UNIVERSIDAD DE SEVILLA
Facultad de Farmacia
Departamento de Bioquímica, Bromatología, Toxicología y Medicina Legal

CARACTERIZACIÓN QUÍMICA Y SENSORIAL DEL AROMA DEL VINAGRE DE VINO

Memoria que presenta la
Licenciada RAQUEL Mª CALLEJÓN FERNÁNDEZ para
optar al título de Doctor en Farmacia con Mención Europea por la Universidad de Sevilla

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Y para que así conste, firmo el presente certificado en Sevilla, Septiembre de 2008.

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Y para que conste, firmamos en Sevilla, Septiembre de 2008

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"Sólo una cosa hace que un sueño sea imposible:
el miedo a fracasar"

Paulo Coelho, El Alquimista

A mis padres y hermanos
A Tomás

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1. RESUMEN

1.1. Justificación y objetivos

El vinagre de vino es el producto resultante de la fermentación acética del vino realizada por las bacterias acéticas. Este producto ha formado parte de la alimentación humana desde la antigüedad más remota como condimento y conservador de los alimentos. El vinagre de vino es un producto cuyo valor y apreciación por parte de los consumidores está experimentando un importante incremento en los últimos años, impulsado por el prestigio que tienen determinados productos en el ámbito gastronómico. Así, las empresas productoras de vinagres se marcan como objetivo obtener vinagres de calidad y aplican toda la tecnología a su alcance para ello.

Las bacterias acéticas son las responsables de los procesos de acetificación, las cuales constituyen un grupo ecológico que comprende bacterias Gram-negativas, aerobias estrictas y muy sensibles al SO_2. Estas bacterias oxidan azúcares y alcoholes, produciendo una acumulación de ácidos orgánicos como producto final, por lo que crecen en medios azucarados y alcoholizados ligeramente ácidos como los vinos y los vinagres. Cuando el sustrato es etanol, producen ácido acético, lo que ha originado el nombre que reciben estas bacterias.

Desde un punto de vista tecnológico, se puede hablar de dos métodos de elaboración del vinagre de vino, bien sea mediante cultivo sumergido, cuyos productos se pueden envejecer o no en madera, o por métodos tradicionales, mucho más lentos, con cultivo superficial, los cuales se llevan a cabo en barriles de madera de diferente capacidad. En los métodos de acetificación con cultivo sumergido, las bacterias acéticas están sumergidas libremente en el líquido a fermentar, en el que constantemente se introduce aire, en condiciones que permitan la máxima transferencia posible desde la fase gaseosa a la fase líquida. Así se obtienen de forma rápida los vinagres comerciales actuales de menor precio. Por otro lado, en los métodos tradicionales con cultivo superficial, las bacterias acéticas se encuentran en contacto directo con el oxígeno gaseoso, situadas bien en el interfase líquido/gas o bien fijadas a soportes de materiales tales como virutas de madera. La combinación del empleo de distintos sustratos vínicos

y métodos de elaboración da lugar a una amplia gama de productos de muy diferente calidad. Los vinagres elaborados mediante métodos tradicionales son de mayor calidad debido a su complejidad aromática y extraordinarias propiedades organolépticas, bien reconocidas por los consumidores. Por este motivo, estos vinagres alcanzan mayores precios en el mercado. Hoy en día, vinagres tan apreciados y selectos como los vinagres de Jerez o los vinagres Tradicionales Balsámicos de Módena, entre otros, se producen con métodos tradicionales.

El aroma es uno de los indicadores más importantes de la calidad de los vinagres. Por eso, los productores eligen las mejores materias primas y el proceso de acetificación óptimo para incrementar la calidad aromática del vinagre de vino, ya que se ha demostrado que ambos factores influyen de manera decisiva en la composición final.

La caracterización de vinagres de vino incluye la determinación de parámetros fisicoquímicos y sensoriales. Los aminoácidos constituyen la principal fuente de nitrógeno para las bacterias acéticas, las cuales son responsables de la conversión del etanol en ácido acético. Debido a que el vino de partida procede de una fermentación previa realizada por levaduras, es esencial asegurarse de que haya una cantidad adecuada de nitrógeno para que se lleve a cabo la fermentación acética. Por tanto, la composición y contenido de aminoácidos es de gran importancia en la producción de vino y vinagre. En estudios realizados en vinos se ha demostrado que muchos aminoácidos experimentan una serie de biotransformaciones, dando lugar a alcoholes de alto peso molecular, aldehídos y ácidos cetónicos, los cuales tienen un gran impacto en las propiedades organolépticas del vino. Por este motivo a los aminoácidos se les atribuye el papel de precursores del aroma.

El aroma del vinagre de vino esta determinado por una serie de compuestos volátiles que tienen tres posibles orígenes: los que proceden del vino, los formados durante la acetificación y aquellos que aparecen durante la maduración o envejecimiento en madera. De este modo, el aroma del vinagre es una fracción compleja que contiene muchos componentes con un amplio margen de volatilidad, polaridad y concentración. La cuantificación de los compuestos del aroma de vinagre de vino ha sido siempre un reto debido a la complejidad de la matriz y la baja concentración de los

compuestos de aroma. Hasta la fecha se han identificado más de setenta 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 del vinagre.

La industria ha dirigido sus esfuerzos a producir vinagres con las mejores propiedades organolépticas, a fin de satisfacer la creciente demanda del consumidor hacia los vinagres de alta calidad. Además, la caracterización de las propiedades sensoriales típicas de los productos tradicionales es muy necesaria, no sólo para la industrialización de la producción de alimentos, sino también para las leyes sobre seguridad alimentaria e incluso para el desarrollo de productos innovadores. Sin embargo, hay muy pocos estudios sobre la contribución de los compuestos individuales a las características del aroma del vinagre de vino. Por ello, la identificación de compuestos que tienen un gran impacto en el aroma de los vinagres de vino, constituye una de las tareas más desafiantes en este campo de investigación.

El objetivo de la Tesis Doctoral que aquí se presenta es caracterizar química y sensorialmente el aroma de diferentes vinagres de vino: vinagres de Jerez, vinagres de vino tinto y blanco.

Este objetivo general incluye los siguientes objetivos parciales:

1. Puesta a punto y aplicación de un método de determinación de aminoácidos por Cromatografía de Líquidos empleando el agente derivatizante 6-aminoquinolil-N-hidroxisuccinimidil carbamato (AQC) en vinagres de vino y seguimiento durante la fermentación acética (cultivo superficial y sumergido).

2. Determinación de compuestos volátiles por Cromatografía de Gases acoplada a Espectrometría de Masas empleando la técnica de "Extracción por absorción con barras magnéticas agitadoras en espacio de cabeza": puesta a punto y aplicación durante el proceso de acetificación (cultivo superficial y sumergido) y de envejecimiento de vinagres de vino.

3. Descripción del perfil aromático del vinagre de Jerez, correlacionando los resultados sensoriales con los datos químicos, e identificación de compuestos volátiles con gran impacto sobre el aroma típico del vinagre de Jerez mediante diferentes técnicas olfatométricas.

1.2. Estructura de la Tesis

La Tesis Doctoral se presenta en forma de compendio de publicaciones científicas y opta a la Mención de "Doctor Europeo".

Esta memoria presenta la siguiente estructura: los dos primeros capítulos "Resumen" e "Introducción" recogen los fundamentos en los que esta Tesis se ha basado para su desarrollo. En el tercer capítulo se describen los objetivos de la Tesis doctoral, justificando la temática de la misma y en el cuarto capítulo se presentan los resultados obtenidos, los cuales han dado lugar a 7 trabajos de investigación: dos publicados, dos aceptados, dos enviados para su publicación y uno en la última fase de redacción. Finalmente, en el último capítulo se incluyen las conclusiones fundamentales que han podido extraerse a partir de todos los resultados obtenidos.

1. SUMMARY

1.1. Justification and objectives

Wine vinegar is a product obtained by the acetic fermentation of wine carried out by different acetic acid bacteria. This product has been part of human diet since antiquity as a condiment and preservative for food. Wine Vinegar has become a highly appreciated product among consumers, specially in last years. Hence, one of the main goals of vinegar-making companies is to produce high quality vinegar by applying successful technologies and quality control.

Acetic acid bacteria are responsible of acetification and constitute an ecological group comprising Gram-negative, obligate aerobes and very sensitive to SO_2. They oxidize sugars and alcohol, producing an accumulation of organic acids as end products. They can grow in a sweet, fortified and slightly acidic environment such as wine and vinegar. They produce high quantities of acetic acid from ethanol.

From a technological point of view, there are two well defined methods for vinegar production: submerged methods, whose products may or not be aged in wood, and traditional slow processes, carried out in wood barrels of different capacity. In the submerged methods, acetic bacteria are freely submerged in the liquid to be fermented. A stream of air is constantly introduced to allow the maximum oxygen transfer from the gas phase to the liquid phase. On the other hand, in the traditional methods, acetic acid bacteria are in direct contact with oxygen gas. They are placed either at the interface liquid/gas or fixed to supports such as wood chips. According to the wine substrates and the acetification method employed there are a lot of products of very different qualities in the market. The different combination of wine substrates and methods of production leads to a wide range of products.

Vinegars obtained by slow traditional surface methods in wood barrels have a higher quality due to its sensory complexity and extraordinary organoleptic properties, well recognized by consumers. For this reason, these vinegars generally reach higher

prices in the market. Nowadays, traditional and selected vinegars such as Sherry vinegars or Traditional Balsamic vinegars from Módena, among others, are produced following this method.

Aroma is one of the most important indicators of vinegar quality. For this reason, it is important to choose the best raw materials as well as the optimum acetification conditions to increase the aromatic quality of wine vinegar, since both factors have an impact on the final aroma.

The characterization of vinegar includes a wide range of values obtained from physicochemical and sensory analysis. The determination of amino acids has also been directed to the characterization of wine vinegars. Amino acids are the main nitrogen source for acetic acid bacteria, which are responsible of the conversion of ethanol into acetic acid. Since for the special case of vinegar, the initial substrate comes from a previous alcoholic fermentation, it is important to ensure enough available nitrogen for the acetic fermentation. Therefore, the amino acid composition of the substrate is of great importance in the production of wine and vinegar. Many amino acids undergo a series of biotransformations during alcoholic fermentation yielding higher alcohols, aldehydes, acids and ketones which have an impact on the organoleptic properties of wine. For this reason the amino acids are considered as flavour precursors.

The flavor of wine vinegars is determined by a series of volatile constituents with three different origins: wine substrate, acetification and aging. Thus, aroma of vinegar is a complex fraction containing many components with a wide range of polarities, solubilities and volatilities.

Quantification of aroma compounds in wine vinegar has been challenging due to complexity of the matrix and low concentrations expected for the aroma compounds. To date, more than seventy compounds have been identified in the volatile fraction of wine vinegars including alcohols, esters, carbonyls, ethers, acids, volatile phenols, lactones and acetals which contribute to a greater or lesser extent in the aroma of vinegar.

The industry has directed their efforts to the production of vinegars with the highest sensory quality, in order to meet consumers increasing demand for high quality

vinegars. Furthermore there is a need for the characterization of the typical sensory properties of traditional products not only for the industrialization of food production, but also for laws on food safety and even for the development of innovative products. However, there are very few systematic studies about the contribution of individual compounds to the final aroma of wine vinegar. Hence, the identification of compounds with large impact on the perceived quality of a food product constitutes one of the most challenging tasks in flavor research.

This Doctoral Thesis aims to characterize by chemical and sensory data the aroma of different wine vinegars: Sherry, red and white vinegars.

This general objective includes the following specific objectives:

1. Validation of a method for the determination of amino acids by Liquid Chromatography (HPLC) in wine vinegars using the reagent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). Evaluation of changes in amino acids during acetic fermentation (surface and submerged culture).

2. Determination of volatile compounds by Gas Chromatography and Mass Spectrometry using the technique "Headspace Sorptive Extraction": Validation of the method. Application of this methodology to follow the acetification process (surface and submerged culture) and to evaluate changes during aging in different wood barrels.

3. Description of the aroma profile of Sherry vinegar, correlating the sensory results with the chemical data, and identification of volatiles with large impact on the perceived tipicity of Sherry vinegar by using different olfactometric techniques.

1.2. Structure of the Thesis

The Doctoral Thesis is presented as a compilation of scientific publications for the title “Doctor Europeo”.

The Thesis report presents the following structure:

The first two chapters, "Summary" and "Introduction", reflects the theoretical basis on which this research has been focused for its development. In the third chapter, the objectives of the Thesis are depicted, justifying the unity of its topic and in the fourth chapter, the results of this research are presented, which suppose 7 original research papers: two of them published, two accepted, two sent for publication and one in the final stage of drafting. Finally, the last chapter, includes the main conclusions which can be drawn from the results obtained.

2. INTRODUCCIÓN

2.1. EL VINAGRE

Según la Reglamentación Técnico Sanitaria (*Presidencia del Gobierno, 1993)* con la denominación genérica de vinagre se designa: *"el líquido apto para el consumo humano resultante de la doble fermentación, alcohólica y acética de productos de origen agrario que contengan azúcares o sustancias amiláceas, con una riqueza mínima de 50 g/L"*. Se entiende por grado de acidez de los vinagres su acidez total expresada en gramos de ácido acético por 100 mL, a 20 ºC. El contenido permitido por la norma para los vinagres, expresado en ácido acético, no será inferior a 50 g/L; excepto para el vinagre de vino, que será, al menos, de 60 g/L.

2.1.1. Tipos de vinagre

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

Según la materia prima originaria se establecen los siguientes tipos (*Presidencia del Gobierno, 1993*):

- *Vinagre de vino*: Es el producto obtenido exclusivamente por fermentación acética de vino.
- *Vinagre de frutas*: Es el producto obtenido a partir de frutas o bayas.
- *Vinagre de alcohol*: Es el producto obtenido por la fermentación acética de alcohol destilado de origen agrario.
- *Vinagre de cereales*: Es el producto obtenido sin destilación intermedia por el procedimiento de doble fermentación alcohólica y acética, de cualquier cereal en grano, cuyo almidón se ha desdoblado en azúcares mediante un procedimiento distinto de la diastasa de la cebada malteada.

- *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.
- *Vinagre de miel*: Es el producto obtenido a partir de la miel.
- *Vinagre de suero de leche*: Es el producto obtenido a partir de suero de leche.

2.1.2. Historia

El vinagre ha formado parte de la alimentación humana desde la antigüedad más remota como condimento y conservador de alimentos, así como base de remedios sencillos para hombres y animales. La fermentación alcohólica seguida de la acética se produce espontáneamente sobre cualquier sustrato azucarado expuesto al polvo y a los insectos que transportan levaduras y bacterias. Duddington (1961) afirma que la elaboración de vino es un arte que data al menos de hace unos 10.000 años, por lo que podemos suponer la existencia del vinagre desde este tiempo. Las referencias más antiguas al uso del vinagre se hallan en la cultura babilónica (5000 a.C.) sobre la obtención de vinagre de dátiles.

En 1732, el holandés Boerhaave hace notar que la llamada "madre del vinagre" es un organismo vivo, aunque sin precisar su papel en la acetificación. Lavoisier demuestra que la acetificación consiste en la oxidación de etanol, pero sin sospechar la existencia de bacterias acéticas. *Persoon, (1822*) describe las películas grasas que se forman en la superficie del vino, la cerveza o el vinagre como sustancias de naturaleza vegetal, y en la "Micología europea" añade nuevas especies de *Micoderma: ollare, mesentericum, lagenoe y pergameneum.* También Chaptal había observado que la producción de vinagre va bien cuando en la superficie del vino aparecen las llamadas "flores de vino", cuya aparición anuncia y precede a la acetificación, pero sobre esto Berzelius advertía que en todas las materias orgánicas en descomposición, expuestas al aire aparece el mismo tipo de vegetación. La acetificación también llega a formar parte de la controversia entre químicos como Berzelius y Liebig quienes mantienen que el proceso era puramente químico (Berzelius 1829-1833) y aquellos que afirmaban que en

dicha transformación intervenía un "ser organizado". En cuanto a la "madre del vinagre", Kützing observó que la débil película que recubre la superficie del líquido acidificado está formada por glóbulos seis veces más pequeños que los de las levaduras; se trataba de bacterias acéticas que fueron observadas al microscopio por primera vez por Kützing (1837) por lo que, en las primeras clasificaciones taxonómicas de estos microorganismos, se denominó *Acetobacter kützingianum* a una especie de ellos (*Llaguno, 1991*).

Pasteur publicó en el año 1864 una amplia memoria sobre la fermentación acética "Études sur le vinaigre, sa fabrication, ses maladies, mohines de les prévenir" que recoge la conferencia que pronunció en Orleáns en 1867. Pasteur afirma que siempre que el vino se transforma en vinagre, es debido a la acción de un velo de *Micoderma aceti* desarrollado en su superficie.

2.1.3. Las bacterias acéticas

Las bacterias acéticas fueron observadas por primera vez al microscopio por Kützing en 1837. Estas bacterias constituyen un grupo ecológico que comprende bacterias gram-negativas (gram-positivas en cultivos viejos), aerobias estrictas y muy sensibles al SO_2. Son catalasa positiva y oxidasa negativa, pueden presentar pigmentación en cultivos sólidos y producir diferentes tipos de polisacáridos (*De Ley et al., 1984*).

