UNIVERSIDAD DE SEVILLA

FACULTAD DE FARMACIA

DEPARTAMENTO DE NUTRICIÓN Y BROMATOLOGÍA, TOXICOLOGÍA Y MEDICINA LEGAL

"CARACTERIZACIÓN QUÍMICA Y SENSORIAL DE CONDIMENTOS DE FRUTA OBTENIDOS MEDIANTE DOBLE FERMENTACIÓN"

Memoria que presenta la Licenciada CRISTINA ÚBEDA AGUILERA para optar al título de Doctor por la Universidad de Sevilla con la Mención de "Doctor Internacional"

Sevilla, 2012

UNIVERSIDAD DE SEVILLA

ÁREA DE NUTRICIÓN Y BROMATOLOGÍA

FACULTAD DE FARMACIA
C/ Profesor García Gonzalez, 2.
41012 Sevilla (España)
Teléfono: 954 55 67 61
Fax: 954 55 12 45

Dña. ANA Mª CAMEÁN FERNÁNDEZ, Catedrática de Universidad y Directora del Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Facultad de Farmacia de la Universidad de Sevilla.

INFORMA:

Que la Tesis Doctoral titulada "**CARACTERIZACIÓN QUÍMICA Y SENSORIAL DE CONDIMENTOS DE FRUTA OBTENIDOS MEDIANTE DOBLE FERMENTACIÓN"**, presentada por la Licenciada CRISTINA ÚBEDA AGUILERA para optar al grado de Doctor por la Universidad de Sevilla con la Mención de "Doctor Internacional", ha sido realizada en el Área de Nutrición y Bromatología de este Departamento bajo la dirección de la Dra. Ana Mª Troncoso González, la Dra. Mª Lourdes Morales Gómez y la Dra. Raquel Mª Callejón Fernández, durante el tiempo requerido y reuniendo los requisitos exigidos en este tipo de trabajo.

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

Fdo. Ana María Cameán Fernández

UNIVERSIDAD DE SEVILLA

ÁREA DE NUTRICIÓN Y BROMATOLOGÍA

FACULTAD DE FARMACIA
C/ Profesor García Gonzalez, 2.
41012 Sevilla (España)
Teléfono: 954 55 67 61
Fax: 954 55 12 45

ANA Mª TRONCOSO GONZÁLEZ, Catedrática de Universidad del Área de Nutrición y Bromatología de la Universidad de Sevilla, Mª LOURDES MORALES GÓMEZ, Profesora Titular del Área de Nutrición y Bromatología de la Universidad de Sevilla, RAQUEL Mª CALLEJÓN FERNÁNDEZ, Profesora Ayudante Doctor del Área de Nutrición y Bromatología de la Universidad de Sevilla,

INFORMAN: Que la Tesis Doctoral titulada "**CARACTERIZACIÓN QUÍMICA Y SENSORIAL DE CONDIMENTOS DE FRUTA OBTENIDOS MEDIANTE DOBLE FERMENTACIÓN**", ha sido realizada por la Licenciada CRISTINA ÚBEDA AGUILERA 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 con la Mención de "Doctor Internacional".

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

Fdo. Ana Mª Troncoso González

Fdo. Mª Lourdes Morales Gómez

Fdo. Raquel Mª Callejón Fernández

AGRADECIMIENTOS

La realización de esta Tesis ha sido posible gracias a las siguientes ayudas económicas y logísticas prestadas por el Ministerio de Ciencia e Innovación del Gobierno de España a través del proyecto AGL2007-66417-C02-01:

- Beca predoctoral de Formación al Personal Investigador (FPI).

- Ayuda para realización de estancia breve de investigación en la Facultad de Ciencias de la Universidad de Lisboa, Portugal.

- Ayuda para realización de estancia breve de investigación en la Facultad de Ciencias de la Universidad de Zaragoza, España.

Asimismo, esta tesis ha podido llevarse a cabo gracias a las muestras a las muestras y material proporcionado por distintas empresas e instituciones:

- Hudisa S.A., Agromedina y el Grupo Alconeras por el suministro de caquis, fresas y pasta de fresa.

- Grupo de Investigación del Dr. Albert Mas perteneciente al Departamento de Bioquímica y Biotecnología de la Facultad de Enología de la Universidad Rovira i Virgili de Tarragona por el suministro de muestras.

- Grupo de Investigación del Dr. Francisco Peña perteneciente al Centro de la Alameda del Obispo del IFAPA, por permitirnos el uso de sus equipos para los análisis olfatométricos.

Gracias…

Me gustaría comenzar dando las gracias a todas aquellas personas que de una manera u otra me han ayudado en la realización de este trabajo, comenzando por mis Directoras de Tesis.

En primer lugar, a la Doctora Mª Lourdes Morales Gómez, ya que sin su ayuda gran parte de este trabajo no habría sido posible, por su constancia y dedicación. Por la paciencia en las largas jornadas de discusión de resultados que, a veces, parecían interminables.

A la Doctora Ana Mª Troncoso González, por darme la oportunidad de comenzar esta carrera científica integrándome en su Grupo de Investigación interesándose por mi y estando siempre disponible.

A la Doctora Raquel Mª Callejón Fernández, por su dedicación, comprensión y sus siempre útiles consejos.

A la Doctora Mª Carmen García Parrilla, por acompañarme en esta etapa ofreciéndome siempre su ayuda.

A todos mis compañeros de laboratorio, Ana Belén, María, Mábel, Michele, Melanie, Mª Antonia, Ruth, José Luis que habéis pasado tantos momentos buenos y malos conmigo, ayudándome en lo posible y haciendo mucho más amenas las horas en el laboratorio.

A los técnicos de laboratorio, Félix y Eva, por ser tan resolutivos y estar siempre dispuestos a echarme una mano en lo que hiciera falta.

Al Área de Toxicología, nuestros vecinos, por los buenos ratos compartidos juntos, y por darme siempre la posibilidad de usar vuestro material y equipos de laboratorio.

Al Grupo de Investigación del Dr. Albert Mas, por proveernos de las muestras y datos necesarios para este trabajo.

A la Doctora Purificación Hernández-Orte, por acogerme con cariño en su Grupo de Investigación en Zaragoza, enseñarme tanto y encontrar soluciones para todo.

A todos mis amigos, que me han aguantado muchos momentos de desánimo, escuchándome, interesándose por mi trabajo y celebrando junto a mí todos los éxitos. Carmen y Lola, por tantos momentos juntas, por seguirme y visitarme en mis estancias de investigación fuera del laboratorio. A mi amiga Tere, por ser mi persona incondicional, estando siempre ahí, aconsejándome y viviendo conmigo a la vez este largo proceso.

Finalmente, quería hacer una mención especial a mi familia, que han vivido día a día mi evolución, apoyándome y celebrando de manera muy especial cada logro conseguido. A mi

Madre, que siempre me anima y me ayuda en cualquier cosa, quitándole hierro al asunto. A mi Hermana, por motivarme en todo momento, y a la que debo la gran portada de este trabajo. A mi Padre, que despertó en mí el interés por la investigación, por estar siempre disponible, enseñarme miles de cosas y darme los consejos más acertados.

ÍNDICE

1- INTRODUCCIÓN……………………………………………………………………………………………...….**1**

1.1. El vinagre ……………………………………………………………………………………….....................1

1.1.1. Tipos de vinagre ……………………………………………………………………………………......1

1.1.1.1. Tipos de vinagre según la materia prima …………………………………………......….1

1.1.1.2. Tipos de vinagre según el método de elaboración ……………………………………...…3

1.1.2. Métodos de elaboración del vinagre ……………………………………………………………...…..4

1.1.2.1. Métodos de acetificación con cultivo superficial ……………………………………………4

1.1.2.2. Métodos de acetificación con cultivo sumergido ……………………………...…………7

1.1.3. Innovación en la elaboración del vinagre: Desarrollo de nuevos condimentos ……………………8

1.2. Compuestos nitrogenados ………………………………………………………………………….…….11

1.2.1. Compuestos nitrogenados: Aminoácidos y Amonio ……………………………………...…….11

1.3. Análisis y caracterización del aroma ……………………………………………………………….………16

1.3.1. Precursores aromáticos ………………………………………………………………...……….17

1.3.2. Aromas libres ………………………………………………………………………….………………19

1.3.2.1. Compuestos volátiles en caqui ……………………………………………………………19

1.3.2.2. Compuestos volátiles en fresa ……………………………………………………...………20

1.3.2.3. Compuestos volátiles en vinagre ………………………………………………..…………21

1.3.2.4. Determinación de compuestos volátiles ………………………………………….………24

1.3.2.4.1. Técnicas de aislamiento o extracción de compuestos volátiles ……..……24

1.3.2.4.2. Técnicas de cromatografía de gases para la determinación de compuestos volátiles …………………………………………………………………..…..…26

1.3.2.5. Caracterización del aroma mediante análisis olfatométrico ……………………………27

1.4. Evaluación de la calidad: Análisis sensorial ………………………………………...……………………31

1.4.1. Los mapas de preferencia o cartografía de preferencia …………………...………………………..34

1.5. Medida de la actividad antioxidante y compuestos fenólicos …………………...…………………………36

1.5.1. Oxidación, daño oxidativo y radicales libres ……………………………….………………………36

1.5.2. Clasificación de las sustancias antioxidantes de la dieta ……………………...…………………36

1.5.3. Tratamiento de muestras para la determinación de la actividad antioxidante y compuestos fenólicos ……………………………………………………………..………………………38

1.5.4. Métodos de medida ……………………………………………………………...…………………..….40

1.6. Evaluación de la seguridad de los condimentos ……………………………………………….……………45

1.6.1. Seguridad Alimentaria ………………………………………………………..……………………45

1.6.2. Determinación de carbamato de etilo ……………………………………………………………45

2- JUSTIFICACIÓN Y OBJETIVOS ………………………………………………………………………………….51

2.1. Justificación …………...…………………………………………………………………………………..51

2.2. Objetivos …………………………………………………….……….……………………………52

3- RESULTADOS Y DISCUSIÓN ……………………….……………………………………………………55

3.1. Determinación de aminoácidos y amonio ……………..…………………………………………….55

3.1.1. Resumen 55

3.1.2. *Determination of amino acids with AQC derivatization during the production of strawberry and persimmon vinegars* ……...57

3.2. Análisis de compuestos volátiles mayoritarios, precursores aromáticos y caracterización aromática de los condimentos 91

3.2.1. Resumen 91

3.2.2. *Determination of major volatile compounds during the production of fruit vinegars by static headspace gas chromatography–mass spectrometry method* 95

3.2.3. *Characterization of odour active compounds in strawberry vinegars* 105

3.2.4. *A comparative study of aroma profiles of srtawberry vinegars obtained by different production processes* 115

3.2.5. *Glycosidically bound aroma compounds and impact odorants of four strawberry varieties* …...145

3.3. Análisis sensorial y pruebas de preferencia ……………………………………………………………153

3.3.1. Resumen 153

3.3.2. *Evaluating consumer acceptance of new strawberry vinegars by preference mapping* …..........155

3.4. Medida de la actividad antioxidante, contenido de fenoles totales y antocianinas monoméricas ……...175

3.4.1. Resumen 175

3.4.2. *Evaluation of antioxidant activity and total phenols index in persimmon vinegars produced by different processes* 177

3.4.3. *Employment of different processes for the production of strawberry vinegars: Effects on antioxidant activity, total phenols and monomeric anthocyanins* 183

3.5. Determinación de carbamato de etilo ……………………………………………………..…………191

3.5.1. Resumen 191

3.5.2. *Validation of an analytical method for the determination of ethyl carbamate in vinegars* 193

4. SUMMARY OF RESULTS...**199**

4.1. Determination of amino acids and ammonium…………………………………………………………….………..199

4.2. Analysis of major volatile compounds, aromatic precursors and aromatic characterization of vinegars…..200

4.3. Sensory analyses and preference tests…………………………………………………………………….….….202

4.4. Determination of antioxidant activity, total phenols content and monomeric anthocyanins…………..………..203

4.5. Determination of ethyl carbamate………………………………………………………………………………204

5- DISCUSIÓN FINAL……………………………………………………………………..…………………………**205**

6- CONCLUSIONES/CONCLUSIONS………………………………..……….……………………………**207/211**

7- BIBLIOGRAFÍA..**215**

8- ANEXO I: Proceso de obtención de condimentos y muestras…………………..…………………………..**239**

1. INTRODUCCIÓN

1.1. El vinagre

El vinagre *es el líquido apto para el consumo humano resultante de la doble fermentación alcohólica y acética de productos de origen agrario s*egún la normativa más reciente publicada sobre vinagres *(R.D. 661/2012 Ministerio de la Presidencia). Según dicha normativa el producto terminado debe contener un mínimo de 50 g/L de ácido acético excepto para el vinagre de vino, que será, al menos, de 60 g/L.*

En la primera etapa de obtención de un vinagre, tiene lugar la fermentación alcohólica. En ésta, los azúcares pasan a alcohol y CO_2 por medio de las levaduras. Luego, este alcohol es utilizado por las bacterias acéticas en una etapa posterior y es transformado en ácido acético. Las bacterias acéticas son gram-negativas (gram-positivas en cultivos viejos), aerobias estrictas y muy sensibles al SO_2. Son, además, catalasa positiva y oxidasa negativa, pueden presentar pigmentación en cultivos sólidos y producir diferentes tipos de polisacáridos *(De Ley et al., 1984)*.

1.1.1. Tipos de vinagre

Cualquier sustrato azucarado o amiláceo puede ser utilizado en la elaboración de vinagres. Asimismo, los métodos de elaboración pueden ser diferentes. Por tanto, los vinagres se pueden clasificar en función del tipo de sustrato empleado o del método usado en su elaboración.

1.1.1.1. Tipos de vinagre según la materia prima

Las materias primas que pueden emplearse según lo establecido por el Ministerio de la Presidencia en el Real Decreto de 2012, pueden ser:

a) Vinos, sidras, zumo de frutas, aguardientes, destilados de origen agrícola, alcoholes etílicos de origen agrícola y bebidas alcohólicas.

b) Otros productos de origen agrícola que contengan almidón, azúcares o almidón y azúcares, incluyendo, entre otros, granos de cereales y malta de cebada.

Entre los ingredientes facultativos esta normativa contempla la posible adición de:

a) Plantas y/o partes de plantas aromáticas y/o especias y/o frutas, enteras o no, y/o aromas que cumplan los requisitos establecidos en el Reglamento (CE) n.º 1334/2008

del Parlamento Europeo y del Consejo, de 16 de diciembre de 2008, sobre aromas y determinados ingredientes alimentarios con propiedades aromatizantes utilizados en los alimentos.

b) Mosto de uva y zumos de fruta.

c) Miel, azúcar, sal.

d) Aditivos autorizados por la normativa de aplicación horizontal sobre esta materia, incluido el caramelo para la coloración.

Con respecto a las materias primas permitidas para la elaboración de vinagres, según el RD de 2012 se establecen los siguientes tipos:

1. Vinagre de vino: Es el producto obtenido exclusivamente por fermentación acética del vino.

2. Vinagre de frutas o vinagre de bayas: Es el producto obtenido a partir de frutas o de bayas de fruta mediante fermentación alcohólica y acética.

3. Vinagre de sidra: Es el producto obtenido a partir de sidra, mediante fermentación acética.

4. Vinagre de alcohol: Es el producto obtenido por la fermentación acética de alcohol destilado de origen agrícola.

5. Vinagre de cereales: Es el producto obtenido, sin destilación intermedia, por el procedimiento de doble fermentación alcohólica y acética, a partir de cualquier cereal en grano, cuyo almidón se haya transformado en azúcares mediante la diastasa de la cebada malteada o por cualquier otro proceso.

6. Vinagre de malta: Es el producto obtenido, sin destilación intermedia, por el procedimiento de doble fermentación alcohólica y acética, a partir de la cebada malteada, con o sin adición de grano, cuyo almidón se ha desdoblado en azúcares mediante la diastasa de la cebada malteada.

7. Vinagre de malta destilado: Es el producto obtenido mediante la destilación del vinagre de malta, a presión reducida, que sólo contiene los componentes volátiles del vinagre de malta del que se deriva.

8. Vinagre balsámico: Es el producto obtenido por adición de mosto de uva, mosto de uva concentrado o mosto de uva concentrado rectificado al vinagre de vino, dando lugar a un vinagre dulce, con un contenido mínimo de azúcar total de 150 g/l, procedente exclusivamente de los mostos indicados.

9. Vinagre balsámico de sidra: Es el producto obtenido por adición de zumo concentrado de manzana al vinagre de sidra, dando lugar a un vinagre dulce con un contenido mínimo de azúcar total de 150 g/l, procedente exclusivamente del zumo concentrado de manzana.

10. Otros vinagres: Vinagres obtenidos a partir de productos de origen agrícola no contemplados en los apartados anteriores por doble fermentación.

1.1.1.2. Tipos de vinagre según el método de elaboración

Atendiendo a la posición que ocupan las bacterias acéticas en el sustrato a acetificar, los vinagres se clasifican en dos grandes grupos:

-Vinagre obtenido por acetificación con cultivo superficial: las bacterias acéticas se encuentran en contacto directo con oxígeno gaseoso, situadas bien en la interfase líquido/gas o bien fijadas a soportes de materiales tales como virutas, elaborándose así la mayoría de los vinagres tradicionales.

-Vinagre obtenido por acetificación con cultivo sumergido: las bacterias acéticas están sumergidas libremente en el seno del líquido a fermentar, en el que constantemente se introduce aire, (solo o enriquecido con oxígeno), en condiciones que permitan la máxima transferencia posible desde la fase gaseosa a la fase líquida. Así se obtienen de forma rápida vinagres de menor calidad que los anteriores y que alcanza un menor precio en el mercado.

Por tanto, en general existen en el mercado dos grandes grupos de vinagres. En uno se engloban los productos de la acetificación con cultivo superficial y en el otro los obtenidos mediante cultivo sumergido. En los países mediterráneos, el vinagre de vino se produce de forma mayoritaria en biorreactores y con cultivo sumergido, que puede después envejecerse o no en madera.

1.1.2. Métodos de elaboración de vinagre

1.1.2.1. Métodos de acetificación con cultivo superficial

Dentro de este grupo se engloban los métodos tradicionales, los cuales se llevan a cabo en toneles de madera de diferente capacidad y suponen un menor volumen de producción *(García-Parrilla et al., 1998).* Utilizando este método se obtienen vinagres de mayor calidad que alcanzan altos precios en el mercado como es el caso del Vinagre de Jerez o el Vinagre balsámico. Entre los métodos de acetificación con cultivo superficial se encuentran:

a) Método de Orleans: En este se emplean toneles de gran capacidad que se apilan en filas de forma horizontal y superpuesta, provistos de 2 agujeros de aproximadamente 5 cm en cada extremo de los fondos de cada barril a 2/3 de la altura del fondo, que se rellenan con estopa para evitar la entrada de las moscas del vinagre, pero que dejan pasar aire. Además, en el lateral superior se hace otro orificio que se tapa con un tapón de corcho por donde penetra un tubo de vidrio, recto, que llega casi hasta el fondo del líquido permitiendo renovar el sustrato sin alterar el velo bacteriano situado en la superficie. Se trata de un procedimiento estático donde el líquido a acetificar es una mezcla de vino de bajo grado alcohólico con un 20% de vinagre turbio.

b) Método Luxemburgués: En este caso, a diferencia del anterior, se emplean virutas de haya que periódicamente quedan sumergidas en el líquido que está acetificándose. Así se consigue aumentar la superficie de acetificación y mejorar la transferencia de oxígeno, por lo que la velocidad de acetificación aumenta.

En este método se utilizan cubas giratorias que están divididas en 2 partes desiguales por un falso fondo, agujereado, con numerosos y finos orificios. La parte menor del tonel está llena de virutas de haya sobre las que crecerán las bacterias acéticas. En el proceso de elaboración, la cuba se hace girar media vuelta repitiendo este proceso sucesivamente cada cierto tiempo, permitiendo que el sustrato esté en contacto con las virutas de forma intermitente. Una vez terminado el proceso, el vinagre elaborado se extrae de las cubas y se sustituye por porciones iguales de vino, continuando la elaboración indefinidamente *(Xandri-Tagüeña, 1977).*

c) Método de Schüzenbach o Método Alemán: Se emplean toneles o generadores verticales de encina con doble fondo. El primero se rellena con virutas de haya impregnadas en vinagre de buena calidad. Sobre el borde superior lleva un diafragma perforado con los orificios obturados con algodón. Al pasar el vino por el diafragma, burbujea aire que existe entre las virutas, y el vinagre se recoge por la parte inferior.

d) *Métodos de obtención de vinagres artesanales de alta calidad*

Entre los vinagres artesanales de alta calidad se encuentran el "Aceto Balsamico Tradicionale de Módena", "Aceto Balsamico Tradicionale de Reggio Emilia", ambos producidos en Italia, el Vinagre de Jerez, el Vinagre del Condado de Huelva y el Vinagre de Montilla-Moriles, estas tres últimas pertenecientes a Andalucía. Todos ellos cuentan con la Denominación de Origen Protegida.

Para producir el Aceto Balsamico Tradicionale se usa la variedad de uva Trebbiano, cuyo mosto se concentra por calentamiento hasta al menos un tercio de su volumen inicial, mediante ebullición suave, lo que producirá la eliminación de la flora espontánea y un incremento del 30% del contenido en azúcares. El mosto concentrado es fermentado por levaduras del género *Zygosaccharomyces* y simultáneamente las bacterias acéticas metabolizan el etanol a ácido acético. Durante la elaboración de este vinagre se emplea una serie de barriles de diferentes tipos de maderas y tamaños *(Seidemann, 1996)*. El vinagre terminado después de un proceso que dura varios años (al menos 12), se extrae del último barril de la serie que es de madera de moral o morera y de 20 litros de volumen (Figura 1).

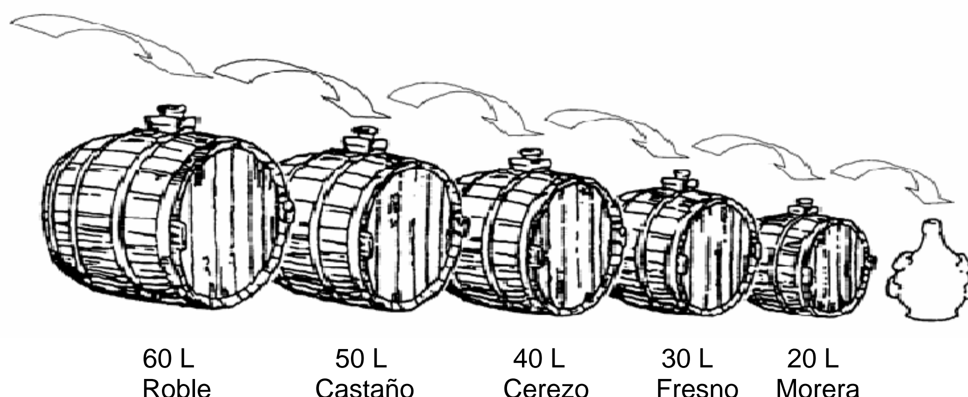


Figura 1. *Proceso de elaboración del vinagre balsámico de Módena (Bergonzini, 1979).*

El Vinagre de Jerez es obtenido a partir de vinos procedentes de las variedades de uva cultivadas en la zona de producción con la Denominación de Origen "Jerez-Xérès-Sherry" y "Manzanilla-Sanlúcar de Barrameda". El Vinagre de Jerez se obtiene tras un periodo de envejecimiento que da lugar a los tres tipos de vinagre de esta Denominación:

- Vinagre de Jerez: es el vinagre sometido a un tiempo de envejecimiento mínimo de seis meses.

- Vinagre de Jerez Reserva: es el vinagre sometido a un tiempo de envejecimiento mínimo de dos años.

- Vinagre de Jerez Gran Reserva: es el vinagre sometido a un tiempo de envejecimiento mínimo de diez años.

Este vinagre se envejece mediante un sistema estático de añadas, o por medio de un sistema dinámico característico y peculiar de “criaderas y solera” (Figura 2). Este último es el más difundido y generalizado. En este sistema ocurre simultáneamente el proceso de acetificación y envejecimiento. El sistema está formado por una serie de botas, agrupadas en filas horizontales o escalas cuyo número puede oscilar entre tres y cinco. La escala que está a ras del suelo recibe el nombre de “solera”, sobre ella se encuentra la primera criadera, sobre la cual se sitúa la segunda y así sucesivamente.

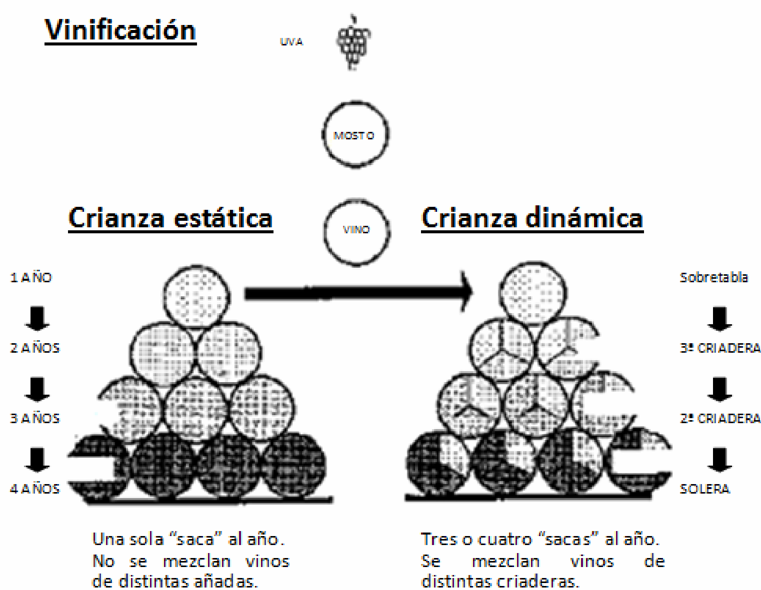


Figura 2. *Sistema de criaderas y solera.*

La edad del vinagre que contienen las botas aumenta conforme descendemos en el sistema, siendo la solera la de mayor envejecimiento. El vinagre acabado se obtiene de la solera, no pudiendo exceder la cantidad que se saca de la bota en un tercio del contenido de la primera criadera. Ésta, a su vez se nutre de la segunda y así sucesivamente, ingresando el

sustrato de partida en el sistema por la criadera superior. El sustrato de partida puede ser mosto del año no alcoholizado o vino que haya sufrido una crianza mínima de dos años.

Para la elaboración de un vinagre amparado con la Denominación de Origen Protegida "Vinagre del Condado de Huelva" debe emplearse un vino con la Denominación de Origen "Condado de Huelva". Este tipo de vino puede dar lugar a dos vinagres diferentes: Vinagre Condado de Huelva y Vinagre Viejo Condado de Huelva. Este último es producto del envejecimiento del primero. Dependiendo del tipo de envejecimiento y el tiempo se distinguen tres subtipos del Vinagre Viejo: Solera, Reserva y Añada. Para su producción se utiliza el sistema de "criaderas y solera" o el de "añadas".

Para la elaboración de un vinagre amparado con la Denominación de Origen Protegida "Vinagre del Condado de Huelva" debe emplearse un vino con la Denominación de Origen "Condado de Huelva". Este tipo de vino puede dar lugar a dos vinagres diferentes: Vinagre Condado de Huelva y Vinagre Viejo Condado de Huelva. Este último es producto del envejecimiento del primero. Dependiendo del tipo de envejecimiento y el tiempo se distinguen tres subtipos del Vinagre Viejo: Solera, Reserva y Añada. Para su producción se utiliza el sistema de "criaderas y solera" o el de "añadas".

De los vinagres mencionados, "Vinagre de Montilla-Moriles" ha sido el último en obtener la calificación de Denominación de Origen Protegida (año 2008). Este vinagre se elabora a partir de vino Denominación de Origen "Montilla-Moriles". Los vinos utilizados para su producción son Fino, Amontillado, Oloroso y Pedro Ximénez. Dependiendo del sistema de producción y el sustrato empleado existen diferentes tipos: Vinagres de envejecimiento, Vinagres dulces, Vinagres dulces al Pedro Ximénez, Vinagres dulces al Moscatel.

1.1.2.2. Métodos de acetificación con cultivo sumergido

En estos métodos no se utiliza material poroso o soporte, sino que se hacen circular pequeñas burbujas de aire a través de la biomasa, con lo que se favorece el proceso fermentativo.

Las acetificaciones se llevan a cabo en biorreactores que se llenan con el vino y se introduce una fuerte corriente de aire, que provoca una acetificación muy rápida. Este proceso es muy utilizado en la actualidad debido a sus buenos rendimientos de transformación de alcohol en acético (hasta el 94 %) y por su velocidad (25-30 horas).

Los primeros métodos de acetificación sumergida surgieron en 1878, cuando Heinrich Frings fundó en Aquisgrán una sociedad productora de vinagre, de la que nació el Acetator Frings, base de la biotecnología vinagrera actual. Una mayor población bacteriana así como la disponibilidad de oxígeno para los microorganismos permiten obtener un mayor rendimiento de la transformación de etanol en ácido acético. Estos métodos requieren estricta vigilancia de 3 parámetros: la temperatura, la presión parcial de oxígeno y de los ciclos de carga y descarga. Este modelo sirvió de base para el desarrollo de los biorreactores tipo Frings que se usan en la actualidad.

1.1.3. Innovación en la elaboración de vinagre: Desarrollo de nuevos condimentos

Antiguamente, el vinagre se consideraba un subproducto de la fabricación del vino usado para limpieza de heridas, remedio contra la tos o incluso para el alivio de torceduras o varices *(Llaguno, 1991)*. Su producción a gran escala ha supuesto un medio para poder utilizar subproductos de frutas. Este es el caso de EEUU, donde la producción de vinagre de sidra era una forma de aprovechamiento de excedentes de manzanas.

En la actualidad, la innovación en la producción de vinagres se puede conseguir por un lado, con la mejora de los procesos de elaboración, y por otro, empleando nuevas materias primas.

Respecto a los procesos de elaboración, se han introducido mejoras tecnológicas con el objeto de automatizar al máximo el proceso de acetificación y permitir un mejor seguimiento de los parámetros del mismo *(García-García et al., 2007).* De esta manera se aumenta la velocidad de producción del vinagre sin perder el rendimiento ni la calidad del producto final. También se ha estudiado el uso de cultivos iniciadores de bacterias acéticas *(Ndoye et al., 2009)* y el empleo de nuevas maderas para la obtención de vinagres por métodos tradicionales *(Callejón et al., 2009; Cerezo et al., 2008)*.

En relación al uso de materias primas innovadoras, se están introduciendo en el mercado vinagres obtenidos a partir de numerosos sustratos, siendo la mayoría de ellos frutas (manzana, kiwi, limón, melocotón, etc.). La producción de vinagres obtenidos a partir de diferentes sustratos puede tener varias aplicaciones:

- Dotar al producto de un valor añadido como pueden ser las características saludables del material de partida.

- Aprovechamiento de fruta de segunda calidad y excedentes.

- Aportar al mercado nuevas variedades de condimentos.

La producción de frutas y otros productos vegetales presenta excedentes con una gran facilidad debido a diversos factores como la estacionalidad, sobreproducción agrícola o el empleo de modernos sistemas de explotación. Por otro lado, si la fruta no presenta el tamaño o forma adecuada, es desechada aún siendo apta para el consumo humano. Esta fruta, se conoce como fruta de segunda calidad y puede suponer hasta un 30% del total de la producción. A pesar de que la tecnología de alimentos ha desarrollado sistemas efectivos de conservación y producción de nuevos alimentos derivados de dichos productos y con muy buena aceptación por el consumidor (mermeladas, frutas desecadas), esos sistemas se muestran insuficientes para evitar los excedentes de algunos productos agrícolas.

Algunos de los problemas que causa el rechazo de gran parte de la producción frutícola se pueden solventar con la generación de nuevos productos que tengan mayor durabilidad y que conserven al máximo sus características para ser consumidas. Entre los sistemas de conservación de los alimentos se pueden destacar las transformaciones fermentativas que permiten el mantenimiento de los productos en alcohol o ácido acético.

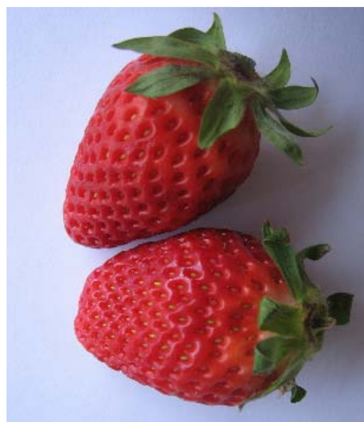
Para seleccionar las frutas más adecuadas para producir vinagre es necesario tener en cuenta las características favorables para su procesado: piel fina, que no tengan hueso, etc. En este sentido, el caqui y la fresa son dos candidatos potenciales para producir este tipo de condimentos.

El caqui (*Diospyros kaki L. f.*), perteneciente a la familia botánica *Ebanaceae*, género *Diospyros*, cuenta con más de 300 especies y son originarios de China. Los principales países productores son: China, Japón, EEUU, Brasil, Nueva Zelanda, Australia, Rusia, Israel y España (principalmente en las regiones de la Comunidad Valenciana, Cataluña y Andalucía). Desde el punto de vista comercial, las variedades de caqui se clasifican en astringentes (Rojo Brillante, Triumph, Tomatero, etc.) y no astringentes (Fuyu, Hana-Fuyu, Jiro, etc.). Esta astringencia es debida al contenido en taninos. Las variedades astringentes tienen una gran cantidad de taninos solubles que van disminuyendo a lo largo de la madurez.

Sin embargo, los taninos de las variedades no astringentes están insolubilizados, por lo que se pueden consumir sin notar la sensación áspera en boca que causa la astringencia. Hoy en día se están desarrollando y aplicando numerosas técnicas de desastringentación en

postcosecha que permiten el consumo de estas variedades astringentes como no astringentes. Este es el caso de la variedad Triumph, comercializada como sharoni. Tras la recolección, se realizan métodos artificiales para eliminar la astringencia, tales como la exposición a vapores alcohólicos o a acetileno, etileno o tratamiento en almacén durante 24 horas a 20-25 ºC en atmósfera al 95% de CO_2.

Por otro lado, la fresa (*Fragaria ananassa*), perteneciente a la familia botánica *Rosaceae* es otra candidata idónea a ser materia prima para la elaboración de nuevos productos, debido a su alto grado de apreciación por el consumidor. Según los últimos datos de la FAO (*FAOSTAT, FAO 2011*), España es en la actualidad el primer país del mundo exportador de fresas y el segundo en producción, sólo por detrás de Estados Unidos, y una gran parte de esta producción se cosecha en Huelva (Andalucía). En el año 2011, nueve de cada 10 fresas españolas se distribuyeron entre los países de la Unión Europea. En España se cultivan diferentes variedades como *Fuentepina, Candonga, Festival, Camarosa,* etc. Esta última es la variedad de fresa más cultivada, alrededor del 60% de la producción mundial y 95% de la española.



La fresa es una fruta muy valorada debido a su aroma, sabor y propiedades saludables. Esta fruta se consume normalmente fresca (75% de la producción total), y también se utiliza en la industria alimentaria como un ingrediente importante en la producción de mermeladas, yogures, té, zumos, helados y otros productos alimenticios (25% de la producción total) (*Borris et al., 2006*).

Debido a diversas razones, cada año se producen excedentes de estas dos frutas, sobretodo de fresa, siendo desechadas aún siendo aptas para el consumo humano. Por ello, la utilización de esta parte de la producción frutícola para la elaboración de vinagre se presenta como una oportunidad para ofrecer al mercado nuevos productos con características diferentes y saludables.

1.2. Compuestos nitrogenados: Aminoácidos y Amonio

El análisis de los compuestos nitrogenados encuentra aplicación en muchos campos de la investigación siendo uno de los más importantes la estimación del valor nutritivo de alimentos gracias a la determinación aminoacídica. La concentración de estos compuestos en el fruto puede causar alteraciones del gusto y de la calidad. Por otra parte, el análisis de aminoácidos y amonio puede ayudar a detectar posibles adulteraciones de alimentos y bebidas. Además, es interesante su determinación ya que son precursores de moléculas potencialmente tóxicas, como es el caso de las aminas biógenas o el carbamato de etilo.

Aminoácidos esenciales como lisina, isoleucina, leucina, metionina, valina, fenilalanina, triptófano y cisteína contribuyen de manera significativa a la calidad nutricional del alimento. Además, algunos aminoácidos libres pueden influir en el sabor de la fruta. El ejemplo más conocido es el L-ácido glutámico, responsable del sabor "umami" y es utilizado en muchos alimentos como potenciador del sabor *(Lindemann, 2001).* La alanina y lisina están altamente correlacionados con el dulzor, y la fenilalanina y tirosina son amargas *(Belitz, Grosch, y Schieberle, 2001).*

Además de ser responsables de una parte del sabor, los aminoácidos participan en la calidad y aceptación de un producto ya que son precursores de aromas importantes en los alimentos. Esto es debido a que muchos de los aminoácidos por medio de una serie de biotransformaciones pueden dar lugar a alcoholes superiores, aldehídos, ésteres y ácidos cetónicos *(Hernández-Orte et al., 2002).*

Debido a las razones antes mencionadas, a lo largo del tiempo se ha estudiado la composición aminoacídica de diferentes frutas como es el caso de la uva *(Nicolini et al., 2001),* melocotón *(Caldwell et al., 1986),* naranja *(Simo et al., 2004)* o manzana *(Ackermann et al., 1992).*

La composición en aminoácidos del caqui ha sido muy poco investigada, parece ser que únicamente *Komiyama y Tsuji (1986)* lo hicieron. Analizaron 6 variedades de tipo astringente, en las que determinaron 22 aminoácidos, siendo los mayoritarios la glutamina, ácido glutámico y citrulina. Además, encontraron en cantidades considerables la asparagina, treonina, valina, leucina e isoleucina.

Sin embargo, los aminoácidos de la fresa han sido estudiados por diferentes autores, entre ellos, *Pérez et al. (1992)* determinaron los aminoácidos mayoritarios de la fresa durante el

proceso de maduración de la fruta. Establecieron como aminoácidos mayoritarios en la fresa a la asparagina, glutamina y alanina. Concluyeron que existía una relación importante entre la desaparición de la alanina y el aumento de ésteres etílicos, que son los compuestos volátiles mayoritarios en todas las etapas de maduración de la fresa y son aromas de impacto. Unos años más tarde, *Pérez et al. (2002),* incubaron fresas con diferentes aminoácidos, estableciendo una relación directa entre los aminoácidos y la generación de nuevos aromas de tipo éster.

Zhang et al. (2009) estableció como aminoácidos mayoritarios a la serina, arginina, ácido glutámico, histidina, ácido aspártico y prolina. Además, cuantificó la pérdida de aminoácidos a medida que avanzaba la maduración.

Cuando la fruta se utiliza como sustrato para la producción de alimentos fermentados y bebidas, los compuestos nitrogenados adquieren mayor importancia al ser fuente de nitrógeno para los microorganismos implicados. Por ejemplo, las levaduras y las bacterias acéticas los consumen durante la fermentación alcohólica y acética respectivamente *(Hernández-Orte et al., 2002; Hernández-Orte et al., 2003; Callejón et al., 2008).* Además, el contenido en nitrógeno es el factor responsable de la parada de la fermentación alcohólica *(Monteiro y Bisson, 1992).* Estos autores han investigado los efectos de la composición en aminoácidos la uva sobre la fermentación alcohólica y acética, estableciendo relaciones entre estos compuestos y los aromas resultantes.

En otros trabajos se adicionó amonio y aminoácidos al mosto para estudiar su efecto en la composición aromática y en las propiedades sensoriales de los vinos obtenidos *(Hernández-Orte et al., 2002; 2006).* Aunque el factor determinante de la composición volátil de los vinos es la cepa de levadura, la adición de nitrógeno a los mostos también influye.

Además, la composición de aminoácidos ha servido para la diferenciación de vinos de diferentes variedades de uva, regiones geográficas y vendimias *(Csomos y Simon-Sarkadi, 2001; Brescia et al., 2002; Soufleros et al; 2003).*

En el campo de investigación del vinagre, el análisis de aminoácidos se ha dirigido a la caracterización de vinagres de vino *(Kutlán y Molnár-Perl, 2003)*; a investigar las diferencias entre acetificaciones con cultivo sumergido y superficial *(Callejón et al., 2008)* o diferencias entre sustratos *(Valero et al., 2005),* y además, a conocer las transformaciones químicas y

bioquímicas que se producen en los vinagres de Jerez durante las diferentes fases de envejecimiento *(Palacios et al., 2002).*

Entre las técnicas analíticas que se han empleado para el análisis de aminoácidos se encuentran:

-Separación por cromatografía líquida de intercambio iónico *(Csomos y Simone, 2002)* o en fase reversa *(Soufleros et al., 2003; Martínez-Rodríguez et al., 2002).*

-Cromatografía de gases *(Abe et al., 2002; Erbe y Brüecker, 1998).*

-Electroforesis capilar *(Kosir y Kidric, 2002).*

-Resonancia magnética nuclear *(Brescia et al., 2002).*

En el caso de la cromatografía líquida, hay que señalar que los aminoácidos pueden ser detectados directamente con un detector de ultravioleta, ya que absorben a una longitud de onda entre 190 y 210 nm. Sin embargo, la mayoría de los disolventes y otros componentes de las muestras también absorben en esta región del espectro; por lo que normalmente se recurre a la formación de derivados detectables a otras longitudes de onda o fluorescentes *(Cáceres et al., 1986)*, y a esto se le denomina derivatización.

Tal derivatización puede llevarse a cabo tanto antes (pre-columna), o después de la separación cromatográfica (post-columna) de aminoácidos, y más raramente, en la columna. Cada manera de obtener el derivado tiene sus propias ventajas y desventajas. En la derivatización post-columna, la separación de los aminoácidos se realiza con una resina de intercambio catiónico y un gradiente tampones ácidos. Después de la separación, los aminoácidos se convierten en derivados coloreados de ninhidrina para la detección colorimétrica, o en ortho-aldehido (OPA) para la detección de fluorescencia. A pesar de que la derivatización pre-columna presenta más ventajas, los métodos tradicionales post-columna no están totalmente descartados.

Por lo general se prefiere la cromatografía líquida con derivatización pre-columna debido al ahorro de tiempo y a que la instrumentación utilizada es simple.

Existe una gran variedad de reactivos empleados para la derivatización pre-columna, teniendo cada uno sus ventajas y desventajas. Algunos de los más utilizados son: fenilisotiocianato (PITC), O-ftalaldehído (OPA), 9-fluorenilmetil-cloroformato (FMOC-CL), cloruro

de dansilo o, más recientemente, se ha descrito un nuevo método que utiliza como agente de derivatización dietil etoximetilenemalonato (DEEMM) *(Gómez-Alonso et al., 2007).* Otro de los más usados es el 6-aminoquinolil-N-hidroxisuccinimidil carbamato (AQC) *(Hernández-Orte et al., 2003).* Éste es un agente para la derivatización de grupos aminos, específicamente diseñado para el análisis de aminoácidos, con la idea de simplificar la reacción de derivatización, aumentar los rendimientos de la reacción e incrementar la sensibilidad y selectividad de los derivados formados cuando se trabaja con detección por fluorescencia (*Cohen y Michaud, 1993; Cohen y Antonis, 1994; Wandelen y Cohen, 1997*). *Hernández–Orte et al. (2003)* optimizaron las condiciones de separación propuestas por *Cohen y Michaud (1993)* para conseguir una cuantificación simultánea de los aminoácidos libres de los mostos y vinos sin problemas de interferencia debidos al contenido de azúcar. Este compuesto reacciona rápidamente con los aminoácidos primarios y secundarios formando productos altamente estables con una fuerte fluorescencia a 395 nm (Figura 3). Los derivados que resultan son estables a temperatura ambiente durante, al menos, una semana y se separan fácilmente por cromatografía líquida en fase reversa utilizando una columna de C18.

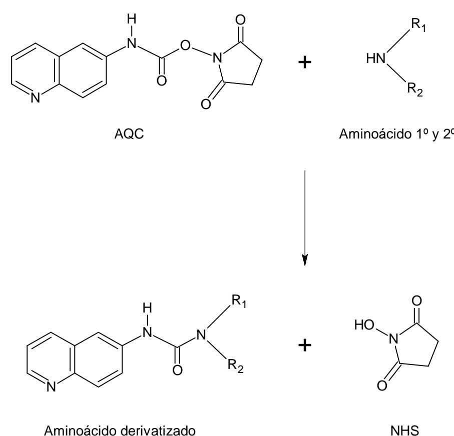


Figura 3. *Reacción de derivatización del AQC.*

El exceso del reactivo se hidroliza durante la reacción para formar 6-aminoquinolina (AMQ), cuyas características espectrales son netamente diferentes a cualquiera de los aminoácidos derivatizados. Ello permite programar una longitud de onda que maximice la respuesta de emisión de los derivados y reduzca al mínimo la respuesta del AMQ. En esta hidrólisis del reactivo también se forma N-hidroxisuccinimida (NHS) y dióxido de carbono pero

éstos no interfieren en el análisis cromatográfico. La destrucción de exceso del reactivo es completa en un minuto.

Este método ha sido optimizado para su uso con un detector de fluorescencia con el fin de lograr límites de detección de 50-300 fentomoles para los aminoácidos existentes en péptidos e hidrolizados de proteínas (*Hernández-Orte et al., 2003*) o para la determinación de aminoácidos y amonio con el fin de comparar la acetificación con cultivo sumergido y superficial *(Callejón et al., 2008).*

1.3. Análisis y caracterización del aroma

El aroma es una propiedad organoléptica debida a las diferentes sustancias volátiles presentes en los productos. Es una mezcla de moléculas pequeñas y ligeras, en concentraciones muy bajas en el aire inhalado, que, al entrar en contacto con el sistema sensorial humano (receptores olfatorios), es capaz de estimular una respuesta. La percepción experimentada es el olor o aroma *(Craven et al., 1996).*

El aroma es sin duda uno de los indicadores más importantes de la calidad de los alimentos y con gran responsabilidad en la aceptación de los mismos *(Mestres et al., 2000)*. Así, dada la importancia del aroma en la aceptabilidad del producto por los consumidores, conocer qué compuestos influyen en el aroma de un producto y cuál es su origen puede ser una herramienta muy útil para propiciar la aparición de los mismos mediante la aplicación de la tecnología conveniente.

La peculiaridad del aroma de un producto es debida a la infinita variedad de combinaciones y concentraciones de diferentes compuestos volátiles. Sin embargo, no todos los compuestos volátiles que lo forman contribuyen de igual manera al aroma. La importancia en el aroma de un compuesto depende de la concentración y el umbral de percepción por los sentidos humanos. Además, entre ellos pueden tener lugar interacciones de diferente naturaleza: se puede producir enmascaramiento, efectos de supresión y de sinergia *(Ortín, 2006)*. Por tanto, la presencia, concentración, umbral de los compuestos volátiles y la interacción entre ellos y con los compuestos no volátiles de la matriz, dan origen al aroma final del producto.

Así, para conocer el aroma de un producto habrá que estudiar su composición volátil y posteriormente establecer la contribución de los mismos al aroma. Este segundo paso conlleva la identificación de odorantes y aromas de impacto, la caracterización de sus propiedades sensoriales (umbrales,…) y, en una fase más avanzada, conocer las interacciones mediante ensayos de omisión y reconstitución.

Los compuestos de impacto son aquellos que van a transmitir de manera efectiva sus características aromáticas específicas sin la necesidad del soporte de más componentes químicos *(Ferreira, 2007).*

Los compuestos volátiles que dan lugar al aroma tienen muy diferentes características químicas y orígenes. Así, parte de ellos pueden proceder de la materia prima, estos pueden

aparecer en forma libre o como precursores aromáticos. Los precursores aromáticos son compuestos inodoros, susceptibles de liberar moléculas olorosas bajo la influencia de diversos factores biológicos, biotecnológicos y fisicoquímicos y tienen un gran interés porque determinan el potencial aromático del producto.

Otra parte importante de los compuestos del aroma de un producto se generan en las etapas de elaboración del mismo. En el caso particular del vinagre, durante la fermentación alcohólica se forman gran cantidad de compuestos, algunos de los cuales sufrirán posteriormente en la fermentación acética diversas transformaciones. El contacto con madera, ya sea durante el proceso de acetificación o de envejecimiento, conlleva un enriquecimiento con los aromas extraídos de la madera producidos por procesos de condensación química o reacciones de oxidación.

1.3.1. Precursores aromáticos

Hace más de 40 años, *Francis y Allcock* (1969) detectaron formas glucoconjugadas de alcoholes monoterpénicos en los pétalos de rosa, y esto abrió un nuevo campo en la investigación en aromas: el estudio de los precursores aromáticos. Entre los precursores aromáticos encontramos diferentes tipos: compuestos glicosilados (terpenos, C_{13}-norisoprenoides y compuestos del metabolismo del ácido shikímico), que son los más abundantes; S-cisteína conjugados y precursores unidos a glutatión que son los precursores de tioles; y carotenoides que dan lugar a C_9- C_{11} y C_{13} norisoprenoides.

Los precursores que se han descrito más recientemente han sido los derivados azufrados ligados a cisteína o al glutatión. Se han determinado en frutas y vegetales tan diferentes como la cebolla *(Starkenmann et al., 2008),* espárrago *(Parry el al., 1985)*, fruta de la pasión *(Tominaga y Dubourdieu, 2000)* o uva *(Tominaga et al., 1998).*

Los aromas derivados de los carotenoides se forman mediante oxidación enzimática y foto-oxidación de los mismos, encontrándose en plantas, flores y frutas. Este es el caso del melón, en el que varios aromas de impacto son generados a partir de carotenoides *(Rodrigo et al., 2010).*

Los precursores aromaticos mencionados tienen una presencia importante en la naturaleza, pero los más abundantes y, sin duda, los más estudiados, son los precursores de aromas de tipo glicosídico. Esto es así porque la glicosidación es la forma más común mediante

el cual se acumulan en los aromas en frutas, ya que este paso bioquímico es el último paso del proceso de biosíntesis *(Hösel, 1981).*

Está claramente establecido que los compuestos no volátiles en forma de glicósidos son una fuente natural de aromas en frutas y otros tejidos vegetales. A medida que avanza la maduración se escinde el enlace β-glicosídico que une el azúcar a la aglicona, aumentando los azúcares solubles en el medio y liberando el aroma (*Bood y Zabetakis, 2002)*. La estructura de algunos de los precursores aromáticos más importantes se pueden observar en la Figura 4.

Los glicósidos de las plantas participan en muchos procesos diferentes. Los compuestos glicoconjugados son una importante forma de acumulación y almacenamiento, así como una manera de transporte de sustancias hidrofóbicas. Además, se les atribuye un papel general como intermediarios de productos del metabolismo secundario en las plantas.

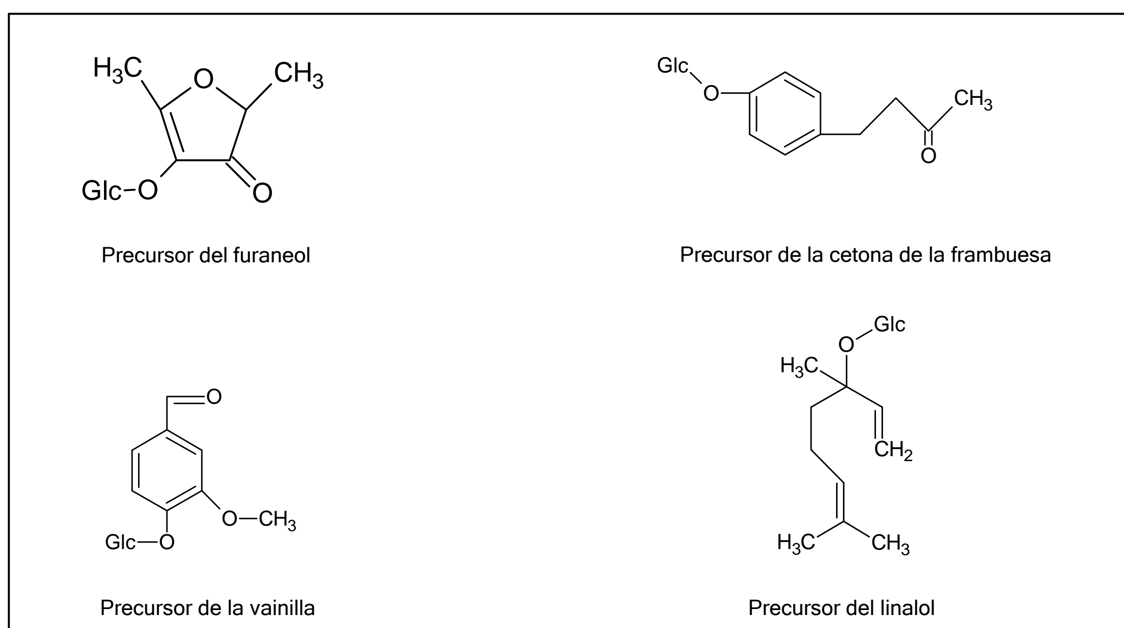


Figura 4. *Ejemplos de compuestos aromáticos glicoconjugados.*

La metodología para el análisis de los precursores comienza con el aislamiento de los glicoconjugados realizando la técnica de retención en fase sólida con adsorbentes de fase inversa C18 *(Williams et al., 1995)*, Amberlita *(Wang et al., 2000)* o con resinas poliméricas LiChrolut EN *(Ibarz et al., 2006).* A continuación se realiza la elución o recuperación de los

compuestos glicoconjugados retenidos usando diferentes solventes como acetato de etilo o metanol. A partir de aquí se pueden seguir dos caminos diferentes dependiendo de cuál sea el objeto de nuestro estudio.

Para analizar las agliconas de los glicoconjugados se realiza una hidrólisis ácida o enzimática y se analiza a continuación por cromatografía de gases acoplada a espectrometría de masas (GC-MS) *(Günata et al., 1985; Ibarz et al., 2006; Loscos et al., 2009; Pedroza et al., 2010).* Por otro lado, si se quieren analizar los glicoconjugados completos se utiliza cromatografía líquida (HPLC), cromatografía a contracorriente (CCC), HPLC-MS/MS *(Herderich et al., 1996; Winterhalter, 1993)* o incluso GC-MS *(Wang et al., 2000).*

Estos compuestos no volátiles se han estudiado en las uvas *(Ibarz et al., 2006; Loscos et al., 2009; Hernández-Orte et al., 2009)* y en otras frutas como el lichi, acerola, mora, piña y mango entre otras *(Humpf y Schreier, 1991; Wu et al., 1991; Boulanger y Crouzet., 2001; Chyau et al., 2002; Lalel et al., 2003).* Sin embargo, no hay apenas estudios sobre los precursores aromáticos de la fresa.

Las investigaciones en esta fruta comenzaron cuando Mayerl et al. describieron la presencia del 2,5-dimetil-4-hidroxi-2H-furan-3-ona β-D-glucopiranósido en fresa en el año 1989. Dos años más tarde, Wintoch et al. (1991) analizaron los compuestos aromáticos glicosídicos de dos especies de fresa *(Fragaria vesca semperflorens y Fragaria ananassa Korona)* utilizando Amberlite XAD-2 como adsorbente. Determinaron 37 agliconas y también analizaron los glicósidos a los que se encontraban ligados dichos precursores aromáticos. Posteriormente los estudios se centraron en conocer la evolución de los precursores durante la maduración de esta fruta *(Pérez et al., 1996; Groyne et al., 1999).* Pérez et al. (1996) se centraron únicamente en la evolución de los precursores del furaneol y del mesifuraneol, mientras que Groyne et al (1999). estudiaron mayor número de compuestos en la maduración de *Fragaria ananassa* de la variedad *Elsanta.*

1.3.2. Aromas libres

1.3.2.1. Compuestos volátiles en caqui

Los compuestos aromáticos del caqui han sido poco estudiados. Los primeros estudios fueron llevados a cabo por Horvat et al. (1991) en caqui de la variedad *Virginiana*. En ellos se establecieron como mayoritarios el acetato de bornilo y el (E)-2-hexenal. Más tarde, Taira et al. (1996) estudiaron el efecto del tratamiento de la fruta con CO_2 para eliminar la astringencia en

la composición volátil de tres variedades de caqui: *Hiratanenashi, Yokono* y *Atago.* Aislaron e identificaron 23 compuestos volátiles, siendo 9 comunes a las 3 variedades: butanol, 3-metil-1-butanol, hexanol, (Z)-3-hexen-1-ol, 2-metil-1-hexanol, acetoína, y ácido acético.

Muy recientemente se ha publicado un estudio mucho más completo en el que se determinaron los aromas del caqui de la variedad *Triumph* por GC-MS y la descripción de sus aromas de impacto por cromatografía de gases con detector olfatométrico *(Wang et al., 2012).* Los resultados de este trabajo señalan a los aldehídos como el grupo responsable en mayor medida del típico olor a caqui. Además es el grupo de compuestos que domina cuantitativamente. Como aromas de impacto describen al metional, (E)-2-hexenal, fenilacetaldehído, (E,Z)-2,6-nonadienal, hexanal y furaneol.

1.3.2.2. Compuestos volátiles en fresa

La composición volátil de la fresa ha sido muy estudiada en los últimos 50 años, identificándose más de 360 compuestos volátiles *(Latrasse, 1991).* Este conjunto de compuestos comprende una combinación de ésteres, que dominan cualitativa y cuantitativamente, furanonas, compuestos azufrados, lactonas, alcoholes y compuestos carbonílicos *(Larsen et al., 1992; Zabetakis y Holden, 1997).*

Los ésteres que se pueden encontrar son el butanoato de etilo y de metilo, hexanoato de etilo, acetato de hexilo y trans-2-hexenil-acetato *(Pyssalo et al., 1979).* Pérez et al. (1992) establecieron que los principales ésteres en fresa madura eran el butanoato de etilo y hexanoato de etilo. Además, como se mencionó en el capítulo 2, en este estudio se investigó la procedencia de estos compuestos, y se observó que el contenido de ésteres en la fresa era directamente proporcional a la concentración del aminoácido alanina, por lo tanto establecieron el papel de éste como precursor de ésteres en esta fruta.

Las furanonas son el otro grupo más importante de volátiles en esta fruta aún siendo un componente minoritario. Esto es debido a que su umbral de percepción es muy bajo y tiene una influencia muy fuerte en el aroma global de la fresa. Las furanonas son 4 isoformas de la molécula 2,5-dimetil-4-hidroxi-2H-furan-2-ona (furaneol), y se pueden encontrar como glucósido de furaneol, mesifuraneol, malonil glucósido de furaneol o como la aglicona libre, furaneol (Figura 5). Muchos autores han descrito al mesifuraneol y furaneol como los aromas de impacto en fresa *(Sanz et al., 1995; Zabetakis et al., 1999)*

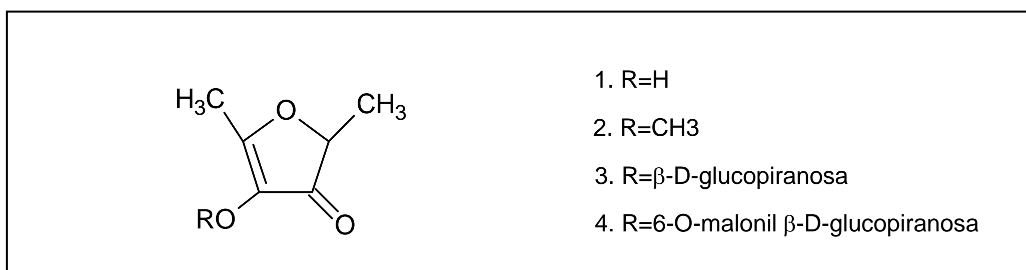


Figura 5. *Estructuras químicas del furaneol (1), mesifuraneol (2), glucósido de furaneol (3) y malonil glucósido de furaneol (4).*

Gran parte del trabajo de investigación sobre los compuestos volátiles de esta fruta se ha destinado a conocer los compuestos de impacto de su aroma. Para ello la fresa ha sido analizada mediante cromatografía gaseosa acoplada a detector olfatométrico y en la mayoría de los casos haciendo uso de un detector de masas. Entre los compuestos que mayor influencia tienen en el aroma global de esta fruta están el furaneol, mesifuraneol, butanoato de metilo, gamma-decalactona, linalol y ácido hexanoico *(Fukuhara et al., 2005; Du et al., 2011).*

1.3.2.3. Compuestos volátiles en vinagre

Los compuestos volátiles del vinagre tienen un efecto decisivo en la calidad de los mismos (Morales et al., 2002). Estos compuestos pueden tener tres orígenes: el vino, materia prima de la que procede, los formados durante la acetificación y aquéllos que aparecen durante la maduración o envejecimiento en madera (Morales et al., 2002).

El aroma del vinagre es una fracción compleja que contiene muchos componentes con un amplio margen de volatilidad, polaridad y concentración *(Blanch et al., 1992).* Hasta la fecha se han identificado más de 100 compuestos volátiles entre los que podemos encontrar tanto compuestos carbonílicos como éteres, acetales, lactonas, ácidos, alcoholes, fenoles volátiles y ésteres, que participan en mayor o menor medida en el aroma final *(Blanch et al., 1992; Morales et al., 2001-2004; Callejón et al., 2009).*

Varios autores han señalado la importancia del proceso de producción en el aroma final de los mismos y por lo tanto en sus cualidades organolépticas *(Morales et al., 2002; Morales et al., 2001; Callejón et al., 2009),* de ahí la importancia del control de este parámetro.

Por otro lado, durante el envejecimiento en las barricas de madera tienen lugar varios fenómenos responsables del incremento de la complejidad aromática del vinagre *(Morales et al., 2004; Callejón et al., 2010)*.

Dentro del grupo de los compuestos carbonílicos el principal aldehído en vinagres es el acetaldehído o etanal, y es además es un precursor para la síntesis de otros compuestos aromáticos *(Etiévant, 1991; Moreno et al., 2005).* Se forma durante la fermentación acética bien sea por la oxidación química del etanol o como metabolito intermedio en la conversión oxidativa del etanol al ácido acético, de donde deriva la mayor parte del acetaldehído presente en vinagres *(Parés y Juárez, 1997).* Otro posible origen de este compuesto es la oxidación del ácido láctico a ácido pirúvico, el cual es transformado posteriormente a acetaldehído. En vinagres, la concentración del acetaldehído esta comprendida entre 10 y 100 mg/L *(Blanch et al., 1992).*

La acetoína es un compuesto característico que se acumula durante el proceso de acetificación, por lo que está íntimamente relacionado con la calidad *(Llaguno y Polo, 1991).* Este compuesto nos sirve para diferenciar los vinagres de fermentación de aquellos artificiales elaborados con acético de síntesis *(Morales et al., 2002).*

El diacetilo, es responsable de un olor característico a mantequilla, y su presencia en vinagres es indicativa del proceso de acetificación y maduración *(Casale et al., 2006).*

Otros compuestos carbonílicos que pueden estar presentes en vinagres son: fenilacetaldehído, 2-furfuraldehído (o furfural), 5-metil-2-furfuraldehído, 5-acetoximetil-2-furfuraldehído y 5-hidroximetil-2-furfuraldehído.

La presencia de alcoholes en vinagre está dominada por el etanol y metanol, ya que son los alcoholes más abundantes en el vino de partida. El metanol no sufre cambios importantes durante el proceso de acetificación, presentando concentraciones del orden de 20-30 mg/L y 30-90 mg/L en los vinagres de vinos blancos y tintos, respectivamente *(Nieto et al., 1993).* En vinagres envejecidos en barriles de madera presentan concentraciones mayores de este alcohol, hasta 213 mg/L *(Callejón et al., 2010)* debido a un fenómeno de concentración.

Los alcoholes superiores se producen durante la fermentación alcohólica y durante la acetificación la bacteria acética los puede metabolizar dando lugar a ácidos *(Nieto et al., 1993; Callejón et al., 2010)*. Entre los alcoholes superiores encontramos en el vinagre el propanol (alcohol propílico) o el isobutanol (2-metil-1-propanol). Otro tipo son los alcoholes isoamílicos

(2-metil-1-butanol y 3-metil-1-butanol), que son los constituyentes más importantes de los alcoholes superiores, representando una fracción abundante dentro de los compuestos volátiles del vinagre de vino. De hecho, constituye aproximadamente el 6-7 % del total de éstos, siendo su concentración superior en productos considerados de alta calidad, como ocurre en los vinagres de Jerez *(Morales et al., 2001).* Estos alcoholes se originan a partir de la isoleucina y leucina *(Bayonove et al., 2000).* Los alcoholes isoamílicos pueden ser transformados por las bacterias acéticas en el correspondiente ácido isovalérico.

En relación al contenido en ácidos orgánicos volátiles, los vinagres contienen ácidos como el acético o isovalérico. Entre ellos, el ácido que identifica el producto es el ácido acético. El ácido mayoritario en vinagres después del acético es el ácido isovalérico.

Los ésteres son responsables de importantes notas frutales y florales del vino *(Charles et al., 2000),* jugando un papel importante en el vinagre producido a partir de éste y se forman se forman por la condensación entre un ácido y un alcohol.

El contenido de acetato de etilo depende casi exclusivamente del contenido de alcohol y de la acidez del vinagre *(Palacios et al., 2002).* En el vinagre común, que contiene una pequeña cantidad de etanol residual, la concentración de acetato de etilo es similar a la que existía en el vino de partida, mientras que en los vinagres de calidad, que se caracterizan por una concentración de etanol residual mayor (del orden de 0,6-1 %), la cantidad de acetato de etilo puede ser más elevada alcanzando valores superiores a 1000 mg/L *(Blanch et al., 1992).* De aquí la mayor fragancia y perfume de éstos vinagres frente a los de fabricación y composición normal.

Otros dos ésteres a destacar son el acetato de metilo y el acetato de isoamilo. Estos compuestos tienen un aroma afrutado (plátano, manzana...etc) y contribuyen a la complejidad aromática de vinos y vinagres *(Ribéreau-Gayon et al., 2006).*

Aparte de estos grupos de compuestos hay otro como los acetales, terpenos, fenoles volátiles y lactonas que tienen una participación muy importante en el aroma del vinagre.

La evolución de los ésteres a lo largo de la acetificación fue estudiada por Callejón et al. (2009), estableciendo que los ésteres acéticos aumentan a lo largo de la fermentación acética y los ésteres etílicos disminuyen.

Con el fin de estudiar en profundidad el aroma del vinagre, se han llevado a cabo algunos estudios olfatométricos. En el año 2000, Charles et al. establecieron la gran importancia del vino de partida en el aroma final del vinagre. Más tarde, Callejón et al., (2008a; 2008b), detallaron los componentes de impacto del aroma del Vinagre de Jerez, Vinagre de Jerez Reserva, Vinagre de Jerez Gran Reserva, señalando al diacetilo, acetato de isoamilo, ácido isovalérico, sotolona y acetato de etilo como los aromas con mayor impacto en estos vinagres. Recientemente, Aceña et al., (2011) describieron nuevos aromas de impacto de este producto.

1.3.2.4. Determinación de los compuestos volátiles

Según la técnica de análisis empleada y el tipo de matriz que se analiza, la determinación de los compuestos volátiles puede requerir una fase previa de extracción.

