentación alcohólica. Existen diferentes cepas comerciales disponibles y resulta interesante estudiar su capacidad para producir melatonina. Las levaduras pueden ver afectado su metabolismo por las condiciones del medio y si se encuentran en fase de crecimiento celular o realizando la fermentación alcohólica. En el estudio se plantearán algunos de los factores que pueden afectar a la concentración de melatonina final. Para ello se seleccionarán cepas comerciales de la especie *Saccharomyces* y se estudiará si son capaces de formar melatonina en diferentes medios de cultivos (medio de cultivo YPD y en mosto sintético) que incluyen distintas concentraciones de azúcares reducidos (20 y 200 g L^{-1}) y la presencia o no del aminoácido precursor de la melatonina, el triptófano (Capítulo 3).

4. Además de la melatonina, ¿se producen otras aminas biógenas?

La melatonina y algunas aminas biógenas como triptamina o serotonina presentan gran similitud estructural. Estas comparten ruta biosintética debido a que todas ellas se sintetizan a partir del aminoácido triptófano. Por otra parte, la presencia de compuestos como histamina, tiramina, cadaverina, etc. puede afectar a las propiedades organolépticas de los vinos. Además, es importante que tener en cuenta que las aminas biógenas pueden afectan negativamente a la salud dependiendo de la sensibilidad del individuo. Por tanto, resulta necesario evaluar ambos aspectos: el aumento en el contenido de melatonina como bioactivo que dé un valor añadido al vino y el contenido en otras aminas biógenas con las que comparte precursor (Capítulo 4) que permita evaluar la seguridad del producto.

5. ¿Presentan la melatonina y compuestos relacionados actividad antioxidante con los métodos *in vitro*?

Con el fin de avanzar en el conocimiento de la actividad de este compuesto debido a su estructura, se propuso un estudio comparativo entre compuestos con la misma estructura indólica. Además de la melatonina, compuestos como la serotonina, triptamina, *N*-acetil-serotonina o el ácido indol-3-acético se encuentran de forma natural en animales y plantas pero su presencia en alimentos es poco estudiada. Con el fin de poder comparar entre ellos y con otros

compuestos de probada actividad biológica, como los compuestos fenólicos, presentes en vino, se realizará un estudio de la actividad antioxidante con métodos tradicionalmente usados para valorar el poder antioxidante de una matriz alimentaria (Capítulo 5).

En resumen, los **objetivos** a los que se ha llegado a la hora de realizar esta Tesis se pueden son:

1. Poner a punto y validación de un método de análisis sensible, específico y reproducible para la determinación de melatonina en vinos. Se realizará la evaluación de distintas técnicas: ELISA (Enzyme-Linked ImmunoSorbent Assay), cromatografía líquida de alta eficacia con detector de fluorescencia (HPLC-FL) y espectrometría de masas (LC-MS/MS) para el análisis de melatonina en vinos y el estudio de sus ventajas e inconvenientes.

2. Determinar melatonina en uvas cultivadas y en vinos producidos en Andalucía.

3. Estudiar la evolución de la melatonina a lo largo de la vinificación y plantear tratamientos y métodos de vinificación para aumentar su contenido en vinos.

4. Estudiar la síntesis de melatonina llevada a cabo por distintas cepas de la especie *Saccharomyces*.

5. Estudiar la evolución conjunta de la melatonina y las aminas biógenas para poder identificar procesos que mejoren la calidad de los vinos.

6. Comparar la actividad antioxidante entre compuestos indólicos y compuestos fenólicos presentes en los vinos.

DISEÑO EXPERIMENTAL

Con el propósito de cumplir los objetivos marcados en el apartado anterior se planteó el siguiente diseño experimental.

CAPÍTULO 1. MÉTODOS DE ANÁLISIS DE MELATONINA EN VINOS: PUESTA A PUNTO Y VALIDACIÓN.

Los viñedos donde se recolectaron las uvas y la bodega experimental donde se elaboraron los vinos pertenecen al centro "Rancho de la Merced" del Instituto de Formación Agraria y Pesquera de Andalucía (IFAPA). Este centro está situado en el término municipal de Jerez de la Frontera (Cádiz), posee un clima mediterráneo con influencias oceánicas, de inviernos templados y húmedos y veranos calurosos y muy secos, y está influenciado por fuertes vientos de levante y poniente.

Las uvas para elaborar los vinos monovarietales se recogieron en su madurez óptima durante la cosecha del 2007, y pertenecían a las variedades: *Cabernet Sauvignon, Jaen Tinto, Merlot, Palomino Negro, Petit Verdot, Prieto Picudo, Syrah* y *Tempranillo.* Las condiciones de vinificación fueron estrictamente controladas en la bodega experimental en tanques de 100 L de acero inoxidable y control de temperatura a 27º C. Los principales parámetros enológicos de los vinos se midieron en las distintas etapas de vinificación siguiendo los métodos oficiales (OIV, 1990).

Para la determinación de melatonina en los vinos se estudiaron 3 métodos analíticos:

- *ELISA (Enzyme-Linked ImmunoSorbent Assay):* se utilizó el Kit ELISA para la determinación de melatonina en fluidos biológicos (Ref. RE54021. IBL Immune-Biological Laboratorios, Hamburg, Germany) que sigue los principios básicos de los inmunoensayos competitivos.
- *Cromatografía líquida con detector de fluorescencia (HPLC-FL):* se puso a punto el método optimizando los parámetros cromatográficos y los de detección. Y se validó la técnica.
- *Cromatografía líquida acoplada a un espectrómetro de masas (LC-MS/MS):* se puso a punto el método cromatográfico (basado en el de HPLC-FL) y se

optimizaron los parámetros del detector. Se utilizó un método en modo *Multiple Reaction Monitoring* (MRM), basado en la preconcentración del analito teniendo en cuenta una transición seleccionada entre dos de sus iones. En el caso de la melatonina se tuvo en cuenta las transiciones (233/216 y 233/174). Esta técnica se puso a punto durante una estancia de tres meses en el grupo de Calidad, Seguridad y Bioactividad de Alimentos Vegetales del Centro de Edafología y Biología Aplicada del Segura CEBAS-CSIC (Murcia), bajo la dirección del Dr. Ángel Gil Izquierdo.

La *validación del método* consistió en la evaluación de varios parámetros del método: la linealidad de la respuesta del detector frente a la concentración, los límites de detección y cuantificación, la precisión (repetitividad y precisión intermedia) (Huber, 1998), la exactitud (estudios de recuperación) (Harris, 1995), y la resolución del pico cromatográfico (Schoenmakers, 1988).

CAPÍTULO 2. DETERMINACIÓN DE MELATONINA EN UVAS, VINOS Y DURANTE EL PROCESO DE VINIFICACIÓN EN BLANCO Y TINTO.

Las muestras utilizadas en este trabajo fueron: trece variedades de uva, nueve variedades de uva para vinificación (*Cabernet Sauvignon, Merlot, Nebbiolo, Palomino Fino, Pedro Ximenez, Petit Verdot, Syrah, Tempranillo* y *Tintilla de Rota*) y cuatro variedades de uva de mesa (*Flame Seedless*, *Moscatel Itálica*, *Red Globe* y *Superior Seedless)*, recosechadas durante la vendimia de 2008. Todas ellas procedentes del Centro IFAPA "Rancho de la Merced".

Los vinos tintos se elaboraron con las variedades de uva *Cabernet Sauvignon, Merlot, Syrah, Tempranillo* y *Tintilla de Rota*. Y para el vino blanco se utilizó la variedad *Palomino Fino*. A continuación se muestran las distintas etapas del proceso de vinificación a escala piloto de los vinos tintos y blancos (Figuras 6 y 7):

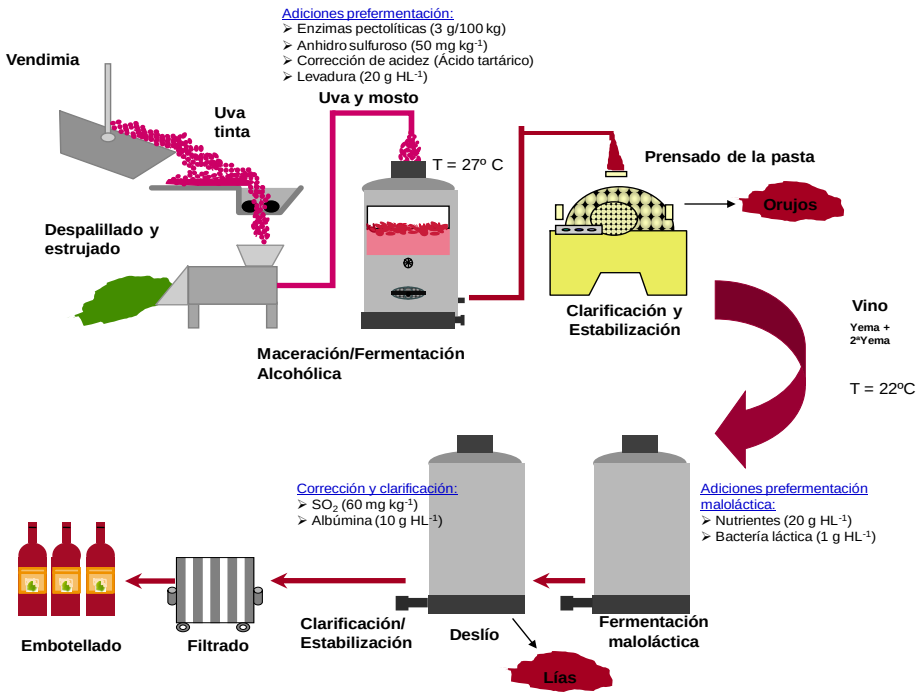

Figura 6. Procesos de la vinificación de vino tinto (cedida por el Dr. R.F. Guerrero).

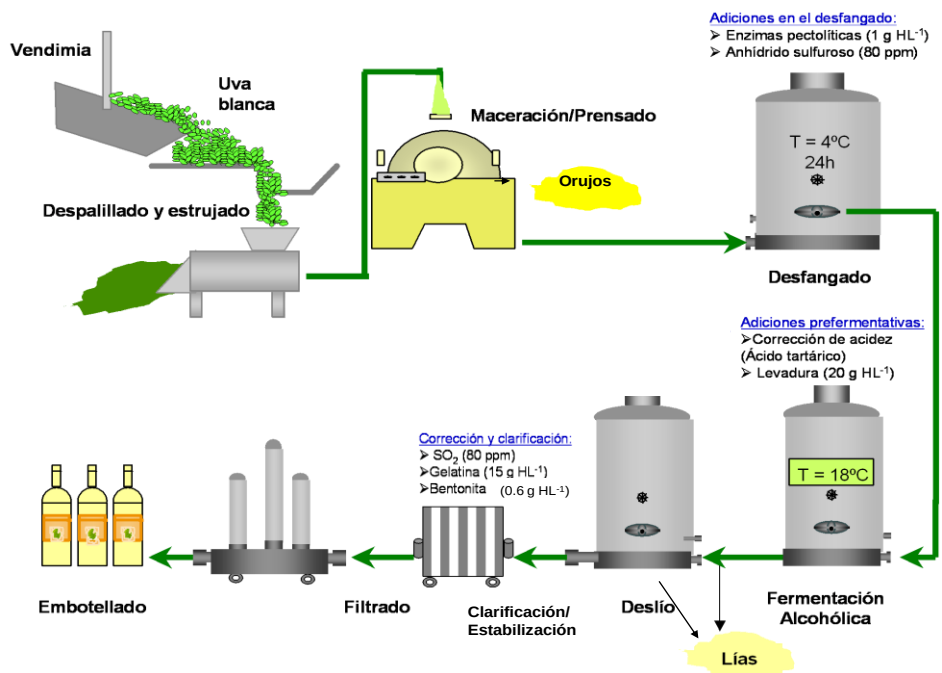

Figura 7. Procesos de la vinificación de vino blanco (cedida por el Dr. R.F. Guerrero).

Durante las vinificaciones se tomaron muestras para determinar la melatonina en el mosto inicial y en las etapas de prensado y deslío de vinos tintos. Igualmente, en el mosto y en las etapas de desfangado y deslío del vino blanco. En los vinos finales (embotellado) se analizaron los parámetros enológicos: etanol, acidez total, pH, acidez volátil, índice de polifenoles totales y azúcares (OIV, 1990).

Después, se llevó a cabo una nueva vinificación con uvas obtenidas en el mercado de la variedad *Alpha red* para el estudio la evolución de la melatonina en el proceso de fermentación alcohólica. Las vinificaciones se realizaron a escala de laboratorio en tres tanques de 10 L de acero inoxidable en condiciones estrictamente controladas. Para comprobar la influencia de la concentración de triptófano sobre el contenido de melatonina, se adicionó 60 mg Kg^{-1} de triptófano a dos de los tanques y el tercero sirvió de control. En este caso se analizó la presencia de melatonina desde el mosto hasta el final de la fermentación alcohólica (días 1, 2, 5, 6 y 7).

Por último, se analizaron un total de veintitrés vinos blancos procedentes de crianza biológica (velo de flor): doce muestras de vino embotelladas y once muestras de vino muestreadas directamente de botas con velo de flor (sin filtrar), todas ellas suministradas por los Consejos Reguladores de Condado de Huelva, Jerez y Montilla-Moriles.

CAPÍTULO 3. ESTUDIO DE LA PRODUCCIÓN DE MELATONINA POR CEPAS DE *SACCHAROMYCES*.

Este trabajo se desarrolló en el Departament de Bioquímica i Biotecnologia de la Facultat d'Enologia de la Universitat Rovira i Virgili (Tarragona) bajo la dirección de los Dres. Albert Mas y Mª Jesús Torija.

Se tomaron doce levaduras comerciales y tras su realizar su tipificación con la técnica de RFLP del ADN mitocondrial (Querol et al., 1992), se verificó que sólo ocho eran distintas. Para el estudio se utilizaron estas las ocho cepas de *Saccharomyces* que resultaron ser distintas: seis *S. cerevisiae* (CLOS, ICV-D254,

QA23, ARM, RVA, TTA), una *S. uvarum* (S6U) y una *S. cerevisiae* var. *bayanus* (Uvaferm BC), todas ellas comerciales.

La técnica de tipificación de levaduras nombrada se ha empleado durante el transcurso de todo el proceso experimental para la identificación y comprobación de las distintas cepas de levadura. Para cada una de las muestras tomadas se analizó la imposición de la levadura correspondiente por la técnica de RFLP del ADN mitocondrial y se realizó la medida del crecimiento de la levadura por contaje de viables en placas de medio YPD sólido.

Todos los análisis de melatonina se hicieron con el método de LC-MS/MS puesto a punto (Rodriguez-Naranjo et al., 2011).

Medios: se usaron distintos medios de crecimiento de las levaduras para comprobar si las levaduras producían melatonina en estas condiciones. Los medios utilizados fueron:

i) Yeast extract pentose dextrose (YPD) con dos concentraciones de triptófano distintas: normal (1x) y cinco veces mayor al normal (5x).

ii) Mosto sintético (SM) (Riou et al., 1997) con modificación de la concentración de azúcares reductores (glucosa:fructosa, 1:1) a 20 g L^{-1} y con tres concentraciones diferentes de triptófano (0, 1x y 5x).

Se crecieron las cepas de levaduras QA23, ARM y S6U en estos cinco medios distintos. Se recogió muestra del medio después de 0, 1, 2, 4, 6 y 24 horas de inocular los medios, y se determinó el contenido de melatonina en cada uno de elllos.

Monitorización de la producción: se llevó a cabo el seguimiento de la producción de melatonina por la cepa QA23 en los medios YPD y SM (20 g L^{-1} de azúcares reductores y 1x Trp). Se muestreó cada hora, de 0 a 12 horas, en YPD y cada 2 horas, de 0 a 46 horas en SM, y se determinó el contenido de melatonina en el medio.

Fermentación alcohólica: se realizaron ocho fermentaciones (por triplicado) utilizando cada una de las cepas seleccionadas para este estudio. La fermentación

se llevó a cabo en medio SM con 200 g L^{-1} de azúcares reductores y 5x Trp. Se tomó muestra cada día desde el primer día hasta el final de la fermentación alcohólica.

CAPÍTULO 4. SEGUIMIENTO DEL CONTENIDO DE AMINAS BIÓGENAS Y MELATONINA DURANTE LA VINIFICACIÓN EN TINTO Y BLANCO.

Las muestras analizadas pertenecen a la cosecha de 2010 y se elaboraron en el centro IFAPA "Rancho de la Merced". Las variedades *Merlot, Syrah, Tempranillo* y *Tintilla de Rota* se utilizaron en vinos tintos y *Palomino Fino* en el vino blanco, y se siguieron las etapas de los procesos de vinificación mostradas anteriormente. Las etapas analizadas fueron: mosto inicial, a mitad de fermentación alcohólica (densidad 1020 g L^{-1}), prensado, deslío y filtración para vino tinto y prensado, desfangado, mitad de fermentación alcohólica (densidad 1020 g L^{-1}) y deslío para vino blanco.

La *melatonina* se determinó utilizando el método de LC-MS/MS (Rodriguez-Naranjo et al., 2011) mencionado anteriormente.

Aminas biógenas: se analizaron diez aminas biógenas en las diferentes etapas de las cinco vinificaciones (agmatina, cadaverina, histamina, metilamina, 2-feniletilamina, putrescina, espermidina, espermita, tiramina y triptamina) (Anexo I). Se utilizó un método de análisis de aminas biógenas que incluye una extracción previa en fase sólida (SPE), seguida de una derivatización pre-columna y análisis por HPLF-FL (Peña-Gallego et al., 2009). El reactivo utilizado para la derivatización es el 6-aminoquinolil-*N*-hidroxisuccinimidil carbamato (ACQ) (AccQ Fluor reagent kit, Waters, Milford, MA, USA).

CAPÍTULO 5. ESTUDIO COMPARATIVO DE LA ACTIVIDAD ANTIOXIDANTE DE LA MELATONINA Y OTROS COMPUESTOS INDÓLICOS.

Para este estudio se utilizaron trece patrones de compuestos indólicos: ácido 3-indolacético, ácido 5-hidroxi-3-indolacético, ácido 5-metoxi-3-indolacético, melatonina, *N*-acetil-5-hidroxitriptamina, serotonina, triptamina, triptófano, triptofol, 5-hidroxitriptófano, 5-metoxitriptamina, 5-metoxitriptofol y 6-metoximelatonina (Anexo II). Todos ellos compuestos están relacionados estructuralmente y/o son intermediarios en las diferentes rutas sintéticas de la melatonina.

Los métodos *in vitro* empleados para la determinación de la actividad antioxidante fueron:

- *FRAP (Ferric Ion Reducing Antioxidant Power*): método descrito por Benzie y Strain (1996).
- *Método DPPH (Diphenyl-1-picrylhydrazyl*): proceso descrito por Sánchez-Moreno et al. (1998).
- *Método ORAC (*Oxygen Radical Absorbance Capacity): Publicado por Ou et al. (2001).

Además, el método DPPH sirvió para determinar los parámetros cinéticos (efectividad antirradical AE, la EC_{50} y la T EC_{50}) y la estequiometría de la reacción (n_T), y con el que se realizó un estudio detallado de la cinética de la reacción.

RESULTADOS

CAPÍTULO 1.
MÉTODOS DE ANÁLISIS DE MELATONINA EN VINOS: PUESTA A PUNTO Y VALIDACIÓN.

MELATONIN: A NEW BIOACTIVE COMPOUND IN WINE.
M. Isabel Rodriguez-Naranjo, Ángel Gil-Izquierdo, Ana M. Troncoso, Emma Cantos y M. Carmen Garcia-Parrilla

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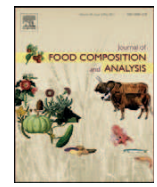

Original article

Melatonin: A new bioactive compound in wine

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ABSTRACT

Melatonin (N-acetyl-3-(2-aminoethyl)-5-methoxyindole) is biologically active as a neurohormone and a chronobiotic and antioxidant agent. Its concentration in plant material and foods is usually determined by ELISA. However, commercial ELISA kits are not validated for those matrixes. This paper aims to accurately detect melatonin in wines. The advantages and pitfalls of the methods currently used to assay melatonin in wines (ELISA, LC-fluorescence and LC-ESI-MS/MS) are presented. The LC-FL method was validated as reliable for the quantitative analysis of MEL in wine samples that met AOAC requirements: LOD = 51.72 ng/mL; LOQ = 172.39 ng/mL; intraday accuracy as RSD = 0.35% and interday accuracy as RSD = 13.46%. The linearity showed a correlation coefficient of 0.9999, and peak resolution ranged from 0.96 to 1.52. Melatonin in wines was identified by LC-ESI-MS/MS, comparing its MS and MS2 spectra with its corresponding authentic commercial marker. LC-ESI-MS/MS revealed another compound with an identical fragment pattern (positive-mode ESI) but a different retention time as melatonin. Major mass fragmentation ions were (*m/z*) 216 and 174, tentatively identified as a melatonin isomer not previously described in wines. This compound appears in certain monovarietal wines (*Jaen Tinto*, *Merlot* and *Palomino Negro*). Only melatonin is present in others (*Petit Verdot* and *Syrah*), and a third group contains both melatonin and the new compound (*Cabernet Sauvignon*, *Prieto Picudo* and *Tempranillo*).

1. Introduction

Melatonin (N-acetyl-3-(2-aminoethyl)-5-methoxyindole) (MEL) is an indoleamine synthesized from L-tryptophan metabolism via serotonin. It is considered a neurohormone, a chronobiotic and an antioxidant (Van Tassel and O'Neill, 2001; Kolar and Machackova, 2005; Posmyk and Janas, 2009). In vertebrates, MEL is produced mainly by the pineal gland and secreted into the blood stream in a rhythmic manner. MEL levels are higher in young people and diminish gradually with age (Cardinali et al., 2008). MEL has been found in many of the organs of higher plants, including the leaves, fruits and seeds, at concentrations from picograms to nanograms per gram of tissue (Burkhardt et al., 2001; Reiter et al., 2005; De la Puerta et al., 2007; Paredes et al., 2009). Its function in plants is not well known yet. Its chemical structure is similar to that of indole-3-acetic acid (IAA), which suggests that MEL could have a role as a growth factor (Posmyk and Janas, 2009). Because of its presence in vegetable tissue, MEL has been evaluated as a food component (Garcia-Parrilla et al., 2009) with biological activity. To determine its contribution as a bioactive compound, suitable methods must be developed and validated for its quantitative analysis in food.

MEL can be determined by high-performance liquid chromatography (HPLC) with fluorescence (FL) detection (Arnao and Hernández-Ruiz, 2007; Mercolini et al., 2008); gas chromatography with mass spectroscopy (GC–MS) (Van Tassel et al., 2001); mass spectroscopy (MS) (Dubbels et al., 1995; Yang et al., 2002); radioimmunoassay (RIA) (Van Tassel et al., 2001); enzyme-linked immunosorbent assay (ELISA) (Iriti et al., 2006; Kollmann et al., 2008; Maldonado et al., 2009) and immunoprecipitation (Pape and Luening, 2006).

In recent years, MEL has been found in eight grape varieties using ELISA (Iriti et al., 2006). However, only one published study concerns the MEL content in wine (Mercolini et al., 2008). The present work aims to determine the presence of MEL in wines

Abbreviation: MEL, melatonin (N-acetyl-3-(2-aminoethyl)-5-methoxyindole).

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produced from common grape varieties. Analytical techniques previously used to assess MEL in plant material were tested: ELISA and HPLC with FL. An accurate identification was achieved by LC-ESI-MS/MS with the application of suitable conditions. The MEL content and the possible occurrence of MEL isomers were explored in different monovarietal wines.

2. Materials and methods

2.1. Chemicals and reagents

N-acetyl-3-(2-aminoethyl)-5-methoxyindole (MEL) standard was purchased from Sigma (Ref. M5250), N-acetyl-3-(2-aminoethyl)-6-methoxyindole (isomer 2) from Wako (Ref. 328-24313), methanol from Merck (Darmstadt, Germany) and formic acid from Panreac (Barcelona, Spain). Solutions were prepared by dilution with Milli-Q water. All reagents were of analytical grade.

2.2. Samples

The grape cultivars and the winery that produced the wines for this study belong to the Instituto de Investigación y Formación Agraria y Pesquera (IFAPA, Jerez de la Frontera, Spain). Grapes were harvested at their optimum maturity (data not shown) during the 2007 vintage. The varieties studied were: *Cabernet Sauvignon*, *Jaen Tinto*, *Merlot*, *Palomino Negro*, *Petit Verdot*, *Prieto Picudo*, *Syrah* and *Tempranillo*. Of the above, only *Jaen Tinto* and *Palomino Negro* are produced locally; the others are common in Spain. Winemaking conditions were strictly controlled at 25 °C. Table 1 provides the main enological parameters of the wines (OIV, 1990).

2.3. Sample preparation

The ELISA kit provides C18 SPE cartridges for extracting MEL from samples, standards and controls. Briefly, the cartridges were conditioned with 2 mL of methanol and 2 mL of distilled water, and 0.5 mL of wine was placed in a cartridge. The cartridge was then washed with 2 mL 10% methanol/water. MEL was recovered with 1 mL of methanol, which was evaporated to dryness. Samples, standards and controls were reconstituted with 0.15 mL assay buffer.

Wine sample treatment prior to LC-fluorescence analyses consisted of an extraction with C18 SPE cartridges using the procedure previously described for the ELISA test. The reconstituted extract was filtered through a 0.45-μm syringe filter (13 mm Millex®-LCR syringe filter, 0.45 μm, Ref. SLCR O13 NL) before injection.

For LC-ESI-MS/MS, wine samples were previously dried and re-diluted in methanol:water (1:1), up to a 3:1 concentration. The reconstituted extracts were passed through 0.45-μm filters before the LC-ESI-MS/MS analyses.

A total of eight monovarietal wines were analyzed in duplicate by ELISA, and in triplicate by the other methods.

Table 1
Enological parameters such as ethanol, total acidity (TA), pH, volatile acidity (VA), total polyphenols index (TPI) and sugars, were measured according to official methods (OIV, 1990).

Wine sample	Ethanol (vol.%)	TA (g/L TH_2)	pH	VA (g/L AcH)	TPI	Sugars (g/L)
Cabernet Sauvignon	13.80	5.82	3.56	0.60	41.31	2.65
Jaen Tinto	9.47	5.32	3.41	0.34	21.8	2.53
Merlot	13.63	4.72	3.65	0.31	47.40	3.15
Palomino Negro	9.62	5.11	3.70	0.35	15.28	2.45
Petit Verdot	13.50	5.15	3.89	0.74	68.70	3.30
Prieto Picudo	13.86	4.68	3.74	0.73	49.37	2.31
Syrah	13.78	4.34	3.83	0.55	48.64	3.21
Tempranillo	12.88	4.20	3.70	0.80	23.83	2.82

TA: total acidity expressed as g/L tartaric acid (TH_2); VA: volatile acidity expressed as g/L acetic acid (AcH); TPI: total phenolic index.