Al microscopio óptico las bacterias acéticas se presentan como pequeñas células cilíndricas, frecuentemente en parejas cocobacilares, cortas y algo gruesas, alineadas o en cadenas, y a menudo agrupadas en forma de ocho. Constituyen un grupo de morfología variable, que se presentan en forma elipsoidal o de bastoncillos (*Suárez e Iñigo, 1990*). Las bacterias acéticas crecen en medios azucarados y alcoholizados, ligeramente ácidos como flores, frutas, cerveza, vino, sidra, vinagre, zumos de fruta agrios y miel. En estos sustratos, las bacterias acéticas oxidan los azúcares y los alcoholes, produciendo una acumulación de ácidos orgánicos como producto final. Cuando el sustrato es etanol, producen ácido acético, lo que ha originado el nombre que reciben estas bacterias. La oxidación del etanol tiene lugar en dos pasos. En el primero,

el etanol es oxidado a acetaldehído y en el segundo el acetaldehído es oxidado a acetato. En ambas reacciones, los electrones son transferidos y el último aceptor es el oxígeno (Figura 2.1). Esta oxidación de etanol a ácido acético es la característica mejor conocida de la bacteria acética, pero estas bacterias también pueden oxidar glucosa a ácido glucónico, etc. Algunas de estas transformaciones resultan interesantes desde el punto de vista de la biotecnología. La aplicación industrial mejor conocida de las bacterias acéticas es la producción de vinagre pero también se usan para producir sorbosa, sorbitol y celulosa.

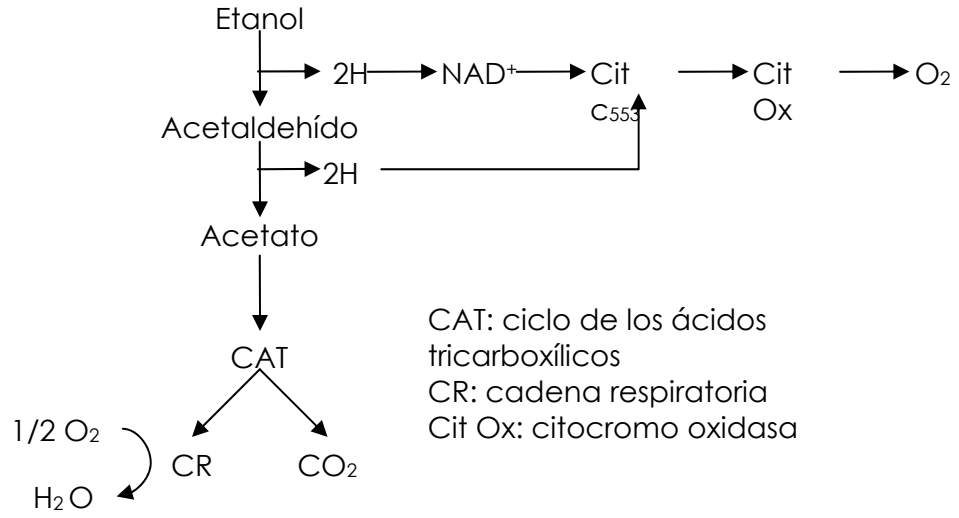


Figura 2.1. *Proceso de oxidación del etanol.*

Las primeras clasificaciones de bacterias acéticas se hicieron atendiendo exclusivamente a criterios morfológicos. Visser`t Hooft (1925) toma en consideración también su fisiología, y más tarde Asai (1968) clasifica las bacterias acéticas según su capacidad para metabolizar la glucosa y el ácido acético. Frateur (1950) propone un sistema de clasificación del género *Acetobacter* en cuatro grupos, basado en los caracteres bioquímicos siguientes: producción de catalasa, oxidación de acetato y lactato a carbonato, formación de compuestos cetónicos, producción de ácido glucónico y capacidad para crecer en un medio con alcohol y sales de amonio como única fuente de nitrógeno (medio de Hoyer). Propuso los siguientes grupos: *Acetobacter peroxydans, Acetobacter mesoxydans, Acetobacter oxydans y Acetobacter suboxydans.* El nombre de

cada grupo alude fundamentalmente a su capacidad para oxidar el etanol a ácido acético.

Durante largo tiempo se aceptó que las bacterias acéticas móviles, pertenecientes al género *Acetobacter*, tenían flagelos polares monotricos, y dicho género estaba incluido por lo tanto en la familia *Pseudomonodaceae*. Sin embargo, Leifson (1954) descubrió que 30 cepas de *Acetobacter* no mostraban este tipo de flagelos, poniendo de manifiesto otro tipo de disposición de flagelos: flagelación perítrica. Propuso entonces separar *Acetobacter* en dos géneros: *Acetobacter y Acetomonas* género nov., incluyendo en este último las especies con flagelos polares multitricos y las no flageladas, todas incapaces de oxidar el acetato y el lactato a CO_2 y H_2O.

Según la clasificación recogida en el manual de Bergey, en su 8ª edición (1974), las bacterias acéticas pertenecen al orden *Pseudomonodales*, familia *Pseudomonodaceae*, incluyendo en esta última dos géneros distintos al género *Pseudomonas* (sobre todo por su capacidad de crecimiento a pH inferior a 4.5): el género *Gluconobacter* y el género *Acetobacter*, que incluye a su vez tres especies importantes: *Acetobacter aceti, Acetobacter pasteurianus* y *Acetobacter peroxydans*. El género *Acetobacter* puede variar en su forma de elipsoide a barra derecha, o ligeramente curva, 0.6-0.8 μm x 1.0-1.4 μm. Se encuentran no agrupadas, en pares o formando cadenas. Las células pueden ser móviles o no. Si son móviles, los flagelos son peritricos o laterales, oxida acetato y lactato a CO_2 y H_2O. El pH óptimo para el crecimiento es de 5.4-6.3 (*De Ley et al., 1984*). Sin embargo, estas bacterias pueden crecer a pH bajos, entre 3-4. El género *Gluconobacter* es capaz de producir grandes cantidades de ácido glucónico a partir de glucosa, tiene incapacidad de formar películas en medios líquidos y pobre crecimiento en sustratos que contienen etanol. Además, es incapaz de oxidar el acetato.

En una serie de trabajos publicados por diversos autores, principalmente Shinwell y Carr (1960), se pone de manifiesto la clara tendencia de las especies de *Acetobacter* a dar cepas mutantes, originándose especies distintas a las ya existentes. Carr (1968) publicó un trabajo en el que pone de manifiesto las observaciones hechas por Shimwell, y afirma que en general, las bacterias acéticas no son genéticamente estables, aunque unas especies sean más estables que otras.

La taxonomía tradicional de los microorganismos, basada fundamentalmente en criterios morfológicos y fisiológicos, se ha visto sometida a continuas reordenaciones y cambios. Esto se ha debido fundamentalmente a la aplicación de técnicas moleculares muy potentes al estudio taxonómico como son la hibridación ADN-ADN, la secuenciación de diferentes regiones del genoma bacteriano, etc. La familia *Acetobacteriaceae* no ha sido una excepción a este proceso de reordenación de géneros y especies. En el año 1997, a los dos géneros mencionados, *Acetobacte*r y *Gluconobacter*, hay que sumarle la definición de dos nuevos géneros de bacterias acéticas: *Gluconoacetobacter* y *Acidomonas* (*Yamada et al.,1997*); y posteriormente se han definido otros dos nuevos géneros más: *Asaia* (*Yamada, 2000*) y *Kozakia* (*Lisdiyanti et al., 2002*).

Por tanto, en estos momentos, la familia *Acetobacteriaceae* está formada por 6 géneros y 34 especies de bacterias acéticas (Tabla 2.1). *Acetobacter* y *Gluconoacetobacter*, con 14 y 11 especies respectivamente, son los géneros donde existe una mayor diversidad de especies (*Guillamón et al., 2003*).

El conocer qué cepas y bacterias acéticas están implicadas en la transformación de etanol en ácido acético es de gran importancia por su repercusión y aplicación a la producción industrial. En general, la producción de vinagres sólo se ha asociado con las especies: *A. aceti, A. pasteurianus, Ga. europaeus Gax.* No obstante, es probable que la especie *G. oxydans* se encuentre asociada a la producción de "condimentos alimentarios" que parten directamente del mosto para la producción de acético, como sería el "Aceto Balsamico Tradizionale" (*Guillamón et al., 2003*).

Tabla 2.1. *Géneros y especies de bacterias acéticas descritas según Yamada, 2003.*

Acetobacter	***Gluconoacetobacter***	***Gluconobacter***
A. aceti	*Ga. liquefaciens*	*G. oxydans*
A. pasteurianus	*Ga. diazotrophicus*	*G. frateurii*
A. pomorum	*Ga. xylinus*	*G. assaii*
A. peroxydans	*Ga. hansenii*	
A. indonesiensis	*Ga. obodiens*	
A. tropicalis	*Ga. intermedius*	
A. syzygii	*Ga. sacchari*	
A. cibinongensis	*Ga. entanii*	
A. orientalis	*Ga. johannae*	
A. orleanensis	*Ga. azotocaptans*	
A. lovaniensis	*Ga. europaeus*	
A. estunensis		
A. malorum		
A.cerevisiae		
Acidomonas	***Asaia***	***Kozakia***
Ac. methanolica	*A. bogorensis*	*K. baliensis*
	A. siamensis	
	A. indonesiensis	
	A. krungthepensis	

La microbiología del proceso no se comprende todavía en su totalidad (*Giudici et al., 2003*). Mediante la aplicación de las distintas técnicas de biología molecular se pretende alcanzar el objetivo de identificar las bacterias viables responsables del proceso sin necesidad de que sean previamente cultivadas. Como técnicas específicas para la cuantificación e identificación de la bacteria en el proceso de acetificación se aplican en la actualidad las siguientes:

- PCR cuantitativa: Esta técnica ya ha sido aplicada para la cuantificación del número total de bacterias (mediante el uso de cebadores universales) en una muestra biológica (*Franke et al., 2000*). En el caso de bacterias acéticas se podrían utilizar cebadores universales de bacterias acéticas diseñados ya para los métodos de identificación (*Ruiz et al., 2000*), así como cebadores particulares de especie, lo que permitiría una segunda cuantificación a nivel de especie.

- Sistemas basados en el uso de marcadores fluorescentes, como epifluorescencia y FISH (*Fluorescent In Situ Hybridisation*). La primera de ellas ha sido utilizada para la cuantificación de bacterias acéticas totales (*Mesa et al., 2003*). La técnica de FISH podría permitir la identificación de bacterias acéticas a nivel de especie lo que sería de gran utilidad y posiblemente complementaria de la PCR cuantitativa (*Franke et al., 1999*).

2.1.4. Métodos de elaboración de vinagre

En el mercado existen dos tipos de vinagres. El primero se obtiene como producto de la fermentación o 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. El segundo tipo se elabora por la acetificación o fermentación con cultivo sumergido, donde 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 los vinagres comerciales actuales de menor precio.

El vinagre de vino, se produce en los países mediterráneos de forma mayoritaria en biorreactores (tanques de acero inoxidable) y con cultivo sumergido, que puede después envejecerse o no en madera. Los métodos tradicionales, lentos, con cultivo

superficial se llevan a cabo en toneles de madera de diferente capacidad y suponen un menor volumen de producción (*Garcia-Parrilla et al., 1998*).

2.1.4.1. Métodos tradicionales de acetificación con cultivo superficial

a) *Método de Orleáns*

Es uno de los métodos más antiguos para fabricar vinagres. Emplea toneles de aproximadamente 250-300 litros de capacidad, que se colocan tumbados en filas horizontales y superpuestas, 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 (Figura 2.2).

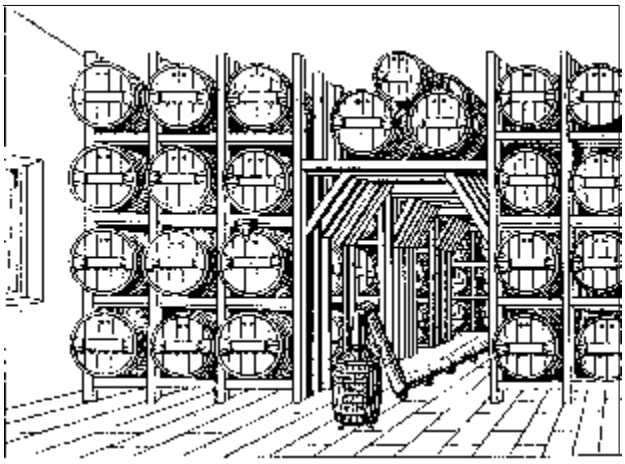


Figura 2.2. *Pilas de botas para la acetificación en una fábrica de vinagre por el método de Orleáns.*

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 (Figura 2.3). 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. Los rendimientos de la transformación de etanol en acético son bajos y el

proceso dura de 8 a 10 días una vez comenzada la acetificación, la velocidad depende de la temperatura, ya que la temperatura de 30 ºC es la óptima para el crecimiento de la bacteria acética.

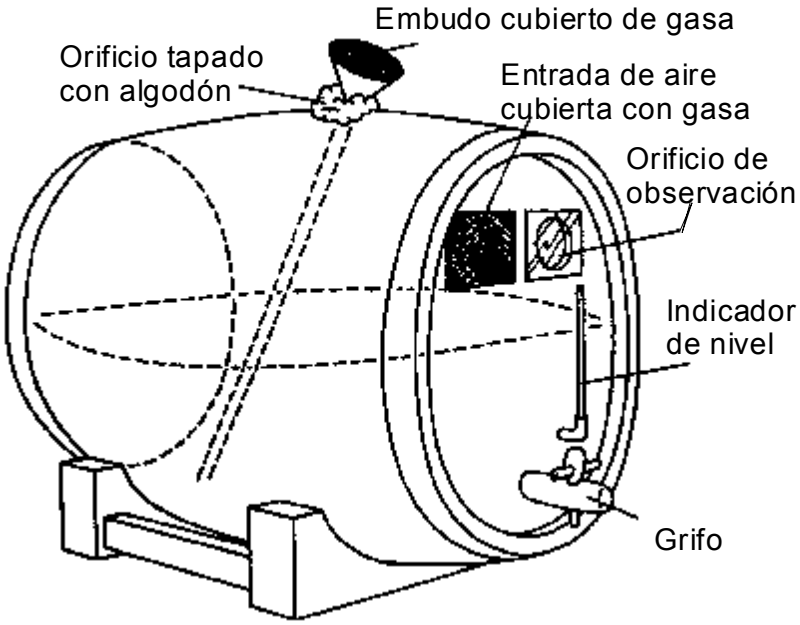


Figura 2.3. *Acetificación por el método de Orleáns (Adams, 1985).*

b) *Método Luxemburgués*

El fundamento de este método y su diferencia fundamental con el método de Orleáns estriba en emplear virutas de haya que periódicamente quedan sumergidas en el líquido que está acetificándose. Así se consigue aumentar la superficie de acetificación de la bacteria y mejorar la transferencia de oxígeno, por lo que aumenta la velocidad de acetificación.

La cuba giratoria más elemental (Figura 2.4) se prepara con un orificio grande en el centro de uno de los fondos, para procurar la entrada de aire. En uno de los costados de esta cuba, en la parte más alejada de la abertura, se practica un orificio estrecho, que puede obturarse con un tapón; es una canilla de madera o vidrio para vaciado del envase. El tonel está dividido en dos partes desiguales por un falso fondo, agujereado, con numerosos y finos orificios. La parte menor del tonel está llena de

virutas de haya. En este compartimento penetra un largo termómetro para controlar la temperatura, aspecto muy interesante para todo método rápido o semirrápido. Se obtienen cantidades de vinagre, que pueden llegar como máximo, cada cuarenta y ocho horas, a la cuarta parte del contenido de un tonel. El vinagre elaborado, que se extrae de las cubas, se sustituye por porciones iguales de vino, continuando la elaboración indefinidamente (*Xandri-Tagüeña, 1977*).

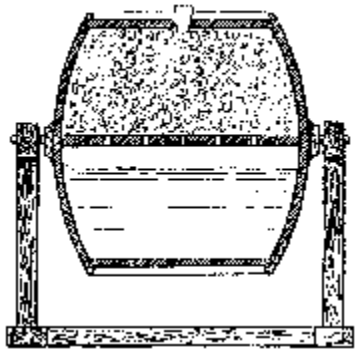


Figura 2.4. *Cuba rotatoria del método Luxemburgués*.

c) *Método de Schüzenbach o Método Alemán*

Se emplean toneles o generadores verticales de encina con doble fondo (Figura 2.5). Sobre el primero, agujereado, se colocan una serie de capas de virutas de madera de haya, impregnadas de 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. El vinagre se extrae por la parte inferior. Se pueden emplear barriles de roble giratorios, parcialmente llenos de virutas, consiguiéndose así una mejor aireación. Las ventajas que se destacan de este proceso son la regulación de oxígeno y su uso para la producción continua de vinagre.

El vinagre obtenido con el método de cultivo superficial tiene el aroma y el gusto propio de la lentitud de la acetificación que se ve favorecido por el simultáneo envejecimiento (*Llaguno y Polo, 1991*).