1.3.2.4.1. Técnicas de aislamiento o extracción de compuestos volátiles

Cuando para el análisis es necesario extraer los compuestos volátiles de la matriz pueden aplicarse diversas técnicas. Las más utilizadas son:

a) Extracción en espacio de cabeza estático *(Static Headspace Extraction, HS):* Este método se basa en el siguiente principio: las moléculas volátiles del producto migran al espacio de cabeza o fase gaseosa que está sobre él hasta alcanzar el equilibrio. De esta manera, esta técnica permite a los analistas tomar una alícuota de la fase gaseosa, la cual está en equilibrio con una fase sólida o líquida *(Ettre, 2002),* que es representativa del aroma de la muestra. El principal factor limitante de esta técnica es su limitada sensibilidad, por ello es utilizada principalmente para el análisis de compuestos mayoritarios. Es por esto que es una técnica es muy representativa del aroma real de la muestra.

b) Extracción en espacio de cabeza dinámico *(Dynamic Headspace Extraction, DHS):* A diferencia del HS, se aplica una corriente de gas que fuerza el paso de los compuestos volátiles al espacio de cabeza. Los volátiles arrastrados al espacio de cabeza son retenidos en algún material acumulándose para su posterior análisis *(B´Hymer, 2003).* Los materiales más utilizados son: Tenax®, Chromosorb®, Porapak®, o resinas XAD Amberlite®. Este sistema tiene más capacidad de extracción que el anterior y por tanto más sensibilidad.

c) Extracción líquido-líquido *(Liquid-Liquid Extraction, LLE):* En este tipo de extracción se pone en contacto un solvente orgánico con la muestra líquida. Para la extracción de

compuestos volátiles se utilizan indistintamente embudos de decantación *(Cobb y Bursey, 1978),* extractores en continuo *(Gutiérrez et al., 1990),* así como técnicas con ultrasonidos *(Mecozzi, 2002).* Esta técnica extrae gran cantidad de compuestos aromáticos, sin embargo el principal problema reside ahí, en el arrastre de compuestos que no son de interés y que dificultan el análisis objetivo de la muestra. Además, requiere del uso de disolventes orgánicos contaminantes ambientales.

d) Destilación y extracción simultáneas: No requiere paso de concentración del extracto y permite extraer los mismos compuestos que la extracción líquido-líquido. Sin embargo, debido al calentamiento de la muestra con las altas temperaturas puede dar lugar a la aparición de compuestos indeseables.

e) Extracción en fase sólida *(Solid Phase Extraction, SPE):* El principio en el que se basa esta técnica es la diferente afinidad de los analitos entre una fase sólida, que es el adsorbente, y una líquida, que es la muestra. Así, se hace pasar la muestra sobre una fase sólida que los adsorbe de manera específica. Se diferencian básicamente tres tipos de adsorbentes: sílices enlazadas, carbón y poliméricos. La elución de los analitos se realiza con unos pocos mililitros de un solvente orgánico como por ejemplo metanol, diclorometano o acetato de etilo. Esta técnica se ha utilizado satisfactoriamente para la extracción de volátiles de vinagre de vino *(Gerbi et al., 1992; Morales et al; 2004).*

f) Microextracción en fase sólida *(Solid Phase Microextraction, SPME):* Fue desarrollada por Arthur y Pawliszyn (1990). Se basa en la extracción de los analitos de la matriz de la muestra mediante una fibra de sílice fundida que está recubierta de un adsorbente o absorbente, en la mayoría de los casos polimérico, seguida de la desorción de los analitos mediante temperatura o disolvente orgánico. La fibra se encuentra ensamblada en un portafibra, cuya estructura es similar a una jeringa ligeramente modificada. La muestra se puede tomar del espacio de cabeza o mediante inmersión en ella. Es simple, muy sensible y reproducible, requiere un bajo coste y pequeños volúmenes de muestra. Se puede utilizar con muestras sólidas, líquidas o gaseosas. Los recubrimientos pueden ser homogéneos (polidimetilsiloxano, poliacrilato) o heterogéneos (divinilbenceno o carboxen sobre una fase entrelazada de polidimetilsiloxano o carbowax).

g) Extracción por absorción con barras magnéticas agitadoras *(Stir Bar Sorptive Extraction, SBSE):* Esta técnica fue desarrollada por Baltussen y Sandra *(Baltussen et al., 1999; Baltussen et al., 2002)* y se basa en los mismos principios de extracción que la SPME. El

absorbente está unido a una barra magnética agitadora. El más usado es el comercialmente conocido como Twister®. Consiste en una barra magnética agitadora recubierta de vidrio y ésta a su vez por una capa de polidemetilsiloxano (PDMS). En este caso los analitos pueden ser extraídos introduciéndolos directamente en la muestra (inmersión) o bien en el espacio de cabeza de la misma *(Callejón et al., 2008).* Otros autores han testado con éxito diferentes fases poliméricas como las de poliuretano, que, unidas a una barra magnética agitadora realizan una función similar al PDMS *(Neng et al., 2007).* Al igual que en el caso de la SPME, la muestra se puede tomar del espacio de cabeza o mediante inmersión directa en la en la misma, y a continuación introducir los compuestos en la columna tras aplicar una desorción térmica. Además, se puede aplicar una retroextracción con solventes orgánicos e inyectar el solvente con los compuestos arrastrados *(Coelho et al., 2008).*

1.3.2.4.2. Técnicas de cromatografía de gases para la determinación de compuestos volátiles

La técnica más ampliamente utilizada hoy en día para el estudio de los compuestos volátiles es la cromatografía de gases acoplada a un detector de espectrometría de masas, sustituyendo al tradicional detector de ionización de llama (FID), ya que tiene mayor sensibilidad y permite identificar los compuestos empleando bases de datos de espectros. Su principal limitación se encuentra en la labilidad de los analitos, los cuales deben ser estables a la temperatura requerida para su volatilización.

Últimamente se ha avanzado mucho en la separación de los compuestos volátiles con la aparición de la cromatografía de gases multidimensional en la cual se emplean dos columnas analíticas situadas consecutivamente. En esta técnica el efluente de la primera columna cromatográfica es reanalizado por una segunda columna que tiene una fase estacionaria de diferente selectividad. Normalmente la primera columna es de naturaleza apolar y la segunda polar. Hay dos tipos de cromatografía multidimensional: convencional (GC-GC) o exhaustiva (GCxGC). En la convencional lo que se eluye de la primera columna es analizado con un detector (FID, MS….) y sólo una parte seleccionada pasa a la segunda columna siendo analizado por un detector de masas. En el caso de la exhaustiva todo lo que se eluye de la primera columna va a la segunda y se vuelve a analizar, habiendo un solo detector de masas.

1.3.2.5. Caracterización del aroma mediante análisis olfatométrico

En general, el aroma está compuesto por uno o más compuestos volátiles que están presentes en concentraciones superiores a las de su umbral de detección olfativo en su correspondiente matriz *(Delahunty et al., 2006).* Así, sustancias con umbral de detección muy bajo pueden contribuir enormemente al aroma, aún en concentraciones muy bajas, y es posible que otras sustancias presentes en concentraciones altas no contribuyan al olor, al ser su umbral de olfacción alto. Por tanto, para poder comprender la contribución de cualquier compuesto volátil sobre el aroma, no basta con saber si ese compuesto está presente o ausente, sino también conocer cómo se percibe a una concentración dada *(Delahunty, et al., 2006).* La técnica empleada para dicho fin es la cromatografía de gases con detector olfatométrico (CG-O). Esta técnica usa la nariz humana para detectar y evaluar los compuestos volátiles que se eluyen de una previa separación por cromatografía de gases *(Delahunty et al., 2006).* La determinación del aroma del analito es posible gracias a la presencia de un accesorio especial, llamado portal olfatorio, situado a la salida de la columna cromatográfica. Además, el cromatógrafo de gases está conectado en paralelo a detectores convencionales como el detector de ionización de llama (FID) o el espectrómetro de masas (MS) *(Plutowska et al., 2008).*

Para la identificación los compuestos volátiles responsables de cada aroma, se hace uso de los Índices de Retención (LRI) de cada compuesto. Dichos índices se calculan empleando la fórmula de Kovats (Figura 6), para la que se necesita el tiempo de elución del compuesto cuyo LRI se quiere determinar y el de una serie de alcanos. Por tanto es necesaria la inyección de una disolución mezcla de diferentes alcanos en las mismas condiciones analíticas.

$$LRI= \left[\frac{t_{r(desconocido)} - t_{r(n)}}{t_{r(N)} - t_{r(n)}} \right] \times (100 \times z) + (100 \times n)$$

LRI= índice de retención lineal
n= nº de átomos de carbono del alcano más pequeño
z= diferencia del nº de átomos de carbono entre el alcano más pequeño y el más grande
t_r= tiempo de retención ajustado
$t_{r(n)}$= tiempo de retención del alcano más pequeño
$t_{r(N)}$= tiempo de retención del alcano más grande

Figura 6. *Fórmula de Kovats.*

Para cada compuesto que emerge del cromatógrafo de gases, el detector humano tiene la capacidad de medir la duración del olor (desde que se detecta hasta que desaparece), describir la cualidad del olor percibido y cuantificar su intensidad. Basándose en estos tres principios, se han desarrollado varias técnicas olfatométricas para determinar la importancia relativa de los odorantes u olores activos de una muestra, los cuales se pueden clasificar en tres categorías: técnicas de dilución, técnicas de tiempo-intensidad y técnicas de frecuencia de impacto.

a) Técnicas de dilución: Estas técnicas se usan para cuantificar la potencia del olor de un compuesto, basándose en la relación entre su concentración y el umbral de detección en el aire *(Delahunty et al., 2006).* Dentro de estas técnicas se pueden distinguir el análisis AEDA (acrónimo de "Aroma Extract Dilution Analysis") y el análisis CHARM ("Combined Hedonic Aroma Response Measurement"), cuya principal diferencia se encuentra en la manera de registrar los datos.

El análisis AEDA mide la dilución máxima de un extracto en la que el olor del compuesto analizado aún puede ser percibido. Este valor se usa para calcular el llamado Factor de Dilución *(van Ruth, 2001).* El AEDA ha demostrado ser una técnica muy poderosa para evaluar el impacto y contribución de los odorantes en el aroma de diferentes alimentos.

Por otro lado, en el análisis CHARM se anota la duración del aroma para generar picos cromatográficos. Esta técnica es adecuada para la determinación de la importancia de cada uno de los compuestos odorantes en una muestra dada, aunque con una menor precisión *(Plutowska et al., 2008).*

b) Técnicas de tiempo-intensidad: en estas técnicas se registra la presencia o ausencia de un odorante y también la intensidad con la que se percibe. Para medir las intensidades de percepción de los odorantes, se van a utilizar diferentes escalas de medida. La forma de registrar las intensidades es lo que diferencia a las técnicas, así tenemos, Intensidad Posterior, Osme y Finger-Span. En la Intensidad Posterior, los panelistas registran la intensidad máxima del compuesto una vez que sale de la columna y se toma como señal la media de los valores dados por el panel.

En general, el principal inconveniente de las técnicas tiempo-intensidad es el intenso entrenamiento que los panelistas requieren para obtener resultados reproducibles.

c) Técnicas de frecuencia de impacto o citación (NIF): NIF es el acrónimo de "Nasal Impact Frecuency". En las técnicas de frecuencia de detección, un panel formado entre 6-12 personas analiza el mismo extracto de la muestra, anotando la ausencia/presencia del odorante y describiendo su aroma. Después, se calcula el porcentaje de panelistas que son capaces de detectar el odorante en un tiempo de retención concreto *(Pollien et al., 1997).*

Se considera que los compuestos que se detectaron con mayor frecuencia son los que tienen una mayor importancia relativa en el aroma de la muestra. Además, se asume que los resultados obtenidos están relacionados con la intensidad del olor percibido en la concentración a la que está presente el analito en el extracto *(van Ruth 2001).* Así, un odorante que ha sido detectado por todo el panel tendrá un valor de intensidad del 100%. Esta técnica ha sido ampliamente utilizada para la determinación de odorantes con gran impacto en varias matrices como, por ejemplo, vinos *(Falcâo et al., 2008)*, vinagres de vino tinto *(Charles et al., 2000)* y aceites esenciales *(Clausen et al., 2005).*

El beneficio fundamental de la frecuencia de impacto es su simplicidad. Además, consume menos tiempo que las otras técnicas, se obtienen muy buenas reproducibilidades y los panelistas no requieren mucho entrenamiento *(Plutowska et al., 2008).* Por el contrario, su limitación principal está relacionada con la escala de medida, ya que un compuesto a una concentración concreta puede ser percibido por todos los panelistas, alcanzando una frecuencia máxima, pero si se incrementa su concentración, su intensidad aromática probablemente también aumentará, y sin embargo, no podrá hacerlo la frecuencia de detección *(Delahunty et al., 2006).* No obstante, la limitada capacidad de discriminación de esta técnica puede mejorarse si se tiene en cuenta la intensidad aromática, aunque ello requiere un intenso entrenamiento del panel sensorial. Algunos autores emplean la llamada "frecuencia modificada", la cual tiene en cuenta tanto la frecuencia como la intensidad de cada odorante, y por tanto, se puede considerar como una técnica híbrida entre ambas *(Campo et al., 2006; Escudero et al., 2007; Pet´ka et al., 2006).*

Hasta el momento, podemos decir que no hay un método o técnica universal para determinar la importancia relativa de los compuestos volátiles identificados como "aromas activos", ya que cada una de las técnicas olfatométricas tiene ventajas e inconvenientes tal como se observa en la Tabla 2 siguiente:

Tabla 2. *Ventajas e inconvenientes de las diferentes técnicas olfatométricas.*

	Ventajas	Inconvenientes
Técnicas de dilución	-Capacidad de discriminación de muestras -Permite conocer las variaciones del aroma en las diferentes concentraciones -Sólo necesita 1 ó 2 panelistas -Se evitan problemas de co-elución	-Requiere bastante tiempo en completar el análisis -Resultados menos precisos -No tiene en cuenta la ley de Steven y asume que la intensidad es proporcional a la concentración
Técnicas tiempo-intensidad	-Resultados reproducibles y precisos -Discrimina muestras -Tiene en cuenta ley Steven	-Requiere mucho entrenamiento, el cual conlleva bastante tiempo -Pueden aparecer problemas de co-elución
Frecuencia de impacto	-Simplicidad -Menor tiempo de análisis -No requiere mucho entrenamiento -Resultados reproducibles y representativos de la población	-Escala de medida: consideran el 100% de intensidad cuando un compuesto es detectado por todo el panel. -Incapaz de discriminar muestras -Requiere entre 6-12 panelistas -Pueden aparecer problemas de co-elución

1.4. Evaluación de la calidad: Análisis sensorial

La calidad de un alimento viene determinada principalmente por su calidad nutricional, sensorial e higiénico-sanitaria. La calidad sensorial de un alimento no es una característica propia, sino el resultado de la interacción entre el alimento y el hombre, por lo que puede ser definida como la sensación humana provocada por determinados estímulos procedentes del alimento, mediatizada por las condiciones fisiológicas, psicológicas y sociológicas de la persona o grupo de personas que la evalúa *(Sancho et al., 1999).*

El éxito de un alimento depende de las reacciones totalmente subjetivas del consumidor. El hombre desde su infancia y de una forma consciente, acepta o rechaza los alimentos de acuerdo con las sensaciones que experimenta al consumirlo *(Sancho et al., 1999).*

Para evaluar la calidad sensorial de un alimento existe una herramienta específica, el análisis sensorial que se basa en observaciones subjetivas y no en medidas objetivas de un instrumento. Según la UNE-87-001-86 (AENOR, 2007), el análisis sensorial es el examen de las propiedades organolépticas de un producto, realizable con los sentidos. Stone y Sidel (2004) lo definieron como la disciplina científica usada para evocar, medir, analizar e interpretar aquellas reacciones o respuestas a las características de los productos percibidas a través de los sentidos vista, olfato, gusto, tacto y oído. Por tanto, según esta definición, el análisis sensorial es una ciencia cuantitativa en la cual se recogen datos numéricos que permitirán establecer relaciones específicas entre las características del producto y la percepción humana. El análisis apropiado de estos datos es crítico para obtener resultados concluyentes de las pruebas sensoriales, para ello se usan diferentes métodos estadísticos.

La evaluación sensorial se ha convertido en una herramienta de gran importancia en prácticamente todas las etapas de producción y desarrollo de la industria alimentaria, para conocer las características organolépticas así como la aceptabilidad del producto. Así, se aplica en el control de calidad y estandarización de alimentos. Además, permite la mejora del producto ya que pone de manifiesto aquellos defectos sensoriales que evitar o atributos sensoriales deseables que potenciar. Por otro lado, facilita la comparación con otros productos similares del mercado para conocer lo competitivo que puede resultar nuestro producto en el mismo *(Barcina, 2001).*

Existen diferentes tipos de análisis sensorial en función de la finalidad u objetivo que se pretende alcanzar. Los tipos de ensayos o análisis son discriminantes, descriptivos y afectivos (Tabla 3).

Tabla 3. *Resumen de los tipos de pruebas sensoriales (Lawless y Heymann, 2010).*

Clase	Objetivo	Características del panel
Discriminante	Averiguar si los productos son sensiblemente diferentes de alguna manera.	Seleccionados por la agudeza sensorial, orientados al tipo de prueba, a veces entrenados.
Descriptiva	Estudiar cómo de diferentes son los productos respecto a una característica específica.	Seleccionados por la agudeza sensorial y motivación, entrenados o altamente entrenados.
Afectivas	Averiguar cómo de buenos son los productos o cuál prefieren.	Seleccionados por ser consumidores de los productos, no entrenados.

La evaluación sensorial comprende un conjunto de pruebas con pautas y técnicas establecidas de presentación del producto, obtención de las respuestas, así como sobre los métodos estadísticos a aplicar y las pautas para la interpretación de resultados. Una correcta aplicación de las técnicas sensoriales implica una correcta correspondencia del método con el objetivo de la prueba. Los análisis discriminante y descriptivo requieren un buen control experimental y una maximización de la precisión de la prueba. Por su parte, las pruebas afectivas requieren usar consumidores del producto representativos y unas condiciones de la prueba que permitan generalizar las impresiones reales que causa el producto en el consumidor.

El buen funcionamiento de un programa de evaluación sensorial es muy útil para las empresas productoras de alimentos ya que permite cubrir las expectativas del consumidor y asegurar una mayor probabilidad de éxito en el mercado *(Lawless y Heymann, 2010).*

Entre los diferentes tipos de análisis sensorial, los ensayos discriminantes tienen por objeto establecer si entre dos o más muestras existen o no diferencias. El análisis se basa en la frecuencia estadística o proporción de respuestas correctas o erróneas. Dentro de este tipo de ensayos existe una gran variedad de pruebas, las que se usan más frecuentemente son las pruebas triangulares, la de comparación pareada y dúo-trío. Este tipo de pruebas suele preceder a los otros tipos de ensayos, el análisis descriptivo o el afectivo *(Lawless y Heymann, 2010).*

El análisis descriptivo es la metodología más sofisticada y potente de la que se dispone para el análisis sensorial. En este tipo de ensayos se cuantifica la intensidad percibida de la característica sensorial del producto. Los resultados proporcionan una descripción sensorial completa de los productos y la base del mapa de similitudes y diferencias del producto, y además, establecen la base para determinar los atributos sensoriales que van a ser importantes para la aceptación *(Stone y Sidel, 2004).*

La finalidad del análisis descriptivo es describir, con un número mínimo de palabras y un máximo de eficacia, el producto a analizar, de manera que tenga una carta de identidad precisa, reproducible y comprensible para todos. Esta descripción deberá tender a ser independiente del grupo de sujetos que la ha generado y deberá, igualmente, ser comparable a otros análisis del mismo tipo efectuados sobre otros productos de la misma familia *(Torre Hernández, 2001).*

El análisis descriptivo puede emplearse para alcanzar diferentes objetivos como pueden ser: definir un estándar de fabricación, mejorar o desarrollar nuevos productos; estudiar la influencia de factores como la materia prima, el proceso de elaboración o conservación del producto; y finalmente, comparar con productos del mismo tipo ya comercializados *(Torre Hernández, 2001).* Este tipo de pruebas requiere un panel sensorial bien entrenado. Los resultados suelen ser representados gráficamente siendo el tipo de grafica más empleado el de tela de araña que proporcionan una especie de huella o mapa del producto. De este modo se consigue una rápida visualización del perfil descriptivo del producto y facilita su comparación con otros productos.

Los ensayos afectivos son el tercer tipo de análisis sensorial, en ellos se intenta cuantificar el grado de gusto o disgusto de un producto, también se denominan hedónicos *(Lawless y Heymann, 2010).* Este tipo de ensayos se lleva a cabo con consumidores.

La aceptación de un producto por parte del consumidor está condicionada, si no por todas, por algunas de las características sensoriales del producto. Por ello, es fundamental realizar ensayos sensoriales con consumidores tanto en estudios destinados a mantener las características de un producto como para la mejora u optimización de procesos y productos. Este tipo de prueba sensorial debería ser una práctica habitual durante el desarrollo de nuevos productos y en estudios de mercados potenciales. Estas pruebas son una buena indicación de su aceptación potencial en el mercado, ya que permiten predecir el comportamiento de los

consumidores frente a un producto. Para obtener unos resultados representativos y fiables se recomienda un número mínimo de 30 sujetos.

Los ensayos hedónicos cuantitativos pueden clasificarse en pruebas de preferencia, aceptación y de consumo *(Pérez-Elortondo, 2001).*

La preferencia es la expresión que señala la elección de un producto frente a otro. Se puede medir directamente por comparación de dos o más productos (prueba de comparación por pares) o indirectamente determinando qué producto ha alcanzado mayor calificación que otros en un test con varios productos o el que ha sido puntuado mejor por mayor número de personas (escala hedónica) *(Pérez-Elortondo, 2001).*

En las pruebas de comparación por pares, la más simple consiste en comparar dos productos y pedir al sujeto que señale la que más le gusta o prefiere. Estas no nos proporcionan información sobre la magnitud de la preferencia *(Stone y Sidel, 2004).*

Las pruebas de aceptación se llevan a cabo bien para evaluar simultáneamente más de dos muestras, o bien para obtener más información sobre el producto. No requiere necesariamente la comparación con otro producto como las de preferencia. En ellas se mide el grado de satisfacción del consumidor. Para llevar a cabo las pruebas de aceptación se emplean las escalas hedónicas *(Pérez-Elortondo, 2001).* La escala hedónica de 9 puntos es probablemente la más usada *(Stone y Sidel, 2004).*

Las pruebas de consumo, consisten en encuestas de consumidores. Estas pruebas pueden englobar a las de preferencia y aceptación por lo que realmente se constituyen en una prueba de estudio de mercado *(Pérez-Elortondo, 2001).*

1.4.1. Los mapas de preferencia o cartografía de preferencia

Para la correcta interpretación de los datos procedentes del análisis sensorial es necesaria la aplicación de diferentes tratamientos estadísticos. Por otro lado, la combinación de datos de análisis descriptivo con los de aceptación del consumidor o con los de medidas instrumentales diversas, mediante las técnicas estadísticas, es una parte esencial para las estrategias de mercado de las empresas alimentarias. Todo ello resulta especialmente útil para saber qué atributos del producto se adaptan mejor a las expectativas del consumidor y así poder optimizar la formulación de esos atributos.

Así, para el análisis e interpretación de los datos del análisis hedónico de los alimentos existe una herramienta denominada mapas de preferencia o cartografías de preferencia. Estos mapas son representaciones gráficas obtenidas aplicando diferentes procedimientos de análisis estadístico multivariante.

Los mapas de preferencia van a permitir averiguar qué atributos del producto influyen en las diferentes tendencias del consumidor hacia diferentes productos *(Michon et al., 2010; Sinesio et al. 2010)*; o conocer los grupos de productos y consumidores *(Oupadissakoon et al., 2010; Sveinsdóttir et al., 2009; Young et al., 2004).*

Existen diferentes tipos de mapas de preferencias, los mapas de preferencia internos y externos *(Meullenet et al., 2007).* Los mapas de preferencia internos analizan sólo los datos de aceptabilidad de los consumidores para determinar el patrón de preferencia de los consumidores. Por su parte, los mapas de preferencia externos permiten relacionar las preferencias de los consumidores con las características físico-químicas, sensoriales o económicas del producto.

El mapa de preferencia interno es la representación multidimensional de los productos y consumidores en un mismo espacio, esta representación es obtenida mediante el análisis de componentes principales de la matriz de covarianza con los productos como filas y la aceptabilidad de los consumidores como variables o columnas *(Meullenet et al., 2007).* Se recomienda emplear la matriz de covarianza para que los consumidores con pequeña o cero preferencia, o con baja desviación estándar no influyan en la estructura del mapa más de lo debido *(Schlich, 1996).* El mapa de preferencia interno proporciona información sobre la principal dirección de la preferencia y la asocia a segmentos o grupos de consumidores *(Greenhoff y MacFie, 1994).*

Los mapas de preferencia externa permiten relacionar datos del análisis descriptivo realizado por expertos con los datos de aceptabilidad del consumidor pudiendo proporcionar información muy valiosa sobre los atributos que condicionan las preferencias del consumidor. Los mapas de preferencia externos se construyen mediante la regresión de los datos de aceptabilidad de los consumidores en el conjunto o espacio externo de datos del análisis descriptivo *(Guinard, 1998).*

1.5. Medida de la capacidad antioxidante y compuestos fenólicos

1.5.1. Oxidación, daño oxidativo y radicales libres

Durante la respiración, la mayor parte del oxígeno que ingresa en el cuerpo es reducido hasta agua. Sin embargo, un pequeño porcentaje, aproximadamente un 5%, se reduce parcialmente, formándose especies reactivas de oxígeno (ROS). Dentro de estas ROS se incluyen los radicales libres, que son especies que poseen uno o más electrones desapareados que son los responsables de su efecto agresivo. Estas ROS son esenciales para mantener la homeostasis celular, pero un exceso puede producir daños en los lípidos, proteínas y ácidos nucleicos de las células *(Magalhaes et al., 2008)*. Para ello, la mayoría de los seres vivos tienen sistemas de defensa endógenos contra el "estrés oxidativo". Sin embargo, a medida que el organismo envejece estos sistemas de defensa no son suficientes y es necesario un aporte exógeno de antioxidantes. Los antioxidantes son sustancias que reducen a los pro-oxidantes dando lugar a compuestos que no tengan o que tengan menos toxicidad *(Magalhaes et al., 2008)*.

Numerosos estudios han constatado una relación inversa entre la toma de frutas y vegetales y la aparición de enfermedades como cardiopatías, cáncer, trastornos asociados a la edad e inflamación (*Willett, 2001*). Los responsables de la mayoría de estos beneficios para la salud son las sustancias presentes en frutas que poseen capacidad neutralizadora de los radicales libres (*Cao et al., 1996; Velioglu et al., 1998).* Estos hallazgos han despertado el interés de la comunidad científica, la cual considera primordial conocer el efecto saludable de los alimentos mediante el estudio de la actividad antioxidante de los mismos.

1.5.2. Clasificación de las sustancias antioxidantes de la dieta

Hay numerosas clasificaciones publicadas de las sustancias antioxidantes. Según *Diplock* et al. *(1998)* se pueden dividir fundamentalmente en nutrientes y no nutrientes:

-Antioxidantes nutrientes:

- Ácido ascórbico (vitamina C)
- Tocoferol (vitamina E)
- Carotenoides (como beta-caroteno, luteína, zeaxantina y licopeno)
- Selenio

-Antioxidantes no nutrientes: Fenoles.

Los fenoles son un grupo de compuestos de naturaleza vegetal, producto del metabolismo secundario de las plantas, encontrándose por tanto en frutas, verduras y alimentos procedentes de ellas. Como se observa en la Figura 8, se caracterizan por tener al menos un grupo fenol como sustituyente (anillo aromático con grupo hidroxilo).

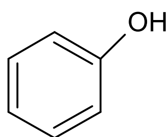


Figura 8. *Estructura fenólica básica.*

Su distribución varía en cada uno de los órganos de la planta, así como también varía su contenido a lo largo de la maduración dependiendo de la zona de cultivo, las condiciones de cosecha, la variedad y el clima.

La síntesis de los compuestos fenólicos en las plantas tiene lugar por dos rutas metabólicas. La vía de los policétidos, minoritaria en plantas superiores, y la vía del ácido shikímico. A veces ambas vías pueden participar en la formación de fenoles complejos. Los polifenoles se sintetizan en las plantas como metabolitos secundarios y cubren funciones de protección contra los rayos ultravioleta. Muchas veces son responsables de la pigmentación de la planta favoreciendo así la atracción del insecto polinizador a la misma o actuando como molécula señal en la interacción de la planta con los microorganismos *(Schijlen et al., 2004)*. Además, son causa de la astringencia, asociada al sabor amargo de algunos vegetales. En general, suelen presentar más de un grupo fenol, por lo que reciben el nombre de polifenoles.

Dentro de este grupo podemos encontrar:

-No flavonoides: como los ácidos fenólicos, estilbenos y taninos hidrolizables (oligómeros de ácido gálico o elágico).

-Flavonoides: flavanoles, antocianos y flavonoles entre otros.

Estos compuestos han sido ampliamente descritos en frutas y verduras como en uva, tomate, fresa, arándano y naranja entre muchos otros *(Aaby et al., 2007a; Slimestad y Verheul, 2009; Giovanelli y Buratti, 2009; Stella et al., 2011)*. Sus características como antioxidantes y beneficios para la salud han sido extensamente estudiados y están claramente demostrados. Poseen efecto como protector cardiovascular, antiinflamatorio, antidiabético, antiproliferativo de

células cancerosas o como preventivo para la inflamación entre otras muchas propiedades *(Duthie et al., 2000; Stoclet, et al., 2004; Arts y Hollman, 2005; Fawzy et al., 2008)*.

1.5.3. Tratamiento de muestras para la determinación de la actividad antioxidante y compuestos fenólicos

Para poder determinar la actividad antioxidante de los alimentos, en ocasiones es necesario un tratamiento previo de los mismos. Estos tratamientos van a depender de la naturaleza de la matriz objeto de estudio. Pueden comprender desde una simple dilución de la muestra o desalcoholización como en el caso de los vinos *(Fernández-Pachón et al., 2006)*, hasta procedimientos más complejos en los que se somete a la muestra a distintas técnicas de extracción (Tabla 4). La finalidad los procesos de extracción es recuperar la mayor cantidad posible de moléculas activas *(Spigno et al., 2007)*. Con este fin se diseñan procedimientos de extracción teniendo en cuenta la labilidad de los antioxidantes frente a la luz, al oxígeno o a la temperatura, evitando técnicas agresivas como el calentamiento, etc. Además, las distintas etapas deben ser optimizadas con el objetivo de obtener los máximos niveles de actividad antioxidante.

Estos procesos de extracción se pueden llevar a cabo directamente sobre las muestras aunque hay autores que las someten previamente a un proceso de liofilización. Hoy en día, la mayoría de los métodos de extracción de compuestos antioxidantes conllevan el uso de algún tipo de solvente. A partir de esta premisa, diversas técnicas son utilizadas para favorecer la salida de los compuestos al exterior de las células. Éstas van desde el empleo de ultrasonido hasta técnicas más complejas como la extracción supercrítica con dióxido de carbono (Tabla 4).

Otros autores son partidarios de obviar la extracción y determinan directamente en la matriz la actividad antioxidante ya que consideran que no hay un solvente único o mezcla de solventes que puedan solubilizar todos los compuestos antioxidantes presentes en un alimento. Este procedimiento ha sido aplicado en galletas, cereales, patatas fritas y carne (*Serpen et al. 2007; Serpen et al., 2008; Morales et al., 2009: Serpen et al., 2012).* Estos autores pretenden eliminar el error que se comete al pensar que el extracto resultante de una extracción sólido-líquido es representativo del total. En otros trabajos, los autores solventaron este problema obteniendo un extracto lipofílico e hidrofílico y así asegurar la completa extracción de compuestos antioxidantes *(Corral-Aguayo et al., 2008).*

Tabla 4. *Resumen de algunas de las técnicas de extracción de compuestos antioxidantes*

TÉCNICA DE EXTRACCIÓN	MUESTRA	MÉTODOS DE MEDIDA	REFERENCIA
Extracción asistida por un extractor de inmersión	Uva	TPI (Índice de Polifenoles Totales) y DPPH	*Pinelo et al., 2005a*
Pulverizado y hervido	Café	ORAC (Capacidad de absorbancia de los radicales de oxígeno)	*Del Castillo et al., 2005*
Extracción simple sólido-líquido	Pepino	DPPH, TPI	*Chuah et al., 2008*
	Arándanos	DPPH, TPI y TA (antocianinas totales)	*Su et al., 2005*
	Caqui	TPI	*Gorinstein et al., 1999*
Extracción asistida por ultrasonido	Caqui	ABTS, TPI y DPPH	*Chen et al., 2008*
	Fresa	TA, TPI y FRAP	*Aaby et al., 2007b*
Extracción asistida por microondas	Salvado de trigo	TPI	*Oufnac et al., 2007*
Extracción asistida por enzimas	Grosella negra	TPI, TA, Inhibición de la oxidación frente al LDL	*Landbo et al.,2001*
Extracción líquida por presión (PLE)	Pulpa de manzana	TPI y DPPH	*Wijngaard et al.,2009*
Extracción supercrítica con dióxido de carbono	*Rosmarinus officinalis* L. y *Salvia officinalis* L.	DPPH y DMPO (5,5-Dimethyl-1-Pyrroline-N-Oxide)	*Ivanovic et al., 2009*

Casi con independencia del método utilizado, es necesario optimizar algunos parámetros para conseguir los mejores rendimientos del proceso. Éstos varían en función del diseño del proceso de extracción pudiendo ser: tipo de solvente utilizado, ratio solvente-muestra, temperatura de extracción (*Pinelo et al., 2005b)*, porcentaje de agua del sistema, tiempo de ultrasonido (*Ghafoor et al., 2009*), tamaño de partícula, etc.

El tipo de solvente es quizás el parámetro más estudiado, y sin embargo sigue presentando muchas dificultades a la hora de su elección. Los rendimientos de la extracción y la resultante actividad antioxidante del extracto son fuertemente dependientes de la naturaleza

del solvente de extracción, debido a la existencia de diferentes compuestos antioxidantes con distintas características químicas y polaridades que pueden o no ser solubles en un solvente en particular (*Sultana et al., 2009*). Para la extracción de antioxidantes en caqui algunos autores utilizan etanol o acetato de etilo como *Gorinstein et al. (2001)* o *Chen et al. (2008)*; para arándano se ha usado una mezcla de HCl al 1% en metanol (*Su y Silva, 2005*), y para el caso de la fresa se ha usado metanol (*Dávalos et al., 2003*) o mezclas acuosas con acetona y metanol (*Henriquez, 2008*). Para algunas frutas tropicales como la piña o el plátano se ha utilizado metanol, etanol y acetona (*Alothman et al., 2009*). Entre todas estas posibilidades la más extendida son las mezclas acuosas con etanol, metanol, acetona y acetato de etilo.

Otro parámetro a optimizar es la proporción solvente-agua. *Sultana et al. (2009)* ensayaron diluciones del 80% y 100% de metanol y etanol con agua, dando mejores resultados el solvente menos concentrado.

La utilización de ultrasonido es muy común en la mayoría de los procesos de extracción ya que es una forma muy adecuada de acelerarlo, además, presenta unas características idóneas respecto a facilidad, eficiencia, precio y simplicidad. Su eficiencia se le atribuye por una parte al efecto de la cavitación acústica producida en el solvente por el paso de la onda ultrasónica (*Wang et al., 2008*) y al efecto mecánico que permite una mejor penetración del solvente en la matriz de la muestra (*Rostagno et al., 2003*). Por otra parte, las ondas rompen la pared de las células y éstas liberan su contenido al exterior *(Vinatoru et al., 1997),* siendo recogido por el solvente. Todo ello hace que el tiempo de ultrasonido sea otro parámetro importante a optimizar.

1.5.4. Métodos de medida de la actividad antioxidante

Hay numerosos métodos para la medida *in vitro* de la actividad antioxidante, pero todos se basan en evaluar si la molécula de estudio es más o menos antioxidante. Como ha sido comentado anteriormente, un pro-oxidante es una sustancia que produce daño oxidativo en varias moléculas diana como ácidos nucleicos, lípidos y proteínas. Una sustancia antioxidante es aquella que consigue reducir al pro-oxidante formando de forma concomitante productos sin toxicidad o con una menor toxicidad (*Magalhaes et al., 2008*).

Actualmente se utilizan tanto métodos *in vitro* químicos como celulares, estos últimos más recientes, que nos dan una idea del potencial antioxidante de una molécula o extracto.

En los ensayos de tipo químico, se evalúa la capacidad de la molécula o muestra de estudio para neutralizar la acción de las especies reactivas de oxígeno (ROS) y especies reactivas de nitrógeno (RNS), que son pro-oxidantes. En la mayoría de estos métodos se realiza una cuantificación comparativa con respecto a otra molécula de reconocida actividad antioxidante que puede ser Trolox, ácido gálico, etc. Los métodos más usados son:

a) Ensayos de reacciones de transferencia de átomos de hidrógeno: La mayoría de estas son reacciones cinéticas de competición y la cuantificación se suele obtener por las curvas descritas a lo largo de esta cinética. Generalmente, en estas reacciones participan un generador de radicales libres, una molécula oxidable y el antioxidante. Entre este tipo de ensayos se encuentran:

- Ensayo ORAC (Capacidad de absorbancia de los radicales de oxígeno): Evalúa la capacidad de una molécula o extracto antioxidante de inhibir la acción de un radical libre sobre una proteína diana fluorescente. Cuando esta proteína sufre un daño oxidativo cambia su conformación y desciende la intensidad de su emisión. El área bajo la curva generada en presencia del antioxidante, frente a la generada al probar un blanco, es interpolada en una curva de calibrado de Trolox, que es un análogo de la vitamina E soluble en agua. La medida obtenida, por tanto, contempla de manera conjunta el grado de inhibición de la fluorescencia ejercido por el antioxidante, así como el tiempo necesario para conseguirlo.

Como fuente generadora de radicales peroxilo se utiliza el AAPH. Se han utilizado diversas moléculas diana o sustratos oxidables. En un principio se usó la ficoeritrina *(Cao y Prior, 1999),* luego el BODIPY (ácido 4,4-difluoro-5-(4-fenil-1,3-butadienil)-4-bora-3a,4a-diaza-s-indacene-3-undecanoico) *(Naguib, 2000),* y más tarde fluoresceína *(Ou et al., 2001),* que es la más usada actualmente y 100 veces más sensible que el BODIPY. Estudios más recientes han comprobado la eficacia del pirogalol rojo como alternativa a la fluoresceína *(López-Alarcón y Lissi, 2006).*

- Ensayo TRAP (Captura total de radicales peroxilos): Se determina el consumo de oxígeno al añadir un generador de radicales libres a la muestra y comenzar éste a oxidar los compuestos presentes en la misma. Durante un periodo de inducción, esta oxidación es inhibida por los antioxidantes, la longitud de este periodo de incubación se compara con la de un patrón a una concentración conocida.

- Ensayo de inhibición de la oxidación de lipoproteínas: Para este ensayo se aíslan lipoproteínas de baja densidad (LDL) de sangre y se incuban junto con un inductor de la oxidación, como por ejemplo el sulfato de cobre, y el antioxidante o mezcla en estudio. Los ácidos grasos presentes en la partícula LDL comienzan a oxidarse formándose los dienos conjugados, que son productos intermedios de oxidación. Estos se detectan y se cuantifican en un espectrofotómetro a 234 nm. El control se realiza incubando las LDL con el inductor de la oxidación en ausencia del antioxidante. Los resultados se expresan como porcentaje de inhibición de la oxidación de las LDL respecto al control.

- Ensayo de inhibición de la oxidación del ácido linoleico: El fundamento es el mismo que el método anterior pero el inductor de la oxidación es un compuesto nitrogenado.

b) Ensayos de reacciones de transferencia de electrones: En estas tiene lugar una reacción redox en la cual el oxidante actúa como indicador del punto final de la reacción. Dentro de este tipo de ensayos se encuentran:

- Ensayo TEAC (Capacidad antioxidante equivalente al trolox): Se basa en la inhibición por antioxidantes de la absorbancia del radical catiónico $ABTS^{\bullet+}$ y mide la habilidad de un compuesto puro (o mezcla) para reducir a este radical.

- Ensayo FRAP (Poder antioxidante de reducción del férrico): Mide la capacidad de un compuesto antioxidante para reducir al Fe^{3+} (férrico) a Fe^{2+} (ferroso) a un pH bajo. Para cuantificar esta fuerza reductora se añade a la reacción redox 2, 4, 6-tripiridil-s-triazina (TPTZ), que provoca una reacción colorimétrica, cuya absorbancia se mide a 593 nm.

- Ensayo CUPRAC (Capacidad antioxidante reductora del ión cúprico): El fundamento de este método es muy parecido al FRAP, y se basa en la reducción de Cu^{2+} (azul claro) a Cu^{+} (amarillo anaranjado). La principal ventaja que presenta es que el cromóforo utilizado para la reacción, $Cu(Nc)_2^{+}$, es soluble en solventes acuosos y orgánicos pudiendo determinar al mismo tiempo antioxidantes de naturaleza lipófila e hidrófila.

- Ensayo DPPH (2,2-difenil-1-picrilhidrazil): Consiste en la medida de la capacidad de captura de un radical libre $DPPH^{\bullet}$ por parte de los compuestos

antioxidantes presentes en la muestra. La molécula del DPPH tiene un espectro característico en un intervalo de UV-visible, con un máximo de absorción a 515 nm y coloración morada. El color cambia de morado a amarillo a medida que la absortividad molar del DPPH medido a 515 nm pasa de 9660 a 1640, es decir, cuando se reduce por efecto de las moléculas antioxidantes y el electrón desapareado del radical se aparea con el hidrógeno del antioxidante.

c) Otros ensayos:

- Ensayo TOSC (Capacidad secuestradora total de oxiradicales): Consiste en la oxidación del ácido α-ceto-γ-metiolbutírico a etileno por radicales peroxilos producidos por 2,2'-Azobis (2-metilpropionamidina) dihidrocloruro (AAPH). La formación de etileno, la cual es parcialmente inhibida por la presencia de antioxidantes, se registra por cromatografía de gases por espacio de cabeza y se compara con la reacción basal.

- Ensayo de quimioluminiscencia: Estos procedimientos utilizan luminol, compuesto que emite luz cuando es oxidado por radicales libres en presencia de oxígeno.

La mayoría de los ensayos mencionados son utilizados frecuentemente, pero presentan una limitación muy importante, que es la falta de estandarización para cada ensayo. Por ello, debido a la heterogeneidad del proceso, reactivos utilizados, concentraciones y cantidades de los mismos, se hace muy difícil la comparación de los resultados obtenidos por diferentes grupos de investigación.

Por otro lado están los ensayos *in vitro* de tipo celular, que sirve a modo de paradigma de lo que ocurriría en organismos superiores.

-CAA (cellular antioxidant activity): Fue el primero de los ensayos de tipo celular, y fue realizado por *Wolfe* y *Liu* en 2007. Este experimento se realizó en células tumorales usando diacetato de 2′,7′-diclorofluoresceína (DCFH-DA) como molécula diana, que al entrar en la célula se hidroliza a DCFH por acción de las esterasas. A continuación el AAPH oxida al DCFH hasta DCF que es fluorescente. Al comparar con las células control, se puede comprobar la actividad antioxidante celular de un compuesto o extracto.

-Método CAP-e: Este ensayo es una versión del anterior, en el que se intercambiaron las células tumorales por eritrocitos (*Honzel et al., 2008; Jensen et al., 2008).* Esto se debe a que los eritrocitos son más baratos y fáciles de conseguir, además de ser más representativo ya que son células presentes en el organismo en todos los casos, y al no tener núcleo ni mitocondria se eliminan las interferencias producidas por la transcripción genética y las especies reactivas de oxígeno producidas en la mitocondria.

-CAA-RBC: Este método está basado en el CAP-e pero la manera de calcular la actividad antioxidante es diferente. Tiene como objetivo calcular la actividad antioxidante de fitoquímicos puros y extractos botánicos (como la uva) comparándolo con los datos obtenidos por el ensayo ORAC clásico *(Blasa et al., 2011).*

-Método utilizando levaduras: Este ensayo es muy reciente y utiliza cepas concretas de *Saccharomyces cerevisiae* para evaluar la actividad antioxidante de moléculas puras *(Wu et al., 2011).* Se basa en el hecho de la levadura detiene su crecimiento al ser sometida a una dosis no letal de oxidante *(Flattery-O´Brien & Dawes, 1998; Alic et al., 2001; Fong et al., 2008).* Primero hay que calcular la concentración de oxidante que produce esa parada y a continuación añadir cada uno de los antioxidantes a testar. Cuanta menos concentración de la molécula se necesite para reactivar el crecimiento, mayor actividad antioxidante tendrá la molécula.

Por otra parte, algunos de los métodos *in vitro* anteriormente citados han sido utilizados para la medida de actividad antioxidante *in vivo* en numerosos estudios tanto en animales como en humanos *(Fernández-Pachón et al., 2005; Shi et al., 2010; Sun et al., 2012).*

1.6. Evaluación de la seguridad de los condimentos

1.6.1. Seguridad Alimentaria

Generalmente se espera de los alimentos que sean agradables al paladar y nutritivos, pero además se requiere que sean sanos, seguros e inocuos. Tras diferentes escándalos alimentarios como el del aceite de colza o las vacas locas, la Comisión Europea publicó el Libro Blanco en el año 2000 *(Comisión de la Comunidad Europea, 1999).* En éste se marcaron las directrices por las que se regiría la política en seguridad alimentaria. Más tarde, en el año 2002, se crearon la *European Food Safety Authority* (EFSA) y la Autoridad Española en Seguridad Alimentaria y Nutrición (AESAN) con el fin de garantizar la salud de los ciudadanos, la información a los consumidores y al sector productivo.

Para lograr estos objetivos, la Unión Europea establece y vela por el cumplimiento de unas normas de control en materia de higiene de los productos alimenticios, de salud y bienestar de los animales, de fitosanidad y de prevención de los riesgos de contaminación por sustancias externas. Además, establece normas para el etiquetado adecuado de productos.

Para garantizar un nivel elevado de seguridad de los productos alimenticios a lo largo de toda la cadena de producción y distribución, a principios de los años 2000 se impuso en la Unión Europea la política en Seguridad Alimentaria basada en el principio «de la granja a la mesa». Esto se aplica tanto a productos fabricados dentro de la Unión Europea como a los importados de terceros países.

Es por esto por lo que antes de la salida al mercado de un nuevo producto alimentario hay que garantizar la seguridad del mismo. Cuando la Comisión Europea recibe una solicitud de autorización de un alimento nuevo o su uso como ingrediente, la Autoridad Europea de Seguridad Alimentaria (EFSA) lo evalúa para saber si presenta algún peligro para los consumidores o pueda inducirles a engaño. En este sentido, se hace necesaria la identificación de riesgos potenciales para la salud como es el caso de la formación de compuestos perjudiciales que aparezcan debido al propio proceso de elaboración del producto.

1.6.2. Determinación de carbamato de etilo

El carbamato de etilo, también denominado uretano (Figura 8), es el éster del ácido carbámico y se encuentra de manera natural en la mayoría de los alimentos fermentados en baja concentración (μg/L). Sin embargo, aún siendo baja su presencia en los alimentos, fue

reconocido y clasificado en 2007 como probablemente carcinógeno para humanos por la Agencia Internacional de Investigación en Cáncer *(Baan et al., 2007).*

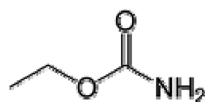


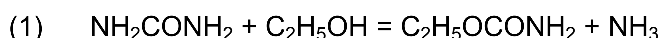
Figura 8. *Molécula de carbamato de etilo.*

A lo largo de la historia, este compuesto ha tenido diversas aplicaciones comerciales como la preparación y modificación de amino resinas, como cosolvente para la fabricación de pesticidas y fármacos, y como intermediario químico en la industria textil *(IARC, 1974).* Además, se ha utilizado como antineoplásico, hipnótico en humanos y anestésico para ratones de laboratorio *(Paterson et al., 1946; University of California, 2003).*

Fue en 1943 cuando se describió su toxicidad y carcinogenicidad *(Nettleleship et al., 1943).* A partir de entonces se ha investigado mucho este compuesto, demostrando ser genotóxico y carcinogénico en especies de seres vivos como ratones, ratas, hamsters y monos *(Beland et al., 2005; Sakano et al., 2002).* Debido a esto y al creciente interés del consumidor por la Seguridad Alimentaria, el estudio de esta molécula, sus posibles vías de formación y técnicas de determinación han aumentado acusadamente.

El carbamato de etilo se forma por la reacción del etanol con diferentes compuestos nitrogenados (urea, citrulina, cianuro de hidrógeno, glicósidos cianogénicos, y otros compuestos N-carbamilados) *(Perestelo et al., 2010).*

La vía de formación más común, es la a partir de la urea, ésta se encuentra en numerosos alimentos fermentados como el yogurt, queso, pan, bebidas alcohólicas y no alcohólicas *(Francis et al., 2002; Larsen, 2006).* En el caso del vino, la vía más habitual de formación es la reacción directa de la urea, procedente de la arginina, con el etanol *(Schehl et al., 2007)* (Eq.1).



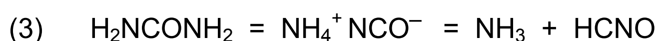
(1) $NH_2CONH_2 + C_2H_5OH = C_2H_5OCONH_2 + NH_3$

La levadura metaboliza la arginina durante la fermentación de la uva y se forma urea, que reacciona con el etanol, alcanzando concentraciones de mg/L. Esta reacción tiene una cinética moderada a temperatura ambiente *(Aresta et al., 2001),* sin embargo, puede

incrementar la formación de carbamato de etilo al aumentar la temperatura (cocción, hervido o tostado).

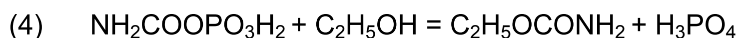
Otras vías de formación de uretano serían a partir de los productos de descomposición de la urea. Así, la urea se descompone en isotiocianato y cianato en soluciones alcohólico-acuosas entre 60 y 100ºC (Eq.2) *(Boulton, 1993),* o en amonio y ácido cianhídrico debido a un calentamiento, formándose carbamato de etilo por reacción del acido cianhídrico con el etanol (Eq.3) *(Schaber et al., 2004).*

(2) $NCO^{-} + C_2H_5OH + H^{+} = C_2H_5OCONH_2$



(3) $H_2NCONH_2 = NH_4^{+} NCO^{-} = NH_3 + HCNO$

Además de la formación a partir de la urea, es muy habitual, aunque en menor medida que en el caso anterior, la producción de carbamato de etilo es a partir de citrulina. Ésta tiene lugar en la fermentación maloláctica especialmente al reaccionar con el etanol *(Arena et al., 1999)* (Eq.4).



(4) $NH_2COOPO_3H_2 + C_2H_5OH = C_2H_5OCONH_2 + H_3PO_4$

Otra manera de formación natural del carbamato de etilo es a partir del anión cianuro. Más de 2000 especies de plantas producen cianoglicósidos y éstos pueden descomponerse en azúcar y cianhidrina, este último compuesto se descompone fácilmente dando cianuro de hidrógeno *(Vetter 2000),* el cual es un precursor del carbamato de etilo.

Existen otras vías de formación del carbamato de etilo pero son menos comunes y aún poco estudiadas.

A los precursores del carbamato de etilo anteriormente citados, hay que añadir a algunos aditivos utilizados en la elaboración de alimentos, los cuales han demostrado producir carbamato de etilo. Este es el caso del carbonato de dietilo y la azodicarbonamida *(Aresta et al., 2001; Schaefer et al., 2003).*

A pesar de haberse demostrado sus efectos tóxicos en animales de experimentación, aún no se han observado consecuencias directas en el ser humano y no se ha establecido un único nivel máximo en alimentos a nivel internacional. Canadá fue el primer país en establecer niveles máximos de este compuesto en bebidas alcohólicas, estableciendo en 1985 el límite permitido en 30 μg/L en los vinos de mesa, 100 μg/L en los vinos generosos, 150 μg/L en

bebidas alcohólicas destiladas, 200 μg/L en el vino de arroz (sake), y 400 μg/L en brandys de frutas y licores *(Conacher et al., 1986).* A partir de 1988, en Estados Unidos se impuso como norma obligatoria determinar el nivel de carbamato de etilo presente en las bebidas fermentadas. Actualmente en los Estados Unidos el límite en vino está entre los 15-60 μg/L *(EFSA, 2007).* Por otro lado, el Comité Codex de la FAO/OMS sobre aditivos alimentarios y contaminantes estableció en 1993 un máximo de referencia de 30 μg/L en vinos de mesa. Recientemente, el 2 de marzo de 2010, la Unión Europea publicó unas recomendaciones que recogen la necesidad de tomar medidas para disminuir lo máximo posible los niveles de carbamato de etilo en bebidas espirituosas por debajo de 1 mg/L *(European Commission, 2010).*

Debido a la creciente preocupación actual por la Seguridad Alimentaria, el carbamato de etilo en los alimentos es un compuesto muy estudiado. Así, ya ha sido descrito en muchos alimentos como el pan, el yogurt, el queso y bebidas alcohólicas como vino, cachaça, cerveza, sidra, ginebra y condimentos como el vinagre *(Lim y Lee, 2011; Hasnip et al., 2007; Nóbrega et al., 2011).* De estos alimentos, el vino ha sido muy estudiado por diversos autores *(Stevens y Ough, 1993; Jagerdeo et al., 2002; Perestrelo et al., 2010),* sin embargo, la presencia de uretano en el vinagre se está investigando más recientemente y hay muy poca información *(Kim et al., 2000; Lim y Lee, 2011).* Al ser el vinagre un producto obtenido mediante doble fermentación, este compuesto puede estar presente bien por provenir del vino sustrato de partida o bien por formarse durante el proceso de elaboración. Algunos autores han comprobado la formación de urea durante la fermentación acética *(Maestre et al 2008)*, lo cual podría conducir a la formación de carbamato de etilo en el vinagre ya que ésta se encuentra favorecida en medio ácido.

Con el fin de la determinación del uretano en los alimentos, se han utilizado y puesto a punto diversas técnicas adaptadas a cada matriz. La extracción líquido-líquido usando diclorometano o acetato de etilo como extractante ha sido utilizada por algunos autores para bebidas *(Ma et al., 1995; Kim et al., 2000).* Sin embargo, las técnicas de extracción más utilizadas son la extracción en fase sólida (SPE) y la microextracción en fase sólida (SPME). Para ello, se han testado varias columnas o materiales de extracción, siendo las más usadas las de tipo copolímero estireno-divinilbenceno (ENV+) *(Jagerdeo et al., 2002; Mirzoian y Mabud, 2006)* o las que contienen tierra de diatomea *(Lim y Lee, 2011).*

El contenido de carbamato de etilo en los alimentos puede oscilar desde algunos ng/L a cientos de μg/L. Debido a las bajas concentraciones de este éster en la matriz y debido a la posible presencia de moléculas que puedan interferir en su determinación, se hace necesario el uso de técnicas muy sensibles y específicas de análisis. La más usada es la cromatografía gaseosa simple (con columna polar) o multidimensional, ambas con diferentes tipos de detectores (FID, MS, MD/MS, etc.). También se ha utilizado la cromatografía líquida con detector de fluorescencia con un paso previo de derivatización que se empleó para el análisis de sidras *(Herbert et al., 2002; Madrera y Suárez, 2009).* Un estudio realizado por *Abreu et al. (2005)* en el que se comparaban las técnicas HPLC y GC-MS, para la determinación de uretano entre varios laboratorios, estableció que las desviaciones más pequeñas entre análisis se obtenían con la técnica GC-MS. Aunque menos utilizada, también cabe mencionar el uso de otros tipos de técnicas tales como espectroscopía FTIR *(Lachenmeier, 2005)* o HPLC-ESI-MS/MS *(Park et al., 2007)*.

2. JUSTIFICACIÓN Y OBJETIVOS

2.1. Justificación

Actualmente existe una creciente demanda de innovación de productos alimenticios por parte de la sociedad. El desarrollo de nuevos productos debe ir encaminado a adaptarse a los nuevos gustos, estilos de vida y preferencias del consumidor. La innovación es una herramienta de gran importancia para mantener o aumentar la competitividad de las industrias alimentarias.

Tradicionalmente, los vinagres de calidad han sido elaborados a partir de vino de uva. Hoy en día, podemos encontrar en el mercado vinagres de uva que se aromatizan con concentrados de otras frutas en la última etapa de elaboración. La demanda de nuevos productos de calidad por parte de los consumidores, encamina la producción de vinagre hacia la diversificación mediante el empleo de nuevas materias primas.

La fruta es una materia prima perecedera, y transformándola mediante un proceso biotecnológico podemos obtener un nuevo producto característico, diferenciado, de larga duración, que tenga aspectos favorables sobre la salud del consumidor, y a su vez posea nuevas y adecuadas propiedades sensoriales. Así, la producción de condimentos a partir de frutas como la fresa y el caqui aportarían al producto final notas aromáticas características además de cualidades saludables debido a la gran actividad antioxidante de éstas, especialmente en el caso de la fresa. Por otro lado, la propuesta de utilización de frutas de segunda calidad para la elaboración de condimentos por doble fermentación permitiría aprovechar los excedentes frutícolas que se generan en cada cosecha.

Por tanto, la obtención de vinagres de frutas mediante doble fermentación se vislumbra como una estrategia innovadora y una alternativa a los métodos de producción actuales. Los productos obtenidos de esta forma han de ser estudiados para conocer sus características químicas y sensoriales, y evaluar las modificaciones que se produzcan durante los procesos de obtención.

2.2. Objetivos

El objetivo general de esta tesis es caracterizar química y sensorialmente los condimentos de fresa y caqui obtenidos mediante doble fermentación, alcohólica y acética. Dentro de este objetivo general se pueden definir una serie de objetivos más concretos:

1- Determinar el contenido de aminoácidos y estudiar los posibles cambios durante el proceso de elaboración de los condimentos.

2- Conocer la composición y evolución de los compuestos volátiles mayoritarios:

2.1. Poner a punto y optimizar un método de análisis empleando la técnica de muestreo del espacio de cabeza seguida de cromatografía de gases acoplada a espectrometría de masas.

2.2. Determinar los compuestos volátiles mayoritarios en los diferentes sustratos, vinos y condimentos.

3- Caracterizar el aroma mediante estudios olfatométricos determinando los compuestos de impacto y odorantes activos.

4- Evaluar la calidad sensorial de los condimentos:

5.1. Establecer el perfil sensorial mediante análisis sensorial descriptivo.

5.2. Conocer la aceptabilidad de los condimentos mediante ensayos hedónicos con consumidores.

5.3. Interpretar los datos sensoriales construyendo mapas de preferencia.

5- Estudiar la actividad antioxidante y parámetros relacionados tanto en los condimentos como en las diferentes etapas del proceso de producción:

1.1. Diseñar y optimizar un método de extracción para el análisis de estos parámetros.

1.2. Determinar la actividad antioxidante utilizando los métodos ORAC (Oxigen Radical Absorbance Capacity) y DPPH (2,2-difenil-1-picrilhidrazil).

1.3. Medir el índice de polifenoles totales.

1.4. Determinar la cantidad de antocianos monoméricos totales, en el caso concreto de la fresa.

6- Estudiar la seguridad de los condimentos determinando su contenido en carbamato de etilo:

6.1. Poner a punto de un método para la determinación de carbamato de etilo empleando cromatografía de gases con detector de espectrometría de masas.

6.2. Cuantificar el contenido de carbamato de etilo en los condimentos.

3. RESULTADOS Y DISCUSIÓN

3.1. Determinación de aminoácidos y amonio

3.1.1. Resumen

Los aminoácidos son fuente de nitrógeno para los microorganismos implicados en los procesos de fermentación, pudiendo ser el motivo de problemas en dichos procesos. Por otra parte, los aminoácidos son precursores de aromas. Por todo ello, decidimos estudiar los cambios en el contenido de 22 aminoácidos y amonio a lo largo de la doble fermentación hasta la obtención de los productos finales. Este trabajo se hizo empleando las muestras de la campaña de fresa del 2008, 2009 y 2010, y las muestras procedentes del caqui.

Las muestras se centrifugaron, se derivatizaron utilizando 6-aminoquinolil-N-hidroxisuccinimidil carbamato (AQC), y se analizaron por cromatografía de líquidos (HPLC) con detector de fluorescencia.

El patrón de consumo de aminoácidos tanto por parte de las levaduras como de las bacterias acéticas está más condicionado por el contenido de aminoácidos de la fruta, que por la cepa de microorganismo. Los resultados indicaron que el microorganismo se adapta a la fuente, ya que siempre consumen los aminoácidos más abundantes en los sustratos de partida. Por otra parte, la cantidad de nitrógeno en el medio fue suficiente para que tuvieran lugar las fermentaciones de manera adecuada. Sin embargo, en la fermentación alcohólica, el nitrógeno total consumido sí se vio condicionado por la cepa de levadura utilizada, ya que la cepa RP1, aislada durante la fermentación espontánea de la pasta de fresa, tuvo una mayor tasa de consumo de nitrógeno total que la otra cepa inoculada (QA23).

Con respecto a los aminoácidos mayoritarios en los diferentes condimentos, ácido gamma-aminobutírico y prolina fueron los más abundantes en el condimento de caqui y en el de fresa procedente de la fermentación alcohólica inoculada de la campaña del 2008. Por el contrario, en el vinagre elaborado a partir de los vinos fermentados espontáneamente, la cisteína fue el mayoritario. Finalmente, la arginina fue el más abundante en los condimentos de fresa de las campañas 2009 y 2010.

Al comparar el contenido de nitrógeno en los sustratos iniciales y el consumo del mismo a lo largo de las dos fermentaciones (alcohólica y acética), se pudo observar que en el caso del caqui se consumió alrededor del 50% del nitrógeno y en el de la fresa de la campaña del 2008 y 2009-2010, un 74% y un 93%, respectivamente.

Determination of Amino Acids with AQC derivatization during the production of strawberry and persimmon vinegars

Journal:	*Journal of Food Science*
Manuscript ID:	Draft
Section:	4 Food Chemistry
Date Submitted by the Author:	n/a

Complete List of Authors:	CALLEJON, R; University of Seville, Food Science and Nutrition Ubeda, C Hidalgo, C Mateo, E Troncoso, A M Morales, M.L.; Universidad de Sevilla, Bioquímica, Bromatología, Toxicología y Medicina Legal

Keywords:	amino acid, fermentation, fruit, HPLC

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1 # Determination of Amino Acids with AQC derivatization during the
2 production of strawberry and persimmon vinegars

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4 R M Callejón[1], C Ubeda[1], C Hidalgo[2], E Mateo[2], A M Troncoso[1], M L
5 Morales[1]

6

7 [1]Área de Nutrición y Bromatología. Facultad de Farmacia. Universidad de
8 Sevilla.
9 C/ P. García González nº2, E- 41012. Sevilla. Spain
10 [2]Departamento de Bioquímica y Biotecnología. Facultad de Enología. Universitat
11 Rovira i Virgili.
12 C/ Marcel•lí Domingo s/n. E- 43007-Tarragona. Spain.
13 *Corresponding author: R M Callejon; e-mail: rcallejon@us.es; Tel.: 34-954-
14 556761 Fax.: 34-954-233765

15

16 **Short version of title:** Amino Acids in fruit vinegars production

17

18 **Choice of journal/section**:
19 Food Chemistry
20

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21 **ABSTRACT**:

22 Changes in amino acids and ammonium were investigated during the alcoholic
23 and acetous fermentation of strawberry and persimmon purees, testing different
24 conditions at each stage of production of these vinegars. A total of 22 amino
25 acids and ammonium were determined by high-performance liquid
26 chromatography (HPLC), employing 6-aminoquinolyl-N-hydroxysuccinimidyl
27 carbamate (AQC) as precolumn derivatization reagent. The most abundant
28 amino acids in strawberry puree were asparagine, glutamine and proline, while in
29 persimmon puree were γ-aminobutyric acid, glutamine, threonine and tyrosine. A
30 significant decrease in the amino acid content was observed during alcoholic
31 fermentations and some acetifications. In the alcoholic fermentation of
32 persimmon and strawberry purees, the Saccharomyces cerevisiae strain used
33 had a great influence on the amino acid profile of wines. Although some amino
34 acids increased during the acetifications, many of them were consumed by
35 acetic acid bacteria. Principal Component Analysis (PCA) reveals that samples
36 of the same harvest can be separated in substrates, wines and final vinegars by
37 using amino acid compounds as variables. In addition, this statistic analysis
38 allowed us to separate final vinegars according to the type of fruit, year of
39 harvest and fresh fruit or commercial puree.

40 **Keywords:** Amino acid, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate,
41 strawberry vinegar, persimmon vinegar, acetification

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43 **Introduction**

44 Vinegar has been used as a seasoning in cooking since ancient times being one
45 of the most widespread and common product in the world because of its
46 availability in many countries in several different varieties (Mazza and Murooka
47 2009). There is a wide variety of vinegars according to the raw materials
48 employed in the production, although the most widely marketed vinegar is the
49 wine vinegar (Ubeda and others 2011a).
50 Currently, consumer interest in the health benefits of food is increasingly
51 important, motivating more research in this area (Ubeda and others 2011b).
52 Recent researches have shown that, in addition to its well-known anti-bacterial
53 activity, vinegar (when consumed as a drink) confers considerable health
54 benefits such as lowering blood pressure (Kondo and others 2001), alleviating
55 the effect of diabetes (Johnston and others 2004), preventing cardiovascular
56 diseases (Sugiyama and others 2003) or acting as an antioxidant (Tagliazucchi
57 and others 2010). Therefore, in addition to the traditional use of vinegar as food
58 flavouring, there is a growing demand for fruit vinegar products that are sold as
59 healthy food (Ou and Chan 2009). Besides, the production of these vinegars
60 provides a use for surpluses of second quality fruit (Ubeda and others 2011a).
61 Amino acids are compounds present in food and beverages which represent the
62 most important form of total nitrogen and are consumed as a nitrogen source
63 during alcoholic and acetous fermentation by yeast or acetic acid bacteria,
64 respectively (Hernández-Orte and others 2003; Callejón and others 2008). In
65 addition, these compounds affect the quality of foodstuff such as aroma since

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66 microbial catabolism of amino acids produces flavour compounds of importance
67 for foods (Anklam 1998; Ardo 2006). For this reason, amino acids are
68 considered precursors of aroma compounds such as higher alcohols, aldehydes,
69 esters, and ketonic acids (Hernández-Orte and others 2002).

70 Many analytical methods have been proposed for the analysis of amino acids
71 (Callejón and others 2011). Reverse-phase high performance liquid
72 chromatography (HPLC) with precolumn derivatization is generally preferred
73 because it is less time-consuming than other procedures and the instrumentation
74 used is simple. The most widely used reagents for precolum derivatization are:
75 phenylisothiocyanate (PITC), O-phthalaldehyde (OPA), 9-fluorenylmethyl-
76 chloroformate (FMOC-CL), dansyl chloride or 6-aminoquinolyl-N-
77 hydorxysuccinimidyl carbamate (AQC) (Hernández-Orte and others 2003). In this
78 work, the AQC reagent was chosen since it has been successfully used for the
79 determination of amino acids in wine and vinegar (Hernández-Orte and others
80 2003; Callejón and others 2008). It reacts with all the primary and secondary
81 amino acids, giving stable fluorescent compounds and the excess is hydrolyzed
82 during the derivatization reaction, avoiding interferences (Callejón and others
83 2008).

84 The aim of this study was to monitor the evolution of amino acids in the
85 production process of fruit vinegars. For this purpose, a total of 22 amino acids
86 and ammonium were determined during the double fermentation (alcoholic and
87 acetous) of strawberries from three different harvests (2008, 2009 and 2010) and
88 persimmons.

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89 **Materials and Methods**

90 **Chemicals**

91 Reagents and standards “AccQ-Fluor” Kit supplied by Waters (Milford, MA, USA)
92 consisted of 6-aminoquinolyl-N-hydrosysuccinimidyl carbamate reagent (AQC),
93 acetonitrile to dissolve the reagent and 0.2 mM sodium borate buffer, pH 8.8.
94 Most of the amino acid standards were purchased from Fluka (Buchs,
95 Switzerland) except for aspartic acid, glutamic acid, histidine, alanine, lysine and
96 γ-aminobutiric acid supplied by Sigma-Aldrich (Steinheim, Germany) and glycine,
97 ornithine obtained from Merck (Darmstadt, Germany). Ammonium sulfate,
98 calcium disodium EDTA and phosphoric acid were supplied by Sigma-Aldrich
99 (Steinheim, Germany). Sodium acetate and trihydrate triethylamine (TEA) were
100 obtained from Fluka (Buchs, Switzerland). Acetonitrile, hydrochloric acid 32%
101 (v/v) and ammonia 25% (v/v) from Merck (Darmstadt, Germany). Ultrapure water
102 was obtained from a Milli-Q water purification system (Millipore, Bedford, MA,
103 USA).

104 **Samples**

105 In this work a total of 32 samples, taken throughout alcoholic and subsequent
106 acetification of persimmons and strawberries from three different harvests (2008,
107 2009 and 2010) were studied. Hence, 8 fruit substrates, 11 wines and 13
108 vinegars were analysed. Codex of each sample is shown in Table 1.

109 Strawberries (Fragaria ananassa var. camarosa) and persimmons (Diospyros
110 kaki var. Sharoni) were provided from different companies from Andalucía
111 (Spain) and vinegars elaboration was performed in the laboratories of the

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112 Department of Biochemistry and Biotechnology (Faculty of Enology, University
113 Rovira i Virgili, Tarragona).
114 The initial processing to obtain the substrate for the elaboration of the wine was
115 performed as follows: 60 g/L of sulphur dioxide were added. Then, 50, 75 and
116 65 g/L of sucrose were added to 2008, 2009 and 2010 strawberry purees,
117 respectively to ensure an adequate final acidity in the resulting vinegars.
118 Additionally, two pectolytic enzymes were incorporated: Depectil extra-garde
119 FCE® and Depectil clarification® (Martin Vialatte Oenologie, Epernay, France),
120 both at a concentration of 15 mg/L.
121 Persimmon and 2008 strawberry fruit substrates were distributed in glass vessels
122 (6L in each recipient) and then divided in two groups: in one group a
123 spontaneous alcoholic fermentation took place and the other was inoculated with
124 the wine yeast Saccharomyces cerevisiae QA23 at the concentration of 2x106
125 cells/mL. The resulting wines were acetified by a spontaneous process to
126 produce the vinegars.
127 In 2009 case, the procedure was similar but the inoculated alcoholic
128 fermentation was carried out with Saccharomyces cerevisiae RP1, isolated from
129 spontaneous fermentation of strawberry in the 2008 process. All wines obtained
130 from spontaneous alcoholic fermentation were mixed and dispensed in different
131 containers: glass vessel and wood barrels (oak and cherry) and left for
132 spontaneous acetification. Wines from inoculated fermentations were distributed
133 in the same way and then were inoculated with a strain of acetic acid bacteria
134 isolated from the 2008 acetification procedure.