2.4. ELISA

MEL was measured using a commercial ELISA kit (Ref. RE54021, IBL Immune-Biological Laboratories, Hamburg, Germany), which includes a microtiter plate with wells coated with goat polyclonal melatonin antiserum and goat anti-biotin antibodies conjugated to alkaline phosphatase. Standards and controls with certified precise concentrations, assay buffer (phosphate buffer with Tween and stabilizer), substrate PNPP (*p*-nitrophenyl phosphate) tablets and PNPP stop solution containing 1 M NaOH with 0.25 M EDTA (ethylene diamine tetra-acetic acid) were used.

The ELISA test is based on the principle of competition for the binding sites of the antibodies coated on the wells between an unknown amount of MEL in the sample and a fixed amount of enzyme-labeled antigen. Incubation times, shaking processes and temperatures were set according to the directions provided in the kit. Finally, absorbance was recorded at 405 nm.

The supplier provides the kit's analytical sensitivity, expressed as a limit of detection of 1.6 pg/mL and intra-assay and inter-assay precision values ranging from 3% to 11.4% and from 6.4% to 19.3%, respectively.

Instruments required to perform ELISA are a SPE vacuum manifold (Supelco), an orbital shaker (Bühler), an evaporator centrifuge (Speed-Vac) and a microtiter plate reader (Biotek) to read absorbance at 405 nm.

2.5. LC-fluorescence

The HPLC apparatus used was a Waters 600E system controller connected to a Waters 474 scanning fluorescence detector. Data treatment was performed in a Waters Millennium 2.0 data station. Separations were obtained on a Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase comprised formic acid/water 0.1% (A) and methanol (B), with 40% of A and 60% of B, and the isocratic flow was 0.8 mL/min for 9 min. The mobile phase was first degassed in an ultrasonic bath. The injected volume was 10 μL.

The fluorescence detector recorded wavelengths of 285 nm for excitation and 345 nm for emission.

2.6. LC-ESI-MS/MS for qualitative analyses of melatonin and its isomers in wine

Chromatographic separation was performed using an Agilent 1200 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a mass detector. The HPLC system consisted of a binary capillary pump (G1376A), a degasser (G1379B), an autosampler (G1377A), a sample cooler (control temperature 4 °C) (G1330B) and a photodiode array detector (G1315D) controlled by Chemstation software (v.B.0103-SR2). The column used was an Agilent 150 mm × 0.5 mm i.d., 5-μm Zorbax Sb-18 column (DE48102564). Chromatographic separation was achieved using a binary gradient consisting of (A) water and (B) methanol as HPLC grade solvents, both containing 0.1% formic acid (v/v). The elution profile was: 40% B (2 min), 85% B (4 min), 90% B (9 min). The flow rate was 10 μl min^{-1}. The injection volume was 3 μl.

The mass detector was a Bruker (Bremen, Germany) ion trap spectrometer equipped with an electrospray ionization (ESI) system and controlled by Bruker Daltonics Esquire *m/z* software (v.6.1). The nebulizer gas was nitrogen. The pressure and the flow rate of the dryer gas were set at 11 psi and 5 L/min respectively.

The full scan mass covered a range from 100 to 250. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 to 2 V. The heated capillary and voltage were maintained at 350 °C and 4.275 kV, respectively. Some parameters have an important effect on sensitivity. Thus, different analytical conditions were applied to optimize MEL detection.

To optimize the ESI source, flow injection analyses (FIA) with a MEL standard (5 μM) and different nebulizer and dryer gas flow and temperatures were tested. The optical MS and trap parameters were optimized by direct infusion to the ESI source. The automatic optimization option of the MS for the standard was used. The common parameters for MEL were as follows: capillary voltage, −4275 V; skimmer, 40 V; capillary exit, 108.5 V; octopole 1 DC, 12 V, octopole 2 DC; 1.7 V; octopole RF, 138.8 Vpp; Lens 1, −5 V and Lens 2, −60 V. MS data were acquired in the positive mode. The MS data obtained were compared with the authentic markers to produce a final MEL identification and investigate another MEL isomer present in wine. In this manner, the major and minor fragment ions during an MS2 event play a relevant role by measuring their corresponding relative abundances. Previous studies have remarked the importance of relative abundances of fragment ions to identify position isomers in different compounds (Ferreres et al., 2004, 2007).

2.7. LC-ESI-MS/MS for quantitative analyses of MEL and its isomers in wine

The LC-ESI-MS/MS conditions for quantitative analyses were the same as those described above. However, we compared the different MS analyzing modes to determine the best sensitivity and reproducible conditions. We tested multiple reaction monitoring (MRM) and selected ion monitoring (SIM) modes. MRM was tested on some of the MS/MS transitions (233/216, 233/174) of the corresponding ions, and we concluded that MRM was a better mode for collecting higher MEL signal intensities. The area of the 233/216 transition was selected to quantify MEL because of its specificity and its better signal-to-noise ratio. MEL standard external calibration was used to quantify MEL and its isomer in wines.

3. Results and discussion

3.1. ELISA

Although the melatonin ELISA kit has been validated for biological samples and used to determine MEL in food, it has not been validated for use in food samples. Because it has been previously used for grapes and beer (Iriti et al., 2006; Maldonado et al., 2009), we used this kit to assay MEL in wines. Table 2 shows the results obtained with the ELISA test. As can be seen, *Palomino Negro* presented the highest MEL content. *Cabernet Sauvignon*, *Jaen Tinto*, *Merlot*, *Petit Verdot*, *Prieto Picudo* and *Syrah* showed similar concentrations of MEL, ranging from 162 to 230 pg/mL.

Table 2
MEL concentration determined by ELISA in the eight red wines.

Wine sample	MEL pg/mL (SD)
Cabernet Sauvignon	226.5 (9.2)
Jaen Tinto	162.0 (8.5)
Merlot	212.0 (15.6)
Palomino Negro	277.5 (2.1)
Petit Verdot	224.5 (6.4)
Prieto Picudo	192.5 (10.6)
Syrah	215.0 (21.2)
Tempranillo	140.5 (9.2)

Data calculated as mean ($n = 2$); standard deviation (SD) indicated in parentheses.

Table 3
Optical density (at 405 nm) obtained by ELISA for different wine dilutions. Undiluted wine samples and diluted wine samples at 1:100, 1:10 000, and 1:1000 000 were assayed.

Wine sample	Optical density (OD)			
	Undiluted	1/100	1/10 000	1/1 000 000
Cabernet Sauvignon	0.37	0.27	0.37	0.36
Jaen Tinto	0.51	0.27	0.34	0.29
Merlot	0.39	0.41	0.39	0.35
Palomino Negro	0.30	0.27	0.44	0.32
Petit Verdot	0.39	0.37	0.33	0.31
Prieto Picudo	0.43	0.62	0.35	0.30
Syrah	0.39	0.33	0.44	0.35
Tempranillo	0.59	0.43	0.34	0.32

To validate the ELISA assay for MEL in wine samples, we aimed to establish the linear range of measurement. Undiluted wine samples and wine samples diluted at 1:100, 1:10 000, and 1:1000 000 were assayed using the ELISA kit. Similar responses were achieved with very different dilutions of wines (Table 3). Additionally, we spiked wine with MEL standard to reach final concentrations between 10 and 300 pg/mL, within the range of linearity described by the ELISA kit. However, similar absorbance values were recorded with wine spiked with very different MEL concentrations, and optical density (OD) values ranged from 0.18 to 0.21 in every case (data not shown). These results did not fulfill requirements for linearity. Therefore, no linear response was obtained with either standard addition or sample dilution.

Wine, like other plant materials, is a complex matrix with primary and secondary metabolites; in many cases, these molecules may interfere with ELISA determinations due to their capacity to either mimic MEL or cross-react within immunoassays (Paredes et al., 2009). This can result in false-positives (Hernández-Ruiz and Arnao, 2008). According to Horwitz (Horwitz, 1982), the appearance of either false positive or false negative results were typical of trace analysis. Concentrations determined by ELISA can be considered within this category. In fact, among the replicates with the solution provided by the ELISA kit (MEL = 0.00 pg/mL), we determined a 25.0% of false-positive rate and a 28.6% of false-negative rate. Thus, the main drawbacks of the ELISA test are a lack of linearity, false positive and false negative results and the relatively high price of the kit. Indeed, the ELISA kit could not be validated for accurate MEL quantitation in wines.

3.2. LC-fluorescence

Fig. 1 shows a chromatogram recorded for a wine sample spiked with MEL standard to reach a 232 ng/mL concentration. LC-FL results show that MEL standard t_R is 5.4 min.

Linearity was assessed with standard solutions at concentrations ranging from 58 to 5807 ng/mL of MEL standard and performed in triplicate. Calibration curves were obtained by plotting the peak areas against different MEL concentrations. A good correlation coefficient ($r = 0.9999$) was achieved.

Limits of detection (LOD) and quantitation (LOQ) were obtained as 3 and 10 times the signal-to-noise ratio, respectively (Huber, 1998); LOD (S:N = 3) was 51.72 ng/mL, and LOQ (S:N = 10) was 172.39 ng/mL. These data contrast with those obtained by other authors (Mercolini et al., 2008), who achieved lower LOD and LOQ (0.01 and 0.03 ng/ml respectively).

Both repeatability and reproducibility were determined by analyzing four different concentrations of the standard (58, 202, 581, 2020 ng/mL). Ten replicates in one working session were performed to calculate intraday repeatability. Analyses were

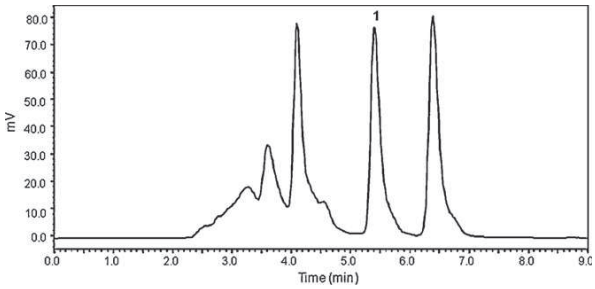


Fig. 1. HPLC-FL chromatographic profile recorded for a wine sample spiked with MEL standard (peak 1) up to 232 ng/mL concentration.

performed during six different working sessions in one week to calculate intermediate interday accuracy. Both values are expressed as RSD (%). Results were 0.35% for intraday and 13.46% for interday accuracy. Both are acceptable according to the AOAC (Huber, 1998).

Accuracy was evaluated by means of recovery assays. The standard addition method (MOSA) (Harris, 1995) to wine samples (*Tempranillo* and *Palomino Negro*) was performed with MEL concentrations ranging from 58 to 5807 ng/mL. Recovery = $(b_{MOSA}/b_{EC}) \times 100$, where b is the slope of the calibration curve obtained with the spiked wine; MOSA is the method of standard addition and EC is external calibration. The recoveries (%) of *Tempranillo* and *Palomino Negro* were 84.70% and 83.34%, respectively.

MEL peak resolution in wine was determined by adding MEL standard solutions to the wine samples. Resolution was calculated as $Rs = 2\Delta t/(W1 + W2)$, where Δt is the difference in retention times between the two peaks and W1 and W2 are the widths of the two peaks (in time units) (Schoenmakers, 1988). The MEL peak resolution in wines ranged from 0.96 to 1.52 for the entire set of samples. These data meet the AOAC requirements.

The LC-FL method used was validated as reliable for the quantitative analysis of MEL in wines samples, following AOAC recommendations (Huber, 1998).

The LOD of MEL in LC-FL was 51.72 ng/mL. However, the LOQ was not low enough to quantify MEL in wines, so a prior-step sample treatment was required. An SPE extraction procedure was applied to concentrate the sample, and its recovery was 98%. Mercolini et al. (2008) used an SPE to extract MEL in wines and determined 0.04 ng/mL of MEL in Trebbiano wine. They reported a recovery higher than 90% and achieved a LOD of 0.01 ng/mL, which is lower than ours. These differences are probably due to their higher injection loop volume (100 μL) whereas our loop volume was 10 μL. Moreover, they determined LOD using peak height instead of peak area as in the present study. However, they carried out the quantitation using peak area. In our opinion, area should be used in LOD calculation if this is the parameter involved in the quantitation procedure.

Table 4
MS fragmentation ions of $[M+H]^+$ at m/z 233 of melatonin, and two isomers.

Nature of sample	Compound	Retention time	216	174	191	161	159	141	146	203	130
Authentic marker	N-acetyl-3-(2-aminoethyl)-5-methoxyindole (MEL)	7.8	X	X	X	X	X	X	X	X	X
Authentic marker	N-acetyl-3-(2-aminoethyl)-6-methoxyindole (isomer 2)	7.5	X	X	X	nd	X	X	X	X	X
Wine	N-acetyl-3-(2-aminoethyl)-5-methoxyindole	7.8	X	X	X	X	X	X	X	X	X
Wine	Melatonin isomer (ISO)	5.1	X	X	nd	nd	nd	X	X	X	X

X: detected; nd: not detected.

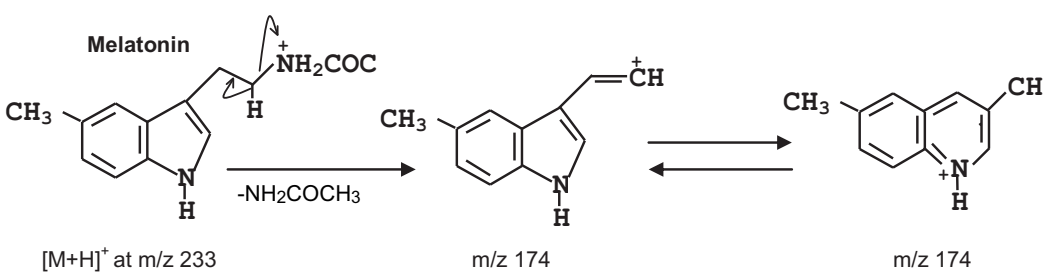
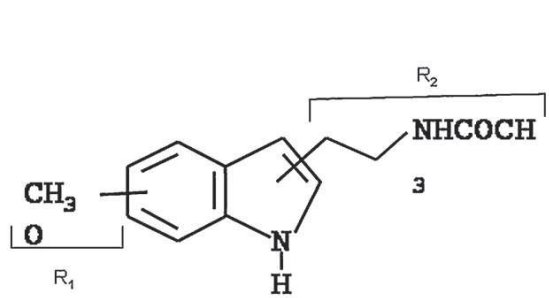


Fig. 2. Main MS2 fragmentation of melatonin and aromatic stability of its fragment ion at m/z 174.



Compound	Substituent position	
	R_1	R_2
Melatonin	5	3
Isomer 1	4	3
Isomer 2	6	3
Isomer 3	4	2
Isomer 4	5	2
Isomer 5	6	2
Isomer 6	7	2
Isomer 7	6	1
Isomer 8	5	4

Fig. 3. Melatonin structure and its possible isomers.

3.3. Qualitative analyses of MEL and its isomer in wine with LC-ESI-MS/MS

Three type samples, two authentic commercial markers (MEL and Isomer 2) and several types of monovarietal wines were tested with LC-ESI-MS/MS (Table 4). The MEL standard MS2 event showed two main fragments at m/z 216 and 174 (base peak). Such fragment types are found in melatonin; 174 originated from the molecular ion by the cleavage of the amide substituent at 3, and 216 was detected as a result of water loss from $[M+H]^+$ (Diamantini et al., 1998; Almeida et al., 2004). The ion at m/z 174 was the main base peak as a result of its aromaticity, which produces stability (Fig. 2). As the authentic maker is not available to identify the isomer by MS2 or MS3 fingerprint, ion trap data is appropriate for tentative identification by comparing relative abundance of minority fragments ions to identify position isomers (Ferreres et al., 2004, 2007). However, the total identification of a novel compound requires the isolation and NMR identification in addition to ion trap mass spectrometer.

MEL is able to present up to 9 isomers (including MEL itself). Those losses are common in all types of MEL isomers (Fig. 3) (Diamantini et al., 1998). For example, Isomer 2 presented those two main fragments, according to Table 4. In addition to those ions, seven minor fragment ions were detected in MEL standard. However, those fragments were not present in all types of isomers and, according to previous studies, their relative abundances may reveal the position of methoxyl and amide groups on the main skeleton (Table 4 and Fig. 3) (Diamantini et al., 1998). The major and minor fragments of MEL coincided with the data presented in the aforementioned report; however, the relative abundances cannot be applied to our study because they were obtained by electron ionization mass spectrometry and mass-analyzed ion kinetic energy spectrometry (MIKES). However, this is not a triple quadrupole neither an ion trap mass spectrometer. For the quantitation goals, the triple quadrupole mass spectrometer provides the best results, but ion trap results are also valid when trap conditions are properly adjusted as it is our case (very accurate LOQ = .0.13 ng/mL and LOD = 0.44 ng/mL).

Tempranillo and *Jaen Tinto* wines were selected for the identification of MEL and MEL isomers because they showed the highest intensities for the compounds analyzed. Wines showed the same main fragment ions from m/z 233 (174 and 216) and the same minor fragment ions at the same retention time as MEL standard; MEL was therefore detected in wine (Table 4). Another compound (ISO) at m/z 233 was detected at 5.1 min of retention time (Fig. 4). It showed 174 (base peak) and 216, just as the main fragments but the minor fragments did not totally coincide with those found in MEL (191, 161 and 141 were not detected) (Table 4). Previous studies and Isomer 2 data demonstrated the importance of 174 as the main fragment ion, and the relative abundances of minor fragment ions may show the position of methoxyl and amide groups (Table 4) (Diamantini et al., 1998). This compound may be tentatively identified as a MEL isomer or a related compound. Previous reports described a typical oxidation product of MEL known as N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) (Almeida et al., 2004; Rosen et al., 2006). This type of compound and other oxidized compounds from MEL have never been described in wine, and they present parent ions similar to those of MEL on MS (Almeida et al., 2004; Rosen et al., 2006).

3.4. Quantitative analyses of MEL and its isomer in wine with LC-ESI-MS/MS

To quantify MEL in wines, LC-MS/MS was performed, using MRM mode to record transitions 233/216, based on MEL standard. Under LC-MS/MS conditions, MEL standard eluted at 7.8 min. MEL

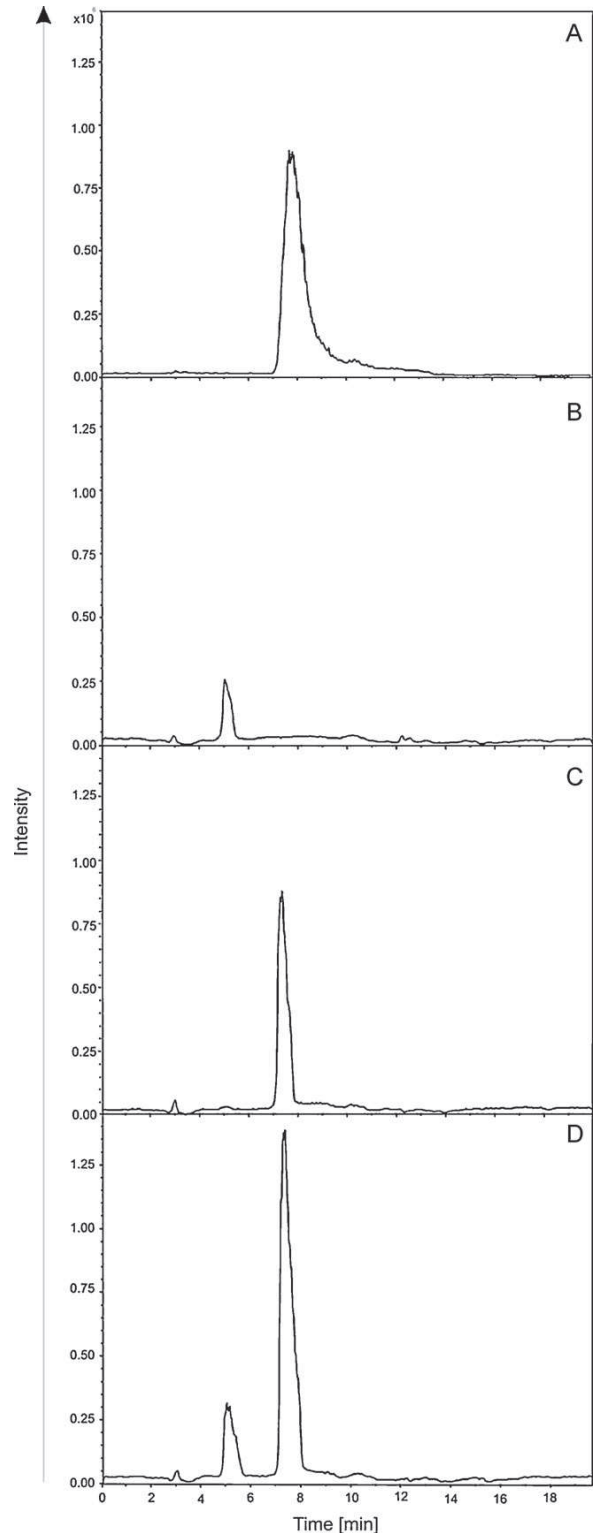


Fig. 4. Chromatographic profile recorded by using liquid chromatography with mass/mass spectrometry (LC–MS/MS) with the multiple reaction monitoring (MRM) mode for the 233/216 transition, of (A) MEL standard 145 ng/mL, (B) *Palomino Negro* concentrated wine (3:1), (C) *Petit Verdot* concentrated wine (3:1) and (D) *Prieto Picudo* concentrated wine (3:1).

Table 5
MEL and MEL isomer contents were measured by HPLC-MS/MS in eight monovarietal wines.

Wine sample	MEL ng/mL (SD)	ISO[a] ng/mL (SD)
Cabernet Sauvignon	14.2 (0.2)	6.1 (0.8)
Jaen	–	21.9 (2.9)
Merlot	–	5.2 (0.4)
Palomino Negro	–	16.7 (1.8)
Petit Verdot	5.1 (0.6)	–
Prieto Picudo	49.0 (4.7)	6.5 (0.9)
Syrah	86.5 (2.6)	–
Tempranillo	129.5 (3.5)	9.3 (2.1)

Data calculated as mean ($n = 3$); standard deviation (SD) indicated in parentheses.
[a] ISO amount calculated by use of MEL standard.

standard concentrations ranged from 9 to 290 ng/mL and were performed in triplicate. A correlation coefficient ($r = 0.998$) was obtained. LOD and LOQ were 0.13 ng/mL and 0.44 ng/mL, respectively (Huber, 1998). This method was more sensitive and selective than HPLC-FL; therefore, it was used to further quantify the sample.

Table 5 shows the MEL and ISO contents obtained in the analysis. *Tempranillo* provided the largest amount of MEL and a significantly higher concentration (129.5 ng/mL) than the other wines tested. *Cabernet Sauvignon*, *Petit Verdot*, *Prieto Picudo*, and *Syrah* also contained MEL at very different concentrations, but MEL was not detected in *Jaen Tinto*, *Merlot* or *Palomino Negro*. On the other hand, the highest ISO was found in *Jaen Tinto* (32.6 ng/mL), which represented a quarter of the amount of MEL found in *Tempranillo*. *Cabernet Sauvignon*, *Merlot*, *Palomino Negro*, *Prieto Picudo* and *Tempranillo* showed small amounts of ISO, which was not detected in *Petit Verdot* or *Syrah*. We may conclude that the occurrence of MEL in monovarietal wines was qualitatively and quantitatively diverse. In fact, in other varietal wines, much lower contents have been reported (i.e., 0.5 ng/ml for Sangiovese and 0.4 ng/ml for Trebbiano; Mercolini et al., 2008).

Moreover, MEL and ISO may appear as single compounds or appear together, depending on the type of wine. This fact may be attributed to variations in MEL in wine grapes, or it may be modulated by one or many treatment processes related to wine-making. Further investigations are needed to make this point clear. ISO's structure, its possible biological activity and its contribution to the health-promoting properties of wines also need to be elucidated.

4. Conclusion

According to our results, ELISA is not suitable for assaying MEL in wines, but LC-FL method was validated for this purpose. It could be used to initially check the presence of MEL in a routine analysis. This method is more affordable than LC-ESI-MS/MS; however, a sample concentration treatment is recommended, as good recovery (98%) was obtained with SPE extraction. Finally, MEL has been unequivocally identified by comparing MS and MS2 spectra. ISO has been tentatively identified as a MEL isomer using the minor ion fragments provided by the parent ion. For complete identification, isolation and NMR analysis are required. The LC-ESI-MS/MS analysis may be used to obtain an exact identification of MEL and an accurate MEL quantitation in wines.

Acknowledgments

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CAPÍTULO 2.
DETERMINACIÓN DE MELATONINA EN UVAS, VINOS Y DURANTE EL PROCESO DE VINIFICACIÓN EN BLANCO Y TINTO.

MELATONIN IS SYNTHESISED BY YEAST DURING ALCOHOLIC FERMENTATION IN WINES.

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Melatonin is synthesised by yeast during alcoholic fermentation in wines

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ABSTRACT

Melatonin (*N*-acetyl-5-methoxytryptamine) is a neurohormone produced in the pineal gland. Its biological properties are related to the circadian rhythm. Recently, the European Food Safety Authority (EFSA) accepted the health claim related to melatonin and the alleviation of subjective feelings of jet lag. This molecule has been detected in some foods. In this work, 13 grape varieties were studied; 7 monovarietal wines were produced in an experimental winery under strictly controlled conditions and were sampled in different steps. The grape varieties used to make the wines were: *Cabernet Sauvignon, Merlot, Syrah, Tempranillo, Tintilla de Rota, Palomino Fino* and *Alpha red*. Liquid chromatography tandem mass spectrometry (LC-MS/MS) unequivocally confirmed the presence of melatonin in wines. The main contribution of this paper is the results that clearly show that melatonin is synthesised during the winemaking process, specifically after the alcoholic fermentation. Indeed, melatonin is absent in grapes and musts and is formed during alcoholic fermentation.

1. Introduction

The determination of the chemical composition of wines aims to improve the winemaking process, establish a relationship with the sensory properties of wines, enable the study of the health benefits or facilitate characterisation. During the last two decades, research in the field has encompassed the development of new, more sophisticated, more sensitive analysis methods (Pereira, Câmara, Cacho, & Marques, 2010; Saurina, 2010), a full description of chemical composition (Polaskova, Herszage, & Ebeler, 2008) and the targeting of molecules with an impact on organoleptic properties (Vilanova, Genisheva, Masa, & Oliveira, 2010). Additionally, a large effort has been made in determining the bioactivity of compounds present in wine and in describing new molecules with biological activity.

The study of the health benefits of wine has focused on polyphenols. Studies have reviewed their content in grapes as a source of bioactive compounds (Chira, Suh, Saucier, & Teissèdre, 2008; Nassiri-Asl & Hosseinzadeh, 2009), their role in the winemaking process (Pérez-Serradilla & Luque de Castro, 2008), their reactivity and influence on the organoleptic properties (Paixão, Perestrelo, Marques, & Câmara, 2007). The described biological activities are varied and include antioxidant, cardioprotective, anti-inflammatory, anti-ageing and antimicrobial properties, among others (Xia, Deng, Guo, & Li, 2010). Bertelli (2007) discussed the possible health benefits of wine consumption on the basis of the concentration of bioactive compounds in wine and the dose needed to achieve the different activities. Indeed, these considerations should be taken into account when evaluating the possible effect of a certain bioactive compound.