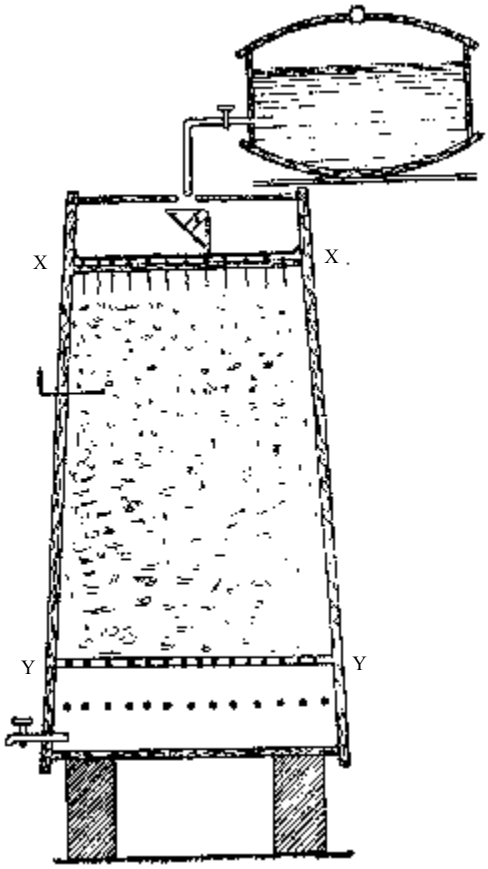


Figura 2.5. *Esquema de un generador vertical para la producción de vinagres vínicos.*

Los principales inconvenientes de los métodos de acetificación en cultivo superficial que emplean las virutas como soporte son:

- Acumulación de bacterias muertas sobre las virutas (debido a falta de aireación o aumentos de temperaturas).
- Desarrollo de bacterias productoras de celulosa.
- La infección por anguílulas (pequeños nematodos) que son imposibles de combatir una vez que se desarrollan.
- Aumentos de temperatura difícilmente controlables, pérdidas de alcohol por evaporación en la corriente ascendente de aire caliente, con bajada del rendimiento.

- Necesidad de gran espacio (generadores de relleno).

Entre los vinagres artesanales producidos por fermentación con cultivo superficial con denominación de origen y reconocimiento internacional destacan el "Aceto Balsámico Tradizionale" de la ciudad italiana de Módena y el no menos prestigioso "Vinagre de Jerez".

2.1.4.2. Métodos de acetificación con cultivo sumergido

Se entiende por fermentación sumergida aquella en la que 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. Emplea toneles de madera o tanques acero inoxidable quedando siempre una parte del vinagre de la operación anterior como inóculo para iniciar el ciclo siguiente. Se llena con el vino, y se introduce posteriormente una fuerte corriente de aire. La acetificación es muy rápida. Este proceso se utiliza ampliamente en la actualidad. Los rendimientos de la transformación del alcohol en ácido acético (hasta el 94 %) resultan ser muy elevados. La velocidad a la que se desarrolla el proceso es mayor (25-30 horas), así como la uniformidad del producto y, sobre todo, se puede lograr la acetificación de iguales volúmenes de alcohol en mucho menor volumen de instalación, con el consiguiente ahorro de espacio. Se puede trabajar con dispositivos automáticos que no sólo regulen el control de la aireación, sino también los ciclos de carga y descarga (*Llaguno y Polo, 1991*).

Modelos Frings

En 1878, Heinrich Frings fundó en Aquisgrán una sociedad productora de vinagre, que más tarde, en 1950, incorpora las patentes de invención resultantes de la investigación del proceso de fermentación sumergida, alcanzando un alto grado de desarrollo. Nació así el "Acetator Frings", base de la biotecnología vinagrera actual.

A partir de las investigaciones de Hromatka y Ebner (1949), en los años 40 fue construido el "Acetator Frings" (Figura 2.6) que se mantiene funcionando, con algunas modificaciones, en gran parte de las industrias vinagreras actuales y en el que se elabora la mayor parte de la producción.

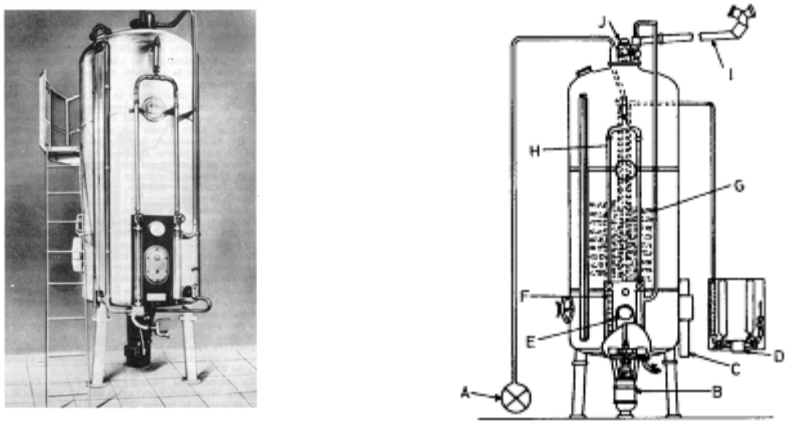


Figura 2.6. *Acetator Frings en acero inoxidable*
A, bomba de carga; B, aireador y motor; C, dispositivo para determinación de alcohol residual; D, válvula entrada agua refrigeración; E, termostato reguladora ; F, rotámetro; G, serpentín de refrigeración; H, entrada de aire; I, salida de gases; J, dispositivo antiespuma.

El fundamento es la presencia de cultivo sumergido en el seno del líquido a acetificar, que se satura constantemente de pequeñas burbujas de aire. 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 (del 94 %) y una mayor velocidad del proceso (25-30 horas). Este procedimiento requiere la estricta vigilancia de tres parámetros: la temperatura, la presión parcial de oxígeno y los ciclos de carga y descarga.

La bacteria acética es viable entre 28-33 ºC, pero la velocidad de fermentación varía en función de la temperatura. La temperatura de la fermentación debe estar comprendida dentro del intervalo entre 30-31 ºC (*Ormaechea, 1991*) que es la temperatura óptima para obtener un mayor rendimiento. Es obvio que la oxidación de etanol a ácido acético es una reacción exotérmica que puede producir alrededor de 8,4 MJ por cada litro de etanol que se oxida (*Adams, 1985*) elevando la temperatura del depósito. Por otra parte, cuando la temperatura es elevada aumentan las pérdidas de

alcohol y productos volátiles y, en menor cuantía, de ácido acético, pero quizás lo más importante, es que puede ocurrir la parada del proceso por la muerte de bacterias.

Un elevado suministro de aire puede causar el fenómeno de sobreoxidación y arrastre de los componentes volátiles y, por otro lado, su carencia puede paralizar la acetificación dado el carácter aerobio de las bacterias acéticas. Además de la cantidad de aire, se ha de tener en cuenta su calidad y pureza, ya que las bacterias acéticas son sensibles a los contaminantes de este.

Todos los parámetros están interrelacionados en la acetificación; el etanol no debe llegar a agotarse totalmente ya que las bacterias acéticas mueren rápidamente y se pierde el cultivo. Por ello en este tipo de sistema de producción de vinagre se suele llevar a cabo la acetificación de forma discontinua realizándose ciclos de descarga-carga. Así se impide que las bacterias acéticas metabolicen el ácido acético formado convirtiéndolo en CO_2 y agua. Se descarga aproximadamente el 40-45 % del volumen de líquido, que se repone con nueva materia prima suministrándole sustrato a la bacteria. Por eso, se puede trabajar de forma automatizada con dispositivos que regulen el control de la temperatura y de la aireación, así como los ciclos de carga y descarga. El acetificador está constituido por un depósito de acero inoxidable de capacidad entre 100 y 300 HL. Los conductos están rodeados por un intercambiador de calor de agua para disipar el calor producido en el proceso y mantener la temperatura a 30 ºC. Para evitar las pérdidas de compuestos volátiles se ha desarrollado un sistema cerrado (*Cantero et al., 1996*), mejorando sensiblemente los resultados del proceso fermentativo. El fermentador consta de un sistema de recirculación de aire y un sistema de control automatizado que inyecta oxígeno en dicha corriente a medida que éste es consumido por la biomasa. Se alcanzan así rendimientos cercanos al 100 % (*Gómez et al., 1993*).

2.1.5. Vinagres tradicionales: Denominaciones de Origen

Entre los vinagres tradicionales, con denominación de origen y reconocimiento internacional destacan el "Aceto Balsámico Tradizionale" de la ciudad italiana de Módena, el prestigioso "Vinagre de Jerez", el "Vinagre del Condado de Huelva" y el más reciente "Vinagre de Montilla-Moriles".

a) Vinagre de Módena

El vinagre balsámico tradicional de la región de Módena y Reggio Emilia fue reconocido como producto de una región específica en el año 1986 (Ley No. 93, 1986). En el año 2000 este producto obtuvo el certificado de denominación de origen protegida por la Comisión Europea debido a su típico y único procedimiento de producción y a su bien definida área de producción (Council Regulation EC Nº 813/2000).

La materia prima para la fermentación consiste en un mosto de uva local, variedad Trebbiano, que se concentra por calentamiento hasta al menos un tercio de su volumen inicial. Este mosto se concentra mediante ebullición suave, lo que producirá la eliminación de la flora espontánea y un incremento del 30 % del contenido en azúcares. De este modo, el mosto es fermentado por levaduras osmófilas pertenecientes al género *Zygosacharomyces*. Simultáneamente las bacterias acéticas metabolizan el etanol a ácido acético, en un proceso lento y tradicional, el cual se lleva a cabo en una batería de barriles de distinta capacidad y construidos tradicionalmente con diferentes tipos de madera, de distinta porosidad (*Cocchi et al., 2008; Consonni et al., 2008*).

El vinagre terminado, después de un proceso que dura varios años, se extrae del último barril de la escala (de madera de moral o morera) de 20 L de capacidad, el cual se rellena de vinagre procedente del anterior, de 30 L construido de madera de fresno, que a su vez se rellena de otro barril de madera de cerezo de 40 L de volumen. El último barril de la serie, de 50 L de capacidad y de madera de castaño, recibe el vinagre de un barril de 60 L construido en madera de roble, el cual se rellena con mosto de uva concentrado para compensar el volumen extraído y la pérdida acumulada de la evaporación de todas las barricas anteriores (Figura 2.7). La porosidad de cada tipo de madera y la entrada de oxígeno que permite, influye tanto en el desarrollo de la acetificación como en el aroma y color del vinagre obtenido. La saca se efectúa cada año, extrayendo un máximo de 2 litros de vinagre. Los periodos tiempo de envejecimiento son de al menos doce años (*Giudici et al., 1992; Consonni et al., 2008*; *Cocchi, et al., 2008*). El producto resultante es un líquido marrón oscuro, de aspecto siruposo, con sabor agridulce.

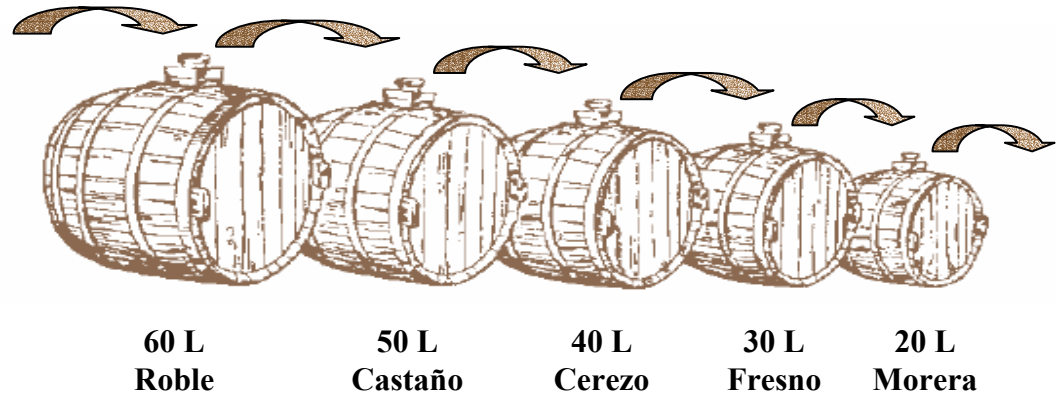


Figura 2.7. *Proceso de elaboración del vinagre "Aceto Balsámico Tradizionale" de Módena*

Existen dos categorías comerciales de vinagre de balsámico en el mercado: "Aceto Balsámico Tradizionale di Módena" y "Aceto Balsámico di Módena" (producido por métodos rápidos industriales). El "Aceto Balsámico di Módena" se produce a partir de vino acetificado por métodos rápidos con cultivo sumergido y se le añade mosto cocido. La diferencia fundamental entre el vinagre de vino y el "Aceto Balsámico Tradizionale" (ABT) está ligada a los microorganismos involucrados en el proceso biológico. En el ABT, la fermentación alcohólica del mosto calentado y concentrado es incompleta, superándose con dificultad los 7º alcohólicos; por tanto los azúcares están siempre presentes en gran cantidad en la masa fermentativa en dos etapas bien definidas: fermentación alcohólica llevada a cabo por las levaduras del género *Zygosaccharomyces*, ya que, a diferencia de las *Saccharomyces* son capaces de crecer con elevadas concentraciones de azúcares y/o en presencia de ácido acético, y oxidación acética en la que están involucradas fundamentalmente las bacterias del género *Gluconacetobacter* (*Giudici et al., 2003*).

b) Vinagre de Jerez

El vinagre de Jerez es el producto resultante de la fermentación acética de vinos procedentes de las variedades de uvas cultivadas en la zona de producción de las Denominaciones de Origen de "*Jerez-Xérès-Sherry*" y "*Manzanilla-Sanlúcar de Barrameda*", consideradas aptas para la producción de los vinos amparados por las

mismas (*Consejería de Agricultura y Pesca, 1995*). El vinagre de Jerez puede elaborarse a partir de mosto, de vino encabezado, o de mezcla de ambos (*Gálvez et al., 1995*).

Los vinagres de Jerez amparados por la Denominación de Origen "Vinagre de Jerez" presentan un color entre oro viejo y caoba y un aspecto denso y untuoso. Su aroma es intenso, ligeramente alcohólico, con predominio de las notas vínicas y de la madera. Su sabor es agradable, a pesar de la acidez, con una gran persistencia en boca.

El vinagre de Jerez se envejece bien mediante un sistema estático de añadas o bien mediante un sistema dinámico peculiar y característico de "criaderas y solera" (Figura 2.8). Este último es el más difundido y generalizado. En el sistema de "criaderas y solera" tiene lugar simultáneamente la acetificación y el envejecimiento, así la aireación del proceso es relativamente mayor en comparación con el sistema estático.

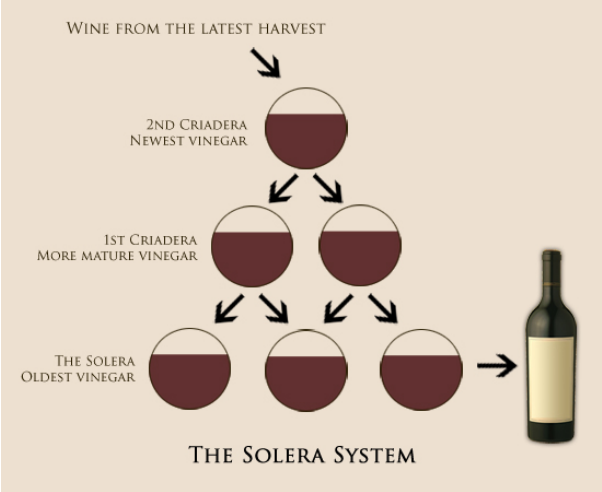


Figura 2.8. *Esquema de un sistema de "criaderas y solera"*

El sistema de "criaderas y solera" está compuesto por una serie indeterminadas de botas, usualmente de roble, agrupadas en filas horizontales o escalas cuyo número puede oscilar entre tres y cinco (Figura 2.9). La fila que está a ras de suelo recibe el nombre de "solera", sobre ella se encuentra la primera criadera, sobre ésta la segunda y así sucesivamente. La edad del vinagre que contienen las botas aumenta conforme descendemos en el sistema, siendo la solera la de mayor envejecimiento.

El vinagre elaborado se obtiene de la solera, no pudiendo exceder la cantidad que se saca de la bota un tercio del contenido de la misma. Esta cantidad se repone con el contenido de la primera criadera. Ésta a su vez se completa con el de la segunda criadera y así sucesivamente ingresando el sustrato de partida en la criadera superior. Estas operaciones se llaman "sacas" y "rocíos". La periodicidad con la que se efectúan es variable según la bodega en la que se realicen, pero lo más frecuente es que sean tres o cuatro veces al año.

Figura 2.9. *Vista de una bodega donde se puede apreciar el sistema de "criaderas y solera"*

Considerando las características del envejecimiento en soleras es imposible determinar la edad exacta de un vinagre, y se habla de una edad media aproximada. Según los periodos de envejecimiento a que son sometidos los vinagres de Jerez, se distinguen las siguientes categorías (*Ministerio de Agricultura y Pesca, 2008a*):

- Vinagre de Jerez: es el vinagre amparado por esta denominación, sometido a un tiempo de envejecimiento mínimo de 6 meses.
- Vinagre de Jerez Reserva: es el vinagre amparado por esta denominación, sometido a un tiempo de envejecimiento mínimo de 2 años.
- Vinagre de Jerez Gran Reserva: es el vinagre amparado por esta denominación, sometido a un tiempo de envejecimiento mínimo de 10 años.

Adicionalmente, en función de la utilización de vinos las variedades correspondientes, se distinguen los siguientes tipos de Vinagre de Jerez semi-dulce, los cuales pueden corresponder a cualquiera de las categorías descritas en el apartado anterior:

- Vinagre de Jerez al Pedro Ximénez: es el vinagre amparado por esta denominación, al que se le adiciona durante el proceso de crianza vinos del tipo Pedro Ximénez.
- Vinagres de Jerez al Moscatel: es el vinagre amparado por esta denominación, al que se le adiciona durante el proceso de crianza vinos del tipo Pedro Moscatel.