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135 In 2010 harvest, the raw material used was a commercial puree provided by the
136 Hudisa Company (Huelva). The elaboration process was performed on a semi-
137 pilot scale; thereby the alcoholic fermentation took place in a stainless steel
138 container after the inoculation with Saccharomyces cerevisiae RP1 strain yeast.
139 Before it, 2 g/L $CaCO3$ were added to adjust the pH to 3.5 and, in this case, 12
140 mg/hL of pectolytic enzimes Rohapect®. After alcoholic fermentation 800 µl/L of
141 dimethyl dicarbonate (Velcorin®) were added to wine. The acetous fermentation
142 was performed inoculating with the above mentioned acetic acid bacteria strain
143 in a cherry wood barrel.

144 **Derivatization and HPLC analysis of samples**

145 Samples analysis was done according to the method previously reported by
146 Callejón and others (2008). Due to the different consistence of the samples, they
147 were centrifuged. 125 µL of the sample supernatant and 50 µL of internal
148 standard (α-aminobutyric acid, 64.45 mg/L) were mixed and Milli-Q water was
149 added to a final volume of 2.5 mL. Afterwards, 20 µL of the previous mixture was
150 derivatized by "AccQFluor" kit according to the instructions provided by the seller.
151 HPLC analysis was performed in a Waters equipment consisting of an
152 autosampler injector Waters 717, a Waters 600E system controller connected to
153 a fluorescence detector, Waters 474. Data treatment was performed in Waters
154 Millennium32 data station. The column was a Luna C18, 5 µm, 250 x 4.6 mm
155 and guard column 4.0 x 3.0 mm from Analytical Phenomenex, (Torrance, CA,
156 USA). Detection was carried out by fluorescence with excitation at 250 nm and
157 emission at 395 nm. The injection volume was 20 µL and the separation was

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158 obtained at a flow rate of 1 mL/min at 34℃ with a quaternary gradient program
159 (Callejón and others 2008).
160 Quantification was performed according to Callejón and others (2008),
161 normalizing the chromatograms by using the relative area to the internal
162 standard. Samples were analysed by triplicate.

163 **Statistical analysis**

164 All statistical analyses were performed by means of Statistica software (StatSoft,
165 2001). One-way ANOVA was performed to evaluate significant differences
166 (significance levels $p<0.05$). Principal Component Analysis (PCA) was carried
167 out as unsupervised method to ascertain the degree of differentiation between
168 samples and which compounds were involved.

169 **Results and Discussion**

170 The aim of this work was to study the changes in the amino acids profile
171 throughout the production process of fruit vinegars. These products were
172 obtained by a double fermentation process (alcoholic and acetous). Different
173 conditions were tested at each stage of production. Hence, we will discuss the
174 results considering the effect of each stage on the concentration of amino acids.

175 **Pre-treatments of fruit puree**

176 According to Kunerman and others (1988), asparagine (Asn) was the most
177 abundant amino acid in the all initial strawberry purees followed by glutamine
178 (Gln) and proline (Pro) (Tables 2a, 3a and 4). On the contrary, histamine (His),
179 metionine (Met), ornithine (Orn) and triptophan (Trp) were not present in any of
180 strawberry puree samples. The strawberry puree from 2008 harvest showed the

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181 highest total content of available nitrogen (sum of amino acids and ammonium)
182 (340 mg/L) while the lowest amounts were observed in strawberry puree from
183 2010 (173 mg/L). Strawberry purees from 2008 and 2009 harvests were
184 obtained in our laboratory from strawberries; however, the 2010 strawberry puree
185 was directly gotten from the manufacture. This may explain the differences in the
186 total available nitrogen amount. After the addition of SO2 and pectolytic enzymes
187 we also observed differences in the total content of available nitrogen. In
188 strawberry purees from 2008 and 2009 this treatment gave rise to a decrease in
189 the total amount of available nitrogen while it increased in purees from 2010
190 (Tables 2a, 3a and 4). The explanation to this result is not clear but could be due
191 to the different characteristic of the raw material used in 2010.
192 On the other hand, Gln was also present in high concentrations in the initial
193 persimmon purees, as occurs in other fruit juices (Özcan and Senyuva 2006).
194 However, the most abundant amino acid was γ-aminobutyric acid (GABA). As in
195 the strawberries purees, His, Met, Orn and Trp were not present in the
196 persimmon samples and, contrary to the strawberry, Asn was not detected in any
197 persimmon purees. Another difference respect to the amino acid profile of
198 strawberry purees was phenylalanine (Phe), which, unlike strawberries, could be
199 quantified in persimmon purees. In general, persimmon showed a lower total
200 amount in available nitrogen (Table 5) which increased significantly after the
201 addition of SO2 and pectolytic enzymes.
202 **Alcoholic fermentation**

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203 In general, a significant decrease in the available nitrogen content was observed
204 during alcoholic fermentation (Tables 2-5). This decline ranged between 200 and
205 300 mg/L in strawberries samples while it was lower, around 95 mg/L, in
206 persimmon wines. This result was expected since amino acids are used as
207 nutrients for yeast growth (Hernández-Orte and others 2003). As shown in
208 Tables 2a, 3a, 4 and 5, most amino acids decreased significantly. Generally, the
209 most consumed amino acids were those most abundant in the fruit purees.
210 Hence, Asn, and Gln were the most consumed in strawberry wines while Gln in
211 the persimmon ones. A curious case was Pro. As mentioned above, it was one
212 of the major amino acid in strawberry purees, representing among 18-25 % of
213 the total amount. This compound decreased significantly during alcoholic
214 fermentation but its consumption was not 100% as Asn and Gln. It could be due
215 to Pro is hardly metabolised by the yeast, according to Hernández-Orte and
216 others (2003). GABA showed also a similar trend in persimmon wines, since it
217 represented 21% of total amino acid amounts but it was not totally consumed
218 during alcoholic fermentation.
219 On the contrary, the concentration of some amino acids increased during
220 alcoholic fermentation. In strawberry wines, arginine (Arg) augmented
221 significantly in all the samples, and others such as glycine (Gly), cysteine (Cys),
222 tyrosine (Tyr), alanine (Ala), lysine (Lys), isoleucine (Ileu) and Met increased in
223 some cases (Tables 2a, 3a and 4). Regarding persimmon wines, all samples
224 showed higher concentrations in Gly, valine (Val), Met and Lys than its initial
225 substrate. The increases of these amino acids could be due to the fact that some

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226 of them are excreted into the medium by the yeast at the end of fermentation
227 (Hernández-Orte and others 2003; Lethonen 1996).
228 Comparing the two types of fermentations (inoculated and spontaneous), the
229 rate of consumption of amino acids in inoculated alcoholic fermentation of 2008
230 strawberry and persimmon puree was lower than in the spontaneous
231 fermentations. In fact, as shown in Tables 2a and 5, there are significant
232 differences of concentration for most of the amino acids. However, this
233 behaviour was not observed in 2009 strawberry wines, since those produced by
234 spontaneous fermentation were richer in amino acids than inoculated wines
235 (Tables 2a y 3a). In our previous work where we studied the major volatile
236 compounds during the production of the same fruit vinegars (Ubeda and others
237 2011a), observing that the changes in higher alcohols for the inoculated 2009
238 wines were similar to the 2008 spontaneous wines and vice versa. The amount
239 of higher alcohols was more abundant in the 2008 spontaneous and 2009
240 inoculated processes. These results were expected taking into account that
241 higher alcohols are formed by yeast from the amino acids (Ribereu-Gayón and
242 others 2006).
243 As mentioned above, the yeast strain used in the production of 2009 inoculated
244 strawberry wines was isolated from 2008 spontaneous wines. Many authors
245 have described differences in the amounts of amino acids consumed by different
246 yeast strains during fermentation and the pattern of consumption of these amino
247 acids (Pérez-Coello and others 1999). Therefore, we could say that the strain
248 involved in the fermentation process has also a strong influence on the amino

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249 acids levels of wines in addition to volatile compounds (Ubeda and others 2011a,
250 Torrea and others 2003; Ribereu-Gayón and others 2006).
251 Just as in grape wines (Hernández-Orte and others 2003; Martínez and others
252 1998), the major amino acid in all strawberry wines was Pro, regardless of
253 substrate, year of harvest or type of fermentation employed (Tables 2a, 3a and
254 4). In the inoculated persimmon wines the most abundant amino acid was also
255 Pro while in the spontaneous, GABA was the major amino acid followed by Pro.
256 As mentioned above, yeasts have a great difficulty to metabolize Pro during
257 alcoholic fermentation. For this reason Pro was the most abundant amino acid in
258 all wines, since yeasts prefer other amino acids, considered as good nitrogen
259 sources, instead of Pro (Callejón and others 2008).

260 **Acetous fermentation**

261 As in alcoholic fermentation, we also carried out two types of acetification
262 processes: spontaneous and inoculated with acetic acid bacteria. Persimmon
263 and 2008 strawberry wines underwent spontaneous fermentation while 2010
264 strawberry wines were inoculated. 2009 strawberry wines, underwent both types
265 of acetifications.
266 Regarding the evolution of total amounts of available nitrogen, we did not
267 observed a similar trend during acetification. In some of them, the total content
268 decreased such as in 2010 strawberry acetification, in all 2009 spontaneous
269 processes and in the inoculated process performed in oak barrels (Tables 3b
270 and 4). In the remaining acetifications the total content increased significantly.

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271 Although the total content of available nitrogen increased during the acetification
272 of persimmon wines, some of these compounds were consumed by acetic acid
273 bacteria, such as glutamic acid (Glu), Pro, GABA and Met. Besides, considering
274 the different substrates, inoculated and spontaneous wines, we observed that in
275 the first ones Gly was also consumed and threonine (Thr) in the second ones
276 (Table 5). Despite Pro was the major amino acid in the inoculated wines and
277 GABA in the spontaneous ones, Glu was the amimo acid most consumed during
278 all acetous fermentations (Table 5). Hence, in persimmon vinegars, the most
279 abundant amino acids were GABA and Pro, and all of them showed similar final
280 content of available nitrogen (Table 5). This is in agreement with Callejón and
281 others (2008) since Pro was also the most abundant amino acid in wine vinegars
282 produced by surface acetification.
283 Regarding 2008 strawberry vinegars, we observed significant differences in the
284 final available nitrogen content, being higher in those obtained from inoculated
285 wines. This was expected since the total amounts of available nitrogen were
286 higher in the inoculated wines. The major amino acids were also different, GABA
287 and Pro in vinegars from inoculated wines and Cys in those from spontaneous
288 ones (Table 2b). As shown in Tables 2a and 2b, most amino acids augmented
289 during acetification. Only Ala decreased significantly during acetous fermentation
290 of inoculated wines and GABA and Ala in acetification of spontaneous ones.
291 Both types of acetification in strawberry samples from 2009 harvest were carried
292 out in different containers: glass vessels, cherry and oak wood barrels. Among
293 them, vinegars produced in oak barrels showed the highest decrease in amino

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294 acids. Although some amino acids increased during acetifications, most of them
295 decreased reaching even concentrations under their detection limits in some
296 cases such as Asn and Gly, which were not detected in any 2009 strawberry
297 vinegars (Tables 3a and 3b). Some differences were observed with respect to
298 the most consumed amino acid in both types of acetifications. Hence, Pro and
299 Asn were the most consumed compounds in all the spontaneous processes,
300 according again to Callejón and others (2008). However, GABA was one of the
301 most consumed amino acid in the inoculated acetifications. Besides GABA,
302 whose consumption was remarkable in glass container fermentation, Pro was,
303 again, the most consumed in vinegars produced in wood barrels (Table 3a and
304 3b). At the end of all 2009 strawberry acetifications, Arg and Cys were the amino
305 acids most abundant, although Pro also reached high concentrations in
306 inoculated vinegars performed in glass vessels.

307 According to this, Arg was also the major amino acid in 2010 strawberry
308 vinegars. Most amino acids of these vinegars also decreased significantly during
309 acetification, being Pro, aspartic acid (Asp) and GABA the most consumed by
310 acetic acid bacteria (Table 4).

311 In most cases, as well as we observed in alcoholic fermentations, the amino acid
312 most consumed by acetic acid bacteria was the most abundant in wine
313 substrate.

314 **Principal Component Analysis (PCA)**

315 PCA was performed to evaluate whether the changes in amino acids produced
316 during the elaboration of the vinegars were high enough to distinguish the

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317 different samples obtained throughout the production process based on
318 substrate, production stage or production method.
319 PCA applied to persimmons and 2008 and 2010 strawberry sample data allowed
320 us to separate the samples into three groups: the substrate, wines and vinegars,
321 with the first three components accounting for 93.8%, 95% and 100% of the
322 cumulative variance, respectively. Their corresponding scores and loadings are
323 plotted into the plan made up of the first two principal components in Figures 1-3.
324 Figures 1 and 3 show that PC1 successfully separates the substrates from the
325 rest of samples. In addition, PC2 separates the substrates after the addition of
326 enzymes and SO2 from the initial substrates. This result takes into account the
327 influence of the treatment on the profile of amino acids. Besides, in the case of
328 strawberries samples from 2010 harvest (Figure 2), PC2 is also able to separate
329 vinegar from wine.
330 Regarding PCA of 2008 strawberry samples (Figure 1), PC1 is able to separate
331 the inoculated wines from the spontaneous ones. Thus, the wines obtained using
332 the same yeast strain appears together in the same quadrant.
333 In addition, when we performed a PCA of strawberry wines obtained in 2008 and
334 2009 (Figure 4), we observed that PC1 separate 2009 spontaneous and 2008
335 inoculated wines from 2009 inoculated and 2008 spontaneous wines. This fact
336 was expected since, as mentioned before, 2009 wines were inoculated with the
337 yeast strain isolated from 2008 spontaneous wines. These results confirm the
338 great influence of the employed yeast strain on the amino acid profile of wine, as
339 well as, on the volatile profile as reported by Ubeda and others (2011a).

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340 The result of PCA on the data obtained from the 2009 strawberry samples
341 showed that the principal three components explained 94% of the cumulative
342 variance. However, as according to Ubeda and others (2011a), the separation of
343 these samples was not as clear as in the other cases. Whilst in 2008 and 2010
344 PCAs (Figures 1 and 3) the samples appear distributed in three separated
345 groups (substrate, wine and vinegar), in 2009 only substrates were successfully
346 separated by PC1 from wines and vinegars (data not shown). Respects to
347 vinegars, the only ones separated in a different quadrant were the inoculated
348 vinegars produced in glass vessels and in cherry wood barrels. Hence, we can
349 say that the influence of the type of recipient in which acetification is carried out
350 is not as important as showed on the volatile compounds (Ubeda and others
351 2011a).
352 On the other hand, a PCA was performed on the amino acids of all final vinegars
353 obtained (Figure 5). Vinegars were successfully separated in different groups
354 according to type of initial substrate (persimmons or strawberries) and year of
355 harvest (2008, 2009 or 2010), with the first three components accounting for
356 96% of variance. As shown in Figure 5, among strawberries vinegars, PC1 was
357 able to separate the 2008 strawberry vinegars from 2009 and 2010 vinegars,
358 since they turned out to be a bit more different. 2009 and 2010 vinegars are very
359 close in the graph, but in different quadrants. These results confirm again the
360 importance of raw material on the amino acids profile of final vinegars (type of
361 fruit, year of harvest and fresh fruit or commercial puree). Thus, the initial content

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362 of amino acids in the substrate is what determinates mainly the amino acid
363 profile of the final product.

364 **Conclusion**

365 As it was expected, a significant decrease in the amino acid content was
366 observed during alcoholic fermentations and some acetifications. In general, the
367 most consumed amino acids were those most abundant in the fruit purees. The
368 highest consumption of amino acid took place during the alcoholic fermentation.
369 Besides, S. cerevisiae strain used had a great influence on the amino acid profile
370 of wines. We could say that the S. cerevisiae strain isolated from strawberry
371 produces the most suitable wines substrates for the production of vinegars, since
372 these wines were richer in amino acids.

373 On the other hand, although some amino acids increased during acetifications,
374 many of them were consumed by acetic acid bacteria. Some differences
375 between inoculated and spontaneous acetifications were observed respect to the
376 most consumed amino acids. So, the strain employed in inoculated acetifications
377 showed, unlike the spontaneous ones, a preference for GABA.

378 GABA and Pro were the most abundant amino acids in the final persimmon
379 vinegars. However, there were differences regarding the major amino acids in
380 the strawberry vinegars. Hence, GABA and Pro were the most abundant
381 compounds in 2008 strawberry vinegars obtained from inoculated wines, Cys in
382 those from spontaneous wines, and Arg in strawberry vinegars from 2009 and
383 2010 harvest.

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384 PCA reveals that in most cases, using amino acid compounds as variables, we
385 can group the samples of the same harvest in substrates, wines and final
386 vinegars. In addition, this analysis was useful to confirm the great influence of
387 the employed yeast on the amino acid profile of wines. Finally, this statistic
388 analysis allowed us to separate the vinegars according to type of fruit, year of
389 harvest and fresh fruit or commercial puree.

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452 **Acknowledgments**

453 This research was made possible through the financial support from the Spanish
454 Government by means of a research project AGL2007-66417-C02-01. Moreover,
455 the researchers are grateful to the enterprises Hudisa S.A., Agromedina and
456 Grupo Alconeras for providing the fruit substrates.

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Tables :

Table 1. Samples codex and description.

Fruit and harvest	Treatment	Puree Sample	Treatment	Sample substrate	Alcoholic fermentation	Wine Sample	Acetification	Recipient	Vinegar sample
Persimmon 2007	Crushed	K7Z1	SO2 Pectolytic enzymes	K7Z2	Inoculated	K7WI	Spontaneous	Glass vessel	K7VI
					Spontaneous	K7WE	Spontaneous	Glass vessel	K7VE
Strawberry 2008	Crushed	F8P1	SO2 Pectolytic enzymes sucrose	F8P2	Inoculated	F8WI1-F8WI2	Spontaneous	Glass vessel	F8VI1-F8VI2
					Spontaneous	F8WE1-F8WE2			F8VE1-F8VE2
Strawberry 2009	Crushed	F9P1	SO2 Pectolytic enzymes sucrose	F9P2	Inoculated	F9WI1-F9WI2	Inoculated	Glass vessel	F9VI
								Oak barrel	F9VIR
								Cherry barrel	F9VIX
					Spontaneous	F9WE1-F9WE2	Spontaneous	Glass vessel	F9VE
								Oak barrel	F9VER
								Cherry barrel	F9VEX
Strawberry 2010	Crushed	F10P1	SO2 Pectolytic enzymes sucrose $CaCO_3$	F10P2	Inoculated	F10WI	Inoculated	Cherry barrel	F10VI

Table 2a. Mean concentrations of amino acids (mg/L) of 2008 strawberry purees and wines

Aa	Puree		Wine			
	F8P1	F8P2	F8WI1	F8WI2	F8WE1	F8WE2
Asp	6.91 ± 0.05	5.674 ± 0.012[a]	n.q.[b]	n.q.[b]	n.q.[b]	n.q.[b]
Asn	97.01 ± 0.21	91.4 ± 0.8[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]
Ser	5.69 ± 0.08	5.29 ± 0.09[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]
Glu	6.67 ± 0.19	7.6 ± 0.3[a]	2.024 ± 0.003[b]	1.86 ± 0.15[b]	n.q.[b,c]	n.q.[b.c]
His	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gln	81.60 ± 0.82	90.7 ± 0.6[a]	1.52 ± 0.04[b]	n.d.[b]	n.d.[b]	n.d.[b]
Gly	0.505 ± 0.022	0.582± 0.005[a]	1.014 ± 0.017[b]	0.84 ± 0.08[b]	1.56 ± 0.09[b,c]	1.62 ± 0.06[b.c]
Arg	1.745± 0.016	1.70 ± 0.07	2.85 ± 0.04[b]	2.80 ± 0.10[b]	2.67 ± 0.06[b]	2.838 ± 0.008[b]
NH_4^+	2.65 ± 0.09	n.d.[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]
Thr	7.781 ± 0.013	7.99 ± 0.11[a]	0.35 ± 0.04[b]	0.33 ± 0.04[b]	0.263 ± 0.012[b]	0.329 ± 0.006[b.c]
Ala	0.561 ± 0.017	0.529 ± 0.018[a]	0.396 ± 0.008[b]	0.38 ± 0.03[b]	0.3815 ± 0.002[b]	0.43 ± 0.03[b]
Pro	68.7 ± 1.3	70.29 ± 0.09[a]	14.38 ± 0.07[b]	15.02 ± 0.21[b]	6.74 ± 0.08[b,c]	7.05 ± 0.07[b.c]
GABA	34.92 ± 0.024	29.76 ± 0.08[a]	29.82 ± 0.012	29.40 ± 0.15	2.26 ± 0.11[b,c]	2.47 ± 0.07[b.c]
Cys	4.9 ± 0.6	1.94 ± 0.11[a]	2.95 ± 0.21	3.07 ± 0.12[b]	2.50 ± 0.07	2.688 ± 0.021[b.c]
Tyr	11.80 ± 0.11	1.237 ± 0.012[a]	2.657 ± 0.015[b]	2.56 ± 0.08[b]	2.08 ± 0.12[b,c]	2.34 ± 0.03[b.c]
Val	3.874 ± 0.004	n.d.[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]
Met	n.d.	n.d.	1.023 ± 0.005[b]	1.04 ± 0.03[b]	n.d.[c]	n.d.[c]
Orn	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lys	1.95 ± 0.11	4.88 ± 0.04[a]	2.73 ± 0.09[b]	2.41 ± 0.07[b]	1.49 ± 0.15[b,c]	1.79 ± 0.08[b.c]
Ileu	1.32 ± 0.03	1.12 ± 0.04[a]	n.q.[b]	n.q.[b]	n.d.[b]	n.d.[b]
Leu	1.542 ± 0.018	1.637 ± 0.011[a]	1.53 ± 0.12	0.74 ± 0.04	1.19 ± 0.06[b]	1.32 ± 0.02[b]
Phe	n.q.	n.d.	n.q.	n.q.	n.q.	n.q.
Trp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total available nitrogen	340.1 ±1.3	322.3 ± 1.6[a]	62.2 ± 0.4[b]	59.4 ± 0.4[b]	21.13 ± 0.04[b,c]	22.88 ± 0.11[b,c]

n.q.: Concentration under quantification limit n.d.: Concentration under detection limit
[a] Significant differences ($p<0.05$) with respect to the initial fruit puree (ANOVA)
[b] Significant differences ($p<0.05$) with respect to its substrate (ANOVA)
[c] Significant differences ($p<0.05$) with respect to inoculated process (ANOVA)

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Table 2b. Mean concentrations of amino acids (mg/L) of 2008 strawberry vinegars

Aa	Vinegar			
	F8VI1	F8VI2	F8VE1	F8VE2
Asp	2.340 ± 0.004[b]	2.362 ± 0.016[b]	2.45 ± 0.05[b,d]	2.636 ± 0.021[b,d]
Asn	1.72 ± 0.03[b]	1.9 ± 0.05[b]	0.66 ± 0.09[b,d]	0.85 ± 0.03[b,d]
Ser	n.d.	n.d.	n.d.	n.d.
Glu	5.10 ± 0.09[b]	5.101 ± 0.005[b]	4.67 ± 0.08[b,d]	4.66 ± 0.04[b,d]
His	n.d.	n.d.	n.d.	n.q.
Gln	n.d.	n.d.	n.d.	n.d.
Gly	1.67 ± 0.017[b]	1.711 ± 0.013[b]	1.43 ± 0.04[b,d]	1.441 ± 0.007[b,d]
Arg	7.61 ± 0.08[b]	7.60 ± 0.024[b]	7.285 ± 0.018[b,d]	7.17 ± 0.06[b,d]
NH_4^+	n.d.	n.d.	n.d.	n.d.
Thr	1.710 ± 0.022[b]	1.76 ± 0.012[b]	1.68 ± 0.045[b]	1.832 ± 0.003[b]
Ala	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]
Pro	16.45 ± 0.03[b]	16.21 ± 0.07[b]	10.02 ± 0.08[b,d]	10.15 ± 0.22[b,d]
GABA	17.1 ± 0.3[b]	18 ± 1[b]	1.036 ± 0.021[b,d]	1.185 ± 0.028[b,d]
Cys	11.83 ±0.18[b]	11.85 ± 0.09[b]	13.15 ± 0.11[b,d]	14.31 ± 0.13[b,d]
Tyr	10.24 ± 0.044[b]	9.90 ± 0.049[b]	9.80 ± 0.19[b]	10.65 ± 0.05[b]
Val	0.804 ± 0.006[b]	0.88 ± 0.01[b]	0.85 ± 0.08[b]	1.074 ± 0.001[b]
Met	0.80 ± 0.04[b]	0.878 ± 0.013[b]	n.d.[d]	n.d.[d]
Orn	n.d.	n.d.	n.d.	n.d.
Lys	7.75 ± 0.09[b]	8.04 ± 0.04[b]	10.6 ± 0.5[b,d]	10.5 ± 0.4[b,d]
Ileu	1.592 ± 0.007[b]	1.551 ± 0.004[b]	2.28 ± 0.05[b,d]	2.487 ± 0.001[b,d]
Leu	5.96 ± 0.06[b]	6.1 ± 0.3[b]	6.9 ± 0.5[b,d]	7.08 ± 0.17[b,d]
Phe	4.087 ± 0.005[b]	4.041 ± 0.012[b]	4.60 ± 0.15[b,d]	4.991 ± 0.007[b,d]
Trp	n.d.	n.d.	n.d.	n.d.
Total available nitrogen	96.8 ± 0.6[b]	97.9 ± 0.5[b]	77.4 ± 0.8[b,d]	81.0 ± 1.5[b,d]

n.q.: Concentration under quantification limit n.d.: Concentration under detection limit
[b] Significant differences ($p<0.05$) with respect to its substrate (ANOVA)
[d] Significant differences ($p<0.05$) with respect to vinegars obtained from inoculated wines (ANOVA)

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Table 3a. Mean concentrations of amino acids (mg/L) of 2009 strawberry purees and wines

Aa	Puree		Wine			
	F9P1	F9P2	F9WI1	F9WI2	F9WE1	F9WE2
Asp	9.3 ± 0.3	7.627 ± 0.01[a]	n.q.[b]	n.q.[b]	2.23 ± 0.04[b,c]	2.23 ± 0.02[b,c]
Asn	87 ± 3	64.4 ± 0.4[a]	n.d.[b]	n.d.[b]	14.5 ± 0.2[b,c]	14.33 ± 0.09[b,c]
Ser	6.00 ± 0.11	4.7 ± 0.3[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]
Glu	11.19 ± 0.05	9.9 ± 0.4[a]	n.q.[b]	n.q.[b]	5.4 ± 0.09[b,c]	5.39 ± 0.11[b,c]
His	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gln	65.495 ± 0.002	79 ± 9[a]	n.d.[b]	n.d.[b]	n.d.[b]	n.d.[b]
Gly	1.2 ± 0.3	1.60 ± 0.25	0.36 ± 0.04[b]	n.q.[b]	0.476 ± 0.013[b]	0.46 ± 0.03[b]
Arg	n.d.	n.q.	3.38 ± 0.01[b]	3.306 ± 0.004[b]	3.412 ± 0.024[b]	3.30 ± 0.04[b]
NH_4^+	0.73 ± 0.17	n.q.[a]	n.d.	n.d.	n.d.	n.d.
Thr	4.458 ± 0.005	3.8 ± 0.1[a]	0.20 ± 0.04[b]	0.130 ± 0.003[b]	0.296 ± 0.005[b,c]	0.298 ± 0.001[b,c]
Ala	0.4 ± 0.05	0.256 ± 0.013[a]	n.q.	n.q.[b]	n.q.	n.q.[b]
Pro	36.6 ± 2.2	42 ± 3[a]	4.3 ± 0.9[b]	4.2 ± 0.04[b]	19.32 ± 0.15[b,c]	19.02 ± 0.14[b,c]
GABA	2.46 ± 0.15	4.0 ± 0.3[a]	2.43 ± 0.06[b]	2.442 ± 0.002[b]	1.373 ± 0.023[b,c]	1.31 ± 0.04[b,c]
Cys	1.33 ± 0.03	1.697 ± 0.017[a]	n.d.[b]	1.98 ± 0.03[b]	3.48 ± 0.08[b,c]	3.00 ± 0.17[b,c]
Tyr	6.24 ± 0.05	6.0 ± 0.3	n.d.[b]	1.371 ± 0.002[b]	2.676 ± 0.001[b,c]	2.53 ± 0.10[b,c]
Val	n.d.	n.d.	n.q.	n.d.	n.d.	n.d.
Met	n.d.	n.d.	n.d.	n.d.	n.q.	n.q.
Orn	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lys	n.d.	n.d.	n.q.	n.q.	2.724 ± 0.005[b,c]	2.609 ± 0.006[b,c]
Ileu	0.59 ± 0.05	0.31 ± 0.06[a]	n.d.[b]	n.d.[b]	0.29 ± 0.01	n.d.
Leu	0.81 ± 0.04	0.88 ± 0.15	n.q.	1.04 ± 0.013	1.758 ± 0.001[b,c]	1.709 ± 0.022[b,c]
Phe	n.d.	n.d.	n.q.	n.d.	n.q.	n.q.
Trp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total available nitrogen	233.8 ± 1.2	226.2 ± 8.3	10.7 ± 0.2[b]	14.47 ± 0.02[b]	57.9 ± 0.5[b,c]	56.9 ± 0.7[b,c]

n.q.: Concentration under quantification limit n.d.: Concentration under detection limit
[a] Significant differences ($p<0.05$) with respect to the initial fruit puree (ANOVA)
[b] Significant differences ($p<0.05$) with respect to its substrate (ANOVA)
[c] Significant differences ($p<0.05$) with respect to inoculated process (ANOVA)

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Table 3b. Mean concentrations of amino acids (mg/L) of 2009 strawberry vinegars

Aa	Vinegars					
	F9VI	F9VIR	F9VIX	F9VE	F9VER	F9VEX
Asp	1.430 ± 0.014	n.q.	n.q.	n.q.[b]	n.q.[b]	n.q.[b]
Asn	n.d.	n.d.	n.d.	n.d.[b]	n.d.[b]	n.d.[b]
Ser	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glu	2.135 ± 0.013[b]	n.d.	0.746 ± 0.031[b]	n.d.[d]	n.d.	n.q.[d]
His	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gln	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gly	n.d.	n.d.	n.d.	n.d.[b]	n.d.[b]	n.d.[b]
Arg	5.33 ± 0.03	2.3 ± 0.03	4.62 ± 0.03	1.88 ± 0.05[b,d]	1.402 ± 0.008[b]	2.150 ± 0.002[b,d]
NH_4^+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Thr	0.835 ± 0.005[b]	n.d.	0.249 ± 0.0129[b]	n.d.[b]	n.d.[b]	n.d.[b]
Ala	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pro	4.28 ± 0.03	0.82 ± 0.03[b]	1.570 ± 0.022[b]	n.q.[b,d]	n.q.[b]	1.86 ± 0.001[b]
GABA	0.770 ± 0.016[b]	n.d.[b]	1.01 ± 0.016[b]	n.d.[b,d]	n.d.[b]	0.53 ± 0.06[b,d]
Cys	6.9 ± 0.2[b]	2.43 ± 0.06	7.13 ± 0.03[b]	1.58 ± 0.13[b,d]	1.50 ± 0.15[b]	2.64 ± 0.03[b,d]
Tyr	4.87 ± 0.10[b]	n.d.	3.05 ± 0.02[b]	n.d.[b,d]	n.d.[b]	1.14 ± 0.01[b,d]
Val	n.q.	n.d.	n.d.	n.d.	n.d.	n.d.
Met	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Orn	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lys	3.70 ± 0.03[b]	n.q.	3.27 ± 0.03[b]	1.10 ± 0.03[b,d]	n.q.[b]	1.826 ± 0.015[b,d]
Ileu	0.615 ± 0.006[b]	n.d.	0.42 ± 0.04[b]	0.23 ± 0.02	n.d.[b]	n.d.[b,d]
Leu	3.70 ± 0.05	0.97 ± 0.02	2.435 ± 0.002	0.821 ± 0.005[b]	0.742 ± 0.006	1.158 ± 0.006[d]
Phe	2.56 ± 0.03[b]	n.d.	2.131 ± 0.011[b]	n.d.	n.d.	n.q.[d]
Trp	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total available nitrogen	37.1 ± 0.3[b]	6.52 ± 0.07	26.63 ± 0.07[b]	5.61 ± 0.10[b,d]	3.64 ± 0.13[b,d]	11.30 ± 0.04[b,d]

n.q.: Concentration under quantification limit n.d.: Concentration under detection limit
[b] Significant differences ($p<0.05$) with respect to its substrate (ANOVA)
[d] Significant differences ($p<0.05$) with respect to vinegars obtained from inoculated process (ANOVA)

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Table 4. Mean concentrations (mg/L) of amino acids during the elaboration of 2010 strawberry vinegars

Aa	**Puree**		**Wine**	**Vinegar**
	F10P1	F10P2	F10WI	F10VI
Asp	6.55 ± 0.21	8.00 ± 1.23[a]	1.7 ±0.3[b]	n.q.[b]
Asn	71.92 ± 1.14	86 ± 8[a]	n.d.[b]	n.d.
Ser	5.612 ± 0.007	7 ± 1[a]	n.d.[b]	n.d.
Glu	7.8 ± 0.3	10.0 ± 1.1[a]	n.q.[b]	n.d.
His	n.d.	n.d.	n.d.	n.d.
Gln	15.5 ± 0.3	17.8 ± 1.4[a]	n.d.[b]	n.d.
Gly	n.d.	n.q.	n.d.	n.d.
Arg	n.q.	1.51 ± 0.12[a]	3.16 ± 0.09	3.6 ± 0.5
NH_4^+	2.6 ± 0.3	3.7 ± 1.0[a]	n.d.[b]	n.d.
Thr	5.0 ± 0.3	5.3 ± 0.3	2.5 ± 0.6[b]	1.95 ± 0.05
Ala	n.q.	n.q.	0.32 ± 0.02[b]	n.d.[b]
Pro	46.90 ± 1.03	52.1 ± 2.3[a]	3.2 ± 0.7[b]	n.q.[b]
GABA	2.24 ± 0.09	2.37 ± 0.13	1.5 ± 0.3[b]	n.d.[b]
Cys	1.85 ± 0.19	2.126 ± 0.007[a]	n.q.[b]	2.192 ± 0.014[b]
Tyr	6.04 ± 0.07	5.24 ± 0.12[a]	n.d.[b]	n.d.
Val	n.d.	n.d.	n.d.	n.d.
Met	n.d.	n.d.	n.d.	n.q.
Orn	n.d.	n.d.	n.d.	n.d.
Lys	1.23 ± 0.08	1.9 ± 0.8	2.2 ± 0.4	3.57 ± 0.16[b]
Ileu	n.d.	n.d.	0.421 ± 0.005[b]	n.d.[b]
Leu	n.q.	n.q.	n.d.	n.d.
Phe	n.d.	n.d.	n.d.	n.d.
Trp	n.d.	n.d.	n.d.	n.d.
Total available nitrogen	173 ± 15	203 ± 17	15.0 ± 1.9[b]	11.3 ± 1.1[b]

n.q.: Concentration under quantification limit n.d.: Concentration under detection limit
[a] Significant differences ($p<0.05$) with respect to the initial fruit puree (ANOVA)
[b] Significant differences ($p<0.05$) with respect to its substrate (ANOVA)

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Table 5. Mean concentrations (mg/L) of amino acids during the elaboration of persimmon vinegars

Aa	Puree		Wine		Vinegar	
	K7Z1	K7Z2	K7WI	K7WE	K7VI	K7VE
Asp	8.6 ± 0.1	10.4 ± 0.1[a]	2.116 ± 0.013[b]	1.9 ± 0.3[b]	2.28 ± 0.18	1.89 ± 0.03[d]
Asn	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ser	4.59 ± 0.23	n.d.[a]	n.d.	n.d.	n.d.	n.d.
Glu	1.42 ± 0.03	10.18 ± 0.19[a]	5.05 ± 0.10[b]	4.01 ± 0.15[b,c]	0.81 ± 0.21[b]	0.745 ± 0.013[b]
His	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Gln	25.4 ± 0.3	27.6 ± 0.1[a]	n.d.[b]	n.d.[b]	3.1 ± 0.5[b]	1.53 ± 0.06[b,d]
Gly	n.d.	n.d.	0.82 ± 0.02[b]	0.56 ± 0.13[b,c]	0.380 ± 0.019[b]	0.98 ± 0.02[b,d]
Arg	6.171 ± 0.016	6.1 ± 0.08	2.12 ± 0.03[b]	1.92 ± 0.12[b,c]	2.61 ± 0.21[b]	6.78 ± 0.14[b,d]
NH_4^+	0.97 ± 0.07	0.618 ± 0.017[a]	n.d.[b]	n.d.[b]	n.d.	n.d.
Thr	16.8 ± 0.5	14.1 ± 0.3[a]	2.3 ± 0.04[b]	6.0 ± 0.7[b,c]	3.5 ± 0.3[b]	2.33 ± 0.06[b,d]
Ala	4.985 ± 0.007	5.03 ± 0.03[a]	1.06 ± 0.07[b]	0.661 ± 0.006[b,c]	1.25 ± 0.03[b]	0.91 ± 0.04[b,d]
Pro	10.8 ± 0.8	15.9 ± 0.6[a]	15.29 ± 0.05[b]	12.05 ± 1.06[b,c]	12.4 ± 0.5[b]	10.43 ± 0.06[b,d]
GABA	36.6 ± 0.3	33.35 ± 0.16[a]	8.54 ± 0.10[b]	15.94 ± 0.08[b,c]	6.60 ± 0.14[b]	12.90 ± 0.04[b,d]
Cys	3.53 ± 0.12	5.66 ± 0.03[a]	8.137 ± 0.022[b]	5.2 ± 0.3[b,c]	10.6 ± 0.5[b]	8.7 ± 0.3[b,d]
Tyr	14.348 ± 0.017	16.54 ± 0.04[a]	5.91 ± 0.03[b]	3.6 ± 0.3[b,c]	7.6 ± 0.3[b]	5.98 ± 0.12[b,d]
Val	n.d.	n.d.	0.546 ± 0.013[b]	n.q.[c]	0.653 ± 0.013[b]	0.68 ± 0.03[b]
Met	n.d.	n.d.	2.98 ± 0.03[b]	3.4 ± 0.2[b,c]	n.d.[b]	1.33 ± 0.05[b]
Orn	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lys	1.05 ± 0.03	2.401 ± 0.014[a]	4.1 ± 0.04[b]	3.06 ± 0.07[b,c]	6.5 ± 0.8[b]	5.03 ± 0.04[b,d]
Ileu	2.02 ± 0.03	3.3 ± 0.02[a]	0.75 ± 0.01[b]	0.33 ± 0.08[b,c]	1.63 ± 0.04[b]	0.93 ± 0.13[b,d]
Leu	2.73 ± 0.11	3.63 ± 0.04[a]	3.44 ± 0.04[b]	2.586 ± 0.006[b,c]	4.90 ± 0.12[b]	4.08 ± 0.05[b,d]
Phe	3.383 ± 0.007	3.762 ± 0.012[a]	1.84 ± 0.09[b]	n.q.[b,c]	2.66 ± 0.03[b]	2.01 ± 0.01[b,d]
Trp	n.q.	n.d.	n.d.	n.d.	n.d.	n.q.
Total available nitrogen	143.4 ± 2.4	158.6 ± 1.7[a]	65.0 ± 0.4[b]	61 ± 3[b]	67.5 ± 0.6[b]	67 ± 4[b]

n.q.: Concentration under quantification limit n.d.: Concentration under detection limit
[a] Significant differences ($p<0.05$) with respect to the initial fruit puree (ANOVA)
[b] Significant differences ($p<0.05$) with respect to its substrate (ANOVA)
[c] Significant differences ($p<0.05$) with respect to inoculated process (ANOVA)
[d] Significant differences ($p<0.05$) with respect to vinegars obtained from inoculated wines (ANOVA)

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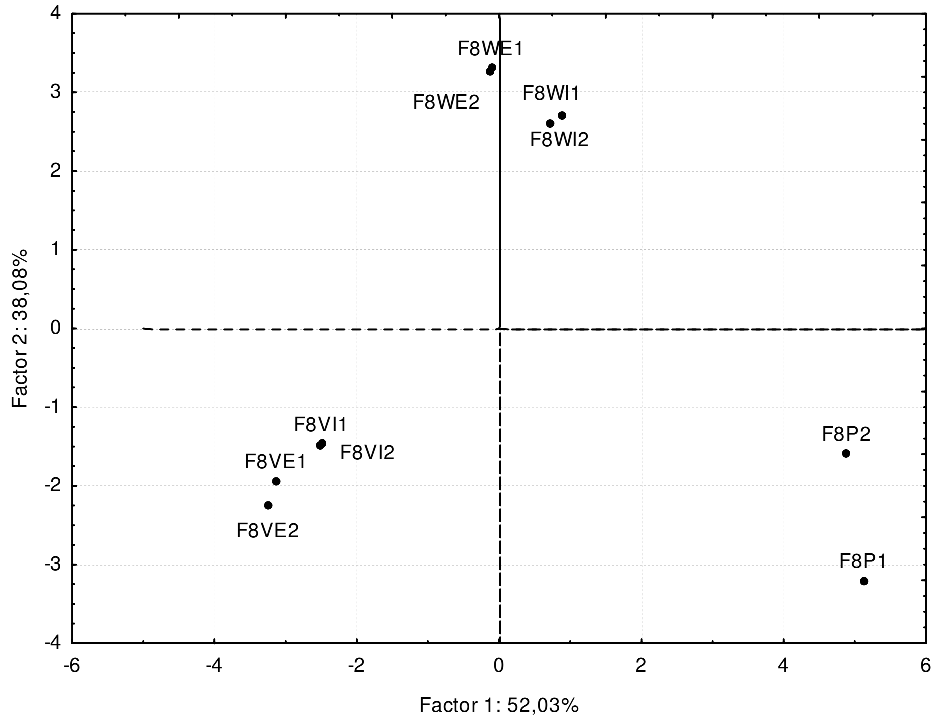
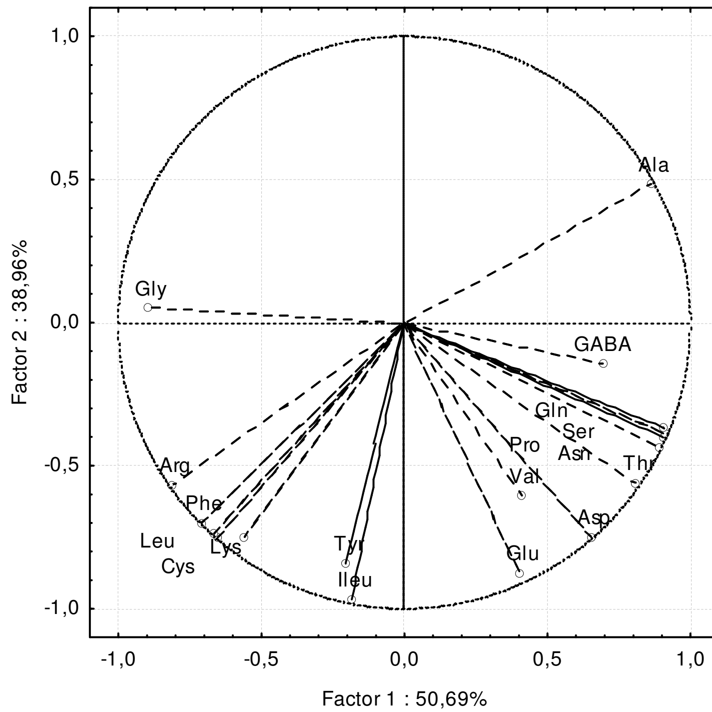


a)



b)

Figure 1. Graphs of the first two principal components (PC1 against PC2) of 2008 strawberry samples: a) Scores of samples; b) Loadings of variables.

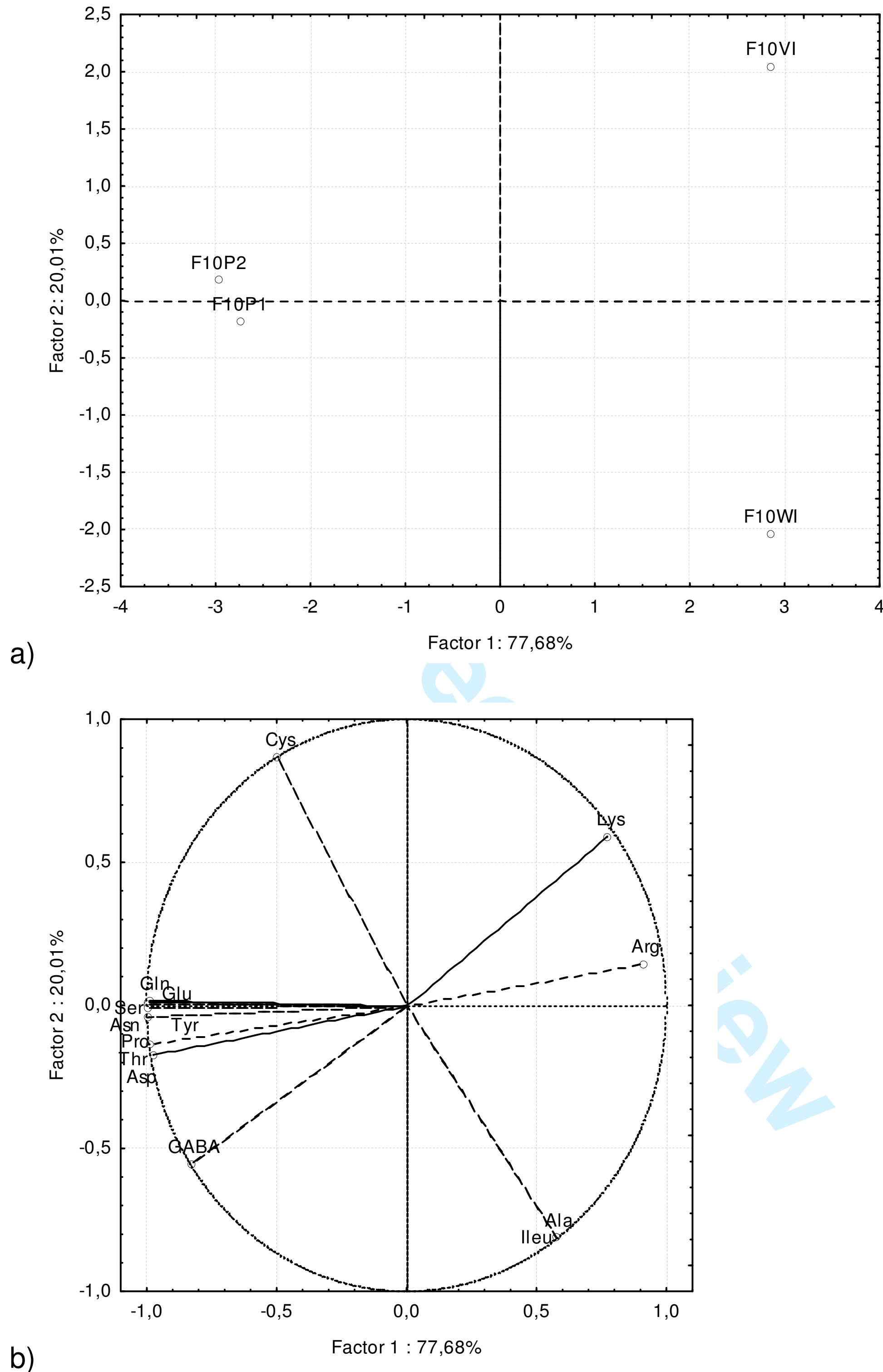
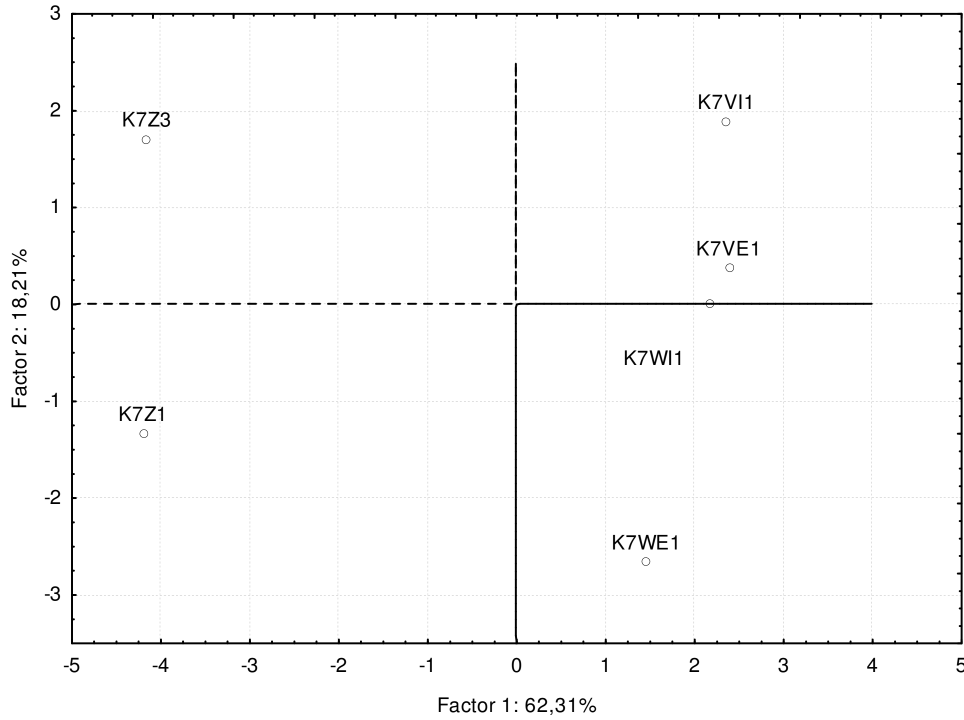
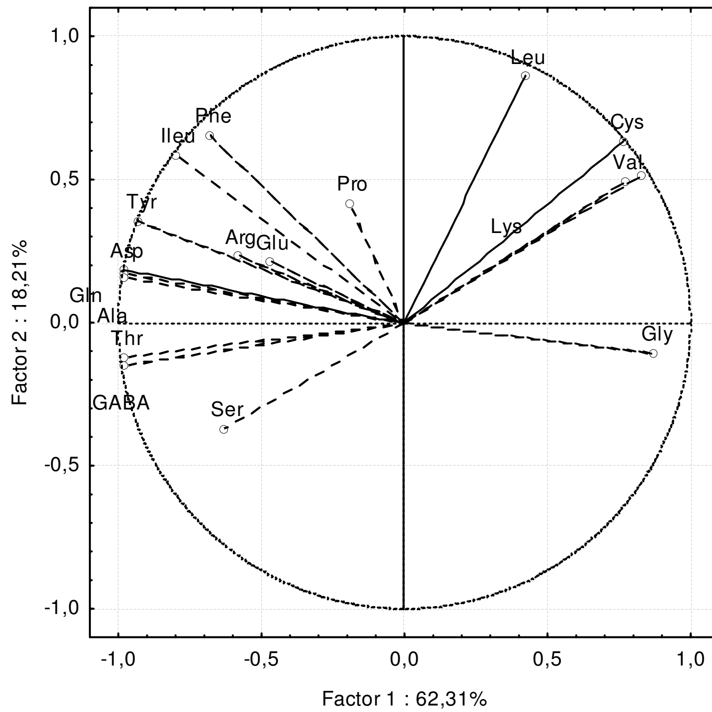


Figure 2. Graphs of the first two principal components (PC1 against PC2) of 2010 strawberry samples: a) Scores of samples; b) Loadings of variables.



a)



b)

Figure 3. Graphs of the first two principal components (PC1 against PC2) of persimmon samples: a) Scores of samples; b) Loadings of variables.

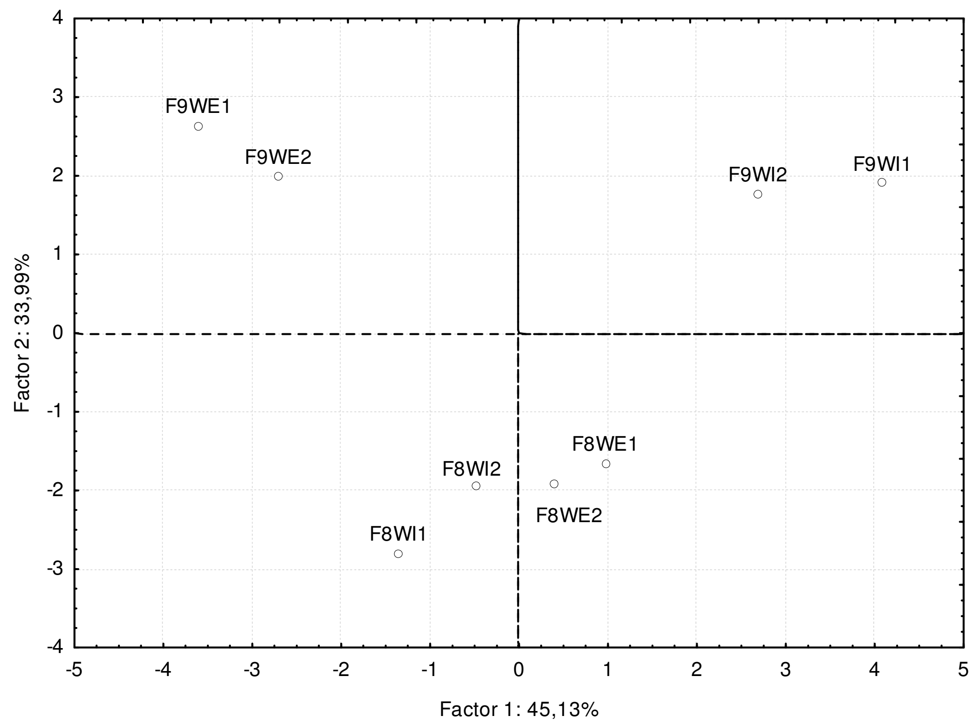


Figure 4. Graph of 2008 and 2009 strawberry wines data score on the plan of the first two principal components (PC1 against PC2).

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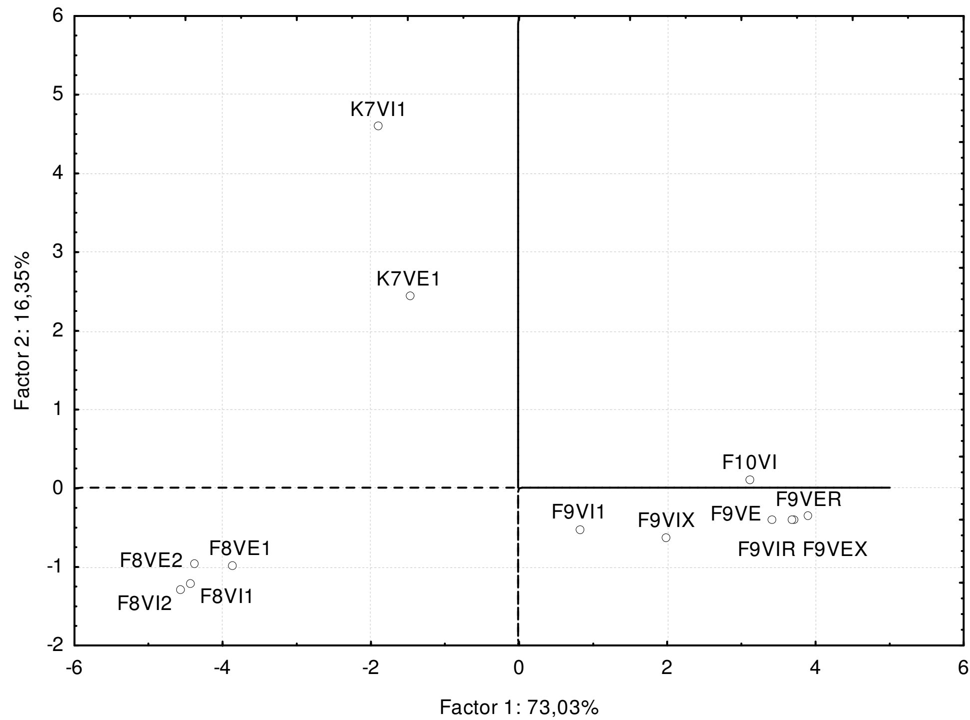

Figure 5. Graph of strawberry and persimmon vinegars data score on the plan of the first two principal components (PC1 against PC2).

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3.2. Análisis de compuestos volátiles mayoritarios, precursores aromáticos y caracterización aromática de los condimentos

3.2.1. Resumen

En este capítulo se muestran los resultados del estudio de la evolución de los compuestos volátiles mayoritarios a lo largo de la fermentación alcohólica y acética del caqui y la fresa. Además, se presenta la caracterización olfatométrica de los sustratos de partida de la fresa y de sus correspondientes condimentos.

A continuación, se incluye el estudio de los precursores aromáticos en cuatro variedades de fresa y los resultados del análisis olfatométrico de cada variedad, para evaluar la posible utilización de variedades de fresa diferentes a la *Camarosa.*

El primer trabajo presenta los resultados de la determinación de los compuestos volátiles mayoritarios (acetato de etilo y de metilo, isobutanol, acetaldehído, metanol, 3-metil-1-butanol, 2-metil-1-butanol, acetato de isoamilo y propanol) desde el sustrato inicial hasta los condimentos finales. De esta manera podemos identificar cuáles procesos son los más indicados para obtener el mejor perfil volátil de los condimentos y cómo afecta cada fermentación a estos compuestos. Para ello, se optimizó un método de muestreo en espacio de cabeza (HS) seguido del análisis mediante cromatografía de gases acoplada a un detector de espectrometría de masas. El método resultó ser lineal, sensible, repetitivo, reproducible y exacto resultando válido para el fin propuesto.

Los resultados mostraron que la adición de enzimas pectolíticas y SO_2 produjo un aumento de acetaldehído y metanol. Además, la cepa de *Saccharomyces cerevisiae* aislada en la campaña del 2008 (RP1) demostró tener gran influencia en la producción de acetaldehído y alcoholes superiores. Podemos decir que los vinos que produce esta cepa tienen un perfil volátil más adecuado para ser utilizado como sustrato para la obtención de los condimentos.

Los análisis olfatométricos se realizaron en los condimentos de fresa obtenidos en las campañas 2008 y 2009 y sus correspondientes sustratos de partida. Además, los extractos se analizaron por GC-MS para identificar los compuestos responsables de las zonas aromáticas percibidas. Con el análisis de las muestras del 2008 se perseguía conocer los cambios de los odorantes activos desde el sustrato al condimento que habían sido producidos a partir de vinos inoculados con levadura o mediante fermentación alcohólica espontánea. El objetivo del segundo estudio fue evaluar cuál de los sustratos de partida (puré y mosto cocido) y recipientes empleados para la acetificación resultaban mejores para el perfil aromático.

Dadas las características de las muestras y las herramientas disponibles, para la preparación de los extractos representativos se aplicó la técnica de extracción líquido-líquido con diclorometano. El análisis olfatométrico fue llevado a cabo por tres panelistas expertos. Con los resultados se calcularon las frecuencias modificadas.

Tras los análisis de las muestras de la campaña del 2008, se pudo observar que el perfil aromático de los condimentos finales era una mezcla entre los procedentes del sustrato y los que se formaron a lo largo de la acetificación. Por una parte, tenían como aromas activos algunos característicos de la fresa como γ-decalactona, furaneol, mesifuraneol, β-damascenona y el etil-2-metilbutirato, que habían sido conservados a lo largo de la doble fermentación, y por otro, tenía los propios que se generan en la acetificación como son los ácidos acético, isovalérico y butírico, además de otros compuestos característicos de algunos vinagres como la sotolona y el diacetilo. Se encontraron varias diferencias entre los dos tipos de condimentos. El que se elaboró a partir del vino que había sido inoculado presentó más zonas aromáticas que el que procedía del vino que se había dejado fermentar espontáneamente. Por tanto, el condimento de elección sería el procedente de la inoculación. Finalmente, concluimos que los aromas de impacto en los condimentos de fresa fueron los ácidos isovalérico, butírico y acético, pantolactona+furaneol, ácido fenilacético, 3-nonen-2-ona, 2-feniletanol, p-vinilguaiacol, vainilla, sotolona y metional debido a la alta frecuencia modificada que alcanzaron en el producto final.

El análisis olfatométrico y sensorial de las muestras de la campaña de fresa del 2009 puso de manifiesto que el uso de diferentes sustratos de partida y recipientes para la acetificación produce grandes diferencias aromáticas en los condimentos finales. Así, el condimento obtenido del mosto cocido era sensorialmente muy diferente a los producidos a partir de la pasta de fresa sin concentrar, por tanto se han obtenido dos condimentos totalmente diferentes. Entre ellos el que consiguió mayor puntuación en impresión general fue el procedente del mosto cocido. Entre los condimentos obtenidos del puré de fresa sin concentrar, los elaborados en barriles de madera presentaron más zonas odorantes que el elaborado en recipiente de cristal. Por otra parte, al comparar el análisis sensorial descriptivo de los condimentos acetificados en barriles de madera, se observó que el elaborado en barril de roble tiene un perfil aromático de mayor calidad y por tanto sería el recipiente elegido para la acetificación.

Para el estudio de los precursores aromáticos se optimizó un método de extracción en fase sólida (SPE) y se analizaron las diferentes variedades. Los resultados pusieron de manifiesto que la resina LiChrolut® demostró ser más efectiva como adsorbente para la

extracción de precursores aromáticos de la fresa que la hasta ahora utilizada Amberlita. De esta manera se pudieron determinar 51 agliconas, 38 de ellas no descritas con anterioridad en esta fruta. De las cuatro variedades estudiadas, *Fuentepina* fue la que tenía más variedad de aromas glicosilados.

Adicionalmente, se llevó a cabo el estudio olfatométrico de las variedades. El extracto representativo se obtuvo por la técnica de SPE con corriente de nitrógeno, y se analizó por cromatografía de gases con detector olfatométrico (GC-O) por un panel de 6 expertos. Los resultados permitieron la identificación por primera vez en fresa de (Z)-1,5-octadien-3-ona, un posible aroma de impacto descrito como geranio/verde/pimiento/lechuga con una frecuencia modificada de más de 80 en dos de las variedades *(Fuentepina y Candonga).* En la variedad *Sabrina* predominaron las notas aromáticas dulces sobre las herbáceas.

Tras el estudio de los resultados obtenidos en este trabajo se concluye que la variedad *Sabrina* podría ser una alternativa a la variedad *Camarosa* para su utilización como sustrato en la obtención de productos fermentados. La variedad *Sabrina* tiene en altas concentraciones los aromas característicos de la fresa en forma de precursores aromáticos.

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Food Research International 44 (2011) 259–268

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Determination of major volatile compounds during the production of fruit vinegars by static headspace gas chromatography–mass spectrometry method

C. Ubeda [a], R.M. Callejón [a], C. Hidalgo [b], M.J. Torija [b], A. Mas [b], A.M. Troncoso [a], M.L. Morales [a,*]

[a] *Área de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Sevilla, C/P García González no. 2, E-41012, Sevilla, Spain*
[b] *Departamento de Bioquímica y Biotecnología, Facultad de Enología, Universitat Rovira i Virgili, C/Marcel·lí Domingo s/n, E-43007, Tarragona, Spain*

ARTICLE INFO

Article history:
Received 16 July 2010
Accepted 17 October 2010

Keywords:
Volatile compounds
Persimmon
Strawberry
Vinegar
Wine
SHS–GC–MS

ABSTRACT

A static headspace gas chromatography coupled to mass spectrometry (SHS–GC–MS) method was validated to determine several major volatile components during the production process of fruit vinegars. The method is simple, fast, linear in the working range, suitably sensitive, repeatable and reproducible, and has a good degree of accuracy for most of the compounds studied. Different conditions were tested in the production process of vinegars by means of double fermentation. The addition of SO_2 and pectolytic enzymes produced a considerable increase in methanol and acetaldehyde, especially in strawberry purees, whereas pressing led to a loss of these volatile compounds. In the alcoholic fermentation of persimmon and strawberry purees, the *Saccharomyces cerevisiae* strain used had a great influence on the production of acetaldehyde and higher alcohols in wines. Considering the influence of these studied compounds in the final profile of the vinegars, our results showed that the *S. cerevisiae* strain isolated in this study produced the most suitable wine substrates for the production of vinegars. Moreover, semisolid fruit substrate provides better results than liquid substrate. Inoculated acetification in wood recipients yielded vinegars with a better volatile profile, as these contained higher levels of most compounds except acetaldehyde.

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1. Introduction

Vinegar is one of the most widespread and common products in the world because it is available in every country in several different varieties (Mazza & Murooka, 2009). The traditional use and integration of vinegars in numerous cultures can be traced back to ancient times. Today, the most widely marketed vinegar is wine vinegar, although vinegar can be produced from a variety of very different raw materials.

In today's market, there is a growing demand for fruit vinegar sold as a health food product (Ou & Chang, 2009). This consumer trend has led to the development of new products with the aim of expanding the range of vinegars available on the market. Furthermore, the production of these vinegars provides a use for surpluses of second quality fruit.

Different quality parameters should be studied in selecting the best production procedure for new fruit vinegars. Such parameters should include volatile compounds responsible for aroma and close attention should be paid to which of these compounds might be influenced by the production process.

Aroma is certainly one of the most important determinants of food quality and acceptance. The particular aroma of vinegar is the result of high quantities of volatile compounds. These compounds may come from the raw material or may be formed during the production process. Different authors have pointed out the importance of the production process in the final aroma of vinegars and therefore in their organoleptic qualities (Morales et al., 2001; Callejón et al., 2009). Moreover, the content of several major volatile compounds found in vinegar such as methanol is restricted by Spanish legislation (<1 g/L) (Presidencia del Gobierno, 1993).

Gas chromatography coupled to a mass spectrometry detector is widely used in the study of volatile compounds. To analyse these constituents in a liquid sample, the sample is introduced into a gas chromatograph, the volatile components are evaporated, and their vapour is carried through the column by the mobile phase (Ettre, 2002). However, the non-volatile matrix remains in the injector, thereby contaminating it. Researching volatile components present in a solid sample is even more complicated. This type of sample obviously cannot be introduced into an instrument; it requires an elaborate sample preparation procedure that includes extracting the volatile components, among other steps (Ettre, 2002).

Headspace is a fast, simple, efficient and environmentally friendly sampling method used with capillary GC for the analysis of volatile fractions in many food samples. Headspace (HS) is essentially a sampling method that permits analysts to take an aliquot of the gas phase in equilibrium with a liquid or solid phase (Ettre, 2002). During static HS analysis, equilibrium between the sample and the headspace above is achieved, and a fraction of this headspace gas phase is

* Corresponding author. Tel.: +34 954 556760; fax: +34 954 233765.
E-mail address: mlmorales@us.es (M.L. Morales).

0963-9969/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodres.2010.10.025

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withdrawn for GC analysis (Bylaite & Meyer, 2006). In equilibrium, the distribution of the analytes between the two phases depends on their partition coefficients. The composition of the original sample can therefore be established from the analytical results of this aliquot (Ettre, 2002).

Static HS-GC works well with high precision and accuracy for liquid samples since calibration can be performed easily by either external or standard addition without any serious problems (Li et al., 2009). With static headspace sampling, sample headspace volatiles are automatically brought directly to the GC, thus offering good validation as well as the possibility for a high number of samples to be processed (Sriseadka et al., 2006). The main disadvantage of static HS-GC compared to dynamic HS-GC is its relatively low sensitivity (Snow & Slack, 2002). However, sensitivity can be increased by salting-out, pH control or increasing the equilibration temperature during sample heating (B'Hymer, 2003). Static headspace GC is mostly useful for applications in the high-ppb to percent concentration ranges (Wang et al., 2008). In the headspace analysis, parameters such as temperature and equilibrium time, headspace volume and instrumental conditions must be carefully standardized (Ariseto & Toledo, 2008).

The overall goal of this work was to develop and to optimize a simple and fast method based on GC–MS to monitor the evolution of major volatile compounds in the production process of fruit vinegars. Firstly, to monitor changes in these compounds a sampling method had to be selected that was suitable for all three products studied: raw material (fruit puree), fruit wine and fruit vinegar[1], which all have very different consistencies. We decided to test headspace sampling. Next, we optimized the static headspace sampling and injection conditions. Finally, the method was successfully applied to determine the major volatile compounds in these kinds of matrices.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals used were analytical-reagent grade and provided from the following sources: acetaldehyde, methyl acetate, methanol, ethyl acetate, 1-propanol, isobutanol, isoamyl acetate, 2-methyl-1-butanol, 3-methyl-1-butanol, ethanol, acetic acid and 4-methyl-2-pentanol (IS) from Merck (Darmstadt, Germany); sodium chloride from Sigma–Aldrich (Madrid, Spain); and water from a Milli-Q purification system (Millipore, Bedford, USA).

2.2. Standards and sample preparation

6 g of sample saturated in sodium chloride (2 g) and 10 µL of internal standard (391 µg kg^{-1}) were placed into a 20 mL HS vial and sealed immediately with a white silica/PETF lined septum and aluminium crimp cap (VWR International Eurolab S.L., Barcelona, Spain) and then placed in the autosampler tray for HS sampling.