In addition to phenolic compounds, indoleamines possess antioxidant activity and are naturally present in wines (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, & Garcia-Parrilla, 2010). Indeed, melatonin has already been reported in grapes and wines (Iriti, Rossoni, & Faoro, 2006; Mercolini et al., 2008). Melatonin (*N*-acetyl-5-methoxytryptamine) (MEL), a neurohormone discovered in the pineal gland, is also produced as secondary metabolite in plants. The amino acid tryptophan is the precursor of all 5-methoxytryptamines (or indoleamines), including MEL, and the biosynthetic pathway is via serotonin (5-hydroxytryptamine) in the case of yeasts, plants and mammals (Chattoraj, Liu, Zhang, Huang, & Borjigin, 2009; Posmyk & Janas, 2009; Sprenger, Hardeland, Fuhrberg, & Han, 1999). MEL has been detected in the roots, leaves, seeds and fruits of a considerable variety of plants (Paredes, Korkmaz, Manchester, Tan, & Reiter, 2009). The role of this molecule in plants is as a phytohormone regulated by light, as an UV irradiation protector and as an antioxidant (Paredes et al., 2009; Posmyk & Janas, 2009). Like other secondary metabolites, MEL has antioxidant properties as a direct free radical scavenger and by stimulating

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antioxidant enzymes (Hardeland & Pandi-Perumal, 2005; Reiter, Tan, & Maldonado, 2005). Moreover, the biological activities of MEL metabolites (N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK)) have been described previously (Guenther et al., 2005; Schaefer & Hardeland, 2009; Than, Heer, Laatsch, & Hardeland, 2006).

MEL has been reported to be present in foods (De la Puerta et al., 2007; Iriti et al., 2006; Maldonado, Moreno, & Calvo, 2009). The EFSA has recently accepted the link between MEL and the alleviation of subjective feelings of jet lag based on a list of health claims in relation to MEL (EFSA, 2010). The MEL dose should be between 0.5 and 5 mg per day (EFSA, 2010). However, there are not enough scientific data on the MEL content in foods to evaluate dietary intakes. Previous work on MEL in foods includes analysis of beer, cherries, tomatoes, rice and olive oil (De la Puerta et al., 2007; González-Gómez et al., 2009; Maldonado et al., 2009; Paredes et al., 2009).

Research of the MEL in grapes is in its beginning as it has only just been detected by ELISA. However, its distribution in grapes and its synthesis during ripening needs to be studied. To the best of our knowledge, the influence of the winemaking process on MEL content in wines has not yet been studied from the perspective of oenology, even though it is essential to do so to understand why MEL is present in wines. As a starting point, reliable analytical methods are required to obtain these data (Garcia-Parrilla, Cantos, & Troncoso, 2009).

This paper aims to determine the presence and content of MEL in different parts of the grapes (skin, pulp and seeds) and monitor the winemaking process (initial must for all wines, pressed and racked for red wines, and dejuice for the white wine) to check the influence of these steps on the content of MEL throughout the winemaking process.

2. Materials and methods

2.1. Chemicals and reagents

N-acetyl-5-methoxytryptamine standard was purchased from Sigma (Ref. M5250), L-tryptophan from Fluka (Ref. 93659), methanol from Merck (Darmstadt, Germany) and formic acid from Panreac (Barcelona, Spain). Solutions were prepared by diluting with Milli-Q water. All reagents were of analytical grade. Pectolytic enzymes (Vinozym® Vintage FCE, Novozymes, Bordeaux, France) and *Saccharomyces cerevisiae* yeast (Actiflore® F5, LAFFORT, France) were used for winemaking.

2.2. Grape samples

The grapes used for this study were from experimental cultivars or the winery at the Rancho de la Merced research centre (Instituto de Investigación y Formación Agraria y Pesquera, IFAPA, Jerez de la Frontera, Spain). Grapes were taken from the 2008 vintage and harvested at their optimum ripeness. Winemaking grapes and table grapes were collected to study the presence of MEL in different parts of the grape. The winemaking grape varieties studied were as follows: *Cabernet Sauvignon*, *Merlot*, *Nebbiolo*, *Palomino Fino*, *Pedro Ximénez*, *Petit Verdot*, *Syrah*, *Tempranillo* and *Tintilla de Rota*. The table grape varieties were also included: *Flame Seedless*, *Moscatel Itálica*, *Red Globe* and *Superior Seedless*.

2.3. Winemaking procedure. Pilot and laboratory assays

Pilot red winemaking: *C. Sauvignon*, *Merlot*, *Syrah*, *Tempranillo* and *Tintilla de Rota* grapes were de-stemmed, crushed and placed in a 50 litre steel vessel. Peptolitic enzymes (3 g/100 kg, Vinozym Vintage FCE, Novozymes, Spain) and sulphur dioxide (70 mg/kg) were added to maximise extraction and to protect the must. One day later, the fermentation was started after yeasting (Actiflore F5, Laffort, Spain), and the temperature was maintained at 27 °C ± 1 during alcoholic fermentation. As soon as the tumultuous fermentation had finished (density 999 g/l), the wine was pressed (Willmes, Germany). For the malolactic fermentation, lactic bacteria *Oenococus lacti* (100 g/l, Challenge Easy ML, Sepsa-Enartis, Spain) and nutrients (2 kg/l Nutriferm ML, Sepsa-Enartis, Spain) were used. When this stage of the process was finished, the wines were racked and clarification (1 kg/l egg white albumen, Laffort, Spain) was performed. Finally the wines were bottled. A diagram of the process is shown in Fig. 1.

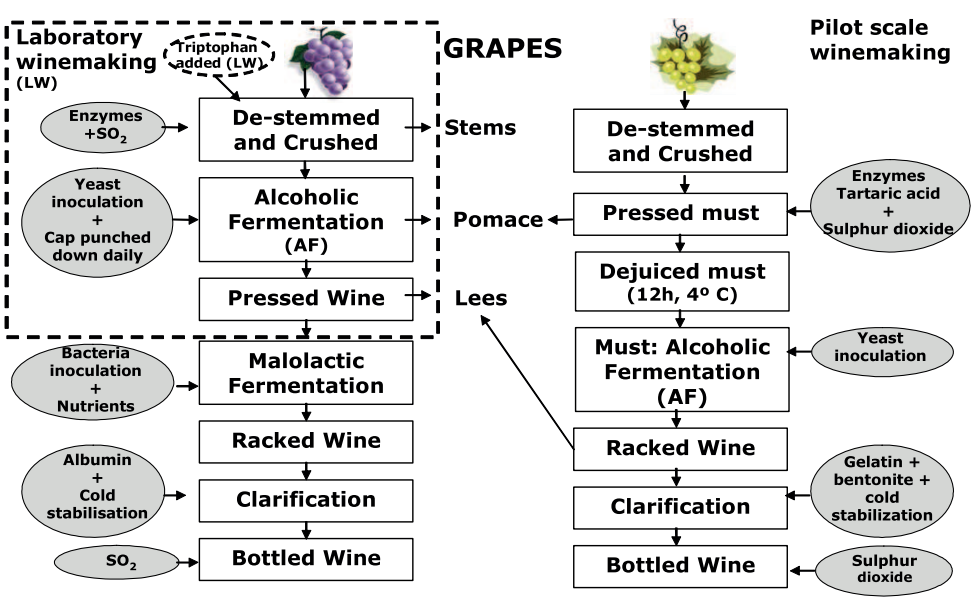

Fig. 1. Pilot and laboratory winemaking procedure scheme following the traditional methods for red and white wines. Solid white line: winemaking procedures at pilot scale; broken white line: laboratory winemaking assay.

Table 1
Enological parameters such as ethanol, tritatable acidity (TA), pH, volatile acidity (VA), total polyphenols index (TPI) and sugars, were measured according to Official Methods (OIV, 1990).

Wine sample	Ethanol (%vol.)	TA[a] (g/l TH2)	pH	VA[b] (g/l AcH)	TPI[c]	Sugars (g/l)
Cabernet Sauvignon	12.28	4.98	3.87	0.49	40.83	2.84
Merlot	13.23	4.99	3.50	0.27	53.98	2.79
Palomino Fino	12.66	3.95	3.26	0.33	md[d]	2.03
Syrah	12.74	4.68	3.73	0.27	59.03	2.29
Tempranillo	12.36	4.89	3.84	0.28	25.07	2.35
Tintilla de rota	15.94	5.73	3.44	0.46	68.94	3.76

[a] TA: tritatable acidity expressed as g/l tartaric acid (TH2).
[b] VA: volatile acidity expressed as g/l acetic acid (AcH).
[c] TPI: total phenolic index.
[d] md: missing data.

Pilot white winemaking: *P. Fino* grapes were de-stemmed, crushed, pressed and sulphur added (70 mg/kg). After pressing, the must was dejuiced for 12 h at 4 °C, and the alcoholic fermentation was then started after yeasting (Actiflore PM, Laffort, Spain). The alcoholic fermentation was carried out at control temperature (18 ± 1 °C), and finished at a density of 995 g/l. Afterwards, the wine was maintained under control conditions (T^a = 18 °C) until racked. Then, the wine was stored in a cold chamber (at 0 °C) until clarification using gelatine (1.5 kg/l, Laffort, Spain) and bentonite (60 g/l, Laffort, Spain). Finally, the wine was bottled (Fig. 1). The stages sampled in the winemaking process were: initial must, pressed and racked for red wines; initial must, dejuice and racked for the white wine. Samples were frozen and stored at −20° C for further analysis. Table 1 gives the main oenological parameters of the wines obtained (OIV, 1990).

Alpha Red grapes (Dole SA) purchased in the market were used in a laboratory winemaking study performed to analyse the occurrence and evolution of melatonin production during alcoholic fermentation. An experimental study in which tryptophan was added was designed to test whether the concentration of the precursor of MEL might affect the production as well as to identify the agent responsible for its synthesis during alcoholic fermentation. The must had the following parameters: 250 g/l sugar, 3.55 pH and tritatable acidity of 4.89 g tartaric acid/l. Three batches of grapes (3.5 kg each) were placed into three 10 l stainless steel tanks; two of those (TRP1 and TRP2) were spiked with 60 mg/kg of L-tryptophan and the third batch of untreated grapes was used as a control batch (CNT). Samples of 5 ml of grape juice were taken from each tank daily during alcoholic fermentation and stored (at −80° C) for subsequent analysis.

2.4. Sample preparation

All grape parts were studied separately: the skin (both lyophilised and fresh), pulp and seeds. Three solvents, already reported in the literature on melatonin extraction, were assessed: chloroform, ethyl acetate and methanol:water (1:1, v:v). Tissues were extracted as previously described by Arnao and Hernández-Ruiz (2009), with some modifications: 1 g of skin, pulp or seed was cut and placed into vials containing 3 ml of chloroform, ethyl acetate or methanol:water (1:1, v:v). Samples were soaked for 18 h at 4° C in darkness, after a brief ultrasonic treatment for 15 min at room temperature. Supernatant from each sample was evaporated to dryness under vacuum. The residue was re-dissolved in 100 μl of methanol:water (1:1, v:v), then filtered (0.45 μm) and analysed using LC-ESI-MS/MS.

The winemaking stage samples were previously dried and re-diluted in methanol:water (1:1, v:v), up to a concentration of 3:1 (v:v). The reconstituted extracts were passed through filters (0.45 μm) before the LC-ESI-MS/MS analysis was performed.

2.5. LC-MS/MS analysis

A chromatographic separation was performed using an HPLC system equipped with an Agilent 1200 series (Agilent Technologies, Waldbronn, Germany) and coupled to a mass detector in series. The HPLC system consisted of a binary capillary pump (G1376A), a degasser (G1379B), an autosampler (G1377A), a sample cooler (control temperature 4° C) (G1330B), and a photodiode array detector (G1315D) controlled by Chemstation software (v.B.0103-SR2). The mass detector was a Bruker (Bremen, Germany) ion trap spectrometer equipped with an electrospray ionisation (ESI) system and controlled by Bruker Daltonics Esquire software (v.6.1).

Melatonin separation was performed using an Agilent 150 × 0.5 mm i.d., 5 μm, Zorbax Sb-18 column (DE48102564). Chromatographic separation was performed using a binary gradient consisting of (A) water and (B) methanol as HPLC grade solvents, both containing 0.1% (v/v) formic acid. The elution profile was: 40% B (2 min), 85% B (4 min), 90% B (9 min). The flow rate was 10 μl min^{-1}. The injection volume was 3 μl. The mass spectrometer settings were as follows: the pressure and flow rate of the dryer gas were set at 11 psi and 5 l/min respectively. The full scan mass spectrum was from *m/z* 100 to 250. Collision-induced fragmentation experiments were performed in the ion trap using He as collision gas, with voltage ramping cycles from 0.3 to 2 V. The heated capillary and voltage were maintained at 350° C and 4.275 kV, respectively. Some parameters have an important effect on sensitivity. Thus, ion optics and trap conditions were optimised for the detection of MEL.

For the optimisation of the ESI source, flow injection analyses (FIA) were performed with a MEL standard (5 μM) and a different nebulizer (nitrogen) and a dryer gas flow, and temperatures were tested. Ion optics and trap parameters were optimised through direct infusion to the ESI source. The automatic optimisation option of the MS for the standard was used. The common parameters for MEL were as follows: capillary voltage, −4275 V; skimmer, 40 V; capillary exit, 108.5 V; octopole 1 DC, 12 V; octopole 2 DC, 1.7 V; octopole RF, 138.8 Vpp; lens 1, −5 V; lens 2, −60 V. MS data were acquired in the positive mode.

The MS data obtained were compared with their authentic marker in order to perform a final identification of the MEL present in the samples. Major and minor fragment ions for the MS2 events play an important role in the identification of MEL since their corresponding relative abundances can be measured. For quantitative analyses, the multiple reaction monitoring (MRM) mode was used. MEL quantification was based on the 233/216 MRM transition resulting from MS/MS fragmentation ions. The 233/216 MRM transition was selected to quantify MEL because of its specificity and its better signal-to-noise ratio. Six MEL concentrations (18, 36, 72, 145, 290 and 580 ng/ml) were used for setting up a calibration curve.

3. Results and discussion

3.1. Analyses of melatonin in grapes

Three solvents (chloroform, ethyl acetate and methanol:water (1:1, v:v) were used to extract MEL from the seeds, pulp and skin of grapes. These solvents had previously proved to be suitable for melatonin analysis (Arnao & Hernández-Ruiz, 2009) on plant material (*Lupinus albus* L. and *Hordeum vulgare* L.) with a good recovery (94%). The LC-MS/MS method was used for the quantification of MEL in grapes. However, melatonin was not found in the grape parts analysed (pulp, seeds or skin), whatever solvent was used for their extraction. In a previous study, ELISA tests found MEL to be present in the grape skins of eight different varieties of grape (Iriti et al., 2006), of which only *Nebbiolo* (428.3 ng/ml) and *C. Sauvignon* (183.6 ng/ml) were included in the present study. In our study, MEL was not found in the samples. The LC-MS/MS method used in our analyses provided a detection limit of 0.13 ng/ml.

3.2. Analyses of melatonin during the winemaking process

In order to quantify MEL in the different stages of the winemaking process (pilot scale), the MRM mode was used to record transitions of the MEL standard using LC-MS/MS analysis. The calibration curve was generated with a 233/216 transition area for each of the six MEL concentrations (from 18 to 580 ng/ml). Good linearity was obtained ($r = 0.998$).

Fig. 2 shows a chromatographic profile recorded using LC-MS/MS with the multiple reaction monitoring (MRM) mode for the 233/216 transition of the MEL standard (Fig. 2A) and a wine stage sample (Fig. 2B). LC-MS/MS results show that the MEL 233/216 transition t_R is 7.8 min.

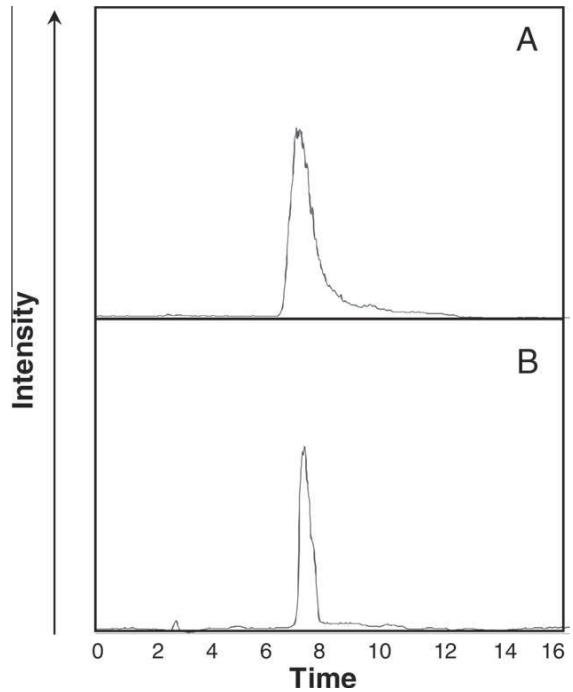

Fig. 2. Chromatographic profile recorded by using liquid chromatography with mass/mass spectrometry (LC-MS/MS) with the multiple reaction monitoring (MRM) mode for the 233/216 transition, of (A) MEL standard 145 ng/ml, (B) MEL present in *Tempranillo* pressed wine.

Table 2 shows the MEL content observed in six grape varieties at different stages of the winemaking process. MEL was not detected in the initial musts of any variety. These results for musts, obtained at the first stage of the winemaking process, were very coherent with the results obtained for grapes. However, MEL appeared during the course of the winemaking process for the sampling steps analysed (press for red wines and rack for white wine). This implies that the presence of MEL in wines is due to the winemaking process rather than to the grapes content.

Table 2
MEL content in different stages of winemaking process (pilot scale) for one white (*Palomino Fino*) and five red (*Cabernet Sauvignon*, *Merlot*, *Syrah*, *Tempranillo* and *Tintilla de Rota*) wines.

Sample	Stage	MEL ng/ml (SD)[a]
Cabernet Sauvignon	Initial must	nd[b]
	Pressed wine	74.13 (2.65)
Merlot	Initial must	nd[b]
	Pressed wine	241.22 (8.86)
	Racked wine	245.46 (14.16)
Palomino Fino	Initial must	nd[b]
	Dejuiced must	nd[b]
	Racked wine	390.82 (10.05)
Syrah	Initial must	nd[b]
	Racked wine	423.01 (1.60)
Tempranillo	Initial must	nd[b]
	Pressed wine	77.72 (9.74)
	Racked wine	306.86 (1.64)
Tintilla de Rota	Initial must	nd[b]
	Pressed wine	322.68 (2.58)

[a] Data calculated as mean ($n = 3$); standard deviation (SD) indicated in brackets.
[b] nd: not detected.

For both white and red grape varieties, MEL appears after yeast has been added. For instance, *P. Fino* did not contain MEL at the dejuice stage, but its MEL content at the racking stage was within the same range as for red wines. There was no difference in the MEL content between white and red varieties (Table 2), but it shows that the addition of yeast had a clear effect. According to Sprenger et al. (1999), *S. cerevisiae* can form MEL in a growth medium and its concentration can be fairly high. MEL has been shown to be synthesised from tryptophan *via* 5-hydroxytryptophan, serotonin and *N*-acetylserotonin and to be metabolised by deacetylation to 5-methoxytryptamine (Fig. 3). However, MEL can also be formed by *O*-methylation of serotonin followed by *N*-acetylation of 5-methoxytryptamine in the yeast (Hardeland, Reiter, Poeggeler, & Tan, 1993; Sprenger et al., 1999).

In order to confirm the role of yeast addition in the occurrence of MEL in wines, a laboratory-scale experimental winemaking process as described above was set up. Table 3 shows the results obtained. As in previous studies, MEL was not found at the initial stage nor on the second day of fermentation in any experiments. MEL could be found on the fifth day, when the sugar content was 63 g/l, and its content increased until the seventh day. Indeed, on the fifth day, the MEL content was statistically higher ($p < 0.05$) in the sample in which tryptophan was added despite the sugar content being similar (Table 2). The growth of yeast is therefore similar and cannot account for the differences in the MEL content. Despite the fact that our results confirms that MEL occurrence in wines is related to the *S. cerevisiae* addition, the role of other microorganisms, such as acetic acid bacteria, lactic acid bacteria or other yeasts present in the medium needs to be elucidated.

Wine was pressed and analysed at the end of alcoholic fermentation and no statistical differences ($p < 0.05$) were observed before or after pressing (Table 3), since the grape skins presented no MEL. Therefore, similar results (Table 2) were obtained for white (not pressed) and red wines (pressed).

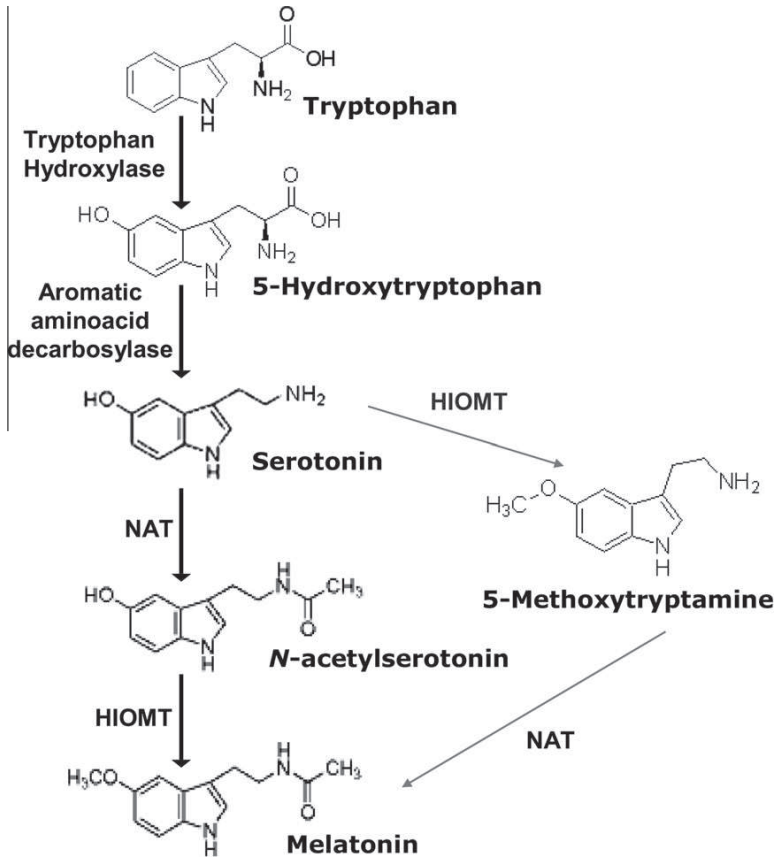

Fig. 3. Biosynthesis of melatonin from the tryptophan aminoacid and serotonin. Thick arrows: normal biosynthetic pathway; grey thin arrows: alternative biosynthetic pathway (Sprenger et al. (1999)). NAT: Indoleamine *N*-acetyltransferase; HIOMT: Hydroxyindole *O*-methyltransferase.

Table 3
MEL content in different stages of samples, control (CNT) and tryptophan added (TRP) sample, in the experimental winemaking process (laboratory scale) designed to monitor melatonin evolution during alcoholic fermentation.

Day	Density (g/l)	MEL ng/ml (SD)[a]	
		CNT	TRP
1 (must)	1074	nd[b]	nd[b]
2	1054	nd[b]	nd[b]
5	1017	87.50 (3.92)	105.78 (11.30)
6	998	127.01 (9.66)	130.79 (11.93)
7 BP[c]	997	146.24 (8.78)	141.59 (8.54)
7 AP[d]	997	153.25 (9.33)	152.32 (9.47)

[a] Data calculated as mean ($n = 3$); standard deviation (SD) indicated in brackets.
[b] nd: not detected.
[c] BP: before pressing.
[d] AP: after pressing.

On the seventh day there were no statistically significant differences in the MEL content between those samples in which tryptophan was added and that in which it was not added. The fact that MEL appeared earlier in samples in which tryptophan was added may be related to the results obtained by Sprenger et al. (1999), where a short period of exposure to tryptophan (30 min) increased MEL to detectable levels. Sprenger stated that MEL appears more quickly when there is a precursor (tryptophan, serotonin or *N*-acetylserotonin) in the growth medium than when there is no precursor. Table 3 shows that, even though tryptophan was added, the MEL content was similar when the same variety, conditions and yeasts were used. However, between wines from different varieties, there was a statistically significant difference in the MEL concentration ($p < 0.05$), even when the yeast and conditions were identical (Table 2). Further research on the role of yeasts and MEL formation are needed.

4. Conclusions

This paper proves unequivocally the presence of MEL in wines and the effect of yeast on its presence and concentration. MEL is absent in grapes and musts and is synthesised during the alcoholic fermentation process. Indeed, in both white and red wines, MEL concentration increases after the addition of yeast and during fermentation.

Acknowledgements

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RESULTADOS DE LOS VINOS DE CRIANZA BIOLÓGICA (CAPÍTULO 2)

Andalucía es productora de vinos, principalmente de blancos generosos y dulces, elaborados mediante el sistema de crianza denominada "*criaderas y soleras*". Los vinos andaluces tienen un estilo propio y se caracterizan por su enorme diversidad dando lugar a los finos y manzanillas, amontillados, olorosos, palo cortado, dulces y moscateles, entre otros. Dentro de los vinos producidos en Andalucía, los vinos de crianza biológica merecen una mención especial. Se trata de vinos blancos con una crianza que se produce a partir de levaduras *Saccharomyces* autóctonas y filmógenas que forman el velo de flor. La crianza bajo velo de flor tiene dos principales repercusiones en los vinos: se evita la oxidación del vino por estar protegido con el velo de flor y absorbe rápidamente el oxigeno presente en el vino debido a los trasiegos, favoreciendo la conservación de su color pálido-verdoso. Este tipo de crianza pertenece a las denominaciones de origen Condado de Huelva, Jerez-Manzanilla-Sanlúcar y Montilla-Moriles.

Teniendo en cuenta que la levadura influye en la producción de melatonina, se decidió analizar los vinos elaborados mediante crianza biológica, que presentan un mayor contacto con levaduras. El análisis de la melatonina en estos vinos se llevó a cabo con el mismo método analítico que para el resto de vinos (Rodriguez-Naranjo et al., 2011).

A diferencia del resto de vinos analizados incluyendo *Palomino fino*, no se encontró melatonina en ninguno de ellos. La crianza biológica se lleva a cabo por cepas autóctonas de *Saccharomyces*, mayoritariamente *rouxi, montuliensis, cheresiensis* y *beticus*, que forman el velo de flor (Esteve-Zarzoso et al., 2001). La ausencia de melatonina en estos vinos podría deberse a que estas levaduras pueden utilizarla como nutriente, debido a que puede ser una fuente de nitrógeno a falta de aminoácidos como el triptófano.