Según la resolución del 24 de Marzo de 2008, de la Dirección General de Industrias y Calidad Agroalimentaria, los vinagres protegidos por la Denominación de Origen "Vinagre de Jerez" deben presentar las siguientes características analíticas (*Ministerio de Agricultura y Pesca, 2008a*):

- Su contenido en alcohol residual no debe superar el 3 % en volumen, salvo en el caso de los vinagres al Pedro Ximénez o al Moscatel, en los que no deberá superar el 4 % en volumen.
- La acidez total en ácido acético debe alcanzar como mínimo los 70 g/L, con la excepción de los vinagres al Pedro Ximénez o al Moscatel, que podrán ser de 60 g/L. En el caso de los vinagres de tipo Gran Reserva, la acidez total mínima debe ser de 80 g/L.
- Debe presentar un extracto seco mínimo de 1,30 g/L y grado acético, debiendo alcanzar como mínimo los 2,3 g/L y grado acético en la categoría de Vinagre de Jerez Gran Reserva.
- Un contenido en cenizas de entre 2 y 7 g/L, con la excepción de la categoría del Vinagre de Jerez Gran Reserva que debe ser entre 4 y 8 g/L.
- Su contenido de carbono 14 debe ser el correspondiente a su origen biológico.

- Para las categorías de Vinagre de Jerez al Pedro Ximénez o al Moscatel, deberán presentar un contenido en materias reductoras, procedentes de estos tipos de vinos, de al menos 60 g/L.

c) Vinagre del Condado de Huelva

El vinagre del Condado de Huelva es el producto obtenido por la fermentación acética de un vino amparado por la Denominación de Origen (D. O.) "Condado de Huelva" (*Consejería de Agricultura y Pesca, 2002*).

La elaboración y las características analíticas que debe cumplir los vinagres amparados por la Denominación de Origen "Vinagres del Condado de Huelva" están establecidas y detalladas en su correspondiente Reglamento (Orden de 31 de Julio de 2002, Boja nº 99 de 24.08.02).

Según el tiempo y el método de envejecimiento se definen tres tipos de Vinagres Viejos Condado de Huelva:

- Solera: "Vinagre Viejo Condado de Huelva" envejecido mediante el tradicional sistema de "criaderas y solera", durante un tiempo superior a seis meses e inferior a un año y enriquecido con vino Condado Viejo, no siendo superior el contenido de alcohol residual al 3 % vol.
- Reserva: "Vinagre Viejo Condado de Huelva" envejecido mediante el tradicional sistema de "criaderas y solera", durante un tiempo superior a un año y enriquecido con vino Condado Viejo, no siendo superior el contenido de alcohol residual al 3 % vol.
- Añada: "Vinagre Viejo Condado de Huelva" envejecido mediante el tradicional sistema de añadas, durante un tiempo superior a tres años y enriquecido con Condado Viejo, no siendo superior el contenido de alcohol residual al 3% vol.

d) Vinagres Montilla-Moriles

El vinagre de Montilla-Moriles es el producto obtenido exclusivamente de la fermentación acética de vinos de crianza que proceden de la zona amparada por la

Denominación de Origen "Montilla-Moriles" (*Ministerio de Aguicultura y Pesca, 2008b*). Estos vinagres empezarán a comercializarse en Septiembre/Octubre de 2008 bajo la Denominación de Origen "Montilla-Moriles".

Atendiendo al tipo de elaboración particular y envejecimiento, en la zona Montilla-Moriles se pueden distinguir los siguientes vinagres:

- Vinagres de envejecimiento: aquellos que son sometidos a una crianza en madera. Entre ellos podemos distinguir: "Vinagre de Montilla-Moriles Añada" (envejecimiento estático superior a 3 años), "Crianza" (periodo de envejecimiento en criaderas y solera mínimo de 6 meses), "Reserva" (envejecimiento mínimo de 2 años) y "Gran Reserva" (mínimo de 10 años).
- Vinagres Dulces: aquellos vinagres, que siendo envejecidos a través del sistema tradicional de "criaderas y solera", tienen la peculiaridad de poseer una adición de mosto concentrado. Según la procedencia del mosto se distinguen los "Vinagres Dulces al Pedro Ximénez" (si el mosto procede de uvas pasificadas Pedro Ximénez) y los "Vinagres Dulces al Moscatel (si el mosto procede de uva pasificada o no de la variedad Moscatel). Estos vinagres dulces también se someten a envejecimiento en "criaderas y solera" obteniéndose vinagres "Crianza", "Reserva" y "Gran Reserva".

2.2. DETERMINACIÓN DE AMINOÁCIDOS EN PRODUCTOS DERIVADOS DE LA UVA

2.2.1. Aplicaciones del análisis de aminoácidos en enología

El análisis de aminoácidos encuentra aplicación en muchos campos de investigación siendo uno de los más importantes la estimación del valor nutritivo de alimentos para humanos y para animales. La elevada demanda de información relacionada con el valor nutritivo ha dado lugar al desarrollo de métodos precisos para aminoácidos. Además, es creciente el número de aplicaciones como la detección de

posibles adulteraciones en alimentos y bebidas o la determinación de aminoácidos, péptidos o derivados potencialmente tóxicos producidos por las nuevas técnicas de procesado de alimentos (*White yHart, 1992a*).

Es conocida la importancia de los aminoácidos del mosto como nutrientes para el desarrollo de las levaduras que realizan la fermentación alcohólica, así como el papel que se les atribuye como precursores de los compuestos del aroma. Por otra parte, la composición en aminoácidos del mosto está muy relacionada con la madurez de las uvas (*Cáceres et al., 1986*), y a su vez, el contenido de aminoácidos de éstas depende de varios factores como fertilización, condiciones climáticas y duración de la maceración de la piel en el mosto (*Soufleros et al., 2003*). Los aminoácidos libres representan la parte más importante del nitrógeno total en los mostos y vinos, aproximadamente entre el 30 y 40 % (*Hérberger et al., 2003*). Estos aminoácidos tienen varios orígenes. Algunos proceden de la uva y pueden ser metabolizados parcial o totalmente por levaduras al final de la fermentación o liberados de las levaduras muertas y otros son producidos por la degradación enzimática de las proteínas de la uva.

Por tanto, la composición de aminoácidos es de gran importancia en la producción de vino (*Kosir y Kidric, 2001*). Muchos aminoácidos experimentan una serie de biotransformaciones, dando lugar a alcoholes de alto peso molecular, aldehídos, ésteres y ácidos cetónicos, los cuales tienen un gran impacto en las propiedades organolépticas del vino. Por este motivo a los aminoácidos se les atribuye el papel de precursores del aroma (*Soufleros et al., 2003*).

A pesar de los diversos factores que afectan a los aminoácidos presentes en el vino, muchos investigadores los han empleado para la diferenciación del producto (*Csomos et al., 2001*). Con dicho fin, así como para establecer la autenticidad del vino, se han aplicado diferentes procedimientos quimiométricos: análisis de cluster, análisis de componentes principales y análisis discriminante. *Hérberger et al. (2003)* encontraron que la aplicación de técnicas quimiométricas al contenido de aminoácidos y aminas biógenas resultó ser una buena herramienta para clasificar vinos húngaros en función de la tecnología de elaboración empleada, sin embargo, la diferenciación de las mismas según su origen geográfico, variedad y cosecha no fue del todo satisfactoria.

Asimismo, *Soufleros et al.* (*2003)* estudiaron el perfil de aminoácidos de vinos blancos griegos procedentes de siete variedades de uva, seis regiones geográficas, y tres vendimias y aplicando el análisis discriminante los clasificaron en función de dichas variables (variedad, origen geográfico y vendimia).

Brescia et al. (2002) diferenciaron vinos procedentes de pequeñas áreas de producción de la misma región aplicando estadística multivariante a las determinaciones de aminoácidos por resonancia magnética nuclear (RMN). Con esto se consiguió un método útil para la protección de la denominación de origen controlada (DOC) contra la adulteración.

Por otro lado, ya que las levaduras juegan un importante papel en el contenido de aminoácidos en el vino, otra de las aplicaciones ha sido el estudio de la influencia de diferentes cepas de levadura sobre los cambios que sufren los aminoácidos, péptidos y proteínas durante el envejecimiento del cava (*Martínez-Rodríguez et al., 2002*). En este estudio se utilizó el mismo vino base y cinco cepas de levaduras. Con los datos obtenidos se dedujo que los cambios que se producen en el proceso de envejecimiento del cava ocurren en al menos cuatro fases claramente diferenciadas y además, se observó que el empleo de una cepa de levadura u otra influye en el contenido de aminoácidos libres y péptidos.

Pérez-Coello et al. (1999) analizaron 15 vinos elaborados en La Mancha con varias cepas de *Saccharomyces cerevisiae,* utilizando la composición volátil y el perfil de aminoácidos con el objeto de determinar la cepa más adecuada para inocular los mostos de esta región.

En otros trabajos se adicionó amonio y aminoácidos a los mostos para estudiar su efecto en la composición aromática y en las propiedades sensoriales de los vinos obtenidos (*Hernández-Orte et al., 2002; 2005; 2006*). A pesar de que el factor determinante de la composición volátil de los vinos es la cepa de levadura, la adición de nitrógeno a los mostos también influye, reduciendo el contenido de β-feniletanol, metionol e isoamilalcohol y aumentando el ácido propiónico. Además, se observó que los vinos que habían sido enriquecidos con amonio eran más ricos en lactato de etilo y *cis*-3-hexenol mientras que los vinos enriquecidos con aminoácidos eran más ricos en γ-

butirolactona e isobutanol. Desde el punto de vista sensorial, la adición de amonio dio lugar a un descenso en notas sulfhídricas y a un incremento en olor cítrico. Por otro lado, se observó que las fermentaciones son mucho más rápidas en los mostos enriquecidos (*Hernández-Orte et al., 2005*).

En el campo de los vinagres, el análisis de aminoácidos se ha dirigido a la caracterización de vinagres de vino (*Kutlán y Molnár-Perl, 2003*), a la comparación de vinagres obtenidos a partir de diferentes substratos vínicos (*Valero et al., 2005*) y a las transformaciones químicas y bioquímicas que se producen en los vinagres de Jerez durante las diferentes fases de envejecimiento (*Palacios et al., 2002*).

La formación de vinagre implica esencialmente la conversión del etanol en ácido acético. Los microorganismos responsables de dicha transformación son bacterias acéticas pertenecientes a los géneros *Acetobacter y Gluconobacter* y los aminoácidos presentes en el medio constituyen la principal fuente de nitrógeno para estas bacterias. Debido a que el sustrato inicial procede de una fermentación previa realizada por levaduras, es esencial asegurarse de que haya una cantidad adecuada de nitrógeno para que se lleve a cabo la fermentación acética (*Valero et al., 2005*).

Además, se han desarrollado métodos para la determinación conjunta de aminoácidos y otras sustancias de interés como las aminas biógenas y poliaminas en vinos y en vinagres (*Bauza et al., 1995; Herbert et al., 2000; Kutlán y Molnár-Perl., 2003; Lozanov et al., 2004)*. Las aminas biógenas son compuestos que afectan a la salud y pueden ser indicadores de unas condiciones de producción antihigiénica, formándose a partir de ciertos aminoácidos por descarboxilación. Sin embargo, la cuantificación rutinaria de estas sustancias no se emplea en el control cualitativo de los vinos debido principalmente a las dificultades del análisis. De ahí la importancia de desarrollar métodos sencillos que permitan determinar simultáneamente las aminas biógenas y aminoácidos en vinos o en vinagres. Las poliaminas, por el contrario, tienen múltiples funciones en los organismos vivos, tales como factores de crecimiento, antioxidantes, estabilizadores de ADN y ARN, reguladores metabólicos, nutrientes y segundos mensajeros.

Los estudios de racemización constituyen otra área en el análisis de los aminoácidos. Los L-aminoácidos de las proteínas de los alimentos se isomerizan parcialmente a D-isómeros por tratamiento alcalino o por calor. Este tratamiento puede afectar al valor nutritivo y seguridad de los alimentos, ya que la mayoría de los D-aminoácidos no pueden ser utilizados por los humanos y algunos son tóxicos. Los isómeros tienen idénticas propiedades químicas y por tanto, deben ser convertidos en dipéptidos diastereométricos mediante una reacción con un agente quiral antes de la cromatografía. Alternativamente, se puede emplear una fase estacionaria o fase móvil quiral. Para conseguir la separación en un solo cromatograma de todos los aminoácidos en sus formas D y L, es necesario utilizar columnas largas y tiempos de análisis de incluso horas.

Se han propuesto diferentes métodos para la determinación aminoácidos enantiómeros y aminas quirales tanto en vinos, vinagres, como en alimentos, cada uno de ellos con sus ventajas e inconvenientes (*Brüeckner et al., 1995; Dongri et al., 1999; Voss y Galensa, 2000; Toshimasa et al., 2001; Abe et al., 2002; Erbe y Brüeckner, 1998*).

2.2.2. Métodos de análisis de aminoácidos

Existen diferentes técnicas empleadas en el análisis de aminoácidos en vinos, cada una con sus respectivas ventajas e inconvenientes, las cuales se muestran en la Tabla 2.2.

2.2.3. Derivatización de aminoácidos

Los aminoácidos pueden ser detectados directamente en el ultravioleta, ya que absorben a una longitud de onda entre 190-210 nm. Sin embargo, en esta región del espectro también absorben la mayoría de los disolventes y otros componentes de las muestras, por lo que normalmente se recurre a la formación de derivados detectables a otras longitudes de onda o fluorescentes (*Cáceres et al., 1986*).

Tabla 2.2. *Ventajas e inconvenientes de las distintas técnicas empleadas en la determinación de aminoácidos y sus aplicaciones.*

Técnica	Ventajas	Inconvenientes	Aplicaciones
Cromatografía de líquidos de intercambio iónico	-Buena fiabilidad -Excelente poder de resolución	-Tiempos de análisis demasiado largos -Sensibilidad limitada -Picos cromatográficos demasiado anchos -No permite la cuantificación de cisteína, la cual es un precursor de los tioles característicos de variedades de uva (*Pripis-Nicolau et al., 2001*) -Problemas con las interferencias de la matriz y con los límites de detección (*Herbert et al., 2000*)	-Esta técnica se ha empleado extensamente en la caracterización de vinos en función del contenido de aminoácidos (*Csomos y Simone, 2002*)
Cromatografía de líquidos en fase reversa	-Facilidad de derivatización -Menor tiempo de análisis -Tiempos de retención cortos -Gran reproducibilidad -Velocidad de muestreo -Amplio campo de aplicación	-Solubilidad de la fase estacionaria en la fase móvil y deterioro de la columna -Control cuidadoso del flujo y temperatura	-Caracterización de vinos (*Soufleros et al., 2003*) -Diferenciación de vinagres en función de la composición en aminoácidos (*Valero et al.,2005*) -Estudio de la evolución de los aminoácidos en el proceso de elaboración del vino (*Martínez-Rodríguez et al.,2002*) -Relación del contenido en aminoácidos con los compuesto volátiles del vino (*Pozo-Bayón et al., 2005*) -Determinación simultánea de aminas biógenas y aminoácidos en vinos y vinagres (*Krause et al. 1995; Lozanov et al., 2004; Kutlán y Molnár-Perl, 2003*) -Determinación de isómeros L y D de los aminoácidos en vinos (*Brüeckner et al., 1995; Jin et al., 1999; Voss y Galensa, 2000; Toyo´oka et al., 2000)*
Cromatografía de gases	-Rápida -Alto poder de resolución y sensibilidad	-Necesita gran experiencia -Complejidad del proceso de derivatización -Minuciosa extracción y preconcentración de la muestra (*Herbert et al., 2000*)	-Determinación de D-aminoácidos y separación de enantiómeros tanto en vinos (*Abe et al., 2002*) como en vinagres (*Erbe y Brüeckner, 1998*)
Electroforesis capilar	-Bajos límites de detección	-Requiere un proceso de derivatización y preconcentración previo análisis (*Kosir y Kidric, 2002*)	- Separación y detección de aminoácidos, péctidos y proteínas (*Male y Luong, 2001; Kilgore y Smith, 2003*)
Resonancia magnética nuclear	-Técnica no destructiva -Sensible -Detecta simultáneamente gran número de compuestos -Fácil preparación de muestra	- Menos sensible	-Determinación de aminoácidos en vino (*Kosir y Kidric, 2001; 2002*) -Caracterización de la autenticidad del vino, determinación del origen geográfico y año de producción (*Brescia et al., 2002*)

La derivatización puede llevarse a cabo antes de la separación cromatográfica (precolumna), inmediatamente después de la elución (postcolumna) o, menos frecuente, en la misma columna. Cada forma de obtener el derivado presenta ventajas e inconvenientes (Tabla 2.3).

Tabla 2.3. *Ventajas e inconvenientes de la derivatización precolumna y postcolumna (Cáceres et al., 1986)*

Derivatización	Ventajas	Inconvenientes
Postcolumna	-Es posible utilizar varios sistemas de detección y eluir los compuestos de la columna detectándolos por métodos no destructivos antes de derivatizarlos. -La reacción es reproducible sin necesidad de formar un único derivado.	-Presencia de interferencias debidas al exceso de reactivo o a productos de degradación. -Pérdida de resolución producida por el ensanchamiento de la banda cromatográfica en el reactor donde se produce la reacción. -No se pueden utilizar tiempos de retención muy largos. -Puede resultar un método caro debido a las modificaciones que hay que realizar en el equipo instrumental para llevar a cabo la reacción.
Precolumna	-La única limitación a las condiciones de la reacción es que se complete en un periodo de tiempo razonable y que sea cuantitativa. -La reacción se puede desarrollar en un disolvente no compatible con la fase móvil utilizada en la separación cromatográfica. -Se pueden separar los productos secundarios formados, en la misma columna o antes de la separación cromatográfica.	-Presencia de picos interferentes en los cromatogramas debidos al mismo reactivo, a productos de reacción o de degradación o a impurezas de los reactivos. -Conviene eliminar el exceso de reactivo, disolvente u otros componentes de la mezcla de reacción antes de la inyección en el cromatógrafo. - Una parte sustancial de todos los derivados será idéntica. Pequeñas diferencias de la cadena lateral de los aminoácidos tendrán un efecto menor en el comportamiento cromatográfico de los derivados, dando una separación más dificultosa.