A standard mix was used to establish the best injection volume. A dearomatised fruit puree spiked with standards was used to select sample incubation temperature and time. Fruit was dearomatised as follows: 5 mL of dichloromethane were added to 20 g of fruit puree. This mixture was stirred with a stir bar over night, and then was centrifuged at 4000 rpm for 10 min and the dichloromethane was withdrawn. This procedure was repeated. To eliminate remains of dichloromethane, the puree was submitted to a nitrogen stream for 20 min. After this, 5 mL of acetone were added and the mixture was stirred for 3 h, followed by centrifugation (4000 rpm for 10 min), the solvent was withdrawn and a nitrogen stream was subsequently applied for 20 min. We spiked a commercial fruit puree and vinegar with the analytes for repeatability, intermediate precision and recovery assays.

2.3. Vinegars production and samples studied

Fruit processing and pre-treatment was performed as follows: fruit was crushed with a beater; 60 mg L^{-1} of sulphur dioxide were added to prevent the growth of undesirable micro-organisms; 15 mg L^{-1} of each of two kinds of pectolytic enzymes (Depectil extra-garde FCE® and Depectil clarification® from Martin Vialatte Oenologie, Epernay, France), were then added to the puree. 50 g L^{-1} and 75 g L^{-1} of sucrose were also added to 2008 and 2009 strawberry puree respectively to ensure an appropriate final acidity in the resulting vinegar. Samples of fruit puree were taken before and after the addition. One portion of 2008 strawberry fruit puree was pressed to study the effect of two types of starting substrates (semisolid and liquid) (Table 1).

The alcoholic fermentation of the fruit substrate was similar in persimmons and 2008 strawberries and slight modifications were made in the case of 2009 strawberries. 6 L of fruit puree was distributed into various glass recipients: six for persimmons, eight for 2008 strawberries (four of purees and two of liquid substrate) and eight for 2009 strawberries. These recipients were then divided into two groups: half of them were inoculated with the oenological yeast *Saccharomyces cerevisiae* QA23 at a concentration of 2×10^6 cells mL^{-1}, and spontaneous alcoholic fermentation was allowed to take place in the other half. The inoculated fermentation in the 2009 strawberries was performed with the yeast strain *S. cerevisiae* RP1, isolated during the spontaneous alcoholic fermentation of the 2008 strawberry puree.

Acetification was carried out in glass vessels by spontaneous processes except for strawberry wines from the 2009 harvest. These wines were acetified in three different containers: a glass vessel, and oak and cherry wood barrels. Each of them was filled with 5.5 L of wine. All the wine obtained from inoculated alcoholic fermentation was mixed and dispensed in the recipients mentioned earlier and inoculated with acetic acid bacteria. The wines from spontaneous alcoholic fermentation were processed in the same way and acetified spontaneously.

All vinegars obtained in 2007 and 2008 were pressed. Additionally two different final treatments were applied to strawberry vinegars from the 2008 harvest: some were centrifuged and others pasteurized. Strawberry vinegars from 2009 were only pasteurized. The 2007 persimmon vinegars presented an average acetic degree between 4.4 (from inoculated wines) and 4.5 (from spontaneous wines). The acetic acid contents average in 2008 strawberry vinegars were 4.8 (from spontaneous wines) and 4.9 (from inoculated wines). Finally, inoculated vinegars from 2009 harvest reached an acetic degree of 5.5 (glass vessel), 6.6 (oak barrel) and 6.3 (cherry barrel).

Furthermore, part of the puree from the 2009 strawberries was concentrated by heating to test another form of increasing the sugar content and prevent having to add it in; the resulting product was a cooked must (Table 1). One liter of this substrate was fermented by a spontaneous process and 1 L by inoculating it with RP1 strain yeast. Finally, the inoculated wines were acetified by adding the selected acetic acid bacteria and the spontaneous wines were left to acetify spontaneously.

Different samples were taken throughout these production processes and a total of 53 samples were analysed: 6 fruit purees and 1 liquid substrate, 22 wines and 24 vinegars. All the samples were stored in 30 mL amber glass flasks at −20 °C until the analysis. The codes and characteristics of the samples are shown in Table 1.

2.4. Optimization of static headspace conditions and method validation

Several headspace conditions were optimized: spit ratio, injection volume, time and temperature of incubation. Different split ratios

[1] Given the acidic nature of these products and the lack of a suitable alternative term, we have decided to refer to these products as vinegars throughout the text, despite the fact that according to Spanish regulations, some of these products are not sufficiently acidic to be classified as vinegars.

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Table 1
Treatment and codex of samples.

Fruit and harvest	Treatment	Puree sample	Treatment	Substrate sample	Alcoholic fermentation	Wine sample	Acetification	Treatment o recipient	Vinegar sample
Persimmon 2007	Crushed	K7Z1	SO2 Pectolytic enzymes	K7Z2	Inoculated	K7WI1–K7WI3	Spontaneous	Pressing Centrifugation	K7VE1–K7VE3
					Spontaneous	K7WE1–K7WE3	Spontaneous	Pressing Centrifugation	K7VI1–K7VI3
Strawberry 2008	Crushed	F8P1	SO2 Pectolytic enzymes sucrose	F8P2	Inoculated	F8WI1–F8WI3	Spontaneous	Centrifugation Pasteurization	F8SVI1C–F8SVI2C F8SVI1P–F8SVI2P
					Spontaneous	F8WE1–F8WE3		Centrifugation Pasteurization	F8SVE1C–F8SVE2C F8SVE1P–F8SVE2P
	–	F8P2	Pressing	F8L	Inoculated Spontaneous	F8LWI F8LWE	–	–	–
Strawberry 2009	Crushed	F9P1	SO2 Pectolytic enzymes sucrose	F9P2	Inoculated	F9WI1–F9WI4	Inoculated	Glass vessel Oak barrel Cherry barrel	F9SVIG F9SVIO F9SVIX
					Spontaneous	F9WE1–F9WE4	Spontaneous	Glass vessel Oak barrel Cherry barrel	F9SVEG F9SVEO F9SVEX
		–	Heating Concentrated	F9MC	Inoculated Spontaneous	– –	Inoculated Spontaneous	Glass vessel Glass vessel	F9MCVI1–F9MCVI2 –

(2, 5, 10, 15, 20 and 40) and injection volumes (250 and 350 μL) were tested.

We studied different incubation times (10, 20, 30 and 40 min) and temperatures (55, 65, 75 and 85 °C). A sample of commercial fruit puree was spiked with all the compounds studied for these trials. The quantities added were roughly 25 mg kg^{-1} except for ethyl acetate, which was 150 mg kg^{-1}.

The method was validated with respect to linearity, sensitivity (LOQ), precision (repeatability and intermediate precision) and accuracy.

The quantification limits were obtained injecting successive dilutions of standards and were calculated as the concentration which would result in a signal-to-noise ratio higher than or equal to 10. These values were determined for liquid and semisolid matrices.

Repeatability and intermediate precision were checked using a dearomatised commercial fruit puree and vinegar spiked with the analytes. These spiked samples were injected six times in a single day for the repeatability assay and three times a day on six different days for the intermediate precision assay. The results, expressed as relative standard deviation (%RSD).

The accuracy of the method was evaluated only in the case of vinegar since the calibration lines were built using hydroacetic solutions instead of a real matrix. A commercial vinegar was spiked with standards at three levels of concentration.

2.5. Static headspace GC–MS instrumentation and conditions

Analyses were conducted using an Agilent 6890 GC system coupled to an Agilent 5975inert quadrupole mass spectrometer and equipped with a Gerstel MP2 headspace autosampler (Müllheim an der Ruhr, Germany).

Static headspace equilibration was performed at 65 °C for 20 min, while a low shaking at 250 rpm was applied during sample heating. 350 μL of headspace gas were injected using a heated (85 °C) gastight syringe (1 mL) in split mode 10:1. The split/splitless inlet temperature was 200 °C. Syringe injection speed was 50 μL s^{-1}.

Separation was performed on a CPWax-57CB column (50 m× 0.25 mm, 0.20 μm film thickness, Varian, Middelburg, The Netherlands). The carrier gas was He at a constant flow rate of 1 mL/min. The column oven temperature was initially set at 35 °C for 5 min, and then was increased to135 °C at 4 °C min^{-1} and then at 10 °C min^{-1} to 200 °C and held for 5 min.

The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 250 °C, respectively. Electron ionization mass spectra in SIM mode were recorded at 70 eV electron energy. A solvent delay of 3.0 min was used and the following ions were monitored: 31, 43, 44, 45, 55, 57, 61 and 74. All data were recorded using an MS ChemStation. The samples were analyzed in triplicate and blank runs were done before and after each analysis.

2.6. Qualitative and quantitative analyses

Compounds were identified based on the comparison of the retention times of individual standard and computer matching with the reference mass spectra from the NIST 98 library. Acquisition was performed in selected ion monitoring mode (SIM). Initially, standard solutions and several samples were analysed in full scan mode (mass range: 29–350 amu). These data were acquired to identify the compounds and determine appropriate ions for the later acquisition in SIM mode.

The quantitative determination of volatile compounds was performed by using the relative area calculated as the ratio between the target ion of each compound and the internal standard (4-methyl-2-pentanol). Calibration curves at seven levels and three replicates per level were built by adding a standard mixture of all compounds in both matrices: a commercial dearomatised fruit puree enriched with ethanol and hydroacetic solution. This procedure was performed in keeping with that described in Mestres et al. (2002) in order to obtain a matrix that was as representative as possible and to ensure that the calibration graphs were applicable to the majority of the real sample. The range of the calibration curves was chosen to cover the possible concentrations in real samples (Tables 2 and 3).

2.7. Statistical analysis

All statistical analyses were performed using Statistica software (StatSoft, 2001). One-way ANOVA was used to evaluate significant differences (significance levels $p<0.05$).

A principal component analysis (PCA) was carried out as an unsupervised method in order to ascertain the degree of differentiation between samples and which compounds were involved. Data were auto-scaled before PCA.

3. Results and discussion

The main aim of this work was to explore the possibility of using the headspace sampling method in major volatile GC–MS analysis. Headspace gas chromatography (HS-GC) is a powerful technique for the analysis of volatile compounds in food and non-food products (Linssen et al., 1995). There are many instrumental parameters of the headspace autosampler that can affect the sensitivity, precision and

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Table 2
Analytical characteristics of the method for vinegar.

Compound	Retention time (min)	m/z	Linear range (mg kg^{-1})	r^2	LOQ (μg kg^{-1})	Added (mg kg^{-1})	Recovery (%)	Mean recovery (%)	Repeatability (%RSD)	Intermediate precision (%RSD)
Acetaldehyde	3.95	44	1–200	0.998	0.30	37	65.1	68.0 ± 3.6	1.88	3.80
						50	67.0			
						62	72.0			
Methyl acetate	5.01	74	2–500	0.998	0.15	15	103.5	102.3 ± 2.0	2.64	4.10
						20	100.0			
						25	103.5			
Ethyl acetate	6.03	61	74–2002	0.9995	0.095	450	82.0	73.7 ± 7.3	3.90	1.60
						600	70.8			
						750	68.3			
Methanol	6.54	31	10–700	0.9992	4.0	150	90.0	88.5 ± 2.5	1.65	2.22
						200	90.0			
						250	85.6			
Propanol	10.8	31	1–75	0.9999	0.24	3.37	90.3	88.8 ± 3.3	2.09	3.00
						4.50	91.0			
						5.62	85.0			
Isobutanol	12.7	43	1–124	0.9998	0.21	9	96.0	102.6 ± 6.9	1.54	1.97
						12	109.7			
						15	102.0			
Isoamyl acetate	13.3	55	0.57–20.5	0.9999	0.015	0.375	84.1	83.5 ± 6.2	4.92	5.20
						0.500	89.4			
						0.625	77.0			
2-Methyl-1-butanol	16.9	57	1–75	1.000	0.11	2.62	102.7	98.1 ± 4.1	0.87	2.52
						3.50	95.0			
						4.37	96.5			
3-Methyl-1-butanol	17.0	55	1–76	0.9993	0.13	10	99.0	108.2 ± 9.0	2.54	3.97
						14	108.7			

accuracy of static headspace analysis. We therefore optimized this sampling technique by evaluating the effect of the following parameters: injection volume, temperature and equilibrium time. The addition of salt into the aqueous extract determined an increment of the ionic strength for the analytes resulting in an increase of their diffusion into the headspace and of the sensitivity (Pawliszyn, 1997). Although the effect of salting-out may play a key role in headspace sampling, taking into account our previous work (Callejón et al., 2008) in which the saturation of samples with salt gave the best results, it was not considered among parameters to optimize and we decided to use an enough amount of sodium chloride to saturate the samples. Good chromatographic data, maximum recovery, sensitivity, and time saving were selected as criteria for optimization. The method was then validated and, finally, applied to the analysis of real samples.

3.1. Optimization of static headspace conditions: the effect of injection volume, equilibrium temperature and time

Among the different split ratios tested, the lowest (2:1 or 5:1) provided poorly defined peaks and the highest resulted in small peaks. The best results were obtained with 350 μL injection volume and a 10:1 split ratio.

After the injection conditions were selected, we studied the incubation parameters. As shown in Fig. 1, we found that the higher the extraction time, the lower all relative areas of chromatographic peaks. However, no significant differences were found among relative areas obtained between 10 and 20 min of extraction. Between 10 and 30 min we found significant differences for isoamyl acetate, and between 10 and 40 min for ethyl acetate and isoamyl acetate. Therefore, we considered 20 min to be an appropriate extraction time. On the other hand, incubation temperature showed different trends depending on the compound (Fig. 2). Relative areas of 1-propanol and 2-methyl-1-butanol clearly increase as temperature rises. However, the values of relative areas for ethyl acetate, isoamyl acetate and acetaldehyde decrease as temperature increases. These decreases begin to be statistically significant for isoamyl acetate when the temperature rises from 65° to 75 °C.

An increase in temperature entailed a loss of sensitivity in some of the compounds studied; because no significant losses were observed at 65 °C, this is the incubation temperature we chose. In summary, the best incubation conditions were established at 20 min at 65 °C.

3.2. Method validation

The method was evaluated with respect to linearity, sensitivity (LOQ), precision (repeatability and intermediate precision) and

Table 3
Analytical characteristics of the method for wine and puree of fruit.

Compound	Linear range (mg kg^{-1})	r^2	LOQ (mg kg^{-1})	Repeatability (%RSD)	Intermediate precision (%RSD)
Acetaldehyde	1–200	0.9986	4.63	4.85	5.75
Methyl acetate	0.9–170	0.9982	2.77	3.12	4.19
Ethyl acetate	61–4500	0.9960	3.1	4.24	4.30
Methanol	51–3000	0.9991	38.1	4.26	5.19
Propanol	1–200	0.9989	2.40	4.96	6.00
Isobutanol	1–200	0.9991	1.54	4.70	6.88
Isoamyl acetate	0.05–10.4	0.9989	0.17	2.86	7.08
2-Methyl-1-butanol	1–200	0.9989	0.27	6.93	8.15
3-Methyl-1-butanol	1–202	0.9967	0.30	0.83	5.73

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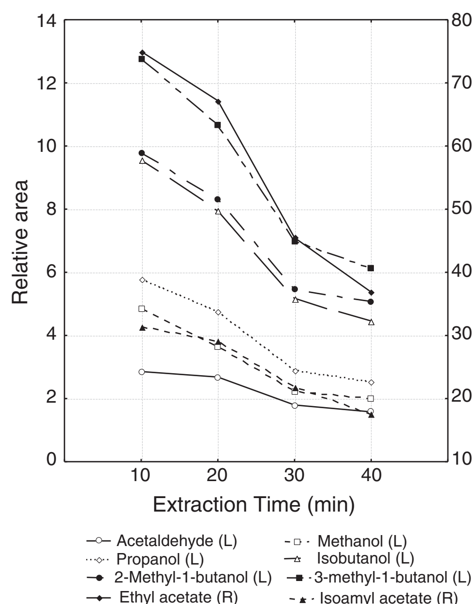

Fig. 1. Optimization of headspace conditions. Effect of incubation time on relative areas of volatiles compounds.

accuracy. The relationship between detector response measured in terms of relative area and amount of standard was linear as suggested by the correlation coefficient obtained (0.996–1.000). The linearity ranges, the equation of linear regression and the correlation coefficient are shown in Tables 2 and 3.

The quantification limits obtained were low enough to quantify the different kinds of samples of this study.

Repeatability and intermediate precision results are in agreement with the values proposed by AOAC (1993) for both kinds of matrices (fruit puree and vinegar).

The recovery percentage obtained in the accuracy assays ranged between 68.0 and 108.2. In general, a good degree of accuracy was achieved for most of the compounds, except for acetaldehyde and ethyl acetate.

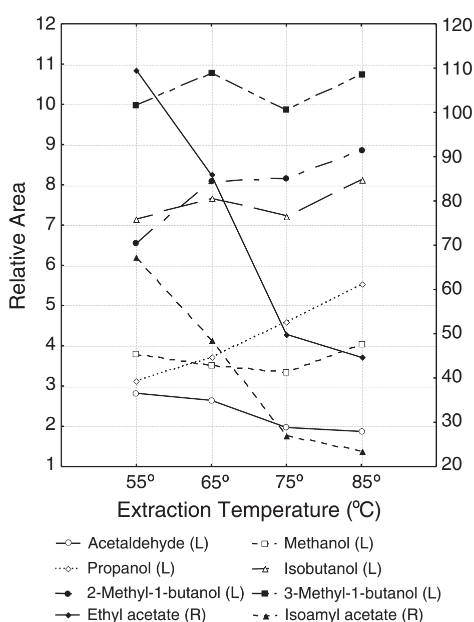

Fig. 2. Optimization of headspace conditions. Effect of incubation temperature on relative areas of volatiles compounds.

3.3. Sample analysis

The optimized method was applied to study the changes in nine major volatile compounds throughout the production process of fruit vinegars. These products were obtained through a double fermentation process (alcoholic and acetic). Different conditions were tested at each stage of production. We will discuss the results considering the effect of each stage on the concentration of these compounds. They are involved directly in the aroma of products because they either provide particular aromatic notes such as ethyl acetate or isoamyl acetate or contribute to the overall aromatic profile. Moreover, some of them are also precursors of other volatile compounds present in vinegars. For example, acetaldehyde undergoes condensation reactions to produce acetoin, a volatile compound characteristic of vinegar. On the other hand, vinegars have a considerable content of volatile acids formed from higher alcohols, especially isovaleric acid from 3-methyl-1-butanol. This alcohol is also a precursor of isoamyl acetate.

3.3.1. Pre-treatments of fruit puree

Methanol was the most abundant compound in the initial fruit puree, especially in the persimmon puree (Tables 4–6). The addition of SO_2 and pectolytic enzymes gave rise to a notable increase in this compound (about 100 mg kg^{-1}) in the strawberry samples. Added pectolytic enzymes act as hydrolysing pectins releasing methoxyl groups and producing an increase in methanol, as Ribéreau-Gayon et al. (2006) described for red wines. The second compound that underwent a considerable change in concentration was acetaldehyde. This aldehyde is a natural aroma component in almost all fruits. This compound appears as a result of fruit metabolism during ripening (Pesis, 2005). In our case, the fruit puree (persimmon and strawberry) presented values between 5.4 and 10.4 mg kg^{-1}. These amounts increased after the addition of SO_2 and pectolytic enzymes, especially in the strawberry samples. In grape must, SO_2 combines with acetaldehyde to form a stable compound (Ribéreau-Gayon et al., 2006). Therefore, the addition of this substance may cause a loss of acetaldehyde. However, we observed an increase, leading us to deduce that pectolytic enzyme may favour the release of acetaldehyde. This effect seems to be stronger than the loss caused by combination with SO_2.

The remaining compounds increased in most cases, the highest changes were found in the strawberry samples except for methyl acetate, which mainly increased in persimmon puree.

One portion of strawberry puree from the 2008 harvest was pressed to obtain a liquid substrate. The pressing process resulted in a decrease in all the compounds (Table 5), especially ethyl acetate and acetaldehyde, which diminished by up to 80%.

3.3.2. Alcoholic fermentation

Two types of alcoholic fermentations were performed. One part of the fruit puree was spontaneously fermented and the other part was inoculated with a selected strain of *S. cerevisiae* yeast.

In general, as can be seen in Tables 4–6, the higher alcohols increased in all cases as expected; in some cases, reaching concentrations close to the lowest values of the content range found in grape wine (Ribéreau-Gayon et al., 2006). During alcoholic fermentation, yeast can synthesize these compounds through two metabolic pathways, one of which is amino acid metabolism (Ribéreau-Gayon et al., 2006; Bayonove et al., 2000). Just as occurs in grape wines, the higher alcohol that reached the largest amounts was 3-methyl-1-butanol (Romano et al., 2003; Garde-Cerdán & Ancín-Azpilicueta, 2007).

If we compare the two kinds of fermentations, the inoculated alcoholic fermentation of persimmon puree produced higher alcohol contents than spontaneous fermentation, except for isobutanol, which reached a similar concentration in both types of fermentations. However, in 2008 strawberry wines produced by spontaneous fermentation

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Table 4
Changes in volatile compounds during the elaboration of persimmon vinegars.

Samples	Mean concentration of compounds (mg kg^{-1}) ± SD								
	Acetaldehyde	Methyl acetate	Ethyl acetate	Methanol	1-Propanol	Isobutanol	Isoamyl acetate	2-Methyl-1-butanol	3-Methyl-1-butanol
K7Z1	10.4 ± 0.3	9.5 ± 0.9	n.q.	343 ± 9	2.27 ± 0.01	1.340 ± 0.003	0.1177 ± 0.0004	0.140 ± 0.004	n.q.
K7Z2	28.2 ± 1.9[a]	18.1 ± 1.7[a]	n.q.	376 ± 39	2.97 ± 0.08[a]	1.99 ± 0.05[a]	0.140 ± 0.004	0.31 ± 0.02[a]	n.q.
K7WE1	32.1 ± 1.9	36.1 ± 1.3[b]	1221 ± 45[b]	551 ± 17[b]	8.9 ± 0.6[b]	15.8 ± 1.3[b]	0.94 ± 0.03[b]	7.69 ± 0.25[b]	27.98 ± 1.04[b]
K7WE2	25.1 ± 3.2	34 ± 3[b]	1046 ± 107[b]	554 ± 41[b]	8.5 ± 0.5[b]	15.6 ± 1.3[b]	0.82 ± 0.06[b]	8.5 ± 0.4[b]	33 ± 3[b]
K7WE3	30.8 ± 1.9	42.2 ± 0.7[b]	1459 ± 17[b]	758 ± 18[b]	11.10 ± 0.05[b]	20.5 ± 0.5[b]	1.33 ± 0.08[b]	8 ± 1[b]	38 ± 4[b]
K7WI1	39.2 ± 0.7[b,c]	38.8 ± 0.9	1094 ± 58[b]	581 ± 40[b]	14.8 ± 1.2[b,c]	15.3 ± 0.9[b]	1.31 ± 0.15[b]	10.466 ± 0.024[b,c]	40.6 ± 0.5[b,c]
K7WI2	40.47 ± 0.14[b,c]	67 ± 5	1942 ± 90[b]	695 ± 6[b]	15.46 ± 0.15[b,c]	16.67 ± 0.03[b]	2.87 ± 0.19[b]	10.93 ± 0.03[b,c]	42.1 ± 0.3[b,c]
K7WI3	36.8 ± 1.6[b,c]	47.8 ± 0.9	1354 ± 140[b]	539 ± 74[b]	16 ± 2[b,c]	16.3 ± 1.7[b]	1.86 ± 0.07[b]	9.3 ± 0.9[b,c]	41 ± 3[b,c]
K7VE1	37 ± 3	103 ± 7[b]	1447 ± 152[b]	471 ± 42	3.07 ± 0.07[b]	7.01 ± 0.03[b]	1.25 ± 0.16	5.19 ± 0.11[b]	16.0 ± 0.6[b]
K7VE2	32.81 ± 0.19	79.89 ± 0.17[b]	1203 ± 24[b]	444 ± 31	3.42 ± 0.14[b]	7.59 ± 0.15[b]	0.89 ± 0.04	5.3 ± 0.4[b]	17.9 ± 0.3[b]
K7VE3	47 ± 3	86 ± 6[b]	1278 ± 100[b]	464 ± 12	3.37 ± 0.07[b]	8.17 ± 0.13[b]	0.90 ± 0.07	5.91 ± 0.16[b]	17.5 ± 0.3[b]
K7VI1	61 ± 4	86.10 ± 5.03[b]	1094 ± 59[d]	374 ± 19[b,d]	4.8 ± 0.1[b,d]	5.83 ± 0.04[b,d]	0.9980 ± 0.0001	4.9 ± 0.4[b]	17.55 ± 0.24[b]
K7VI2	33.4 ± 2.1	67 ± 4[b]	921 ± 70[d]	326 ± 22[b,d]	4.47 ± 0.15[b,d]	5.38 ± 0.13[b,d]	0.89 ± 0.03	5.14 ± 0.07[b]	17.1 ± 0.3[b]
K7VI3	38.1 ± 2.4	87 ± 6[b]	1024 ± 84[d]	385 ± 8[b,d]	4.169 ± 0.002[b,d]	5.11 ± 0.12[b,d]	0.95 ± 0.12	4.6 ± 0.1[b]	15.3 ± 0.3[b]

n.q.: concentration under quantification limit.
[a] Significant differences ($p<0.05$) with respect to the initial fruit puree (ANOVA).
[b] Significant differences ($p<0.05$) with respect to its substrate (ANOVA).
[c] Significant differences ($p<0.05$) with respect to spontaneous process (ANOVA).
[d] Significant differences ($p<0.05$) with respect to the vinegars obtained from spontaneous wines (ANOVA).

were richer in isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol than inoculated wines, with the latter containing higher levels of 1-propanol than those produced with spontaneous fermentation. Persimmon and strawberry purees were inoculated with the same yeast strain, but the only common trend found was the production of 1-propanol in greater proportion than any other higher alcohol. This alcohol is synthesized by yeast in relation to the metabolism of amino acid sulphur (Bayonove et al., 2000). Otherwise, the observed increases in 2-methyl-1-butanol and 3-methyl-butanol in the inoculated processes were similar in both substrates. These results suggest that the production of 1-propanol could be further conditioned by the type of substrate and the production of the other two alcohols by the yeast strain. Ibarz et al. (2005), pointed out that the production of higher alcohols in grape wines depends on both factors: the yeast and must used.

Interestingly, the results of the 2009 wines showed opposite changes in higher alcohols to those observed in 2008 wines, being these changes for the inoculated 2009 wines similar to the 2008 spontaneous wines and vice versa (Tables 5 and 6). As explained in Section 2.3, the yeast strain used in the production of 2009 inoculated strawberry wines was isolated from 2008 spontaneous wines. Therefore, the strain involved in the fermentation process has a strong influence on the end levels of these compounds in wines (Torrea et al., 2003; Ribéreau-Gayon et al., 2006).

Methanol levels increased in persimmon and 2008 strawberry during alcoholic fermentation, although these differences were only statistically significant in the case of persimmon. Methanol is a non-fermentative alcohol; therefore, the only source of this compound during alcohol fermentation is the hydrolysis of pectins. In these reactions, ester bonds between galacturonic acid and methanol are

Table 5
Changes in volatile compounds during the elaboration of strawberry vinegars in harvest 2008.

Samples	Mean concentration of compounds (mg kg^{-1}) ± SD								
	Acetaldehyde	Methyl acetate	Ethyl acetate	Methanol	1-Propanol	Isobutanol	Isoamyl acetate	2-Methyl-1-butanol	3-Methyl-1-butanol
F8P1	9.9 ± 0.5	6.9 ± 0.6	96 ± 10	190 ± 14	4.46 ± 0.11	11.6 ± 0.5	0.249 ± 0.004	3.4 ± 0.1	15.5 ± 0.9
F8P2	52.9 ± 2.2[a]	9.30 ± 0.11[a]	140 ± 7[a]	292. ± 9[a]	5.97 ± 0.14[a]	16.9 ± 0.7[a]	0.360 ± 0.007[a]	5.3 ± 0.4[a]	22.5 ± 1.2[a]
F8L	7.2 ± 0.3[d]	1.93 ± 0.08[d]	53 ± 2[d]	244 ± 7[d]	3.51 ± 0.09[d]	10.3 ± 0.2[d]	0.107 ± 0.015[d]	3.7 ± 0.4[d]	12 ± 9[e]
F8LWE1	9.2 ± 0.6	2.1 ± 0.4[e]	59 ± 9[e]	489 ± 33	49.9 ± 2.1	65.0 ± 2.4[e]	0.96 ± 0.07[e]	16 ± 2[e]	80.9 ± 1.8[e]
F8LWI1	27.4 ± 2.4[c,e]	2.9 ± 0.4[e]	86 ± 6[c,e]	530 ± 14[e]	90.3 ± 0.4[c]	40.44 ± 0.08[c,e]	0.678 ± 0.024[c,e]	11.0 ± 0.3[c]	72.0 ± 1.1[c,e]
F8WE1	21.8 ± 0.9[b]	9.2 ± 0.7	170 ± 11	462 ± 9	37 ± 2[b]	83 ± 3[b]	0.95 ± 0.06[b]	34.0 ± 0.3[b]	108.3 ± 0.6[b]
F8WE2	19.0 ± 0.6[b]	7.62 ± 0.22	127 ± 5	290.1 ± 2.3	24.9 ± 0.9[b]	61.2 ± 2.3[b]	1.14 ± 0.08[b]	24.3 ± 1.3[b]	80.1 ± 1.9[b]
F8WE3	19.8 ± 1.4[b]	8.1 ± 0.4	129 ± 11	303 ± 22	26.0 ± 1.7[b]	69 ± 7[b]	0.969 ± 0.021[b]	26.2 ± 1.9[b]	92 ± 7[b]
F8WI1	51 ± 5[c]	8.47 ± 0.09	173 ± 6[b]	305 ± 3[b]	43.9 ± 0.8[b,c]	33.9 ± 0.4[b,c]	1.382 ± 0.007[b,c]	14.4 ± 0.3[b,c]	62 ± 5[b,c]
F8WI2	46 ± 4[c]	8.9 ± 0.5	184 ± 11[b]	317 ± 16[b]	44.8 ± 1.7[b,c]	35 ± 1[b,c]	1.50 ± 0.12[b,c]	12.1 ± 0.7[b,c]	64 ± 3[b,c]
F8WI3	52.5 ± 1.4[c]	9.07 ± 0.25	207 ± 17[b]	327 ± 28[b]	45 ± 4[b,c]	34.3 ± 2.4[b,c]	1.52 ± 0.15[b,c]	12.3 ± 1.6[b,c]	58.0 ± 1.9[b,c]
F8SVE1C	34.3 ± 0.4[b]	17.7 ± 0.5[b]	439 ± 31[b]	259 ± 13	4.40 ± 0.09[b]	11.93 ± 0.04[b]	0.610 ± 0.023[b]	5.74 ± 0.21[b]	13.7 ± 0.4[b]
F8SVE1P	40 ± 4[b]	19.4 ± 1.9[b]	483 ± 47[b]	246 ± 33	4.3 ± 0.3[b]	11.7 ± 0.5[b]	0.61 ± 0.08[b]	5.1 ± 0.3[b]	13.7 ± 0.3[b]
F8SVE2C	75.9 ± 3.0[b]	13.00 ± 0.11[b]	368 ± 15[b]	195 ± 12	4.16 ± 0.23[b]	11.6 ± 0.6[b]	0.46 ± 0.07[b]	5.47 ± 0.16[b]	14.2 ± 0.6[b]
F8SVE2P	79 ± 4[b]	14.2 ± 0.9[b]	374 ± 34[b]	181 ± 14	4.05 ± 0.13[b]	11.4 ± 0.3[b]	0.31 ± 0.03[b]	5.4 ± 0.3[b]	14.4 ± 0.3[b]
F8SVI1C	67.9 ± 2.3[b]	14.0 ± 0.5[b]	374 ± 11[b]	174 ± 10[b]	6.78 ± 0.24[b,c]	5.32 ± 0.22[b,c]	0.271 ± 0.003[b,c]	3.0 ± 0.4[b,c]	9.7 ± 0.4[b,c]
F8SVI1P	77.4 ± 2.3[b]	16.5 ± 0.9[b]	446 ± 24[b]	180 ± 5[b]	7.3 ± 0.4[b,c]	5.8 ± 0.3[b,c]	0.251 ± 0.013[b,c]	2.98 ± 0.19[b,c]	10.5 ± 0.8[b,c]
F8SVI2C	89.2 ± 1.6[b]	15.50 ± 0.19[b]	424.11 ± 2.23[b]	179 ± 5[b]	7.72 ± 0.25[b,c]	5.87 ± 0.19[b,c]	0.260 ± 0.016[b,c]	2.93 ± 0.03[b,c]	10.85 ± 0.15[b,c]
F8SVI2P	98 ± 4[b]	18.2 ± 0.8[b]	498 ± 34[b]	181 ± 15[b]	8.24 ± 0.13[b,c]	6.3 ± 0.3[b,c]	0.248 ± 0.014[b,c]	2.9 ± 0.3[b,c]	11.6 ± 0.3[b,c]

[a] Significant differences ($p<0.05$) with respect to the initial fruit puree (ANOVA).
[b] Significant differences ($p<0.05$) with respect to its substrate (ANOVA).
[c] Significant differences ($p<0.05$) with respect to spontaneous process (ANOVA).
[d] Significant differences ($p<0.05$) with respect to F8P2 sample (ANOVA).
[e] Significant differences ($p<0.05$) with respect to semisolid wines obtained with similar alcoholic process (spontaneous or inoculated) (ANOVA).

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Table 6
Changes in volatile compounds during the elaboration of strawberry vinegars in harvest 2009.

Samples	Mean concentration of compounds (mg kg^{-1}) ± SD								
	Acetaldehyde	Methyl acetate	Ethyl acetate	Methanol	1-Propanol	Isobutanol	Isoamyl acetate	2-Methyl-1-butanol	3-Methyl-1-butanol
F9P1	5.4 ± 0.1	3.21 ± 0.05	n.q.	159 ± 12	2.34 ± 0.02	1.371 ± 0.011	0.118 ± 0.001	0.211 ± 0.002	n.q.
F9P2	95 ± 8^{a}	4.6 ± 0.4^{a}	n.q.	293 ± 31^{a}	3.47 ± 0.12^{a}	2.60 ± 0.08^{a}	0.133 ± 0.001	1.37 ± 0.07^{a}	3.9 ± 0.3^{a}
F9WE1	65.1 ± 0.7^{b}	11.5 ± 0.3^{b}	639 ± 11^{b}	237 ± 10^{b}	14.4 ± 0.5^{b}	25.0 ± 0.7^{b}	2.94 ± 0.04^{b}	16.5 ± 0.5^{b}	48.8 ± 0.9^{b}
F9WE2	55.1 ± 0.6^{b}	12.4 ± 0.1^{b}	761 ± 8^{b}	254 ± 11^{b}	15.0 ± 0.5^{b}	26.0 ± 0.8^{b}	2.85 ± 0.04^{b}	13.4 ± 0.4^{b}	48.6 ± 1.3^{b}
F9WE3	44 ± 1^{b}	11.3 ± 0.4^{b}	667 ± 31^{b}	239 ± 8^{b}	14.4 ± 0.4^{b}	25.0 ± 0.4^{b}	2.66 ± 0.18^{b}	15.19 ± 0.11^{b}	47.6 ± 0.7^{b}
F9WE4	49 ± 4^{b}	11.1 ± 0.7^{b}	633 ± 47^{b}	222 ± 3^{b}	13.6 ± 0.3^{b}	23.9 ± 0.3^{b}	2.55 ± 0.11^{b}	12.69 ± 0.15^{b}	46.0 ± 0.5^{b}
F9WI1	23.6 ± 1.3^{b,c}	4.72 ± 0.07^{c}	n.q.	303 ± 4	12.81 ± 0.22^{b,c}	69.7 ± 0.5^{b,c}	2.64 ± 0.06^{b}	52.7 ± 1.3^{b,c}	171 ± 7^{b,c}
F9WI2	25.1 ± 1.9^{b,c}	4.45 ± 0.15^{c}	n.q.	279 ± 16	12.05 ± 0.22^{b,c}	67.6 ± 1.1^{b,c}	2.60 ± 0.17^{b}	42.4 ± 0.8^{b,c}	167 ± 10^{b,c}
F9WI3	23.2 ± 1.3^{b,c}	4.02 ± 0.12^{c}	n.q.	235 ± 5	11.1 ± 0.1^{b,c}	59.4 ± 0.7^{b,c}	1.98 ± 0.06^{b}	39 ± 3^{b,c}	152 ± 5^{b,c}
F9WI4	20.0 ± 0.6^{b,c}	4.52 ± 0.03^{c}	n.q.	277 ± 12	11.9 ± 0.5^{b,c}	67.2 ± 2.4^{b,c}	2.72 ± 0.08^{b}	44 ± 3^{b,c}	173 ± 11^{b,c}
F9SVEG	1.43 ± 0.07	7.0 ± 0.5	45 ± 5	120 ± 1	0.71 ± 0.01	1.569 ± 0.022	n.q.	2.111 ± 0.003	2.739 ± 0.004
F9SVEO	23.6 ± 0.6	16.2 ± 0.5	148 ± 5	165.6 ± 0.4	1.16 ± 0.01	3.036 ± 0.012	0.065 ± 0.007	2.914 ± 0.008	5.64 ± 0.07
F9SVEX	63.15 ± 0.11	14.22 ± 0.02	439 ± 17	198.2 ± 1.1	2.001 ± 0.003	5.176 ± 0.014	0.158 ± 0.014	4.67 ± 0.07	9.5 ± 0.3
F9SVIG	129 ± 5	3.4 ± 0.3	83 ± 5	146.7 ± 0.9	1.493 ± 0.024	11.5 ± 0.3	0.27 ± 0.04	8.81 ± 0.22	27.0 ± 0.7
F9SVIO	42 ± 3	20.4 ± 1.4	682 ± 41	276 ± 5	2.364 ± 0.012	24.7 ± 0.9	1.4 ± 0.1	21.2 ± 0.6	47.5 ± 0.06
F9SVIX	64.4 ± 1.0	17.3 ± 1.1	663 ± 5	278 ± 16	2.82 ± 0.09	26.2 ± 0.8	1.282 ± 0.023	23.3 ± 1.0	52.1 ± 0.4
F9MCVI1	719 ± 58	22.8 ± 2.1	341 ± 17	318 ± 15	11.3 ± 0.4	9.9 ± 0.6	0.57 ± 0.03	9.1 ± 0.6	43 ± 3
F9MCVI2	410 ± 17	25.4 ± 2.1	452 ± 39	370 ± 27	15.1 ± 0.8	11.3 ± 0.4	0.65 ± 0.05	11.3 ± 1.0	48.9 ± 1.9

n.q.: concentration under quantification limits.
[a] Significant differences ($p < 0.05$) with respect to the initial fruit puree (ANOVA).
[b] Significant differences ($p < 0.05$) with respect to its substrate (ANOVA).
[c] Significant differences ($p < 0.05$) with respect to spontaneous process (ANOVA).

cleaved, releasing this alcohol into the medium, which is carried out by pectin esterases (Fernandez-Gonzalez et al., 2005). Several authors have shown that some *S. cerevisiae* strains have pectin-esterase activity (Pretorius & Van der Westhuizen, 1991; Gainvors et al., 1994; Fernandez-Gonzalez et al., 2005). Thus, the increase in methanol in this fermentative stage may have come from two possible hydrolytic pathways: due to the pectin-esterase activity of the yeast and/or to the pectolytic enzymes added to the substrate that continued to act.

Acetaldehyde is a secondary product of yeast alcoholic fermentation; it is produced during the first days of fermentation (Bosso & Guaita, 2008). This aldehyde increased in persimmon case, being slightly higher in inoculated fermentations than in spontaneous fermentations, although the changes were not statistically significant. Meanwhile, in strawberry alcoholic fermentation acetaldehyde values decreased, especially in spontaneous fermentation. Strawberries are rich in anthocyanins, which are responsible for the berry's red colour. In the production of red wines, these compounds undergo condensation reactions in which different molecules are linked by acetaldehyde bridges (Bosso & Guaita, 2008). These reactions involve a loss of this aldehyde. These types of reactions could explain the diminution of acetaldehyde in strawberry wine production. Opposing trends were found in terms of the final amount of this compound in strawberry wines depending on the year of harvest. In 2008, strawberry wines from inoculated fermentation, “inoculated wines,” were found to have higher values than “spontaneous wines.” However in 2009 strawberry wines, the highest results for acetaldehyde were found in spontaneous wines. As mentioned earlier, the yeast strain employed for the production of 2009 inoculated wines was the same as that used for 2008 spontaneous wines. Furthermore, these 2008 spontaneous wines and 2009 inoculated wines presented similar values for this compound. The influence of the *S. cerevisiae* strain on the differing production of acetaldehyde has been reported by several authors (Antonelli et al., 1999; Regodon et al., 2006).

Among the esters studied, the most abundant in our fruit wines was ethyl acetate followed by methyl acetate and isoamyl acetate, this last related to a fruity aroma.

Ethyl acetate is the most prevalent ester in grape wines (Ribéreau-Gayon et al., 2006). In persimmon puree, the concentration of this ester was below the quantification limit; however the wines presented extremely high levels compared to the normal values in grape wines (30–110 mg kg^{-1}, Regodon et al., 2006). In 2008 strawberry, ethyl acetate underwent a slight increase only during alcoholic fermentation in the inoculated wines. Although the starting concentrations in 2009 wines were very low (below the quantification limit), the wines obtained through spontaneous fermentation presented high concentrations (633–761 mg kg^{-1}) while in those obtained through inoculated fermentation this compound was not detected. Several authors have shown that the formation of esters during alcoholic fermentation is closely related to the enzymatic activity of the yeast strain (Barre et al., 2000). In keeping with this, we observed that this compound was not produced in the 2009 inoculated process and it was only produced in one case in 2008 spontaneous wines (Tables 5 and 6). The ester isoamyl acetate increased in all cases studied.

Methyl acetate is formed by the condensation of methanol and acetic acid. We found that during the alcoholic fermentation of persimmon the amount of this ester doubled. This is consistent with the high levels of methanol found in persimmon substrate.

This compound remained practically unchanged in strawberry wine production except in the case of the 2009 spontaneous process, in which the levels of methyl acetate concentration increased. Finally, all compounds were found to have increased in the alcoholic fermentation of strawberry liquid substrate. Figuring among the most outstanding changes, we might mention a considerable increase (up to 70%) in acetaldehyde, higher alcohols and isoamyl acetate. The liquid substrate was fermented in the absence of solid colorants so the binding reaction between acetaldehyde and monomeric anthocyanins did not frequently occur. This is a likely explanation for why levels of this aldehyde were found to increase in wines from this substrate. Furthermore, the largest increase in acetaldehyde occurred in inoculated alcoholic fermentation. We observed the same behaviour for higher alcohols as in the fermentation of semisolid substrate, showing the highest contents of 1-propanol in inoculated wines and the other three higher alcohols in spontaneous wines. These results again indicate the relevance of the yeast strain in the production of higher alcohols.

Comparing the final content of the volatile compounds analysed in wines from different substrates (liquid and semisolid), it is clear that methanol and 1-propanol reached higher values in liquid wines than in wines from semisolid substrate. Wines from liquid resulted in

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lower values of methyl and ethyl acetate than wines from the other type of substrate.

3.3.3. Acetic fermentation

In the acetic fermentation of persimmon wine, levels of acetaldehyde increased in most cases. In 2008 strawberry vinegar, concentrations of this compound increased in all cases. The transformation of ethanol to acetic acid takes place in two steps, with acetaldehyde being the intermediary product. These reactions can be performed by acetic acid bacteria as well as by chemical oxidation. When performed by a micro-organism, each step is catalyzed by different enzymes (alcohol deshidrogenase and aldehyde deshydrogenase, respectively). In chemical oxidation, the step from acetaldehyde to acetic acid depends on the presence of oxygen (Ribéreau-Gayon et al., 2006).

The acetification process in samples from the 2009 harvest was carried out in different containers (glass vessels, cherry and oak wood barrels). In the vinegar from glass vessels, we noticed a remarkable amount of acetaldehyde together with lower levels of ethanol and acetic acid than in vinegar produced in wood barrels. The main difference between these kinds of recipients is the better oxygen transference that occurs through wood pores. This might suggest that ethanol is being transformed into acetaldehyde while the second reaction is not taking place at a similar rate, probably due to the lower proportion of oxygen in the glass vessel. This result coincides with that reported by other authors on the accumulation of this aldehyde during acetification due to oxygen impoverishment (Polo & Sanchez-Luengo, 1991). Acetaldehyde tends to accumulate under low oxygen conditions instead of being oxidized to acetic acid (Zoecklein et al., 1995). Furthermore, we have observed increases in acetaldehyde in previous studies during glass bottle aging of red vinegars in which acetification and aging processes took place simultaneously (Callejón et al., 2010). And during accelerated aging in glass vessels with wood chips we observed an increase in acetaldehyde due to the chemical oxidation of ethanol (Tesfaye et al., 2004). Although these studies prove that the accumulation of acetaldehyde in vinegars can take place by means of the two pathways mentioned earlier (microbiological or chemical oxidation), in our case, microbiological transformation is the most likely cause of the accumulation of this compound.

The samples from cherry wood barrels had higher concentrations of acetaldehyde than those from oak barrels, regardless of the type of acetification. This compound may be released into the liquid medium from this type of wood, as this phenomenon has been observed in white wine vinegars aged in different kinds of wood (oak, cherry, chestnut and acacia) (Callejón et al., 2010).

A loss of higher alcohols occurred during the acetification stage. Callejón et al. (2009) showed that acetic acid bacteria consume other alcohols apart from ethanol, with 3-methyl-1-butanol being the most frequently consumed followed by isobutanol and 2-methyl-1-butanol, in keeping with the abundance order in the substrate. In our case, a similar behaviour was observed, and in agreement with these authors, the pattern of higher alcohols consumption varied depending on the abundance of these alcohols in the starting wines. In other words, the higher the concentration of the alcohol, the more it was consumed.

The 2009 strawberry wines were divided into two groups: one underwent spontaneous fermentation and the other was inoculated with acetic acid bacteria. In the inoculated processes the vinegars reached 6°Ac while spontaneous processes they only reached 4°Ac as a consequence of the unexpected halt of the acetification process. Therefore, in terms of the changes in higher alcohols, the consumption of these compounds was more pronounced in vinegars produced using selected acetic acid bacteria.

Although the consumption of methanol by acetic acid bacteria has not been previously reported, the acetification process implied a decrease in this alcohol. Generally, these micro-organisms have a defence mechanism that transforms alcohols into less toxic products such esters. Persimmon vinegars showed a reduction in the concentration, with about 150 mg kg^{-1}, and a similar diminution was observed for 2008 strawberry samples. In the 2009 acetification processes, spontaneous fermentation produced a larger decrease in methanol than did inoculated fermentation and this difference was more pronounced in samples produced in glass vessels. The concentration of methanol in all final products was below the legal level allowed for vinegars (Presidencia del Gobierno, 1993).

On the other hand, methanol is involved in the synthesis of methyl esters, in this case, especially of methyl acetate. We observed higher levels of methyl acetate in persimmon vinegars, and as in alcoholic fermentation, during the production process the content of this ester doubled. In 2008 strawberry samples, acetic fermentation produced significant increases in this compound. However, these condensation reactions alone are not sufficient to explain the diminution of methanol mentioned earlier.

In samples from the 2009 harvest, both strawberry vinegars produced in glass vessels experienced a similar decrease in methyl acetate. However, an increase in methyl acetate was found in the vinegar produced in wood barrels, with slightly higher levels recorded in the case of oak barrels, which may be due to concentration phenomena. Furthermore, we might point out a considerable increase in inoculated processes in barrels. In general, despite the different evolutions observed, the final concentrations of methyl acetate in vinegars were correlated with initial concentrations of methanol ($r = 0.7$).

Different trends were found in levels of ethyl acetate, a characteristic compound of vinegar, which were especially conditioned by the fruit substrate used. In persimmon, the concentrations of this ester in the resulting vinegars were similar to those in wines and no clear tendency was observed (Table 4). In 2008 strawberry vinegars, ethyl acetate reached more than twice the concentration of that in wines. From the 2009 harvest, the vinegars obtained through inoculated acetification showed values between 83 for glass vessels and 663–682 for the others. This indicates a considerable formation along with a slight concentration of this compound in wood recipients. The results of the spontaneous acetifications in the 2009 samples were the opposite because a hydrolysis of ethyl acetate was taking place. This behaviour has been observed by several authors who have shown that the active consumption of ethanol by acetic acid bacteria induces the hydrolysis of most ethyl esters (Callejón et al., 2009).

Isoamyl acetate usually increases during surface acetification processes, however, in our vinegars in most cases it was found to diminish. This might be explained again by a hydrolysis reaction due to the consumption of alcohol 3-methyl-1-butanol by acetic acid bacteria.

Comparing the two final treatments applied to the 2008 strawberry vinegars, pasteurization and centrifugation, no statistically significant differences in the volatile compounds studied between them were found (Table 5).

Special vinegars were also produced for this study which used cooked strawberry must (Table 6). Only inoculated acetifications we obtained final products. The main difference in these heated strawberry vinegars was the high levels of acetaldehyde compared to vinegars obtained from uncooked strawberry fruit puree. These high levels would adversely affect the organoleptic properties of the end product.

3.4. Principal component analysis

The compounds studied underwent a series of changes during the production of the vinegars. Several principal component analyses were performed to evaluate whether these changes were great enough to distinguish the different samples obtained throughout the production process based on substrate, production stage or production method. In the case of persimmons, the PCA allowed us to

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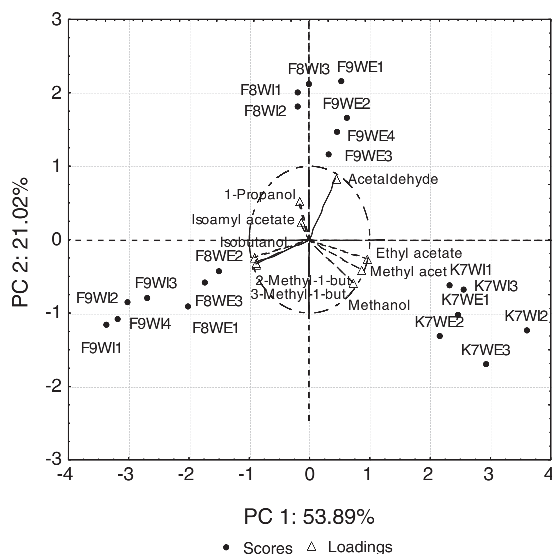

Fig. 3. Data scores and variable loadings plot on the plan made up of the first two principal components (PC1 against PC2) of wine samples.

separate the samples into three groups: the substrate, wines and vinegars, with the first three components accounting for 93.9% of the variance. Similar results were obtained when the PCA was applied to the 2008 strawberry sample data. However, in the products obtained from the 2009 harvest the separation was not so clear.

Moreover, this analysis was applied to the data of the strawberry puree substrates to study the influence of the addition of enzymes and SO_2. Each sample appears in a different quadrant in the plan of the two principal components. The PC1 is able to separate the substrates depending on the harvest and the PC2 separates the samples with and without treatment.

PCA of strawberry wines from 2008 harvest reveals that substrate pressing affects more than the inoculation. This is deducted from the samples separation into the plan of two first PC. The liquid wines inoculated and spontaneous appear in the same quadrant whist the group of wines from inoculated semisolid substrate are separated in different quadrant from the spontaneous group.

On the other hand, the result of this analysis on the data obtained from all the wine samples showed that the principal three components explained 92.6% of the variance. Data scores and variable loadings are plotted simultaneously into the plan made up of the first two principal components in Fig. 3. This figure shows that the samples are distributed into three groups. The figure shows that PC2 successfully separates the 2008 strawberry spontaneous and 2009 inoculated wines from the other strawberry wines. Thus, the wines obtained through the use of the same yeast strain appear together in the same quadrant. This reinforces the theory that the yeast strain has a strong influence on these compounds of the aromatic profile. We confirmed a high degree of association between strawberry wines inoculated with the RP1 strain and the production of higher alcohols such as 2-methyl and 3-methyl-1-butanol and isobutanol. Moreover, if we consider only the persimmon and 2008 strawberry wines, the PCA revealed that PC1 allows us differentiates between persimmon wines and strawberry wines and PC2 distinguishes between inoculated and spontaneous wines. PC1 was positively correlated with acetaldehyde, the three acetates and methanol, and PC2 was positively correlated with acetaldehyde, isoamyl acetate and propanol. In the analysis of the final vinegars, the score plot obtained by selecting the first two PCs as axes showed that the samples were distributed in three groups, one formed by persimmon vinegars, another which included 2008 strawberry vinegars and 2009 strawberry vinegars produced in a glass vessel, and a third group, very far from the previous ones, comprised of the 2009 strawberry vinegars produced in barrels. This shows the importance of the type of recipient in which the acetification is carried out on the final content of these compounds.

4. Conclusions

The headspace sampling method proposed has proved to be a valuable methodology for the determination of major volatile compounds during the production process of fruit vinegars. From a practical point of view, this method does not require any complicated sample preparation. The validation of the method was satisfactory, recovery values and limits detection are acceptable for most of the compounds studied, and the method was successfully applied to real samples.

The addition of SO_2 and pectolytic enzymes produced a considerable increase in methanol and acetaldehyde, especially in the strawberry samples. However, pressing led to a loss of these volatile compounds. In alcoholic fermentation, the *S. cerevisiae* strain used had a great influence on the production of acetaldehyde and higher alcohols in wines. Taking into account the influence of these compounds studied in the final profile of vinegar, the results show that the *S. cerevisiae* strain isolated in this study produces the most suitable wine substrates for the production of vinegars. Moreover, the use of semisolid fruit substrate provides better results than the use of a liquid substrate.

In terms of acetic fermentation, inoculated acetifications in wood recipients resulted in vinegars with better volatile profiles as these presented higher levels of most compounds except acetaldehyde.

Acknowledgments

This research was made possible through the financial support from the Spanish Government by means of a predoctoral grant and the research project AGL2007-66417-C02-01 funded by the Ministry of Science and Innovation. Moreover, the researchers are grateful to the enterprises Hudisa S.A., Agromedina and Grupo Alconeras for providing the fruits used in the vinegar production.

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Special Issue Paper

Flavour and Fragrance Journal

Received: 27 December 2011; Revised: 16 April 2012; Accepted: 28 April 2012; Published online in Wiley Online Library: 24 May 2012

(wileyonlinelibrary.com) DOI 10.1002/ffj.3103

Characterization of odour active compounds in strawberry vinegars†

C. Ubeda,[a] R. M. Callejón,[a] A. M. Troncoso,[a] J. M. Moreno-Rojas,[b] F. Peña[b] and M. L. Morales[a]*

ABSTRACT: Odour-active compounds in strawberry vinegars were determined by gas chromatography equipped with an olfactometer using the modified frequency (MF) technique. The initial strawberry substrate was also analysed showing that ethyl 2-methylbutyrate, mesifurane, β-damascenone, furaneol and γ-decalactone were preserved during the double fermentation process, presenting high MF values. The final aromatic profile of strawberry vinegars is formed both by compounds from the substrate and by those formed during alcoholic and acetous fermentation. Due to their high MF, a total of 12 odour zones, identified as acetic, butyric and isovaleric acids, methional, 3-nonen-2-one, 2-phenylethanol, pantolactone + furaneol, *p*-vinylguaiacol, sotolon, phenylacetic acid and vanillin, were considered as possible impact odorants of strawberry vinegars. Finally, all potential impact odorants with similar sensory descriptors were grouped into eight categories, these being: fruity, sweet, grassy, spicy, butter–lactic–cheesy, chemical, empyreumatic and miscellaneous. According to the MF percentage of these categories, grassy, fruity, sweet and spicy aroma seem to have the highest influence on the overall impression of strawberry vinegars. **Copyright © 2012 John Wiley & Sons, Ltd.**

Keywords: impact odorant; olfactometry; vinegar; strawberry; modified frequency

Introduction

Because of its worldwide availability and great array of varieties, vinegar is one of the world's most widespread and common products. In Spain, vinegar is mainly produced by the double fermentation (alcoholic and acetous) of grapes. At present, innovations in vinegar production falls into two areas: improving production processes and employing different raw materials.

Strawberries are prized for both their aroma and flavour. Spain is the world's second largest strawberry producer. Since there are surpluses of second quality strawberry, this makes it an attractive candidate for use as a raw material in producing new types of vinegars.

Aroma is certainly one of the most important determinants in food quality and acceptance.[1] Therefore, when introducing a new food product, the characterization of its aroma is an important aspect to be considered. Aroma is determined by a large number of compounds that are involved in different ways. Among the above, odour-active compounds play an important role in perceived aroma and, especially, impact odorants, that directly influence in it.

An essential tool when characterizing the olfactory impact of an odorant (its odour descriptor and intensity) is the coupling of gas chromatography with olfactometry (GC-O).[2] Among the different types of olfactometric analysis, the three main ones are: dilution methods, perceived intensity methods and frequency of detection methods. The frequency of detection has been proved to provide quantitative estimates of the sensory importance of a compound[3] and has even been proved to be accurate enough to provide real quantitative data,[4] but it is limited because once the concentration becomes higher than the least sensitive member of the panel, the signal becomes saturated.[5] This limitation can be partly overcome by combining the frequency of detection with a simple measurement of intensity, which also has been proved to provide reliable quantitative data.[6] As concentration increases, odour intensity may continue to increase; however, the frequency of detection might not increase when all members of the panel are finally able to detect the odorant.[5]

The combination of intensity and frequency of detection can be done through the so-called modified frequency (MF) which is the geometric mean of the detection frequency of an aromatic zone (expressed as a percentage) and the average intensity (expressed as a percentage of the maximum intensity).[7] This concept is useful because the discriminative capabilities of the detection frequency may be improved by taking intensity into account[8] and, moreover, because the members of a tasting panel can have widely divergent sensitivities.[7] MF, therefore, provides more reliable results.

The volatile composition of strawberry has been the subject of extensive study. The aroma of this fruit is generally a complex mixture of esters, furanones and terpene alcohols with smaller amounts of lactones, aldehydes, alcohols and sulfur compounds.[9–16] Among the above, furaneol and mesifurane are considered to be the two major flavour contributors due to

* Correspondence to: M. L. Morales, Área de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Sevilla, c/P García González nº 2, E- 41012 Seville, Spain. E-mail: mlmorales@us.es

† *This article is published in Flavour and Fragrance Journal as Part I of Special Issue: 13th Weurman Flavour Research Symposium, Zaragoza, Spain, 27th – 30th September 2011, edited by Vicente Ferreira (University of Zaragoza).*

[a] Área de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Sevilla, c/P García González nº 2, E- 41012 Seville, Spain

[b] IFAPA Centro Alameda del Obispo, Avda. Menéndez Pidal s/n, E-14004 Córdoba, Spain

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their low odour threshold and their high quantities.[17] These compounds have been confirmed in several studies on the odour-active compounds in different strawberry varieties. In these works, the authors have identified a considerable number of odour-active compounds and they agree on furaneol, mesifurane and γ-decalactone as being impact odorants in strawberry.[18,19]

In this work we have determined odour-active compounds and the possible impact odorants by GC-O in strawberry vinegars obtained through double fermentation. Moreover, an olfactometric analysis of the starting substrate has been also performed to ascertain whether the key odorants of raw material remain in the final products.

Experimental

Chemicals

Dichloromethane and anhydrous sodium sulfate were purchased from Merck (Darmstadt, Germany), and all were of analytical quality.

Strawberry Vinegar Samples

Vinegars analysed in this study were produced from second quality strawberries of 'Camarosa' variety. For that purpose, fruit was crushed and mixed with pectolytic enzymes and sulfur dioxide. Additionally, 50 g/l of sucrose was added to ensure an appropriate final acidity in the resulting vinegar. This starting substrate (F8P2) was submitted to two types of alcoholic fermentations, spontaneous and inoculated with the yeast *Saccharomyces cerevisiae* QA23 at a concentration of 2×10^6 cells/ml. All resulting wines were spontaneously acetified by surface culture. Thus, we obtained two different vinegars: F8VI (from inoculated alcoholic fermentation) and F8VE (from spontaneous alcoholic fermentation).

Sample Extraction

The liquid–liquid extraction method was performed to obtain a representative extract of the samples.[8] The procedure followed was: 5 g of anhydrous sodium sulfate was added to 50 ml of each sample and was extracted twice for 5 min with 5 ml of dichloromethane using a magnetic stir bar. Then, 2.5 ml of the organic phase was concentrated five times under a stream of nitrogen.

Gas Chromatography–Olfactometry Conditions

Analyses were conducted using a Varian 3800 GC (Middelburg, The Netherlands) equipped with a flame ionization detector and an OP275 olfactometer (GL Science Inc., Tokyo, Japan). Two microlitres of each extract were injected in splitless mode into a DB-WAX column with 60 m $\times$ 0.25 mm $\times$ 0.22 μm film thickness (J & W Scientific, Agilent Technologies Inc., Santa Clara, CA, USA). The oven temperature program was as follows: 40° for 1 min, increasing to 220°C at a rate of 2°C/min and held for 30 min. The column effluent was split 2:3 into a flame ionization detector and a heated sniffing port. The injector and detector temperature was 220°C. The carrier gas was hydrogen at a flow rate of 1 ml/min. The sensory panel was composed of three trained tasters, all of whom sniffed each sample twice, assigning to each perceived odour an intensity level: 1, 2 or 3. Results were expressed as 'modified frequency' (MF), calculated with the formula proposed by Dravnieks.[20]

Gas Chromatography–Mass Spectrometry Conditions

Analyses were conducted using an Agilent 6890 GC system coupled to an Agilent 5975inert quadrupole mass spectrometer and equipped with a Gerstel MP2 headspace autosampler (Müllheim an der Ruhr, Germany). The analytical column was a CP-Wax 57CB column of 50 m $\times$ 0.25 mm and 0.20 μm film thickness (Varian, Middelburg, The Netherlands). The injector port was heated to 220°C and 2 μl of sample extract were injected in splitless mode with a purge flow of 70 ml/min and purge time of 2 min. The carrier gas was helium at a flow rate of 1 ml/min. The oven temperature program was the same as employed in GC-O. Quadrupole, source and transfer line temperatures were 150, 230 and 250°C, respectively. Electron ionization mass spectra data from *m/z* 29–350 were collected in the scan mode, with an ionization voltage of 70 eV.

Identification of Aroma Compounds

In the GC-MS analysis, volatile compounds were identified based on the comparison of the retention times of individual standard and computer matching with the reference mass spectra from the NIST 98 library.

In the GC-O analysis, compounds were identified by the comparison of their linear retention indices (LRIs) with those obtained in GC-MS analysis of extract and standards as well as with LRIs and odour qualities from Flavornet (www.flavornet.org)[21] and Pherobase (www.pherobase.com)[22] online data bases and literature.[8,23–37]

LRIs were calculated on the basis of retention times of *n*-alkanes (C10–C32) under identical conditions for each instrument.

Statistical Analysis

All statistical analyses were performed using Statistica software version 7.0 software package (Statsoft, Tulsa, OK, USA). A principal component analysis was carried out as an unsupervised method in order to ascertain the degree of differentiation between samples and which compounds were involved.

Results and Discussion

In this study, we only considered those odour zones which were detected in at least half of the total sniffing trials as odour active. The odour zones and their corresponding identification are listed in Table 1, some of them were only tentatively identified (TI). Thus, GC-O analyses showed 79, 55 and 49 odour-active zones in substrate, F8VI and F8VE vinegars, respectively.