CAPÍTULO 3.
ESTUDIO DE LA PRODUCCIÓN DE MELATONINA POR CEPAS DE *SACCHAROMYCES*.

PRODUCTION OF MELATONIN BY *SACCHAROMYCES* STRAINS UNDER GROWTH AND FERMENTATION CONDITIONS.

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Production of melatonin by *Saccharomyces* strains under growth and fermentation conditions

Abstract: Melatonin is a bioactive compound that is present in wine because it is contained in vinification grapes and synthesized by yeast during alcoholic fermentation. The purpose of this study was to determine the capacity of various *Saccharomyces* strains to form melatonin during its growth and alcoholic fermentation. A selection of yeasts including six *S. cerevisiae* (Lalvin CLOS, Lalvin ICV-D254, Enoferm QA23 Viniferm ARM, Viniferm RVA, and Viniferm TTA), one *S. uvarum* (Lalvin S6U) and one *S. cerevisiae* var. *bayanus* (Uvaferm BC) were tested to determine whether they produce melatonin in yeast extract peptose dextrose and synthetic must media in a variety of conditions. Two *S. cerevisiae* strains (ARM, and QA23), the *S. uvarum* and the *S. cerevisiae* var. *bayanus*, synthesized melatonin. The conditions in which they did so, however, were different: the QA23 strain produced melatonin best in a medium with a low concentration of reducing sugars and Lalvin S6U and Uvaferm BC required a synthetic must under fermentation conditions. Melatonin synthesis largely depended on the growth phase of the yeasts and the concentration of tryptophan, reducing sugars and the growth medium. These results indicate that melatonin may have a role as a yeast growth signal molecule.

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Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is an indoleamine found in plants, microorganisms and humans [1–3]. Melatonin modulates many physiological processes including the sleep/wake cycle and the reproductive physiology via a receptor-mediated mechanisms [4] and as an antioxidant via nonreceptor processes [5, 6].

The intake of foods containing melatonin increases its level in plasma and the number of melatonin-derived metabolites [7, 8]. Many studies have been carried out to identify melatonin in edible plants [9, 10], fruits such as strawberries and grapes [11, 12] and beverages such as beer and wine [13–15]. In addition to their polyphenol content, the presence of melatonin in some foods and drinks should be taken into account when their health benefits are estimated.

The reported concentration of melatonin in grapes (*Vitis vinifera* L.) varies from 0.005 ng/g in Cabernet Franc [16] to 150 μg/g in Merlot [17]; in wine, the concentration ranges from 0.16 ng/mL in Chardonnay [18] to 130 ng/mL in Tempranillo [14]. The melatonin level in barley (*Hordeum vulgare* L.) is 0.58 ± 0.05 ng/g [19] and in beer it ranges from 52 to 170 pg/mL [13].

Melatonin concentration increases during the fermentation step of the wine-making process [20]. Therefore, the role played by yeasts seems to be crucial. Microorganisms use tryptophan as the nitrogen source and yield metabolites such as indolacetic acid, tryptophol or biogenic amines. The ability of yeasts and bacteria to produce indole has been screened as they are related with off flavors in wine [21]. Indeed *S. cerevisiae* and *S. bayanus* form indole compounds in a Chardonnay grape juice at concentrations between 126 and 257 μg/L depending on the yeast strain used [21]. Lactic acid bacteria can also produce tryptamine from tryptophan [21]. However, little is known about the role that microorganisms play in melatonin production in fermented products.

Commercial yeast starters are commonly used in the wine-making process to prevent possible problems during fermentation and to obtain standardized products. *Saccharomyces cerevisiae* is the main species used as a starter for wine fermentations, although in some conditions other *Saccharomyces* species such as *S. cerevisiae* var. *bayanus* or *S. uvarum* are also used. Previous studies have shown that *S. cerevisiae* can synthesize high amounts of melatonin and other methoxyindoles in a standard yeast growth and salt medium. Production increases if the precursor is available

[22]. The role of melatonin in *Saccharomyces* has yet to be elucidated but is not apparently related to circadian rythms [2]. Recently, the role of small aromatic molecules with an indole ring such as tryptophol and indole-3-acetic acid has been revised [23]. Depending on nitrogen availability, *S. cerevisiae* can use these compounds as signals to modulate population growth. Nitrogen starvation stimulates tryptophol and phenylethanol synthesis to inhibit yeast growth [24].

Both the amino acid concentration in the must and the strain of *Saccharomyces* involved in the fermentation determine the formation of biogenic amines [25]. Melatonin and tryptamine are formed from the same precursor, tryptophan. The literature related to biogenic amines in wines is extensive because of their impact on organoleptic properties and their negative effects on health [26, 27]. However, the role played by different yeast strains in melatonin synthesis has not been previously explored even though it is known that it affect positively health [28, 29].

The aim of the present work was to study the ability of different *Saccharomyces* strains to form melatonin during its growth and alcoholic fermentation, and how yeast growth conditions can affect their capacity to produce melatonin. To eliminate variables from the natural medium, we have used synthetic media and selected strains.

Materials and methods

Yeast strains

The following wine yeasts were used in this study: six *S. cerevisiae* (Lalvin CLOS (CLOS), Lalvin ICV-D254 (D254) and Enoferm QA23 (QA23) from Lallemand Inc., Montréal, QC, Canada; Viniferm ARM (ARM) from Agrovin, Viniferm RVA (RVA), and Viniferm TTA (TTA), Spain)), one *S. uvarum* (Lalvin S6U (S6U) from Lallemand Inc.) and one *S. cerevisiae* var. *bayanus* (Uvaferm BC from Lallemand Inc.). The yeasts were rehydrated in water at 37°C for 30 min before inoculation.

Media

The media used in this study were: yeast extract pentose dextrose (YPD) (1% yeast extract, 2% glucose and 2% peptone) supplemented with two different amounts of standard tryptophan (Trp) (Table 1); and synthetic grape must (SM) [30] modified with two different reducing sugar concentrations (20 and 200 g/L) and supplemented with three different amounts of standard tryptophan, although in all the cases the available nitrogen was maintained at 382.56 mg/L (Table 1).

Conditions of melatonin production

Media

QA23, ARM and S6U strains were grown in five media (YPD 1× Trp, YPD 5× Trp, SM 0× Trp, SM 1× Trp, and SM 5× Trp) so that yeast growth and melatonin production could be tested. Experiments were performed in 50-mL flasks filled with 30 mL of each medium and covered with a cotton cap. The initial inoculated population in every flask was 1×10^6 cell per mL and cells were grown at 28°C in a shaking incubator (150 rpm). A total of 1 mL of each medium was sampled every hour from 0 to 6 hr and at 24 hr. Each sample was then transferred to 1.5-mL Eppendorf tubes to centrifuge at 21,910 g for 3 min at room temperature. The supernatant of these samples was stored at −20°C for melatonin analysis. Yeast growth was calculated by counting the viable yeast cells after they had been plated on solid YPD medium (1% yeast extract, 2% glucose, 2% peptone and 1.7% agar) at an appropriate dilution and incubated for 2 days at 28°C. Imposition of the inoculated strains was checked by restriction analysis of the mitochondrial DNA (RFLP of mtDNA) [31] at the beginning and end of each test.

Table 1. Reducing sugars, tryptophan (Trp) and available nitrogen in the medium used for yeast growth, the monitoring of melatonin production, and alcoholic fermentation in this study

Medium	Name	Reducing Sugars (g/L)		Trp (g/L)	Available nitrogen (mg N/L)
		Glucose	Fructose		
Yeast Extract Peptone Dextrose	YPD 1× Trp	20	–	0.59	1032.94
	YPD 5× Trp	20	–	2.94	1194.04
Synthetic Must	SM 0× Trp	10	10	0	382.56
	SM 1× Trp	10	10	0.17	382.56
	SM 5× Trp	10	10	0.87	382.56
	SM Ferm	100	100	0.87	382.56

Monitoring melatonin synthesis

The QA23 strain was inoculated in YPD 1× Trp and SM 1× Trp. A total of 40 mL of each medium was inoculated in a 50-mL flask to a population of 1×10^6 cell/mL. The flasks were shaken at 28°C and were sampled every 1 hr from 0 to 12 hr in the case of YPD and every 2 hr from 0 to 46 hr in the case of SM. At each timepoint, 1 mL was removed and centrifuged at 21,910 g in Eppendorf tubes for 3 min at room temperature and the supernatant was stored at −20°C for melatonin analysis. Cell cultures were plated on solid YPD medium at the same points to determine the viable population and to check the imposition of the inoculated strain by RFLP of mtDNA analysis [31].

Alcoholic fermentation

Fermentations were performed with eight different strains (ARM, CLOS, D254, QA23, RVA, S6U, TTA, and Uvaferm BC) in SM Ferm medium (Table 1). A total of 450 mL of medium, inoculated with at 1×10^6 cell/mL, was placed in 500-mL bottles. Fermentations were carried out under stirring at 28°C.

Every day 5 mL of each medium was sampled and the density measured. Then 1 mL was transferred to 1.5-mL Eppendorf tubes and centrifuged at 21,910 g for 3 min at room temperature. The supernatant was stored at −20°C for melatonin analysis. Viable yeasts were determined every day by plating them on solid YPD medium at an appropriate sample dilution. To check the imposition of

the inoculated strain, ten colonies were randomly taken and isolated from each plate and mtDNA restriction analysis was performed [31]. At the end of fermentation, enzymatic kits (Roche Applied Science, Mannheim, Germany) were used to assay the reducing sugars. Fermentations were considered to have finished when the residual sugars were below 2 g/L.

Melatonin detection

Samples were analysed by liquid chromatography-mass spectrometry as previously described by Rodriguez-Naranjo et al. [14]. The apparatus consisted of high performance liquid chromatography coupled to a triple quadrupole mass spectrometer (Agilent G6410A; Agilent Technologies, Waldbronn, Germany). Separations were obtained on a Phenomenex Luna C18 column (250 × 4.6 mm, 5 μm). Melatonin was identified by comparing the 233/174 transition MS data of the samples and the melatonin standard.

Results and dicussion

The QA23, ARM and S6U strains were grown in five media (YPD 1× Trp, YPD 5× Trp, SM 0× Trp, SM 1× Trp, and SM 5× Trp) to determine whether the yeast strains under study produced different melatonin contents. Melatonin was identified using LC-MS/MS analysis in MRM mode to record the ion transition 233/174 (m/z) of the melatonin standard and samples. Melatonin was found in neither YPD 5× Trp nor SM 0× Trp media whatever the yeast strain used (data not shown). Therefore, these media were not further considered for any experiment. According to Sprenger et al. [22], yeast growth conditions can influence levels of methoxyindoles which, in turn, can lead to dramatic changes in methoxyindole concentration. Table 2 displays the melatonin production in the media with positive results. It shows the detection and relative abundance of melatonin during the first 0 to 6 hr and then 24 hr after inoculation. The QA23 strain produced melatonin in YPD 1× Trp, SM 1× Trp and SM 5× Trp media and the other two yeast strains (ARM and S6U) only in SM 5× Trp. The QA23 strain produced melatonin in the three media. It formed quickest in YPD 1× Trp (2 hr) but the highest content at the final fermentation was in SM 5× Trp (Table 2). Arevalo-Villena et al. [21] described a higher indole accumulation in grape juice medium containing 100 mg/L of Trp. Conversely, trace amounts of indole were detected in the absence of Trp. The Trp content had a crucial influence: melatonin did not form at all when it was absent from the medium (SM 0×Trp), as it is the principal precursor. The effect of Trp enrichment depended on whether the medium was YPD or SM: melatonin was not produced at all in YPD 5× Trp and was produced in SM 5× Trp. Moreover, melatonin was detected earlier (at 2 hr) and at higher concentrations than in nonenriched SM (Table 2). Therefore, the melatonin produced by yeast might be affected by both the culture medium and the presence of Trp in the medium as was the case of wines enriched with Trp [20].

Table 2. Relative abundance of melatonin produced during 0–6 hr and 24 hr after inoculation of yeast (QA23, ARM, and S6U) into different media

Yeast		QA23			ARM	S6U
Media		*YPD 1x Trp*	*SM 1x Trp*	*SM 5x Trp*	*SM 5x Trp*	*SM 5x Trp*
Time (h)	0	–	–	–	–	–
	1	+	–	–	–	+
	2	++	–	+	+	+
	4	+	+	+	+	+++
	6	+	+	+	+	+
	24	–	++	+++	+++	+

Although the three yeast strains synthesized melatonin in SM 5× Trp medium, differences were found for *S. cerevisiae* (QA23 and ARM) and *S. uvarum* (S6U). The maximum concentration of melatonin was determined at 4 hr in the case of *S. uvarum* (S6U) and 24 hr in the case of *S. cerevisiae*.

The QA23 strain was selected to check melatonin production over time. In YPD, melatonin was detectable after 1 hr of growth and its concentration was four times higher 1 hr later. It was also found at 4 hr at a much lower concentration (Fig. 1). Sprenger et al. [20] studied the formation of endogenous melatonin by yeast that synthesized large amounts after 2 hr in YPD. Melatonin could protect against reactive oxygen species (ROS) inside the yeast, as has been demonstrated in the dinoflagellate *Gonyaulax polyedra* [32]. Additionally, *S. cerevisiae* had the ability to metabolize N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), a kynuric melatonin metabolite, to form N^1-acetyl-5-methoxykynuramine (AMK) [33]. However, lower concentrations of melatonin were released into the YPD medium by the yeast and these concentrations may have functions different from the functions they have inside the yeast.

For comparative purposes, the sugar concentration of the SM medium was low (20 g/L). Fig. 2 displays the evolution of melatonin production, QA23 population, and reducing sugars over 46 hr in SM 1× Trp. Melatonin concentration was dependent on the yeast growth phase. The first part of the growth phase (0–4 hr) may somehow be crucial for yeast as only essential molecules were formed while the yeast was adapting to the conditions in the new medium. In this early phase, melatonin was not synthesized. The average ratio (Mel area mL per cell) was $3.73 \pm 0.35 \times 10^{-6}$ area mL per cell between 4 and 6 hr. This increase in melatonin at low cell density could act as a signal for the start of yeast growth. After 8 hr, melatonin no longer increased, and the average ratio was $1.25 \pm 0.32 \times 10^{-6}$ between 8 and 12 hr and $< 1 \times 10^{-6}$ between 14 and 20 hr. In the log phase, when metabolic machinery was working to support exponential yeast growth, melatonin accumulation was quite constant. Then, in the stationary phase, the melatonin area changed from 11 to 58 between 20 and 26 hr while the population was more or less constant. In this step, the synthesis of melatonin by cells was higher and its concentration peaked at 26 hr when cell density was 4.7×10^7 cell per mL. In the stationary phase, yeast growth slowed down and secondary

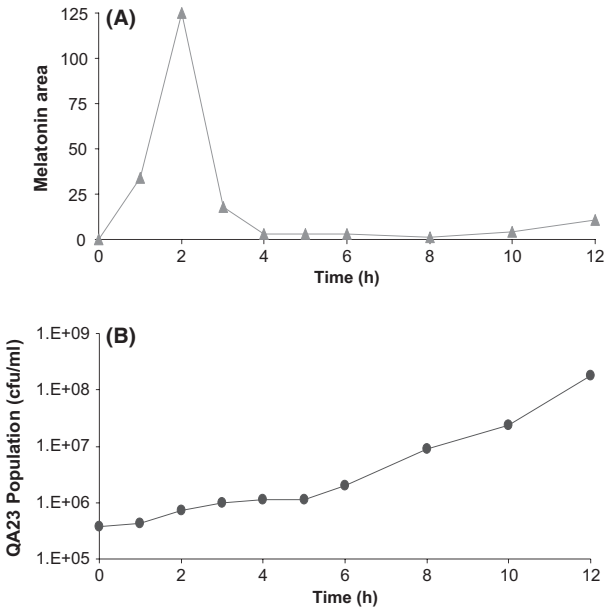

Fig. 1. Evolution of melatonin production expressed as melatonin area (A) and QA23 strain population (cfu/mL) under growth conditions in YPD medium.

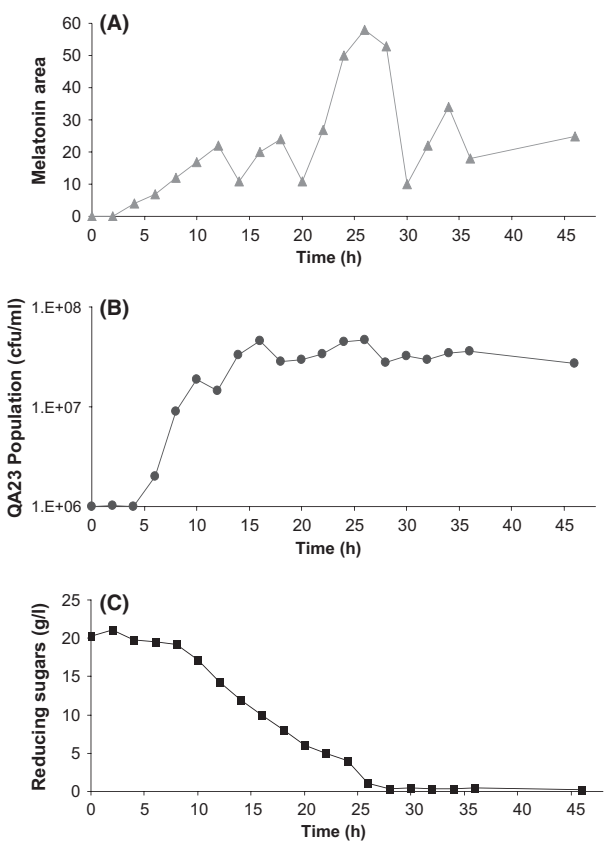

Fig. 2. Evolution of melatonin production expressed as melatonin area (A), QA23 strain population (cfu/mL) and (C) reducing sugars (g/L) under growth conditions in SM 1 × Trp medium.

metabolites were formed. The fact that melatonin production increased might also be related to a possible signaling mechanism. The concentration of reducing sugars was < 5 g/L after 20 hr. In addition, when the area of melatonin was largest (26 hr), the sugar level was < 2 g/L. This triggering of melatonin synthesis could be linked to the appearance of tryptophol, which has been described as a signal molecule [24]. Chen and Fink [24] attributed the greater synthesis of aromatic alcohols such as tryptophol and tyrosol to their role as metabolic signals. Indeed, cells at high density produce higher concentrations of these alcohols than cells at low density and their accumulation in the medium was growth dependent. Melatonin synthesis was undoubtedly related to the yeast growth phase. The beginning of the log phase and the stationary phase are the two most important stages in the production of melatonin. Finally, during the decline phase melatonin levels also decreased.

Eight fermentation experiments were carried out in triplicate with the eight aforementioned strains. None of the *S. cerevisiae* strains synthesized melatonin under fermentation conditions during the 7 days of fermentation. Melatonin was only produced by Lalvin S6U (*S. uvarum*) and Uvaferm BC (*S. cerevisiae* var. *bayanus*) (Fig. 3). The concentration of melatonin reached its maximum during the second day of fermentation, which is consistent with the maximum indole accumulation (2nd day of fermentation) described for Chardonnay grape juice by Arevalo-Villena et al. [21]. According to the data in Table 2, the melatonin concentration decreased after this maximum and by the end of fermentation was up to four times lower than the 2nd day. This is consistent with the fact that the melatonin concentration of wine is lower than at some stages during the wine-making process (e.g. 129.5 ± 3.5 ng/mL in *Tempranillo* wine and 306.9 ± 1.6 ng/mL in *Tempranillo* racked wine [14, 20]). The same applies to the accumulation of indole, which is structurally related to melatonin, during the alcoholic fermentation and in terms of its final content in wine [21].

Medium conditions and yeast strain both determine melatonin synthesis. Only the S6U strain produced melatonin at both sugar concentrations. Surprisingly, the QA23 strain, which synthesized the highest concentrations at 20 g/L of reducing sugars (Fig. 2), did not synthesize it under alcoholic fermentation despite a 5× Trp concentration. This suggests that one way to produce a wine or fermented beverage rich in this bioactive compound would be inoculating a suitable yeast strain able to synthesized melatonin.

This paper reports a comparative study on melatonin formation in different yeast strains under conditions of grown and fermentation. The study demonstrates the ability of two *S. cerevisiae* strains (ARM, QA23), one *S. uvarum* (S6U) and one *S. cerevisiae* var. *bayanus* (Uvaferm BC) to produce melatonin. The composition of the medium is crucial for the synthesis. The presence of Trp is essential, because it is its principal precursor, it increases increases final melatonin content and it accelerates its formation. The synthesis of melatonin largely depends on the growth phase of the yeast and the concentration of the reducing sugars. This is the first indication that this indoleamine may play a role as a growth signal.

The growth medium also limited the production of melatonin. The QA23 strain was the best producer of

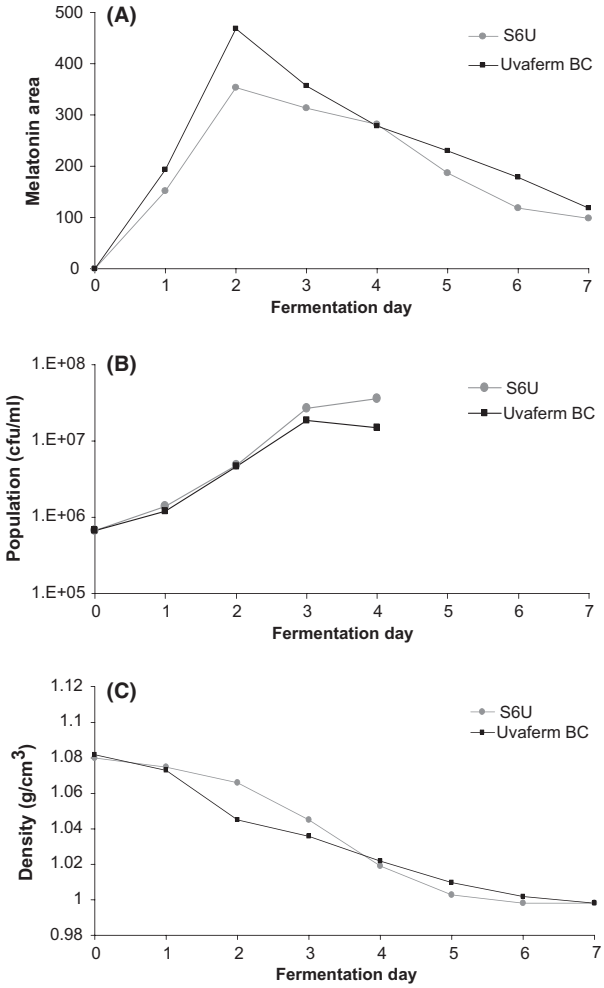

Fig. 3. Evolution of melatonin production expressed as melatonin area (A), population (cfu/mL) and, (C) density (g/cm^3) of S6U and Uvaferm BC strains under fermentation conditions in SM Ferm medium.

melatonin when the medium had a low concentration of reducing sugars. However, S6U (*S. uvarum*) and Uvaferm BC (*S. cerevisiae* var. *bayanus*) formed melatonin in synthetic must under fermentation conditions. Thus, both the yeast strain and the must composition equally determined the concentration of the bioactive melatonin in wines.

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CAPÍTULO 4.
SEGUIMIENTO DEL CONTENIDO DE AMINAS BIÓGENAS Y DE MELATONINA DURANTE LA VINIFICACIÓN EN TINTO Y BLANCO.

MONITORING BIOGENIC AMINES AND MELATONIN FROM MUST TO WINE IN DIFFERENT GRAPE VARIETIES

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Preparado para enviar a Food Chemistry

Abstract

The European Food Safety Authority (EFSA) accepted health claims in relation to the food constituent melatonin as scientific evidence shows it is effective reducing sleep onset latency, and it alleviates subjective feelings of *jet lag*. According to risk assessment data, EFSA published that histamine and tyramine are the most toxic and food safety relevant biogenic amines. Potential biogenic amines (BA) formation concerns to fermented foods due to intensive microbial activity. Conversely, *Saccharomyces cerevisiase* produces melatonin during fermentation in the winemaking process. This work aims to evaluate the production of the potential healthy melatonin and the toxic BA during the winemaking process.

For this purpose, 11 biogenic amines (agmatine, cadaverine, histamine, metilamine, 2-phenyletylamine, putrescine, spermidine, spermine, tyramine, tryptamine and melatonin), have been monitored during the winemaking of 5 monovarietal wines (*Merlot, Palomino Fino, Syrah, Tempranillo* and *Tintilla de Rota*). Both alcoholic and malolactic fermentation are crucial for the formation of these compounds.

Keywords: Biogenic Amines, Melatonin, Alcoholic Fermentation, Malolactic Fermentation, LC-MS/MS, winemaking, grape varieties

1. INTRODUCTION

Biogenic amines (BA) are organic bases of low molecular weight with aliphatic, aromatic or heterocyclic structures which can be present in fermented foods (cheeses and sausages) and beverages (beer and wine) (Silla Santos, 1996). These compounds are formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones that occur during the microbial, vegetable, and animal metabolism (Askar, & Treptow, 1986). BA are present in grapes and musts (Del Prete, Costantini, Cecchini, Morassut, & García-Moruno, 2009). They are formed by the action of the yeasts during alcoholic fermentation (Goñi, & Azpilicueta, 2001), by the lactic acid bacteria during the malolactic fermentation (Arena & Manca de Narda, 2001; Lonvaud-Funel, 2001), and/or by the presence of other microorganisms responsible for the spoilage of wine (Costantini, Vaudano, Del Prete, Danei, & Garcia-Moruno, 2009). The BA concentration in wine depends on factors such as the content of nitrogenous compounds in the grape, its level of maturation, the nitrogenous fertilization of the soil and the use of good hygiene in the winemaking process among others (Ancín-Azpilicueta, González-Marco, & Jiménez-Moreno, 2008).

The total level of amines in wines ranges from 0 to 130 mg L^{-1} (Soufleros, Barrios, & Bertrand, 1998). Histamine, tyramine, putrescine and cadaverine are the most frequent amines but nearly 25 have been described in wines (Önal, 2007). HPLC coupled with fluorimetric or spectrophotometric detection and mass spectrometry are the most used methods for the analysis of wines in recent years (Anlı & Bayram, 2008). However, the difficulty of the detection of amines in wines relies on the complexity of the matrix and the low concentrations expected (Anlı & Bayram, 2008; García-Marino, Trigueros, & Escribano-Bailón, 2010). Therefore the use of pre or post column derivatization methods with different reagents (dansyl chloride, *p*-phthalaldehyde, and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) is generally carried out in the analysis of amines to increase the sensitivity of the detection and prevent interferences (Önal, 2007; García-Marino, Trigueros, & Escribano-Bailón, 2010).