En la derivatización postcolumna la separación de aminoácidos no derivatizados se lleva a cabo con una resina de intercambio catiónico con un gradiente de tampones acídicos. Después de la separación se convierten en derivados de ninhidrina coloreados para la detección colorimétrica, o en derivados de ortoftaldehído para la detección por fluorescencia.

Aunque la derivatización precolumna ofrece más ventajas, los métodos tradicionales postcolumna no se han eliminado totalmente.

Los agentes derivatizantes más utilizados en el análisis de aminoácidos, con sus correspondientes ventajas e inconvenientes se muestran en la siguiente tabla:

Tabla 2.4. *Agentes empleados en la derivatización de aminoácidos*

Reactivo	Derivatiz.	Método separación	Tipo detección	Ventajas	Inconvenientes
Ninhidrina[a] *(White y Hart, 1992b)*	Postcolumna	Intercambio iónico	UV	-Reaciona con aa primarios y secundarios -Capaz de detectar picomoles	-No reproducible en cantidades menores a 100 picomoles -Problemas de interferencias con la matriz -Sensible a la luz, O_2, cambios T[a] y pH
DNS-Cl[a b] *(White y Hart, 1992b)*	Precolumna y Postcolumna	Fase reversa	Fluorescencia UV	-Reacciona con aa primarios y secundarios -Capaz de detectar picomoles o fentomoles -Derivados estables a la hidrólisis -Buena reproducibilidad para todos aa menos para His -Muy adecuado para determinar Cys	-El exceso de reactivo interfiere en el cromatog. -Los derivados son fotosensibles -Reacción lenta -His forma picos múltiples -No es específico y reacciona con otros compuestos
DABS-Cl *(White y Hart, 1992b)*	Precolumna	Fase reversa	Visible	-Reacciona con aa primarios y secundarios. -Derivados muy estables (4 semanas a T[a] ambiente) -Capaz de detectar picomoles	-El exceso de reactivo interfiere en el cromatog. -Las sales y detergentes presentes en las muestras interfieren -Produce múltiples derivados
DNFB (White y Hart, 1992b)	Precolumna	Fase reversa	Fluorescencia	-Reacciona con aa primarios y secundarios -Capaz de detectar bajos niveles de picomoles -Determina aa que otros agentes resuelven peor	-Los derivados son fotosensibles -Reacción muy lenta. -Método destructivo para péptidos
OPA[a b] (*Cáceres et al., 1986*)	Precolumna y Postcolumna	Fase reversa e Intercambio iónico	Fluorescencia UV	-El reactivo se puede usar en exceso porque no interfiere -Reacción rápida -Derivados altamente fluorescentes -10 veces más sensible que reacción con Ninhidrina	-Los derivados son inestables -No reacciona con aa secundarios (prolina) -Necesita una completa automatización de la reacción -Respuesta de la Lys, Hidroxilisina y Cys baja -Derivados se descomponen

[a] *Aplicado en muestras de vino* [b] *Aplicado en muestras de vinagre*

DNS-Cl: Cloruro de Dansilo *DBS-Cl: Cloruro de Dabsilo*

DNFB: Dinitrofluorobenceno *OPA: Ortoftaldehido*

Tabla 2.4. *Agentes empleados en la derivatización de aminoácidos (Continuación)*

Reactivo	Derivatiz.	Método separación	Tipo detección	Ventajas	Inconvenientes
PITC[a b] *(White y Hart, 1992b)*	Precolumna	Fase reversa	UV	-Reacciona con aa primarios y secundarios -Determina Prolina e Hidroxiprolina con una sensibilidad de picomoles -Reacción rápida -Forma derivados más estables que otros reactivos -El reactivo se puede usar en exceso porque no interfiere	-Problemas de interferencias con la matriz en el análisis de mostos -No adecuado para su automatización -Sensibilidad menor a otros métodos (50 veces menor que OPA y FMOC) -El proceso es lento porque necesita un paso de evaporación a sequedad
FMOC[a] *(White y Hart, 1992b)*	Precolumna	Fase reversa	Fluorescencia	-Reacciona con aa primarios y secundarios -Reacción rápida (30 s) -Derivados estables y altamente fluorescentes -Muy sensible	-Produce múltiples derivados -El reactivo es por sí mismo fluorescente e interfiere en el cromatog. -El proceso de derivatiz. es lento ya que se tiene que eliminar el reactivo
EMMDE (*Alaiz et al., 1992*)	Precolumna	Fase reversa	UV-Visible	-Reacciona con aminoácidos primarios y secundarios -Derivatización directa sin previa preparación -El exceso de reactivo no interfiere -Capaz de detectar picomoles	-Proceso de derivatización lento -Inestablilidad de los derivados de prolina e hidroxiprolina -No sensible a niveles inferiores a picomoles
AQC[a] *(Wandelen y Cohen, 1997)*	Precolumna	Fase reversa	Fluorescencia UV	-El exceso de reactivo no interfiere -Reacciona con aa primarios y secundarios -Derivados estables y altamente fluorescentes -Muy sensible (50-300 fentomoles) -Reacción rápida -Derivados se inyectan sin previa preparación de la muestra -Las sales y detergentes de muestras no interfieren	-Lys y Trp muestran una menor fluorescencia por el llamado "quenching interno" -Si el amonio no se derivatiza totalmente, el exceso de éste puede distorsionar el análisis de Arg y Thr

[a] *Aplicado en muestras de vino* [b] *Aplicado en muestras de vinagre*

PITC: Fenilisotiocianato

FMOC: 9-Fluorenilmetil cloroformato

EMMDE: Etoximetilenmalonato de dietilo

AQC: 6-aminoquinolil-N-hidroxisuccinimidil carbamato

El 6-aminoquinolil-N-hidroxisuccinimidil carbamato (AQC) 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 2.10). Los derivados que resultan son estables a temperatura ambiente durante, al menos, una semana y se separan fácilmente por cromatografía de líquidos en fase reversa utilizando una columna de C18.

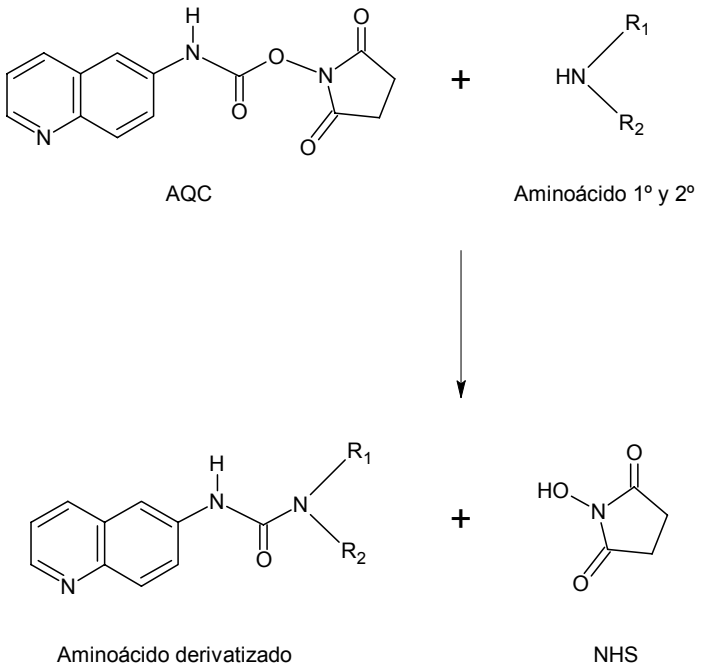


Figura 2.10. *Reacción de derivatización del AQC*

El exceso del reactivo se hidroliza durante la reacción para formar 6-aminoquinolina (AMQ) (Figura 2.11), cuyas características espectrales son netamente diferentes a cualesquiera 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.

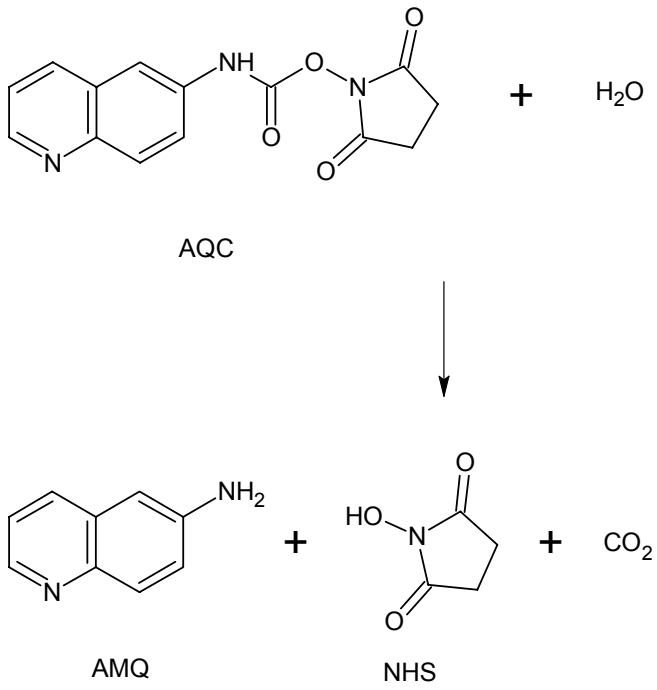


Figura 2.11. *Hidrólisis del exceso de AQC*

El protocolo de derivatización, agregado del reactivo y calefacción de la muestra previamente tamponada, es simple y directo. Los derivados de los aminoácidos se inyectan directamente sin preparación adicional de la muestra, mientras que las sales (presentes en la muestra) prácticamente no causan interferencias en la reacción o en la reproducibilidad de los resultados (*Wandelen y Cohen, 1997*).

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*).

Utilizando un detector de UV, a 250 nm, el AMQ absorbe alrededor de 200 veces más que cualquiera de los aminoácidos derivatizados y esto puede ocasionar dificultades en la cuantificación del ácido aspártico, que es el primero de los aminoácidos en eluir. Esto no ocurre cuando la detección se lleva a cabo por fluorescencia, ya que la señal del AMQ es mucho menor a la obtenida en el UV. Otra característica del AMQ es que su tiempo de retención puede variar dependiendo del pH de la fase móvil.

La presencia de dos grupos fluorescentes en una misma molécula puede disminuir considerablemente su fluorescencia debido a un fenómeno conocido como "quenching interno". Este efecto se observa muy débilmente en el caso de la molécula de lisina, pero es bien notorio con el triptófano. En este caso la sensibilidad del método se ve ampliamente favorecida por el uso de un detector UV. Tanto el análisis de muestras con triptófano, como el análisis de aminoácidos libres en una solución intravenosa, puede ser eficazmente realizado usando ambos detectores en serie, primero el de fluorescencia para la mayoría de los aminoácidos y luego el detector UV para el triptófano.

Este reactivo también se ha utilizado como una alternativa al reactivo de derivatización más común, OPA, para determinar aminas biogénicas en vinos (*Busto et al., 1996*) y para estudiar la evolución de los aminoácidos y péptidos durante la fermentación alcohólica y autolisis de los vinos (*Alexandre et al., 2001*).

Aunque la derivatización precolumna ofrece más ventajas, ninguno de los agentes prederivatizantes estudiados se considera el agente universal ya que ninguno cumple todos los requisitos:

- Reaccionar rápidamente bajo condiciones suaves para obtener el derivado de forma cuantitativa.

- Reaccionar con aminoácidos primarios y secundarios.

- Los derivados deberían ser estables durante varios días, preferiblemente a temperatura ambiente para permitir el análisis automatizado de múltiples muestras.

- Respuesta lineal en los niveles de concentración típicos de la mayoría de las aplicaciones.

- El exceso de reactivo o productos secundarios no deberían interferir en el análisis de aminoácidos.

2.3. COMPUESTOS VOLÁTILES DEL VINAGRE DE VINO

Los compuestos volátiles del vinagre tienen un efecto decisivo en la calidad de los mismos (*Morales et al., 2002*). La fracción volátil esta influenciada por varios factores, como la materia prima usada, el proceso de elaboración empleado y, en algunos casos, el envejecimiento en madera (*Pizarro et al., 2008; Natera et al., 2002*). Por ello, los productores eligen las mejores materias primas y el proceso de acetificación óptimo para incrementar la calidad aromática del vinagre y presentar nuevos productos a los consumidores (*Morales et al., 2002*).

Además, el perfil volátil del vinagre también puede suministrarnos información acerca del estado de integridad o de alteración química, enzimática o biológica del mismo, por lo que su análisis es una eficiente medida para evaluar su autenticidad (*Pizarro et al., 2008; Guerrero et al., 2007*). De hecho, se han realizado diversos estudios que describen la aplicación efectiva del análisis de compuestos volátiles para la clasificación y diferenciación de diferentes vinagres (*Blanch et al., 1992; Cocchi et al., 2007*).

Los compuestos aromáticos del vinagre de vino 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*). Cuando se intenta describir sensorialmente este producto, se hace referencia a estos tres factores que vienen representados por la vinosidad, es decir, aquella fracción del vino que permanece en el vinagre, los aromas de la fermentación acética, representados por la sensación punzante del ácido acético, el olor a pegamento del acetato de etilo y el olor a madera, entre otros (*Tesfaye et al., 2002*). Así pues, aunque la mayoría de los compuestos volátiles están presentes en el vino de partida, el contenido final está íntimamente relacionado con las características genuinas del vinagre (*Troncoso y Guzmán, 1987*).

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 setenta 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., 2004*). Son muchos los autores que han estudiado tanto el perfil volátil del vinagre de vino (*Aurand et al., 1966; Khan et al., 1972; Blanch et al. 1992; Gerbi et al., 1992; Morales et al. 2001-2004; Guerrero et al., 2006; 2007*) como los cambios que se producen durante la conversión del vino en vinagre (*Morales et al.,2001a; Valero et al., 2005; Guerrero et al.,2006*).

De manera general podemos decir que durante la acetificación, los compuestos volátiles procedentes del vino pueden sufrir importantes transformaciones. Las bacterias acéticas pueden metabolizar los alcoholes superiores, de manera similar al etanol, produciéndose, por tanto, incrementos en la concentración de los respectivos ácidos. Además, los ésteres etílicos pueden hidrolizarse al mismo tiempo que se forman los ésteres de acético, como el acetato de isoamilo o de metilo (*Palacios et al., 2002; Morales et al., 2001a*).

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; Jarauta et al., 2005*):

- Aumento de concentración de los compuestos volátiles debido a las pérdidas de agua que se producen a través de los poros de la barricas.

- Aparición de compuestos nuevos debidos a procesos de oxidación (como es el caso de la acetoína y el diacetilo), de condensación (formándose fundamentalmente ésteres de bajo umbral de percepción), y a la extracción de algunos compuestos de la madera (principalmente aldehídos).

- Desaparición de otros compuestos debidos a fenómenos de adsorción en la madera

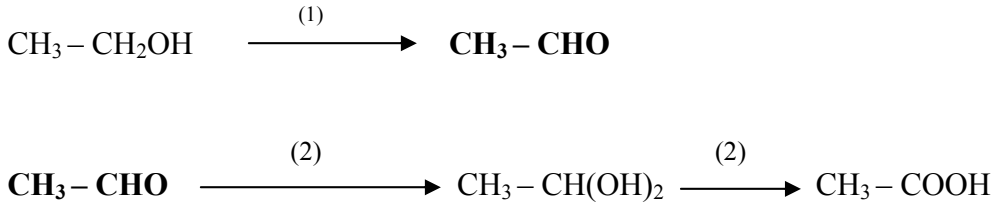
2.3.1. Compuestos carbonílicos

Los compuestos carbonílicos del vino están implicados en los aromas primarios y de fermentación. Los aromas primarios dan notas vegetales y herbáceas al vino y son debidos a los aldehídos de seis carbonos (hexanales y hexenales). Los aromas de fermentación son debidos en parte a compuestos dicarbonilos sintetizados durante la fermentación por levaduras y bacterias lácticas, las cuales reducen el grupo carbonilo al final de la fermentación (*Bayonove et al., 2000*).

El principal aldehído en vinagres es el acetaldehído o etanal. Este compuesto está presente en el vino, sobre todo en aquellos con crianza biológica. Recientemente se ha demostrado que este compuesto influye en el aroma distintivo de los vinos "Finos" debido a las altas concentraciones en las que se encuentra (*Campo et al., 2008*). Estos altos niveles de acetaldehído permite diferenciar, de un modo fácil, los vinos "Finos" de otros tipos de vinos de Jerez producidos por crianza oxidativa (*Zea et al., 2001; Campo et al., 2008*). El acetaldehído, a su vez, es un precursor para la síntesis de otros compuestos aromáticos (*Etiévant, 1991; Moreno et al., 2005*).

Este compuesto 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, según las siguientes reacciones (*Parés y Juárez, 1997*).