In the substrate, the odour zones that presented maxima MF (100) were those which were identified as methyl butyrate (LRI 990, TI), isoamyl alcohol (LRI 1208), *cis*-2-nonenal (LRI 1504, TI), γ-decalactone (LRI 2150), γ-dodecalactone (LRI 2382) and vanillin (LRI 2564, TI). Some have been previously reported as important components in strawberry aroma. Thus, methyl butyrate is one of the major esters present in this fruit.[38,39] According to Gomes da Silva and Chaves das Neves,[40] prominent lactones were γ-decalactone and γ-dodecalactone, giving fruity notes. *cis*-2-Nonenal has been described as a frequent odorant present in different strawberry varieties.[41] On the other hand, the presence of vanillin among the aroma compounds in strawberry has been scarcely observed by other authors[42,43] and until now it had been described as an odour-active compound in one variety only.[44]

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Table 1. Odour active compounds in strawberry substrate and the vinegars obtained

LRI DB-Wax	LRI CP-Wax and/or literature	Odour descriptor	Odorant[d]	Substrate		Inoculated		Spontaneous		Identification[c]
				F^a	MF[b]	F^a	MF[b]	F^a	MF[b]	
934	—	Strawberry acid	Unknown	—	—	—	—	3	41	—
963	961[e]	Plastic, synthetic	Ethyl propionate	—	—	3	41	3	41	MS, OD
970	969 [8];970 [23,24]	Butter	Diacetyl	—	—	4	67	4	67	LRI, OD
990	990 [21]	Strawberry	Methyl butyrate[1]	6	100	—	—	6	100	LRI, OD
1012	1012[e]; 1014 [25]	Medicinal	Isobutyl acetate	4	47	—	—	—	—	LRI, MS, OD
1020	1022 [21]	Fruit, strawberry, pineapple	Methyl isovalerate[2]	6	71	—	—	—	—	LRI, OD
1029	1029[e];1032 [24]	Banana, strawberry, soap	Ethyl butyrate[3]	5	75	5	53	—	—	LRI, MS, OD
1040	1040[e],1041 [26]	Banana, fruit	Ethyl 2-methylbutyrate[4]	6	82	6	58	6	82	LRI, MS, OD
1055	1055[e],1056 [26]	Fruit, strawberry, banana	Ethyl isovalerate	4	67	3	41	3	41	LRI, MS, OD
1066	1067 [22];1069 [27]	Metallic, rubber, sweat	Hexanal[5]	5	75	—	—	—	—	LRI
1069	—	Toasted bread, coffee	Unknown[6]	5	75	—	—	—	—	—
1083	1083[e],1084 [8,28]	Rubber, sweat, latex	Isobutanol	4	67	—	—	—	—	LRI, MS, OD
1105	1105[e]	Banana	Isoamyl acetate	3	41	—	—	—	—	MS, OD
1144	1146[e],1145 [21]/1145 [21]	Boiled potato, vegetable, grass	1-Butanol/myrcene[7]	6	82	3	58	3	41	LRI, MS, OD/LRI, OD
1166	1169 [29]	Green, plastic, rubber	Limonene	3	41	—	—	—	—	LRI, OD
1183	1181 [30]/1185 [25]	Sweet, medicinal, aspirin	Methyl hexanoate/ethyl 3-methylpentanoate	4	47	—	—	—	—	LRI, OD
1208	1205,1208 [21];1212[e]	Rancid, rubber, chemical	Isoamyl alcohols (2-methyl-1-butanol + 3-methyl-1-butanol)[8]	6	100	4	47	6	58	LRI, MS, OD
1220	1220 [21]	Plastic	2-Hexenal	4	67	—	—	—	—	LRI, OD
1224	—	Chicken, cooked, synthetic, vegetable	Unknown	—	—	3	41	—	—	—
1234	1230[e],1240 [30]	Strawberry, blackberry, citrus, violet	Ethyl hexanoate[9]	5	75	—	—	—	—	LRI, MS, OD
1247	—	Vegetable, plastic, flower	Unknown	3	41	—	—	—	—	—
1264	1269 [31]	Boiled potato, boiled vegetable	α-Terpinolene	4	58	—	—	—	—	LRI,OD
1289	—	Metallic, paint	Unknown	—	—	—	—	3	41	—
1302	1303 [25];1306 [24]	Plastic, cooked vegetable, metallic	1-Octen-3-one	5	53	—	—	—	—	LRI, OD
1305	1308 [21]	Toasted corn, boiled potato	1-Octen-3-one + 2,6-dimethylpyrazine[10]	5	75	4	47	5	53	LRI, OD/LRI, OD
1317	1315 [25]	Barbecue	2-Methyl-3-furanthiol	—	—	3	58	5	53	LRI, OD
1327	1331 [32]	Toasted, chicken soup, dairy product, plastic	2-Acetyl-1-pyrrolidone	—	—	—	—	6	58	LRI, OD
1347	1345 [21]/1345[21]	Vegetable, grass	2-Octenal/3-nonenal	3	41	—	—	—	—	LRI, OD
1350	1350 [28]; 1355 [33]; 1357[e]	Plastic	1-Hexanol	3	58	—	—	—	—	LRI, MS, OD
1373	1368 [24];1377 [33]/1377 [21]	Green leaf, fish	Dimethyl trisulfide/*trans*-2-hexenol[11]	6	71	—	—	—	—	LRI, OD/LRI, OD

(Continues)

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Table 1. *(Continued)*

LRI DB-Wax	LRI CP-Wax and/or literature	Odour descriptor	Odorant[d]	Substrate		Inoculated		Spontaneous		Identification[c]
				F^a	MF^b	F^a	MF^b	F^a	MF^b	
1391	—	Baked potato, vegetable	Unknown	3	58	—	—	—	—	—
1396	1394 [25,30]; 1396[e]	Baked potato, mushroom	*cis*-3-Hexenol[12]	5	75	3	41	3	41	LRI, MS, OD
1405	1405[e]	Pungent	Acetic acid[13]	—	—	6	82	6	82	MS, OD
1419	1424 [25]	Anise, sweet, plastic doll	Ethyl cyclohexanoate	5	53	—	—	—	—	LRI, OD
1433	1436 [25]	Toast, coffee	2-Furfurylthiol[14]	6	82	—	—	—	—	LRI, OD
1435	1432[e];1436 [21]	Fruit, strawberry, lemon	Ethyl octanoate	3	58	3	41	—	—	LRI, MS, OD
1450	1449 [34]/1452 [25]	Baked potato	Methional[15]	—	—	6	100	4	82	LRI, OD
1463	1568	Strawberry, banana, vanilla, sweet	Benzaldehyde	—	—	3	41	—	—	MS, OD
1500	—	Toast, burned, hot iron	Unknown	3	41	—	—	—	—	—
1502	—	Vegetable, baked potato, orange	Unknown	3	58	—	—	—	—	—
1504	1502 [34]	Toasted, vegetable, river water	*cis*-2-Nonenal[16]	6	100	4	47	3	41	LRI, OD
1508	1511 [35]	River water, vapour	3-Nonen-2-one[17]	4	67	5	91	5	91	LRI, OD
1535	1534[e]	Plastic, rancid	2-(Methylthio)ethanol	3	58	—	—	—	—	MS, OD
1560	1560 [30]	Grass, boiled green beans, flower	Linalool	4	67	—	—	—	—	—
1563	1563 [21]; 1565[e]	Cheese, vomit	Isobutyric acid	—	—	—	—	4	58	LRI, MS, OD
1581	1575 [21]	Violet, flower, vegetable, soap	(*E*,*Z*)-2,6-Nonadienal[18]	6	82	4	75	3	71	LRI, OD
1594	1584 [22]; 1592[e]	Caramel, sweet, cotton candy	Mesifurane[19]	5	75	3	41	6	71	LRI, MS, OD
1611	—	River water, rancid	Unknown	3	58	—	—	—	—	—
1616	—	Cut grass, river water	Unknown	3	50	—	—	—	—	—
1619	1621 [25]	Toasted, peanut, rancid	2-Acetylpyrazine	3	58	—	—	—	—	LRI, OD
1623	1623[e]; 1632 [34]	Cheese, vomit	Butyric acid	6	82	6	82	6	82	LRI, MS, OD
1648	1645 [21];1647[e]	River water, fruit compote, sweet, plastic	Acetophenone	3	41	—	—	3	41	LRI, MS, OD
1669	1665 [21]	Cheese	Isovaleric acid[20]	5	53	6	100	6	100	LRI, OD
1692	1692[e]	Fruit, flower, plastic, vapour	γ-Hexalactone	3	41	—	—	—	—	MS, OD
1700	1702[e]; 1711 [22]	Cut grass, soap, plastic, flower	α-Terpineol	4	67	4	47	—	—	LRI, MS, OD
1715	1718[e];1706 [36]	Plastic doll, anise	Benzyl acetate[21]	5	75	—	—	—	—	LRI, MS, OD
1721	1720 [21]	Mint, lemon, vegetable	Carvone	5	65	5	53	3	58	LRI, OD
1731	1731[e];1738 [37]	Plastic, river water	Methionol	3	58	4	67	—	—	LRI, MS, OD
1740	—	Plastic, bitumen, pneumatic	Unknown	4	67	—	—	4	58	—
1766	1765 [21]/1762 [21];1769 [33]	Rancid, flower, citric, fresh	Linalyl valerate/citronellol[22]	5	75	—	—	3	41	LRI, OD/LRI, OD
1785	1784 [21]	Mint, plastic	Ethyl salicylate	3	58	—	—	—	—	LRI, OD
1810	—	Plastic, peanut, barbecue	Unknown[23]	3	58	5	75	4	67	—
1821	1818 [25]	Fruit preserve, quince compote, roast apple	β-Damascenone[24]	6	82	5	75	3	58	LRI, OD

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1824	1823[e];1829 [21]	Mint, flower, jasmine	2-Phenylethanol acetate	—	—	4	58	—	—	LRI, MS, OD
1836		Licorice, curry, spicy	Unknown[25]	—	—	6	82	—	—	—
1844	1838[e];1842 [21]	Plastic, sweat, dung, rancid	Ethyl dodecanoate[26]	5	75	—	—	—	—	LRI, MS, OD
1861	1864 [25]	River water, olive, clove, barbecue	Guaiacol[27]	6	82	4	67	4	67	LRI, OD
1877	1880[e]	Boiled potato, metallic, mint, violet	Benzyl alcohol	4	58	—	—	4	58	MS, OD
1915	1916 [25]	Rose, hyacinth	2-Phenylethanol[28]	5	53	6	71	5	75	LRI, MS, OD
1919	1920[e]	Coconut, sweet	Hydroxycinnamyl acetate	—	—	3	41	—	—	MS, OD
1926	—	Cut grass, lima beans, gasoline	Unknown [29]	5	75	—	—	—	—	—
1941	—	Chamomile, urine	Unknown	—	—	4	67	—	—	—
1957	—	Lemon, baked potato	Unknown	3	41	—	—	—	—	—
1967	1967 [33]	Ripe fruit, quince compote, roast apple	δ-Octalactone	—	—	4	47	—	—	LRI, OD
1971	1972[e]	Lemon, washing powder, green beans, grass	6,7-Dihydro-7-hydroxylinalool[30]	6	82	—	—	—	—	MS
1988	—	Caramel, metallic, sweet, honey	Unknown	3	41	—	—	4	58	—
2003	2009 [21]/2006 [27]2010 [22]	Soap, vegetable	*trans*-Nerolidol/*cis*-nerolidol[31]	5	75	3	41	—	—	LRI, MS, OD
2034	2034[e];2033 [21]/2033 [32]	Cotton candy, caramel, quince	Pantolactone + furaneol[32]	6	91	6	100	5	75	LRI, MS, OD
2054	—	Sweat, grass, stagnant water	Unknown[33]	5	75	—	—	—	—	—
2064	—	Green beans, baked potato, rancid oil	Unknown[34]	5	75	—	—	—	—	—
2085	2084 [34]/2091 [25]	Tempera, burned plastic	*p*/*m*-Cresol[35]	—	—	5	75	—	—	LRI, OD
2150	2157[e];2165 [37]	Fruit, blackberry, peach	γ-Decalactone[36]	6	100	6	100	4	67	LRI, MS, OD
2155	—	Mentholated	Unknown	—	—	3	58	2	47	—
2167	2170[e];2159 [28];2176 [25]	Clove, sponge cake	Eugenol[37]	6	82	3	41	—	—	LRI, MS, OD
2183	2182 [34];2185 [25]	Tempera, cucumber	4-Ethylphenol[38]	4	75	5	75	3	58	LRI, OD
2196	2198 [21]	Coconut, clove, toasted	*p*-Vinylguaiacol[39]	4	75	6	82	5	91	LRI, OD
2203	2203 [22];2204 [25]	Licorice, curry	Sotolon[40]	3	71	6	82	4	82	LRI, OD
2219	2216 [22]	Liquor	δ-Decalactone[41]	—	—	—	—	3	71	LRI, MS, OD
2230	2223 [21]/2234 [37]	Grass, banana, fruit, honey	*o*-Aminoacetophenone	—	—	3	41	3	58	LRI, OD
2241	2247 [21]	Spice, coconut, flower, roast vegetable	Abhexone[42]	4	67	5	75	3	58	LRI, OD
2270	2269[e]	Tempera, barbecue, burned plastic	Ethyl hexadecanoate[43]	4	75	5	75	—	—	MS, OD
2319	—	Mint, lemon, toothpaste, fruit	Unknown	4	47	3	58	3	58	—
2382	2384 [21]2385[e]	Apple, apricot, strawberry, sweet, milk	γ-Dodecalactone[44]	6	100	4	58	—	—	LRI,MS, OD
2396	—	Banana, flower, clove	Unknown	4	47	—	—	3	58	—
2437	2426 [21]	River water, clove, spicy, barbecue	δ-Dodecalactone[45]	4	82	6	58	—	—	LRI, OD
2455	2452[e]	Sweet, cinnamon	Coumaran	—	—	4	58	—	—	MS, OD

(*Continues*)

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Table 1. *(Continued)*

LRI DB-Wax	LRI CP-Wax and/or literature	Odour descriptor	Odorant[d]	Substrate		Inoculated		Spontaneous		Identification[c]
				F^a	MF[b]	F^a	MF[b]	F^a	MF[b]	
2490	—	Plastic, bleach, rancid	Unknown	4	67	—	—	—	—	—
2545	—	Sweet, coconut	Unknown	—	—	4	67	—	—	—
2556	2251 [21]; 2553[e], [36]	Rose, honey	Phenylacetic acid[46]	4	75	6	100	5	91	LRI, OD
2564	2569 [22]	Vanilla, caramel	Vanillin[47]	6	100	5	75	4	75	LRI, OD
2600	2598 [21]	Honey, vanilla	Methyl vanillate	—	—	3	58	—	—	LRI, OD
2636	—	Compote, banana, strawberry	Unknown	3	41	—	—	—	—	—
2644	2640 [21]	Honey	Acetovanillone	—	—	3	71	—	—	LRI, OD

[a]*F*, frequency of occurrence.
[b]MF, modified frequency.
[c]LRI, identified by lineal retention index; MS, identified by matching mass spectra of GC-MS analysis of extract with those from the NIST 98 library.
[d]Corresponding number of this compound in Figure 1.
[e]LRI in column CP-Wax 57CB of compound identified by GC-MS.
OD, odour descriptor.

As expected, furaneol (LRI 2034) and its methoxy derivative,[45] mesifurane (LRI 1594), the strawberry components most quoted as character impact compounds, also reached high MF: 91 and 75, respectively.

Within those odour zones which reached high MF, we can highlight those that we have tentatively identified for the first time in strawberry such as 2,6-dimethylpyrazine (LRI 1305), 2-furfurylthiol (LRI 1433), guaiacol (LRI 1881), 6,7-dyhydro-7-hydroxylinalool (LRI 1971), 4-ethylphenol (LRI 2183), sotolon (LRI 2203) and ethyl hexadecanoate (LRI 2270). The first two odorants and sotolon are typical compounds produced by the Maillard reaction.[46,48] The presence of these compounds may be due to the same formation pathway, since strawberry has reducing sugars as well as amino acids.

Moreover, sniffers also perceived some off-odour attributes tentatively identified as volatile phenols with high MF: guaiacol and 4-ethylphenol. Several authors point to these compounds as off-flavours in different food matrices.[49,50] Moreover, they are related to fruit contamination caused by different microorganisms.[51,52]

In addition to the above-mentioned compounds, we found other important odour zones with MF higher than 70, providing fruity notes, corresponding to typical strawberry esters [ethyl butyrate (LRI 1029) and ethyl hexanoate (LRI 1234, TI)]. Furthermore, we identified linalyl valerate/citronellol (LRI 1766, TI) and nerolidol (LRI 2003) around this MF value, giving grassy and citric aroma characters.

Among the 38 odour zones with a significant contribution to the aroma (MF $>$ 70), we were, even tentatively, unable to identify four of them.

The comparison of olfactometric analysis results of substrate and vinegars showed that a total of 29 odorants present in the strawberry puree were detected both in vinegars produced from wines whose substrate had been inoculated and those which had been obtained from spontaneous fermentation. These odorants were, therefore, preserved throughout the double fermentation process. Most of them, however, presented lower MF values in vinegar than in the substrate due to the losses and transformations undergone during the alcoholic and acetous fermentations.[53,54] The aromatic profile of wine vinegars is the result of the permanence of those volatile compounds from the raw material (grape varietals aroma), synthesized by yeast during alcoholic fermentation and those formed during the acetous fermentation. The results showed that after the double fermentation, some characteristic compounds of strawberry[27,55] such as γ-decalactone, furaneol, mesifurane, β-damascenone (LRI 1821) and ethyl 2-methylbutyrate (LRI 1040) were preserved from the raw material to both vinegars. Surprisingly, moreover, they reached high MF values.

On the other hand, the impact odorants of strawberry, ethyl butyrate and γ-dodecalactone, were only perceived in vinegar produced from wine obtained by inoculation of the substrate while methyl butyrate was perceived only in vinegar from wine obtained by spontaneous fermentation.

These results indicate that the overall odour impression of vinegar would evoke aromatic notes of the raw strawberry material used.

Moreover, we observed the appearance of odour zones, or an increase in the MF scores of other odour zones corresponding to compounds usually formed in acetous fermentation.[8,56] Therefore, aromatic notes such as butter (diacetyl, LRI 970) and pungent (acetic acid, LRI 1405), appeared in the vinegars. The

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cheese odour, identified as isovaleric acid (LRI 1669), increased its MF (53 in the substrate), reaching the maximum value in both vinegars. Other typical compounds produced in surface acetification are the acetic esters;[56] however, they were either not perceived (isobutyl acetate and isoamyl acetate) or were perceived with low MF [2-phenylethanol acetate (LRI 1824) and hydroxycinnamyl acetate (LRI 1919)] in our vinegars. The acetic esters are formed by chemical condensation that occurs slowly, especially during ageing of vinegars.[57] Therefore, those vinegars which undergo a long ageing process have high levels of these compounds. The vinegars analysed in this work did not have an ageing process and therefore, these compounds may indeed have been present in these vinegars, but at imperceptibly low concentrations.

Within the identified compounds that underwent a large MF increase from substrate to vinegars, we found 3-nonen-2-one (LRI 1508, TI), 2-phenylethanol (LRI 1915), *p*-vinylguaiacol (LRI 2196, TI), sotolon (TI) and phenylacetic acid (LRI 2556, TI). After the MF increase in the first three compounds, they then became possible impact odorants in the resulting vinegars. Among the aforementioned compounds, sotolon has already been reported as a key odorant in sherry vinegar.[8]

Another new odour zone that appeared in vinegars, described as 'baked potato', was tentatively identified as methional (LRI: 1450). This compound is produced by *Saccharomyces cerevisiae* in different fermented foods.[58] In our case, therefore, the methional was probably synthesized during alcoholic fermentation and subsequently remained in the vinegar.

It should be observed that there is a relatively high variability in the GC-O profiles between the vinegars, depending on the type of alcoholic fermentation (spontaneous or inoculated). The vinegar produced by inoculation of the substrate has a higher number of odour zones that, in general, are more intense than those found in the vinegar obtain by spontaneous fermentation. Several of them clearly differentiate these two vinegars, since they were only present in one of the vinegars reaching a high MF. Thus, we can highlight strawberry and liquor, identified as methyl butyrate (TI) and δ-decalactone respectively, only detected in F8VS; and liquorice/curry/spicy (unknown, LRI: 1836), burned plastic (*p*-/*m*-cresol, LRI 2085, TI) and honey (acetovanillone, LRI 2644, TI) only perceived in F8VI.

If we consider those compounds with a frequency and modified frequency higher than 3 and 70, respectively, as possible impact odorants, we found 12 possible impact odorants in our strawberry vinegars: acetic, butyric (LRI 1623) and isovaleric acids, methional, 3-nonen-2-one, 2-phenylethanol, pantolactone + furaneol, *p*-vinylguaiacol, sotolon, phenylacetic acid and vanillin, some of which have been previously reported as key odorants in sherry vinegar.[8]

Principal component analysis was performed using the potential impact odorants data ($F > 3$ years, $MF > 70$) to summarize major differences and highlight potential relationships among the substrate and the vinegars obtained from the wines produced by inoculated and spontaneous alcoholic fermentation. The first two components explain 73.42% and 26.58% of the total variance, respectively (Figure 1). Principal component 1 (PC1) separated the substrate from vinegars and PC2 the two vinegars. As we can see in Figure 1, the substrate is related to a great number of potential impact odorants. As expected, odour zones which reached maxima MF (100) in the substrate are placed on the right side of the graph (Figure 1b), being more related with the strawberry substrate (Figure 1a). The aromatic zones identified as characteristic

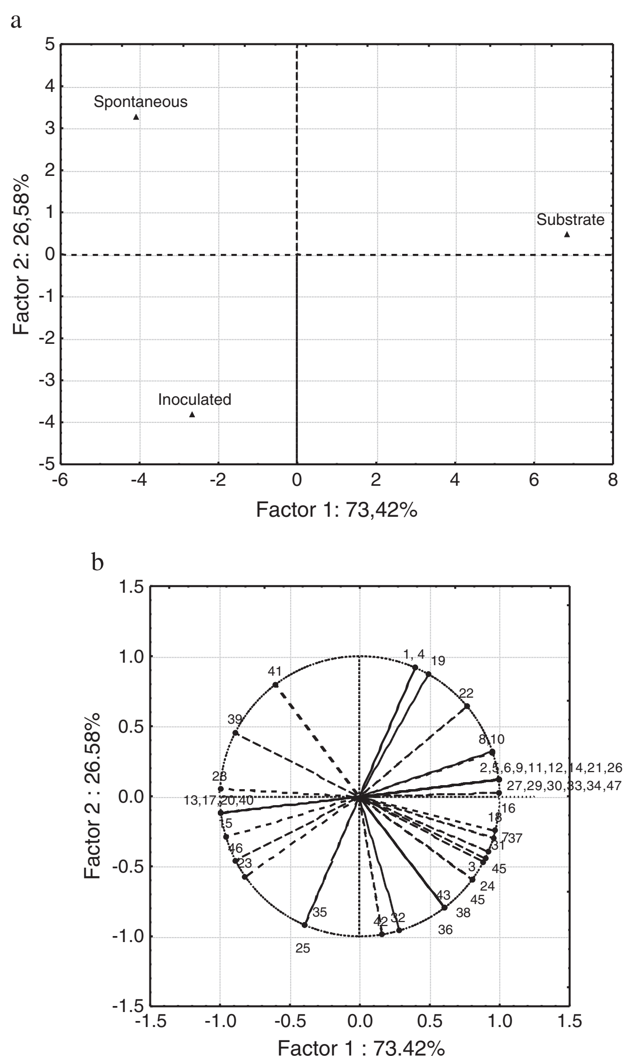

Figure 1. Data scores (MF) (a) and variable loadings (b) plots on the planes made up of the first two principal components (PC1 against PC2). The compound names corresponding to the number of variables are located in Table 1

aroma compounds of vinegars such as acetic and isovaleric acids among others, appear on the left side of the plot. If we compare both vinegars, we observe that some compounds such as *p*-vinylguaiacol and δ-decalactone seem to be more related to those vinegars obtained from spontaneous wines and *p*-/*m*-cresol with those from inoculated ones. In addition, results for principal component analysis confirm the influence of the alcoholic fermentation process (spontaneous or inoculated) in the aroma profile of vinegars. Hence, among the impact odorants of strawberry, ethyl butyrate and γ-dodecalactone are located at the bottom of the graph, and thus are closer to the vinegar produced from inoculated alcoholic fermentation. Meanwhile methyl butyrate is located nearer to those vinegars produced by spontaneous process.

Finally, the potential impact odorants of all samples with similar sensory descriptions were grouped into eight categories on the basis of the character of their aroma: fruity, sweet, grassy, spicy, butter–lactic–cheesy, chemical, empyreumatic and miscellaneous. This last group included odour zones described as tempera, plastic

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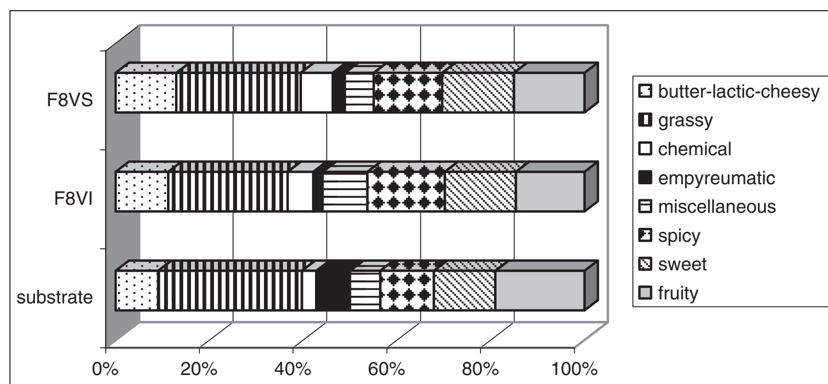

Figure 2. Contribution of each aroma category as a percentage of the total MF of odour zones that might be potential impact odorants in at least one sample

or metallic. Figure 2 shows the contribution of each aroma category as a percentage of the total MF of odour zones that might be potential impact odorants in at least one sample.

In the substrate, the grassy aroma character (30.7% total MF) stood out, followed by fruity, sweet and spicy aroma categories, the remaining aroma groups accounting for approximately 25%. However, in the vinegars, the percentages of the first two categories decreased and increased, principally, the spicy and butter–lactic–cheesy percentages of total MF. For this last aroma group, the result was to be expected since the compounds responsible for this aroma category are the acids typically formed in acetous fermentation. The comparison of the total MF percentages in the vinegars showed that the miscellaneous group, related to negative aromatic nuances, had higher values in F8VI than in F8VS.

Therefore, although aromatic notes that mainly contribute to aroma samples correspond to the grassy category, the sum of aromatic characters – fruity, sweet and spicy – probably results in a major influence in the overall odour impression.

Conclusions

The final aromatic profile of strawberry vinegars was formed by compounds from the substrate and others produced during alcoholic and acetous fermentations. Thus, impact aromatic compounds characteristic of strawberry such as ethyl 2-methylbutyrate, mesifurane, β-damascenone, furaneol and γ-decalactone, were preserved throughout the double fermentation process, presenting high MF values.

The type of alcoholic fermentation (spontaneous or inoculated) had an influence on the number of odour-active compounds. Hence, vinegars from the inoculated process accounted for more odour zones with high MF than the spontaneous process and, moreover, these odour zones had pleasant aromatic nuances.

We can therefore conclude that the MF of odour zones identified as acetic, butyric and isovaleric acids, methional (TI), 3-nonen-2-one (TI), 2-phenylethanol, pantolactone + furaneol, *p*-vinylguaiacol (TI), sotolon (TI), phenylacetic acid (TI) and vanillin (TI) point to these compounds as possible impact odorants in these strawberry vinegars.

Considering the total MF percentage of the different aroma categories of potential impact odorants, the grassy, fruity, sweet and spicy aroma seem to have the highest influence on the overall odour impression of strawberry vinegars. Vinegars produced from spontaneous wines presented a higher total MF percentage of grassy and butter-lactic-cheese aroma categories.

All these results indicate that inoculated alcoholic fermentation could provide vinegars with a higher aromatic quality than those produced by spontaneous process.

Acknowledgements

This research was made possible through the financial support from the Spanish Government by means of a predoctoral grant and the research project AGL2007-66417-C02-01 funded by the Ministry of Science and Innovation. The authors wish to thank Agromedina enterprise for providing the fruit substrates, and the research group of Dr A. Mas from University Rovira i Virgili for providing fruit vinegars.

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A COMPARATIVE STUDY OF AROMATIC PROFILES OF STRAWBERRY VINEGARS OBTAINED BY DIFFERENT PRODUCTION PROCESSES

Journal:	*Journal of Agricultural and Food Chemistry*
Manuscript ID:	Draft
Manuscript Type:	Article
Date Submitted by the Author:	n/a

Complete List of Authors:	Ubeda, Cristina; University of Seville, Callejón, Raquel; University of Seville, Troncoso, Ana M.; University of Seville (Spain), Bioquímica, Bromatología, Toxicología y Medicina Legal Moreno Rojas, Jose-Manuel; IFAPA, Postharvest, technology and Food Industries Peña-Rodríguez, Francisco; IFAPA Centre "Alameda del Obispo", Postharvest and Food Technology Area Morales, M; Universidad Sevilla, Area Nutrición y Bromatología

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A COMPARATIVE STUDY OF AROMATIC PROFILES OF STRAWBERRY VINEGARS OBTAINED BY DIFFERENT PRODUCTION PROCESSES

Authors: Cristina Ubeda[1], Raquel M. Callejón[1], Ana M. Troncoso[1], Jose M. Moreno-Rojas[2], Francisco Peña[2], M.Lourdes Morales[1*]

Address: [1]Área de Nutrición y Bromatología. Facultad de Farmacia. Universidad de Sevilla. c/ P. García González nº2, E- 41012. Seville. Spain.

[2]IFAPA Centro Alameda del Obispo. Avda. Menéndez Pidal s/n, E-14004. Córdoba. Spain.

[*]Corresponding author: e-mail: mlmorales@us.es; Tel.: 34-954-556760; Fax.: 34-954-551245.

1 **Abstract**

2 Odor-active compounds in strawberry vinegars produced in different containers (glass,
3 oak and cherry barrels) were determined by gas chromatography olfactometry (GC-O)
4 using the modified frequency (MF) technique. Beside, their sensory profiles were built
5 employing descriptive sensory analysis. The aromatic profile of vinegar from
6 strawberry cooked must was also studied. This vinegar accounted the highest number
7 of odor-active zones and stood out for its general impression, raisin and liquer aroma
8 scores. Wood barrels provided vinegars with higher number of impact odorants than the
9 glass container. In this last one predominated odorants with grassy character while in
10 vinegars produced in wood barrels were the odorants with sweet and fruity characters.
11 Odor-active zone identified as δ-octalactone was only perceived in vinegars from wood
12 barrels, reaching maximum MF in them. Principal Component Analysis showed that the
13 procedures employed lead to important differences in the main impact odorants,
14 separating the vinegars in the different quadrants.

15 **Keywords**: impact odorant, odor-active compound, olfactometry, vinegar, strawberry,
16 descriptive sensory analysis.

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26 **Introduction**

27 Due to the high level of competitiveness of today's market, the success of a product
28 requires the use of raw material different to the traditionally used, and the application of
29 innovative techniques of production. Regarding vinegar, in Spain, traditionally is
30 produced from grapes by means of double fermentation (alcoholic and acetous). There
31 are several advantages in the use of fruits for vinegars production such as their healthy
32 and organoleptic properties. So, an important task in the production processes is to keep
33 or even enhance these features.

34 On the other hand, due to the current economic situation, the use of fruit surpluses is a
35 good solution to save resources and provide extra income for the agricultural sector.
36 Strawberry is grown in large quantities in Huelva (Spain), and every year, part of the
37 crop is discarded for several reasons, including size, deformations or overproduction,
38 which leads to surpluses. This fact, together with the qualities of this fruit, makes it a
39 good candidate for fruit vinegar production.

40 Among essential aspects to consider when developing a new food product is its aroma.
41 The volatile composition of strawberry fruit has been extensively studied over the past
42 50 years and more than 360 volatile compounds identified[1]. This set of compounds
43 comprises a combination of esters, which dominate qualitatively and quantitatively as
44 well as furanones, sulphur compounds, lactones, alcohols and carbonyls[2,3]. However, all
45 volatile compounds do not contribute to the composition of aroma and, therefore, gas
46 chromatography-olfactometry (GC-O) analysis has become important for clarifying
47 whether a volatile compound has an odor characteristic and for evaluating its
48 contribution to the overall aroma[4]. Among the methodologies for the analysis of
49 samples using GC-O, the modified frequency (MF) combines intensity and frequency
50 detection methods and subsequently provides more reliable results[5].

51 Much work has been done to elucidate the impact odorants of strawberry. Among them,
52 some key odorants of this fruit are furaneol, mesifurane, methyl butanoate, *gamma*-
53 decalactone, linalool and hexanoic acid[6,7,8].
54 In lesser extent, key odorants of vinegar have been studied. In red wine vinegar, Charles
55 et al.[9] found as relevant aroma diacetyl, acetoin, acids such as acetic, isovaleric and
56 butyric and alcohols such as isoamyl and 2-phenylethanol. In the case of Sherry wine
57 vinegar were established as key odorants ethyl acetate, diacetyl, butyl acetate, isoamyl
58 acetate, ethyl octanoate, acetic acid, isovaleric acid and sotolon[10]. In addition, recently,
59 Aceña et al.[11] also described as active-odorants in this type of vinegar the compounds
60 ethyl isobutyrate and ethyl *trans*-cinnamate.
61 It is well known that when vinegars are aged in barrels, the overall aroma is enriched
62 mainly as the result of three important processes: firstly, they are concentrated because
63 water is lost through the wood pores; secondly, the aromatic compounds are transferred
64 from the wood and finally, new compounds are formed by chemical reactions, such as
65 esters[12,13]. On the other hand, the use of barrels of different wood produces differences
66 in the aromatic composition of wine vinegar. This fact is due to the different porosity of
67 wood as well as to their peculiar chemical composition[14]. However, the influence of the
68 type of container on the production of vinegars from fruits different to grape should be
69 researched.
70 So, the main objective of this work was to check the effect of different containers in
71 aroma profile of strawberry vinegars to select the best production procedure. In
72 addition, we studied the use of two kinds of substrate, fruit puree and concentrated fruit
73 puree. For these purposes, olfactometric and descriptive sensory analyses have been
74 performed.
75 **Materials and methods**

76 *Chemicals*

77 Dichloromethane and anhydrous sodium sulphate were purchased from Merck
78 (Darmstadt, Germany), all of them were of analytical quality.

79 *Samples and sample extraction procedure*

80 Strawberry vinegar samples analyzed in this study were produced from second quality
81 strawberries of Camarosa variety harvested in 2009. For this purpose, a strawberry
82 puree (FS) was inoculated with the isolated yeast strain *Saccharomyces cerevisiae* RP1.
83 After alcoholic fermentation, the resulting wine was acetified in three different
84 containers: a glass vessel (FVG), and oak (FVO) and cherry wood barrels (FVC). Each
85 of them was filled with 5.5 L of wine and inoculated with acetic acid bacteria[15]. A part
86 of the strawberry puree was concentrated by heating; the resulting product was a cooked
87 must. 1 L of this must was submitted to alcoholic and acetous fermentation (FVCM) in
88 a glass container following the same procedure as the one without concentration.
89 All fermentation processes were performed by surface culture. The sample codes appear
90 in brackets.
91 For olfactometric analysis representative extracts of the samples were obtained by a
92 liquid-liquid extraction method[16]. The procedure followed was: 5 g of anhydrous
93 sodium sulphate was added to 50 mL of each sample and was extracted twice during 5
94 minutes with 5 mL of dichloromethane using a magnetic stir bar. Then, 2.5 mL of the
95 organic phase was concentrated 5 times under a nitrogen stream.

96 *Gas Chromatography-Olfactometry conditions (GC-O)*

97 Analyses were conducted using a Varian 3800 GC (Middelburg, The Netherlands)
98 equipped with a flame ionization detector (FID) and an OP275 olfactometer (GL
99 Science Inc., Tokyo, Japan). Two microliters of each extract were injected in splitless
100 mode into a DB-WAX column with 60 m x 0.25 mm x 0.22 µm film thickness (J & W

101 Scientific, Agilent Technologies Inc., Santa Clara, USA). The oven temperature
102 program was as follows: 40 °C for 1 min, increasing to 220 °C at a rate of 2 °C/min and
103 held for 30 min. The column effluent was split 2:3 into a FID and a heated sniffing port.
104 The injector and detector temperature was 220 ºC. The carrier gas was H2 at a flow rate
105 of 1 mL/min. The sensory panel was composed of three trained tasters, all of them
106 sniffed each sample twice, assigning to each perceived odor an intensity level: 1, 2 or 3.
107 Results were expressed as "modified frequency" (MF), calculated with the formula
108 proposed by Dravnieks[17].

109 *Gas Chromatography-Mass Spectrometry (GC-MS) conditions*

110 Analyses were conducted using an Agilent 6890 GC system coupled to an Agilent
111 5975inert quadrupole mass spectrometer and equipped with a Gerstel MP2 headspace
112 autosampler (Müllheim an der Ruhr, Germany). The analytical column was a CP-Wax
113 57CB column of 50 m × 0.25 mm and 0.20 μm film thickness (Varian, Middelburg, The
114 Netherlands). The injector port was heated to 220 °C and 2 μL of sample extract were
115 injected in splitless mode with a purge flow of 70 mL/min and purge time of 2 minutes.
116 The carrier gas was He at a flow rate of 1 mL/min. The oven temperature program was
117 the same employed in GC-O. Quadrupole, source and transfer line temperatures were
118 150, 230 and 250 °C, respectively. Electron ionization mass spectra data from m/z
119 29~350 were collected in the scan mode, with an ionization voltage of 70 eV.

120 *Identification of Aroma Compounds*

121 In the GC-MS analysis, volatile compounds were identified based on the comparison of
122 the retention times of individual standard and computer matching with the reference
123 mass spectra from the NIST 98 library. In the GC-O analysis, compounds were
124 identified by the comparison of their linear retention indices (LRIs) with those obtained
125 in GC-MS analysis of extract and standards as well as with LRIs and odor qualities

126 from Flavornet (www.flavornet.org)[18] and Pherobase (www.pherobase.com)[19] online
127 data base and literature[8,10,11,20-38].
128 Linear retention indices (LRIs) were calculated on the basis of retention times of n-
129 alkanes (C10–C32) under identical conditions for each instrument.
130 *Descriptive sensory analysis*
131 The aroma of the four samples of strawberry vinegar was evaluated by an expert panel
132 composed of eight tasters (six females and two males). All members belonging to the
133 laboratory staff were trained according to international protocols[39,40]. Fifteen milliliters
134 of vinegar sample was presented in dark glass covered with a plastic dish. Descriptive
135 sensory analysis was carried out using sixteen sensory terms: pungent, ethyl acetate,
136 herbaceous, red fruit, tropical fruit, ripe fruit, raisin, sweet, cheese/rancid,
137 leather/animal, white wine, toasted caramel, spicy, liquer, aromatic complexity and
138 general impression. The panel selected by consensus these terms to describe the samples
139 during preliminary tasting sessions. The selected attributes were put on a tasting-card
140 and panelists were asked to rank each descriptor on a 10-cm unstructured scale (from
141 unnoticeable to very strong).
142 *Statistical analysis*
143 All statistical analyses were performed using Statistica software version 7.0 software
144 package (Statsoft, Tulsa, USA). ANOVAs were conducted to assess significant
145 differences ($p<0.05$) among scores of each attributes of different vinegar samples. A
146 principal component analysis (PCA) was carried out as an unsupervised method in order
147 to ascertain the degree of differentiation between samples and which odor-active
148 compounds were involved.
149 **Results and Discussion**
150 *GC-O*

151 Among all odor zones detected in this study, we only included in this discussion those
152 ones which were detected in at least half of the total sniffing trials of each sample.
153 These odor zones were considered odor-active and their corresponding identification are
154 listed in Table 1, some of them were only tentatively identified (TI). Thus, GC-O
155 analyses evidenced 82, 71, 67, 66 and 62 odor-active zones in FVCM, FVC, FVG, FVO
156 and substrate respectively. We can highlight that the samples FVCM, FVC and FVO
157 had higher number of odor zones with MF>80 than FVG. Cooked must suffered an
158 important concentration process by heating; therefore a loss of volatile compounds
159 would be expected. However, we observed that the vinegar produced from this raw
160 material accounted the highest number of odor-active zones, losing only 15 of them
161 from substrate.
162 The odor zone (LRI 1666) identified as isovaleric acid was the only one that reached the
163 maximum MF in all the vinegars. This compound has been previously described in
164 vinegars as an impact odorant by different authors[10,11,16].
165 Other odor zones perceived in all the vinegars with very high MF (>90) were identified
166 as pantolactone+furaneol (LRI 2036), p-vinylguaiacol (LRI 2197), phenylacetic acid
167 (LRI 2555) and vanillin (LRI 2564). The MF of these last three odorants in vinegars
168 increased through the double fermentation process. These compounds have been
169 described as glyco-conjugated aroma precursors in strawberry[41] and are released during
170 alcoholic fermentation. Probably, this fact causes the observed increase of MF from
171 fruit puree to vinegars.
172 Beside, in most of the vinegars reached high MF the following odor zones: river
173 water/vapor (3-nonen-2-one, LRI 1508); river water/olive/clove…(guaiacol, LRI 1861);
174 apricot/strawberry/sweet/dairy (gamma-dodecalactone, LRI 2382). The first odor zone
175 presented maximum MF in the substrate.

176 An especial case is the odor zone river water/clove/spicy/barbecue identified as delta-
177 octalactone (LRI 1963) which was only perceived by the tasters in vinegars acetified in
178 wood barrels. This compound has been found in the volatile fraction of several
179 fruits[29,42,43]. In our case, however, it seems that it could be given by the wood, although,
180 until now, as far as we known, this compound has not been described as a characteristic
181 compound of wood.
182 The potential impact odorants (frequency>3 and MF>70) of all samples with similar
183 sensory descriptions were grouped into 8 categories on basis of their aroma character:
184 fruity, sweet, grassy, spicy, butter-lactic-cheesy, chemical, empyreumatic and
185 miscellaneous. This last group included odor zones described as tempera, plastic or
186 metallic. Figure 1 shows the contribution of each aroma category as a percentage of the
187 total MF of odor zones that could be potential impact odorants in at least one sample.
188 Grassy and fruity were the major aromatic characters since these aromatic groups
189 provide the greatest percentage of total MF.
190 The grassy aromatic group was the one that contributed mostly to the aroma, accounting
191 the highest percentage of total MF of the samples studied. In this group stood out the
192 impact odorants 3-nonen-2-one, *trans*-nerolidol/*cis*-nerolidol, phenylacetic acid, 2-
193 phenylethanol, methional, present in all vinegars. The first two were impact odorants
194 also in the substrate. The vinegar produced in glass container presented a grassy
195 character similar to substrate and slightly higher than the others vinegars, showing as
196 impact odorants just in this case 4-methylguaiacol, 3,7-Dimethyl-1,6-octadiene-3,4-diol
197 and isogeraniol. The compounds ethyl dodecanoate and benzyl acetate were also impact
198 odorants in most of vinegars except for the one produced in glass container and oak
199 wood barrel respectively.

200 The percentage of total MF of fruity aroma slightly decreases from the substrate to
201 vinegars. In this category we have identified mainly esters, compounds responsible of
202 this kind of aromatic nuances in a wide variety of matrices[8,44], and also lactones. As
203 expected, the most frequent odor description for the odor zones that form this category
204 was “strawberry”. This would indicate that in these vinegars the strawberry aromatic
205 nuances would be preserved in the overall aroma. Among the compounds of this
206 aromatic group, γ–dodecalactone, abhexone, methyl butyrate and ethyl isovalerate were
207 considered as impact odorants in all vinegars for their MF values. These compounds are
208 typical aroma of strawberry, except abhexone, the ethyl analogue of sotolon. It has been
209 described in aged Sauternes wine[45], coffee beverages[46] and in fruits such as red
210 raspberries[47].
211 Moreover, we could also highlight as impact odorants β-damascenone and ethyl 2-
212 methylbutyrate except for vinegars produced in the glass container. Regarding the effect
213 of the production process, some of the impact odorants of the substrate were kept such
214 as γ–dodecalactone and β-damascenone (the latter in most cases) and other were lost
215 (ethyl butyrate and E,Z-2,6-nonadienal). Callejón et al.[48] observed the hydrolysis of
216 some esters such as ethyl butyrate throughout acetification processes due to the
217 metabolism of acetic acid bacteria.
218 In the sweet aromatic group, were included the odor zones mainly described as cotton
219 candy, caramel or sweet, attributes commonly linked to sweet foods aroma.
220 Pantolactone+furaneol, eugenol and vanillin, included in this group, resulted impact
221 odorants in all vinegars and in the substrate. Furaneol is the typical impact odorant of
222 strawberry[6,8]. Other compound belonging to this category is mesifurane, quoted by
223 many authors as being one of the most significant odorants in strawberry, only reached
224 high MF in vinegar from cherry barrel. The vinegars produced in wood barrels were the

225 only ones in which the sniffers detected δ-octalactone and maltol with a MF of impact
226 odorant. If we compare all production processes, cherry wood provided the vinegar with
227 the highest percentage of total MF for sweet aroma character.
228 Among the odor zones that provided spicy character it was possible to identify the
229 impact odorants p-vinylguaiacol, guaiacol, sotolon and δ–dodecalactone. These last two
230 compounds, impact odorants in the substrate, stop being in the vinegar from cooked
231 must and in that one from glass container respectively.
232 In the empyreumatic aromatic group were included the odor zones related to the toasted
233 aroma. As expected, the vinegar from cooked must accounted higher total MF
234 percentage of this category than the vinegar from the substrate without heating. The
235 odor zone more important of this group (LRI 1808), described as plastic, peanut,
236 barbecue was identified as (*E,E*)-2,4-decadienal.
237 On the other hand, characteristic compounds of acetous fermentation are included in the
238 group butter-lactic-cheesy. So the three compounds identified in this group, isovaleric
239 acid, butyric acid and diacetyl, can be considered impact odorants for their MF, except
240 the last two in vinegars produced in oak barrel.
241 Chemical is the least intense aroma category except to vinegar from oak barrel
242 (miscellaneous category). In this group, we may highlight isoamyl alcohols and acetic
243 acid, impact odorants in all vinegars.
244 Finally, in the category miscellaneous are included compounds chemically very
245 different. The two more important would be 2-(methylthio)ethanol and ethyl 9-
246 hexadecanoate, that were already impact odorant in the substrate.
247 In summary, we count 40 impact odorants in the vinegar obtained from cooked must.
248 Among them, linalyl valerate/citronellol, ethyl propionate, dimethyl trisulfide/trans-2-

249 hexenol and four odor zones unidentified with LRI 934, 1501, 1610 were impact
250 odorants exclusively in this vinegar.
251 The vinegar produced in cherry barrel presented 37 impact odorants, differing with the
252 other vinegars in benzyl alcohol, mesifurane and wine lactone, and two unknown odor
253 zones coconut, honey, sweet (LRI 2547) and honey (LRI 2679). Although vinegar from
254 oak barrel had only one impact odorant less than the previous one, presented several
255 exclusive impact odorants such as ethyl hexanoate, γ-decalactone, and two unknown
256 odor zones flower, grass, boiled potato (LRI 1548) and clove, barbecue, river water
257 (LRI 2268).
258 Finally, the vinegar with less impact odorants was that one produced in glass container
259 (28), differentiating it from the other in impact odorants 4-methylguaiacol, 3,7-
260 Dimethyl-1,6-octadiene-3,4-diol, isogeraniol and the odor zone leather, iron, tempera
261 (LRI 2277, tentatively identified as ethyl-9-hexadecanoate). These results were
262 expectable due to in this recipient are not taking place concentration and compounds
263 transference phenomena as those occurring in wood barrels.
264 *Descriptive sensory analysis*
265 We completed this study with the sensory analysis of the overall aroma of vinegars. The
266 aroma of the vinegars was assessed by eight tasters, using sixteen descriptors (pungent,
267 ethyl acetate, herbaceous, red fruit, tropical fruit, ripe fruit, raisin, sweet, cheese/rancid,
268 leather/animal, white wine, toasted caramel, spicy, liquer, aromatic complexity and
269 general impression) previously agreed upon as the best for describing sensory
270 characteristics of studied vinegars. A "spider-web" graph of the results obtained in
271 descriptive analysis is showed in Fig.2. As can be seen, vinegar from cooked must and
272 from oak barrel presented the highest scores in general impression and aromatic
273 complexity. These attributes reflect the overall aromatic quality. Moreover, FVCM

274 sample stood out for its raisin and liquer aroma, FVC for its unpleasant cheese aroma
275 and FVO for its red fruit character. With respect to raisin aroma we can highlight that
276 among six odor zones described with this attribute four of them were odor-active only
277 in vinegar from cooked must.
278 In spite of the great number of grassy odor zones detected with high MF in olfatometric
279 analysis, in the description of the overall aroma, the attribute herbaceous reached low
280 score. Probably, the compounds responsible of fruity and sweet nuances have more
281 influence on the overall aroma and masked the herbaceous character.
282 *Principal Component Analysis*
283 Principal component analysis (PCA) was performed using the potential impact odorants
284 with MF>90 to summarize major differences among the different vinegars studied. The
285 first two components explain 48.2% and 28.8% of the total variance, respectively (Fig.
286 3). The different procedures employed to produce these vinegars lead to important
287 differences in the main impact odorants, enabling to separate the samples in the
288 different quadrants.
289 Finally, comparing olfactometry and sensory analysis results, the vinegar from cooked
290 must had sensory characteristic very different to the vinegars from strawberry puree
291 without heating. These last ones resembled more to the starting fruit substrate;
292 therefore, we could say that we have obtained two different types of vinegars. Within
293 them, the vinegar from cooked must presented the highest number of impact odorants
294 and the best general impression.
295 Among vinegars from strawberry puree, the vinegars produced in wood barrels
296 accounted more impact odorants than vinegar from glass container. If we compare the
297 first ones, they have similar number of impact odor zones but different odorants. Both
298 of them have strawberry impact odorants such as mesifurane, in vinegar from cherry

299 barrel, and ethyl hexanoate and γ-decalactone in the case of vinegar from oak barrel.
300 However, the descriptive sensory analysis revealed that the vinegar from oak barrel had
301 an aromatic profile of best quality than the cherry one. Therefore, to conclude, using
302 oak wood barrels would provide the best vinegars from strawberry puree.

303 **Acknowledgements**

304 The authors wish to thank Grupo Alconeras enterprise for providing the fruit substrates
305 and Dr. A. Mas' research group from University Rovira i Virgili for providing fruit
306 vinegars.

307 *Footnote*

308 This research was made possible through the financial support from the Spanish
309 Government by means of a predoctoral grant and the research projects AGL2007-
310 66417-C02-01 and AGL2010-22152-C03-01 funded by the Ministry of Science and
311 Innovation.

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451 **Figure Captions**

452 Figure 1. Contribution of each aroma category as a percentage of the total MF of odor
453 zones that might be potential impact odorants in at least one sample.
454 Figure 2. Sensory profiles of studied vinegars. Significant difference ($p<0,05$) between
455 samples are indicated with the following superscripts: a) FVO-FVC; b) FVO-FVG c)
456 FVO-FVCM; d) FVC-FVG; e) FVC- FVCM; f) FVG- FVCM.
457 Figure 3. Data scores (MF>90) (a) and variable loadings (b) plots on the planes made up
458 of the first two principal components (PC1 against PC2). The compound names
459 corresponding to the number of variables are located in Table 1.
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Table 1. Odor-Active Compounds in Strawberry Substrate and Obtained Vinegars.

LRI[a]	LRI[b]	Odor Descriptor	Odorant[c]	F[d]	MF[e]	F[d]	MF[e]	F[d]	MF[e]	F[d]	MF[e]	F[d]	MF[e]	Identification[f]
				FS		FVG		FVC		FVO		FVCM		
934	-	Strawberry, sweet	Unknown	6	58	-	-	-	-	-	-	5	75	-
942	-	Bleach, rancid, alcohol	Unknown	-	-	-	-	-	-	3	41	4	47	-
963	961[g]	Plastic, synthetic, grass	Ethyl propionate	-	-	4	67	3	41	3	41	5	75	MS, OD
970	969[10];970[11,18]	Butter	Diacetyl	-	-	6	71	6	82	4	67	6	82	LRI, OD
990	990[18]	Strawberry	Methyl butyrate[1]	6	58	5	75	6	100	6	91	6	82	LRI, OD
1012	1012[g];1014[20]	Plastic, gum, grass, rancid	Isobutyl acetate	-	-	4	47	-	-	-	-	3	41	LRI, MS, OD
1020	1022[18]	Fruit, strawberry, pineapple	Methyl isovalerate	5	53	-	-	-	-	-	-	-	-	LRI, OD
1023	-	Sweet	Unknown	-	-	-	-	-	-	-	-	4	47	-
1029	1029[g];1032[11]	Strawberry, banana, fruit	Ethyl butyrate	6	100	3	41	6	58	3	41	6	58	LRI, MS, OD
1040	1040[g];1041[21]	Banana, strawberry, fruit	Ethyl 2-methylbutyrate	4	47	6	58	6	82	6	82	5	75	LRI, MS, OD
1055	1055[g];1056[21]	Fruit, strawberry, banana	Ethyl isovalerate	4	58	5	75	6	82	5	75	4	75	LRI, MS, OD
1069	-	Toasted bread, coffee	Unknown	-	-	-	-	-	-	-	-	4	47	-
1083	1083[g];1084[22]	Grass, rubber, sweat	Isobutanol	6	71	-	-	3	41	4	47	-	-	LRI, MS, OD
1105	1105[g]	Banana	Isoamyl acetate	-	-	-	-	4	67	4	47	4	67	MS, OD
1113	1113[23]	Glass cleaner product, raisin, strawberry	3-penten-2-one	-	-	-	-	-	-	-	-	3	41	LRI, OD
1144	1146[g];1145[18]/ 1145[18]	Cut grass, plastic, fruit	1-Butanol/myrcene	6	82	4	47	-	-	4	47	4	47	LRI, MS, OD/ LRI, OD
1179	1181[8]/1185[20]	Sweet, blackberry, strawberry	Methyl hexanoate/ethyl 3-methylpentanoate	-	-	-	-	-	-	-	-	4	67	LRI, OD/ LRI, OD
1201	1198[24];1204[25]	Cooked vegetable, child's modeling clay	E-2-hexenal	-	-	3	41	-	-	-	-	-	-	LRI,OD
1212	1212[g];1205+ 1208[18]	Rancid, rubber, chemical	Isoamyl alcohols (2-methyl-1-buatnol+3-methyl-1-butanol)[2]	6	58	6	82	6	91	6	100	6	82	LRI, MS, OD
1236	1232 [g];1235-1238[26]	Strawberry, blackberry, violet	Ethyl hexanoate	5	65	-	-	4	47	5	75	5	53	LRI, MS, OD
1247	1247[27]	Plastic, cream, burned, toasted	γ-Terpinene	3	41	-	-	-	-	-	-	4	67	-

1259	-	Burned	Unknown	-	-	-	-	3	41	-	-	-	-	-
1265	1269[28]	Citric, alcohol, plastic, dairy	α-Terpinolene	-	-	3	41	-	-	-	-	3	41	LRI, OD
1275	1270[18];1274[29]	Cologne, grass, dairy	Hexyl acetate	-	-	-	-	3	41	-	-	-	-	LRI, OD
1280	1280[18];1282[30]	Strawberry, raspberry, flower	Octanal	-	-	-	-	-	-	-	-	3	41	LRI, OD
1294	-	Boiled potato, cooked vegetable, dairy, plastic	Unknown	3	41	-	-	4	47	-	-	3	58	-
1297	-	Blackberry, strawberry, cotton candy	Unknown	-	-	3	41	-	-	-	-	-	-	-
1303	1303[20];1306[11]	Plastic, vegetable, grass, metallic	1-octen-3-one	3	41	3	58	5	53	4	58	4	67	LRI, OD
1310	1308[18]	Toasted corn, boiled potato	1-octen-3-one+2,6-dimethylpyrazine	4	58	4	67	-	-	5	75	5	75	LRI, OD
1319	1315[20]	Medicinal, stew, dairy	2-methyl-3-furanthiol	-	-	-	-	-	-	3	41	-	-	LRI, OD
1326	1331[31]	Chocolate milk, fruit, fried	2-acetyl-1-pyrrolidone	-	-	3	58	-	-	-	-	-	-	LRI, OD
1349	1345[18]/1345[18]	Vegetable, grass, citric	2-octenal/3-nonenal	-	-	-	-	4	47	-	-	-	-	LRI, OD
1363	1357[g];1360[18]	Green bean, metallic, cooked vegetable	1-hexanol	4	47	-	-	-	-	-	-	4	58	LRI, MS, OD
1369	1370[18]	Fish, urine, earth, grass	Trimethyltiazole	4	67	-	-	-	-	-	-	-	-	LRI, OD
1375	1377[18]/1377[18]	Crude meat, fish, metallic	Dimethyl trisulfide/*trans*-2-hexenol	3	71	4	47	4	47	4	58	6	82	LRI, OD/ LRI, OD
1394	1398[8]	Strawberry, sweet, plastic	Methyl octanoate	-	-	3	41	-	-	-	-	3	41	LRI, OD
1405	-	Strawberry, raisin, sweet, metallic, soap	Unknown	-	-	4	58	-	-	-	-	3	41	-
1408	1405[g]	Pungent	Acetic acid[3]	-	-	5	75	6	91	6	82	6	82	MS, OD
1420	-	Lemon, mint	Unknown	5	72	-	-	-	-	-	-	-	-	-
1426	1424[20]	Raisin, fruit	Ethyl cyclohexanoate	-	-	-	-	-	-	-	-	3	41	LRI, OD
1434	1432[g];1436[18]	Strawberry, fruit, lemon, dairy	Ethyl octanoate	5	53	-	-	3	58	3	41	4	58	LRI, MS, OD
1462	1460[32]	Baked potato, green bean	Methional[4]	-	-	6	91	6	82	5	75	5	83	LRI, OD
1501	-	River water, vapor	Unknown	-	-	3	71	-	-	-	-	5	71	-
1503	1503[25]	Toasted, vegetable, river water	*Cis*-2-Nonenal	5	65	3	58	-	-	4	67	-	-	LRI, OD
1508	1511[32]	River water, vapor	3-Nonen-2-one[5]	6	100	4	82	6	100	6	100	4	82	LRI, OD
1534	1534[g]	Plastic, rancid ,cut grass	2-(Methylthio)ethanol	6	82	4	58	5	75	6	82	5	75	MS, OD

1548	-	Flower, grass, boiled potato	Unknown	-	-	-	-	3	41	5	75	-	-	-
1562	1560[32]	Green bean	Linalool	3	41	-	-	-	-	-	-	-	-	LRI, OD
1564	1565[g];1563[18]	Cheese, vomit	Isobutyric acid	-	-	-	-	5	53	4	47	-	-	LRI, MS, OD
1585	1585[18]	Blackberry, ripe fruit, violet, flower, cut grass	Nonyl acetate	5	75	4	67	4	58	5	53	4	67	LRI, OD
1596	1592[g];1584[19]	Caramel, sweet	Mesifurane	3	58	3	58	6	82	4	58	4	47	LRI, MS, OD
1610	-	River water, plastic	Unknown	3	58	-	-	5	53	-	-	5	75	-
1625	1623[g]; 1632[29]	Cheese, vomit	Butyric acid	4	58	6	82	5	75	6	58	6	82	LRI, MS, OD
1630	1627[32]	Burned, toasted, flower	2-acetylpyrazine	3	41	3	58	-	-	5	75	5	75	- LRI, OD
1644	-	River water, olive	Unknown	-	-	-	-	-	-	-	-	3	58	-
1648	1647[g];1645[18]	Rose, flower, sweet	Acetophenone	-	-	3	41	-	-	-	-	-	-	LRI, MS, OD
1658	-	Burned, toasted	Unknown	-	-	-	-	4	58	3	58	-	-	-
1666	1665[18]	Cheese	Isovaleric acid	4	58	6	100	6	100	6	100	6	100	LRI, OD
1675	1675[33]	Plastic, toasted	2-Methyl-3-(methyldithio)furan	-	-	-	-	-	-	-	-	4	67	-
1703	1692[g]+1702[g]; 1711[19]	Plastic, barbecue, peanut, cut grass	γ-Hexalactone+α-Terpineol[6]	4	67	5	53	6	100	5	75	3	58	MS, OD/ LRI, MS, OD
1718	1718[g]	Mint, grass	Benzyl acetate[7]	3	41	6	100	6	82	3	58	6	82	MS, OD
1723	1720[18]	Mint, vegetable	Carvone	-	-	-	-	-	-	5	75	5	75	LRI, OD
1732	1731[g];1738[34]	Plastic, river water, compote	Methionol	5	53	4	67	4	67	3	41	5	65	LRI, MS, OD
1737	-	Toasted, burned, sweet, oil	Unknown	5	53	-	-	-	-	-	-	-	-	-
1740	-	Plastic, bitumen, pneumatic	Unknown	-	-	4	67	-	-	-	-	3	41	-
1747	-	Chamomile, flower, sweet	Unknown	-	-	-	-	4	67	-	-	-	-	-
1753	-	Plastic, citric	Unknown	4	67	-	-	-	-	-	-	-	-	-
1757	-	River water	Unknown	-	-	-	-	-	-	-	-	4	67	-
1764	1767[18]	Rancid, toasted corn, plastic	Acetylthiazoline	-	-	-	-	-	-	4	67	6	82	LRI, OD
1766	1765[18]/1762[18]; 1769[29]	Ripe fruit, citric, flower, grass, soap	Linalyl valerate/citronellol	5	75	4	47	4	67	-	-	-	-	LRI, OD/ LRI, OD
1785	1784[18]	Mint, plastic	Ethyl salicylate	-	-	-	-	-	-	-	-	-	-	LRI, OD
1796	-	Sweet, hot milk	Unknown	-	-	-	-	3	41	-	-	-	-	-
1808	1811[20]	Plastic, peanut, barbecue	(*E,E*)-2,4-decadienal[8]	5	91	3	71	5	91	6	82	5	91	LRI, OD
1813	1812[18]	Plastic, flower, jasmine	Isogeraniol[9]	-	-	5	91	-	-	-	-	-	-	LRI, OD

1823	1819[27]	Fruit preserve, quince compote, roast apple	β-Damascenone[10]	6	82	4	67	6	82	6	82	5	91	LRI, OD
1831		Raisin, licorice, metallic	Unknown	-	-	-	-	-	-	-	-	4	58	-
1842		Licorice, spice	Unknown[11]	-	-	6	82	3	41	5	75	6	91	-
1845	1838[g];1842[18]	Vegetable, sweat, rancid, cheese, burned	Ethyl dodecanoate[12]	6	82	3	58	5	75	6	100	6	100	LRI, MS, OD
1851	1850[19;35]	Flower, cologne, citric, burned, rancid, sweet, coconut	Geraniol	-	-	4	47	3	41	-	-	3	58	LRI, OD
1861	1864[20]	River water, olive, clove, barbecue, spice	Guaiacol[13]	6	82	6	100	6	82	6	100	5	91	LRI, OD
1877	1880[g]	Cut grass, flower, metallic, violet, fruit	Benzyl alcohol	4	67	4	58	6	71	-	-	4	75	MS, OD
1917	1916[g;20]	Rose, hyacinth	2-Phenylethanol	-	-	6	82	5	75	6	82	6	82	LRI, MS, OD
1923	1920[g]	Coconut, honey, caramel, plastic	Hydroxycinnamyl acetate	5	53	-	-	4	58	3	41	-	-	MS, OD
1926	-	Grass, metallic, sweat, boiled potato	Unknown	4	47	-	-	-	-	-	-	3	58	-
1939	-	Stew, cooked vegetable, sweat	Unknown	-	-	6	71	-	-	-	-	-	-	-
1963	1967[29]	Clove, coconut, ripe fruit, compote	δ-Octalactone[14]	-	-	-	-	6	100	6	100	-	-	LRI, OD
1971	1972[g]	Lemon, washing powder, green beans, grass	6,7-dihydro-7-hydroxylinalool[15]	4	67	3	58	3	58	5	75	6	100	MS
1990	1995[19]	Caramel, chocolate, cotton candy	Maltol	-	-	4	67	5	75	6	82	-	-	LRI, OD
2003	2007[g] 2009[18];2006[24]	Soap, vegetable	Nerolidol[16]	6	91	5	75	5	75	3	71	6	91	LRI, MS, OD
2036	2034[g]; 2033[18]/ 2033[31]	Cotton candy, caramel, quince	Pantolactone+Furaneol[17]	6	100	5	91	6	91	6	100	6	91	LRI, MS, OD
2059	-	Burned plastic, grass	Unknown	-	-	3	41	3	58	-	-	3	58	-
2084	2084[25]	Tempera, burned plastic	p-Cresol	3	58	-	-	4	58	3	58	4	58	LRI, OD
2095	2091[20;35]	Tempera, toasted	m-Cresol	-	-	-	-	-	-	-	-	3	41	LRI, OD
2136	2139[18]	Sweet, licorice, spicy,	Ethyl cinnamate	-	-	4	58	3	58	-	-	5	53	LRI, MS, OD
2151	2157[g];2165[34]	Peach, ripe fruit, sweet	γ-Decalactone	4	67	4	67	4	67	4	75	4	67	LRI, MS, OD
2159	-	Mentholated, ripe fruit, sweet	Unknown	-	-	-	-	4	67	4	47	4	75	-

2170	2170[g]; 2176[20]	Clove, sweet	Eugenol[18]	6	82	6	82	6	82	6	91	6	82	LRI, MS, OD
2181	2182[25]; 2185[20]	Tempera, plastic, tire	4-Ethylphenol	-	-	-	-	6	82	-	-	6	82	LRI, OD
2197	2198[18]	Coconut, clove, spicy	p-Vinylguaiacol[19]	4	67	6	100	6	100	5	91	6	100	LRI, OD
2203	2203[19]; 2204[20]	Licorice, curry	Sotolon	4	82	4	82	4	82	4	82	-	-	LRI, OD
2209	2209[18]	Quince, sweet, raisin	(3S,3aS,7aR)-3a,4,5,7a-Tetrahydro-3,6-dimethylbenzofuran-2(3H)-one (wine lactone)	-	-	-	-	4	82	3	71	3	71	-
2224	2223[18]	Mint, lemon, sweet, fruit	o-Aminoacetophenone	4	67	-	-	-	-	-	-	-	-	LRI, OD
2233	2230[36]/2233[19]	Roasted, spicy, sweet, raisin	2,3-Dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one (DDMP)/ 3,5-Dihydroxy-2-methyl-4H-pyran-4-one (5-hydroxymaltol)	-	-	-	-	-	-	-	-	4	67	LRI,OD
2242	2247[18]	Coconut, ripe fruit	Abhexone[20]	3	41	5	75	6	100	3	71	6	91	LRI, OD
2263	2260[18]	Spicy, sweet	Methyl anthranilate	-	-	-	-	-	-	-	-	3	58	LRI, OD
2268		Clove, barbecue, river water	Unknown	-	-	-	-	-	-	5	75	-	-	-
2271	-	Tempera, bitumen, stable	Unknown	5	83	5	75	5	75	-	-	6	100	MS
2277	2277[g]	Leather, iron, tempera	Ethyl-9-hexadecanoate[21]	-	-	5	75	-	-	-	-	-	-	MS
2320	2321[19]	Mint, chamomile, grass	3,7-Dimethyl-1,6-octadiene-3,4-diol	4	47	6	82	3	58	-	-	4	58	LRI, OD
2352	2350[18]	Flower, body milk	*trans*-Farnesol	5	75	-	-	4	67	3	58	-	-	LRI, OD
2382	2386[g]; 2384[18]	Apricot, strawberry, sweet, dairy	γ –Dodecalactone[22]	6	91	6	100	6	91	6	82	6	100	LRI, MS, OD
2398	-	Coconut, vanilla, flower	Unknown	-	-	-	-	3	58	-	-	-	-	-
2424	2427[18]	Toasted, grass	4-Vinylphenol	3	41	-	-	-	-	-	-	-	-	LRI,OD
2437	2426[18]	River water, clove, spicy, barbecue	δ –Dodecalactone	6	82	4	58	6	82	6	82	5	75	LRI,OD
2454	2452[g]	Roast apple, compote	Coumaran	-	-	-	-	-	-	3	58	-	-	MS, OD
2469	2466[36]	Quince, sweet	5-Hydroxymethylfurfural	-	-	-	-	-	-	-	-	4	47	LRI, OD
2475	2470[37]	Blackberry, sweet, fruit	Benzophenone	-	-	-	-	5	53	-	-	-	-	-
2484	2480[19]	Flower	Ethyl (Z)-9-octadecenoate	-	-	3	41	-	-	-	-	-	-	LRI, MS

2488	-	Acid, soap	Unknown	-	-	-	-	-	-	3	41	-	-	-
2506	-	Strawberry, compote	Unknown	-	-	-	-	3	41	-	-	-	-	-
2547	-	Coconut, honey, sweet,	Unknown	-	-	3	58	3	75	4	47	-	-	-
2555	2553[g;38]; 2251[18]	Rose, honey	Phenylacetic acid[23]	3	71	5	91	6	100	6	100	6	100	LRI, MS, OD
2564	2569[18]	Vanilla, caramel	Vanillin[24]	4	67	6	100	6	100	6	91	6	91	LRI, OD
2570	2570[18]	Honey, vanilla	4-Allyl-2,6-dimethoxyphenol	3	71	-	-	-	-	-	-	-	-	LRI, OD
2618	2612[18]	Honey, vanilla	Ethyl vanillate	-	-	4	67	-	-	3	58	-	-	LRI, OD
2654	2650[18]	Sweet, apricot, honey rose	Hydrocinnamic acid	-	-	3	41	-	-	3	58	-	-	LRI, OD
2679	-	Honey	Unknown	-	-	-	-	5	75	-	-	-	-	-

[a]LRI in DB-WAX column
[b]LRI in column CP-Wax 57CB and /or literature
[c]Corresponding number of this compound in Figure 1.
[d]F: Frequency of occurrence.
[e]MF: Modified frequency.
[f]LRI: Indentified by Lineal Retention Index; MS: Identified by matching mass spectra of GC-MS analysis of extract with those from the NIST 98 library; OD: Odor descriptor.
[g]LRI in column CP-Wax 57CB of compound identified by GC-MS.

Figure 1.

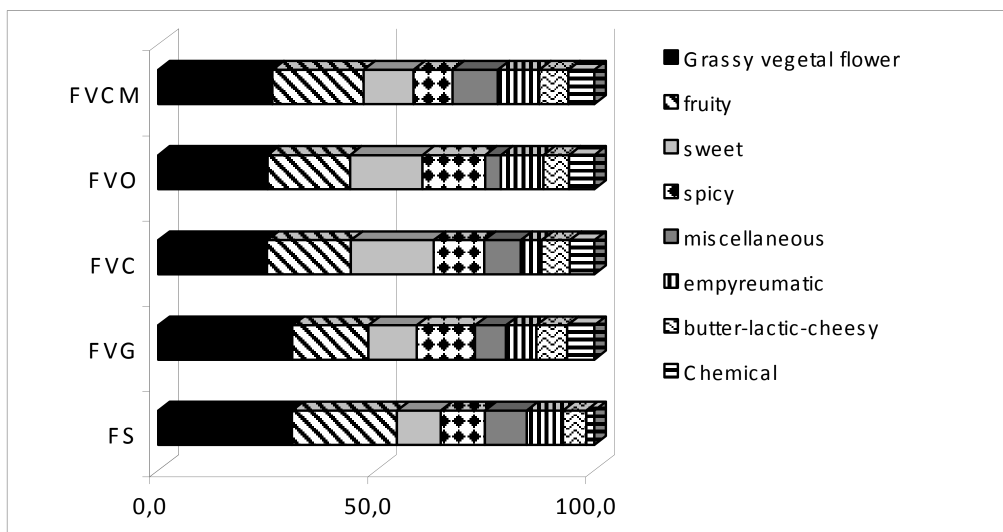


Figure 2.

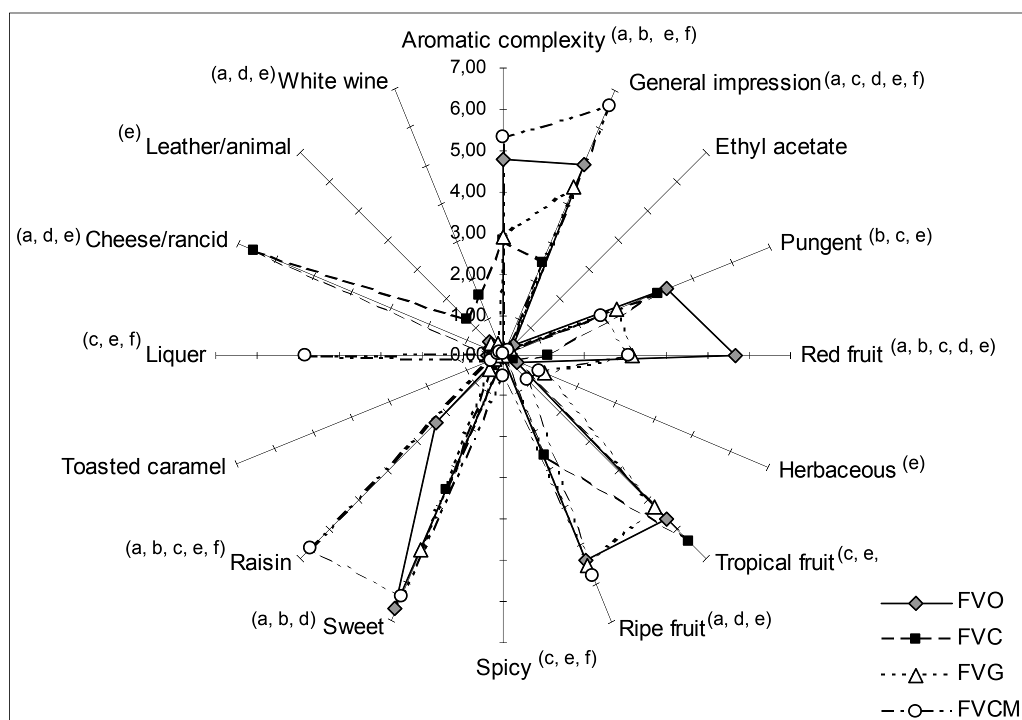


Figure 3a.