Since biogenic amines present negative effects on health they are regulated for safety purposes. The intake of high concentration may cause adverse effects like headaches,

hypo- or hypertension, nausea, cardiac palpitation, renal intoxication, cerebral hemorrhage or even death depending on the sensitive individual and the capacity of detoxification (Silla Santos, 1996; Shalaby, 1996). Additionally to their impact on health, they can present negative effects on the aroma and the flavour (Ancín-Azpilicueta, González-Marco, & Jiménez-Moreno, 2008) being a quality marker (Önal, 2007). The European Food Safety Authority (EFSA) published the scientific opinion on risk based control of biogenic amine formation in fermented foods in 2011. This report states that histamine and tyramine are considered the most toxic amines in foods (EFSA, 2011a). It reveals that further research is needed for estimating safe levels of BA in foods as there is a lack of scientific data for a complete risk assessment.

The amino acid tryptophan is the precursor of all 5-methoxytryptamines such as tryptamine, serotonin and melatonin. Melatonin (*N*-acetyl-5-methoxytryptamine; Mel) is an indoleamine structurally related to tryptamine. This compound is synthesized mainly by the pineal gland and plays an important role in the synchronization of the sleep/wake cycle in humans. In contrast to other BA, EFSA accepted the alleviation of subjective feelings of jet lag in 2010 (EFSA, 2010) and recently concluded that the food constituent melatonin is sufficiently characterised and that a cause and effect relationship has been established between the consumption of melatonin and reduction of sleep onset latency (EFSA, 2011b).

As well as BA, melatonin has already been reported in grapes and wines (Murch, Hall, Le, & Saxena, 2010; Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, & Garcia-Parrilla, 2011a) and it can appears due to the metabolism of *Saccharomyces* during the alcoholic fermentation of the winemaking process (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, & Garcia-Parrilla, 2011b). It amounts ranges from 0.16 µg L^{-1} in Chardonnay (Stege, Sombra, Messina, Martinez, & Silva, 2010) to 130 µg L^{-1} in Tempranillo (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, & Garcia-Parrilla, 2011a).

Saccharomyces produces the bioactive melatonin but other deleterious BA can be formed during fermentation. This effect can not be overlooked. The aim of this work is

to evaluate the formation of melatonin and other BA from the grape must to wine during winemaking process under controlled conditions. This paper intends to assess both melatonin and BA formation from must to wine during red and white winemaking steps of five grape varieties.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

The biogenic amines agmatine (Ag), cadaverine (Cad), histamine (His), metilamine (Met), 2-phenyletylamine (Phe), putrescine (Put), spermidine (Sne), spermine (Spe), tyramine (Tyr), tryptamine (Tne) and melatonin (Mel), and the internal standard 2-aminoheptanoic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium acetate trihydrate and triethylamine (TEA) were supplied by Fluka (Steinheim, Germany), ortophosphoric acid by BDH Prolabo (Barcelona, Spain), acetronitrile and methanol by Merck (Darmstadt, Germany) and formic acid by Panreac (Barcelona, Spain). All reagents were of analytical grade. Milli-Q purification system (Millipore, Bedford, MA, USA) was used to obtain ultrapure water.

The AccQ Fluor reagent kit (contained ACQ reagent, acetonitrile and 0.2 mM sodium borate buffer pH 8.8) was supplied by Waters (Milford, MA, USA).

2.2 Samples

The grapes used for this study were grown under strictly controlled conditions in the experimental cultivars Rancho de la Merced Research Centre (Instituto de Investigación y Formación Agraria y Pesquera, IFAPA) in Jerez de la Frontera, Spain. Grapes were harvested at their optimum ripeness from the 2010 vintage. Winemaking process was carried out in an experimental winery at pilot scale. Red wines were elaborated with *Merlot*, *Syrah*, *Tempranillo* and *Tintilla de Rota* varieties. Red grape varieties were de-stemmed, crushed and placed in a 100 L steel vessel. Peptolitic enzymes (0.03 g kg^{-1}, Vinozym® Vintage FCE, Novozymes, Spain) and sulphur dioxide (50 mg kg^{-1}) were added to maximize polyphenol extraction and to protect the must. One day later, fermentation was started after yeasting (*Saccharomyces cerevisiae* yeast, Actiflore® F5, Laffort, Spain), and temperature was maintained at 27°C ± 1

during alcoholic fermentation (AF). As soon as the tumultuous AF had finished (density 999 g L^{-1}), the wine was pressed (Willmes, Germany). For the malolactic fermentation (MLF), lactic bacteria *Oenococcus oeni* (1g HL^{-1}, Challenge Easy ML, Sepsa-Enartis, Spain) and nutrients (20 g HL^{-1}, Nutriferm ML, Sepsa-Enartis, Spain) were used. When this step of the process was finished, the wine was racked and the clarification (10 g HL^{-1} egg white albumen, Laffort, Spain) was performed. Finally the wines were bottled.

The white wine was produced with *Palomino Fino* grape variety. Grapes were destemmed, crushed, pressed and sulphur added (80 mg kg^{-1}). After pressing must was dejuiced for 12 hours at 4ºC, and AF was then started after yeasting (Actiflore PM, Laffort, Spain). AF was developed at controlled temperature (18 ± 1 ºC). After AF wine was maintained under control conditions (T^a= 18 ºC) until racked. Then, wine was stored in a cold chamber (at 0 ºC) until clarification using gelatine (15 g HL^{-1}, Laffort, Spain) and bentonite (0.6 g HL^{-1}, Laffort, Spain). Finally, the wine was filtered and bottled.

Table 1 shows grape varieties, sampling dates of grapes and the steps of winemaking. Samples were frozen and stored at -20º C for further analysis. Table 2 shows the main oenological parameters of the musts and wines obtained (OIV, 1990).

2.3 Preparation of standard solutions and samples

The biogenic amine solutions were dissolved in methanol up to a concentration of 2 g L^{-1}. The standard solutions were prepared by diluting the standard solutions in water.

One millilitre of must or wine samples was centrifuged at 14000 rpm for 3 min and passed through filters (0.45µm). Then, a solid-phase extraction procedure was performed using Oasis MCX (30 mg) extraction cartridges from Waters (MA, USA) and following the method proposed by Peña-Gallego, et al. (2009).

The derivatization of standards and samples were achieved using the method described by Cohen and De Antonis (1994) with the modifications proposed by Peña-Gallego et al. (2009).

2.4 Biogenic amines analysis by HPLC coupled to fluorescence detection

The HPLC apparatus used was a Waters 600E system controller connected to a Waters 474 scanning fluorescence detector (Waters, Milford, MA, USA). Data acquisition and treatment were performed in a Waters Millennium 2.0 data station. Separations were obtained on a Phenomenex Luna C18 column (250 x 4.6 mm, 5 µm). The fluorescence detector recorded wavelengths of 250 nm for excitation and 395 nm for emission. The injection volume was 5 µL and the flow rate was 1 mL min^{-1}. Solvents and gradient conditions were performed with the method described by Peña-Gallego et al. (2009), including the following BA: Ag, Cad, His, Met, Phe, Put, Sne, Spe, Tyr and Tne. The identification of BA was carried out by comparison of their retention times with those reference standards. Quantification was performed with an external linear calibration curve of each BA.

2.5 Melatonin analysis by LC-MS/MS

An HPLC Perkin Elmer Series 200 system equipped with a binary capillary pump, a degasser, an autosampler, and coupled in series with a QTRAP mass detector equipped with an electrospray ionization (ESI) system. Samples were analyzed by LC-MS/MS as previously described by Rodriguez-Naranjo et al. (2011a). Melatonin identification was carried out by comparing the 233/174 transition MS data of the samples and its standard. An external standard calibration was used for quantifying Mel in samples.

2.6 Statistical analysis

For comparison purposes, data were showed as mean ± standard deviation (SD) of analyses in triplicate and performed using Microsoft Excel (Version 2007). The one-way analysis of variance (ANOVA), with the correlation coefficient of $p \leq 0.05$, was calculated using Statistica package (Version 6.0).

3. RESULTS AND DISCUSSION

The most abundant biogenic amines found at different steps of winemaking process of five grape varieties were histamine, tyramine and putrescine (Fig. 1). They ranged 0.08 – 2.7 mg L^{-1}, 0.032 –0.62 mg L^{-1}, and 0.025 – 0.31 mg L^{-1} respectively. Table 3 shows levels of other BA present at lower concentration in the samples, their content ranged: Ag (0 – 0.18 mg L^{-1}), Met (0 – 0.013 mg L^{-1}), Phe (0 – 0.026 mg L^{-1}), Sne (0 – 0.061 mg L^{-1}), Spe (0 – 0.056 mg L^{-1}), and Tne (0 – 0.07 mg L^{-1}), in all the steps. The amine Sne was present in all varieties and Cad was not found in quantifiable concentration in any step.

Cad and Met were absent or not quantifiable in musts (step 1). Conversely, His, Put, Sne and Spe were detected in all the must and Ag, Phe, Tyr and Tne were only found in some of them (Fig. 1, Table 3). The concentration of BA previously reported in grapes and musts (Sass-Kiss, Szerdahelyi, & Hajós, 2000; Del Prete, Costantini, Cecchini, Morassut, & García-Moruno, 2009; Hajós, Sass-Kiss, Szerdahelyi, & Bradocsz, 2000) depends on factors such as the degree of grape maturation, the soil composition and the climatic conditions during growth (Anlı & Bayram, 2009). Our values of BA in musts of red varieties are rather similar from one variety to another as they were grown in the same vineyard, vintage and harvested at their optimum maturity conditions (Fig. 1, Table 3). Indeed, Del Prete et al. (2009) compared two harvests (2004 and 2005) with very different climate conditions (average temperatures 28 ºC and 19 ºC in August, respectively). The higher the temperature is the lower total BA content in musts and wines. Temperature in our study is even higher (average temperature was 33.12 ºC in August) and the content of total BA in must is therefore lower (1.9 mg L^{-1} in Merlot and 2.3 mg L^{-1} in Syrah) than they reported (14.46 mg L^{-1} in Merlot and 14.57 mg L^{-1} in Syrah at 28 ºC) (Del Prete, Costantini, Cecchini, Morassut, & García-Moruno, 2009).

During alcoholic fermentation (step 2), the BA were those detected in musts (His, Put, Sne and Spe) and Phe and Tne. (Fig. 1, Table 3). The concentrations of Ag, Met, Phe, Put, and Tyr were very small, between 0.01 and 0.2 mg L^{-1} (Fig. 1, Table 3). In agreement with previously published results, it appears that the yeast used could be

somehow responsible for the presence of BA in wines (Torrea & Ancín, 2002). Histamine concentration reaches its maximum level during the alcoholic fermentation (step 2) in red wines (Fig. 1). Buteau et al. (1984) and Bauza et al. (1995) reported the amount of His increase in some red wines during AF. Spe and Spe levels were unchanged (no significant differences, Table 3) reflecting that *Saccharomyces cerevisiae* can not use these amines as a source of energy as other alcoholic fermentative yeasts do (Bover-Cid, Torriani, Gatto, Tofalo, Suzzi, & Belletti, 2009).

MLF (step 4) increases the level of BA as certain strains of *Oenococcus* can produce those amines in variable proportions (Landete, Ferrer, & Pardo, 2007; Alcaide-Hidalgo, Moreno-Arribas, Martín-Álvarez, Polo, 2007). Histamine increases in all varieties after MLF (step 4, Figure 1) whilst Put and Tyr in most of them. Besides, Phe was found in quantifiable amount after MLF in *Tempranillo* and *Tintilla de Rota*. Finally, no relevant changes in the total BA content of wines studied after clarification, filtering and bottling that were carried out after a month the MLF had finished.

Figure 1 displays the concentration of His and Put in each step of all wines studied. Note His, which is the most abundant amine, has a significant lower content in white wine than in red wines (Figure 1), and its content has very similar variation in all red wines analyzed. Therefore it clearly depends on the winemaking process more than the variety. However, the other amines studied did not present the same pattern as His, as can be seen in the example of Put (Fig. 1).

Figure 2 shows an overview of total amines concentration of the wines at each step. The amine levels resulted very low in all cases (0.3 – 3.3 mg L^{-1}) compared with certain published data: 7 – 30 mg L^{-1} (García-Marino, Trigueros, & Escribano-Bailón, 2010) and 15 – 25 mg L^{-1} (Del Prete, Costantini, Cecchini, Morassut, & García-Moruno, 2009). Our results agree with those reported by López et al. (2011) ranging from 3.56 to 3.66 mg L^{-1} for *Tempranillo* variety from La Rioja. The trend of total BA for *Merlot*, *Syrah* and *Tempranillo* varieties shows an increasing during AF and MLF and a decreasing after pressing and racking (Fig. 2). However, the red variety *Tintilla de Rota* remains rather constant concentration regardless the winemaking step is considered.

Figure 3 shows the content of Mel and Tne of the samples analysed. Tne is an intermediate of the auxin indole-3-acetic acid and involved in the biosynthetic pathway of the melatonin (Posmyk, & Janas, 2009). Before yeast inoculation, Tne is detected in three of five musts analysed whilst Mel is absent in all of them. Tne increased during AF (step 2, density 1020 g L^{-1}) in all of wines (especially in *Tempranillo*) whilst Mel occurred just in *Tempranillo*. After the AF (step 3), Tne decreased except in *Tintilla de Rota* which Tne reached the higher amount (125 µg L^{-1}), and Mel was detected in all of wines in a range from 0.4 to 6.1 µg L^{-1}. After MLF (step 4), Tne decreases spectacularly and the content of Mel increases from 5.6 to 18 µg L^{-1} in *Tintilla de Rota*. Tne can be transformed into serotonin by the action of tryptophan 5-hydroxylase which is present in all of kingdoms (Roschina, 2010). Therefore, the biochemical pathway to transform Tne in Mel is a possibility to consider within lactic acid bacteria. *Tintilla de Rota* data show a clear connection between the precursor Tne and the Mel. The main characteristic of this variety is the higher alcoholic degree (Table 2) which might affect to the formation of melatonin. Rodriguez-Naranjo et al. (2012) proved the relevant role of medium in melatonin formation by *Saccharomyces*, however, lactic acid bacteria are unexplored at the moment. Indeed, this is the first paper reporting its formation during MLF in wines and further studies are required.

The amounts of Mel in the wines studied are very small compared with those of BA, between 0 to 18 µg L^{-1}. Similarities in all the vinifications are: Mel was not detected in any grape or must and then it was quantified in all samples after the AF (Fig. 3, step 3) as previously reported (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, & Garcia-Parrilla, 2011b). At pressing (step 3), Mel values present no significant differences regardless the variety considered, excepting *Syrah*, which presented very low concentration. Stabilization process (storage at cold) seems to affect importantly to Mel content in final wines (Fig. 3, step 5). White wine presented Mel amounts similar to red wines as happened in the 2008 vintage (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, & Garcia-Parrilla, 2011b). The Mel content in *Tintilla de Rota* varieties presented a remarkable increase after MLF, three or four folds higher in MLF

(Fig. 3, step 4). Despite all varieties were inoculated with the same commercial *Oenococcus oeni*, Mel values do not follow a pattern as it does during AF.

In our previous work, we carried out our experiments with the same varieties, grown in the same vineyard and identical winemaking process. Values from 2008 are also very similar between them for all the varieties involved and the same year. Surprisingly, vintage from 2008 present values 100 folds higher than those in the present study. In both experiences the inoculated yeast was Actiflore© F5 for red wine and Actiflore© PM for white wine. Therefore, climatic conditions influence somehow the final content of melatonin in wines as to BA, probably changing the concentration of their precursors. The effect of medium concentration on *Saccharomyces* production and final concentration was observed in a previous paper with synthetic musts and our results confirm that in wines.

4. CONCLUSIONS

His, Tyr, and Put are major BA present in analyzed wines. Histamine amounts have a clear dependence of winemaking process; they increase after alcoholic or malolactic fermentation. BA values for these wines elaborated in warm climates are lower than other previously reported. Therefore, it makes these wines are within safe limits for consumption.

This paper confirms that Mel is formed after AF in wines. MLF influences on final concentration of Mel in wines being this bioactive linked to yeasts and bacteria metabolism. As well as microorganisms involved in the winemaking process, climatic conditions can also affect the final amounts of Mel.

To conclude, the bioactive Mel can be present in wines and its formation occurs when BA keep concentrations considered as safe for consumers.

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FIGURE CAPTIONS

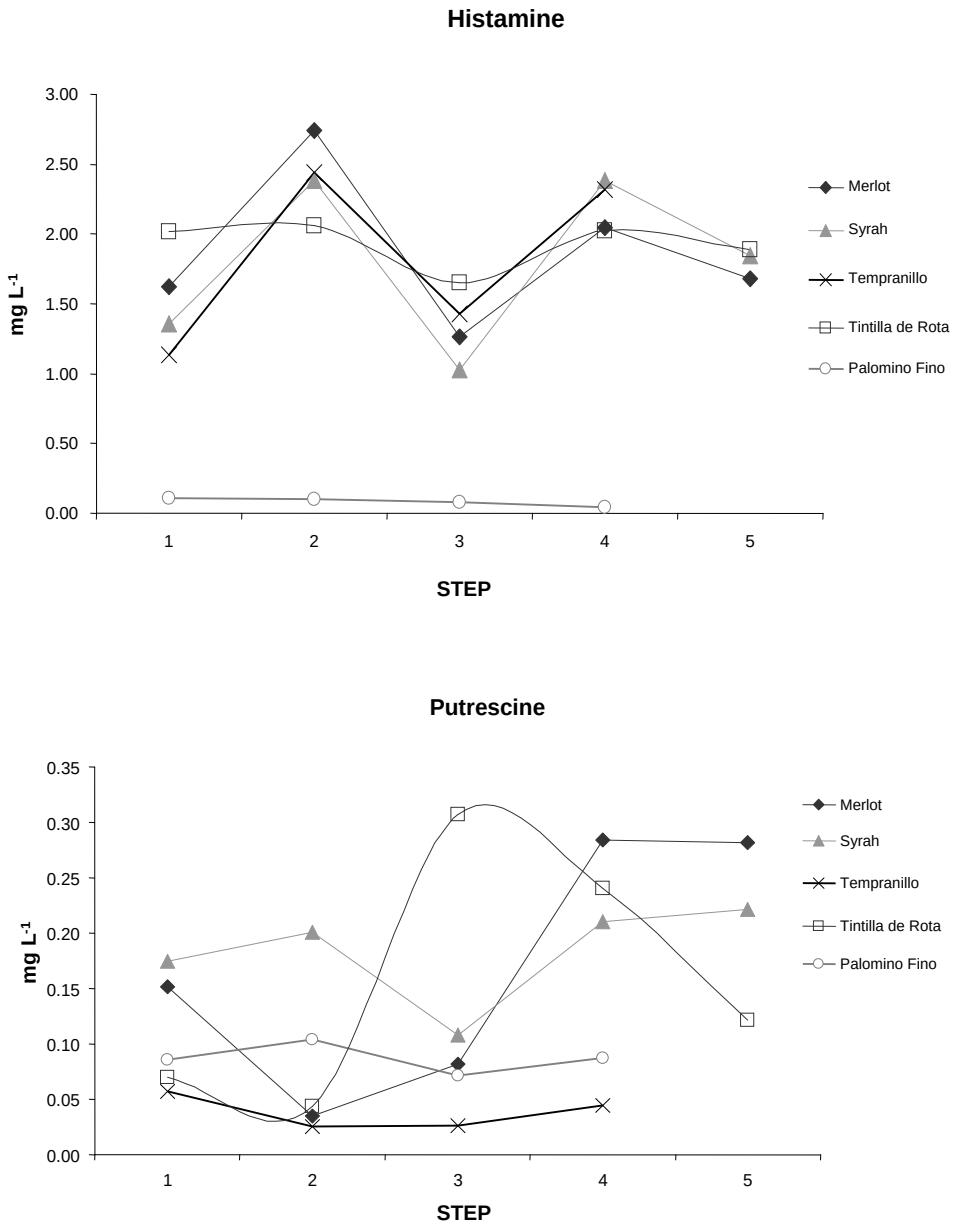

Figure 1. Evolution of histamine and putrescine found in four red wines (*Merlot*, *Syrah*, *Tempranillo* and *Tintilla de Rota*) and one white wine (*Palomino Fino*) during the winemaking process.

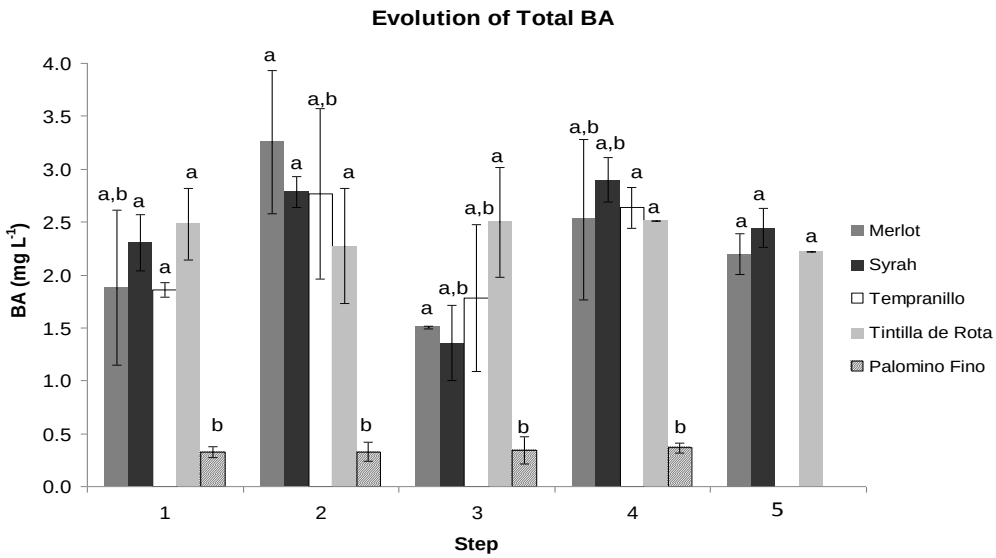

Figure 2. Evolution of total biogenic amine found in four red wines (*Merlot*, *Syrah*, *Tempranillo* and *Tintilla de Rota*) and one white wine (*Palomino Fino*) during the winemaking process. Letters above the bars show significant differences between varieties for the same step.

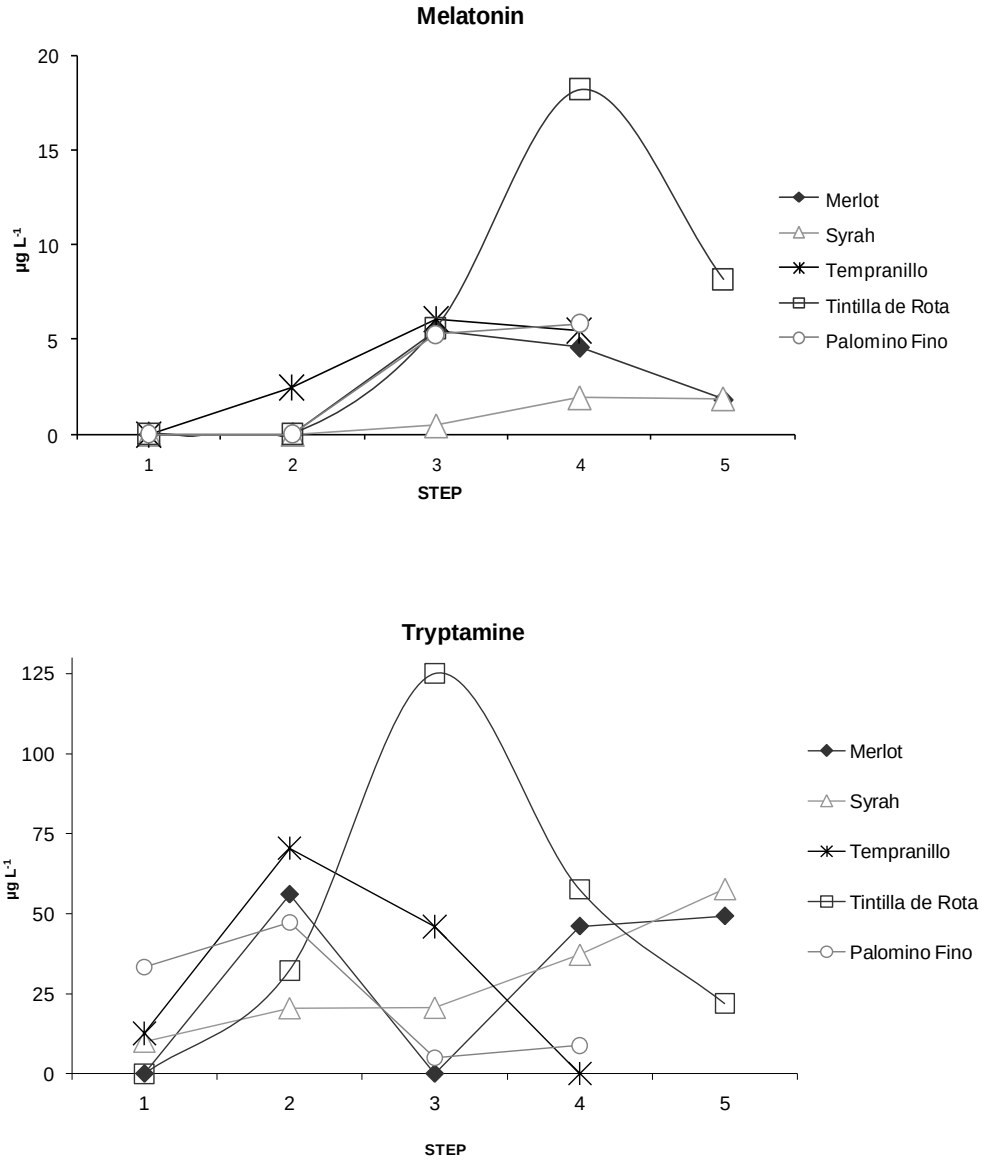

Figure 3. Evolution of tryptamine and melatonin found in four red wines (*Merlot*, *Syrah*, *Tempranillo* and *Tintilla de Rota*) and one white wine (*Palomino Fino*) during the winemaking process.

TABLES

Variety	Code	Step Number	Name of step	Description	Sampling date
Merlot	**M1**				02/09/2010
Syrah	**S1**		Harvest		02/09/2010
Tempranillo	**T1**	Step 1		Grape must	18/08/2010
Tintilla de Rota	**TR1**				09/09/2010
Palomino Fino	**P1**		Press		25/08/2010
Merlot	**M2**				08/09/2010
Syrah	**S2**		Density 1020	Tumultuous AF	07/09/2010
Tempranillo	**T2**	Step 2			23/08/2010
Tintilla de Rota	**TR2**				20/09/2010
Palomino Fino	**P2**		Dejuice	Before the AF	26/08/2010
Merlot	**M3**				14/09/2010
Syrah	**S3**		Press	Before the MLF	13/09/2010
Tempranillo	**T3**	Step 3			27/08/2010
Tintilla de Rota	**TR3**				27/09/2010
Palomino Fino	**P3**		Density 1020	Tumultuous AF	30/08/2010
Merlot	**M4**				22/10/2010
Syrah	**S4**			After the MLF	08/10/2010
Tempranillo	**T4**	Step 4	Racking		15/09/2010
Tintilla de Rota	**TR4**				22/10/2010
Palomino Fino	**P4**			After the AF	06/09/2010
Merlot	**M5**				01/12/2010
Syrah	**S5**	Step 5	Stabilization	Before bottling	01/12/2010
Tintilla de Rota	**TR5**				01/12/2010

Table 1. Varieties, steps of winemaking, and sampling dates of the grapes used to produce the wines in the study.