(1) Alcohol Deshidrogenasa

(2) Aldehído Deshidrogenasa

Otro posible origen de este compuesto es la oxidación del ácido láctico a ácido pirúvico, el cual es transformado posteriormente a acetaldehído, pudiéndose transformar finalmente en ácido acético por la misma vía metabólica de transformación del etanol a acético (*Parés y Juárez, 1997*). Se ha constatado que durante los procesos de acetificación tiene lugar una disminución del contenido en ácido láctico del sustrato de partida, sin embargo, el acetaldehído, al ser un compuesto tan volátil, desaparece en el desarrollo del proceso de acetificación sumergida que cursa con fuerte aireación (*Morales et al., 2001a*). 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*). Cuando un vinagre no contiene acetoína, hay que sospechar de su origen. Por tanto, este compuesto nos sirve para diferenciar los vinagres de fermentación de aquellos artificiales elaborados con acético de síntesis (*Morales et al., 2002*). Este compuesto tiene un olor a lácteo y presenta una contribución positiva en el perfil sensorial del vinagre (*Gonzalez-Sáiz et al., 2008; Bayonove et al., 2000*). Es uno de los compuestos mayoritarios en vinagres y se puede encontrar en un amplio rango de concentración, que puede ir desde 100 mg/L hasta alrededor de 1000 mg/L en vinagres envejecidos (*Morales et al., 2002; Guerrero et al., 2007*). Algunos autores han puesto

de manifiesto que la cantidad de acetoína en vinagre depende de la composición del substrato vínico de partida (*Pizarro et al., 2008*). Este compuesto se puede formar por varias vías, una parte del acetaldehído que se origina en la oxidación del etanol a acético experimenta lo que se denomina la llamada "condensación acetoínica" para dar acetoína (*Bayonove et al., 2000*) y además, diversas cepas de bacterias acéticas son capaces de formar acetoína mediante la descarboxilación del α-acetolactato (*Caligiani et al., 2007*). En dicha transformación tiene lugar una descarboxilación de una molécula de piruvato que posteriormente formará el complejo α-acetolactato al reaccionar con una segunda molécula de piruvato. Finalmente, este α-acetolactato se descarboxila dando lugar a la molécula de acetoína. Por otro lado, la acetoína es el punto intermedio en el proceso óxido-reductor en el que se forman diacetilo y 2,3-butanodiol. De este modo, la acetoína puede transformarse en butanodiol si el medio es reductor o bien oxidarse parcialmente a diacetilo (*Bayonove et al., 2000*). En los vinagres de Jerez se ha observado un aumento en los niveles de acetoína durante el envejecimiento en madera, como consecuencia de la transformación del butanodiol en acetoína y por pérdidas de agua por evaporación (*Palacios et al., 2002*).

El 2,3-butanodiol es un compuesto que está presente de forma constante en los vinagres de fermentación en concentraciones que oscilan entre 0,1 y 1,5 g/L, siendo más abundante en aquellos vinagres producidos por métodos tradicionales (*Morales et al., 2001b*). Este compuesto es un metabolito producido por la enzima butanodiol deshidrogenasa. Se ha demostrado que hay varias butanodiol deshidrogenasa y que todas ellas son esteroespecíficas. Así, a partir de L(+)-acetoína se forma L(+)-2,3-butanodiol y de la D(+)-acetoína se forma el D(+)-2,3-butanodiol. Cuando se hallan presentes las dos deshidrogenas y no hay racemasa se forma el *meso*-2,3-butanodiol (*Parés y Juárez 1997*). Algunos autores han estudiado los cambios estereoisoméricos del 2,3-butanodiol durante el envejecimiento de los vinagres balsámicos tradicionales, obteniendo unos resultados que ayudan a entender las modificaciones que tienen lugar en el envejecimiento (*Caligiani et al., 2007*).

El diacetilo, es un compuesto volátil responsable de un olor característico a mantequilla con un umbral de percepción bastante alto en vinos (0,1 mg/L) (*Moreno et al., 2005*) Este compuesto define el aroma característico de los vinos (*Pretorius y*

Swiegers, 2008) y se encuentra en altas concentraciones en los vinos "Finos", diferenciándose del resto de vinos blancos (*Campo et al., 2008*). Su presencia en vinagres es indicativa del proceso de acetificación y maduración (*Casale et al., 2006*). Hasta el momento no se ha encontrado en vinagres de vino tinto, sin embargo, son varios los autores que ponen de manifiesto la presencia de diacetilo en vinagres de Jerez, en concentraciones superiores a los vinos de esta denominación de origen que oscilan entre 10-55 mg/L (*Morales et al., 2002; Morales et al., 2003*). Además, se ha observado que las concentraciones de este compuesto aumentan durante el envejecimiento, por lo que algunos autores lo consideran como un indicador de la edad de los vinagres de Jerez (*Morales et al., 200*2).

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. El fenilacetaldehído presenta un aroma a miel y se encuentra en concentraciones entre 0.1-1 mg/L (*Blanch et al., 1992*). En vinos también está presente, observándose altos niveles de fenilacetaldehído en los vinos al Pedro Ximénez (*Campo et al., 2008*). Al igual que el acetaldehído se forma por la descarboxilación de los ácidos cetónicos correspondientes llevada a cabo por las levaduras (*Bayonove et al., 2000*). Su presencia se ve favorecida en aquellos vinos en los que hay cantidades apreciables de oxígeno disuelto (*Câmara et al., 2003*). Por otro lado, los otros compuestos carbonílicos (furfural, 5-metil-2-furfuraldehído, 5-acetoximetil-2-furfuraldehído y 5-hidroximetil-2-furfuraldehído) son productos de la Reacción de Maillard, y se han encontrado en altas concentraciones en los vinagres balsámicos de Módena y al Pedro Ximénez. Esto se debe al específico proceso de elaboración de estos vinagres, sobre todo de los balsámicos, donde se lleva a cabo un proceso lento de calentamiento para cocer el mosto y la adición de caramelo en diferentes proporciones (*Pizarro et al., 2008*). Todos estos compuestos contribuyen a las propiedades organolépticas y a la caracterización de estos vinagres (*Cocchi et al., 2008*).

2.3.2. Alcoholes

Los alcoholes más abundantes en los vinagres de vino son sin duda el etanol y el metanol, ya que éstos se encuentran en altas concentraciones en los vinos 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*). Durante el envejecimiento tiende a disminuir debido al metabolismo aerobio de las bacterias que transforma el alcohol en ácido, el cual se transforma seguidamente en su correspondiente éster (*Palacios et al., 2002*).

El contenido de etanol residual en vinagres vínicos, aunque variable, oscila en un estrecho margen (*Guerrero, 19*95). Según la reglamentación vigente el límite máximo de etanol en un vinagre comercial es de 0,5 % v/v. En los vinagres con denominación de origen como el de Jerez se permiten hasta un 3% de alcohol residual (*Ministerio de Agricultura y Pesca, 2008a*). Durante el proceso fermentativo, se procura el mayor rendimiento posible en la transformación de etanol a ácido acético. Pero no resulta rentable agotar por completo el etanol, ya que entonces las bacterias acéticas podrían degradar el ácido acético producido (*Morales, 1999*). Si los vinagres se fabrican por modernos sistemas industriales, lo normal es que al final se observe una pequeña cantidad de alcohol residual. Pero si intervienen métodos artesanales, no se desea agotar todo el etanol ya que durante el proceso de envejecimiento se van a ir formando ésteres de etanol en un proceso lento que puede mejorar el buqué del vinagre (*Morales, 1999*).

El resto de alcoholes presentes en el vinagre pertenecen al grupo de los llamados alcoholes superiores. En enología, el concepto de alcoholes superiores abarca a aquellos alcoholes cuyo número de carbonos es superior a dos y que presentan un peso molecular y punto de ebullición superiores al del etanol. Este grupo se compone de alcoholes alifáticos y aromáticos (*Cañete, 1994*). Los alcoholes alifáticos incluyen al propanol, isobutanol, alcohol amílico e isoamílico, mientras que el 2-feniletanol se considera el principal representante de los alcoholes aromáticos. El alcohol isoamílico, amílico e isobutanol se conocen también como alcoholes ramificados y se consideran productos

de degradación de los aminoácidos ramificados: leucina, isoleucina y valina (*Lambrechts y Pretorius, 2000*).

Los alcoholes superiores son cuantitativamente el grupo mayoritario en muchas bebidas alcohólicas y están dotados de propiedades organolépticas (*Bayonove et al., 2000, Ribéreau-Gayon et al., 2006*) y pueden ser reconocidos por su fuerte y punzante olor y sabor. Algunos de estos alcoholes superiores permanecen en cantidad inalterada o casi inalterada durante el proceso de transformación del vino en vinagre y otros, en cambio, decrecen de forma acusada durante la acetificación (*Nieto et al., 1993*). Esto puede ser debido a que las bacterias acéticas tienen la capacidad de oxidar aquellos alcoholes con un número de carbonos menor o igual a cinco produciendo sus correspondientes ácidos grasos (*Nieto et al., 1993*). Esto unido a las pérdidas que se producen durante los procesos de acetificación con fuerte aireación nos lleva a contenidos finales de alcoholes superiores en vinagre inferiores a los que cabría esperar (*Parés y Juárez 1997*). Por otro lado, estos alcoholes se ven implicados en la formación de ésteres, lo que supone otra causa del descenso que se observa en sus valores (*Palacios et al., 2002*).

El propanol (alcohol propílico) puede oxidarse a ácido propiónico por la acción de *Acetobacter pasteurianus* (*Mecca et al., 1979*). La cantidad de propanol presente en el vinagre de vino oscila entre 1-20 mg/L (*Palacios et al., 2002*). Este compuesto, al igual que los demás alcoholes superiores, disminuye durante el envejecimiento llegando a niveles casi indetectables (*Palacios et al., 1992*).

El isobutanol (2-metil-1-propanol) se origina a partir de la valina y está presente en concentraciones entre 1-150 mg/L en el vinagre de vino (*Blanch et al., 1992*). El valor mínimo se encuentra en productos comerciales convencionales, mientras que el máximo se encuentra en el vinagre de vino envejecido, favoreciéndose su formación, por tanto, en procesos de fermentación lentos en los que la concentración de oxígeno es limitada (*Morales et al., 2001a*).

Los alcoholes isoamílicos (2-metil-1-butanol y 3-metil-1-butanol) 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., 2001b*). 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. El cociente entre 3-metil-1-butanol y 2-metil-1-butanol es un indicador adecuado para juzgar el proceso de acetificación, ya que disminuye conforme avanza la acetificación (*Nieto et al., 1993*). El 2-metil-1-butanol es metabolizado por las bacterias acéticas a un ritmo más lento que el 3-metil-1-butanol, por ello, cuando la velocidad de acetificación es elevada se produce un brusco descenso de dicho cociente, tal como ocurre en las acetificaciones realizadas por cultivo sumergido (*Nieto et al., 1993*).

Por otro lado, en el vinagre de vino se encuentran otros alcoholes superiores aunque en pequeñas dosis, como son: 2-feniletanol, cis-3-hexen-1-ol, alcohol bencílico o alcohol furfurílico entre otros. El 2-feniletanol tiene un olor rosas y es uno de los alcoholes superiores que tienen impacto olfativo en los vinos (*Bayonove et al., 2000*). El cis-3-hexen-1-ol está presente en altas concentraciones en los vinos "Finos" diferenciándolo de los olorosos (*Zea et al., 2001*). Este hecho sugiere que este compuesto es producido por el velo de levaduras implicadas en el proceso de crianza biológica. También está presente en vinagres de Jerez en concentraciones hasta de 1 mg/L, y en vinagres de vinos tintos y blancos entre 0,1 y 96 mg/L (*Guerrero et al., 2007*). El alcohol bencílico está presente en diferentes tipos de vinagres, balsámicos de Módena, vinagres de vinos tintos y blancos y vinagres de Jerez, (*Guerrero et al., 2007*) en concentraciones menores a 0,1 mg/L, al igual que el alcohol furfurílico aunque éste presenta concentraciones más altas en los vinagres de Jerez (*Blanch et al., 1992*).

2.3.3. Ácidos

En relación al contenido en ácidos orgánicos, los vinagres contienen ácidos volátiles (acético, isovalérico...etc), y no volátiles (tartárico, cítrico, málico, succínico...etc). Entre ellos, el ácido que identifica el producto es el ácido acético. Su

concentración puede variar dependiendo del contenido en carbohidratos del substrato vínico de partida (*Natera et al., 2003*).

El ácido mayoritario en vinagres después del acético es el ácido isovalérico. Éste está presente en concentraciones entre 10-1000 mg/L (*Blanch et al., 1992*) siendo mayores en los vinagres de Jerez (*Guerrero et al., 2007*). Al igual que la mayoría de los ácidos orgánicos volátiles, presenta un aroma desagradable a queso o rancio.

Algunos de los ácidos orgánicos volátiles ya están presentes en el vino de partida aunque se forman en gran medida durante el proceso de acetificación. Algunos ácidos orgánicos como el isobutanol, nonanoico y hexanoico se han utilizado para diferenciar 4 clases de vinagres (vinagres de vinos tintos, de vinos blancos, balsámicos de Módena y vinagres de Jerez), por lo que se afirma que el contenido en ácidos orgánicos del vinagre está muy influenciado por la materia prima empleada (*Pizarro et al., 2008*).

2.3.4. Ésteres

Los ésteres son componentes importantes del aroma del vinagre de vino y son responsables de las notas frutales y florales de los mismos (*Charles et al., 2000*). Así, el contenido equilibrado de ésteres juega un papel importante sobre las características organolépticas del vinagre de vino, ya que intervienen en el aroma y sabor, en especial en los envejecidos, dándole la finura y el valor típico de los productos de calidad (*Morales, 1999*). Los ésteres se producen como resultado de la reacción de condensación entre un ácido y un alcohol. Debido a los altos contenidos de ácido acético y etanol, se diferencian fundamentalmente dos tipos de ésteres: los ésteres de acético o acetatos y los ésteres etílicos.

En el caso de los vinagres, pequeñas cantidades de ésteres proceden del vino de partida y en gran medida se forman por la condensación entre un ácido y un alcohol durante la maduración y el envejecimiento (*Charles et al., 2000; Morales et al., 2001a*). Durante la conservación del vinagre, su composición en ésteres sufre un reajuste (hidrólisis/esterificación) muy lento debido a que tienen lugar reacciones puramente

químicas entre los reactivos hasta que se alcanza un estado de equilibrio (*Bayonove et al., 2000*).

Los ésteres se clasifican en volátiles y no volátiles. Los primeros intervienen en el aroma y son principalmente el acetato de metilo, etilo e isoamilo, mientras que los no volátiles influyen en el gusto y los más destacables son el succinato, malato y tartrato de etilo (*Morales, 1999*).

El éster volátil cuantitativamente más importante es el acetato de etilo. Se forma a partir de acético y etanol, previa eliminación de una molécula de agua, reacción catalizada por una esterasa (*Navascués et al., 1995*). Este compuesto tienen un olor característico a "pegamento" y tiene gran influencia en el perfil aromático de los vinagres de Jerez (*Charles et al., 2000*) y en otros tipos de vinagres de vino envejecidos (*Casale et al., 2006*).

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.

El acetato de etilo es un compuesto altamente volátil y de baja solubilidad (*Morales, 1999*). En los sistemas de acetificación abiertos, este compuesto es arrastrado por la corriente de aire observándose importantes pérdidas (*Morales et al., 2001a*). No obstante si la maduración se lleva a cabo en presencia de cantidades de etanol residual del orden de 2º, se consigue que en sólo tres meses de envejecimiento se duplique e incluso triplique la cantidad de acetato de etilo con la que se inicia el envejecimiento del vinagre, llegándose en algunos casos a concentraciones de acetato de etilo del orden de 8 g/L (*Morales et al., 2002*).

Otros dos ésteres a destacar son el acetato de metilo y el acetato de isoamilo. En cuanto al acetato de metilo, su contenido en los vinagres procedentes de vino tinto es mayor que en los vinagres de vino blanco, ya que el contenido en metanol es superior en dichos vinos. Además, la formación de acetato de metilo se ve favorecida en medios ácidos. Así, los vinagres que maduren con un alto grado acético presentarán mayor contenido en dicho éster (*Morales et al., 2002*). El acetato de isoamilo está presente en concentraciones del orden de los 3 mg/L en los vinagres de vino blanco y tinto (*Natera et al., 2003*), sin embargo, se ha observado concentraciones superiores de este compuesto, aproximadamente de 10 mg/L, en los vinagres de Jerez (*Blanch et al., 1992*). La formación de estos compuestos se ve favorecida por la presencia de altos contenidos en alcoholes isoamílicos en los vinos o substratos de partida (*Morales et al., 2002*). Estos compuestos tienen un aroma afrutado (plátano, manzana...etc) y contribuyen a la complejidad aromática de vinos y vinagres (*Ribéreau-Gayon et al., 2006*).

En vinagres, también se han encontrado muchos otros ésteres como acetato de 2-feniletanol, acetato de butilo, acetato de bencilo, fenilacetato de etilo, benzoato de etilo, salicilato de etilo, decanoato de etilo, succinato de dietilo o lactato de etilo entre otros. El succinato de dietilo, acetato de 2-feniletanol y lactato de etilo están presentes en altas concentraciones, estando entre los ésteres mayoritarios (*Natera et al., 2003; Guerrero et al., 2007*). El lactato de etilo, compuesto que tiene un olor a leche agria (*Ribéreau-Gayon et al., 2006*), es característico de los vinos de Jerez y esta presente en los vinagres que tienen un mayor contenido de alcohol residual (*Morales et al., 2002*). Por otro lado, el acetato de bencilo, fenilacetato de etilo, benzoato de etilo y salicilato de etilo tienen una notable influencia en la garantía de la calidad del vinagre y del origen (*Pizarro et al., 2008*). Y por último el decanoato de etilo es uno de los compuestos que más contribuyen al aroma y sabor de la uva y su concentración disminuye durante el envejecimiento (*Casale et al., 2006*).