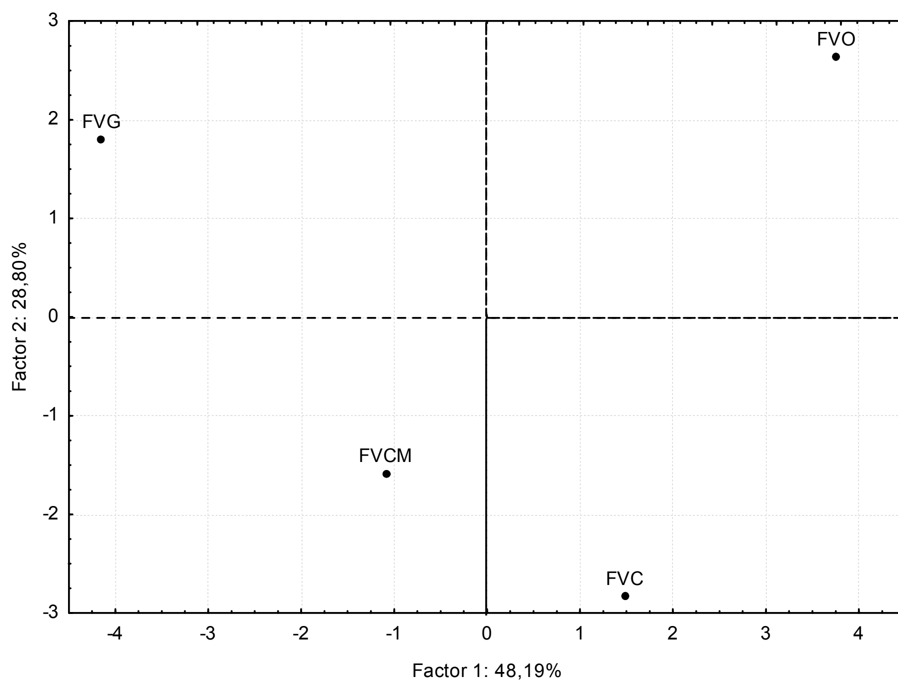
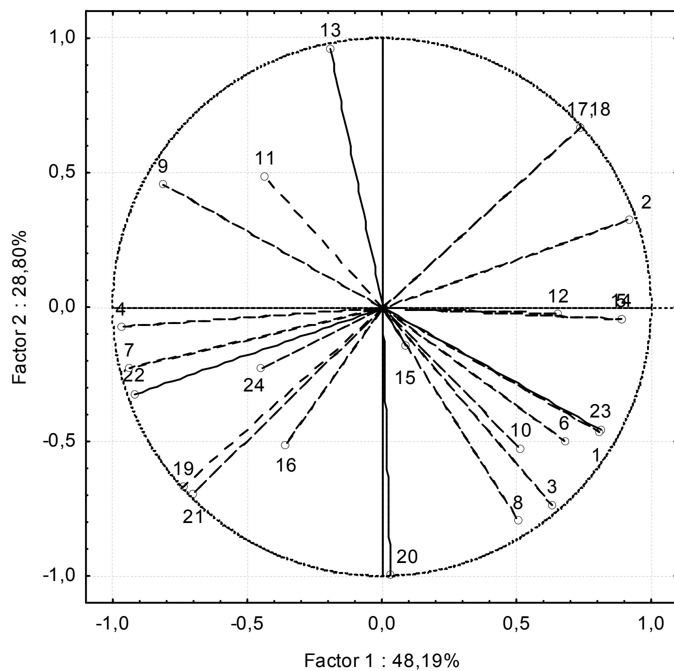


Figure 3b.



JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Glycosidically Bound Aroma Compounds and Impact Odorants of Four Strawberry Varieties

Cristina Ubeda,*[†] Felipe San-Juan,[§] Belén Concejero,[§] Raquel M. Callejón,[†] Ana M. Troncoso,[†] M. Lourdes Morales,[†] Vicente Ferreira,[§] and Purificación Hernández-Orte[§]

[†]Área de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Sevilla, C/P. García González 2, E-41012 Sevilla, Spain

[§]Laboratorio del Análisis del Aroma y Enología, Departamento de Química Analítica, Facultad de Ciencias, Universidad de Zaragoza, 50009 Zaragoza, Spain

ABSTRACT: This paper reports the determination of glycosidically bound aroma compounds and the olfactometric analysis in four strawberry varieties (Fuentepina, Camarosa, Candonga and Sabrina). Different hydrolytic strategies were also studied. The results showed significant differences between acid and enzymatic hydrolysis. In general terms, the greater the duration of acid hydrolysis, the higher was the content of norisoprenoids, volatile phenols, benzenes, lactones, Furaneol, and mesifurane. A total of 51 aglycones were identified, 38 of them unreported in strawberry. Olfactometric analyses revealed that the odorants with higher modified frequencies were Furaneol, γ-decalactone, ethyl butanoate, ethyl hexanoate, ethyl 3-methylbutanoate, diacetyl, hexanoic acid, and (Z)-1,5-octadien-3-one. This last compound, described as geranium/green/pepper/lettuce (linear retention index = 1378), was identified for the first time. Differences with regard to fruity, sweet, floral, and green aroma characters were observed among varieties. In Candonga and Fuentepina, the green character overpowered the sweet. In the other two strawberry varieties sweet attributes were stronger than the rest.

KEYWORDS: *glycosides, strawberry, aroma, flavor, olfactometry*

INTRODUCTION

Strawberry is a much appreciated fruit due to its aroma, taste, and health properties. It is usually consumed fresh (75% of total production) but is also used in the food industry as an important ingredient in jam, yogurt, syrup, tea, juice, ice cream, and other food products (25% of overall production).[1] Aroma is one of the most valued attributes of strawberry. The aroma of this fruit includes volatile compounds, both in their free form and as nonvolatile compounds, present mainly as glycoconjugates formed by a sugar and an aglycone.

There are numerous studies concerning the free volatile compounds of strawberry, with more than 360 volatile flavor compounds[2] identified. To learn more about the volatile composition of strawberry, several olfactometric studies have been undertaken using gas chromatography−olfactometry (GC-O).[3−5]

Nonvolatile compounds are, moreover, potential natural sources of aroma because hydrolysis of the bonds between the sugar and the aglycone turns this molecule into an aromatic compound. As ripening proceeds, the increase in these soluble sugars results in an increase in the availability of precursors capable of producing aroma compounds.[6]

These nonvolatile compounds have been extensively studied in grapes[7−9] and in other fruits such as lychee, acerola, blackberry, pineapple, and mango,[10−14] among others. Strawberry precursors have hardly been studied. After the description of the presence of 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one β-D-glucopyranoside in strawberry,[15] Wintoch et al.[16] analyzed the glycosidical aroma compounds from two strawberry species using Amberlite XAD-2. Other research groups have focused their studies on one aglycone, Furaneol (2,5-dimethyl-4-hydroxy-2*H*-furan-3-one),[17] due to its high influence on the overall flavor. In addition, there have been some studies concerning the evolution of these nonaromatic precursors during ripening. These studies show an increase in their aglycones during the above-mentioned stage.[18] Knowledge of the strawberry aromatic precursors is important because it enables us to predict the final aroma of new strawberry-based products. As a result, there are several different groups studying the production process of strawberry fermentation products.[19,20] Such analyses would enable us to estimate the aromatic potential and therefore select the best raw material. The aim of this study was to determine the aromatic potential of different strawberry varieties with the aim of selecting the most suitable varieties for producing several fermented strawberry-based food products. Therefore, the aroma compounds released by acid hydrolysis of glycosidic precursors isolated from four different varieties have been determined. Free aromas were also studied by GC-O analyses to determine the most important compounds, from a sensory point of view, in these varieties.

MATERIALS AND METHODS

Reagents and Standards. Dichloromethane, ethanol, and methanol were supplied by Merck (Darmstadt, Germany) and ethyl acetate and sodium fluoride by Fluka (Buchs, Switzerland). Sodium dihydrogen phosphate 1-hydrate, L-(+)-ascorbic acid, and citric acid were purchased from Panreac (Barcelona, Spain). Pure water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). LiChrolut EN resins were purchased from Merck. An alkane solution (C8−C28), 20 mg/L in dichloromethane, was used to calculate the linear retention index (LRI) of each analyte. The chemical standards used for the identification and quantification of volatile

Received: March 15, 2012
Revised: May 28, 2012
Accepted: May 31, 2012
Published: May 31, 2012

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Table 1. Concentration (Micrograms per Kilogram of Strawberries Except Where Indicated) of Volatile Compounds Released after Harsh Acid and Enzymatic Hydrolysis of the Strawberry Precursors Pool[a]

	0 min	15 min	1 h	4 h	1 week, 45 °C	enzymatic
terpenes						
α-terpinolene	nd	0.62 ± 0.10 a	2.94 ± 0.36 b	4.51 ± 0.14 c	0.27 ± 0.01 d	1.17 ± 0.09 f
(*Z*)-rose oxide	nd	nd	0.02 ± 0.00 a	0.25 ± 0.01 b	nd	nd
(*R*/*S*)-linalool	nd	75 ± 2 a	3.50 ± 0.30 b	nd	5.07 ± 0.13 c	105 ± 2 d
α-terpineol	nd	27 ± 1 a	111 ± 13 b	50 ± 1 c	77 ± 2 d	1.28 ± 0.14 e
nerol	nd	6.20 ± 0.79 a	12.72 ± 0.39 b	nd	nd	2.18 ± 0.22 c
geraniol	4.46 ± 0.36 a	29 ± 1 b	4.91 ± 0.63 a	nd	3.74 ± 0.35 a	5.95 ± 0.56 a
farnesol	nd	12 ± 1	nd	nd	nd	nd
linalool acetate	nd	0.23 ± 0.04	nd	nd	nd	nd
terpinen-4-ol[b]	nd	nd	3.01 ± 0.27 a	2.84 ± 0.09 a	0.68 ± 0.04 b	nd
δ-terpineol[b]	nd	nd	6.74 ± 0.41 a	6.05 ± 0.38 a	3.93 ± 0.05 b	nd
neric acid	nd	nd	0.20 ± 0.02 a	0.50 ± 0.04 b	nd	1.01 ± 0.03 c
norisoprenoids						
β-damascenone	nd	0.46 ± 0.02 a	1.30 ± 0.11 b	2.28 ± 0.08 c	0.59 ± 0.02 d	nd
β-ionone	0.15 ± 0.01 a	nd	nd	nd	nd	0.25 ± 0.01 b
1,1,6-trimethyl-1,2-dihydronaphthalene (TDN)[b]	nd	0.28 ± 0.01 a	1.68 ± 0.01 b	2.77 ± 0.09 c	0.59 ± 0.02 d	1.11 ± 0.01 e
tert-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB)[b]	nd	0.34 ± 0.01 a	6.35 ± 0.09 b	7.51 ± 0.44 b	2.13 ± 0.04 c	0.50 ± 0.00 d
3-oxo-β-ionone[b]	nd	1.33 ± 0.03 a	4.51 ± 0.42 b	4.65 ± 0.21 b	2.77 ± 0.14 c	nd
actinidols[b]	nd	0.24 ± 0.02 a	5.81 ± 0.52 b	6.62 ± 0.23 b	4.09 ± 0.15 c	0.25 ± 0.02 a
norisoprenoid 1[b]	nd	nd	2.81 ± 0.22 a	4.15 ± 0.10 b	0.27 ± 0.03 c	nd
3-oxo-α-ionol	nd	nd	0.63 ± 0.07 a	nd	nd	75 ± 2 b
volatile phenols						
guaiacol	nd	nd	nd	0.70 ± 0.09 a	nd	0.91 ± 0.04 a
m-cresol	nd	nd	nd	nd	nd	0.22 ± 0.01
eugenol	0.62 ± 0.01 a	0.70 ± 0.08 a	1.35 ± 0.17 b	6.06 ± 0.86 c	1.07 ± 0.02 b	18 ± 1 d
4-ethylphenol	nd	0.08 ± 0.00 a	nd	nd	nd	1.11 ± 0.14 b
4-vinylguaiacol	4.38 ± 0.08 a	5.73 ± 0.79 a	116 ± 11 b	151 ± 14 b	35 ± 2 c	352 ± 8 d
(*E*)-isoeugenol	1.79 ± 0.09 a	1.38 ± 0.07 a	0.91 ± 0.14 b,c	1.33 ± 0.05 ab	0.68 ± 0.01 c	3.70 ± 0.57 d
4-vinylphenol	121 ± 2 a	247 ± 16 b	12606 ± 1440 c	20904 ± 3263 ce	6231 ± 120 d	27863 ± 2764 e
vanillin derivatives						
vanillin	0.50 ± 0.01 a	1.08 ± 0.01 b	2.22 ± 0.23 c	3.81 ± 0.10	2.36 ± 0.06	8.21 ± 0.68
methyl vanillate	0.10 ± 0.00 a	nd	nd	nd	nd	1.16 ± 0.09 b
acetovanillone	nd	nd	nd	0.56 ± 0.09 a	nd	2.19 ± 0.00 b
homovanillyl alcohol	nd	nd	nd	nd	1.18 ± 0.00	nd
homovanillinic acid[b]	5.21 ± 0.10 a	4.10 ± 0.24 b	nd	nd	nd	83 ± 4 c
benzenes						
benzaldehyde	0.74 ± 0.06 a	1.86 ± 0.01 b	3.69 ± 0.35 c	8.13 ± 0.68 d	3.07 ± 0.12 c	11 ± 1 e
phenylacetaldehyde	0.67 ± 0.01 a	0.87 ± 0.10 a	3.24 ± 0.19 b	4.35 ± 0.27 c	nd	4.46 ± 0.43 c
benzyl alcohol	1.69 ± 0.21 a	3.14 ± 0.37 b	21 ± 1 c	59 ± 1 d	10 ± 1 e	1361 ± 40 f
β-phenylethanol	nd	1.90 ± 0.08 a	4.54 ± 0.05 b	9.61 ± 0.27 c	3.21 ± 0.19 d	97 ± 4 e
ethyl cinamate	nd	nd	7.09 ± 0.09 a	23 ± 1 b	18 ± 1 c	3.21 ± 0.19 d
2-phenoxyethanol	1.03 ± 0.04 a	1.38 ± 0.24 ac	0.64 ± 0.11 b	0.96 ± 0.01 a	0.54 ± 0.06 b	1.85 ± 0.14 c
benzoic acid	7.10 ± 0.93 a	10 ± 1 b	113 ± 17 c	210 ± 19 d	44 ± 3 e	240 ± 7 d
dihydromethyleugenol[b]	nd	nd	0.20 ± 0.03 a	0.41 ± 0.01 b	0.18 ± 0.01 a	3.20 ± 0.17 c
lactones						
δ-octalactone[c]	nd	0.47 ± 0.01 a	1.09 ± 0.01 bc	1.47 ± 0.01 b	1.08 ± 0.00 c	nd
γ-nonalactone[c]	nd	0.68 ± 0.01 a	nd	nd	nd	0.86 ± 0.00 b
γ-decalactone[c]	nd	0.10 ± 0.01 a	7.54 ± 0.00 b	17 ± 1 c	nd	1.08 ± 0.03 d
pantolactone	2.49 ± 0.03 a	1.18 ± 0.11 b	6.49 ± 0.49 c	8.47 ± 0.98 c	nd	nd
miscellaneous						
(*Z*)-3-hexen-1-ol	nd	1.16 ± 0.01 a	2.03 ± 0.01 b	2.19 ± 0.09 b	2.17 ± 0.12 b	13 ± 1 c
(*E*)-2-hexen-1-ol	4.37 ± 0.52 a	4.95 ± 0.52 a	2.87 ± 0.15 b	2.90 ± 0.10 b	3.00 ± 0.17 b	5.80 ± 0.17 c
ethyl decanoate	4.34 ± 0.05 a	4.38 ± 0.02 a	4.36 ± 0.02 a	nd	4.28 ± 0.00 a	nd
2-ethylhexanoic acid	1.30 ± 0.22 a	1.20 ± 0.06 a	1.13 ± 0.01 a	1.07 ± 0.01 a	1.87 ± 0.22 ab	2.19 ± 0.09 b
4-methoxy-2,5-dimethyl-3(2*H*)-furanone (mesifurane)[b]	50 ± 5 a	338 ± 4 b	339 ± 19 b	315 ± 23 c	251 ± 6 d	307 ± 9 c
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (Furaneol)[b]	23 ± 1 a	58 ± 2 b	74 ± 1 c	102 ± 3 d	60 ± 1 b	74 ± 3 c
cinnamic acid[b]	338 ± 18 a	586 ± 61 b	2917 ± 226 c	7657 ± 555 d	1828 ± 80 e	6209 ± 119 d

[a] Concentrations of the same compound with different letters show significant differences ($p < 0.05$). nd, not detected. [b] Chemical standard not available; tentatively identified. Data are relative areas (to 4-methyl-2-pentanol × 1000). [c] Data are the relative areas (to 4-methyl-2-pentanol × 1000). Chemical standard available, but the degradation of the products did not allow quantification.

dx.doi.org/10.1021/jf301141f | J. Agric. Food Chem. 2012, 60, 6095−6102

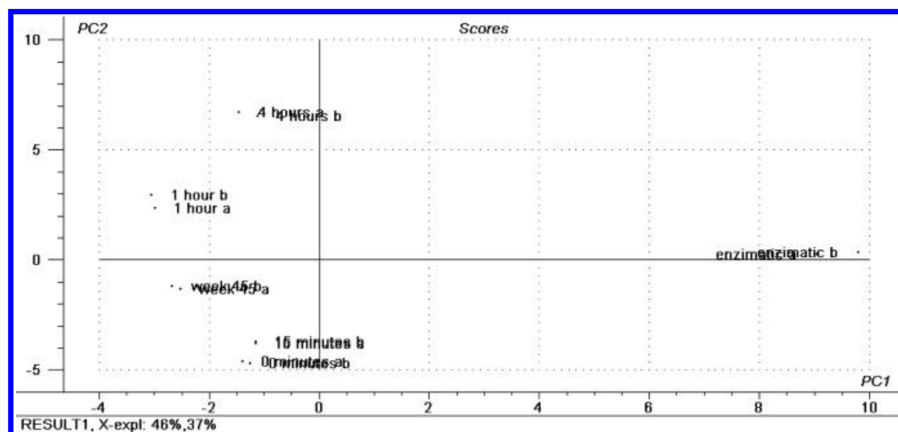

Figure 1. Principal component plot showing the scores for the samples of acid and enzymatic hydrolysis.

compounds were as follows: (*Z*)-rose oxide, linalool, *α*-terpineol, nerol, geraniol, benzaldehyde, *β*-phenylethanol, and 2-phenoxyethanol were purchased from Fluka. *β*-Ionone was sourced from Sigma (St. Louis, MO, USA), and guaiacol, *m*-cresol, eugenol, 4-ethylphenol, 4-vinylguaiacol, methyl vanillate, acetovanillone, zingerone, homovanillyl alcohol, phenylacetaldehyde, benzyl alcohol, ethyl cinnamate, *γ*-nonalactone, *γ*-decalactone, (*Z*)-3-hexen-1-ol were from Aldrich (Gillingham, U.K.). (*E*)-Isoeugenol, 4-vinylphenol, *δ*-octalactone, and *δ*-decalactone were purchased from Lancaster (Strasbourg, France). Finally, *β*-damascenone and vanillin were supplied by Firmenich (Geneva, Switzerland) and Panreac (Barcelona, Spain), respectively.

Samples. We employed freshly purchased *Fragaria ananassa* var. Camarosa strawberries to optimize the extraction method and to obtain the aroma precursors extract. Aromatic precursors were then determined in four different varieties of strawberry: Fuentepina, Camarosa, Candonga, and Sabrina. These strawberries were also employed for the olfactometric studies.

Extraction of Aroma Precursors. To study the effects of different kinds of hydrolysis, we prepared a precursors pool from strawberries of Camarosa variety acquired in the market. The preparation procedure was based on that of Ibarz et al.[7] We used an Ultra Turrax T25 Basic mixer (Ika, Labortechnik) to crush and homogenize 2 kg of strawberries with 1 L of cold Milli-Q water in the presence of 0.13 M NaF, to prevent microbial growth, and 50 mg/L of ascorbic acid (as an antioxidant). This mixture was then centrifuged and filtered, obtaining a strawberry must, which was placed in Pyrex flasks to which 2 g of LiChrolut resins (previously preconditioned with dichloromethane, methanol, and Milli-Q water) per kilogram of strawberry was added. The oxygen of the flasks was evacuated using nitrogen. We left the must in contact with the resins for 16 h in a Heidolph Promax 1020 shaker (Schwabach, Germany) at 90 rpm. We packaged the resin, and each cartridge of 500 mg was washed with 50 mL of water. It was then completely vacuum-dried, and free aromas were extracted with 50 mL of dichloromethane and discarded. Thirty milliliters of an ethyl acetate/methanol solution (9:1) was subsequently percolated through the resin. The solvents were evaporated under vacuum, resuspended in a 50:50 ethanol/water solution, and kept at −20 °C.

To analyze the four different strawberry varieties, we followed the same technique as that utilized for obtaining the precursors pool. In this case we processed 10 g of strawberry because we obtained the best results in previous studies using that quantity (data not shown). The must was percolated through a 200 mg LiChrolut EN cartridge (previously preconditioned with 10 mL of dichloromethane, 10 mL of methanol, and 10 mL of Milli-Q water). After that, the column was washed with 20 mL of Milli-Q water and then was completely dried. To eliminate all free aromatic compounds, we passed 20 mL of dichloromethane through the cartridge. To recover the precursors from the resin we employed 20 mL of a solution of ethyl acetate/methanol (9:1). This eluate was concentrated to 1 mL under vacuum at 40 °C and then taken to dryness under a gentle nitrogen stream. Each sample was extracted in duplicate.

Acid and Enzymatic Hydrolysis. Different hydrolytic conditions were performed to study their influence on the aromatic profile of strawberry using the precursors pool previously obtained. The acid hydrolyses assayed were 15 min and 1 and 4 h at 100 °C and 1 week at 45 °C. For this hydrolysis we mixed 8 mL of citric buffer (0.2 M, pH 2.5), 1 mL of the precursor extract, and 1 mL of an ethanol/water solution (50:50) (to maintain the same concentration of ethanol in all of the acid hydrolysis assays in a 20 mL vial). After this, the vial was sealed and placed in the oven. Moreover, an enzymatic hydrolysis was performed during 16 h at 38 °C. In this case we used 8.7 mL of citrate (0.1 M)/phosphate (0.2 M) buffer solution at pH 5, 1 mL of the precursor extract, which was subjected to vacuum to remove the ethanol, and 800 *μ*L of a pectinase enzyme solution with 200 mg/mL of AR 2000.

Otherwise, for the analysis of the four varieties of strawberry, the dry extract was reconstituted in 10 mL of citric buffer (0.2 M, pH 2.5, 10% EtOH) and was subjected to hydrolysis at 100 °C for 1 h. Before any hydrolysis was undertaken, the remaining oxygen was displaced from the vial with nitrogen to prevent oxidation of the compounds during the process. Each hydrolysis was done in duplicate.

Extraction of Volatiles Released in the Hydrolysis. After the hydrolysis, the solution was percolated through a 50 mg LiChrolut EN cartridge (previously preconditioned with 6 mL of dichloromethane, 2 mL of methanol, and 2 mL of citric buffer solution) and then was washed with 1 mL of Milli-Q water and dried. To elute the aromatic compounds, 700 *μ*L of dichloromethane was passed through the column and collected in a Kuderna Danish (Supelco, Bellefonte, PA, USA); 14 *μ*L of the internal standard 4-methyl-2-pentanol (402.6 *μ*g/g) was added. Finally, the solution was concentrated to 100 *μ*L with a gentle nitrogen stream.

Preparation of the Olfactometry Extract. To obtain a representative extract of each strawberry variety for the olfactometry analyses, we followed the method used by Ferreira et al.[21] Eighty grams of the fruit was crushed and placed in a purge and trap system.[22] A Lichrolut EN cartridge was placed on top of the bubbler flask. A nitrogen stream of 500 mL/min was applied to the sample for 100 min, releasing the free volatile compounds of strawberry in the headspace being trapped by the cartridge. Finally, these compounds were eluted with 3.2 mL of dichloromethane containing 5% methanol. The extract was concentrated to a final volume of 200 *μ*L.

GC-MS and GC-O Analytical Conditions. GC analysis of the volatiles released in the hydrolysis was performed with a CP-3800 chromatograph coupled to a Saturn 2200 ion trap mass spectrometric detection system from Varian (Sunnyvale, CA, USA). A DB-WAXetr capillary column (J&W Scientific, Folsom, CA, USA) (60 m × 0.25 mm i.d., film thickness = 0.5 *μ*m) preceded by a 3 m × 0.25 mm uncoated (deactivated, intermediate polarity) precolumn from Supelco was used. Helium was the carrier gas at a flow rate of 1 mL/min. The oven temperature program was 3 min at 40 °C, ramped at 10 °C/min to 90 °C, ramped at 2 °C/min to 230 °C, and finally held at this temperature for 37 min. Initially, the injector was kept at 35 °C for

Table 2. Concentration (Micrograms per Kilogram of Strawberries Except Where Indicated) of Volatile Compounds Released after Harsh Acid Hydrolysis of the Precursor Extract from Each Strawberry Variety[a]

	Fuentepina	Camarosa	Candonga	Sabrina
terpenes				
α-terpinolene (1)[b]	0.58 ± 0.05 a	0.39 ± 0.01 b	0.24 ± 0.01 c	0.19 ± 0.01 d
(Z)-rose oxide (2)	0.02 ± 0.00	nd	nd	nd
(Z)-linalool oxide[c] (3)	1.16 ± 0.13 a	nd	nd	7.68 ± 0.34 b
(E)-linalool oxide[c] (4)	1.02 ± 0.03 a	nd	nd	4.81 ± 0.46 b
(R/S)-linalool (5)	9.21 ± 0.23 a	13 ± 1 a	32 ± 3 b	48 ± 2 c
α-terpineol (6)	100 ± 4 a	63 ± 6 b	89 ± 10 b	78 ± 5 b
nerol (7)	0.82 ± 0.09 a	0.93 ± 0.13 a	3.83 ± 0.43 b	6.03 ± 0.42 c
geraniol (8)	18 ± 2 a	22 ± 1 ab	28 ± 2 b	45 ± 5 c
farnesol	nd	nd	9 ± 1 a	18 ± 2 b
δ-terpineol[c] (9)	1.19 ± 0.09 a	0.48 ± 0.03 b	0.59 ± 0.01 b	0.34 ± 0.01 c
norisoprenoids				
β-damascenone (10)	2.00 ± 0.18 a	1.75 ± 0.00 a	1.14 ± 0.14 b	0.65 ± 0.01 c
β-ionone	nd	0.92 ± 0.01 a	nd	0.67 ± 0.04 b
1,1,6-trimethyl-1,2-dihydronaphthalene (TDN)[c] (11)	1.09 ± 0.08 a	0.46 ± 0.03 b	0.42 ± 0.01 b	0.08 ± 0.01 c
tert-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB)[c] (12)	3.84 ± 0.07 a	4.34 ± 0.45 a	0.96 ± 0.09 b	0.58 ± 0.02 c
3-oxo-β-ionone[c] (13)	2.01 ± 0.18 a	1.48 ± 0.03 a	0.74 ± 0.04 b	0.44 ± 0.01 c
actinidols[c] (14)	2.84 ± 0.32 a	2.24 ± 0.04 a	0.91 ± 0.01 b	0.75 ± 0.01 c
norisoprenoid 1[c] (15)	0.69 ± 0.04 a	0.73 ± 0.01 a	0.27 ± 0.01 b	0.05 ± 0.01 c
volatile phenols				
m-cresol	nd	0.65 ± 0.02 a	0.47 ± 0.03 b	nd
eugenol	nd	0.91 ± 0.01 a	0.17 ± 0.01 b	0.27 ± 0.03 c
4-vinylguaiacol (16)	76 ± 1 a	31 ± 2 b	31 ± 2 b	26 ± 1 b
4-vinylphenol (17)	8565 ± 92 a	994 ± 73 b	9602 ± 90 c	2426 ± 242 d
vanillin derivatives				
vanillin (18)	2.96 ± 0.08 a	4.12 ± 0.03 b	1.46 ± 0.10 c	1.75 ± 0.19 c
zingerone (19)	0.76 ± 0.01 a	nd	nd	1.07 ± 0.07 b
benzenes				
benzaldehyde (20)	6.82 ± 0.11 a	4.94 ± 0.17 b	3.80 ± 0.35 b	4.74 ± 0.30 b
phenylacetaldehyde (21)	3.60 ± 0.28 a	2.66 ± 0.08 b	2.16 ± 0.03 c	2.16 ± 0.03 c
benzyl alcohol (22)	37 ± 1 a	20 ± 1 b	14 ± 1 c	8.45 ± 0.49 d
β-phenylethanol (23)	9.39 ± 0.62 a	7.55 ± 0.18 a	6.09 ± 0.29 b	6.17 ± 0.37 b
ethyl cinamate (24)	8.71 ± 0.69	nd	nd	nd
2-phenoxyethanol (25)	5.20 ± 0.42 a	7.95 ± 0.67 b	3.29 ± 0.42 c	5.42 ± 0.42 a
benzoic acid (26)	131 ± 12 a	80 ± 7 b	129 ± 3 a	116 ± 5 a
lactones				
δ-octalactone[d] (27)	2.89 ± 0.15 a	2.10 ± 0.18 b	14 ± 0 c	7.65 ± 0.93 d
γ-nonalactone[d] (28)	1.89 ± 0.13 a	1.94 ± 0.16 a	1.42 ± 0.07 b	1.45 ± 0.16 b
γ-decalactone[d] (29)	12 ± 1 a	5.55 ± 0.45 b	23 ± 1 c	26 ± 2 c
pantolactone (30)	1.66 ± 0.01 a	1.28 ± 0.01 a	0.93 ± 0.01 b	0.84 ± 0.01 c
miscellaneous				
(Z)-3-hexen-1-ol (31)	5.26 ± 0.41 a	5.21 ± 0.08 a	4.85 ± 0.04 b	4.10 ± 0.00 c
(E)-2-hexen-1-ol (32)	19 ± 2 ab	24 ± 1 a	17 ± 1 b	18 ± 1 b
ethyl decanoate (33)	16 ± 1 a	17 ± 0 a	16 ± 0 a	16 ± 0 a
2-ethylhexanoic acid (34)	13 ± 1 a	14 ± 1 ab	13 ± 1 a	15 ± 1 b
4-methoxy-2,5-dimethyl-3(2*H*)-furanone (mesifurane)[c] (35)	5.07 ± 0.02 a	22 ± 1 b	34 ± 1 c	42 ± 1 d
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (Furaneol)[c] (36)	8.15 ± 0.01 a	39 ± 1 b	16 ± 1 c	19 ± 1 c
cinnamic acid[c] (37)	1678 ± 36 a	178 ± 24 b	850 ± 49 c	877 ± 61 c

[a]Concentrations of the same compound with different letters show significant differences ($p < 0.05$). nd, not detected. [b]Peak number in Figure 2 is given in parentheses. [c]Chemical standard not available. Tentatively identified. Data are relative areas (to 4-methyl-2-pentanol × 1000). [d]Data are the relative areas (to 4-methyl-2-pentanol × 1000). Chemical standard available, but the degradation of the products did not allow quantification.

0.3 min and a pressure pulse of 25 psi for 2.60 min was applied. The injector was then heated to 250 °C at a rate of 200 °C/min. The splitless time was 2.60 min. The injection volume was 4 μL. The global run time was recorded in full scan mode (m/z 40−220 mass range). The chromatographic data were analyzed by Varian Saturn GC-MS version 6.3 software.[23]

To carry out the olfactometric analyses, we followed the protocol described in Ferreira et al.[21] The sensory panel was composed of six expert sniffers. Each strawberry extract was smelled once a day by each panelist. Sniffing time was approximately 30 min. The experiments were carried out in a Thermo 8000 series GC equipped with a flame ionization detector (FID) and a sniffing port (ODO-1 from SGE) connected by a flow splitter to the column exit. The chromatographic conditions were the same as described in Campo et al.[22] Tasters were asked to score the intensity of each aromatic stimulus using a 4-point scale (0 = not detected, 1 = weak, 2 = clear but not intense note,

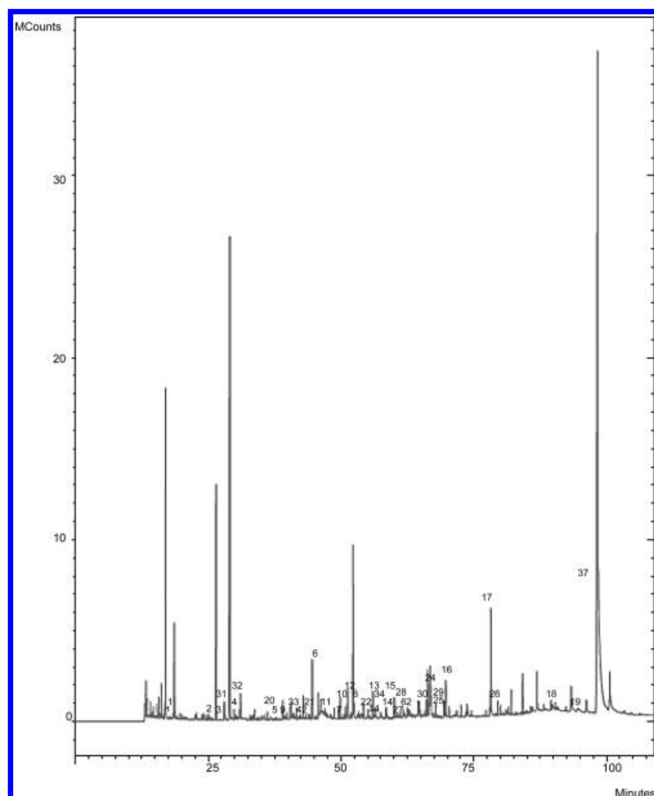

Figure 2. GC-MS chromatographic profile of the strawberry variety Fuentepina.

3 = intense note). Results were expressed as "modified frequency" (MF), calculated with the formula proposed by Dravnieks.[24] The identification of the odorants was done by comparison of their odors, chromatographic retention indices, and MS spectra with those of pure reference compounds.

Data Treatment. Analysis of variance (ANOVA) was performed using the Statistica (version 7.0) software package (Statsoft, Tulsa, OK, USA). Principal component analysis (PCA) was carried out using Unscrambler vs 9.7 from Camo (Oslo, Norway).

RESULTS AND DISCUSSION

Influence of Type of Hydrolysis. In general, the concentrations of the released compounds were very different depending on the type of hydrolysis (Table 1 and Figure 1). PCA was performed to observe which conditions were related to the release of the different compounds. As can be seen in Figure 1, PC1, which explains 47% of the variance, clearly separates the acid hydrolyses from the enzymatic ones. Also, PC2 (35% of the variance) groups the samples as a function of time. As the time of hydrolysis increased, the concentrations of norisoprenoids, volatile phenols, benzenes, and lactones were higher. The behavior of terpenes was heterogeneous. The amounts of α-terpinolene, (*Z*)-rose oxide, and neric acid increased during the harsh hydrolysis, reaching the highest amount after 4 h of the hydrolytic assay. However, the remaining terpenes reached their maximum concentration between 15 min and 1 h of hydrolysis. In the case of vanillin derivatives, each compound followed a different trend. With respect to the miscellaneous group, it is important to mention the cases of Furaneol and cinnamic acid, which increased during hydrolysis, reaching their maximum after 4 h.

Results after leaving the precursors pool for 1 week at 45 °C in citric buffer did not show great differences from the aforementioned hydrolysis. However, hydrolysates from the enzymatic assay were very rich in linalool, 3-oxo-α-ionol, and some volatile phenols such as eugenol, 4-vinylguaiacol, and 4-vinylphenol. Vanillin derivatives were also released more effectively. Moreover, this hydrolysis resulted in an extract with high amounts of benzyl alcohol and β-phenylethanol. With regard to Furaneol, there were no significant differences between 1 h of acid hydrolysis and 1 h of enzymatic hydrolysis. On the other hand, when harsh acid hydrolysis was applied, the release of terpenes, with the exception of linalool, was greater. These results are in accordance with previous studies[8] in which different hydrolytic strategies have been compared. The enzymatic hydrolysis was much more efficient for releasing volatile phenols, vanillin derivatives, and benzenes such as β-phenylethanol and benzyl alcohol than acid hydrolysis.

Despite these results, we decided to apply acid hydrolysis to perform the assays in each strawberry extract due to its similarity with alcoholic fermentation.[8] This was done to compare the results with a hypothetical strawberry fermentation. The time period chosen was 1 h as a compromise between compounds that are degraded after 4 h and those that are not formed earlier than this.

Study of the Aglycones Released from Hydrolysis of Four Strawberry Varieties. With the results obtained after testing the selected strategies taken into account, 1 h of harsh acid hydrolysis was applied for the analysis of minor aromatic compounds released from nonvolatile precursors of the four strawberry varieties.

As can be observed in Table 2, within the analyzed varieties, Fuentepina (Figure 2) proved to have the highest quantity of aromatic compounds present as precursors. After this, Camarosa and Sabrina varieties presented high levels, the Candonga variety being the poorest in these nonaromatic molecules.

In general, among the aglycones quantified, the major ones were linalool, α-terpineol, geraniol, 4-vinylguaiacol, 4-vinylphenol, benzyl alcohol, benzoic acid, γ-decalactone, and cinnamic acid. The presence of 4-vinylphenol in strawberries, especially in Candonga variety, is remarkable because it reached values between 0.9 and 9.6 mg/kg of fruit. This is in agreement with the results obtained by Groyne,[18] who observed a great amount of variability of this compound related to the strawberry variety.

The Sabrina variety was characterized by high amounts of terpenes, presenting discrete values for the rest of the aglycones with respect to the other varieties tested.

One of the most important components of strawberry flavor is 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one (Furaneol),[6] which is responsible for the sweet, caramel, and burnt sugar notes at high concentrations and fruity at lower concentrations. This compound reached the highest levels in Camarosa variety. Another important compound of this fruit is mesifurane, which is described with similar descriptors. In this case, Sabrina showed the highest levels of mesifurane as a glycosidically bound aroma form.

Finally, it is important to remark that XAD-2 Amberlite was the adsorbent employed for the determination of strawberry aromatic precursors in previously published works. In this work we tested the effectiveness of LiChrolut EN cartridges. This resin has been demonstrated as being more efficient that the Amberlite used in previous works by other authors. We identified a total of 51 aglycones with LiChrolut EN resins, 38 of which had previously not been reported in strawberry. Knowing the aromatic potential of the strawberries gives us an idea of the overall final aroma of a product made from this fruit, and therefore we could select the best variety as starting substrate.

Odor Active Compounds Determined Using GC-O. We performed olfactometric analyses of the free aroma compounds of four varieties of strawberry. This extraction technique enables

Table 3. Odor Active Compounds of the Four Strawberry Varieties Analyzed

			% modified frequency[a]			
LRI VF5-MSDBWax	odor descriptor	identity	Fue	Cam	Cdo	Sab
918	solvent, gas, glue	not identified	0	0	31	0
972	dairy product, sweet, buttery	diacetyl	55	61	55	78
1007	fruity, strawberry, sweet	isobutyl acetate	0	48	33	24
1033	fruity, strawberry, sweet	ethyl butanoate	69	59	75	73
1052	fruity, sweet, anise, cream	ethyl 2-methylbutanoate	50	46	29	0
1066	fruity, apple, anise, green, metallic	ethyl 3-methylbutanoate	61	33	69	73
1180	rubber, moisture, gas	not identified	0	0	0	34
1191	fruity, anis	methyl hexanoate	17	0	0	33
1236	fruity, raspberry, strawberry, anise	ethyl hexanoate	43	33	62	55
1303	mushroom, metallic, chlorine, cucumber	1-octen-3-one	51	33	45	53
1312	spicy, green, barbecue, yeast	2-methyl-3-furanthiol	55	0	50	36
1346	floral, sweet, strawberry	(*Z*)-rose oxide	38	0	0	0
1378	geranium, green, pepper, lettuce	(*Z*)-1,5-octadien-3-one	82	51	80	29
1380	tropical, pineapple, citrus, green	methyloctanoate[b]	0	0	61	0
1458	vinegar	acetic acid	67	38	48	38
1548	green, grass, sweet, cucumber	(*E*)-2-nonenal	41	0	0	0
1552	garbage, sulfur, peanuts, barbecue	not identified	0	0	49	0
1563	floral, lemon	(*R*/*S*)-linalool	33	0	0	0
1570	unpleasant, fatty acid, vomit, vinegar	not identified	0	0	0	40
1597	tropical, sweet, caramel, cotton candy	mesifurane[b]	43	33	35	45
1609	strawberry	not identified	31	0	0	0
1626	burnt hair	2-acetylpyrazine	73	61	75	53
1631	cheese, vomit, feet	butyric acid	27	17	35	43
1676	cheese, feet, sweat, milk	isovaleric acid	59	67	61	61
1730	fruity, honey, berry, tropical, sweet, floral	phenyl acetate[b]	0	31	22	38
1826	sweet, floral, rose	β-damascenone	0	26	0	41
1850	soil, green, spicy, pepper, peanuts, dry grass	hexanoic acid[b]	65	54	66	58
1865	camphor, barbecue, spicy	guaiacol	45	76	35	59
2052	caramel, strawberry, sweet	Furaneol	82	82	80	85
2100	leather, animal, stable	*p*/*m*-cresol	31	47	33	36
2170	peach, sweet, strawberry	γ-decalactone	80	26	85	83
2221	animal, spicy, licorice	sotolon	45	76	0	31
2294	latex, spicy, burnt	not identified	0	0	0	53
2420	coconut, vanillin	γ/δ-dodecalactone	0	29	25	33

[a]Fue, Fuentepina; Cam, Camarosa; Cdo, Candonga; Sab, Sabrina. [b]Tentatively identified by lineal retention index and odor descriptor.

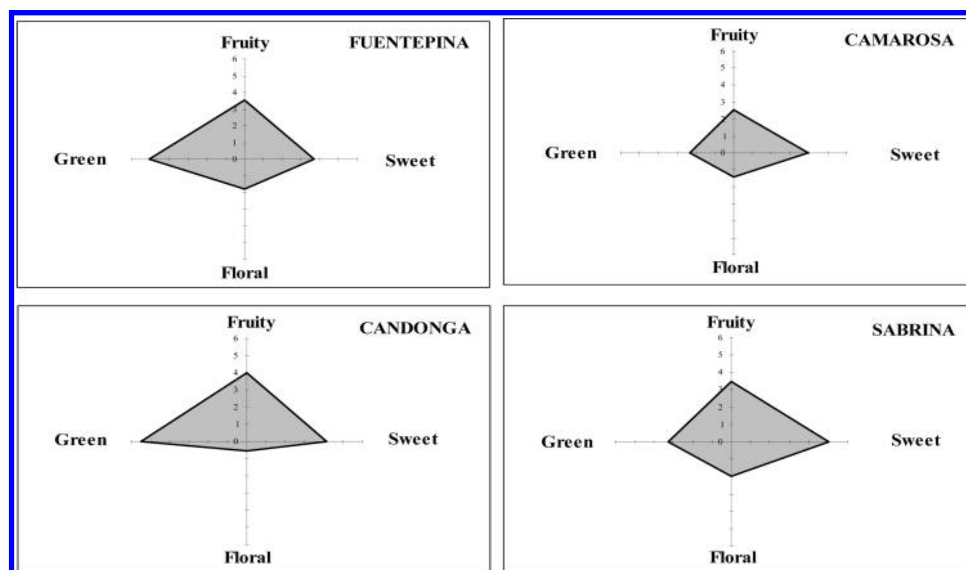

Figure 3. Sensory profile plot of Fuentepina, Camarosa, Candonga, and Sabrina varieties considering fruity, sweet, green, and floral characteres.

us to obtain a more representative extract than other techniques, and therefore it provides a more realistic idea of the overall sample flavor. Thirty-four important odor zones were perceived in the headspace extract. Table 3 shows the modified frequency (MF) of all the perceived odorants; only those with MF > 30 in at least one sample (odor active compounds) are included. Among these perceived aromatic zones, six were not identified.

Within the odor zones that had the greatest impact in the majority of the strawberry varieties, we identified Furaneol, γ-decalactone, ethyl butanoate, ethyl hexanoate, ethyl 3-methylbutanoate, diacetyl, and hexanoic acid, in agreement with other studies.[25,3,4] These compounds, therefore, seem to be responsible for the overall impact aroma of strawberries. They provide caramel-like, fruity, buttery, and sour notes. Furthermore, other odor zones with high MF were perceived in most of the varieties, with unpleasant notes such as cheese/feet/sweat/milk or burnt hair. We identified them as isovaleric acid and 2-acetylpyrazine. Panelists also perceived an odor zone described as geranium/green/pepper/lettuce (LRI = 1378) with an MF > 80 in Fuentepina and Candonga varieties, identified as (*Z*)-1,5-octadien-3-one. This odor zone had been observed by other authors but, to our knowledge, it had not been identified. There are some odor zones that clearly differ one variety from the others. This is the case of the floral/sweet/strawberry (LRI = 1346) and floral/lemon (LRI = 1563) notes identified as (*Z*)-rose oxide and (*R*/*S*)-linalool and which are present in only Fuentepina strawberry. In the Candonga variety tasters perceived a tropical/pineapple/citrus/green (LRI = 1380) odor zone with a high MF (61), tentatively identified as methyl octanoate, which was not perceived in the other strawberries.

As expected, Furaneol reached a high MF (≥80) but mesifurane MF values hovered at 33–45. These compounds, like the rest of the aglycones, are released during the fruit ripening stage, their presence increasing as a free form in ripe strawberry.[18] Depending on the fruit developmental stage, different aglycones will appear. This explains why some data from the precursors analysis (Table 2) do not match the olfactometric results. (*Z*)-Rose oxide is present only as a precursor in the Fuentepina variety and was perceived only in this variety during the olfactometric analysis. Additionally, panelists perceived the peach/sweet/strawberry (LRI = 2170) odor zone identified as γ-decalactone with a very high MF (≥80) in all varieties except for Camarosa. This odor zone reached a low MF (26), a similar situation occurring in the precursors determinations. However, the results obtained in olfactometric and precursors assays for linalool and β-damascenone do not match. As mentioned above, this confirms the staggered release of the aglycones. In conclusion, we could say that there were some odor zones that clearly differ among varieties, being present in only one of the varieties.

We used spider webs to have a general visual comparison of the four strawberry varieties considering fruity, sweet, floral, and green aroma characters (Figure 3). For that purpose, we added the MF of the odor zones of each character type of every strawberry (divided by 10) and then divided by the total of odor zones found for that character during the olfactometric analysis. Differences can be observed among the different strawberry varieties. The Camarosa variety was the least aromatic one because its aromatic zones reached the lowest MF. Green character predominates over sweet in Fuentepina and Candonga; however, in the other two varieties the sweet character is stronger than the other attributes.

In the case of Candonga, the figure shows that the floral character is almost imperceptible compared to the fruity character, which is very high.

In summary, the results suggest that this method is suitable for the determination of glycosidically bound aroma compounds of strawberry. There were several significant differences among varieties with respect to the content in precursors, Fuentepina being the variety that had the highest quantity of aromatic compounds present as precursors. A total of 38 aglycones have been described for the first time in strawberry.

In general, the key odorants were Furaneol, γ-decalactone, ethyl butanoate, ethyl hexanoate, ethyl 3-methylbutanoate, diacetyl, and hexanoic acid. In addition, we could state that the presence of some odor zones clearly differs among varieties. On the other hand, if we consider fruity, sweet, floral, and green aroma characters, the overall aroma of Fuentepina and Candonga varieties presented mainly green notes; however, in the case of Camarosa and Sabrina varieties the aromatic notes were mainly sweet.

AUTHOR INFORMATION

Corresponding Author

*E-mail: c_ubeda@us.es. Phone: 34-954-556761. Fax: 34-954-233765.

Funding

This research was made possible through financial support from the Spanish government by means of predoctoral grant BES-2008-003116 and research project AGL2007-66417-C02-01 funded by the Ministry of Science and Innovation.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to Juan Jesús Medina from Finca El Cebollar for providing the strawberries. The authors are also grateful to the panelists.

ABBREVIATIONS USED

LRI, linear retention index; FID, flame ionization detector; MF, modified frequency; PCA, principal component analysis; ANOVA, analysis of variance; GC-O, gas chromatography−olfactometry; GC-MS, gas chromatography−mass spectrometry.

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3.3. Análisis sensorial y pruebas de preferencia

3.3.1. Resumen

En el presente capítulo se presentan los resultados tras la evaluación de las características sensoriales de los condimentos de fresa obtenidos en la campaña del 2009. Se incluyen los resultados del análisis descriptivo de las muestras realizado por expertos y de las pruebas de preferencia con consumidores potenciales de los mismos.

Los condimentos evaluados fueron los producidos a partir de pasta de fresa en barriles de madera de roble y de cerezo y el elaborado en recipiente de cristal, y el condimento obtenido a partir del mosto cocido. Como referencia se utilizó un vinagre comercial de vino blanco.

En las pruebas de preferencia participaron 52 posibles consumidores a los que se les pidió una valoración de cada muestra (aroma y gusto) haciendo uso de una escala de 11 puntos, siendo 0, muy desagradable, y 10, muy agradable. El análisis descriptivo fue llevado a cabo por un panel experto compuesto por 8 miembros utilizando 16 atributos que habían sido seleccionados por consenso.

Los resultados obtenidos tras la realización de las pruebas de preferencia mostraron que el condimento preferido tanto por el gusto como por el aroma fue el producido a partir del mosto cocido.

Los datos de ambas pruebas sensoriales se relacionaron mediante técnicas estadísticas multivariantes y se construyeron mapas de preferencia. Mediante éstos pudimos averiguar que los descriptores pasa, caramelo tostado, punzante y licoroso eran los que condicionaban la preferencia de los consumidores.

Elsevier Editorial System(tm) for Food Research International
Manuscript Draft

Manuscript Number:

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Article Type: Research Article

Keywords: preference mapping, strawberry vinegar, sensory analysis, acceptability.

Corresponding Author: Dr. M. L. Morales,

Corresponding Author's Institution: University of Seville

First Author: C Ubeda

Order of Authors: C Ubeda; R.M. Callejón; A.M. Troncoso; M. L. Morales

Abstract: Consumer's opinion is very important for launching new products to market. In this work, consumer's preference tests have been carried out to know the sensory acceptance of new strawberry vinegars produced by a double fermentation process. The results showed that vinegars from strawberry cooked must was considered the best for its odor as well as for its taste for the highest percentage of consumer. Moreover, internal and external preference mapping techniques have been applied to interpret the vinegars acceptance results and to establish the relationship between the consumer's preferences and the descriptive sensory attributes. Internal preference mapping confirmed the low consumers' preference for the commercial vinegar in relation to strawberry vinegars. Furthermore, internal preference mapping pointed out that there are slight differences between odor and taste consumers' preferences. Thus, while vinegar obtained from strawberry puree were preferred for their odor, the vinegar from cooked must and the one produced in cherry barrel were preferred for their taste. External preference mapping revealed that the sensory attributes that mainly drive consumers' preferences are raisin, toasted caramel, spicy and liquer aroma.

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Title: EVALUATING CONSUMER ACCEPTANCE OF NEW STRAWBERRY VINEGARS BY PREFERENCE MAPPING

Authors: C. Ubeda, R.M. Callejón, A.M. Troncoso, M.L. Morales*

Address: Área de Nutrición y Bromatología. Facultad de Farmacia. Universidad de Sevilla. c/ P. García González nº2, E- 41012. Seville. Spain.

*Corresponding author: e-mail: mlmorales@us.es; Tel.: 34-954-556760; Fax.: 34-954-551245

Abstract

Consumer's opinion is very important for launching new products to market. In this work, consumer's preference tests have been carried out to know the sensory acceptance of new strawberry vinegars produced by a double fermentation process. The results showed that vinegars from strawberry cooked must was considered the best for its odor as well as for its taste for the highest percentage of consumer. Moreover, internal and external preference mapping techniques have been applied to interpret the vinegars acceptance results and to establish the relationship between the consumer's preferences and the descriptive sensory attributes. Internal preference mapping confirmed the low consumers' preference for the commercial vinegar in relation to strawberry vinegars. Furthermore, internal preference mapping pointed out that there are slight differences between odor and taste consumers' preferences. Thus, while vinegar obtained from strawberry puree were preferred for their odor, the vinegar from cooked must and the one produced in cherry barrel were preferred for their taste. External preference mapping revealed that the sensory attributes that mainly drive consumers' preferences are raisin, toasted caramel, spicy and liquer aroma.

Keywords: preference mapping, strawberry vinegar, sensory analysis, acceptability.

1. Introduction

The development of new products is necessary for food companies to survive in today's highly competitive market. When new commodities are going to be marketed, is very important to know the consumer's opinion about them. A form to answer to this question is by means of the consumer's evaluation of these new products by hedonic sensory test.

On the other hand, the sensory characterization of products using trained judges to determine the food quality properties is also essential in the development and innovation of products.

Preference mapping is a useful tool for the analysis and a deeper understanding of consumer's preferences and, in some cases, it allows to know which descriptive sensory attributes drive consumer's preferences.

Preference mapping is a graphical display created by multivariate analysis methods which allows researchers to understand influences of attributes on consumer liking (Michon, O'Sullivan, Sheehan, Delahunty, & Kerry, 2010; Sinesio et al. 2010), differences among products (Felberg, Deliza, Farah, Calado, & Donangelo, 2010; Villanueva, & Da Silva, 2009), and segments of products and consumers (Oupadissakoon, Chambers, Kongpensook, Suwonsichon, Yenket, & Retiveau, 2010; Sveinsdóttir, Martinsdóttir, Green-Petersen, Hyldig, Schelvis, & Delahunty, 2009; Young, Drake, Lopetcharat, & McDaniel, 2004). Additionally, preference maps are used in predicting new prototypes for industries.

There are different types of preference mapping. For instance, internal preference mapping uses only consumer data to determine consumer's preference patterns, whereas external preference mapping relates consumer's preference data to descriptive sensory information and/or instrumental data (Lawlor & Delahunty, 2000). Hence, the consumer

liking can not only relate with product sensory attributes but also with physicochemical data (Gambado, Ares, Giménez, & Pahor, 2007).

Internal preferences mapping is a principal component analysis of the matrix of hedonic scores across the products and the consumer, which is carried out on a covariance matrix to allow for differences in the strength of the consumer's preferences to be expressed (Guinard, 1998). Thus, internal preference mapping provide a summary of the main preference directions and the associated consumer segments (Greenhoff & Macfir, 1994). External preference mapping regresses the individual consumer's preferences onto a set of descriptive or other analytical ratings across products (Guinard, 1998). The procedure requires an objective characterization of product sensory attributes, achieved by descriptive analysis, which is then related to preference ratings for the product obtained from a representative sample of consumers (Murray & Delahunty, 2000).

Internal and external preference mapping techniques have been implemented in the study of a wide variety of products such as beer (Guinard, Uotani, & Schlich, 2001), tomatoes (Sinesio et al., 2010), cheeses (Drake, Lopetcharat, Clark, Kwak, Lee, & Drake, 2009; Young et al., 2004), chocolate milks (Thompson, Drake, Lopetcharat, & Yates, 2004), filled chocolates (Miquelim, Behrens, & Da Silva Lannes, 2008), hot dogs (Ramirez et al., 2010), soybean paste (Kim, Hong, Song, Shin, & Kim, 2010), soy-coffee beverages (Felberg, Deliza, Farah, Calado, & Marino, 2010) and tropical juice (Serrano-Megías, Pérez-López, Núñez-Delicado, Beltrán, & López-Nicolás, 2005).

The aim of the present work was to assess the sensory acceptance of 4 new strawberry vinegars. Furthermore, we researched consumer's preferences using internal preference mapping and the relationship between the consumer's preferences and the descriptive sensory attributes by external preference mapping techniques. This was done for the

purpose of finding out which sensory properties drive consumer liking for these products.

2. Material and methods

2.1. Samples

The vinegars analyzed in this study were produced from second quality strawberries of Camarosa variety in the laboratory of the Department of Biochemistry and Biotechnology (Faculty of Enology, University Rovira i Virgili, Tarragona). For this purpose, the strawberries were crushed with a beater and then, the alcoholic fermentations were performed inoculating with a selected yeast strain *Saccharomyces cerevisiae* (Ubeda, Callejón, Hidalgo, Torija, Mas, Troncoso, & Morales, 2011). The wine obtained was dispensed in three different containers: a glass vessel, and oak and cherry wood barrels. Each of them was filled with 5.5 L of wine. After that, the wine was inoculated with a selected strain of acetic acid bacteria. Thus, three different vinegars were obtained depending on type of container used: FV9G (glass), FV9C (cherry wood barrel) and FV9O (oak wood barrel).

Furthermore, part of the puree strawberries was concentrated by heating; the resulting product was a cooked must. 1 L of this must was submitted to alcoholic and acetous fermentation in the same way as the one without concentration. All these fermentation processes took place in a glass container. The vinegar obtained was named FV9CM.

In addition, one common commercial wine vinegar (CWV) was used in the consumer's preference test to compare the acceptability of strawberry vinegars with one usually employed by consumers.

2.2. Descriptive analysis

The aroma of the four samples of strawberry vinegar was evaluated by an expert panel composed of eight tasters (six females and two males). All members belonging to the

laboratory staff were trained according to international protocols (ISO, 1983; 1985). Fifteen milliliters of vinegar sample was presented in dark glass covered with a plastic dish. Quantitative descriptive analysis (QAD) was carried out using 16 sensory terms: pungent, ethyl acetate, herbaceous, red fruit, tropical fruit, ripe fruit, raisin, sweet, cheese/rancid, leather/animal, white wine, toasted caramel, spicy, liquer, aromatic complexity and general impression. The panel selected by consensus these terms to describe the samples during preliminary tasting sessions. The selected attributes were put on a tasting-card and panelists were asked to rank each descriptor on a 10-cm unstructured scale (from unnoticeable to very strong).

2.3. Consumer testing

A total of 52 regular consumers of vinegar were recruited from the IFAPA Research Center (Córdoba). The number of consulted consumer was limited by the available sample volume. The ages ranged from 20 to 60 years old, being 61% female and 39% male. Consumers were asked to evaluate their odor and taste liking using an 11-point hedonic scale, where 0= dislike extremely and 10= like extremely. The four vinegars were evaluated by consumers in a single session. Fifteen milliliters of the sample were presented in coded black glasses covered with a plastic dish. First, consumer was asked to smell the samples. Then, for the evaluation of taste as condiment, the consumer put several drops on a piece of lettuce and tried each sample.

2.4. Statistical analysis

ANOVAs were conducted to assess significant differences ($p<0.05$) among consumer's acceptance scores of the different vinegar samples. To interpret the consumer's preferences, a principal component analysis (PCA) based on covariance matrix of consumers (variables) by products (objects) were performed to obtain an internal preference map. Partial least square (PLS) regression model was applied to link

consumer's preferences to sensory data. PLS was carried out using the descriptive variables (16 attributes x 4 samples) as regression matrix (X data) and the consumer's odor preference scores (52 variables x 4 samples) as regressand (Y data). ANOVAs and PCA were performed with Statistica, version 7.0 software (Statsoft, Tulsa, USA) and PLS2 was conducted with the statistical software Unscrumbler v. 9.1 (Camo Process AS, Oslo, Norway).

3. Results and discussion

3.1. Consumers' preferences

The Table 1 presents the mean liking rating for each vinegar sample. The highest acceptance level was for vinegar from strawberry cooked must. However, no significant differences ($p<0.05$) were found among this vinegar and the other strawberry vinegars for any of the parameter evaluated. These results can be explained since the intravariability of consumer's preferences is higher than the intervariability among samples. The commercial vinegars got the lowest mean score with significant differences with respect to the other samples.

In Figure 1, the percentage of consumers who chose each vinegar in first, second, third, fourth and fifth place by preference are given. Regarding odor liking (Figure 1a), the percentages showed that the sample chosen as first by the highest percentage of consumer was vinegar from cooked must matching with the sample which reaches the highest score of preference. Furthermore, for a high percentage of consumers, the second position was for vinegar produced in glass container, the third and fourth for vinegar produced in oak and cherry barrel respectively. For the half of consumers, the commercial vinegar was the least liked.

The order of vinegar concerning to the taste preference of consumers was not so clear, except for commercial vinegar ranked fourth by a large amount of consumers (Figure

1b). However, we can say that vinegar from cooked must was again preferred by consumers since high percentage of them classified it as first and second. On the other hand, it seems that vinegar produced in cherry barrel reached a slightly higher score in the taste than those produced in glass container and oak barrel. Moreover, consumers considered that vinegar produced in cherry barrel present better taste than odor.

3.1. Internal preference mapping

The internal preference maps (Figure 2 and 3) were created by the principal component analysis (PCA) of a data matrix with consumers (variables) and products (objects).

Regarding the odor of vinegars, PCA results showed that the first two principal components explained 66.67% of total variance among acceptation of samples. The Figure 2a shows scores of samples plotted into the plan of the first two principal components. In this graph, we can observe the samples separate into three groups, one formed by commercial vinegar, another by strawberry vinegar produced in glass container and the third one that included strawberry vinegars produced in wood barrels and the other one produced from cooked must. These two last sample groups appear closer in the graph, separated of the remaining vinegars by PC2. In the Figure 2b, each point represents the correlation between the consumer's acceptance data and the first two components. Thus, most of consumers are placed in the right quadrants being positively correlated with the first component and, therefore, they preferred the vinegars obtained from strawberry puree. Although, vinegar FV9CM reached the highest mean acceptability score, the number of consumer correlated with this sample was not higher than with the other strawberry vinegars. However, if we consider PC2 (Figure 2), there was a large number of consumers located in the first two quadrants also showing a high acceptance of the vinegars located in said quadrants (FV9C, FV9O and FV9CM) .

The location of product CWV on the graph confirmed the low consumers' preference for this vinegar.

In relation to the sample taste, the first two PCs explained 63.35% of total variance. In this case, the graph of sample scores into the plan of the first two principal components revealed that the consumers' preference for the vinegars are very different, and the samples are far apart except FV9CM and FV9C (Figure 3a). Regarding loadings of consumer's acceptance (Figure 3b), there is a greater number of consumers negatively correlated with the first component showing a preference for the samples above mentioned.

3.2. External preference mapping

After the analysis of consumers' preferences, we were interested in knowing which aromatic characteristics determined this preference pattern of consumers. Thus, the data of consumer preferences and sensory descriptive analysis of the four strawberry vinegars were use to built the external preference map (Figure 4). PLS2 regression analysis conducted for external preference mapping explained 70% of sensory variance and 34.21% of liking variance. Most consumers are correlated negatively with the PC1, and located in the same direction as FV9CM sample.

These results are consistent with earlier, FV9CM reached the highest mean score of overall odor liking, in order of preference, it was chosen as first by the highest percentage of consumer.

The attributes which were highly related in a positive way to preference were raisin, toasted caramel, general impression, spicy, liquer, aromatic complexity, sweet, ripe fruit, herbaceous, red fruit, and, in a lesser extent, ethyl acetate. Thus, the projection of sensory attributes on external preference mapping allowed the identification of raisin, toasted caramel, spicy and liquer aroma as the major driver of acceptance. Toasted

caramel is the aroma character of two important volatile compounds of strawberry, furaneol and mesifurane, considered strawberry impact odorant due to their low odour threshold and their high quantities (Aubert, Baumann, & Arguel, 2005).

Meanwhile, attributes related to unpleasant aromatic notes such as cheese/rancid, leather/animal or pungent among others, were positioned in the right side of the map, where lower number of consumer were. As expected, these attributes had a negative correlation with preference.

4. Conclusions

Strawberry vinegars reached higher scores than commercial vinegar of white wine in the consumer's preference tests. This fact indicates that strawberry vinegars could be commodities with a good market acceptance.

Internal preference mapping allowed us a quick interpretation of data. Thus, internal preference mapping revealed that there were differences in the odor and taste preferences of consumers with regard to the studied vinegars. Respect to the odor, vinegars from strawberry puree (without concentration) were preferred by more consumers and vinegar from cooked must and that produced in cherry wood barrel were considered the best by a high number of consumers for their taste. These results showed that consumers preferred vinegars from strawberry than commercial white wine vinegar.

External preference mapping, where only strawberry vinegars were considered, showed that the attributes that mainly drive consumers' preferences are raisin, toasted caramel, spicy and liquer aroma.

Acknowledgements

This research was made possible through the financial support from the Spanish Government by means of a predoctoral grant and the research project AGL2007-66417-C02-01 funded by the Ministry of Science and Innovation. The authors wish to thank

Agromedina enterprise for providing the fruit substrates and Dr. A. Mas' research group from University Rovira i Virgili for providing fruit vinegars. We also want to thank Dr. Francisco Peña for his invaluable help in performing the consumer's preference tests.

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Figure Captions

Figure 1. Percentage of consumers who chose each vinegar in descending rank order of preference: ■ FV9CM, ■ FV9G, □ FV9O, ■ FV9C, □ CWV.

Figure 2. Internal preference map obtained by PCA on the consumer's data of overall odor preference: a) sample score plot; b) consumers' loading plot.

Figure 3. Internal preference map obtained by PCA on the consumer's data of overall taste preference: a) sample score plot; b) consumers' loading plot.

Figure 4. External preference map obtained by PLS2 analysis of descriptive data and consumer's overall odor liking of strawberry vinegars: a) sample score plot; b) correlation loadings of sensory descriptive and consumer's overall odor liking data.

Figure

Figure 1a.

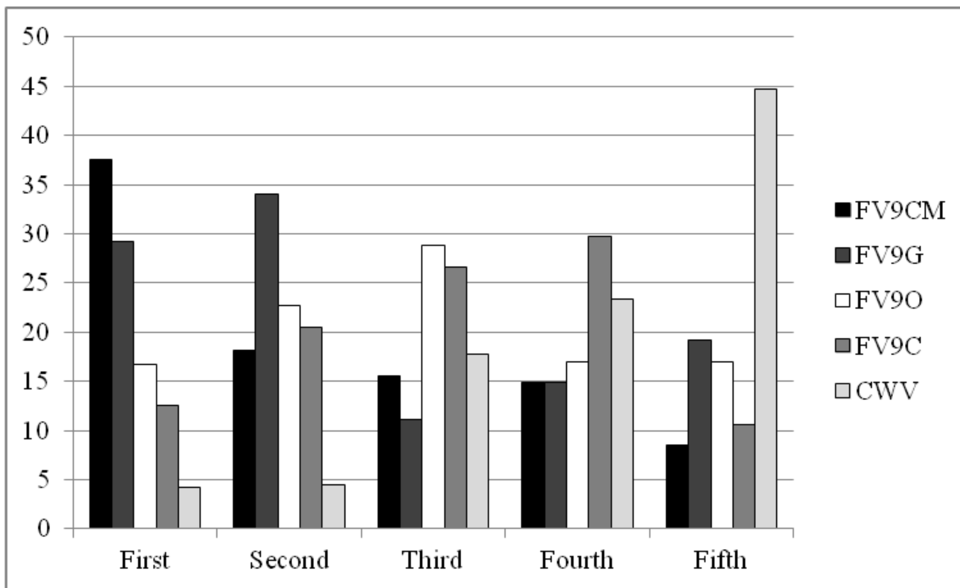


Figure 1b.