		Merlot	*Syrah*	*Tempranillo*	*Tintilla de Rota*	*Palomino Fino*
Musts	Sugars [a]	25.2	24.5	22.2	26.3	19.7
	TA [b] (g L^{-1} TH_2)	4.35	4.88	5.53	3.95	3.79
	pH	3.7	3.62	3.75	3.75	3.8
	Potassium (ppm)	1972	1827	1882	2025	2315
Wines	Ethanol (%vol.)	14.48	14.43	13.67	16.02	11.91
	TA [b] (g L^{-1} TH_2)	5.84	5.42	6.14	7.12	4.72
	pH	3.42	3.60	3.35	3.41	3.27
	VA [c] (g L^{-1} TH_2)	0.43	0.45	0.46	0.43	0.16
	TPI [d]	45.98	51.39	43.40	52.09	6.9
	Sugars (g L^{-1})	2.65	2.55	1.38	3.66	0.67

[a] Sugars measured as Brix degree

[b] TA: total acidity expressed as g L^{-1} tartaric acid (TH_2).

[c] VA: volatile acidity expressed as g L^{-1} acetic acid (AcH).

[d] TPI: total phenolic index.

Table 2. Must and wine oenological parameters such as sugars, total acidity (TA), pH, potassium, ethanol, volatile acidity (VA) and total polyphenols index (TPI), were measured according to Official Methods (OIV, 1990).

Sample	Biogenic Amines (mg L^{-1})							
	Ag	**Cad**	**Met**	**Phe**	**Sne**	**Spe**	**Tyr**	**BA total**
M1	n.d.	n.q.	n.d.	0.014 ± 0.003	0.037 ± 0.005	0.04 ± 0.01	n.d.	1.9 ± 0.7
S1	0.05 ± 0.01	n.q.	n.q.	n.q.	0.03 ± 0.00	0.042 ± 0.005	0.62 ± 0.03	2.48 ± 0.34
T1	0.15 ± 0.05	n.q.	n.d.	0.010 ± 0.001	0.024 ± 0.000	0.020 ± 0.001	0.46 ± 0.02	2.3 ± 0.3
TR1	n.d.	n.q.	n.d.	n.q.	0.027 ± 0.003	0.014 ± 0.001	0.34 ± 0.05	1.9 ± 0.1
P1	0.025 ± 0.002	n.q.	n.q.	0.014 ± 0.004	0.03 ± 0.01	0.023 ± 0.005	n.d.	0.32 ± 0.05
M2	0.18 ± 0.02 [a]	n.q.	0.03 ± 0.01 [a]	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.062 ± 0.001 [a]	3.3 ± 0.7 [a]
S2	0.082 ± 0.002	n.q.	0.011 ± 0.004	0.012 ± 0.002	0.03 ± 0.01	0.042 ± 0.001	n.d.	2.3 ± 0.5
T2	0.07 ± 0.01	n.q.	n.d.	0.014 ± 0.004	0.061 ± 0.005	0.041 ± 0.004	0.04 ± 0.01 [a]	2.8 ± 0.2 [a]
TR2	0.05 ± 0.01 [a]	n.d.	n.d.	0.011 ± 0.003	0.031 ± 0.003	0.014 ± 0.002	0.032 ± 0.002	2.8 ± 0.8 [a]
P2	n.d.	n.q.	n.d.	0.010 ± 0.001	0.030 ± 0.005	0.037 ± 0.001	n.d.	0.3 ± 0.1
M3	0.05 ± 0.01 [a,b]	n.q.	0.013 ± 0.001 [a]	0.015 ± 0.005	0.028 ± 0.005	0.056 ± 0.003	n.d.	1.50 ± 0.01 [b]
S3	0.04 ± 0.01 [b]	n.q.	n.q.	0.014 ± 0.003	0.020 ± 0.002 [a]	0.050 ± 0.003	0.07 ± 0.01 [a,b]	2.5 ± 0.5
T3	0.11 ± 0.03	n.q.	n.d.	n.q.	0.022 ± 0.004	0.024 ± 0.005	0.118 ± 0.002 [a,b]	1.4 ± 0.4 [b]
TR3	0.09 ± 0.02 [a]	n.d.	n.d.	n.d.	0.040 ± 0.005	n.d.	0.28 ± 0.00 [b]	1.8 ± 0.7
P3	0.020 ± 0.004 [d]	n.q.	n.q.	n.q.	0.031 ± 0.005	0.039 ± 0.005	0.08 ± 0.01 [a,b]	0.3 ± 0.1
M4	0.031 ± 0.007 [a,b]	n.q.	n.d.	0.01 ± 0.00	0.04 ± 0.01	0.06 ± 0.01	n.d.	2.5 ± 0.8 [c]
S4	n.d.	n.q.	n.d.	0.011 ± 0.003	0.030 ± 0.005	n.d.	0.23 ± 0.02 [a,b,c]	2.51 ± 0.05
T4	0.04 ± 0.01	n.q.	n.d.	0.026 ± 0.004 [a,c]	0.019 ± 0.001 [a]	0.033 ± 0.002	0.16 ± 0.02 [a,b,]	2.9± 0.2 [c]
TR4	0.09 ± 0.00	n.q.	n.d.	0.014 ± 0.000 [a]	0.04 ± 0.00 [a]	n.d.	0.038 ± 0.001 [c]	2.6 ± 0.2
P4	0.03 ± 0.00 [d]	n.q.	n.d.	n.q.	0.033 ± 0.002	0.054 ± 0.003	0.10 ± 0.01 [a,b]	0.37 ± 0.04
M5	0.064 ± 0.005	n.q.	n.d.	0.010 ± 0.004	0.05 ± 0.01	0.052 ± 0.005	n.d.	2.2 ± 0.2
S5	n.d.	n.d.	n.d.	n.q.	0.025 ± 0.005	0.05 ± 0.01	0.24 ± 0.02 [a,b,c]	2.4 ± 0.2
TR5	0.12 ± 0.00 [b]	n.q.	n.d.	0.013 ± 0.000 [a]	0.014 ± 0.002 [a,c]	n.d.	0.04 ± 0.00 [b,c]	2.22 ± 0.05

Data calculated as mean (n = 3) and expressed as Mean ± SD (standard desviation); n.d.= not detected; n.q.= not quantified.

[a] Significant differences ($p \leq 0.05$) with respect to its initial grape must (ANOVA).

[b] Significant differences ($p \leq 0.05$) with respect to its density 1020 stage (ANOVA).

[c] Significant differences ($p \leq 0.05$) with respect to its press stage (ANOVA).

[d] Significant differences ($p \leq 0.05$) with respect to its dejuice stage (ANOVA).

[e] Significant differences ($p \leq 0.05$) with respect to its racking stage (ANOVA).

Table 3. Biogenic amines (mg L^{-1}) content in the different steps of the winemaking process.

CAPÍTULO 5.
ESTUDIO COMPARATIVO DE LA ACTIVIDAD ANTIOXIDANTE DE LA MELATONINA Y OTROS COMPUESTOS INDÓLICOS.

COMPARATIVE EVALUATION OF THE ANTIOXIDANT ACTIVITY OF MELATONIN AND RELATED INDOLES

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Abstract

Recent scientific evidence suggests that melatonin is present in foods and beverages. Therefore, its antioxidant activity needs to be evaluated and compared with that of other bioactive compounds such as polyphenols. This paper presents a systematic study of the antioxidant activity of melatonin and other compounds involved in the melatonin biosynthetic pathway and chemically related substances. The methods selected were the ferric reducing antioxidant power (FRAP) assay, the oxygen radical absorbance capacity (ORAC) assay and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The reactions depend on the mechanisms involved (electron transfer or hydrogen atom transfer). The kinetic parameters (stoichiometric factors, rate constants) and antioxidant activity show considerable differences within the indole compounds: some compounds do not react at all, melatonin presents a discrete *in vitro* antioxidant activity while the highest values are shown by 5-hydroxy compounds. These compounds have a clear relation between structure and activity when an electron transfer mechanism is involved (FRAP and DPPH assays). Melatonin and indole-3-acetic acid react better through the hydrogen atom transfer mechanism. The radical scavenging ability towards the $DPPH^{\bullet}$ was studied and EC_{50}, T_{EC50} and antioxidant efficacy (AE) were calculated: the 5-hydroxy-indoles present values that are similar to those of ascorbic acid or kaempferol.

Keywords: Melatonin, Indole, Serotonin, Wine, Food composition, Food analysis, Antioxidant activity, Kinetic parameters.

1. INTRODUCTION

The moderate consumption of red wine is related with a decreased risk of cardiovascular diseases. Both alcohol and polyphenols have protective effects (Bertelli, 2007; Bertelli, 2009; Covas et al., 2010), so the bioavailability of polyphenols has become a topic of intense research lately. After the ingestion of wine, these compounds are rapidly metabolized, which means that only a minimal amount of the original polyphenols remains in the circulatory system. Over 80% of these polyphenols are found in methylathed, glucuronated and sulphated conjugated forms in plasma and urine, and their biological activity depends on the effects of the conjugated compounds formed (Covas et al., 2010; D'Archivio et al., 2010). Recent studies have shown that melatonin (*N*-acetyl-5-methoxytryptamine) (Mel) is present in wines and that it is formed during the alcoholic fermentation process (Rodriguez-Naranjo et al., 2011a; 2011b) and certain *Saccharomyces* strains form Mel while they carry out the alcoholic fermentation (Rodriguez-Naranjo et al., 2012).

In humans, Mel exerts different actions in organs and fluids, where it is untransformed. The antioxidant properties of Mel and its ability to scavenge several radical species as part of its bioactivity have been described in the literature (Allegra et al., 2003; Reiter et al., 2003; Estevão et al., 2011), both by direct and indirect mechanisms (Barrenetxe et al., 2004; Bonnefont-Rousselot and Collin, 2010; Milczarek, 2010). Indeed, it scavenges ROS and RNS, and metabolites formed afterwards are also radical scavengers in their turn (Tan et al., 2007). Additionally, melatonin supports the effect of antioxidant enzymes as superoxide dismutase, catalase and glutathione reductase which contributes to their *in vivo* antioxidant effects (Bonnefont-Rousselot and Collin, 2010). Unlike polyphenols, melatonin can cross physiological barriers and reduce the oxidative damage in both lipid and aqueous cell environments (Reiter et al., 2004).

Mel is found throughout the biological kingdoms and it can be formed from tryptophan by either of two different synthetic routes (Fig. 1). In animals, Mel functions as a neurohormone to regulate the light/dark cycle and also protects tissues against oxidative damage (Reiter et al., 2003; Barrenetxe et al., 2004; Reiter et al., 2004). The possible physiological functions of Mel in plants are associated with reproductive and

vegetable developments and circadian rhythms, the protection of cells against free radical damage (Paredes et al., 2009). Other indole compounds that are chemically related to Mel have biological properties: tryptamine, serotonin, and *N*-acetyl-serotonin are also neurohormones and indole-3-acetic acid is a phytohormone. Some of those compounds are present in wine because they are natural components of grapes (for example, the aminoacid tryptophan) whilst others are produced during fermentation (tryptophol, tryptamine, indole-3-acetic acid) and contribute to the wine's sensory properties.

During the last decade scientific literature has focused on the antioxidant properties of phenolic compounds: structure-activity relations have been sought and found, antioxidant activity (AA) has been determined by different methods and the contribution of each class of phenolic compound to the overall antioxidant activity of wine has been evaluated (Fernández-Pachón et al., 2006; Villaño et al., 2007; Villaño et al., 2005). In the last two years, the European Food Safety Authority (EFSA) accepted health claims in relation to melatonin and reduction of sleep onset latency, and the alleviation of subjective feelings of *jet lag* (EFSA, 2010; 2011). Biological properties of indole compounds have been studied in depth (Noda et al., 1999; Poeggeler et al., 2002; Cano et al., 2003; Estevão et al., 2011). Melatonin analogues have been synthesised in order to enhance their antioxidant properties (Suzen et al., 2006; Gurkok et al., 2009; Shirinzadeh et al., 2010; Suzen et al., 2012). Still, there is no evidence that they produce higher clinical advantages without side effects (Shirinzadeh et al., 2010).

The aims of this study are to describe the antioxidant properties of the naturally indole compounds involved in synthetic pathways in plants and animals (Fig. 1) and of other chemically related compounds in wines; to understand the underlying antioxidant mechanism (hydrogen atom transfer (HAT) or electronic transfer (ET)); and to explore the kinetic behaviour. For these purposes three different methods were selected: the ferric ion reducing antioxidant power (FRAP) assay, the oxygen radical absorbance capacity (ORAC) assay, and the diphenyl-1-picrylhydrazyl (DPPH) assay. The FRAP method is based on an electron-transfer reaction in which the antioxidant reduces the

ferric ion. The ORAC assay involves a hydrogen atom transfer reaction in which the antioxidant competes with fluorescein for the flux of peroxyl radicals generated by the thermal decomposition of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). Depending on the medium the reaction occurs in, both ET and HAT reactions can take place in the DPPH method (Huang et al., 2005). The conditions of this assay also make it possible to determine radical scavenging parameters and kinetic constants. In addition, the FRAP assay is carried out under acidic conditions (pH 3.5) in aqueous medium and the DDPH method uses methanol. The ORAC assay is carried out in an aqueous buffered medium at pH 7.4 and it has been used in the food industry to measure antioxidant capacity (Huang et al., 2005). The whole set of methods can provide a good interpretation of the antioxidant properties of these compounds (Fig. 2).

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

The standards of the 13 indole compounds were: indole-3-acetic acid (IAA), *N*-acetyl-3-(2-aminoethyl)-5-methoxyindole (melatonin, Mel), *N*-acetyl-5-hydroxytryptamine (N-Ac-Ser), serotonin hydrochloride (Ser), tryptamine (Tne), indole-3-ethanol (tryptophol) (Tol), *L*-tryptophan (Trp), 5-methoxyindole-3-acetic acid (5-MeOH-IAA), 5-hydroxyindole-3-acetic acid (5-OH-IAA), 5-methoxytryptamine (5-MeOH-Tne), 5-hydroxy-*L*-tryptophan (5-OH-Trp), 5-methoxytryptophol (5-MeOH-Tol), and *N*-acetyl-3-(2-aminoethyl)-6-methoxyindole (6-Mel). They were purchased from Sigma, Aldrich, Fluka and Wako and all of them had a grade of purity higher than 97%. Ferric 2,4,6-tripyridyl-*s*-triazine (TPTZ), ferrous sulfate ($FeSO_4 \cdot 7H_2O$), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), and 1,1-Diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) were provided by Sigma. 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid (Trolox) and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Aldrich and fluorescein (FL) from Fluka. Methanol was obtained from Merck (Darmstadt, Germany) and formic acid from Panreac (Barcelona, Spain). All reagents were of analytical grade.

2.2 Instrumental

Absorbance measurements (FRAP and DPPH assays) were recorded on a Hitachi UV-2800® spectrophotometer thermostated with a Peltier system at 25 ºC (Hitachi High Technologies Corporation, Tokio, Japan). The ORAC assay was carried out on a Synergy HT (Biotek® Instruments Inc., Colmar Cedex, France) multi-detector microplate reader at 37 ºC.

2.3 The FRAP assay

The FRAP reagent consists of acetate buffer (300 mM, pH 3.6), TPTZ (10 mM in HCl 40 mM) and $FeCl_3 \cdot 6H_2O$ (20 mM) (10:1:1, v:v:v). A total of 3 ml of FRAP reagent was mixed with 300 µl Mili-Q water and 100 µl of sample. Absorbance was measured after eight minutes at 593 nm. An aqueous solution of $FeSO_4 \cdot 7H_2O$ in the 0–1 mM range was used for calibration. All determinations were performed in triplicate. Results are expressed as mol of Fe^{+2} per mol of indole (Benzie and Strain, 1996).

2.4 The ORAC assay

The ORAC assay used was one previously reported (Ou et al., 2001) with some modifications. Briefly, 50 µL of sample or Trolox was mixed with 100 µL of FL (90 nM) and 50 µL of AAPH (12 mM). A total of 5 different concentrations of indole in the range 0.78 to 6.25 µM were analyzed in triplicate. The FL fluorescence was recorded for 90 min (excitation and emission wavelengths were set at 485 nm and 528 nm, respectively). Several dilutions of Trolox (0.5 – 15 µM) were used as a calibration standard. The ORAC results were calculated by using a regression equation between the Trolox concentration and the net area of the FL decay curve (area under curve). ORAC values were expressed as mol Trolox equivalents per mol of indole.

2.5 The DPPH assay

The AA of indole compounds was estimated using the described procedure (Sánchez-Moreno, et al., 1998) and the DPPH˙ radical. A total of 70 µL of different indole concentrations ranging from 0 to 3.5 mM dissolved in methanol was added to 2.73 mL of DPPH˙ methanolic solution (25 mg/L). The initial absorbance at 515 nm was close to 0.700 and the stability of the DPPH˙ radical checked for 1 min. Dilutions of Trolox (1.25 – 12.5 mM) were used as the standard. A regression equation was obtained by

plotting the absorbance decrease versus the trolox concentration. The DPPH results obtained by interpolating on the Trolox calibration curve and expressed as mmol Trolox equivalents per mol of indole.

2.5.1 Kinetic measurements

Stock solutions of the indoles were prepared and properly diluted (within the range 0.25-1.5 10^{-3} M in the cuvette) in methanol. Solutions of DPPH$^{\cdot}$ (61.8 10^{-6} M in the cuvette) usually have a maximum absorbance close to 0.700 (methanol, max 515 nm). In all cases the DPPH$^{\cdot}$ concentration was higher than that of the indole (generally 2–10 times higher). The procedure was as follows: 2.73 mL of DPPH$^{\cdot}$ solution was put into the spectrophotometer cuvette. Subsequently, an aliquot of 70 μL of the indole solution was rapidly added and the changes in the DPPH$^{\cdot}$ absorbance were monitored over time. The same lot of methanol was used throughout the kinetic experiment to prevent the impurities in the solvent from interfering in the rates of reaction. Measurements were carried out in triplicate.

2.6 Data analysis

Results were processed by Microsoft® Excel 2007 and Origin® 7.5. One-way analysis of variance (ANOVA) was carried out with STATISTICA® 6.0 software package.

2.6.1 EC_{50}, T_{EC50} and antiradical efficiency

The kinetic reactions of indoles with DPPH$^{\cdot}$ were recorded for each compound (Fig. 3). From these plots, the percentage of DPPH$^{\cdot}$ remaining in the steady state (DPPH$^{\cdot}$ rem) was determined as

$$\%DPPH \cdot rem = \left(\frac{A_f}{A_0} \right) x100 \qquad (1)$$

where A_0 and A_f are the radical absorbances at 515 nm at the beginning and at steady state, respectively. Absorbance at the time of the steady state was used in order to ensure that reaction did not progress further. Concentrations of the indole compounds in the reaction medium were plotted against the percentages of the remaining DPPH$^{\cdot}$ at the end of the reaction in order to obtain the EC_{50} index, defined as the amount of antioxidant needed to decrease the initial DPPH$^{\cdot}$ concentration by 50%.

T_{EC50} is the time taken to reach steady state at the concentration corresponding to EC_{50}. It was obtained by plotting the times taken to reach the steady state against the concentration of the antioxidant compound.

Antiradical efficiency (AE) combines EC_{50} and T_{EC50} factors, and was calculated as $AE = (T_{EC50} \times EC_{50})^{-1}$.

2.6.2 Stoichiometric factor determination

The total number of DPPH˙ radicals quenched per molecule of indole, the total stoichiometry factor n_T, was calculated in the presence of an excess of DPPH˙ as compared to the indole concentration by using the formula:

$$n_T = \frac{A_0 - A_f}{\varepsilon\, c_0\, l} \qquad (2)$$

where c_0 is the initial indole concentration, A_0 and A_f are the radical absorbances at 515 nm at the beginning and at steady state, respectively, ε is the molar extinction coefficient for the DPPH˙ radical at 515 nm in M^{-1} cm^{-1} and l is the optical path of the cuvette in cm. Since the molar extinction coefficient of DPPH˙ depends on the solvent (Goupy et al., 2003; Foti et al., 2004), ε was estimated under our working conditions: a calibration curve was obtained by measuring the absorbance at 515 nm at different DPPH˙ concentrations ranging from 40 to 108 µM and using a methanolic reaction medium and a value of ε=11598 M^{-1} cm^{-1} was found.

n_T values were also calculated as

$$n_T = \frac{1}{2EC_{50}} \qquad (3)$$

where $2EC_{50}$ is the theoretical efficient concentration of antioxidant needed to reduce 100% of DPPH˙ radical (Brand-Williams et al., 1995).

3. RESULTS AND DISCUSSION

3.1 Antioxidant activity: comparison of FRAP, ORAC and DPPH values

The FRAP, ORAC and DPPH values listed in Table 1. The AA of the indole compounds investigated largely depends on the assay used. This behaviour seems to be

peculiar to this class of compounds because other antioxidants such as phenolic compounds present similar activities whatever assay is applied (Villaño et al., 2005). The indole compounds consist in an electron-rich indole heterocycle and side chains, which explain the differences of AA of these compounds. 5-hydroxyindole compounds are the most active with the FRAP assay. This is clearly observed if IAA (0.18), 5-MeOH-IAA (0.84), and 5-OH-IAA (9.62), or Mel (0.11), and N-Ac-Ser (4.20), or Trp (0.01), and 5-OH-Trp (5.05) are compared. The activity is at least 10 times lower if there is a 5-methoxy group in the indole ring. Poeggeler et al. (2002) reported the two 5-hydroxylated indoleamines, N-Ac-Ser and Ser, were stronger suppressors of $ABTS^{\bullet}$ throughout a direct electron donation. For the same 5-hydroxyindole structure, the group in the lateral chain also contributes to the AA being the order: acid (5-OH-IAA (9.62)), primary amide (5-OH-Trp (5.05)), secondary amide (N-Ac-Ser (4.20)) and amine (Ser-HCl (3.70)).

The ORAC results do not show the same structure-activity relations as the FRAP results. The differences between the results of the whole set of compounds are not so large. The most active compounds are 6-Mel, Mel and IAA. The first of these is a synthetic Mel isomer but the other two have hormone activity in both animals and plants respectively. In this case, the 5-OH-substituted compounds also have very similar values (from 0.80 to 0.95 mol Trolox mol^{-1} indole) but they do not have the highest ORAC values. The indoleamines are not good peroxyl radical-trapping antioxidants compared to polyphenols. In fact, Antunes et al. (1999) suggest that compounds with an NH group in a 5-position of the pyrrole ring react only slightly with peroxyl radicals. However, Mel is an effective scavenger of peroxyl radical *in vivo* (Reiter et al., 2003). It may be related to its ability to scavenge the initiating radicals limiting the direct appearance of a peroxyl radical (Reiter et al., 2003). Further the reaction of Mel (*in vitro* and *in vivo*) with radical species as $O_2^{\bullet -}$, H_2O_2, singlet oxygen, carbonate radical and $ONOO^-$ form N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), which is also capable to donate electrons (Tan et al., 2007).

A clear structure-activity relationship can be deduced from DPPH values. The most effective antioxidants against DPPH˙ radicals are those with an 5-hydroxy substituent in the indole ring (Ser-HCl > 5-OH-Trp > 5-OH-IAA > N-Ac-Ser). Their values are in the same range as those reported for phenols (such as gallic acid) or flavonoids (such as (+)-catechin and (–)-epigallocatechin) (Villaño et al., 2005). Those indoles with a 5-methoxy substituent in the indole ring, such as 5-MeOH-IAA, 5-MeOH-Tne and 6-Mel, show some AA. The other antioxidants investigated show negligible AA against DPPH˙ radicals.

Regarding the differences of the values obtained with the three methods (FRAP, ORAC and DPPH), these can be explained by the mechanism involved. The ORAC assay uses a hydrogen atom transfer mechanism and the FRAP assay an electron transfer mechanism (Huang et al., 2005). In the case of the DPPH assay, the reaction between DPPH˙ radicals and the antioxidants can go simultaneously through an HAT and an ET mechanism (Huang et al., 2005), although the contribution of the latter is expected to be substantially less important to the whole process than that of the former. A possible explanation shown in Table 1 is that, the contribution of the ET mechanism is appreciable for the 5-OH-indole compounds investigated. The replacement of 5-methoxy group by a 5-hydroxy group has a greater influence on the results when the ET mechanism reaction (FRAP and DPPH) is involved (N-Ac-Ser >> Mel, 5-OH-IAA >> 5-MeOH-IAA, and Ser >> 5-MeOH-Tne). However, this substitution reduces the lipophility of the compound and it may affect its antioxidant potential. The ORAC values show that Mel and IAA act better through an HAT mechanism. Nonetheless, it is necessary to stress the complexity of the processes taking place in the three assays used. In relation to this, the possibility of the contribution to the AA of processes involving the reaction products cannot be ruled out.

3.2 Radical scavenging parameters: EC_{50}, T_{EC50} and AE.

The AA of a compound is inversely related to its EC_{50}, the parameter that is used to measure the potency of an antioxidant. The higher the EC_{50}, the lower the AA of a compound. As can be seen in table 2, the chemical structure of 5-hydroxyindole compounds shows the most active reactivity. 5-OH- IAA, 5-OH-Trp, N-Ac-Ser and

Ser-HCl have EC_{50} values (4.6 – 6.3) that are similar to those of such phenolic compounds as gallic acid, (+)-catechin and (–)-epigallocatechin (Villaño et al., 2007) which have between 3 and 6 hydroxy groups in their molecules. IAA, 5-MeOH-IAA, and 5-MeOH-Tne needed to be more concentrated for EC_{50} to be calculated and it could not be estimated for the other compounds.

Sánchez-Moreno et al. (1998) use the T_{EC50} value to classify the kinetic behavior of the antioxidant compound as follows: rapid ($T_{EC50} < 5$ min), intermediate ($5 \text{ min} \leq T_{EC50} \leq 30$ min) and slow ($T_{EC50} > 30$ min). According to this classification, 5-OH- IAA, 5-OH-Trp, N-Ac-Ser and Ser-HCl are intermediates (Table 2). These compounds are faster than most of the polyphenols reported in the literature (Villaño et al., 2007; Sánchez-Moreno et al., 1998) and slower than ascorbic acid or Trolox, which react very quickly with DPPH˙ radicals.

The AE parameter involves both EC_{50} and T_{EC50}, and is also used to classify antioxidant power (Villaño et al., 2007). The lower EC_{50} and T_{EC50} are, the higher AE will be. For Ser-HCl, AE is very high, similar to of the value it has for ascorbic acid. The EC_{50} values for 5-OH- IAA, 5-OH-Trp and N-Ac-Ser are as high as for kaempferol.