2.3.5. Acetales

Los acetales se forman por una reacción lenta y reversible entre 2 moléculas de alcohol y un aldehído, tal como se muestra en la siguiente figura:

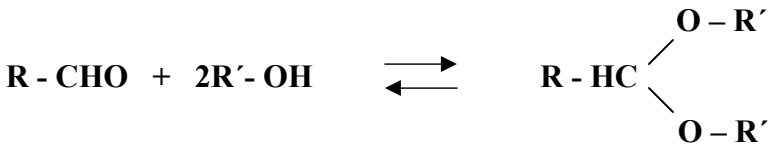


Figura 2.12. *Formación de un acetal.*

Los acetales tienen un olor herbáceo que participa en la complejidad aromática de los vinos (*Ribéreau-Gayon et al., 2006*). Se han identificado más de 20 acetales en vino. De todos ellos el más importante es el 1,1-dietoxietano (o acetaldehído dietilacetal), resultado de la reacción entre el acetaldehído y el etanol. Este compuesto da a los vinos notas a frutas verdes y a licor (*Zea et al., 2007; Moreno et al., 2005*). Además, este compuesto puede ser usado como un marcador analítico de los cambios de los vinos finos durante su envejecimiento biológico (*Moreno et al., 2005*).

El 1,1-dietoxietano también se ha cuantificado en el vinagre de vino en concentraciones entre 1 y 10 mg/L (*Blanch et al., 1992*), debido a los altos contenidos de acetaldehído de los vinagres.

2.3.6. Terpenos

Los terpenos constituyen un buen ejemplo de sustancias de origen varietal, las cuales pueden ser usadas en la tipificación de los vinos (*Pizarro et al., 2008*). El contenido en terpenos de un vino se considera un factor de calidad, ya que estos compuestos contribuyen al aroma aportando notas florales, y puede estar influenciado por un amplio número de factores enológicos incluyendo la variedad de uva y el proceso de maduración. Estos compuestos están presentes principalmente en la piel de las uvas y su concentración varía de una variedad a otra, destacándose el alto contenido de los vinos procedentes de las uvas Moscatel (*Bayonove et al., 2000*).

En la uva y en el vino se pueden citar actualmente alrededor de 70 compuestos terpénicos identificados. La mayor parte de éstos son monoterpernos (limoneno, α-

terpineno), sesquiterpenos (farneseno) y sus alcoholes (linalol, geraniol, α-terpineol) y aldehídos (geranial, citroneral) correspondientes, que son los más interesantes en el plano olfativo (*Bayonove, et al., 2000; Ribéreau-Gayon et al., 2006*).

Hasta el momento los únicos compuestos de este grupo encontrados en vinagres de vino han sido el α-terpineol (*Guerrero et al., 2007*) y eudesmol (*Pizarro et al., 2008*). El α-terpineol se ha cuantificado en vinagres balsámicos, de Jerez, de vino blanco y tintos, alcanzando concentraciones entre 0,9 y 32,64 μg/L (*Guerrero et al., 2007*); los vinagres blancos y los de Jerez presentaron las menores concentraciones (*Guerrero et al., 2007*).

2.3.7. Fenoles volátiles

Los fenoles volátiles son compuestos aromáticos cuya nota olfativa viene a menudo marcada por el olor poco agradable a "farmacia", pero ligada a notas de ahumado, de bosque, cuero, pimienta que con una dilución son más agradables (*Bayonove et al., 2000*). Todos tienen umbrales olfativos muy bajos y son susceptibles de tipificar el vino con su presencia.

Los principales fenoles volátiles producidos por las levaduras *Saccharomyces cerevisiae* a partir de los ácidos p-cumárico y ferúlico son el 4-vinilfenol (olor de aguada, clavel) y el 4-vinilguayacol (olor a clavo). Las cantidades producidas son generalmente mucho más elevadas en vinos blancos que en tintos, siendo igualmente más abundante el primero que el segundo (*Baumes et al., 1986; Bayonove et al., 2000*).

4-Etilfenol y 4-etilguayacol se producen en los vinos a partir de los mismos precursores del 4-vinilfenol y 4-vinilguayacol, ácidos p-cumárico y ferúlico, por acción de las levaduras pertenecientes a los géneros *Brettanomyces/Dekera* (*Chatonnet et al., 1992*). Estos compuestos también son producidos por las levaduras *Brettanomyces* a partir de compuestos fenólicos (*Campo et al., 2008*). Los vinos "finos" se caracterizan por tener altos niveles de 4-etilguayacol. Este hecho confirma que el 4-etilguayacol es también un compuesto aromático característico de los vinos "finos" (*Campo et al., 2008*).

Durante la conservación del vinos se ha observado una disminución en la concentración de los vinilfenoles y, sin embargo, un aumento en la concentración de los etilfenoles que puede llegar a ser incluso perjudicial para la calidad organoléptica del vino (*Bayonove et al., 2000*).

El 4-etilfenol y 4-etilguayacol se han encontrado en diferentes tipos de vinagres como balsámicos, vinagres de Jerez, vinagres de vino blanco y tinto y vinagres al Pedro Ximénez (*Charles et al., 2000; Natera et al., 2002; 2003; Guerrero et al., 2007*). Estos compuestos están presentes en concentraciones entre 33-209 μg/L y 3-57 μg/L, respectivamente, siendo mayores las concentraciones de los vinagres balsámicos y las de los vinagres de Jerez (*Guerrero et al., 2007*). Y al igual que ocurre en vinos, éstos compuestos tienden a aumentar con el envejecimiento (*Natera et al., 2002*).

2.3.8. Lactonas

Las lactonas son componentes significativos del aroma de los vinos, especialmente de los vinos de Jerez en donde se han encontrado la γ-butirolactona, 4-carbetoxi-γ-butirolactona, 2-hidroxi-3,3-dimetil-γ-butirolactona (pantolactona) y 2-hidroxi-3-metil-2-penteno-γ-lactona (sotolón) (*Etiévant 1991*). 4-carbetoxi-γ-butirolactona es responsable de un olor a nuez de coco y parece ser que comunica a los vinos de Jerez un olor característico denominado "sherry like" (*Brock et al., 1984*). Parece ser que de todas estas lactonas, sólo el sotolón tiene un impacto importante sobre el aroma y está presente en altas cantidades en vinos elaborados bajo el velo de levaduras, como el vino de Jerez, el vino de Tokaï de Hungría, y en particular el vino de Jura (*Câmara et al., 2004*).

La γ-butirolactona ha sido detectada en diversos tipos de vinagres (*Charles et al., 2000; Blanch et al., 1992; Morales et al., 2003*) siendo más abundante en aquellos producidos por métodos tradicionales (*Morales et al., 2001b*). Además, se ha observado que la γ-butirolactona aumenta durante el envejecimiento del vinagre, alcanzando concentraciones que oscilan entre 40-100 mg/L (*Morales et al., 2002*). *Jarauta et al.*

(2005) consideran que este compuesto junto con otros como los ácidos butírico, hexanoico, octanoico, isovalérico, metionol y β-etilfenol, son metabolitos que se extraen fácilmente de la superficie de la madera y que se producen como consecuencia de la existencia de microorganismos en dicha superficie.

2.3.9. Compuestos extraídos de la madera

El envejecimiento de los vinagres en madera mejora la calidad de los productos debido al incremento de la complejidad aromática que se produce por el aumento de concentración de los compuestos volátiles y por la formación de compuestos nuevos debidos a procesos de oxidación o de condensación. Además, durante el envejecimiento se extraen algunos compuestos de la madera que también participan en el aroma final del vinagre envejecido (*Morales et al., 2002*).

Aunque se han identificado cientos de compuestos volátiles en la madera de roble sin tostar (*Chatonnet, 1991, Morales et al., 2004*), son pocos los compuestos que pueden ser extraídos en cantidades significativas (*Chatonnet, 1991; Sefton et al., 1993*). Entre esos compuestos se encuentran la vainillina, eugenol, derivados del guayacol, *cis*- y-*trans*-β-metil-γ-octalactona, comúnmente conocidas como "whisky-lactonas" (*Kepner et al., 1972*), derivados del furano y siringaldehído (*Jarauta et al., 2005*).

El eugenol extraído de la madera es producto de la degradación de la lignina, el cual se ha encontrado en vinagres de vino envejecidos en barricas (*Morales et al., 2004*). Este compuesto tiene un aroma a clavo y su concentración en madera puede aumentar en las barricas que han sido tostadas. Por esta razón, el eugenol está presente en los vinagres envejecidos en barricas, y de ahí su lógica asociación con los vinagres producidos por métodos tradicionales (*Pizarro et al., 2008*).

La vainillina es otro compuesto producido por la degradación de la lignina y es el principal componente aromático de la vainilla natural (*Clark, 1990*). Este contribuye a la calidad de los vinos envejecidos en madera (*Sefton et al., 1993*) y su contenido en las barricas depende de las diferencias en la penetración del calor y de la intensidad del tostado (*Spillman et al., 1997*). *Morales et al. (2004)* realizaron un estudio de

envejecimiento de vinagres de vino en barricas y lo compararon con un envejecimiento acelerado con virutas de roble. Las concentraciones de vainillina fueron superiores en los vinagres envejecidos con virutas de madera, ya que cuando las barricas se tuestan sólo se afectan las capas más superficiales, mientras que cuando se calientan las virutas el calor penetra y conduce a un aumento en la formación de vainillina. Por tanto, este compuesto parece ser el principal indicador del envejecimiento con virutas de madera.

El siringaldehído, otro fenol volátil al igual que la vainillina, también se extrae de la madera durante el envejecimiento de los vinos (*Jarauta et al., 2005; Bayonove et al., 2000*). Este compuesto también se ha determinado en los vinagres de vino blanco y en vinagres de Jerez envejecidos en madera (*Natera et al., 2003; García-Parrilla et al., 1999*).

Las "whisky-lactonas", responsables del olor a coco, son unos de los compuestos volátiles más importantes de la madera que contribuyen al aroma de las bebidas envejecidas en barricas y su concentración también pueden aumentar con el tostado de la madera (*Morales et al., 2004*). Se han encontrado amplias diferencias en las concentraciones totales de estas lactonas y en la relación de sus isómeros en maderas de diferentes orígenes. Algunos autores consideran que la relación *cis/trans* es característica del origen de la madera (*Guichard et al., 1995*). De este modo, la relación *cis/trans* de los vinos envejecidos en barricas de roble americano es siempre mayor a 5, mientras que la relación en los vinos envejecidos en roble europeo es siempre igual o menor a 2 (*Towey y Waterhouse 1996*). Sin embargo, los resultados obtenidos en vinagres de vino envejecidos en virutas de madera de roble americano no mostraron esta tendencia, ya que presentaron una relación *cis/trans* entre 2 y 5 (*Morales et al., 2004*).

Los compuestos carbonílicos también están implicados en el aroma de productos envejecidos debido a la extracción de aldehídos fenólicos de la madera de las barricas (*Da Silva Ferreira y Bertrand, 1995; Guillou y Bertrand, 1995*). El 2-furfuraldehído y 5-metil-2-furfural son dos aldehídos que también pueden ser extraídos de la madera, ya que también se forman durante el proceso de tostado por degradación térmica de la hemicelulosa y celulosa, respectivamente (*Bozalongo et al., 2007*). Estos compuestos al igual que el benzaldehído y los derivados del guayacol se han encontrado en vinagres de

vino tinto, blanco y en vinagres de Jerez (*Blanch et al., 1992; Guerrero et al, 2007; Morales et al., 2004*).

Por otro lado, algunos compuestos que se extraen de la madera también pueden formarse a partir de precursores presentes en el vino. Es decir, estos compuestos principalmente vienen de la madera pero también pueden estar presentes, aunque en concentraciones traza, en vinos que no han estado en contacto con la madera. Dentro de este grupo encontramos la acetovainillona, metilvainillato, etilvainillato y furaneol, entre otros (*Jarauta et al., 2005*). Entre ellos la α-ionona se ha determinado en vinagres de Jerez, tanto en los envejecidos en madera como en los no envejecidos aunque en concentraciones más bajas (*Natera et al., 2002*).

2.4. DETERMINACIÓN DE LOS COMPUESTOS VOLÁTILES

2.4.1. Técnicas de aislamiento o separación

El interés por conocer la composición de los aromas ha estado siempre presente en la Enología, y concretamente en el campo del vinagre. Sin embargo, hasta la aparición de las técnicas de cromatografía de gases no se pudo llevar a cabo la separación y cuantificación de la fracción volátil del vinagre. Además, hasta épocas recientes, no se han desarrollado técnicas analíticas que permiten la cuantificación de compuestos minoritarios, que pueden estar presentes en concentraciones que van desde g/L hasta ng/L.

Desde un punto de vista analítico, el aroma del vinagre está formado por una compleja fracción de compuestos con un amplio rango de volatilidades, polaridades y concentraciones (*Blanch et al., 1992*). Por ello, los procesos de aislamiento de los compuestos volátiles del vinagre del resto de la matriz están basados en distintas propiedades físico-químicas como son la volatilidad, la solubilidad en las distintas fases orgánicas inmiscibles con la matriz y la capacidad para ser adsorbidos selectivamente sobre ciertos materiales.

Los compuestos mayoritarios no necesitan ningún proceso de preconcentración, sino que la inyección directa en el cromatógrafo permite detectarlos, ya que tienen una concentración lo suficientemente alta como para poder ser registrados por los detectores habituales. Estos compuestos son muy variados y destacan alcoholes y ésteres. La inyección directa de vinagre ha sido ampliamente utilizada por investigadores como *Jones y Greenshields (1969), Khan et al. (1966), Mecca y Di Vecchio (1977), Cabezudo et al. (1978), Troncoso y Guzmán (1987)) o Morales et al. (2001b),* entre otros.

Sin embargo, hay otro grupo de compuestos, llamados compuestos minoritarios, que se encuentran en concentraciones tan pequeñas que necesitan ser extraídos y concentrados para poder ser determinados (*Blanch et al., 1992*). Estos procesos de pre-tratamiento de muestra, extracción y concentración de los compuestos son los puntos críticos del análisis de volátiles (*Morales y Troncoso, 2003*).

Algunos de los métodos que se utilizan para concentrar los volátiles de baja concentración requieren demasiado tiempo, o suponen una gran manipulación, lo que puede conducir a distintos tipos de error. Otros tienen baja reproducibilidad o bien pueden producir alteraciones de los volátiles originales cuando se superan determinadas temperaturas, también pueden formarse nuevos compuestos, e incluso puede que la fracción de aroma determinada no sea representativa de la muestra (*Núñez y Maarse, 1986*). Por ello se piensa que lo más próximo a la concentración real de un vinagre se conseguirá utilizando un método con la mínima manipulación posible en el que los compuestos analizados sean los que realmente definen el aroma, con alta reproducibilidad, y a ser posible que pueda controlarse de modo automático.

Dentro de las técnicas que han sido aplicadas para la extracción del aroma de vinagre de vino cabe destacar:

a) ***Extracción por espacio de cabeza.*** El vinagre se satura con una sal soluble para disminuir la presión de vapor de agua y facilitar la extracción de los compuestos volátiles a la fase gaseosa. Una vez establecido el equilibrio líquido-vapor se analizaría la fase gaseosa. En vinagres, muchos autores han utilizado esta técnica (*Aurand et al., 1966, Ferrer-Gimenez y Clotet-Ballus, 1979*) a pesar de estar limitada por su baja sensibilidad.

b) ***Extracción líquido-líquido.*** La extracción por medio de un disolvente orgánico es una técnica conocida desde muy antiguo, la cual tiene una amplia aplicación en Enología (*Brander et al., 1980; Pérez-Coello et al., 2000*; *Díaz-Plaza et al., 2002*) aunque está siendo reemplazada por otras técnicas menos contaminantes. Además, también se ha utilizado para extraer los compuestos volátiles de vinagres de vino empleándose distintos disolventes como el éter de petróleo, pentano, triclorofluorometano o diclorometano, así como sus mezclas (*Khan et al., 1972; Gerbi et al., 1992; Morales et al., 2004*). 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*). Las variables a tener en cuenta para obtener buenos resultados son muy numerosas, siendo las más importantes el tipo y cantidad de disolvente, el volumen de muestra a tratar y el tiempo de extracción.

La técnica de extracción líquido-líquido suele consumir bastante tiempo y es bastante laboriosa, ya que incluye múltiples pasos o etapas. Además, requiere disolventes orgánicos tóxicos y se suelen producir pérdidas de volátiles en el paso de concentración del extracto, obteniéndose bajas recuperaciones.

c) ***Destilación y extracción simultáneas.*** Esta técnica fue desarrollada inicialmente para el estudio de aceites esenciales vegetales (*Likens and Nickerson, 1964*) y posteriormente ha sido aplicada a diferentes tipos de muestras (*Núñez y Bemelmans, 1984; de Frutos et al., 1988; Blanch et al., 1991*). En 1992, *Blanch et al.* aplicaron por primera vez esta técnica en vinagres de Jerez.

La destilación y extracción simultánea no requiere el 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 altas temperaturas puede dar lugar a la formación de sustancias no deseadas.

d) ***Extracción en fase sólida (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. Existen diferentes tipos de adsorbentes que se comercializan en forma de cartuchos y que se escogen en función de los analitos que se quieren determinar. Se diferencian básicamente en tres tipos: los de sílices enlazadas, carbón y poliméricos. La elución de los analitos retenidos se realiza mediante unos pocos mililitros de un solvente orgánico como por ejemplo metanol, diclorometano o acetato de etilo. Por tanto, con esta técnica se reduce el uso de disolventes orgánicos y la utilización de material caro y frágil, pero, al igual que la extracción líquido-líquido, la extracción en fase sólida también requiere tiempos de preparación excesivos y es bastante laboriosa, incluyendo múltiple etapas (*López-Ávila, 1999*).