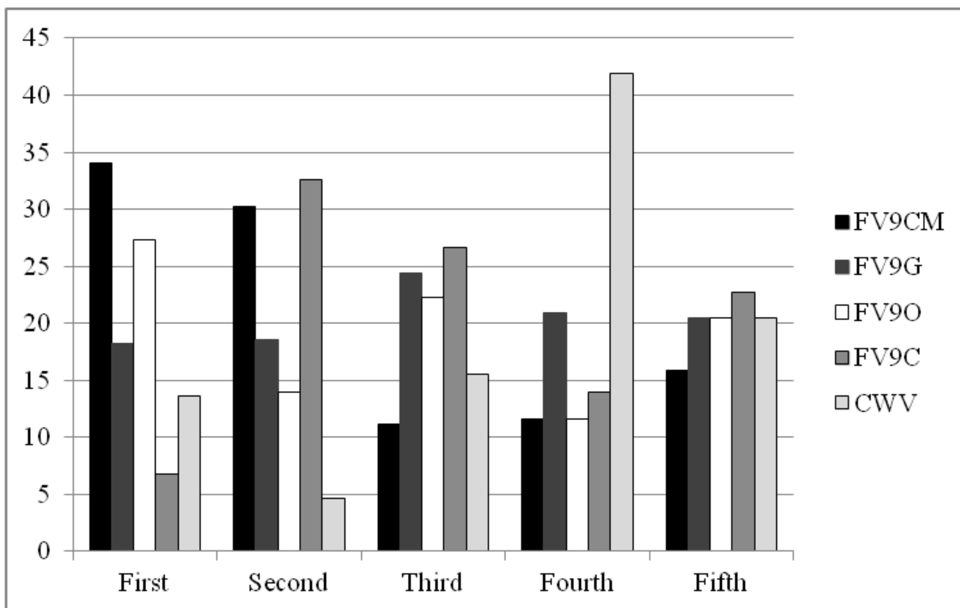
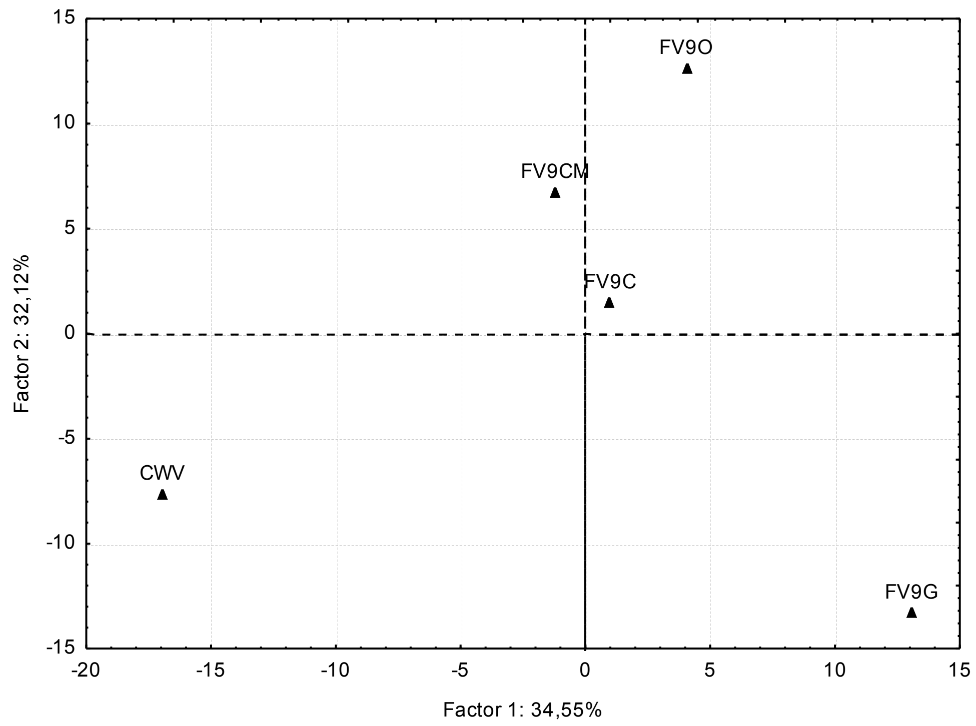


Figure 2a.



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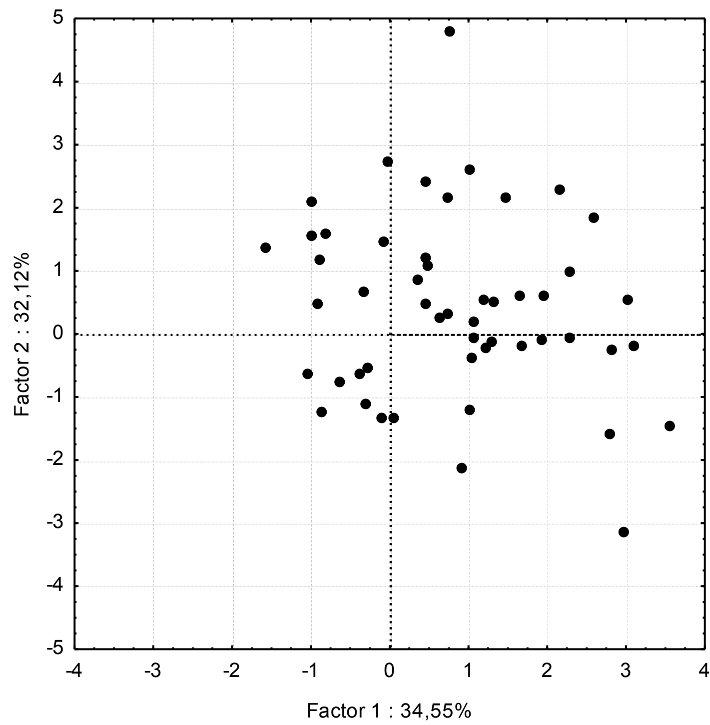

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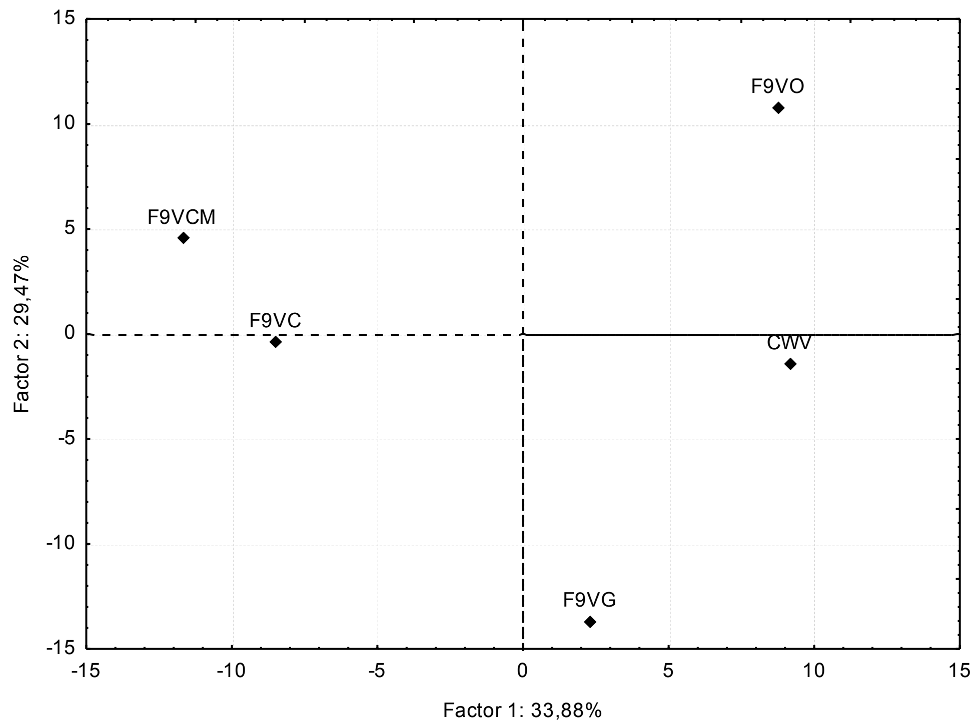


Figure 3b.

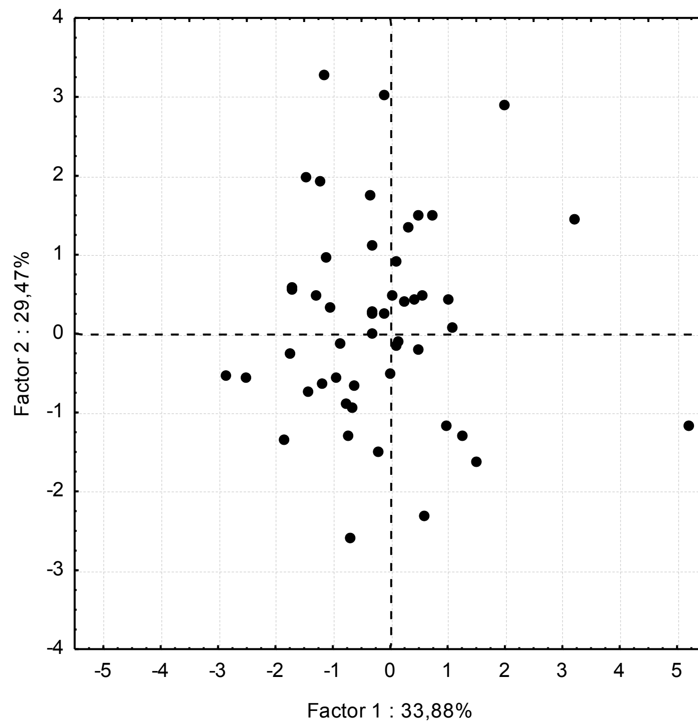


Figure 4a.

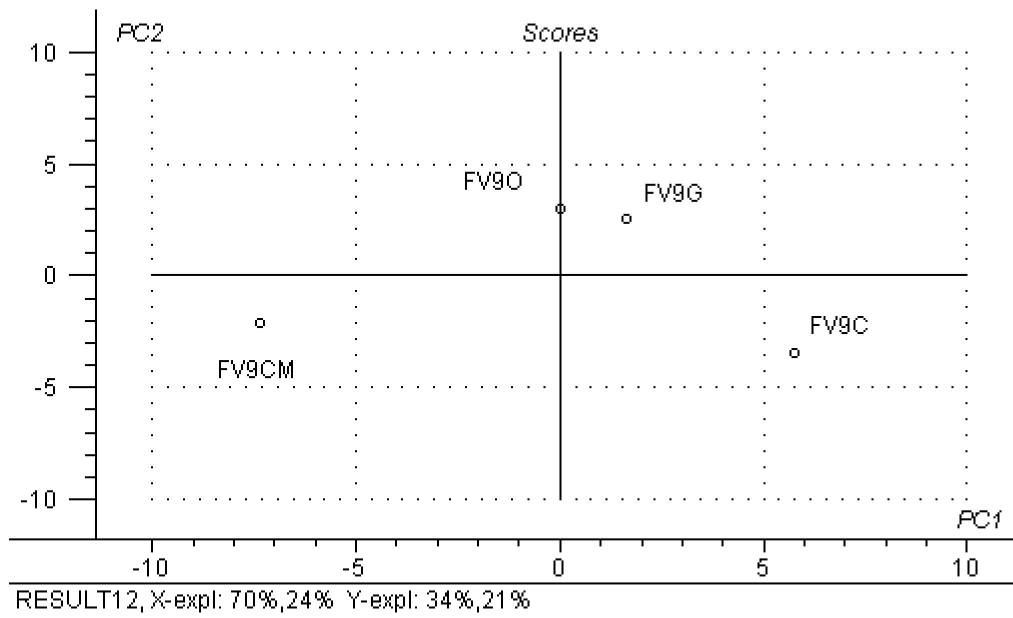
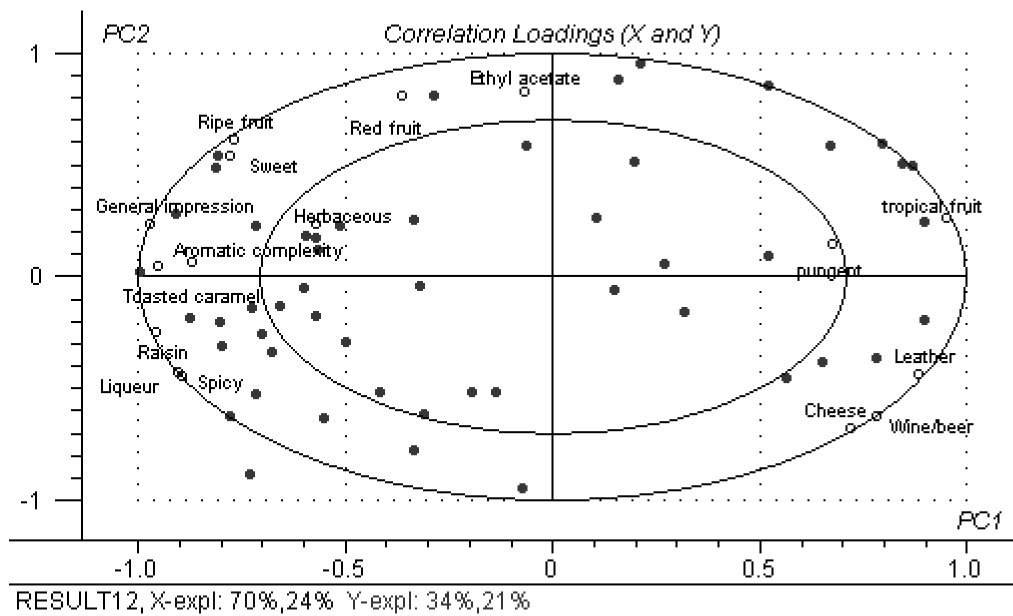


Figure 4b.



Table

	Samples				
	FV9G	FV9C	FV9O	FV9CM	CWV
Overall odor liking	6.2 ± 2.8	5.6 ± 2.2	6.1 ± 2.6	6.6 ± 2.7	3.9[a] ± 2.2
Overall taste liking	6.1 ± 2.5	5.6 ± 2.3	6.4 ± 2.7	6.44 ± 2.5	5.1[b] ± 2.3
Overall acceptance[c]	6.2 ± 2.6	5.6 ± 2.2	6.2 ± 2.6	6.50 ± 2.6	4.6[a] ± 2.3

[a]Significant difference ($p>0.05$) with all other samples.

[b]Significant difference with other samples except FV9C.

[c]This value was a mean between odor and taste liking values

3.4. Medida de la actividad antioxidante, contenido en fenoles totales y antocianinas monoméricas

3.4.1. Resumen

En este capítulo se estudia la evolución de la actividad antioxidante (AA) y el contenido de compuestos fenólicos totales durante la fermentación alcohólica y acética de la fresa y del caqui. De esta manera, podemos diseñar el mejor proceso para obtener los condimentos que conserve al máximo las cualidades saludables del sustrato de partida. Para el análisis de estos parámetros fue necesario establecer un protocolo de extracción y optimizar las variables del mismo. Tras la obtención del extracto, se efectuaron las medidas de actividad antioxidante por los métodos ORAC (Capacidad de absorbancia de los radicales de oxígeno) y DPPH (2,2-difenil-1-picrilhidrazil), y el índice de polifenoles totales (TPI). En el caso de la fresa se estudiaron además las antocianinas monoméricas totales (TA).

Los resultados mostraron que para ambos tipos de frutas, el uso de ultrasonido durante 25 minutos dió lugar a los mejores extractos (mayor actividad antioxidante y contenido en compuestos fenólicos). Con respecto al tipo de extractante, se consiguió una óptima extracción empleando etanol al 80% en el caso del caqui y acetona al 80% en el de la fresa.

Durante la primera etapa de elaboración de los condimentos se observó que la **adición de enzimas pectolíticas y SO_2** al sustrato produjo un incremento en todos los parámetros estudiados en ambas frutas, producido principalmente por la acción de las enzimas al romper las estructuras celulares que contienen las moléculas antioxidantes. Por otro lado, en el caso de la fresa, se compararon tres posibles **tipos de sustrato de partida:** puré, líquido obtenido de la centrifugación del puré, y mosto cocido. Los resultados indicaron que los condimentos obtenidos a partir de mosto cocido fueron los que presentaron los mayores valores de AA y TPI. Para la elaboración a nivel industrial el sustrato más adecuado sería la pasta de fresa, mientras que el mosto cocido podría ser adecuado para la elaboración de un condimento con características especiales a pequeña escala.

Los condimentos que procedían de los vinos en los que se impuso o se inoculó la cepa de *Saccharomyces cerevisiae* RP1 presentaron mayores valores de actividad antioxidante.

Además, se testaron diferentes **recipientes para realizar la fermentación acética** en fresas, y se observó que el uso de barriles de madera, especialmente de cerezo, producía condimentos con alta actividad antioxidante y más ricos en compuestos fenólicos. Esto es

debido a un fenómeno de concentración y de extracción de compuestos con actividad antioxidante procedentes de la madera.

En el caso del caqui, desde la pasta inicial hasta el condimento final, no se constataron pérdidas importantes en los parámetros estudiados. Sin embargo, en la producción de condimentos de fresa, si se produjeron descensos acusados en dichos parámetros, siendo la acetificación la etapa que más les afectó. Finalmente, el aumento de la **escala de producción** a escala semi-piloto repercutió positivamente en las características saludables de los condimentos de fresa al haber menos pérdida en la actividad antioxidante y en los contenidos totales de compuestos fenólicos.

Si comparamos los condimentos obtenidos a partir de caqui y fresa, éstos últimos exhibieron mayores cualidades saludables. Además, al compararlos con otros vinagres comerciales (vinagre de vino tinto, de vino blanco, de manzana, de Jerez, y balsámico de Módena) el condimento de fresa ocupó un segundo lugar, sólo superado por el vinagre balsámico. Esto lo señala como un producto potencialmente competitivo en el mercado en relación a los parámetros estudiados en este capítulo.

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LWT - Food Science and Technology 44 (2011) 1591–1596

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Evaluation of antioxidant activity and total phenols index in persimmon vinegars produced by different processes

C. Ubeda [a], C. Hidalgo [b], M.J. Torija [b], A. Mas [b], A.M. Troncoso [a], M.L. Morales [a,*]

[a] *Área de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Sevilla C/P, García González no 2, E- 41012 Sevilla, Spain*
[b] *Departamento de Bioquímica y Biotecnología, Facultad de Enología, Universitat Rovira i Virgili C/Marcel·lí Domingo s/n, E- 43007 Tarragona, Spain*

ARTICLE INFO

Article history:
Received 15 July 2010
Received in revised form
16 February 2011
Accepted 2 March 2011

Keywords:
Antioxidant activity
Persimmon
Diospyros kaki
Vinegar
Wine
Acetification

ABSTRACT

The total phenols index (TPI) and antioxidant activity of persimmon vinegars produced by different processes were evaluated. A novel extraction method was designed and optimised for this purpose with respect to the type and concentration of solvent and ultrasonication time. The best extraction conditions found were the use of 80% ethanol and 25 min of ultrasonication. Antioxidant capacity was determined by the oxygen-radical absorbance capacity of fluorescein (ORAC-FL) and 2,2′-diphenyl-1-pycrylhydrazyl (DPPH) free-radical assays. The antioxidant activities were the same in the fruit and the vinegar, except in the ORAC assay, which showed a significant decrease during the acetification process. The results showed that using the wild yeast strain native to the persimmon produced vinegars with higher antioxidant activity than that of an inoculated alcoholic fermentation. Finally, a comparison between our vinegars and other commercial examples was made. The TPI and antioxidant activity values of persimmon vinegars were always higher than those obtained from white and red-wine vinegars. The antioxidant activity and total phenols of the final product indicate that persimmon vinegar is a competitive product in the market.

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1. Introduction

Currently, consumer interest in the health benefits of foods is increasingly important, motivating more research in this area in recent years. Furthermore, consumers are demanding value-added products with new characteristics; therefore, the purpose of many investigations has been to elaborate new products providing health benefits. The main raw materials used to obtain these new products are fruits and vegetables. Several studies have shown a negative correlation between the consumption of fruits and vegetables and risks for cardiovascular disease, cancer, inflammation or problems associated with ageing (Dillard & German, 2000; Garcia-Closas, Gonzalez, Agudo, & Riboli, 1999; Joseph et al., 1999; Prior & Cao, 2000; Steinmetz & Potter, 1996; Wargovich, 2000).

Each year a large fraction of every fruit harvested is discarded because their size is outside the standard range, deformations or overproduction. For this reason, we proposed a study of the utilisation surplus fruit for vinegar production. Persimmon was one of the fruits selected for this purpose; it is mainly consumed fresh and the processing industry is scarcely developed. Persimmon is widely consumed in China and traditionally used for medicinal purposes such as coughs, hypertension, dyspnoea, paralysis, burns and bleeding (Mowat, 1990). It has also been demonstrated to have an inhibitory effect on human lymphoid leukaemia cells (Achiwa, Hibasami, Katsuzaki, Imai, & Komiya, 1997), and in some persimmon varieties such as *Mopan* a positive effect on hypercholesterolemia has been reported (Gorinstein et al., 1998). It is assumed that these "nutraceutical" properties are due to the antioxidant components of this fruit, including phenolic compounds (Yokosawa & Okumura, 2007), vitamins and carotenoids.

There are many methods available for the evaluation of antioxidant activity; most are colorimetric assays, so it is necessary to have a sample or extract free of solid particles. Sometimes an extraction method is required due to sample consistency. The established techniques for the extraction of antioxidant compounds differ in some parameters such as the kind of solvent used, but the main objective of the extraction stage is always to recover as much of the bioactive fraction as possible with the highest efficiency (Spigno, Tramelli, & De Faveri, 2007a). Previous studies have reported the influence of several parameters (ultrasonication time, solvent type, temperature and percentage of extractant) in the extraction of phenolic molecules and antioxidant compounds in general (Alothman, Bhat, & Karim, 2009; Pinelo, Del Fabbro, Manzocco, Núñez, & Nicoli, 2005a; Spigno et al., 2007a).

* Corresponding author. Tel.: +34 954 556760; fax: +34 954 233765.
E-mail address: mlmorales@us.es (M.L. Morales).

0023-6438/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.lwt.2011.03.001

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Table 1
Samples description.

Type of sample	Treatment/Fermentation	Sample codex
Puree	No treatment	K7Z1
Puree	Pectolytic enzymes and sulphur dioxide	K7Z2
Wine	From K7Z2 by spontaneous alcoholic fermentation	K7WE1-K7WE3
Wine	From K7Z2 by inoculated alcoholic fermentation	K7WI1-K7WI3
Vinegar	From K7WE made by spontaneous acetification	K7VE1-K7VE3
Vinegar	From K7WI made by spontaneous acetification	K7VI1-K7VI3

The aim of this work was the evaluation of the antioxidant activity and total phenols index of persimmon vinegar[1] at each production step in a double fermentation process (alcoholic and acetic); the effect of spontaneous versus inoculated alcoholic fermentation on these parameters was of special interest. For this purpose, an extraction method was designed in which the following variables were optimised: the kind of solvent, solvent-to-water ratio and ultrasonication time. Finally, the values obtained for our vinegars were compared with some commercial vinegars.

2. Materials and methods

2.1. Samples

In this work we have employed three different persimmon (*Diospyros kaki var. Sharoni*) batches. Persimmons were harvested at commercial ripeness in November, 2007. This variety belongs to the group of non astringent persimmon. Batch 1 and batch 2, were acquired in the market and employed for the extraction process optimization. The batch 3, provided by Agromedina company, was used for the vinegar production. The elaboration process was performed in the laboratories of the Department of Biochemistry and Biotechnology (Faculty of Enology, University Rovira i Virgili, Tarragona), according to the following procedure: ~50 kg of persimmon fruit was crushed with a beater to obtain 45 L of puree. 60 g/L of sulphur dioxide were added to avoid undesirable microbial growth. Additionally, two pectolytic enzymes were incorporated: Depectil extra-garde FCE® for volatiles release and Depectil clarification® to help clarify the product (Martin Vialatte Oenologie, Epernay, France), both at a concentration of 15 mg/L. This puree was then distributed into six glass vessels, with 6 L of sample in each. Three of these vessels were inoculated with the enological yeast QA23 at the concentration of 2×10^6 cells/mL and a spontaneous alcoholic fermentation was allowed to occur in the other three vessels. The resulting wines were acetified by a spontaneous process to produce the persimmon vinegars. At each fermentation stage, samples were taken (Table 1). Samples were stored in 30-mL amber glass flasks at −20 °C until analysis.

For solvent and percentage selection we used puree prepared in our laboratory from persimmon batch 1 and for the ultrasonic extraction time selection we have employed puree from persimmon batch 2.

2.2. Chemicals

The reagents acetone, methanol, Folin-Ciocalteu reagent, ethanol, anhydrous dipotassium hydrogen phosphate, sodium dihydrogen phosphate monohydrate, potassium chloride, sodium acetate and anhydrous sodium carbonate were provided by Merck (Darmstadt, Germany). Fluorescein sodium and gallic acid were supplied by Fluka (Madrid, Spain). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid ("Trolox"), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) free-radical were purchased from Sigma–Aldrich (Steinheim, Germany).

2.3. Sample-extraction process

Due to the different consistencies of the samples studied, it was necessary to establish an extraction system for the determination of total phenols index and antioxidant activity. To design the extraction method, we modified the procedures proposed by Gorinstein et al. (1999) and Chen, Fan, Yue, Wu, and Li (2008). Optimisation of the most influential parameters in the extraction method was required; the parameters optimised were type of solvent (acetone, methanol or ethanol), percentage of solvent (50%, 80% or 100%) and ultrasonic extraction time (15, 25, 35 or 50 min). The selection of the best extraction parameters was made by taking into consideration the maximum values obtained in each assay as well as economy of time and solvent use. The extraction conditions are shown in Fig. 1.

2.4. Antioxidant-activity assays

2.4.1. Oxygen-radical absorbance-capacity assay (ORAC-FL)

ORAC-FL was performed in a black 96-well microplate (BD Falcon, BD Biosciences, UK), following the method described by Dávalos, Gómez-Cordovés, and Bartolomé (2004) with some modifications. This assay was realised with a Multidetection plate reader (Synergy HT, Vermont, USA). Previously, fluorescein (60 nM) and appropriate dilutions of the samples were prepared along with solutions of different Trolox concentrations (0.5, 2, 3.5, 5, 6.5, 8,

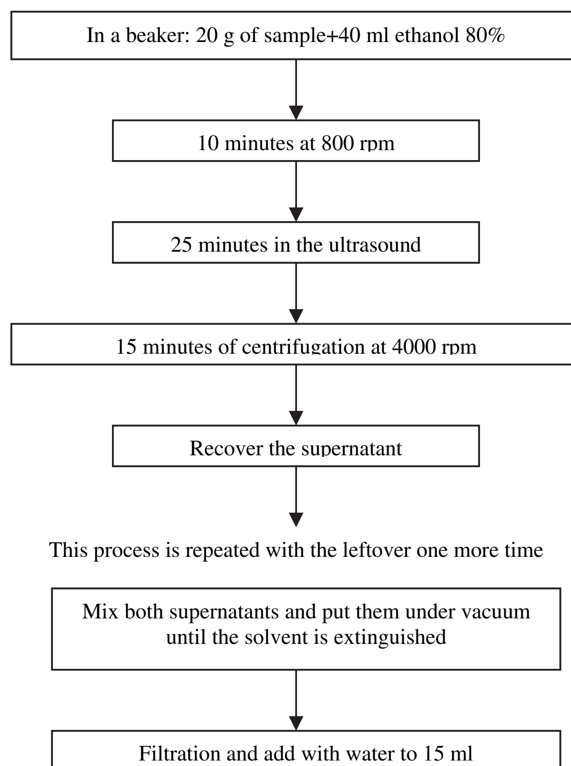

Fig. 1. Extraction process.

[1] Footnotes: Given the acidic nature of these products and the lack of a suitable alternative term, we decided to refer to these products as vinegars throughout the text, despite the fact that according to Spanish regulations some of these products are not sufficiently acidic to be classified as vinegars.

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9.5 μM) used to construct the calibration curve, all in 75 mM phosphate buffer (pH 7.4). First, the wells at the edges of the microplate were filled with 200 μL of buffer to moderate the temperature throughout the assay. Then all of the wells containing 50 μL of the sample (buffer, Trolox or assay sample) at the required dilution plus 50 μL fluorescein were preincubated for 15 min at 37 °C. Afterwards, 50 μL of fresh AAPH (15 mM in phosphate buffer) was rapidly added to the reaction using a multichannel pipette. Fluorescence measures were taken at intervals of 5 min over a period of 90 min. The excitation wavelength was set at 485 nm and the emission wavelength at 528 nm. All the reaction assays were realised in triplicate. The results are expressed as the area under the curve (AUC) as calculated by the Cao and Prior (1999) equation:

$$\text{AUC} = (0.5 + f_5/f_0 + f_{10}/f_0 + f_{15}/f_0 + \ldots f_{90}/f_0) \times 5$$

where f_0 is the initial fluorescence and f_i is the fluorescence at time i (minutes).

The final AUC values were calculated by subtracting the AUC of the blank from all of the results. For each experiment, a blank was assayed and a calibration line with different Trolox concentrations was made to obtain the regression equation and calculate the ORAC-FL final values, expressed as μmol Trolox equivalents (TE)/kg of sample.

2.4.2. DPPH radical-scavenging assay

The DPPH method employed to determine the radical-scavenging capacity of each sample was based on Brand-Williams, Cuvelier, and Berset (1995). Here, 0.1 mL of appropriately diluted sample was added to 3.9 mL of DPPH solution (0.025 g/L in methanol). The absorbance of the mixtures was measured at 515 nm using a cuvette filled with methanol as a blank. Readings were taken at $t_i = 0$ (the time of sample addition) and $t_f = 60$ min (when the reaction reached steady state). A UV/vis spectrophotometer U-2800 Digilab coupled to a Peltier thermostatic system (Hitachi,Tokyo, Japan) was used. Six different concentrations of Trolox (0.02, 0.06, 0.1, 0.14, 0.18 and 0.22 mM) were used in the same sample conditions to construct a calibration curve. The antiradical activity was calculated by considering the variation of the absorbance obtained, given by:

$$\text{Absorbance variation} = \text{Abs}_{t=60} - \text{Abs}_{t=0}$$

This absorbance variation was plotted versus the concentration of Trolox, the regression equation obtained and the sample values found by extrapolation. The final values were expressed as μmol Trolox equivalents (TE)/kg of sample. All the determinations were realised at least in triplicate.

2.5. Total phenols index (TPI)

This parameter was determined using the Folin-Ciocalteu method following the procedure of Waterhouse (2001). The concentrations of standards chosen to create the regression line were 50, 75, 100, 125, 150, 200, 250 and 500 mg/L of gallic acid. The absorbance of each coloured mixture was determined at 765 nm against a blank (distilled water). The assays were performed in triplicate and the results expressed as gallic acid equivalents (mg/L).

2.6. Statistical analysis

All statistical analyses were performed using the Statistica version 7.0 software package (Statsoft, Tulsa, USA).

3. Results and discussion

3.1. Optimisation of the extraction process

The criteria selected for optimisation of the extraction parameters (solvent, percentage of solvent and ultrasonication time) were the maximum values of antioxidant activity, total phenolics, and time and solvent savings.

3.1.1. Selection of the solvent

Despite being probably the most investigated parameter, solvent selection is still a complicated issue because extract yields and resulting antioxidant activities of the sample are strongly dependent on the nature of the extracting solvent. This is due to the presence of different antioxidant compounds of various chemical characteristics and polarities that may or may not be soluble in a particular solvent (Sultana, Anwar, & Ashraf, 2009). In our case, the solvents selected for the assays were acetone, methanol and ethanol. In these assays we used for all cases a mixture solvent: water at 80% and set an ultrasonication time of 15 min.

Fig. 2 shows that when we used ethanol as the solvent we got the best results in the ORAC assay (3630 μmol TE/kg) and TPI determination (330 mg gallic acid/kg). These values were significantly different from those obtained with acetone and methanol. These results may be explained based on the composition of persimmon, this kind of fruit contains different compounds (polyphenols, carotenoids, sugars, polysaccharides, vitamins, etc.) which provide antioxidant activity having different solvent affinity and response to the selected assays.

Using acetone as the extractant, we obtained the maximum antioxidant capacity in the DPPH assay (1730 μmol TE/kg). Significant differences were found between these values and those with the other solvents. However, this solvent gave the worst results in the case of the TPI determination and ORAC assay. With methanol, the extracts obtained the worst results for the DPPH assay and intermediate values for the ORAC and TPI assays.

3.1.2. Effect of solvent percentage

Some studies have suggested that the recovery of phenols is dependent on the fruit type and the kind and percentage of solvent used (Alothman et al., 2009). Because ethanol was the best solvent, we assayed aqueous solutions with the following percentages of ethanol: 50%, 80% and 100%, the ultrasonication time was set at 15 min. As shown in Fig. 3, maximum values for all of the parameters studied were obtained with the solvent:water ratio of 80:20. Our results are in agreement with those of Sultana et al. (2009), who evaluated methanol and ethanol and their mixtures with

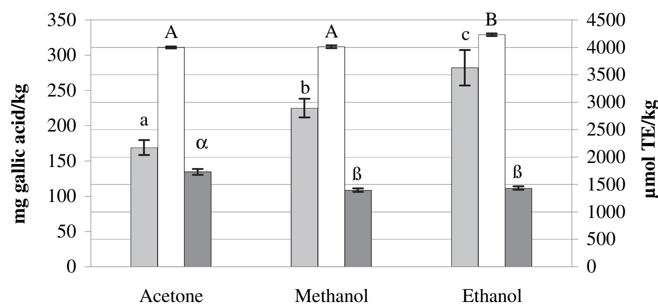

Fig. 2. ORAC, DPPH and TPI values of persimmon puree (batch 1) for the different extraction solvents tested □ TPI (mg gallic acid/kg); ■ DPPH (μmol TE/kg); □ ORAC (μmol TE/kg). The bars in the same assay with different letters show significant differences ($p < 0.05$) (a, b, c: ORAC assay; A, B, C: IPT; α, β, γ: DPPH test). ORAC and DPPH values are on the right axis and TPI values are on the left.

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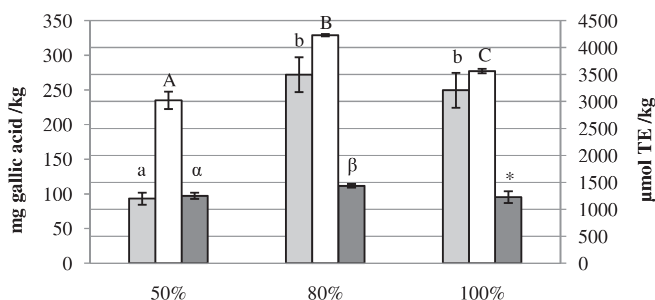

Fig. 3. ORAC, DPPH and TPI values of persimmon puree (batch 1) for the different solvent percentages tested □ TPI (mg gallic acid/kg); ■ DPPH (μmol TE/kg); □ ORAC (μmol TE/kg). The bars in the same assay with different letters show significant differences ($p < 0.05$); *: no significant differences with α and β (a, b, c: ORAC assay; A, B, C: IPT; α, β, γ: DPPH test). ORAC and DPPH values are on the right axis and TPI values are on the left.

water (80%) as extraction solvents for medicinal plants. Aqueous solutions exhibited better antioxidant activities and higher phenolic contents. Pinelo et al. (2005a) also concluded that mixtures of alcohols and water showed better recoveries of phenolic compounds than the corresponding monocomponent solvent systems. Accordingly, in the ethanol extraction of grape-seed powder, Yilmaz and Toledo (2005) reported an increase in extracted phenol content (as gallic acid equivalents) when they increased the amount of water in the mixture from 0% to 30%; phenol contents remained constant for 30, 40 and 50% water and decreased at higher percentages.

3.1.3. Impact of ultrasonication time

The mechanical effects and the acoustic cavitations produced in the solvent by the passage of an ultrasound wave allow for better penetration of the solvent into the sample matrix (Rostagno, Palma, & Barroso, 2003; Wang, Sun, Cao, Tian, & Li, 2008). Hence, the duration of ultrasonication is an important parameter to optimise. The best results with respect to antioxidant capacity were obtained using 25 min of ultrasonication, yielding 3595 (ORAC) and 1230 (DPPH) μmol of TE/kg, respectively (Fig. 4). With respect to TPI, the values were very similar, with no significant differences among the values at different sonication times. The TPI was 264.3 mg/kg of gallic acid at 25 min.

Extraction times longer than 25 min produced significant decreases for the parameters measured. These results agree with those of previous studies on the extraction of flavonoids from plants. Zhang, Shan, Tang, and Putheti (2009) tested different

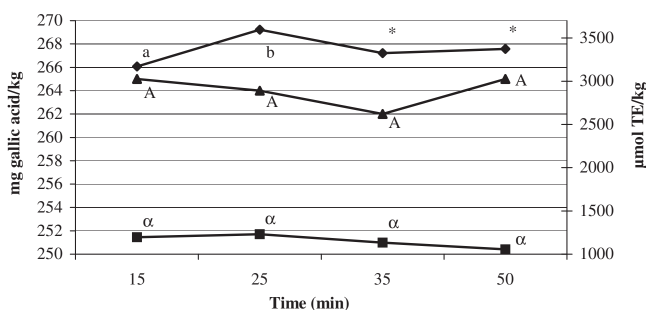

Fig. 4. ORAC, DPPH and TPI values persimmon puree (batch 2) for the different ultrasound times tested —▲—TPI (mg gallic acid/kg) —◆—DPPH (μmol TE/kg) —■— ORAC (μmol TE/kg). The markers in the same assay with different letters show significant differences ($p < 0.05$); *: no significant differences with a and b (a, b, c: ORAC assay; A, B, C: IPT; α, β, γ: DPPH test). ORAC and DPPH values are on the right axis and TPI values are on the left.

extraction times (20, 25, 30, 35, and 40 min) and the recovery of flavonoids decreased as ultrasonication time was extending beyond 25 min. On the other hand, the acoustic cavitations of ultrasound produce a progressive increase of temperature in the internal structure of the sample. Some authors (Pinelo, Rubilar, Jerez, Sineiro, & Nuñez, 2005b; Spigno & De Faveri, 2007b; Yilmaz & Toledo, 2005) have pointed out that temperature increases can denatured some phenolic compounds, so this fact could explain the loss of antioxidant activity too.

3.1.4. Evaluation of the extraction process

To test the efficiency of our extraction method we compared it with a centrifugation process. A persimmon wine sample was divided into two equal portions. One of them was subjected to a centrifugation process and the other was extracted by our method, the final volume of the obtained liquid extracts by both methods were adjusted to 15 mL. We then measured, the antioxidant activity and total phenols index of the supernatants collected from the centrifuged and the extracted samples. The ORAC, DPPH and TPI results using our extraction method was about 70%, 50% and 20% higher, respectively, than with a simple centrifugation.

3.2. Evolution of the antioxidant activity and total phenolics during the production of persimmon vinegars

After the optimisation of the extraction process, antioxidant activity and total phenols index were measured in the fruit puree, wine and vinegar samples.

3.2.1. Substrates

For the production of persimmon vinegars, the starting substrate was a puree of this fruit (batch 3). After obtaining the puree, sulphur dioxide and pectolytic enzymes were added. As can be observed in Table 2, this addition had technological benefits and a positive effect on the antioxidant character of the persimmon puree, increasing it and the phenols in solution. Phenols have been reported to be linked to cell-wall polysaccharides by hydrophobic interactions and hydrogen bonds. The release of these phenols may be improved by cell-wall degradation catalysed by enzymes (Pinelo, Arnous, & Meyer, 2006). The polysaccharides liberated from the cell-wall by the addition of pectolytic enzymes have antioxidant activity, as has been reported by several authors (Aguirre, Isaacs, Matsuhiro, Mendoza, & Zuniga, 2009; Chattopadhyay et al., 2009; Chen, Tsai, Huang, & Chen,

Table 2
Values of ORAC, DPPH and TPI for purees, wines and vinegar analyzed.

Sample	ORAC[a]	DPPH[a]	TPI[b]
Puree			
K7Z1	1891 ± 106	1289 ± 22	277 ± 22
K7Z2	2841 ± 66	1540 ± 39	424.1 ± 2.4
Wine			
K7WE1	2542 ± 215	1758 ± 75	288 ± 13
K7WE2	3192 ± 341	1838 ± 15	295.6 ± 2.2
K7WE3	3557 ± 232	1870 ± 162	320.6 ± 6.6
K7WI1	2816 ± 195	1421 ± 134	245 ± 20
K7WI2	3142 ± 282	1649 ± 88	300.3 ± 4.4
K7WI3	3637 ± 70	1699 ± 44	300.3 ± 4.4
Vinegar			
K7VE1	2111 ± 1	1731 ± 64	317.5 ± 5.1
K7VE2	1894 ± 334	1615 ± 18	268.0 ± 3.2
K7VE3	1854 ± 205	1698 ± 88	273.2 ± 1.9
K7VI1	1780 ± 12	1627 ± 88	303.6 ± 4.5
K7VI2	2022 ± 182	1457 ± 64	384.8 ± 5.5
K7VI3	1479 ± 29	1482 ± 53	397.5 ± 3.9

[a] Expressed in μmol TE/kg.
[b] Expressed as mg gallic acid/kg.

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2009). Moreover, phenols and compounds with antioxidant activity confined in the vacuoles inside the cell could be released; this is the case with grapes (Pinelo et al., 2006). Conversely, sulphur dioxide may act through two different pathways: as a protector against oxidation (Delteil, Feuillat, Guilloux-Benatier, & Sapis, 2000) and as a phenol extractor (Lee & Wrolstad, 2004). One possible explanation was given by Cacace and Mazza (2002), who reported that the addition of SO_2 reduced the dielectric constant of water and consequently increased the solubility of phenols, but the mechanism remains unknown.

3.2.2. Wines

Wines were obtained by two different kinds of alcoholic fermentations: spontaneous and inoculated. The measured values of the three parameters varied among the three replicates from the same type of fermentation (Table 2). For this reason, we did not find significant differences between the inoculated and spontaneous wines with respect to ORAC and TPI, although the average antioxidant activity of inoculated wines was higher. However, for the DPPH assay, the spontaneous wines were significantly higher than the inoculated ones. The inoculation seemed to have no significant impact on persimmon purees with respect to TPI. Moreover, the contrary behaviour of the ORAC and DPPH assays did not allow us to come to a clear conclusion regarding the merits of inoculation of the substrates versus spontaneous fermentation.

3.2.3. Vinegars

Vinegars were obtained from spontaneous and inoculated wines by spontaneous acetification. With respect to the ORAC assay, values for the vinegars from spontaneous wines were higher than those from inoculated wines, but there was no significant difference between them (Table 2). DPPH values for vinegars from spontaneous wines were significantly higher than in the vinegars from the inoculated ones. Concerning the total phenols determination, vinegars from inoculated wines had significantly higher amounts of total phenols than the vinegars from spontaneous wines.

In summary, after the acetification process, vinegars from inoculated wines had higher TPI, whereas the vinegars from spontaneous wines had higher antioxidant activity. It should be noted that the two fermentations (alcoholic and acetic) took place in succession and therefore acetification was carried out in the presence of yeast lees, which might explain the variations in TPI and antioxidant activity values. In microbiological characterization of the whole process, differences were just found in the alcoholic fermentation. The results revealed that different kind of yeasts carried out the alcoholic fermentations: spontaneous and inoculated (data not shown). In spontaneous alcoholic fermentation are involved mainly strains of non-Saccharomyces yeast and the strain used in inoculated was not detected. The yeast may influence in two different ways: capturing polyphenols (Mazauric & Salmon, 2005; Razmkhab et al., 2002) and releasing antioxidant compounds, differently than the polyphenols, from inside cell and from cell-wall (Aredes-Fernández et al., 2010; Jaehrig, Rohn, Kroh, Fleischer, & Kurz, 2007). The way in which the yeast influences the antioxidant activity depends on the yeast strain. Since this was the only factor that varied between both processes this may be the reason for the different values of TPI and antioxidant activity found.

3.2.4. Overall changes

The changes in the studied parameters between the substrate and the vinegars are shown in Fig. 5. Regarding the ORAC assay, we observed an overall decrease of 34.6% from fruit (K7Z2) to vinegar. DPPH values also showed an increment during the alcoholic fermentation followed by a decrease after the acetic fermentation,

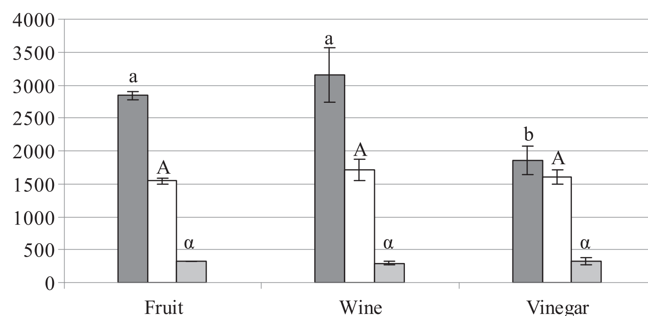

Fig. 5. Evolution of antioxidant capacity parameters (ORAC and DPPH) and TPI in the production process of persimmon vinegars (means values of K7Z2 substrates, wines set and vinegars set) ▢ TPI (mg gallic acid/kg) □ DPPH (μmol TE/kg) ■ ORAC (μmol TE/kg). The bars in the same trial with different letters show significant differences ($p < 0.05$) (a, b, c: ORAC assay; A, B, C: IPT; α, β, γ: DPPH test). ORAC and DPPH values are on the right axis and TPI values are the left.

resulting in an overall increase of 3.8%. With respect to the total phenols index, there was a decrease from the substrate to the wines and an increase from the wine to the final vinegar, for an overall increase of 1.6%. The final content of polyphenols was similar to that obtained by others for Hiratanenashi persimmon vinegar (Sakanaka & Ishihara, 2008).

A likely explanation for the different behaviours of the ORAC and DPPH assays might be the different reaction mechanisms of the substances in the reaction medium. The ORAC assay is a hydrogen-atom-transfer (HAT) reaction which quantifies hydrogen-atom donor capacity, whereas the DPPH method is a single-electron-transfer (ET) reaction which measures the antioxidant reducing capacity (Huang, Ou, & Prior, 2005). The overall balance was positive because only the ORAC underwent a significant decrease while DPPH and TPI had constant values.

3.3. Comparison with commercial vinegars

Several common vinegars were selected from the market to compare them with our persimmon vinegars. In Table 3, it can observed that the average antioxidant values of our vinegars were lower than balsamic, sherry and cider vinegars but always higher than red and white wine vinegars.

The antioxidant activity and total phenols index values in our persimmon vinegars were lower than those reported by previous authors (Sakanaka & Ishihara, 2008). This may be because the persimmon varieties used in their study are astringent, so they have tannins in their composition and the persimmon used to produce our vinegars is a variety non astringent and have a lesser content of tannins. Several studies have shown that tannins are the components mainly responsible for the antioxidant activity of persimmons (Gu et al., 2008).

Table 3
Antioxidant activities and TPI values of different kinds of commercial vinegars and mean values of our persimmon vinegars.

Sample	ORAC[a]	DPPH[a]	TPI[b]
Balsamic vinegar	40049 ± 663	8842 ± 163	2539 ± 6
Apple vinegar	8986 ± 106	2036 ± 75	343 ± 10
Sherry vinegar	7879 ± 270	2066 ± 23	467 ± 6
Persimmon vinegar[c]	1857 ± 220	1601 ± 111	324 ± 55
Red wine vinegar	1462 ± 3	1229 ± 66	229 ± 16
White wine vinegar	973 ± 153	939 ± 29	137 ± 10

[a] Expressed in μmol TE/kg.
[b] Expressed as mg gallic acid/kg.
[c] Mean values of K7VE1, K7VE2, K7VE3, K7VI1, K7VI2 and K7VI3.

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4. Conclusions

We determined that in the case of *Diospyros kaki var. Sharoni* the use of 80% ethanol and 25 min of ultrasonication were the best conditions among the variables assayed to obtain the greatest extraction of phenolic compounds and the highest values of antioxidant activity. The addition of sulphur dioxide and pectolytic enzymes had a positive effect on the antioxidant activity and total phenols index.

Comparing the two kinds of alcoholic fermentation, the spontaneous wines produced vinegars with higher antioxidant activity than the inoculated wines. Therefore, the isolation of the yeast strains involved in the spontaneous alcoholic fermentation and their use in this production process could be an important issue in improving the antioxidant activity of these vinegars.

The DPPH and TPI values remained constant during the processing from the fruit to the final vinegar, and the ORAC assay showed significant decrease after acetification. The antioxidant activity of the final vinegars was lower than what was reported by other authors because the variety used in this work belongs to the group of non astringent persimmons; however, it possessed higher values than other commercial vinegars like white and red-wine vinegars. These results suggest that persimmon vinegar has health-promoting qualities and could be a competitive product in the commercial market.

Acknowledgements

We wish to thank the Ministry of Science and Innovation of the Spanish government for their financial support of project AGL2007-66417-C02-01 and a predoctoral fellowship. We also thank Agromedina for their kind provision of the persimmons.

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LWT - Food Science and Technology xxx (2012) 1–7

Contents lists available at SciVerse ScienceDirect

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Employment of different processes for the production of strawberry vinegars: Effects on antioxidant activity, total phenols and monomeric anthocyanins

C. Ubeda [a], R.M. Callejón [a], C. Hidalgo [b], M.J. Torija [b], A.M. Troncoso [a], M.L. Morales [a,*]

[a] Área de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Sevilla, C/P. García González n°2, E-41012 Sevilla, Spain
[b] Departamento de Bioquímica y Biotecnología, Facultad de Enología, Universitat Rovira i Virgili, C/ Marcel·lí Domingo s/n, E-43007 Tarragona, Spain

ARTICLE INFO

Article history:
Received 9 November 2011
Received in revised form
14 February 2012
Accepted 20 April 2012

Keywords:
Antioxidant activity
Monomeric anthocyanins
Strawberry
Vinegar
Wine

ABSTRACT

The use of strawberry surpluses for the production of added value products seems to be a good solution choice to avoid the waste of this fruit. We produced strawberry vinegars through double fermentation (alcoholic and acetous) from three different harvests of *Fragaria x ananassa var. Camarosa.* The objective was to study the evolution of antioxidant activity, total phenols and monomeric anthocyanins during the vinegar production process. These parameters increased when sulphur dioxide and pectolytic enzymes were added to substrates. Inoculation with the *Saccharomyces cerevisiae* strain RP1 produced wines with half the anthocyanins with respect to the spontaneous fermentations. The use of wood barrels, particularly cherry wood barrels, had a positive effect on all the parameters determined. All measured parameters decreased during the double fermentation process. In general, the acetification stage led to a high loss of antioxidant compounds. Moreover, the production of these vinegars at a semi-pilot scale yielded final commodities with the best values for antioxidant activity, total phenols and monomeric anthocyanins comparing with the vinegars obtained in 2008 and 2009 harvest.

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1. Introduction

Strawberries are a widely researched fruit for their nutritional and health benefits as well as their organoleptic properties. This fruit is rich in vitamins, minerals, fibre and phytochemicals. In addition, strawberries contain potentially bioactive compounds and are a great source of phenolic compounds such as flavonoids and phenolic acids (Aaby, Skrede, & Wrolstad, 2005; Määttä-Riihinen, Kamal-Eldin, & Törrönen, 2004; Seeram, Lee, Scheuller, & Heber, 2006). All of these phenolic compounds have been shown to prevent oxidative processes, particularly those caused by reactive oxygen species (ROS) (Aaby, Ekeberg, & Skrede, 2007; Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, & Troncoso, 2010a). These compounds make strawberries a highly antioxidant fruit (Aaby et al., 2005; Wolfe et al., 2008) with potential health benefits. Among the numerous healthy properties described in the literature are anti-proliferative effects on cancer cells (Meyers, Watkins, Pritts, & Liu, 2003; Olsson, Andersson, Oredsson, Berglund, & Gustavsson, 2006) and the antioxidant and anti-inflammatory effects that have been shown to reduce cardiovascular disease risk factors in several prospective cohort studies (Hannum, 2004).

According to the latest data from the FAO (FAOStat, FAO, 2011), Spain is the second-largest strawberry producer in the world; a large portion of this production is harvested in Huelva (Andalucía). Every year, part of the crop is discarded for various reasons, including size or deformations of the berries, or overproduction which leads to surpluses. Because vinegar is generally an inexpensive product, its production requires low-cost raw materials, such as sub-standard fruit and seasonal agricultural surpluses (Solieri & Giudici, 2009). In addition, there is a growing demand for fruit vinegars, which are sold as a health food (Shau-mei & Chang, 2009). The use of strawberries of second quality, which are still suitable for human consumption, to production healthy vinegars with special organoleptic nuances may be a good method to reduce losses due to discarding the fruit.

For this purpose, we have produced strawberry vinegars using second-quality strawberries employing two-stage fermentation and assessed different conditions and treatments. The aim of this work was to evaluate the changes in the antioxidant activity (AA), total phenols index (TPI) and total monomeric anthocyanins (TA) during the production process of strawberry vinegar. In addition, an adequate extraction method to perform these determinations was designed.

* Corresponding author. Tel.: +34 954 556760; fax: +34 954 233765.
E-mail address: mlmorales@us.es (M.L. Morales).

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doi:10.1016/j.lwt.2012.04.021

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2. Materials and methods

2.1. Chemicals

The reagents acetone, methanol, Folin–Ciocalteu reagent, ethanol, di-potassium hydrogen phosphate (anhydrous), sodium di-hydrogen phosphate 1-hydrate, potassium chloride, sodium acetate and sodium carbonate (anhydrous) were purchased from Merck (Darmstadt, Germany). Fluorescein sodium and gallic acid were supplied from Fluka (Madrid, Spain). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma–Aldrich (Steinheim, Germany).

2.2. Samples

For the optimisation of the extraction process, we used strawberries (*Fragaria ananassa var. camarosa*) acquired at the market. The fruit was crushed in our laboratory, distributed into amber glass flasks and frozen at −20 °C.

For the production of the vinegars, we employed three different batches of strawberries (*Fragaria ananassa var. camarosa*) from the Huelva area (Spain), corresponding to three harvests: 2008, 2009 and 2010. The production processes were performed in the laboratories of the Dept of Biochemistry and Biotechnology, Faculty of Oenology, Univ Rovira i Virgili (Tarragona). In 2008 and 2009, the substrate employed were purees prepared in the laboratory using a beater. In 2010, we used a commercial puree provided by the Hudisa Company (Huelva). Sulphur dioxide (60 mg/L), sucrose and two types of pectolytic enzymes (Depectil extra-garde FCE® and Depectil clarification® from Martin Vialatte Oenologie, Epernay, France), both at a concentration of 15 mg/L, were added to the puree. After this point, the procedures were slightly different in each harvest.

2.2.1. 2008 harvest

One portion of the strawberry puree was pressed to study the effect of two types of starting substrates (semi-solid and liquid) (Table 1). Six glass containers were filled with 6 L of fruit substrate (four purees and two liquids). Half of the containers of each type of substrate were inoculated with the yeast *Saccharomyces cerevisiae* QA23 at a concentration of 2×10^6 cells/ml, and spontaneous alcoholic fermentation was allowed to occur in the other half. All wines were spontaneously acetified keeping it in the same containers. Two final treatments were tested in vinegars: pasteurization or centrifugation. The average acetic degrees in the 2008 strawberry vinegars were 4.8.

2.2.2. 2009 harvest

For the vinegar production in 2009, eight glass vessels were filled with 6 L of strawberry puree each. Half of these vessels were inoculated with the yeast strain *S. cerevisiae* RP1, isolated during the 2008 spontaneous alcoholic fermentation, and spontaneous alcoholic fermentation was allowed to occur in the other half. All of the wines obtained from the inoculated alcoholic fermentation were mixed and dispensed in three different types of containers: a glass vessel and oak or cherry wood barrels. Samples were then inoculated with a strain of acetic acid bacteria isolated from the 2008 acetification. Wines from the spontaneous alcoholic fermentation were processed in the same way and left to acetify spontaneously. The vinegars obtained were pasteurised. Inoculated vinegars from the 2009 harvest reached an acetic degree of 5.5 (glass container), 6.6 (oak barrel) and 6.3 (cherry barrel).

A portion of the puree from the 2009 strawberries was concentrated by heating in a water bath at 80 °C during 10 h, to test another method of increasing the sugar content; the resulting product was a cooked must (Table 1). The sucrose final concentration was 140 g/L. One litre of this substrate was fermented by a spontaneous process and 1 L was inoculated with the RP1 strain of yeast. The inoculated wines (IWs) were acetified with the same acetic acid bacteria isolated in 2008, and the spontaneous wines (SWs) were left to acetify spontaneously.

2.2.3. 2010 harvest

In this harvest, the pectolytic enzymes added were Rohapect® (12 mg/hL) and the pH was adjusted to 3.5 with 2 g/L $CaCO_3$. In this case, 45 L of puree were fermented in a stainless steel container on a semi-pilot scale, after inoculation with *S. cerevisiae* RP1. The acetous fermentation was performed in a cherry wood barrel. The vinegar had an acetic degree of 6.3.

All vinegars from 2009 to 2010 harvest were pasteurized as final treatment.

Forty-one samples, taken throughout these production processes, were analysed. The codes and characteristics of the samples are shown in Table 1. In addition, five commercial vinegars were also

Table 1
Samples description.

Harvest	Treatment	Puree Sample	Treatment	Sample substrate	Alcoholic fermentation (time)	Wine Sample	Acetification (time)	Treatment or Recipient	Vinegar sample
2008	Crushed	F8P1	SO_2 Pectolytic enzymes Sucrose (50 g/L)	F8P2	Inoculated (4 days)	F8WI1–F8WI4	Spontaneous (2 months)	Centrifugation	F8VIC1–F8SVIC2
								Pasteurization	F8SVIP1–F8SVIP2
					Spontaneous (5 days)	F8WE1–F8WE4		Centrifugation	F8SVEC1–F8SVEC2
								Pasteurization	F8SVEP1–F8SVEP2
	–	F8P2	Pressing	F8L	Inoculated (4 days)	F8LWI	–	–	–
					Spontaneous (5 days)	F8LWE			
2009	Crushed	F9P1	SO_2 Pectolytic enzymes Sucrose (75 g/L)	F9P2	Inoculated (5 days)	F9WI1–F9WI4	Inoculated (2 months)	glass vessel	F9SVIG
								oak barrel	F9SVIO
								cherry barrel	F9SVIX
					Spontaneous (8 days)	F9WE1–F9WE4	Spontaneous (2 months)	glass vessel	–
								oak barrel	–
								cherry barrel	–
			Heating Concentrated	F9MC	Inoculated (7 days)	F9MCWI1–F9MCWI2	Inoculated (5 months)	glass vessel	F9MCVI1–F9MCVI2
					Spontaneous (7 days)	F9MCWE1–F9MCWE2	Spontaneous (2.5 months)	glass vessel	F9MCVE1–F9MCVE2
2010	Crushed	F10P1	SO_2 Pectolytic enzymes Sucrose (65 g/L) $CaCO_3$	F10P2	Inoculated (4 days)	F10WI	Inoculated (1.5 months)	cherry barrel	F10VI

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analysed to carry out comparative studies: Aceto Balsamico, red wine and white wine vinegars, apple vinegar and sherry vinegar.

2.3. Sample-extraction procedure

The consistency of the samples (purees, wines and vinegars) made it necessary to establish an extraction system prior to analysis. The method employed was based on the extraction procedures designed and optimised previously by Ubeda, Hidalgo, et al. (2011). Twenty grams of sample were mixed in a beaker with 40 ml of extract for 10 min while shaking at 800 rpm. The sample was then subjected to ultrasonication followed by a centrifugation at 4000 rpm for 15 min. The supernatant was recovered, and the pellet was re-extracted with 40 ml of solvent following the same procedure. Both extracts were subsequently mixed, and the organic solvent was removed under vacuum. Finally, the extract was filtered, and MilliQ water was added to a final volume of 15 ml. Every extraction was performed in duplicate. We tested different condition to get the maximum values of AA, TPI and TA as well as economy of solvent used and time. Thus, the parameters studied to select the best extraction conditions were: type of solvent (acetone, methanol or ethanol), percentage of solvent (80% or 100%) and ultrasonic extraction time (15, 25, 35 or 50 min).

2.4. Assays and methods

2.4.1. ORAC-FL assay

The Oxygen Radical Absorbance Capacity assay (ORAC-FL) was performed in a Black 96-well microplate, following the procedure described in Ubeda, Hidalgo, et al. (2011). This assay was conducted in a Multi-detection plate reader (Synergy HT, Vermont, USA) located at the Centre for Research, Technology and Innovation at the University of Seville (CITIUS). All reaction assays were performed in triplicate. Results were expressed as μmol Trolox equivalents (TE)/kg of sample.

2.4.2. DPPH radical scavenging assay

To determine the radical scavenging capacity, the DPPH assay described by Brand-Williams, Cuvelier, & Berset (1995) was used. For this test, we used an UV/Vis spectrophotometer U-2800 Digilab coupled to a Peltier thermostatic system (Hitachi, Tokyo, Japan). Results were expressed as μmol Trolox equivalents (TE)/kg of sample. The assays were performed in triplicate.

2.4.3. Total phenols index

This parameter was determined in triplicate, using the Folin–Ciocalteu method following the procedure described in Waterhouse (2001). Results were expressed as mg gallic acid/L.

2.4.4. Total monomeric anthocyanins

The determination of total monomeric anthocyanin content (TA) was measured following the pH-differential method described in Giusti & Wrolstad (2001). TA was expressed as pelargonidin-3-glucoside (Plg-3-glu), which is the major anthocyanin in strawberry fruit with a $\lambda_{vis\text{-}max}$ at 510 nm (Swain, 1965). Two buffers were prepared: potassium chloride buffer pH = 1 (0.025 M), and sodium acetate buffer pH = 4.5 (0.4 M). We measured the absorbance at 510 and 700 nm against a cuvette filled with distilled water as a blank.

We then calculated the absorbance of the diluted sample (A) as follows:

$$A = (A_{510} - A_{700})_{\text{pH } 1.0} - (A_{510} - A_{700})_{\text{pH } 4.5}$$

The monomeric anthocyanin concentration in the original sample was calculated using the following formula:

$$\text{TA[Plg} - 3 - \text{glu (mg/L)]} = (A \times \text{MW} \times \text{DF} \times 1000)/(\varepsilon \times 1)$$

where, A = Sample absorbance, MW = Molecular weight of Plg-3-glu (487.5), DF = Dilution factor, ε = Absorption coefficient of Plg-3-glu (17,330).

The results were expressed as mg Plg-3-glu/kg of sample.

2.5. Statistical analysis

All statistical analysis was performed using the Statistica version 7.0 software package (Statsoft, Tulsa, USA).

3. Results and discussion

3.1. Selection of the best extraction conditions

Several factors, such as solvent composition, time of extraction, temperature, pH, solid-to-liquid ratio and particle size, may significantly influence solid–liquid extractions (Azizah, Ruslawati, & Tee, 1999; Pinelo, Del Fabbro, Manzocco, Nunez, & Nicoli, 2005). In our case, the parameters that were evaluated to determine the best extraction conditions were the type of solvent, the solvent–water ratio and ultrasonication time. The criteria used to select the extraction parameters were the maximum values of antioxidant activity, total phenols, anthocyanins and time and solvent savings.

The type of solvent is one of the most influential variables in the extraction process. We tested acetone, ethanol and methanol. The extraction with methanol gave the worst results in all the assays. As shown in Fig. 1, acetone yielded the highest values for DPPH (8327 μmol Trolox equivalents (TE)/kg) and TPI (2090 gallic ac. mg/kg), with significant differences in this last parameter. However, we obtained the best results for the ORAC assay (24,329 μmol TE/kg) and for the TA determination (26.78 mg Plg-3-glu/kg) using ethanol, but no significant differences were found between these values and those with acetone (26.30 mg Plg-3-glu/kg). Henríquez, Carrasco-Pozo, Gomez, Brunser, & Speisky (2008) reported that the antioxidant activity of strawberry extracts obtained with acetone/water was higher than that with ethanol/water and aqueous extracts. Taking into account this and other studies (Garcia-Viguera, Zafrilla, & Tomás-Barberán, 1998; Pinelo et al., 2005) and our results, we selected acetone for the strawberry extractions.

The solvent–water ratios assayed were 100 and 80:20 (acetone:water) (Fig. 2). The best results for all the parameters measured were obtained using a ratio of 80:20.

Finally, the extraction potential of ultrasound technique depends on the application time, so, we assayed 15, 25, 35 and

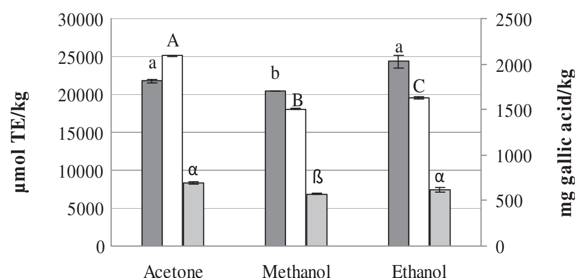

Fig. 1. ORAC, DPPH (left axis) and TPI (right axis) values for the different extraction solvents tested in strawberries acquired at the market. The bars in the same assay with different letters show significant differences ($p < 0.05$) (ORAC assay: a, b, c; IPT: A, B, C; DPPH test: α, β, γ).

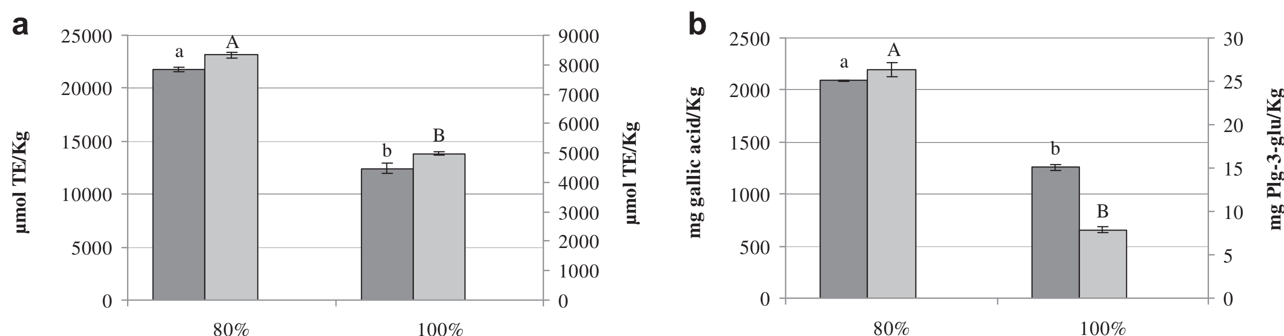

Fig. 2. Effect of solvent percentages. a) ORAC and DPPH values. b) TPI and TA values of strawberries acquired at the market. The bars in the same assay with different letters show significant differences ($p < 0.05$) (ORAC and TPI assays: a, b; DPPH and TA tests: A, B).

50 min. The ultrasonication time chosen was 25 min, since at this time ORAC, TPI and TA reached the highest values (Fig. 3).

3.2. Changes in AA, TPI and TA during the production of strawberry vinegars

3.2.1. Substrate pre-treatments

Three different strawberry purees were employed in this study. These purees presented similar values for all parameters, except the high values of TA in the substrate of the 2009 harvest. After the pre-treatments (pectolytic enzymes and SO_2 addition), we observed significant increases in almost all of the measured parameters, comparing P1 and P2 samples of each harvest (Tables 2–4). Considering the increases percentage, we observed a good correlation between the DPPH with TA ($r^2 = 0.998$) and with TPI ($r^2 = 0.971$) percentages. This could mean that these phenolic compounds are responsible for a percentage of the increases of AA.

Previous studies have shown that pectolytic enzyme treatment is very useful for the release of phenols and anthocyanins from different kinds of berries (Klopotek, Otto, & Boehm, 2005; Meyer, 2002). These enzymes were effective for the release of other phenolic compounds such as ellagic acid, which has been described as the main phenolic compound in berries from the *Fragaria* (strawberry) genus, representing 51% of the compounds analysed (Häkkinen et al., 1999). On the other hand, SO_2 protects against oxidation (Delteil, Feuillat, Guilloux-Benatier, & Sapis, 2000) and may be extracting anthocyanins and phenolic compounds. This effect was observed in blueberries (Lee & Wrolstad, 2004).

The 2008 liquid substrate had significantly lower values for all parameters when compared to the puree substrate.

The cooked must from 2009 harvest had more AA than the original substrate. Because of this result, and taking into account that the starting substrate was concentrated 2.13 times, it seems that the AA was affected by the heating as expected. In addition, anthocyanins were strongly affected by this treatment, decreasing 84%. This same effect was observed by Verbeyst, Oey, Van der Plancken, Hendrickx, & Van Loey (2010), who showed that anthocyanins are more rapidly degraded at higher temperatures on strawberry puree.

3.2.2. Alcoholic fermentation

Alcoholic fermentation was associated with a decrease in all parameters studied. The decline was statistically significant in most cases when the substrate employed was a puree, except in the case of cooked must, in which AA increased obtaining a very high antioxidant product. The decrease in anthocyanins was larger than in the rest of parameters (63–85%). This result is similar to the values obtained in other studies (decrease of 69–79%) (Klopotek et al., 2005). In general, the final values of AA and TPI in wines were similar in the three harvests.

In 2008, we found significantly differences between types of alcoholic fermentation, i.e. inoculation (IW) and spontaneous (SW) for DPPH, TPI and TA values. Total phenolic content was higher in SWs, and anthocyanin contents were higher in IWs, regardless the type of substrate used (semi-solid or liquid). In the wine from the liquid substrate, we observed that the AA and the TPI were lower than semi-solid substrate. However, the levels of anthocyanins in both types of wines were similar.

In the 2009 wines, strawberry SWs had higher significantly values of TA than inoculated wines, even in wines made from cooked must, showing a trend contrary to that observed in the wine production of 2008. It is important to note that the yeast strain (RP1) employed for the production of 2009 IWs was isolated from the 2008 spontaneous alcoholic fermentation. For this reason, we

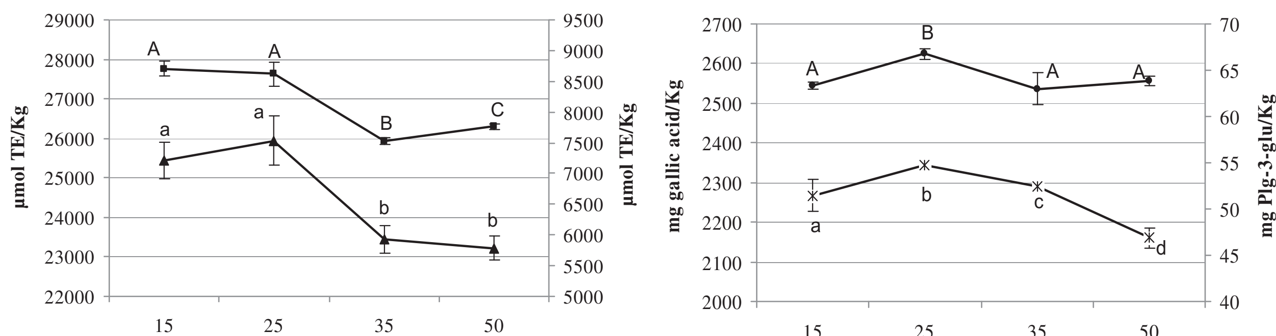

Fig. 3. Effect of different ultrasonication times a) ORAC and DPPH values. b) TPI and TA values of strawberries acquired at the market. The markers in the same assay with different letters show significant differences ($p < 0.05$) (ORAC and TPI assays: a, b, c; DPPH and TA tests: A, B, C).