These results show that the indoles that have a 5-hydroxy group are powerful DPPH˙ radical scavengers. The other indoles tested have a very low response to the radical.

3.3 Study of kinetic reaction of DPPH˙ and indole compounds

The stoichiometric factors in Table 2 are calculated at the end point of the reaction. The two sets of n_T values obtained with eq 2 and 3 match well. This could indicate a fast reaction kinetics in which reaction products are hardly involved. The n_T values are comparable to those of phenolic compounds with several OH-substituents in their structure. This may mean that an ET mechanism is involved as well as the HAT mechanism.

The radical scavenger capacity of the indole was investigated in methanolic media under pseudo first-order conditions, with a DPPH˙ concentration at least ten times smaller than that of the antioxidant compounds. Under pseudo first-order conditions the rate of the reaction was defined as:

$$-\frac{d[DPPH\cdot]}{dt} = n_T k_1 [indol]^{\alpha} [DPPH\cdot]^{\beta} = k_{obs} [DPPH\cdot]^{\beta} \quad (4)$$

where k_{obs} is the experimentally observed rate constant, k_1 is the second-order rate constant for the reaction, n_T is the stoichiometric factor, and α and β are the partial orders of the process studied with respect to each of the reagents. For several antioxidants investigated α and β are equal to unity (Valginigli et al., 1995; Foti et al., 2004; Villaño et al., 2007). However, to the best of our knowledge, the reaction between DPPH˙ radicals and the indols investigated in this study has not been previously studied. Therefore, the dependence of k_{obs} on the concentration of indole compounds in the reaction medium needs to be used to estimate the α and the β values for the processes studied.

In order to calculate the experimentally observed rate constant, k_{obs}, the following equation was used:

$$\ln A = \ln A_0 - k_{obs}\, t \quad (5)$$

where A_0 and A are the absorbances of the radical at 515 nm at the beginning and at time t. The plots of ln A against time were straight lines in all the kinetics investigated, which indicates that the reaction is first order with respect to the DPPH˙ concentration (that is, β=1). To estimate the α value, the following equations must be used:

$$k_{obs} = n_T k_1 [indol]^{\alpha} \quad (6)$$

and

$$\log k_{obs} = \log(n_T k_1) + \alpha \log[indol] \quad (7)$$

Therefore, from the slope of log k_{obs} vs. log [indol] the value of α can be calculated. For the indoles investigated, the value of α was equal to 1: that is, the rate of the reaction between the DPPH˙ radicals and the indole compounds is a second-order reaction, but first-order with respect to each of the reagents. The same result was found for several phenolic compounds (Villaño et al., 2007; Foti et al., 2004; Valginigli et al., 1995). The product n_T x k_1 was obtained, in each case, from the slopes of the plots of k_{obs} vs. [indol]. Subsequently, the stoichiometric factor values were used to estimate the second-order rate constant for the reaction, k_1. The k_1 values are listed in Table 2.

Nonetheless, taking into account the complexity of the reactions studied and the differences in the stoichiometric factors reported in the literature for a given antioxidant, the k_1 values listed in Table 2 have to be considered as approximate.

The k_1 values listed in Table 2 show that the radical scavenger capacity of the four indole compounds studied is similar, within the experimental errors. Only the 5-OH-Trp seems to show a somewhat lower AA.

4. CONCLUSION

This paper reports the *in vitro* AA of indole compounds using FRAP, ORAC and DPPH assays. Indole AA depends largely on the analytical method used and considerable differences are found. Chemical structure plays a significant role: 5-hydroxyindoles are most active, and their values are comparable to those of phenolic compounds if the ET mechanism is the base of the reaction. Both Mel and IAA are discrete antioxidants that are active if a HAT reaction is involved (ORAC value). In addition to its AA, these compounds are interesting because many of them are synthesized naturally in the human body.

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FIGURE CAPTIONS:

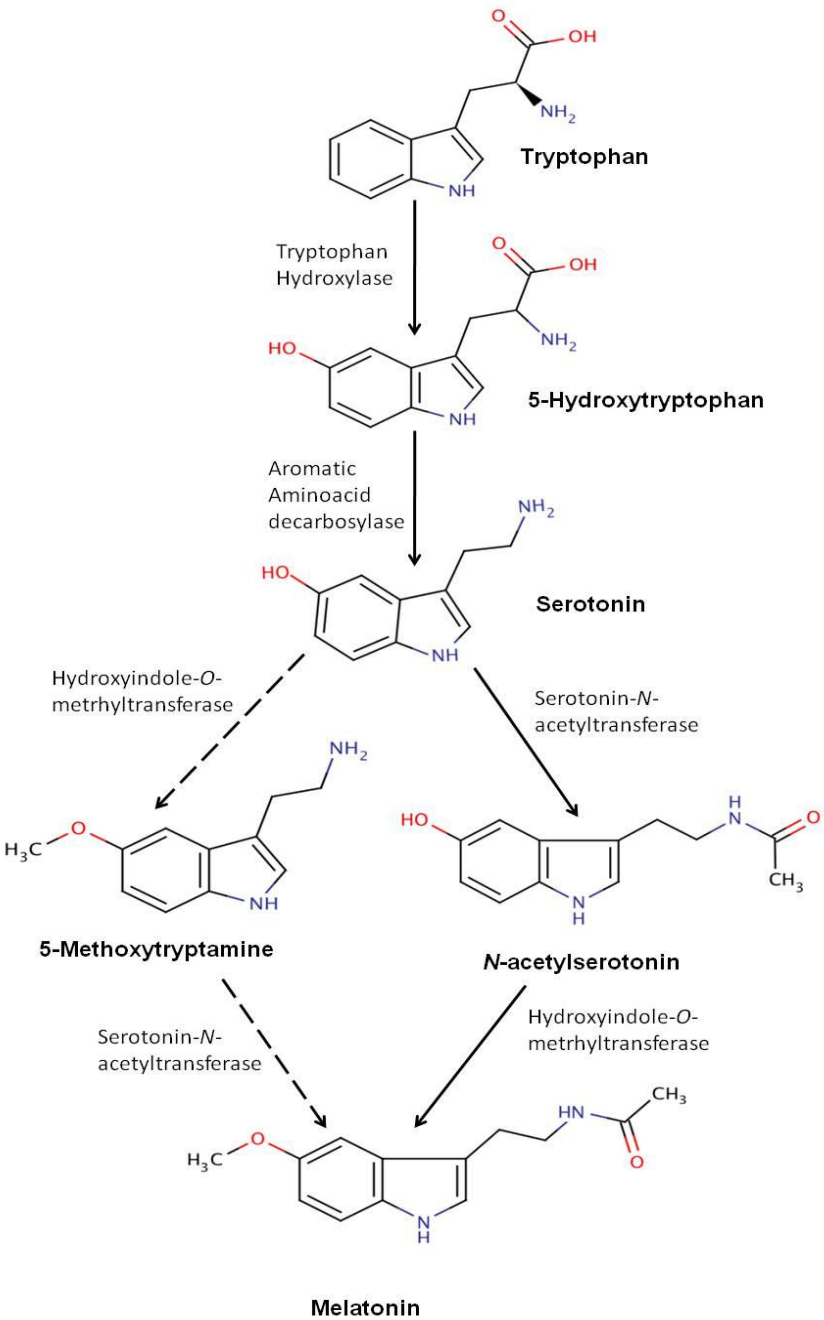

Figure 1. Biosynthetic pathways of melatonin from tryptophan amino acid.

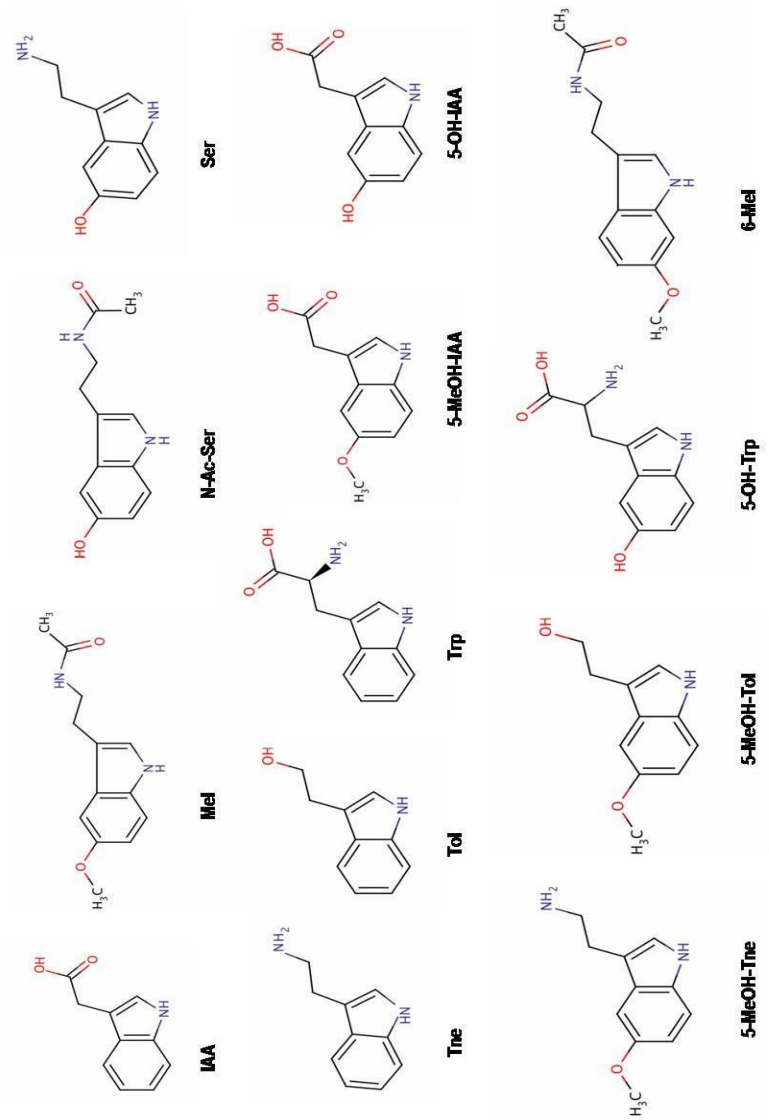

Figure 2. Structures of indole compounds: indole-3-acetic acid (IAA), melatonin (Mel), *N*-acetyl-5-hydroxytryptamine (N-Ac-Ser), serotonin hydrochloride (Ser-HCl), tryptamine (Tne), tryptophol (Tol), *L*-tryptophan (Trp), 5-methoxyindole-3-acetic acid (5-MeOH-IAA), 5-hydroxyindole-3-acetic acid (5-OH-IAA), 5-methoxytryptamine (5-MeOH-Tne), 5-methoxytryptophol (5-MeOH-Tol), 5-hydroxy-*L*-tryptophan (5-OH-Trp), and *N*-acetyl-3-(2-aminoethyl)-6-methoxyindole (6-Mel).

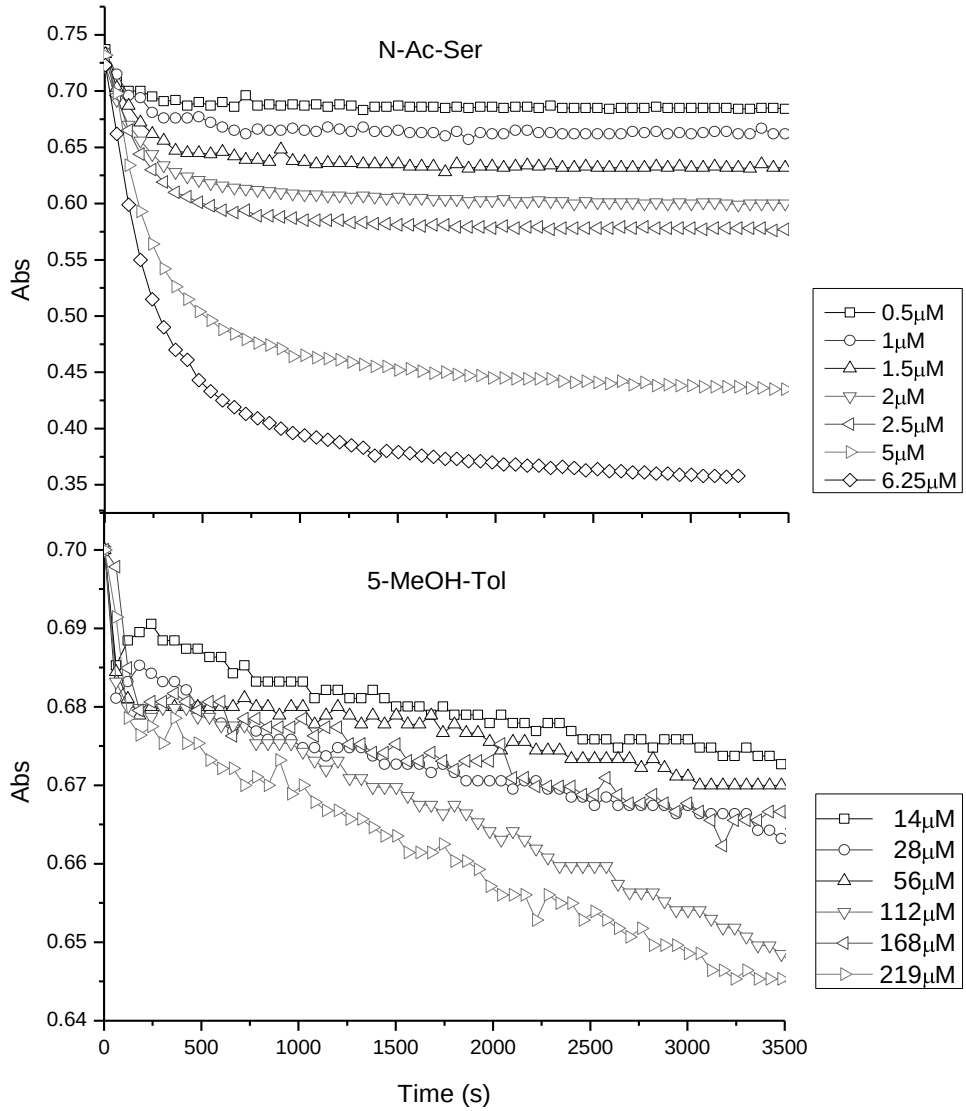

Figure 3. Absorbance decay curve of DPPH radical (61.8 μM) in the presence of different solutions of *N*-acetyl-5-hydroxytryptamine (N-Ac-Ser) and 5-methoxytryptophol (5-MeOH-Tol).

TABLES

Indole	FRAP [a] (mol Fe^{2+} mol^{-1} indole)	ORAC [a] (mol Trolox mol^{-1} indole)	DPPH [a] (mmol Trolox mol^{-1} indole)
IAA	0.18 (0.01) **	1.85 (0.09)	42.05 (3.54) **
Mel	0.11 (0.00)	1.86 (0.05)	3.15 (1.07)
N-Ac-Ser	4.20 (0.08) **	0.81 (0.01) **	$1.8 \cdot 10^3$ (4.49) **
Ser-HCl	3.70 (0.08) **	0.95 (0.04) **	$2.4 \cdot 10^3$ (108.75) **
Tne	0.02 (0.00) **	0.70 (0.02) **	9.20 (1.84) *
Tol	3.42 (0.15) **	0.57 (0.02) **	3.78 (0.58) *
Trp	0.01 (0.00) **	0.69 (0.01) **	0.24 (0.00) **
5-MeOH-IAA	0.84 (0.05) **	0.38 (0.01) **	207.32 (12.5) **
5-OH-IAA	9.62 (0.98) **	0.93 (0.03) **	$2.1 \cdot 10^3$ (21.25) **
5-MeOH-Tne	0.02 (0.00) **	0.54 (0.03) **	90.05 (3.18) **
5-MeOH-Tol	0.27 (0.02) **	0.46 (0.02) **	4.33 (1.47)
5-OH-Trp	5.05 (0.31) **	0.80 (0.01) **	$1.9 \cdot 10^3$ (189.71) **
6-Mel	2.07 (0.14) **	4.79 (0.38) **	32.11 (3.78) **

[a] Data calculated as mean (n=3) and standard deviation (SD) indicated in brackets.
Significantly different from Mel: * $p \leq 0.01$, ** $p \leq 0.001$.

Table 1. Antioxidant activity values of indole compounds: indole-3-acetic acid (IAA), melatonin (Mel), *N*-acetyl-5-hydroxytryptamine (N-Ac-Ser), serotonin hydrochloride (Ser-HCl), tryptamine (Tne), tryptophol (Tol), *L*-tryptophan (Trp), 5-methoxyindole-3-acetic acid (5-MeOH-IAA), 5-hydroxyindole-3-acetic acid (5-OH-IAA), 5-methoxytryptamine (5-MeOH-Tne), 5-methoxytryptophol (5-MeOH-Tol), 5-hydroxy-*L*-tryptophan (5-OH-Trp), and *N*-acetyl-3-(2-aminoethyl)-6-methoxyindole (6-Mel).

Indole	EC_{50} (10^{-6} M)	T_{EC50} [a]	AE [b] (10^{-3})	n_T [c]	n_T [d]	k_1 [e] (M^{-1} s^{-1})
IAA	380	---	---	0.1	0	---
Mel	---	---	---	---	---	---
N-Ac-Ser	6.3	23.15	6.86	4.9	4.4	1.9-2.1
Ser-HCl	4.6	16.82	12.92	6.7	6.4	1.7-1.8
Tne	---	---	---	---	---	---
Tol	---	---	---	---	---	---
Trp	---	---	---	---	---	---
5-MeOH-IAA	139	---	---	0.1	0.1	---
5-OH-IAA	5.4	22.23	8.33	5.7	5.2	2.0-2.2
5-MeOH-Tne	174	---	---	0.1	0.1	---
5-MeOH-Tol	---	---	---	---	---	---
5-OH-Trp	6.3	20.53	7.73	4.9	4.8	0.65-0.68
6-Mel	---	---	---	---	---	---

[a] Obtained graphically
[b] Antiradical efficiency = $(TE_{50} \times EC_{50})^{-1}$
[c] Obtained by using $n_T = (A_0-A_f)/ \varepsilon c_0 l$
[d] Obtained by using $n_T = 1/ 2EC_{50}$
[e] $k_1 = K_{obs}\ (n)^{-1}$

Table 2. Radical scavenging parameters (EC_{50}, T_{EC50}, and AE), stoichiometric factors (n_T) and rate constants (k_1) of indole compounds (indole-3-acetic acid (IAA), melatonin (Mel), *N*-acetyl-5-hydroxytryptamine (N-Ac-Ser), serotonin hydrochloride (Ser-HCl), tryptamine (Tne), tryptophol (Tol), *L*-tryptophan (Trp), 5-methoxyindole-3-acetic acid (5-MeOH-IAA), 5-hydroxyindole-3-acetic acid (5-OH-IAA), 5-methoxytryptamine (5-MeOH-Tne), 5-methoxytryptophol (5-MeOH-Tol), 5-hydroxy-*L*-tryptophan (5-OH-Trp), and *N*-acetyl-3-(2-aminoethyl)-6-methoxyindole (6-Mel)).

DISCUSIÓN GENERAL

El primer requisito para la determinación de melatonina en uvas y vinos es disponer de un método analítico validado. En un principio, uno de los métodos más utilizados fue el ELISA (Enzyme-Linked ImmunoSorbent Assay) debido a que se utilizaba para el análisis de melatonina en plasma y otros fluidos biológicos. Otros autores han empleado este método sin modificación alguna para el análisis en vegetales o alimentos de origen vegetal e incluso vino (De la Puerta et al., 2007; Iriti et al., 2006).

En el **Capítulo 1** de esta Tesis se muestran los resultados obtenidos al aplicar el test *ELISA* a muestras de vino diluidas ($1:10^2$, $1:10^4$ y $1:10^6$) o adicionadas con melatonina (10 – 300 ng L^{-1}) para conseguir concentraciones iguales a las descritas en el intervalo de medida del kit. No hubo respuesta lineal en las medidas en ninguno de los dos casos. La falta de linealidad de la respuesta no permitió la validación del método para análisis de melatonina en vinos.

Además, se determinó el porcentaje de falsos positivos (25.0 %) usando la solución 0.00 ng L^{-1} de melatonina procedente del kit. Y el porcentaje de falsos negativos del kit (28.6 %) empleando distintas diluciones de melatonina patrón dentro del rango de concentraciones detectables del kit. La aparición de falsos positivos y/o falsos negativos son típicos en el análisis de compuestos traza (Horwitz, 1982) y, en este caso, las concentraciones determinadas por ELISA pueden considerarse como trazas (ng L^{-1}).

Por tanto, este método de análisis no puede ser validado para el análisis de melatonina en vinos. También debe tenerse en consideración el alto coste del kit, que lo hace poco conveniente para un análisis de rutina.

La segunda técnica ensayada fue la *cromatografía líquida con detector de fluorescencia (HPLC-FL).* Los laboratorios de análisis de alimentos suelen estar equipados con el instrumental utilizado, por tanto puede resultar un método económico. Se puso a punto un método cromatográfico cuya detección se basa en las propiedades fluorescente de la melatonina ($\lambda_{exitación}$=285 nm y $\lambda_{emisión}$=345 nm) y en condiciones isocráticas (Metanol:Ácido fórmico 0.1%, 60:40). Se trata de un método sencillo y corto (9 min) que puede analizar melatonina de modo rutinario. Y, por tanto, se validó como un método fiable para la cuantificación de melatonina

en vinos, según las recomendaciones de la AOAC (Huber, 1998), con un rango de linealidad de 58-5807 μg L^{-1}, con límites de detección (LDD) y cuantificación (LDC) de 51.72 y 172.39 μg L^{-1} respectivamente. Este límite de detección es alto para cuantificar melatonina en vinos directamente y requiere la concentración del analito en la muestra. Sin embargo, es un método adecuado para determinar melatonina en suplementos que se formulan con dosis de mayores del LDC.

La tercera y última de las técnicas estudiadas fue la *cromatografía líquida acoplada a un espectrómetro de masas (LC-MS/MS).* Una vez optimizados los parámetros del detector, se puso a punto un método para la cuantificación de la melatonina en vinos utilizando el modo multiple reaction monitoring (MRM) para las transiciones 233/216 y 233/174. El rango de linealidad fue de 9 a 290 μg L^{-1} (r=0.998). Este método resultó ser más sensible que el método de HPLC-FL ya que el LDD y el LDC fueron 0.13 y 0.44 μg L^{-1} respectivamente. La gran ventaja es que por comparación del tiempo de retención y del patrón de fragmentación de la melatonina presente en las muestras con el de su estándar comercial, teniendo en cuenta los fragmentos mayoritarios y minoritarios y sus abundancias, es posible la identificación inequívoca de la melatonina.

Se cuantificó la melatonina en los vinos de las variedades *Cabernet Sauvignon*, *Petit Verdot, Prieto Picudo, Syrah* y *Tempranillo* con concentraciones de entre 5.1 a 129.5 μg L^{-1}. Otros estudios han encontrado menores contenidos en melatonina en vinos procedentes de las variedades Sangiovese (0.5 μg L^{-1}) y Trebbiano (0.4 μg L^{-1}) (Mercolini et al., 2008). Sin embargo, en un reciente estudio llevado a cabo por Vitalini et al. (2011), se ha encontrado concentraciones de entre 5.2 - 8.1 μg L^{-1} para las variedades Groppello y Merlot.

Además, en algunos vinos (Cabernet Sauvignon, Jaen Tinto, Merlot, Palomino Negro, Prieto Picudo y Tempranillo) se observó la presencia de un compuesto con fragmentación similar a la melatonina que aparece a distinto tiempo de retención. Este compuesto fue identificado como un posible isómero de melatonina o un compuesto relacionado estructuralmente con la melatonina (ISO). El compuesto ISO (m/z = 233) presentaba los iones fragmento 174 (pico base) y 216 como mayoritarios, al igual que la melatonina, pero los minoritarios no coinciden

totalmente con los de melatonina (m/z = 191, 161 y 159). Esta es la primera vez que se describe la presencia de un isómero de melatonina en vinos. Recientemente, este posible isómero de la melatonina u otro ha sido identificado en el proceso de vinificación de la variedad de uva *Malbec* (Gomez et al. 2012).

Como resultado de este capítulo se dispone de un método fiable y validado que identifica inequívocamente la melatonina con un límite de identificación suficientemente bajo para su determinación en vinos.

En el **Capítulo 2** de esta Tesis se exploró la posible presencia del bioactivo melatonina en diferentes partes de la uva (piel, pulpa y semilla). Se estudiaron trece variedades incluyendo de vinificación (*Cabernet Sauvignon, Merlot, Nebbiolo, Palomino Fino, Pedro Ximénez, Petit Verdot, Syrah, Tempranillo,* y *Tintilla de Rota*) y de mesa (*Flame Seedless*, *Moscatel Itálica*, *Red Globe* y *Superior Seedless)*. Iriti et al. (2006) detectaron melatonina por el método ELISA en ocho variedades de uva, entre las cuales se encontraban *Nebbiolo* (428.3 $\mu g\ L^{-1}$) y *C. Sauvignon* (183.6 $\mu g\ L^{-1}$), analizadas en nuestro estudio.

Se empleó un método de extracción descrito por Arnao y Hernández-Ruiz (2009) para material vegetal con distintos disolventes (cloroformo, acetato de etilo y metanol:agua 1:1) y cuya recuperación era del 94 %. Para analizar las muestras se usó el método anteriormente descrito de LC-MS/MS, de LDD = de 0.13 $\mu g\ L^{-1}$. Y, sin embargo, no se detectó melatonina en ninguna de las partes analizadas de las trece variedades de uvas estudiadas.

De modo que la melatonina estaba presente en ciertos vinos monovarietales (Capítulo 1) pero no en las mismas variedades de uvas de procedencia (Capítulo 2). A la vista de estos resultados, se planteó el estudio del proceso de vinificación sobre el contenido de melatonina en el vino (Capítulo 2) para observar en qué momento se formaba. Del análisis de las diferentes etapas de vinificación de varios vinos monovarietales (mosto inicial, prensado y deslío en tintos; y mosto inicial, desfangado y deslío en blancos) se constató que la melatonina no estaba en el mosto (recién despalillado) de ninguna de las variedades, resultado que coincide con los obtenidos para las uvas, reafirmando la ausencia de la indolamina en el sustrato inicial. Tras analizar las diferentes etapas se vio que la melatonina

aparecía después de la adición de la levadura para la fermentación alcohólica, tanto en vino tinto como en blanco. Por tanto, el metabolismo de la levadura podría ser responsable de la su aparición de melatonina en los vinos. Los valores obtenidos para las cinco fermentaciones están dentro del rango de 74.13 a 423.01 $\mu g\ L^{-1}$.