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Table 2
Changes in 2008 samples on ORAC, DPPH, TPI and TA during strawberry vinegar production (average ± standard deviation).

Samples		ORAC (μmol TE/kg)	DPPH (μmol TE/kg)	TPI (mg gallic acid/kg)	TA (mg plg-3-glu/kg)
Substrates	F8P1	21,792 ± 221	8327 ± 99	2090 ± 10	26.3 ± 0.8
	F8P2	26,714 ± 910[a]	10,116 ± 88[a]	2298 ± 0[a]	69 ± 0[a]
	F8L	20,642 ± 111[b]	5907 ± 516[b]	1615 ± 33[b]	43 ± 0[b]
Wines	F8LWE	12,757 ± 267[b,c]	2837 ± 59[b,c]	868 ± 29[b,c]	12.2 ± 0.2[b]
	F8LWI	13,497 ± 227[b,c]	2898 ± 129[b,c]	858 ± 13[b,c]	17.9 ± 0.2[b,d]
	F8SWE1	25,314 ± 650	8200 ± 58[b]	1907 ± 26	13.1 ± 0.7[b]
	F8SWE2	24,696 ± 70	7879 ± 70[b]	1773 ± 32	12.9 ± 0.6[b]
	F8SWE3	25,458 ± 403	7689 ± 82[b]	1757 ± 45	12.4 ± 0.7[b]
	F8SWI1	27,987 ± 1227[b]	7241 ± 35[b,d]	1670 ± 9[b,d]	16 ± 0[b,d]
	F8SWI2	25,451 ± 429[b]	8004 ± 35[b,d]	1584 ± 19[b,d]	18.0 ± 0.3[b,d]
	F8SWI3	23,745 ± 15[b]	6515 ± 67[b,d]	1548 ± 6[b,d]	17.3 ± 0.6[d]
Vinegars	F8SVE1C	9202 ± 390[b]	3256 ± 205[b]	769 ± 13[b]	0.4 ± 0.0[b]
	F8SVE1P	9849 ± 413[b]	3368 ± 352[b]	774 ± 23[b]	0.5 ± 0.1[b]
	F8SVE2C	9215 ± 338[b]	3210 ± 129[b]	781 ± 0[b]	1.1 ± 0.2[b]
	F8SVE2P	10,869 ± 190[b]	3252 ± 234[b]	683 ± 10[b]	0.6 ± 0.0[b]
	F8SVI1C	10,139 ± 341[b,e]	3227 ± 117[b]	751 ± 16[b]	1.3 ± 0.0[b]
	F8SVI1P	11,611 ± 89[b,e]	3388 ± 64[b]	744 ± 6[b]	0.9 ± 0.1[b]
	F8SVI2C	11,054 ± 40[b,e]	3260 ± 246[b]	694 ± 16[b]	0.8 ± 0.1[b]
	F8SVI2P	11,082 ± 86[b,e]	3380 ± 76[b]	712 ± 9[b]	1 ± 0[b]

Sample codes are located in Table 1.
[a] Significant differences ($p < 0.05$) with respect to the initial fruit puree (ANOVA).
[b] Significant differences ($p < 0.05$) with respect to the sample from which was produced (ANOVA).
[c] Significant differences ($p < 0.05$) with respect to semi-solid wines obtained with similar alcoholic process (spontaneous or inoculated) (ANOVA).
[d] Significant differences ($p < 0.05$) with respect to spontaneous process (ANOVA).
[e] Significant differences ($p < 0.05$) with respect to the vinegars obtained from spontaneous wines (ANOVA).

believe that the diminution of TA may be related in some way to the yeast strain involved in fermentation. There are several possible explanations: the adsorption of anthocyanins to the cell walls of the used yeast strain (Morata, Gomez-Cordoves, Colomo, & Suarez, 2005) and condensation reactions with acetaldehyde (Bosso & Guaita, 2008). Perhaps the *Saccharomyces* strains involved in the 2008 spontaneous fermentations had a greater tendency to adsorb these molecules than the strain used in the inoculated processes.

The condensation reactions involve a loss of the aldehyde and the diminution of anthocyanins. We have previously reported (Ubeda, Callejón, et al., 2011) wines obtained by spontaneous alcoholic fermentations in 2008 and inoculated in 2009 contained less acetaldehyde and TA (mentioned above) than their corresponding opposite type of fermentation. In any case, the yeast strain had a greater influence in TA values than the strawberry harvest.

Finally, in the alcoholic fermentation at semi-pilot scale in a stainless steel tank (2010), the loss of AA, TPI and TA was smaller than the losses in the 2008 and 2009 harvests. Probably, the difference found may be due to the lower volume to size of contact surface with oxygen ratio in the stainless steel tank.

3.2.3. Acetous fermentation

In most cases, the acetification process was associated with a decrease in the parameters studied, being TA the most affected. Some of the loss of anthocyanins can be attributed to polymerisation or condensation reactions with other phenols, as noted in vinous substrates (Andlauer, Stumpf, & Fürst, 2000; Cerezo, Cuevas,

Table 3
Changes in 2009 samples on ORAC, DPPH, TPI and TA during strawberry vinegar production (average ± standard deviation).

Samples		ORAC (μmol TE/kg)	DPPH (μmol TE/kg)	TPI (mg gallic acid/kg)	TA (mg plg-3-glu/kg)
Substrates	F9P1	23,176 ± 868	9964 ± 193	2028 ± 82	173.0 ± 3.7
	F9P2	28,998 ± 1893[a]	10,117 ± 88	2085 ± 67	183.8 ± 3.1[a]
	F9MC	37,472 ± 1419[b]	17,897 ± 176[b]	3741 ± 21[b]	27 ± 1[b]
Wines	F9WE1	24,945 ± 276[b]	6898 ± 132[b]	1853 ± 67	52 ± 1[b]
	F9WE2	25,998 ± 795	6992 ± 299[b]	1683 ± 0[b]	55.3 ± 0.4[b]
	F9WI1	25,723 ± 564	7079 ± 53[b]	1705 ± 123[b]	26.3 ± 0.6[b,c]
	F9WI2	27,771 ± 1086	7135 ± 114	2017 ± 29	30.9 ± 1.1[b,c]
	F9MCWE1	49,755 ± 2015[b,c]	19,413 ± 141[b,c]	3380 ± 87[b,c]	24.1 ± 1.5[c]
	F9MCWE2	46,290 ± 279[b,c]	18,493 ± 105[b,c]	3001 ± 63[b,c]	23.3 ± 2.1[c]
	F9MCWI1	45,446 ± 2536[d]	17,747 ± 105[d]	3026 ± 29[d]	7.4 ± 0.6[c,d]
	F9MCWI2	43,095 ± 2576[d]	20,726 ± 271[d]	3416 ± 53[d]	6 ± 0[f,d]
Vinegars	F9VIG	15,163 ± 341[b]	6235 ± 72[b]	1099 ± 55[b]	3.07 ± 0.17[b]
	F9VIO	17,446 ± 107[b]	6902 ± 31	1844 ± 56	6.5 ± 0.9[b]
	F9VIX	19,077 ± 161[b]	7163 ± 31	1693 ± 45	4.80 ± 0.17[b]
	F9MCVE1	33,779 ± 974	14,907 ± 103	2377 ± 45	2.9 ± 0.5
	F9MCVE2	31,643 ± 1832	14,428 ± 41	2480 ± 56	4.0 ± 0.4
	F9MCVI1	30,685 ± 1377[e]	14,119 ± 305[e]	2536 ± 45[e]	1.70 ± 0.02[e]
	F9MCVI2	26,278 ± 1409[e]	14,283 ± 123[e]	2377 ± 22[e]	1.79 ± 0.15[e]

Sample codes are located in Table 1.
[a] Significant differences ($p < 0.05$) with respect to the initial fruit puree (ANOVA).
[b] Significant differences ($p < 0.05$) with respect to the sample from which was produced (ANOVA).
[c] Significant differences ($p < 0.05$) with respect to spontaneous wines from F9P2 (ANOVA).
[d] Significant differences ($p < 0.05$) with respect to inoculated wines from F9P2 (ANOVA).
[e] Significant differences ($p < 0.05$) with respect to inoculated vinegars from F9WI wines (ANOVA).

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Table 4
Changes in 2010 samples on ORAC, DPPH, TPI and TA during strawberry vinegar production (average ± standard deviation).

Samples		ORAC (μmol TE/kg)	DPPH (μmol TE/kg)	TPI (mg gallic acid/kg)	TA (mg plg-3-glu/kg)
Substrates	F10P1	20,409 ± 431	10,218 ± 171	1800 ± 122	46.4 ± 1.6
	F10P2	23,783 ± 649[a]	10,592 ± 237	1886 ± 79	54.8 ± 1.4[a]
Wine	F10WI	22,910 ± 315	9652 ± 378[b]	1691 ± 36[b]	20.2 ± 0.5[b]
Vinegar	F10VI	19,784 ± 117[b]	9113 ± 331	1605 ± 95	10.6 ± 0.9[b]

Sample codes are located in Table 1.
[a] Significant differences ($p < 0.05$) with respect to the initial fruit puree (ANOVA).
[b] Significant differences ($p < 0.05$) with respect to the sample from which was produced (ANOVA).

Winterhalter, Garcia-Parrilla, & Troncoso, 2010b). Again, as occurred in alcoholic fermentation, we observed the lowest decreases in all of these parameters in the 2010 samples.

In 2008, vinegars were subjected to two different final treatments. In assessing the antioxidant activity (Table 2), we observed that the ORAC and DPPH values were slightly higher in pasteurised vinegars than in centrifuged vinegars. The centrifugation procedure removes suspension particles being able to produce losses of antioxidant compounds. Moreover, this result could also be explained by the formation of Maillard reaction products such as melanoidins that are produced by the heat of pasteurisation. Several authors who have studied vinegar melanoidins have concluded that contribute to the total antioxidant capacity of it (Xu, Tao, & Ao, 2007).

In the 2009 (Table 3), spontaneous and inoculated acetifications were performed. However, the spontaneous fermentation stopped, so we only obtained inoculated vinegars. Regarding the effect of the type of container used in the acetification, the vinegar produced in glass vessel displayed the lowest values for all the parameters studied. These results were expected due to concentration phenomena and compounds extraction in wood barrels. The vinegar from cherry barrel had the highest AA, at levels significantly different from the oak vinegar. From the oak barrel, we obtained vinegar with the highest amount of total phenols and anthocyanins, but significant differences were not found with the vinegar from cherry barrel. These results were similar to those of Cerezo et al. (2008), who reported a generally decreasing trend of TPI and TA in vinegars acetified in cherry and oak barrels, being slightly lower in oak. The lower final levels of TA in vinegar from cherry barrel may be explained by the different porosity of wood (higher in cherry wood than in oak). Oxygen permeation through the wood favours the formation of stable anthocyanin-derived compounds (Cano-López, Pardo-Minguez, López-Roca, & Gómez-Plaza, 2006), decreasing monomeric anthocyanins. According to these results, it seems that cherry wood barrel is the best to produce high antioxidant strawberry vinegars rich in phenols.

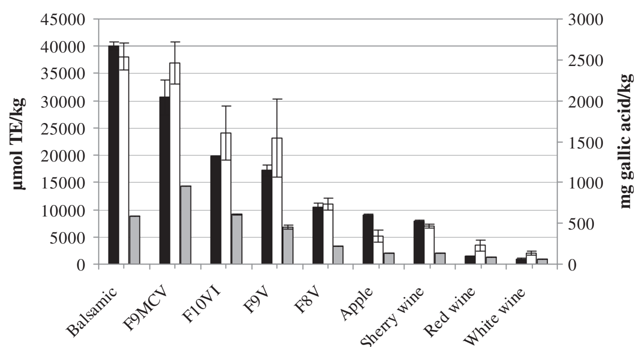

Fig. 4. Comparison of ■ ORAC, □ DPPH (left axis) and ■ TPI (right axis) values of strawberry vinegars with commercial varieties. Sample codes: F9MCV (mean value of all vinegars from cooked must), F9V (mean value of all vinegars from 2009 harvest) and F8V (mean value of all vinegars from 2008 harvest).

Vinegars from cooked must had the highest AA and TPI of all of the vinegars produced.

Otherwise, the 2010 vinegars produced on a semi-pilot scale had the highest AA and TA values of all the vinegars obtained from strawberry purees without heating. As mentioned above, the important losses of TA that occurred in the 2008 and 2009 vinegars did not occur in 2010, where losses were only around 50% from wine to vinegar. These results indicate that the production of vinegars on a semi-pilot scale allowed getting vinegars with better antioxidant properties.

Finally, we compared our vinegars with common vinegars from the market. The results are given in Fig. 4. Vinegars produced in this research project were surpassed only by the Aceto Balsamico. Cooked must vinegar had AA and TPI values close to this one.

4. Conclusions

The addition of SO_2 and pectolytic enzymes to the substrate increased AA, TPI and TA.

Although the cooked must vinegar presented the highest AA and TPI values, this substrate must be discarded for the strawberry vinegars production at an industrial scale because of their obtaining process is very slow and complex. Concerning the acetification stage, the use of wood barrels was an improvement in all of the parameters determined; specifically, cherry barrels were the best to produce high antioxidant strawberry vinegars rich in phenols. The most appropriate final treatment was the pasteurisation with reference to AA. All measured parameters decreased during the double fermentation process. In general, acetic fermentation was associated with higher decreases in AA and polyphenols than alcoholic fermentation, except in the semi-pilot scale case. Moreover, anthocyanins were severely influenced by this process. So, for substrate selection the parameter more important to take into account is the TA content. We also noted that the production of these vinegars on a semi-pilot scale resulted in final products with the best antioxidant properties and phenolic content. The antioxidant properties of these vinegars point to them as products with potential health benefits that could make them competitive commodities in the market.

Acknowledgements

This research was made possible through the financial support from the Spanish Government by means of a predoctoral grant and the research project AGL2007-66417-C02-01. Moreover, the researchers are grateful to the enterprises Hudisa S.A., Agromedina and Grupo Alconeras for providing the fruit substrates.

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Please cite this article in press as: Ubeda, C., et al., Employment of different processes for the production of strawberry vinegars: Effects on antioxidant activity, total phenols and monomeric anthocyanins, *LWT - Food Science and Technology* (2012), doi:10.1016/j.lwt.2012.04.021

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3.5. Determinación de carbamato de etilo

3.5.1. Resumen

En este capítulo se exponen los resultados obtenidos tras la evaluación del contenido de carbamato de etilo para comprobar la seguridad de todos los condimentos obtenidos. Para ello se puso a punto un método empleando la técnica de extracción en fase sólida (SPE) seguida de cromatografía de gases con espectrometría de masas.

Se ensayaron diferentes pH dentro del rango 2,5-7, para obtener una óptima recuperación así como una buena resolución de pico. Estos ensayos pusieron de manifiesto que al basificar el vinagre hasta pH 5,5 se obtenían los mejores resultados.

El método propuesto fue validado ya que resultó ser lineal, sensible, repetitivo, reproducible y exacto, en el que se usaron por primera vez los cartuchos de extracción ISOLUTE ENV+ para la determinación de carbamato de etilo en vinagres. De esta manera se comprobó que los condimentos elaborados presentaban valores por debajo de los límites de cuantificación o detección, siendo seguro su consumo en relación a este compuesto.

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Talanta 89 (2012) 178–182

Contents lists available at SciVerse ScienceDirect

Talanta

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Validation of an analytical method for the determination of ethyl carbamate in vinegars

C. Ubeda, C. Balsera, A.M. Troncoso, R.M. Callejón, M.L. Morales*

Área de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Sevilla, C/P, García González n°2, E-41012, Sevilla, Spain

ARTICLE INFO

Article history:
Received 17 October 2011
Received in revised form
30 November 2011
Accepted 4 December 2011
Available online 8 December 2011

Keywords:
Ethyl carbamate
Vinegar
Solid phase extraction
Gas chromatography–mass spectrometry

ABSTRACT

A solid phase extraction method (SPE) using Isolute ENV+ cartridges was validated for the determination of ethyl carbamate (EC) in different kinds of vinegars. The method proved to be quite sensitive, precise and accurate, improving the recovery and LQD of other existing methods for the same purpose. For the optimization of the method, different pH values of the sample were tested, resulting 5.5 the most adequate. Among the 14 samples analysed, only 5 of them had contents of EC above the quantification limits, ranging between 6.73 μg/L and 56.4 μg/L. The highest value was found in red wine vinegar. Taking into account the amount of vinegar consumed in a meal and the limits established for alcoholic beverages in some countries, the levels of ethyl carbamate in the vinegars tested in this work were acceptable.

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1. Introduction

Ethyl carbamate (EC), or urethane, is genotoxic and carcinogenic in a number of species, including mice, rats, hamsters and monkeys which suggests a potential carcinogenic risk to human [1–4]. This compound is present in many fermented food (yoghurt, cheese or bread) and alcoholic beverages (wine, beer or spirits, particularly in stone-fruit brandies), usually consumed by human population [5]. Ethyl carbamate, potentially toxic, was re-classified in 2007 as probably human carcinogen compound (Group 2A) by the International Agency for Research on Cancer (IARC) [5]. Thus, the presence of ethyl carbamate in beverage and food is a public health concern for government agencies from countries throughout the word [6].

Ethyl carbamate results from the reaction between ethanol and nitrogen-containing compounds (e.g. urea, citrulline, hydrogen cyanide, cyanogenic glycosides, and other N-carbamyl compounds), which has a moderate kinetic formation at room temperature [7]. One of the most common formation pathway of ethyl carbamate production, in acidic medium, is the reaction of urea with ethanol [8,9]. In the case of wine, the yeasts generate urea from the degradation of arginine [10]. Median levels of ethyl carbamate in alcoholic beverages of up to 5 μg/L for beer and wine, 21 μg/L for spirits other than fruit brandy and 260 μg/L for fruit brandy were calculated [11].

There are currently no harmonised maximum levels for ethyl carbamate. In Canada, the first country to introduce maximum levels of ethyl carbamate in a variety of alcoholic beverages, and in the Czech Republic, the limits range from 30 μg/L for wines to 400 μg/L for fruit brandies. The USA has voluntary targets for wines 15–60 μg/L [11]. Recently, the European Union (EU), recommended taking mitigation measures to reduce the levels of ethyl carbamate in stone fruit spirits and stone fruit marc spirits to get levels of ethyl carbamate as low as possible with the aim to achieve the level of 1 mg/L as a target [12].

Ethyl carbamate has been analysed employing different analytical instruments. Most of them require pre-treatments of the sample to avoid interferences and increase the sensitivity. Among them, we can mention liquid–liquid extraction, solid phase extraction (SPE) or solid phase microextraction (SPME). Different solvent in liquid–liquid extraction has been employed, dichloromethane [13] or ethyl acetate [14]. Solid phase extraction (SPE) has been widely applied using different types of cartridges such as ENV+ (hyper cross-linked styrene-divinylbenzene copolymer column) [6,15], or diatomaceous earth column [16–20]. Recently, solid phase microextration (SPME) has also been employed in the analysis of wines and spirits [7,21,22].

The most widespread analytical technique used is gas chromatography simple or multidimensional [6,7,13] with different types of detector (FID, MS, MS/MS, etc.). Mass spectrometer detection in selected ion monitoring mode (SIM) increase significantly the ethyl carbamate detection [23].

Ethyl carbamate has also been analysed by high-performance chromatography with fluorescence detector with a previous

* Corresponding author. Tel.: +34 954 556760; fax: +34 954 233765.
E-mail address: mlmorales@us.es (M.L. Morales).

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doi:10.1016/j.talanta.2011.12.012

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derivatization step [24,25]. Moreover, a rapid method as FTIR spectroscopy for stone-fruit spirits analysis [26] and other methods based on more complex techniques such as HPLC-ESI–MS/MS analysis of samples without [27], or with xanthydrol derivatization technique [28] have also been applied.

The presence of ethyl carbamate in vinegars has been scarcely studied [14,17,20]. However, this compound could be present in vinegars since it is a product obtained from a double fermentation, alcoholic and acetous. Ethyl carbamate could come from the raw material (wine) or be formed during process production. Several authors have reported the formation of urea during the acetous fermentation [29], which could lead to the synthesis of ethyl carbamate that is favoured in acidic medium as vinegar.

The aim of this work was to develop and validate an analytical method for determining ethyl carbamate in different types of vinegars by SPE and gas chromatography–mass spectrometry analysis.

2. Materials and methods

2.1. Chemicals and standard solutions

Methanol, ethyl acetate and sodium hydroxide were purchased from Merck (Darmstadt, Germany), and MilliQ water. The standards employed were ethyl carbamate (EC) (Aldrich) and propyl carbamate (PC) as internal standard (Dr. Ehrenstorfer GmbH Laboratories, Germany). The stock and working standard solutions of EC and PC for validation studies were prepared in ethyl acetate.

On the other hand, for spiked vinegar samples, the stock and working standard solutions were prepared in methanol, since this solvent allows a better solubilization of EC and PC in vinegar matrix than ethyl acetate.

2.2. Samples

Six wine vinegars were analysed: two white wine vinegars (WWV1, WWV2), a red wine vinegar (RWV), and three Sherry vinegars, one from each category: Sherry vinegar (SHV), "Reserva" (RV) and "Gran Reserva" (GRV), with 6 months, 2 years and 10 years of ageing in oak wood barrels, respectively. Also, eight fruit vinegars were analysed: two persimmon vinegars (PV1, PV2) and six strawberry vinegars (SV1, SV2, SV3, SV4, SV5, SV6). For validation studies, one white wine vinegar was employed. Wine vinegars were acquired in the market and fruit vinegars were produced in the lab.

2.3. Solid phase extraction

The SPE method employed was a modification of the one used by Jagerdeo et al. [6]. We used cartridges of 6 mL containing 500 mg of ISOLUTE ENV+ (Biotage, Uppsala, Sweden) as extraction phase. The extraction was carried out in a Visipred vacuum manifold (Supelco, Bellefonte, PA). The cartridge was conditioned with 2 ml of methanol followed by 3 ml of MilliQ water. Then, 25 ml of vinegar were passed through the cartridge at a flow rate of 3 ml/min. Samples were previously adjusted to a pH 5.5 with NaOH and spiked with 100 μL of propyl carbamate (6 mg/L). The sorbent was dried by letting air pass through it at −0.6 Bar. EC and PC were eluted from cartridge with 3 ml of ethyl acetate. The organic phase of the eluate was carefully collected with a pipette and afterwards concentred under vacuum to a final volume of 2 ml. 300 μL of the extract were placed into a vial fitted with an insert that was tightly capped for the injection in the gas chromatograph. This extraction procedure was carried out in duplicate for each sample.

2.4. Quantitative analysis

For the quantification in validation studies, we made calibration curves of both standards employing ethyl acetate solutions and injecting them, in triplicate, directly in the gas chromatograph. Concentration ranges were 3–520 μg/L for EC (five different levels of concentration) and 2.88–1000 μg/L for PC (six different levels of concentration). The calibration curves were built representing the areas of the target ion (m/z = 62, in both cases) againts the concentrations of analyte.

For the samples quantification, a calibration curve was done using one spiked vinegar with EC at five different levels of concentration (3.7–334 μg/L) which was extracted with the same method employed for the samples. Now, the calibration curve was made using the relative area of EC (ratio between the peak area of target ion of EC and the peak area of internal standard) and the concentration of analyte added to the sample.

2.5. Chromatographic conditions

Extracts were analysed in a gas chromatograph Agilent 6890 GC system coupled to an Agilent 5975 inert quadrupole mass spectrometer. For the separation of the compounds we employed a CPWax-57CB (Varian) capilar column of 50 m × 0.25 mm and 0.20 μm film thickness (Varian, Middelburg, The Netherlands). 4 μL of the extract were injected in the splitless mode with a purge flow of 70 mL/min and purge time of 1 min. The injector temperature was 220 °C. The carrier gas was He at a constant flow rate of 1 mL/min. Oven temperature program was as follows: the initial temperature 40 °C and then was increased 2.5 °C/min until 150 °C for 2 min and afterwards increased 15 °C/min until 220 °C. The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 280 °C, respectively. Detection was carried out in the SIM mode, the monitored ions were: 44, 62 y 74. Extracts were injected in duplicate and the identification was done comparing the peak retention times with their respective standards.

2.6. Validation parameters

For method validation the following parameters were evaluated: linearity, sensitivity (LOQ), precision (repeatability and intermediate precision) and accuracy (recovery studies). For the recovery studies, a white wine vinegar was spiked with five different concentration levels of EC in the range of 3.7–161 μg/L.

The linearity of the method was determined by two ways: considering the correlation coefficient obtained from the regression line made with spiked vinegar at five different levels of concentration (described in Section 2.4); and plotting the response factor (relative area of peaks divided by their respective analyte concentrations) as a function of analyte concentrations [30].

The quantification limit (LOQ) was calculated as the concentration of ethyl carbamate in the sample that produces a signal ten times higher than the average of relative area of background noise of the chromatogram baseline.

To study the repeatability of the method, 5 successive extractions of a vinegar sample spiked with 60 μg/L of ethyl carbamate were performed. On the other hand, intermediate precision was evaluated using the same sample referred before and performing the extraction on 5 different days by two different analysts over a month of work.

3. Results and dicussion

3.1. Sample pre-treatments

Some authors which have determined EC in vinegars made a previous neutralization of the samples because this improves the shape of EC peak [14,17,20]. Taking into account this fact, we tested the effect of different pHs in the recovery of EC and PC in vinegar samples spiked with the standards. The pH range assayed was from 2.5, pH of vinegars, to neutrality (pH = 7). The pH value of samples

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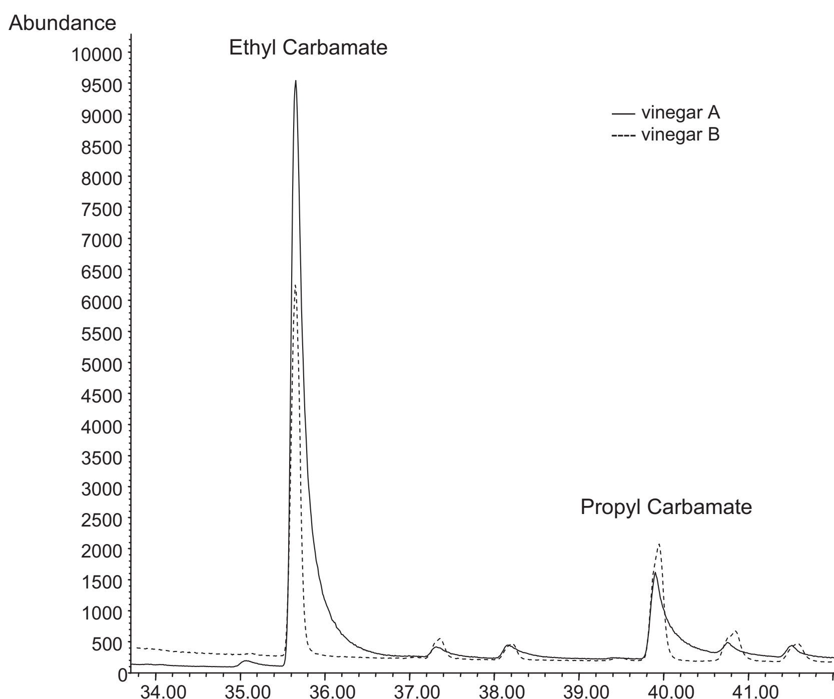

Fig. 1. Overlay of chromatograms from spiked vinegars A and B. Vinegar A: with neutralization (continuous line); and vinegar B: without neutralization (dashed line).

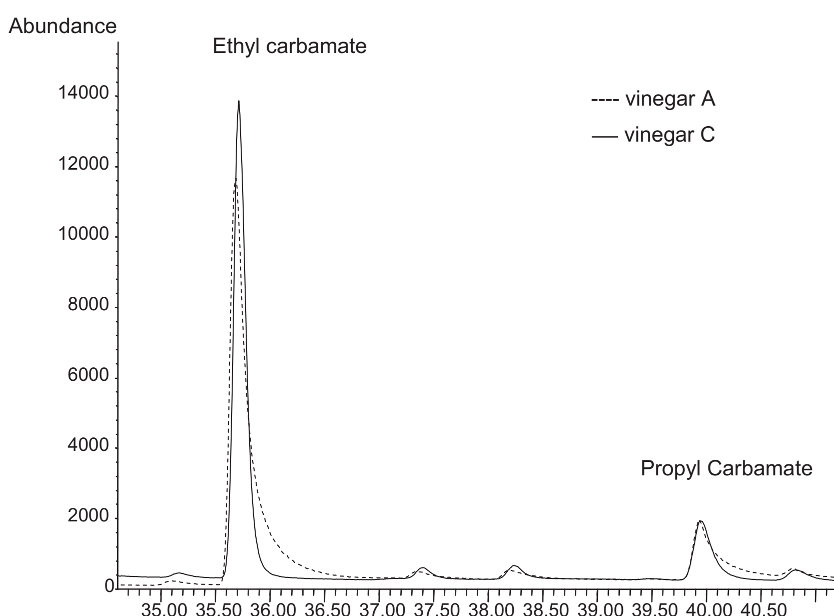

Fig. 2. Overlay of chromatograms from spiked vinegars A and C. Vinegar A: with neutralization (dashed line); and vinegar C: pH 5.5 (continuous line).

was modified with the addition of NaOH. These trials showed that peak areas obtained with vinegar without NaOH addition, were approximately the half that neutralized vinegar (pH = 7) (Fig. 1). However, the peaks in the last case had a pronounced tail. At pH 5.5, the side of peaks area was similar to the neutralized vinegar but the shape of peaks was much better than in the neutralized samples (Fig. 2).

3.2. Method validation

The method was evaluated with respect to linearity, sensitivity (LOQ), precision (repeatability and intermediate precision) and accuracy (recovery studies).

One of the most important issues in a extraction process is the ability to recover the highest amount of the analyte of interest. Thus, the first aspect assessed was the recovery. The average recovery rate (Table 1), in the accuracy assays, was 94.1%, which is a very suitable result according to those proposed by AOAC [16]. Our recovery percentage was higher than those achieved by other methods for EC determination in vinegars (below 83%) [17,20].

The good linearity of the method in the used range of concentration was verified by a 0.9998 correlation coefficient of the regression line between the relative area of EC and the concentration of analyte added to the sample. On the other hand, the line obtained after plotting the response factor as a function of analyte concentrations was horizontal over the concentration range. Two

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Table 1
Values of accuracy assay.

Accuracy assay	EC added (μg/L)	Recovery (%)	Mean recovery (%)
Experimental data	3.7	99.0	94.1 ± 3.1
	35	90.5	
	77	92.6	
	115	94.1	
	161	94.3	
AOAC range of suitable values [16]	10–100	–	60–115

Table 2
Values of precision assay.

Precision assay	EC added (μg/L)	Repeatability (%RSD)	Intermediate precision (%RSD)
Experimental data	60	2.5	6.5
AOAC maximum suitable values [16]	10–100	5.3–7.3	5.3–7.3

Table 3
Ethyl carbamate concentrations in vinegar samples (μg/L).

Sample	Ethyl carbamate (μg/L)
WWV1	nq
WWV2	6.46 ± 0.01
RWV	56 ± 3
PV1	nd
PV2	nd
SV1	nq
SV2	nd
SV3	nq
SV4	nq
SV5	nq
SV6	nq
SHV	6.7 ± 0.9
RV	14 ± 2
GRV	1.68 ± 0.08

nd, peak not detected; nq, concentration under quantification limit.

parallel lines are drawn in the graph at 0.95 and 1.05 times the average values of the response factors and there were no intersections of the points of response factor with these parallel lines. Both results confirmed the linearity of the method.

The LOQ was defined as the lowest concentration of EC in a sample that can be determined quantitatively with acceptable precision and accuracy under the established conditions of the method. This value was 1.26 μg/L. If we compare with the LOQs obtained by other authors that ranged between 9.16 μg/L and 110 μg/L [6,7,20,21,31,32], our method proved to be sensitive enough, improving the values of LOQ achieved up to the present.

The precision of the method was evaluated by repeatability and intermediate precision assays. We checked the repeatability of the method by the relative standard deviation (RSD) obtained after repeating the extraction assay of spiked vinegar 5 times successively, resulting a 2.5% (Table 2). In the intermediate precision evaluation, the RSD obtained was 6.5% (Table 2). Both values are in agreement with the values proposed by AOAC [16], showing that the method is quite precise.

3.3. Samples analysis

Once we set up the method, the procedure was applied to different types of vinegars. Data are presented in Table 3. Among the 14 samples, only 5 of them presented levels above the quantification limits, ranging between 6.73 μg/L and 56.4 μg/L. The highest value was found in red wine vinegar. As mentioned in the introduction, only some countries have established their own maximum limits for the EC content in alcoholic beverages [11], but there are not legal limits for vinegar. Except in the case of red wine vinegar, the EC content in the samples is below those values. Other authors have already described the presence of EC in Sherry vinegar [17], founding concentrations of 33 μg/L. The Sherry vinegars analysed in this study had a lower amount of EC than in the above mentioned work. These levels are far away compared to those found by other researchers in vinegars from Taiwan (107.5–250.5 μg/L) [33].

4. Conclusions

Due to the natural acidity of vinegar, a modification of pH at 5.5 previous to the SPE was necessary in order to get an adequate recovery rate and peak resolution. The present method is quite sensitive, precise and accurate, improving the recovery and LQD of other existing methods for the same purpose. Considering the amount of vinegar consumed in a meal and the limits established for alcoholic beverage in some countries, we could conclude that the levels of ethyl carbamate in the vinegars tested in this work were acceptable.

Acknowledgments

This research was made possible through the financial support from the Spanish Government by means of a predoctoral grant and the research project AGL2007-66417-C02-01 funded by the Ministry of Science and Innovation. The authors wish to thank Dr. A. Mas' research group from University Rovira i Virgili for providing fruit vinegars.

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4. SUMMARY OF RESULTS

In this chapter, a summary of the results obtained in this Thesis is presented, following the same order of the chapter 3 “Resultados y discusión”.

4.1. Determination of amino acids and ammonium

Amino acids are the nitrogen source for the microorganisms involved in fermentation processes, which may be the reason of problems in these processes. Moreover, amino acids are precursors of aromas. Therefore, we studied the changes in the content of 22 amino acids and ammonium along the double fermentation process to the final products.

This work was done using samples from persimmon and from 2008, 2009 and 2010 strawberry harvests. The samples were centrifuged, derivatized using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), and analyzed by liquid chromatography (HPLC) using a fluorescence detector.

The consumption pattern of amino acids by yeasts and acetic acid bacteria is more dependent on the amino acid content of the fruit than the strain of microorganism involved in the process. The results indicated that the microorganism adapts to the source, as they always consume the most abundant amino acids in the starting substrates. Moreover, the amount of nitrogen in the medium was enough for fermentations to take place.

However, in the alcoholic fermentation, the total nitrogen consumed was conditioned by the yeast strain used, because the strain RP1, isolated during the spontaneous fermentation of the strawberry puree, had higher rate of consumption of total nitrogen than the other inoculated strain (QA23).

With respect to the major amino acids in the vinegars, gamma-aminobutyric acid and proline were the most abundant in the persimmon vinegar and in the strawberry vinegar from the alcoholic fermentation inoculated in 2008 harvest. By contrast, in the vinegar made from spontaneous wine, cysteine was the major amino acid. Finally, arginine was the most abundant in strawberry vinegars from 2009 and 2010 harvests.

By comparing the content of nitrogen in the initial substrates and its comsumption along the two fermentations (alcoholic and acetic), it was observed that in the case of the persimmon about 50% of nitrogen was consumed and in strawberry from 2008 and 2009-2010 harvests, these comsumptions were around 74% and 93%, respectively.

4.2. Analysis of major volatile compounds, aromatic precursors and aromatic characterization of vinegars

This chapter shows the results of the study of the evolution of the major volatile compounds during alcoholic and acetic fermentation of the persimmon and strawberry. In addition, the olfactometric characterization of the starting substrates and their resulting vinegars was reported.

Then, it is included the study of aromatic precursors of four strawberry varieties and their olfactometric analyses for each variety, to evaluate the possible use of different strawberry varieties to *Camarosa*.

The first paper presents the results of determination of the major volatile compounds (ethyl acetate and methyl, isobutanol, acetaldehyde, methanol, 3-methyl-1-butanol, 2-methyl-1-butanol, isoamyl acetate and propanol) from the initial substrate to the final vinegars. So, we can identify which processes are the most appropiated to get the best volatile profile of the vinegars and how each fermentation affects to these compounds. For this purpose, a headspace (HS) sampling method followed by analysis by gas chromatography coupled to mass spectrometry detector was optimized. The validation of the method was satisfactory, recovery values and limits detection are acceptable for most of the compounds studied, and the method was successfully applied to real samples.

The results showed that the addition of pectolytic enzymes and SO_2 resulted in an increase in acetaldehyde and methanol. In addition, *Saccharomyces cerevisiae* strain isolated in the 2008 harvest (RP1) proved to have great influence on the production of acetaldehyde and higher alcohols. We can say that the wines produced by this strain had a volatile profile more suitable for using it as a substrate for the production of vinegars.

Olfactometry analyses were performed in the strawberry vinegars obtained in the 2008 and 2009 harvests and their corresponding starting substrates. Furthermore, the extracts were analyzed by GC-MS to identify the compounds responsible for the perceived aromatic zones. The purpose for the analysis of 2008 samples was to study the changes of the active odorants from the substrate to the vinegars produced from wines inoculated with yeast or by spontaneous fermentation. The aim of the second study was to evaluate which of the starting substrates (puree and cooked must) and containers used for acetification were better for the aroma profile.

Due to the characteristics of the samples and the available tools, for the preparation of representative extracts we applied the technique of liquid-liquid extraction with dichloromethane. Olfactometry analyses were carried out by three expert panelists. With all the data colleted we calculated the modified frequencies (MF).

After the analyses of samples from the 2008 harvest, it was observed that the aromatic profile of the final vinegar was a mixture between those from the substrate and those formed during acetification. On the one hand, the vinegar had some of the characteristic active aroma compounds of the strawberry such as γ-decalactone, furaneol, mesifurane, β-damascenone and ethyl-2-methylbutyrate, which had been preserved over the double fermentation process, and secondly, it had the ones generated in the acetification such as acetic, isovaleric and butyric acids, and others that are characteristic compounds of vinegars such as sotolon and diacetyl. There were several differences between the two types of vinegars. The vinegar from the wine that had been inoculated showed more aromatic zones than the one from the wine that had been left to ferment spontaneously. Therefore, the process of choice would be the inoculated one.

Finally, we conclude that the impact aroma compounds of strawberry vinegars would be isovaleric, butyric and acetic acid, pantolactone + furaneol, phenylacetic acid, 3-nonen-2-one, 2-phenylethanol, p-vinylguaiacol, vanillin, methional and sotolon due to the high modified frequency reached in the final product.

Olfactometry and sensory analysis of samples of 2009 strawberry harvest showed that the use of different starting substrates and acetification containers produces important differences in the aromatic profile of vinegars. Thus, the vinegar obtained from the cooked must was sensorially very different to those produced from the puree without concentration. So we really produced two totally different vinegars. Among them, the vinegar that got the highest score in general impression was the one from the cooked must. And among the vinegars obtained from strawberry puree, those produced in wood barrels presented more odor zones than the one produced in glass vessel. Moreover, when we compared the descriptive sensory analysis of vinegars acetified in wood barrels, it was observed that the aromatic profile of the vinegar produced in oak barrel has a higher quality than the vinegar produced in cherry barrel. Therefore this would be the recipient to use for the acetification.

For the study of the aromatic precursors it was optimized a method of solid phase extraction (SPE) and analysed the different varieties. The results showed that the LiChrolut®

resin proved to be more effective as an adsorbent for the extraction of aromatic precursors of the strawberry than the to the hitherto used Amberlite. Thus, we could determine 51 aglycones, 38 of them not previously described in this fruit. Among the four strawberry varieties studied, *Fuentepina* had the highest number of glycosylated aroma precursors.

Additionally, we carried out olfactometric analyses. The representative extract was obtained by the technique of SPE with nitrogen stream, and analyzed by gas chromatography olfactometry detector (GC-O) by a panel of six experts. The results made possible the identification for the first time in strawberry of (Z) -1,5-octadien-3-one, a possible impact aroma described as geranium / green / pepper / lettuce with a modified frequency of more than 80 in two varieties (*Fuentepina* and *Candonga*).

After studying the results obtained in this paper, we could conclude that the variety *Sabrina* could be an alternative to the *Camarosa* variety to use it as a substrate in the production of fermented products. In *Sabrina* variety sweet aromas predominated over green and it had high concentrations of characteristic aromas of strawberry in form of aromatic precursors.

4.3. Sensory analyses and preference tests

This chapter presents the results of the evaluation of sensory characteristics of strawberry vinegars obtained in 2009 harvest. It includes the results of descriptive analysis of samples carried out by experts and preference tests by the potential consumers. The vinegars tested were those produced from strawberry puree in barrels of oak and cherry, the ones produced in glass container, and the vinegar made from cooked must. As a reference we used commercial white wine vinegar.

In preference tests 52 potential were asked for a valuation of each sample (aroma and taste) using a 11-point scale, with 0 being dislike extremely and 10, like extremely. Descriptive analysis was carried out by an expert panel composed of 8 members using 16 attributes selected by consensus.

The results obtained after preference tests showed that the vinegar of choice by both the taste and the aroma was the one produced from cooked must. Data from both sensory tests were related through multivariate statistical techniques and preference maps were made. Using the preference maps we found that the descriptors raisin, toasted caramel, spicy and liquor were those which conditioned the consumer preference.

4.4. Determination of antioxidant activity, total phenols content and monomeric anthocyanins.

This chapter studies the evolution of the antioxidant activity (AA) and the content of total phenolic compounds during alcoholic and acetic fermentations of the strawberry and persimmon. Thus, we can design the best process to retain the healthy qualities of the starting substrate. For the analysis of these parameters was necessary to establish an extraction procedure and optimize the variables of the method. After obtaining the extract, we measured the antioxidant activity by ORAC (absorbance capacity of oxygen radicals) and by DPPH (2,2-diphenyl-1-picrylhydrazyl) methods and total phenol index (TPI). In the case of strawberries were also studied total monomeric anthocyanins (TA).

The results showed that for both types of fruit, the use of ultrasound for 25 minutes yielded the best extracts (high antioxidant activity and phenolic compounds). With regard to the type of extractant, an optimal extraction was achieved using ethanol 80% in the case of the persimmon and 80% acetone in the strawberry case.

During the first processing stage of the vinegar we observed that the addition of pectolytic enzymes and SO_2 to the substrate resulted in an increase in all the studied parameters in both fruits, mainly produced by the action of enzymes breaking down cellular structures containing antioxidant molecules. Furthermore, in the case of the strawberry, we compared four possible types of starting substrate: puree, liquid obtained by centrifugation of the puree, commercial puree and cooked must. The results indicated that the condiments made from cooked must were those with the highest values of AA and TPI. For the industrial production of the vinegars, the most appropriate substrates would be the strawberry purees, while the cooked must could be suitable for the production of vinegars with special characteristics at small-scale.

The vinegars made from the wine inoculated with *Saccharomyces cerevisiae* strain RP1 had higher values of antioxidant activity. Furthermore, different containers were tested for acetic fermentation of strawberries, and it was observed that the use of wood barrels, especially cherry barrels, produced vinegars with high antioxidant activity and richer in phenolic compounds. This is due to a phenomenon of concentration and extraction of compounds with antioxidant activity from the wood.

In the case of the persimmon, from the initial puree to the final vinegars were not observed significant losses in the parameters studied. However, in the production of strawberry vinegars, there were important decreases in these parameters, being the acetification, the stage that more affected them. Finally, the production at semi-pilot scale had a positive impact on the healthy characteristics of strawberry vinegars, having fewer losses in antioxidant activity and total contents of phenolic compounds.

If we compare the vinegars produced from persimmon and strawberry, the latter exhibited greater health benefits. In addition, when we compare them with other commercial vinegars (red wine vinegar, white wine, apple, Jerez, and Balsamic) strawberry vinegar took second place, behind only the Balsamic vinegar. This point to these vinegars as a potentially competitive product in the market regarding the parameters studied in this chapter.

4.5. Determination of ethyl carbamate

This chapter presents the results obtained after evaluating the content of ethyl carbamate to ensure the safety of all produced vinegars. For that purpose, we set up a method using the technique of solid phase extraction (SPE) followed by gas chromatography with mass spectrometry. Different pH values were tested within the range 2.5 to 7, for optimal recovery and an adequate peak resolution. These tests showed that when the vinegar was basified to a pH of 5.5 we obtained the best results. The proposed method was validated and proved to be linear, sensitive, repetitive, reproducible and accurate, using the extraction cartridge Isolute ENV + for the first time for the determination of ethyl carbamate in vinegars. Thus it was found that the vinegars produced had values below the limit of quantification, being safe for comsumption in regard to this compound.

5. DISCUSIÓN FINAL

El objetivo general de esta tesis era evaluar el proceso de elaboración y los condimentos obtenidos a partir de caqui y fresa de segunda calidad o excedentes. El empleo de caqui como sustrato fue descartado debido a las dificultades en su manejo y procesado y a que no presentaron características antioxidantes destacables. Por tanto, se seleccionó la fresa como sustrato de partida para la elaboración de los condimentos.

Los resultados de la evaluación de los condimentos de fresa durante tres campañas nos indican que los sustratos más adecuados son la fresa triturada, mosto cocido y pasta industrial de fresa (aunque el efecto del uso de esta última en el perfil aromático y aceptación del consumidor no ha sido evaluado).

En relación al contenido de precursores aromáticos, se propone el empleo de la variedad de fresa *Sabrina* o una combinación de ésta con *Camarosa,* con el fin de obtener condimentos con notas aromáticas a fresa más pronunciadas.

Debido a las pérdidas de actividad antioxidante producidas durante la doble fermentación, la adición de sulfuroso y enzimas pectolíticas resulta beneficiosa para obtener un sustrato de partida con mayor actividad antioxidante y aromas, siendo importante el control de los niveles de metanol.

La inoculación con microorganismos seleccionados permite un mayor control de la fermentación y reproducibilidad en los productos finales. Por ello, se estudió el empleo de una cepa aislada en las primeras fermentaciones alcohólicas. Esta cepa (RP1), se caracteriza por un mayor consumo de nitrógeno que la cepa comercial (QA23), sin embargo, esto no supone ningún inconveniente para que se lleve a cabo la fermentación acética ya que hemos observado que las bacterias acéticas adaptan su perfil de consumo a la disponibilidad de aminoácidos. Otro aspecto a tener en cuenta es la disminución en el contenido de antocianinas totales cuando se impone la cepa RP1. Todo parece indicar que, aunque la cepa aislada produce condimentos aceptables, el efecto del uso de otras cepas de levaduras sería un aspecto a considerar con objeto de mejorar el producto final.

El recipiente más adecuado para realizar la acetificación de los condimentos de fresa serían los barriles de madera ya que dan lugar a productos más antioxidantes y más ricos en compuestos fenólicos que el recipiente de vidrio y además, con mejores características aromáticas. Por otro lado, teniendo en cuenta el perfil aromático, la opinión de los

consumidores y de los catadores expertos en la evaluación de los dos tipos de madera utilizados (cerezo y roble), la mejor solución tecnológica es el empleo de barriles de roble.

La adaptación del proceso a escala semi-piloto tuvo muy buenos resultados, indicando que la producción a escala industrial de estos condimentos sería viable, obteniéndose productos seguros por su escaso o nulo contenido en carbamato de etilo y potencialmente competitivos en el mercado.

Los ensayos realizados partiendo del sustrato mosto cocido y la evaluación de sus correspondientes productos finales nos ha planteado la posibilidad de la obtención de un producto con características peculiares. Estos condimentos fueron los mejor calificados por los consumidores y el panel de expertos, además de presentar los valores más altos de actividad antioxidante y concentración de fenoles. Sin embargo, la obtención de este sustrato es lenta y compleja, por lo que pensamos que este sustrato sería ideal para la producción a pequeña escala de productos de calidad diferenciada.

6. CONCLUSIONES

De los resultados obtenidos durante el desarrollo de la presente tesis doctoral se extraen las siguientes conclusiones:

1. Respecto a la evaluación de la actividad antioxidante, polifenoles y antocianos podemos concluir que:

1.1. El método de extracción más adecuado para el estudio de estos parámetros comprende la aplicación de ultrasonido durante un tiempo intermedio (25 minutos) y una mezcla de solvente y agua (80:20), siendo el etanol el más adecuado en el caso del caqui y acetona en el de la fresa.

1.2. Considerando las diferentes condiciones testadas a lo largo del proceso de producción de los condimentos comprobamos que:

-La adición de enzimas pectolíticas y SO_2 tiene un efecto positivo ya que permite obtener sustratos de partida con mayor actividad antioxidante y compuestos fenólicos.

-En relación a los tres sustratos empleados en la elaboración de condimentos de fresa (pasta, líquido y mosto cocido), el mejor condimento es el obtenido a partir de mosto cocido al presentar los mayores valores de actividad antioxidante e índice de polifenoles totales.

-El uso de la cepa de levadura seleccionada RP1 parece no ser la más adecuada para la obtención de condimentos ricos en antocianinas monoméricas.

-Los mejores recipientes para llevar a cabo la fermentación acética son los barriles de madera, especialmente de cerezo.

-El aumento de la escala de producción a escala semi-piloto supone una menor pérdida de actividad antioxidante y compuestos fenólicos.

1.3. En el caso particular de los condimentos de fresa, durante el proceso de obtención se produce un importante descenso de la actividad antioxidante y compuestos fenólicos, siendo la acetificación la etapa que afecta de manera más drástica a dichos parámetros.

1.4. Los condimentos de fresa presentan mayores cualidades saludables que los de caqui. Además, al compararlos con otros vinagres comerciales, el condimento de fresa ocupa un segundo lugar, sólo superado por el Aceto Balsámico de Módena. Esto lo señala como un producto potencialmente competitivo en el mercado.

2. El patrón de consumo de aminoácidos, tanto por parte de las levaduras como de las bacterias acéticas, está más condicionado por el contenido de aminoácidos de la fruta, que por el tipo de microorganismo ya que consumen los más abundantes en los sustratos de partida. Sin embargo, en las fermentaciones alcohólicas inoculadas, el nitrógeno total consumido sí está condicionado por la cepa de levadura utilizada. La cepa RP1, aislada durante la fermentación espontánea de la pasta de fresa, presenta una mayor tasa de consumo de nitrógeno total que la cepa comercial (QA23).

3. El método de muestreo en espacio de cabeza para la determinación de compuestos volátiles mayoritarios es lineal, sensible, repetitivo, reproducible y exacto, resultando válido para el fin propuesto. En relación a las condiciones de producción, la adición de enzimas pectolíticas y SO_2 produce un aumento de acetaldehído y metanol, siendo los niveles de este último un valor a controlar por su efecto negativo en la salud y en el aroma. Por otro lado, se ha comprobado que las cepas de *Saccharomyces cerevisiae* empleadas tiene una gran influencia en la producción del acetaldehído y de alcoholes superiores y parece ser que la RP1 produce los vinos más adecuados para obtener los condimentos con mejor perfil volátil.

4. El análisis olfatométrico del sustrato de fresa y sus correspondientes condimentos de la campaña del 2008 pone de manifiesto que a pesar del proceso de doble fermentación los productos obtenidos conservan los aromas característicos y de impacto de la fresa. Por tanto, el perfil aromático de los condimentos finales es una mezcla entre los procedentes del sustrato y los que se forman a lo largo de los procesos de fermentación.

5. Tras el análisis olfatométrico y sensorial de las muestras de la campaña de fresa del 2009 se concluye que el uso de diferentes sustratos de partida y recipientes para la acetificación produce grandes diferencias aromáticas en los condimentos finales:

5.1. El condimento obtenido del mosto cocido es sensorialmente muy diferente a los producidos a partir de la pasta de fresa sin concentrar, por tanto son dos condimentos

totalmente diferentes, teniendo mayor puntuación en impresión general el procedente del mosto cocido.

5.2. Entre los condimentos obtenidos del puré de fresa sin concentrar, los elaborados en barriles de madera presentan más zonas odorantes que el elaborado en recipiente de cristal.

5.3. Al comparar el análisis sensorial descriptivo de los condimentos acetificados en barriles de madera, se concluye que el elaborado en barril de roble tiene un perfil aromático de mayor calidad y por tanto sería el recipiente elegido para la acetificación.

6. En relación al estudio de precursores aromáticos de la fresa:

6.1. Entre las estrategias ensayadas la hidrólisis ácida durante una hora fue la más adecuada para el estudio del potencial aromático de esta fruta.

6.2. El uso por primera vez de resinas LiChrolut® para el análisis de precursores aromáticos permite determinar hasta un total de 51 agliconas, 38 de ellas no descritas anteriormente en fresa.

6.3. El análisis de los precursores aromáticos de las cuatro variedades de fresa estudiadas, pone de manifiesto que la variedad *Fuentepina* es la que tiene la mayor diversidad de precursores. Sin embargo, la posible alternativa a la variedad *Camarosa* es la *Sabrina*, ya que en ella predominan los aromas dulces sobre los herbáceos y tiene altas concentraciones de aromas característicos de la fresa en forma de precursores aromáticos.

6.4. El análisis olfatométrico de las cuatro variedades permitió la identificación por primera vez en fresa de (Z)-1,5-octadien-3-ona, un posible aroma de impacto con una frecuencia modificada de más de 80 en dos de las variedades *(Fuentepina y Candonga).*

7. Los descriptores aromáticos pasa, caramelo tostado, punzante y licoroso son los que condicionan la preferencia de los consumidores. Además, las pruebas de preferencia señalan que el condimento producido a partir del mosto cocido es el preferido por los consumidores.

8. Tras la validación y aplicación de un método para la determinación de carbamato de etilo en vinagres utilizando cartuchos de extracción ISOLUTE ENV+, se comprueba que los

condimentos elaborados presentan valores por debajo de los límites de cuantificación, siendo seguro su consumo en relación a este compuesto.

9. Finalmente, para producir condimentos de fresa de calidad se debe partir de puré o pasta de fresa adicionada de enzimas pectolíticas y sulfuroso, inocular con una cepa de levadura seleccionada y llevar a cabo el proceso de acetificación en barriles de madera de roble con bacterias acéticas seleccionadas. La variedad de fresa empleada así como las cepas de levadura y bacterias acéticas que lleven a cabo las fermentaciones deben ser objeto de futuras investigaciones.

6. CONCLUSIONS

The main conclusions that can be drawn based on the results obtained during the development of this Thesis are as follows:

1. With regard to evaluation of antioxidant activity, polyphenols and anthocyanins we can conclude that:

1.1. The most adequate method for the study of these parameters is the use of ultrasound during an intermediate time (25 minutes) and a mixture of solvent and water (80:20), being ethanol the most suitable in the case of persimmon and acetone in the case of strawberry.

1.2. Considering the different conditions tested during the production process of vinegars we checked that:

- The addition of pectolytic enzymes and SO_2 has a positive effect since it enables to obtain starting substrates with major antioxidant activity and phenolic compounds.

- Regarding to the three substrates employed in the production of strawberry substrates (puree, liquid and cooked must), the best vinegar is the one obtained from cooked must, since it presents the higher values of antioxidant activity and total phenol index.

- The use of the selected yeast strain RP1, seems not to be the most adequate for obtaining rich vinegars in monomeric anthocyanins.

- The best recipients to carry out the acetification are the wood barrels, specially the cherry ones.

- The increase of the production scale to semi-pilot scale means less loss of antioxidant activity and phenolic compounds.

1.3. In the particular case of the strawberry vinegar, a significant decrease in antioxidant activity and phenolic compounds is produced during the elaboration process, being the acetification the step that most affects these parameters.

1.4. The strawberry vinegars present the higher healthy qualities tan the persimmon ones. In addition, if we compare them with other commercial vinegars, the strawberry

one is in the second place, only surpassed by the Aceto Balsamico of Modena. This points it as a potentially competitive product in the market.

2. The consumption pattern of amino acids, by yeast and acetic bacteria, is more conditioned by the amino acid content of fruit tan by the type of microorganism involved in the process, since they consume the most abundant amino acids in the starting substrate. However, in the inoculated fermentations, the total nitrogen consumed is conditioned by the used yeast strain. The RP1 strain, isolated during spontaneous fermentation of strawberry puree presents a higher rate of total nitrogen consumption than the commercial strain (QA23).

3. The sampling static headspace method for the determination of major volatile compounds is lineal, sensitive, repetitive, reproducible and accurate, being suitable for the proposed aim. Regarding the conditions of production, the addition of pectolytic enzymes and SO_2 produce an increase of acetaldehyde and methanol, being the levels of the last one a value to control by its negative effect in the health and aroma. On the other hand, it has been observed that the employed *Saccharomyces cerevisiae* strains have a great influence in the production of acetaldehyde and higher alcohols, and it seems that the RP1 one produces the most adequate wines to obtain the vinegars with the best volatile profile.

4. The olfactometric analyses of the strawberry substrate and its corresponding vinegars from 2008 harvest reveal that, despite the double fermentation process, the obtained products preserve the characteristic and impact strawberry aroma. Hence, the aromatic profile of final vinegars is a mixture of those aroma compounds from the substrate and those formed during the fermentation processes.

5. After olfactometric and sensory analyses of samples from 2009 harvest we can conclude that the use of different starting substrates and recipients for the acetification produces great aromatic differences in the final vinegars:

5.1. The vinegar obtained from cocked must is, from a sensory point of view, very different to those produced from strawberry puree without concentration, hence they are two totally different vinegars, reaching higher score in the general impression attribute the one from cooked must.

5.2. Among the obtained vinegars from strawberry puree without concentration, those produced in wood barrels present more odor-active zones than the one produced in glass recipient.

5.3. If we compare the descriptive sensory analysis of vinegars produced in wood barrels, we conclude that the one produced in oak barrel has an aromatic profile of higher quality and hence it would be the chosen recipient to perform the acetification.

6. With respect to the study of strawberry aromatic precursors:

6.1. Among the hydrolytic strategies assayed, the use of one tour of harsh acid hydrolysis was the most appropiated for the study of the aromatic potencial of this fruit.

6.2. The use of LiChrolut® resins for the analysis of aromatic precursors enable to determine 51 aglycones, 38 of them not previously described in strawberry.

6.3. The analysis of the aromatic precursors of the four varieties studied, shows that the *Fuentepina* is the one that has the higher number of aromatic precursors. However, the alternative to the *Camarosa* variety is *Sabrina* since sweet aroma predominates over green nuances and has high concentration of characteristic strawberry aroma compounds in the precursors form.

6.4. The olfactometric analysis of the four varieties enabled the identification of (Z)-1,5-octadien-3-one for the first time in strawberry, a possible impact odorant with a modified frequency >80 in two of the varieties (*Fuentepina y Candonga).*

7. The aromatic descriptors: raisin, toasted caramel, spicy and liquor are the ones that condition the consumer preference. In addition, preference tests point to the vinegar produced from the cooked must as the most appreciated by the consumers.

8. After the validation and application of a method for the ethyl carbamate determination in vinegars using the ISOLUTE ENV+ cartridges, it is observed that the vinegars produced present values under the quantification limits, being safe for it consumption.

9. Finally, to produce quality strawberry vinegars it is necessary to use the fruit puree or a commercial puree with pectolytic enzymes and sulphur dioxide, inoculate with a selected yeast strain and carry out the acetification in an oak wood barrel with selected acetic acid bacteria. The strawberry variety employed and the yeast and acetic acid bacteria strains used for fermentation should be the aim of future research.

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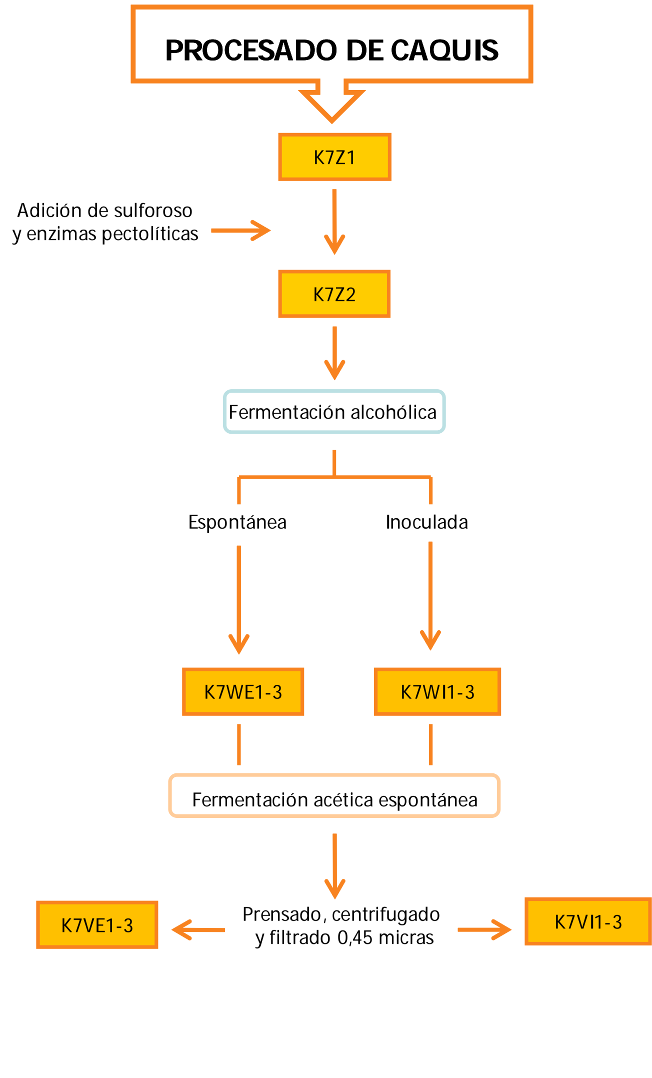

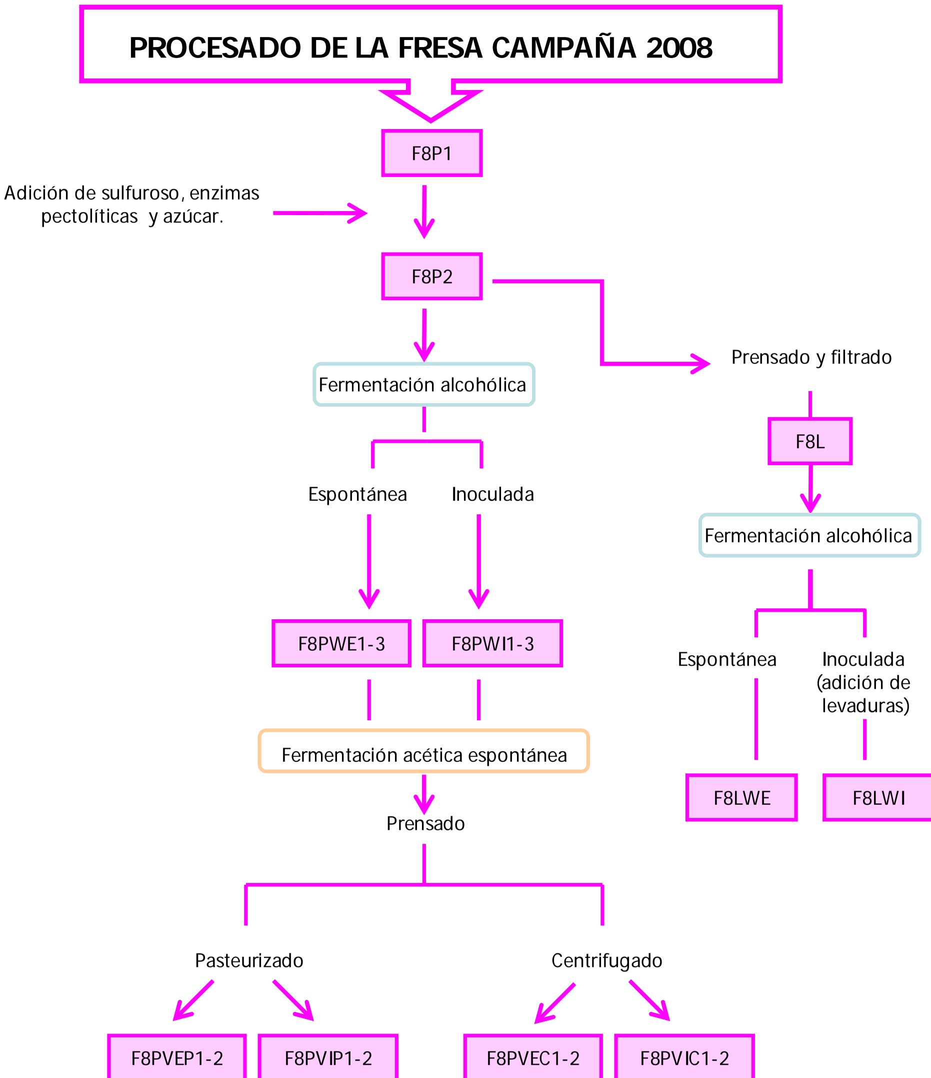

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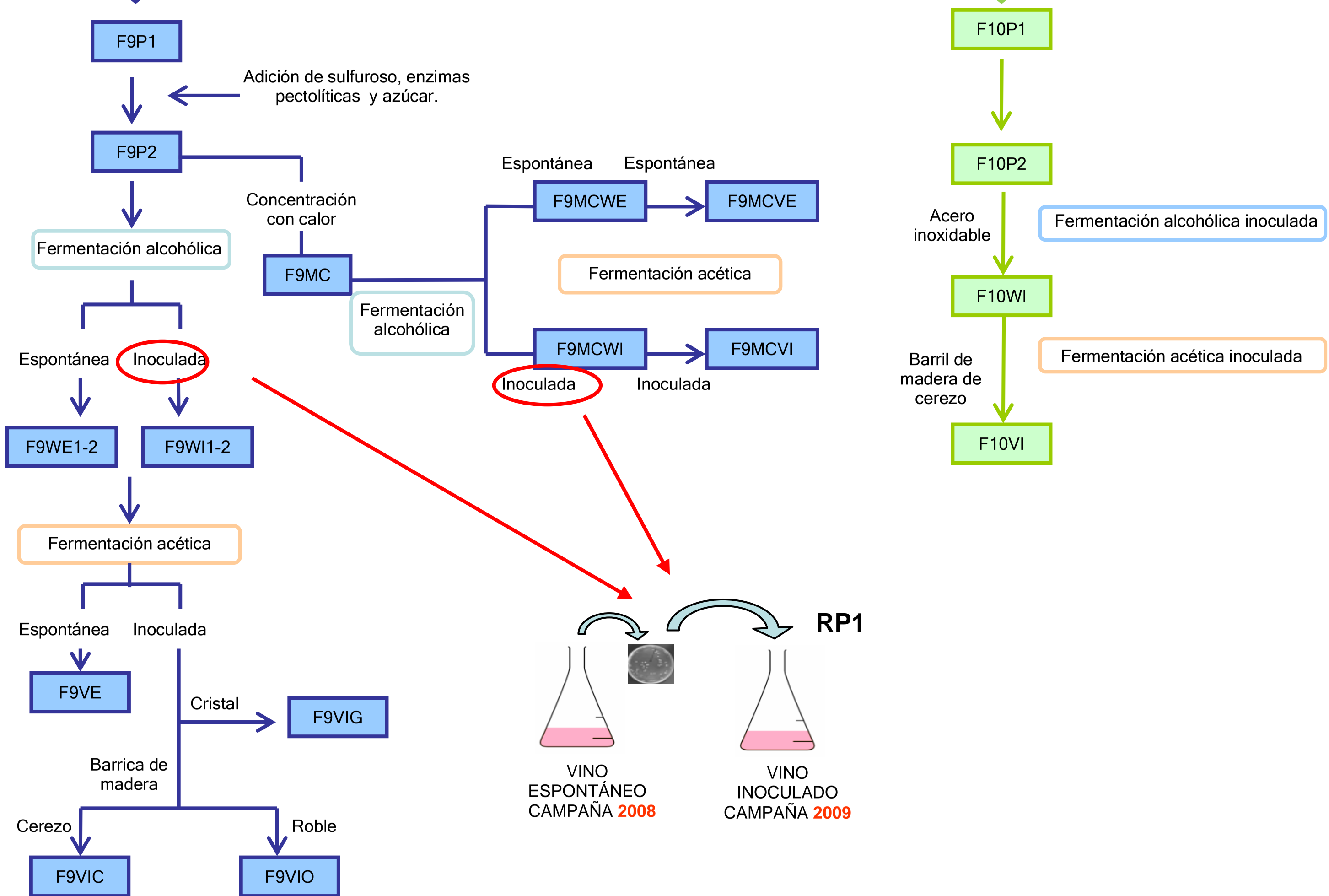

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