Para confirmar el papel de la levadura, se realizó una nueva vinificación tomando muestras desde el mosto hasta el final de la fermentación alcohólica, siete días después, se verificó que las concentraciones aumentaban progresivamente de no detectada a (153.25 ± 9.33) $\mu g\ L^{-1}$. En este estudio también se pudo comprobar la influencia de la adición del aminoácido triptófano, precursor principal de la melatonina, al mosto inicial. En la vinificación con adición de triptófano, el contenido de melatonina (105.78 ± 11.30 $\mu g\ L^{-1}$) fue significativamente mayor ($p<0.05$) en el día 5 de fermentación que en el mosto al que no se había adicionado (87.50 ± 3.92 $\mu g\ L^{-1}$), a pesar de que la densidad era la misma (1017 $g\ L^{-1}$). Sin embargo, al final de la fermentación presentan valores similares (153.25 ± 9.33 $\mu g\ L^{-1}$ y 152.32 ± 9.47 $\mu g\ L^{-1}$, respectivamente). La melatonina se forma antes cuando su precursor está presente en mayor concentración, de acuerdo con el estudio realizado por Sprenger et al. (1999), en el que *Saccharomyces cerevisiae* producía melatonina en un menor periodo de tiempo cuando había una mayor presencia de precursores en el medio que cuando no.

El trabajo de Sprenger et al. (1999) es el único antecedente bibliográfico encontrado para estudiar la implicación de *Saccharomyces* en la síntesis de melatonina cuando la levadura está en condiciones de crecimiento y estrés. Es preciso un mayor conocimiento del papel de la levadura en la producción de melatonina para conseguir un enriquecimiento de este bioactivo en los vinos y poder darles un valor añadido.

El **Capítulo 3** se centró en estudiar la capacidad de sintetizar melatonina de diferentes cepas comerciales de *Saccharomyces*, por ser la especie desde el punto de vista enológico, más utilizada a nivel mundial. Se seleccionaron ocho cepas comerciales (seis *S. cerevisiae* (CLOS, ICV-D254, QA23, ARM, RVA, TTA), una *S. uvarum* (S6U) y una *S. cerevisiae* var. *bayanus* (Uvaferm BC)) para el estudio,

comprobándose que sólo cuatro de las levaduras son productoras (QA23, ARM, S6U y Uvaferm BC).

Sobre la producción de melatonina en condiciones de crecimiento, se utilizaron seis medios distintos: YPD y SM (mosto sintético), con concentraciones de azúcares reductores de 20 g L^{-1} y concentraciones variables de triptófano (0, 1x y 5x de su contenido inicial y SM con 200 g L^{-1} de azúcares reductores para la fermentación.

Los resultados obtenidos mostraron que la presencia del precursor triptófano en el medio es crucial. En su ausencia no hay síntesis de melatonina y su presencia influye en la velocidad de producción de la indolamina. Estos datos coinciden con los descritos para la producción de indol a lo largo del proceso de vinificación con distintas levaduras (Arevalo-Villena et al., 2010).

El seguimiento de la síntesis de melatonina con el tiempo se realizó utilizando la cepa QA23, que resultó ser la más productora en dos medios (YPD y SM con 20 g L^{-1} de azúcares reductores). En el medio YPD, la melatonina empieza a detectarse a partir de 1h y presenta su máximo a las 2h y a partir de la tercera vuelve a disminuir. El mismo resultado encontró Sprenger et al. (1999) para la formación de melatonina en el interior de la levadura, pero en mayor concentración. Puede que el papel que desempeñe dentro de la célula sea protector, como antioxidante (Sprenger et al., 1999; Hardeland et al., 2003). En el seguimiento de la producción de melatonina en SM (20 g L^{-1} de azúcares reductores) se observó una clara dependencia de la producción de melatonina con la población de la levadura durante la fase de crecimiento. Durante la fase estacionaria, justo después de que la concentración de azúcares sea menor a 5 g L^{-1}, la concentración de melatonina vuelve a aumentar. Esta síntesis parece estar involucrada en un mecanismo de señalización, como ocurre con el triptofol, compuesto relacionado estructuralmente con la melatonina (Chen y Fink, 2010). Se ha encontrado una acumulación de alcoholes aromáticos como el triptofol (de estructura indólica) en medios en los que hay una elevada población de levaduras, y esta acumulación es dependiente del crecimiento celular (Chen y Fink, 2010).

En el estudio de la fermentación alcohólica, de las ocho cepas utilizadas en este

estudio, sólo S6U (*S. uvarum*) y Uvaferm BC (*S. cerevisiae* var. *bayanus*) produjeron melatonina. En la vinificación de uva de la variedad *Chardonnay* se produce una acumulación de indol en el segundo día de fermentación (Arevalo-Villena et al., 2010), al igual que ocurre para la melatonina en nuestro estudio. A partir de este máximo, la concentración de melatonina disminuye hasta el final de la vinificación (Arevalo-Villena et al., 2010). Lo mismo ocurre si comparamos los datos obtenidos en la etapa de deslío (306.9 $\mu g\ L^{-1}$) (Capítulo 2, tabla 2) con el obtenido en el vino *Tempranillo* (129.5 $\mu g\ L^{-1}$) (Capítulo 1, tabla 5). Podemos decir que tanto la presencia de precursores en el medio como la cepa de levadura seleccionada son factores determinantes para aumentar la presencia de la melatonina en los vinos.

Teniendo en cuenta que estudios previos habían demostrado que algunas especies de levaduras podían producir aminas biógenas en vinos (Torrea y Ancín, 2001; Caruso et al., 2002), cabe plantearse si la levadura, a la vez que produce melatonina, también forma aminas biógenas que comprometen la seguridad del producto. Por tanto, dado el posible efecto negativo de la presencia de algunas aminas biógenas sobre la salud de los consumidores y sobre las propiedades organolépticas del vino, y el posible beneficio de la presencia de melatonina en vinos, resulta interesante el estudio de estos compuestos durante el proceso de elaboración de los vinos (**Capítulo 4**).

Se vinificaron cinco variedades de uva (*Merlot*, *Syrah*, *Tempranillo, Tintilla de Rota* y *Palomino Fino,*) en la cosecha del 2010, y se analizaron diferentes etapas (mosto inicial, a mitad de fermentación alcohólica, prensado, deslío y filtración para vino tinto; y prensado, desfangado, mitad de fermentación alcohólica y deslío para vino blanco). En general, se puede decir que estos vinos, elaborados en un clima cálido, presentan un bajo contenido en aminas (0.3 – 3.3 $mg\ L^{-1}$) comparado con los datos publicados por otros autores (García-Marino et al., 2010; Del Prete et al., 2009). Además, estas concentraciones resultan muy por debajo de los límites marcados por países como Suiza, que tiene establecido como concentración máxima de histamina 10 $mg\ L^{-1}$ en vino (OSEC, 2002) u otros como Alemania y Bélgica, que tienen valores máximos inferiores recomendados (Millies y Zimlich 1988). Además, la Organización Internacional de la Viña y el Vino (OIV, 2011)

recomienda no sobrepasar el límite de 12 mg L^{-1} de histamina. Histamina y putrescina son las aminas biógenas con mayor abundancia, apareciendo en todas las etapa de la vinificación (0.04 – 2.7 mg L^{-1} y 0.025 – 0.28 mg L^{-1}, respectivamente). Además, la tiramina, aunque apareció en menos ocasiones, alcanzó niveles más elevados que la putrescina (0.04 – 0.62 mg L^{-1}). Se pone de manifiesto que después de la fermentación alcohólica y maloláctica se produce un aumento en la concentración total de aminas biógenas en vinos tintos, principalmente debido al aumento de la concentración de la amina mayoritaria, la histamina. Este incremento probablemente se deba al metabolismo de las levaduras y bacterias que llevan a cabo estos procesos.

Las vinificaciones realizadas en este año (2010) confirman la formación de melatonina durante la fermentación alcohólica. Después de la fermentación alcohólica, los vinos tintos se inocularon con *Oenococcus oeni* para garantizar la fermentación maloláctica. Se observó un aumento de la concentración de melatonina en los vinos de tres de las variedades (*Merlot*, *Syrah* y *Tempranillo*) después de la segunda fermentación. Por tanto, la fermentación maloláctica también influye en el contenido final de melatonina en los vinos. Durante la proceso de vinificación de la variedad *Tintilla de Rota* se observó clara relación entre el precursor triptamina y la melatonina.

La melatonina tiene una reconocida validez como bioactivo. En los dos últimos años, el Panel de Productos Dietéticos, Nutrición y Alergias (NDA) de la Autoridad Europea de Seguridad Alimentaria (EFSA) ha considerado a la melatonina como un constituyente natural de los alimentos, y le ha atribuido dos alegaciones de propiedades saludables: ayudar a aliviar los síntomas de *jet lag* y a reducir el tiempo de concilio del sueño (EFSA, 2010; 2011a). Para que el consumo de melatonina tenga efectos como los descritos anteriormente, se recomienda un consumo de 1 a 5 mg diarios. Las concentraciones halladas en el vino distan de estos valores por lo que no se podría atribuir la citada alegación. No obstante, los alimentos proporcionan concentraciones menores que pueden suponer un beneficio sobre la salud a nivel local actuando como antioxidante (Hardeland y Pandi-Perumal, 2005). Por otra parte, en 2011 se publicó la opinión del Panel de Riesgos Biológicos (BIOHAZ) sobre la evaluación del riesgo de la formación de

aminas biógenas en alimentos fermentados (EFSA, 2011b). Este estudio reveló que la histamina y la tiramina son las aminas biógenas más tóxicas presentes en los alimentos y para estimar la evaluación de riesgo son necesarios más estudios de estos compuestos. El presente estudio demuestra que la formación de melatonina no está vinculada a valores peligrosos de aminas ya que los vinos analizados presentan (0 – 18.23 $\mu g\ L^{-1}$) de melatonina y se pueden considerar seguros por su bajo contenido en aminas (0.3 – 3.3 $mg\ L^{-1}$).

El último capítulo (**Capítulo 5**) de esta Tesis está dedicado al estudio de la actividad antioxidante de la melatonina y de otros compuestos indólicos, que están relacionados por formar parte de su ruta metabólica como es el caso de la serotonina, la *N*-acetilserotonina o el 5-hidroxitriptófano, u otros relacionados como el ácido 3-indolacético, la triptamina, o el triptofol. Estos compuestos pueden encontrarse presentes en vino debido a que forman parte de las uvas o a que se forman durante la fermentación y pueden contribuir a la actividad antioxidante del vino. Por tanto, se planteó el estudio de la actividad antioxidante de 13 compuestos indólicos (ácido 3-indolacético, ácido 5-metoxi-3-indolacético, ácido 5-hidroxi-3-indolacético, melatonina, *N*-acetil-5-hidroxitriptamina, serotonina, triptamina, triptófano, triptofol, 5-hidroxitriptófano, 5-metoxitriptamina, 5-metoxitriptofol y 6-metoximelatonina) utilizando los métodos FRAP, ORAC y DPPH. Además se ha explorado el principal mecanismo de reacción y los parámetros relacionados con la cinética de la reacción. Los resultados se compararon con los obtenidos para los compuestos fenólicos, conocidos antioxidantes, presentes en los vinos.

En general, la actividad antioxidante de los compuestos indólicos está muy relacionada con su estructura química. Los compuestos 5-hidroxiindólicos actúan a través de un mecanismo de reacción de transferencia electrónica, obteniendo los mejores resultados en el método FRAP y DPPH. Los mejores resultados obtenidos con el método ORAC fueron para la neurohormona melatonina y la fitohormona ácido 3-indolacético, que actúan con un mecanismo de transferencia de átomos de hidrógeno. Comparando los resultados con los de compuestos fenólicos, los compuestos 5-hidroxiindólicos tienen valores de EC_{50} parecidos a los obtenidos para el ácido gálico, la (+)-catequina y la (−)-epigalocatequina (Villaño et al.,

2007). Además, algunos de ellos reaccionan con mayor rapidez que muchos de los compuestos fenólicos (Villaño et al., 2007; Sánchez-Moreno et al., 1998). Teniendo en cuenta que un factor importante a tener en cuenta en la actividad antioxidante que puede ejercer los compuestos en el organismo es su biodisponibilidad, los compuestos fenólicos suelen presentar baja biodisponibilidad y se metabolizan rápidamente formando derivados metilados, glucuronidados o y sulfatados en el plasma y orina (Covas et al., 2010; D'Archivio et al., 2010). Sin embargo, muchos de los compuestos indólicos se sintetizan a partir del triptófano en algunos lugares del cuerpo humano. Y, en el caso de la melatonina, se debe absorber en el tracto gastrointestinal, aumentando la concentración de la indolamina en sangre (Hattori et al., 1995). Su carácter anfipático favorece su absorción y le permite atravesar membranas biológicas, siendo identificada en distintas partes celulares: membranas, citoplasma, mitocondria y núcleo (Reiter et al., 2004) por lo que aunque tengan un valor TEAC inferior pueden ejercer una acción antioxidante.

En resumen, los datos obtenidos a lo largo de todo el estudio demuestran que la melatonina es un compuesto que se encuentra en los vinos. Podemos asegurar que la levadura que lleva a cabo la fermentación alcohólica también es responsable de la síntesis de melatonina a partir del aminoácido triptófano, explicando el origen de su presencia. Sin embargo, aunque hemos comprobado que aparece durante la fermentación maloláctica, queda por evaluar el papel de las bacterias lácticas sobre el contenido del bioactivo. Es ésta una de las perspectivas futuras planteada con el fin de optimizar el proceso completo de producción de la indolamina durante la vinificación y poder aplicarlo a la obtención de vinos, aportándoles un valor añadido con el aumento del contenido en este bioactivo.

CONCLUSIONES

Las conclusiones a las que se ha llegado a lo largo de este trabajo de investigación son:

1. El Kit comercial "Melatonin ELISA" (IBL-Hamburg) para muestras biológicas (suero y plasma) no está validado para la determinación de melatonina en vinos (por falta linealidad en la respuesta y elevado número de falsos positivos y falsos negativos). Se validó un método que emplea HPLC-FL para el análisis de melatonina en vino. Sin embargo, presenta límites de detección y cuantificación que obligan a la utilización de pasos previos de concentración del analito para la cuantificación. Por último, se optimizó un método de LC-ESI-MS/MS cuya sensibilidad fue adecuada para la detección de melatonina en las muestras de vino, con límites de detección y cuantificación inferiores a los del método de HPLC-FL. Además, con este método se consiguió la identificación inequívoca de la melatonina, y se empleó a lo largo de toda esta tesis.

2. Se cuantificó la melatonina presente en los vinos de las variedades *Cabernet Sauvignon*, *Petit Verdot, Prieto Picudo, Syrah* y *Tempranillo*. Además, se identificó, por primera vez, un compuesto (ISO) con un patrón de fragmentación muy similar y con relación *m/z=233* igual que la melatonina, a diferente tiempo de retención. Este posible isómero de melatonina estaba presente en los vinos: *Cabernet Sauvignon, Jaen Tinto, Merlot, Palomino Negro, Prieto Picudo* y *Tempranillo.*

3. La melatonina no se encontró ni en las uvas (piel, pulpa y semilla), ni en los mostos de procedencia y se determinó en el vino final. Por tanto, aparece a lo largo del proceso de vinificación de vinos tintos y blancos. Como demuestran los resultados del seguimiento de las distintas vinificaciones, la aparición de la indolamina tiene lugar después de haber añadido la levadura que lleva a cabo la fermentación alcohólica y está relacionada con el metabolismo de la levadura *Saccharomyces.*

4. El tipo de cepa de *Saccharomyces* es determinante para la producción de melatonina en vinos. Además, tanto el medio en el que tiene lugar el crecimiento de la levadura como el contenido en el aminoácido triptófano juegan un importante papel en su formación. De las cepas probadas, QA23 (*S. cerevisiae*) produjo el mayor contenido de melatonina en condiciones de baja concentración

de azúcares reductores (20 g L^{-1}, Glucosa:Fructosa 1:1). Sin embargo, en condiciones de fermentación, las únicas cepas levaduras que sintetizaron melatonina durante la fueron S6U (*S. uvarum*) y Uvaferm BC (*S. cerevisiae* var. *bayanus*).

5. Histamina y putrescina fueron las aminas biógenas más abundantes, y junto a la tiramina, las que alcanzaron las mayores concentraciones. En general, contenido de aminas biógenas total aumenta después de las dos fermentaciones (alcohólica y maloláctica) en vinos tintos. Sin embargo, los valores resultaron ser bajos en estos vinos elaborados en clima cálido, manteniendo así concentraciones que se consideran como seguras para los consumidores.

6. Las levaduras utilizadas para la vinificación los vinos blanco y tintos (Actiflore® PM y F5, Laffort, Spain) formaron de melatonina durante la fermentación alcohólica. Pero la fementación maloláctica también afecta a su contenido final, y por tanto, la levadura y las bacterias lácticas están relacionadas con su formación. Además, las concentraciones del bioactivo se produjeron manteniendo niveles de seguridad razonables de aminas biógenas.

7. La actividad antioxidante de los compuestos indólicos varía dependiendo del método antioxidante que se utilice. La estructura química juega un papel importante, siendo los compuestos 5-hidroxiindólicos los más activos cuando se tiene en cuenta métodos antioxidantes basados en mecanismos de transferencia electrónica como el FRAP y DPPH. Muchos de éstos tienen valores similares a los compuestos fenólicos presentes en vino. La melatonina y el ácido 3-indolacético presentan la mayor actividad antioxidante cuando se utiliza el ORAC, que se basa en mecanismos de transferencia protónica.

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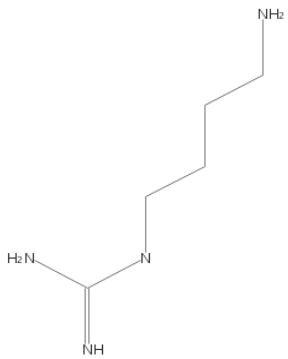
ANEXOS

ANEXO I. Estructuras de las Aminas Biógenas

Putrescina (Put)

Metilamina (Met)



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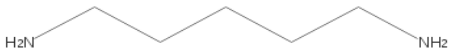
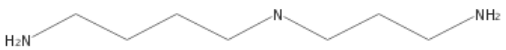


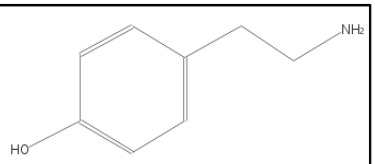
Cadaverina (Cad)



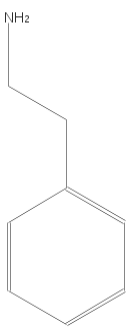
Espermidina (Spe)

Espermina (Spe)

Aminas alifáticas

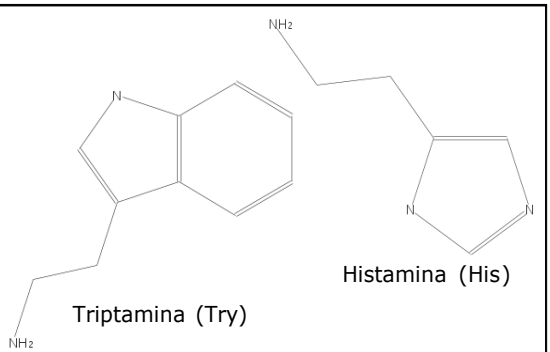


Tiramina (Tyr)



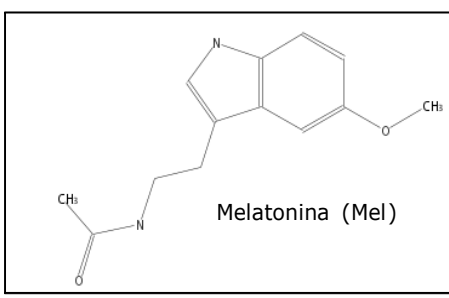
2-feniletilamina (Phe)

Aminas aromáticas



Triptamina (Try)

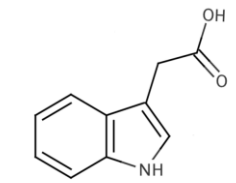
Histamina (His)



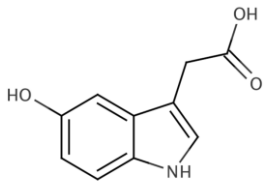
Melatonina (Mel)

Aminas heterocíclicas

ANEXO II. Estructuras de compuestos indólicos



Ácido 3-indolacético



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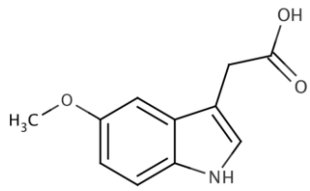

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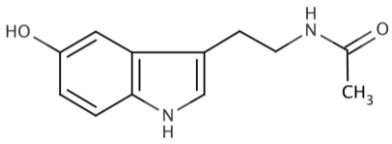
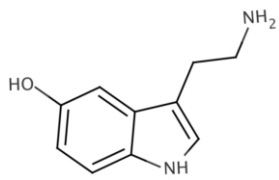
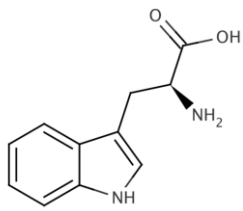


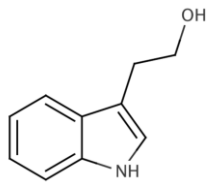
N-acetil-5-hidroxitriptamina



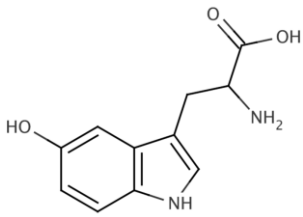
Serotonina



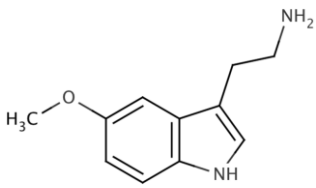
Triptófano



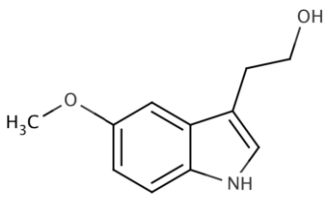
Triptofol



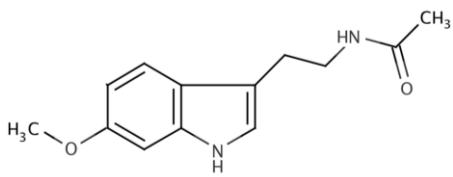
5-hidroxitriptófano



5-metoxiitriptamina



5-metoxiitriptofol



6-metoximelatonina

ANEXO III. Difusión de resultados.

PUBLICACIONES:

Rodriguez-Naranjo, M.I., Gil-Izquierdo, A., Troncoso, A.M., Cantos-Villar, E., Garcia-Parrilla, M.C. (2011). **Melatonin is synthesized by yeast during alcoholic fermentation in wines**. *Food Chemistry,* 126: 1608-1613.

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Rodriguez-Naranjo, M.I., Torija, M.J., Mas, A., Cantos-Villar, E., Garcia-Parrilla, M.C. (2012). **Production of melatonin by *Saccharomyces* strains under growth and fermentation conditions**. *Journal of Pineal Research*. doi: 10.1111/j.1600-079X.2012.00990.x

PUBLICACIONES EN REVISIÓN:

Rodriguez-Naranjo, M.I., Moyá, M.L., Cantos-Villar, E., Garcia-Parrilla, M.C. (2012). **Comparative evaluation of the antioxidant activity of melatonin and related indoles**. *Journal of Composition and Analysis.*

Rodriguez-Naranjo, M.I., Cantos-Villar, E., Garcia-Parrilla, M.C. (2012). **Determination of melatonin and other biogenic amines from must to wine of different grape varieties.** *Para enviar a Food Chemistry.*

CAPÍTULOS DE LIBRO:

Rodríguez Naranjo, M. I. (2011). **Melatonina: propiedades, presencia en alimentos y alegaciones**. *Temas de interés en Seguridad Alimentaria,* 397-413. ISBN: 978-84-8434-600-5.

Rodriguez-Naranjo, M.I., Gil-Izquierdo, A., Cantos-Villar, E., Garcia-Parrilla, M.C. (2011). **Determinación del bioactivo melatonina durante el proceso de vinificación.** *Actualizaciones en Investigación Vitivinícola,* 319-322. ISBN: 978-84-938945-6-6.

COMUNICACIONES A CONGRESOS:

Rodriguez-Naranjo, M.I., Moyá, M.L., Cantos-Villar, E., Garcia-Parrilla, M.C. **Antioxidant Activity of Melatonin and Indolic compounds** (Póster). *7th In Vino Analitica Scientia Conference.* Graz, Austria, Julio de 2011.

Rodriguez-Naranjo, M. I., Gil-Izquierdo, A., Cantos, E., Garcia-Parrilla, M.C. **Determinación del bioactivo melatonina durante el proceso de vinificación** (Póster). *XI Congreso Nacional de Investigación Enológica. Extensivo a Iberoamérica (GIENOL).* Jerez de la Frontera, Cádiz, Junio de 2011.

Garcia-Parrilla, M.C., Rodriguez-Naranjo, M.I., Gil-Izquierdo, A., Troncoso, A.M., Cantos, E. **Free melatonin detected in wines** (Póster). *XXI Congress of Chemists and Technologists of Macedonia.* Ohrid, Republic of Macedonia, Septiembre de 2010.

Garcia-Parrilla, M.C., Rodriguez-Naranjo, M.I., Troncoso, A.M., Cantos, E., Gil-Izquierdo, A. **Melatonin produced at different winemaking steps** (Póster). *XXI Congress of Chemists and Technologists of Macedonia.* Ohrid, Republic of Macedonia, Septiembre de 2010.

Rodriguez-Naranjo, M.I., Gil-Izquierdo, A., Troncoso, A.M., Cantos-Villar, E., Garcia-Parrilla, M.C. **Melatonin and an isomer are present in different**

monovarietal red wines (Póster). *28th Internacional Horticultural Congreso*. Lisboa, Portugal, Agosto de 2010.

Rodriguez-Naranjo, M.I., Garcia-Parrilla, M.C., Troncoso, A.M., Cantos-Villar, E., Gil-Izquierdo, A. **Melatonin is synthesized during vinification process** (Póster). *28th Internacional Horticultural Congreso*. Lisboa, Portugal, Agosto de 2010.

Rodriguez-Naranjo, M.I., Cantos-Villar, E., Garcia-Parrilla, M.C. **Melatonin in vinification grapes: ELISA versus HPLC-Fluorescence analysis** (Póster). *6th In Vino Analitica Scientia Conference.* Angers, Francia, Julio de 2009.

Rodriguez-Naranjo, M.I., Cantos-Villar, E., Garcia-Parrilla, M.C. **ELISA versus HPLC to determine melatonin in wines** (Comunicación Oral). *Future Trends in Phytochemistry in the global era of agri-food and health: A Young Scientists Meeting (PSE and CSIC).* Los Alcázares, Murcia, Mayo de 2009.

Cantos, E., Guerrero, R.F., Puertas, B., Rodriguez-Naranjo, M.I., Garcia-Parrilla, M.C. **Nuevas tecnologías para aumentar la cantidad de bioactivos en vino tinto** (Póster). *II Internacional Conference of food science and technology (Food and Health).* Pamplona, Noviembre de 2008.

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