Esta técnica se ha utilizado para extraer los compuestos volátiles de vinagres de vino (*Gerbi et al., 1992*; *Morales et al., 2004*) obteniéndose un perfil aromático similar al obtenido en la extracción líquido-líquido, aunque algunos compuestos (alcoholes, ésteres y ácidos) alcanzaron concentraciones superiores con la SPE.

e) ***Microextracción en fase sólida "(SPME).*** Esta técnica fue desarrollada a principios de los años 90 por el grupo de investigadores de J. Pawliszyn (*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 (*Alpendurada, 2000*). La fibra de sílice fundida con la cobertura polimérica se encuentra unida a una envuelta metálica y ambas están protegidas por una cobertura de metal evitando que se dañe el polímero mientras no esté en uso. Finalmente, la fibra se encuentra ensamblada en un portafibra, cuya estructura es similar a una jeringa ligeramente modificada (Figura 2.13).

Esta técnica presenta una serie de ventajas frente a las técnicas de preconcentración mencionadas anteriormente ya que es muy simple, tiene una alta sensibilidad y reproducibilidad, presenta un bajo coste, puede ser automatizada, requiere pequeños volúmenes de muestra y generalmente no

precisa del uso de disolventes orgánicos para llevar a cabo la extracción, a diferencia de la extracción líquido-líquido y la extracción en fase sólida (*Alves et al., 2005*). Debido a su diseño, es fácilmente transportable, presenta la posibilidad de utilizarse con todos los tipos de muestras, ya sean gaseosas, líquidas o sólidas y se puede aplicar a la determinación de compuestos de diferente volatilidad. Esta técnica ha sido aplicada satisfactoriamente para la caracterización de vinos (*Flak et al., 2005; López et al., 2007*) y vinagres (*Natera et al., 2002; Natera et al., 2003*).

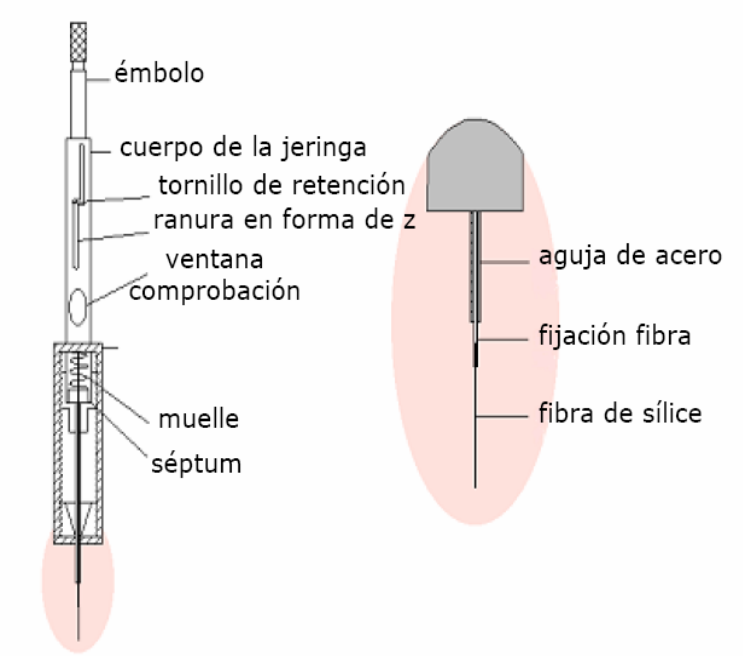


Figura 2.13. *Esquema del dispositivo SPME*

Como inconveniente se puede mencionar que debido a la limitada capacidad de las fibras (la cantidad de recubrimiento es muy pequeña) en ocasiones no se obtienen unos límites de detección bajos, sobre todo si la SPME se utiliza combinada con la cromatografía de líquidos (*Alves et al., 2005*).

En la actualidad hay dos tipos de recubrimientos que se comercializan: los homogéneos (que se comportan como un líquido) y los heterogéneos (que se comportan como un sólido). Tanto la naturaleza del analito como el tipo de

recubrimiento van a determinar la forma de interacción entre ambos, por lo que las propiedades físico-químicas del recubrimiento, como la polaridad y el grosor de la capa de polímero, influyen claramente en la eficacia de la extracción por SPME (Figura 2.14).

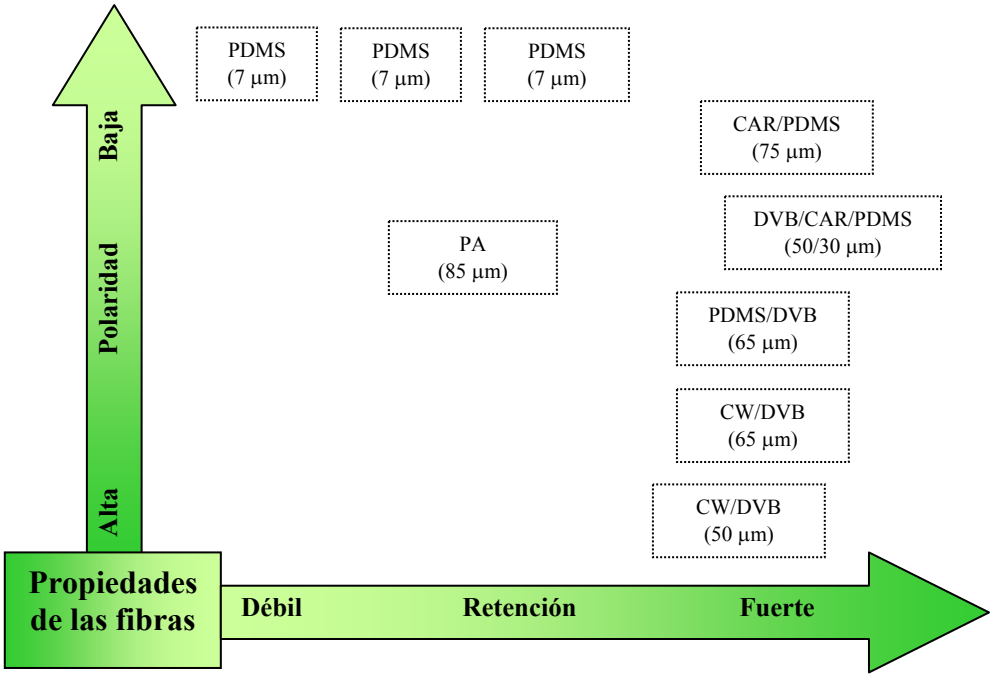


Figura 2.14. *Esquema de las propiedades de las fibras según su polaridad y capacidad de retención de los analitos*

Dentro de los recubrimientos homogéneos se incluyen las fibras compuestas por polidimetilsiloxano (PDMS) y poliacrilato (PA). Ambas fases estacionarias son líquidas y la extracción de los analitos tiene lugar mediante procesos de absorción. Por otro lado, los recubrimientos heterogéneos son fibras en las que se inmovilizan partículas porosas de divinilbenceno (DB) o carboxen (CAR) sobre una fase parcialmente entrelazada de PDMS o carbowax (CW). Aunque tienen una mayor selectividad, generalmente presentan menor estabilidad mecánica que los recubrimientos homogéneos.

La técnica SPME consta de dos etapas fundamentales:

- Extracción: En esta etapa se pone la fibra recubierta del adsorbente/absorbente en contacto con la muestra durante un tiempo y

temperatura determinadas, de manera que se produce el reparto de los analitos entre la matriz de la muestra y la fase estacionaria de la fibra hasta que se alcanza la situación de equilibrio. Existen dos formas básicas de realizar la extracción en SPME tal como se muestra en la Figura 2.15: extracción por inmersión directa o bien en el espacio de cabeza (*Natera et al., 2002*).

- Desorción: Una vez que la fibra ha estado expuesta un tiempo determinado, la fase estacionaria se retrae dentro de la jeringa. Seguidamente la jeringa con el recubrimiento protegido se extrae del vial y se inserta en un portal de inyección de un cromatógrafo de gases o en la cámara de desorción de la interfase para cromatógrafo de líquidos (Figura 2.15). La desorción de los analitos desde la fibra tiene lugar mediante un calentamiento en el portal de inyección o por solvatación mediante empleo de una pequeña cantidad de disolventes en la cámara de desorción.

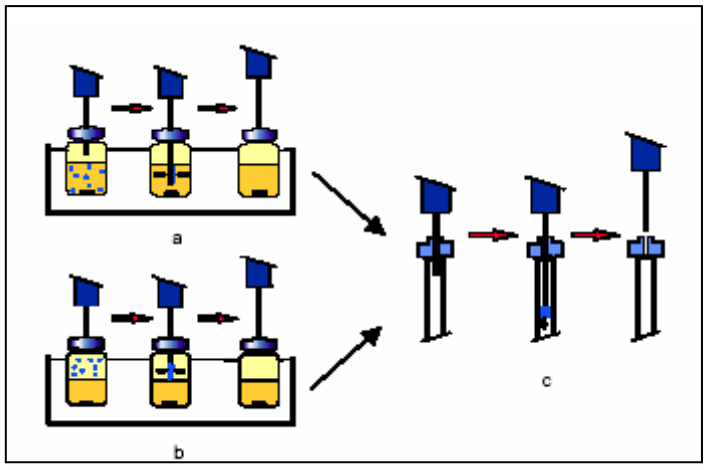


Figura 2.15. *Esquema del proceso de SPME: (a) inmersión directa; (b) espacio de cabeza (c) desorción térmica en el cromatógrafo de gases.*

El modo de extracción en espacio de cabeza tiene la ventaja que permite proteger la fibra de los compuestos de elevado peso molecular que pueden estar presentes en la matriz u otras interferencias no volátiles. Este modo de

extracción es muy útil para el análisis de muestras sólidas como los alimentos o muestras biológicas donde la interferencia de la matriz es importante, pero está restringido al análisis de compuestos volátiles o semivolátiles (*De la Calle et al., 1996*).

f) ***Extracción por absorción con barras magnéticas agitadoras o "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. La extracción de los analitos de la muestra se realiza también mediante un equilibrio de partición de éstos entre la muestra y un absorbente polimérico. En este caso, el absorbente, que es una capa de 1 mm de PDMS, está dispuesto recubriendo una barra magnética agitadora (*Gerstel, 2000*) (Figura 2.16). Estas barras magnéticas agitadoras recubiertas se denominan comercialmente Twister^{TM}.

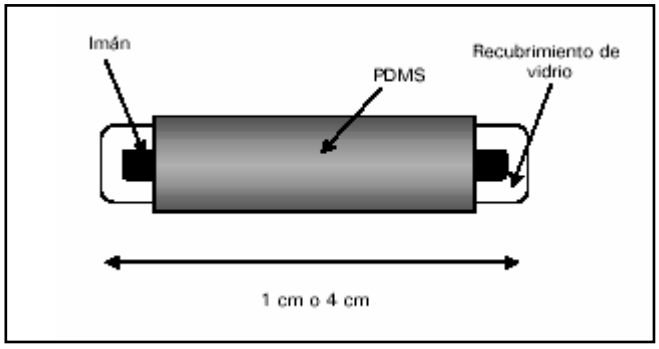


Figura 2.16. *Esquema de las barras magnéticas agitadoras recubiertas de PDMS usadas en SBSE.*

Así pues, en SBSE, los analitos pueden ser extraídos introduciéndolos, directamente en la muestra (inmersión) (*Diez et al., 2004; Marin et al., 2005*) o bien en el espacio de cabeza (*Hayasaka et al., 2003; Bicchi et al., 2000*) de la misma. La extracción en espacio de cabeza es muy ventajosa ya que reduce el riesgo de contaminación y además, incrementa el tiempo de vida de la barra magnética agitadora recubierta de PDMS (*Weldegergis et al., 2007, Rodríguez-*

Bencomo et al., 2003). A continuación la barra magnética agitadora se retira de la muestra, se aclara con agua y se seca con un papel libre de pelusas. Los compuestos extraídos por la barra magnética son desorbidos térmicamente en la unidad de desorción y son introducios en línea en el cromatógrafo de gases o bien mediante un solvente orgánico para su posterior inyección en un cromatógrafo de líquidos o de gases (*David y Sandra, 2007*).

La diferencia fundamental que presenta la SBSE con la SPME es la mayor cantidad de absorbente (PDMS) que contienen las barras magnéticas agitadoras en comparación con las fibras de SPME (*David y Sandra, 2007; Alves et al., 2005*). Este mayor contenido en PDMS permite una mayor recuperación de los analitos tal como se aprecia en la siguiente figura:

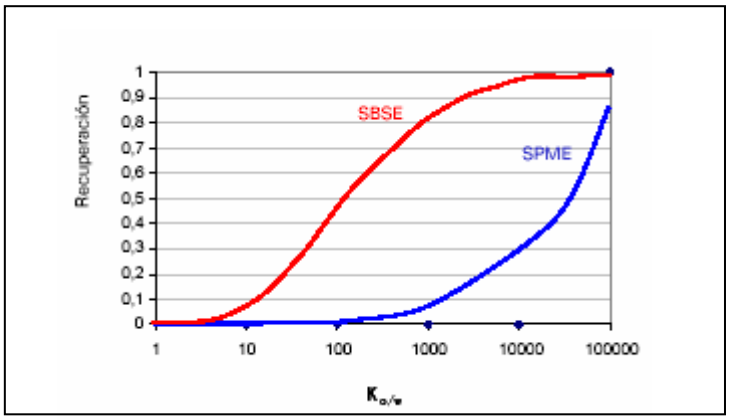


Figura 2.17. *Comparación teórica entre las eficacias de extracción en SPME y SBSE.*

En principio, la gran ventaja que presenta la SBSE frente a la SPME es la posibilidad de llegar a límites de detección más bajos pero también se debe indicar como inconveniente que hasta ahora sólo se comercializan barras magnéticas agitadoras para SBSE con recubrimiento de PDMS por lo que el campo de aplicación está restringido a compuestos apolares a no ser que se realice una derivatización in situ para extraer los compuestos polares (*Kawaguchi et al., 2006*). Es de esperar, sin embargo, que en el futuro se amplíe el número de absorbentes de las barras magnéticas agitadoras para poder aplicarlas a la determinación de mayor número de compuestos (*David y Sandra,*

2007). *Bicchi et al. (2005)* propusieron el empleo de twisters de doble fase, como una posible alternativa para mejorar o solucionar la limitación de la SBSE. Estos twisters de doble fase combinan la capacidad de concentración de dos o más materiales de muestreo (PDMS y diferentes tipos de carbono activo) que operan de forma diferente (absorción y adsorción). Sin embargo, esta nueva propuesta analítica presentó un limitado rango de aplicación, la cual demostró ser adecuada para el análisis en espacio de cabeza de algunas matrices o ciertas clases de compuestos. Por otro lado, otra reciente tentativa ha sido el empleo de twisters que contienen una nueva fase polimérica (poliuretano) capaz de extraer compuestos polares, los cuales una vez realizada la extracción se someten a desorción líquida *(Neng, et al., 2007*).

La aplicaciones de la SBSE en análisis de alimentos pueden dividirse en tres categorías: análisis de compuestos volátiles minoritarios, determinación de compuestos traza responsables de olores desagradables (*Hayasaka et al., 2003*) y análisis de contaminantes traza (pesticidas) (*Bicchi et al., 2003*).

Respecto al análisis de compuestos volátiles, la extracción por absorción con barras magnéticas agitadoras se ha aplicado satisfactoriamente, tanto en el modo de inmersión como en el espacio de cabeza, en frutas (*Kreck et al., 2001*; *Luan et al., 2006; Caven-Quantrill y Buglass, 2006*) en diferentes bebidas como café (*Bicchi et al., 2002; Bicchi et al., 2005*), cerveza (*Kishimoto et al., 2005*), vino (*Weldegergis et al., 2007; Alves et al., 2005; Hayasaka et al., 2003, Diez et al., 2004; Marin et al., 2005*), whisky (*Demyttenaere et al., 2003*) y sake (*Isogai et al., 2005*), y en vinagres (*Guerrero et al., 2006; 2007*). En general, la extracción por absorción con barras magnéticas agitadoras permite la detección de importantes compuestos aromáticos presentes en concentraciones por debajo de los umbrales de detección (*David y Sandra, 2007*).

Una aplicación especial de esta técnica ha sido el uso de la barra magnética, situada en un recipiente especial, para la detección de compuestos aromáticos liberados en la boca durante la cata de vino (*Buettner y Welle, 2004*).

2.4.2. Técnicas de determinación

Una vez que se han obtenido los extractos y que contienen los compuestos a estudiar, el siguiente paso es su determinación.

La cromatografía de gases es una técnica analítica de separación que ha experimentado un gran desarrollo desde sus inicios. Además, sus posibilidades han ido ampliándose a medida que se ha mejorado la instrumentación (columnas capilares, integradores computerizados, sistemas de gradiente de temperatura, nuevos detectores, etc.). Esta técnica es la que ofrece mejor poder de resolución para los compuestos volátiles. Su principal limitación se encuentra en la labilidad de los solutos, los cuales deben ser estables a la temperatura requerida para su volatilización.

La inyección cromatográfica es un parámetro importante a valorar, ya que la precisión del proceso cromatográfico viene determinada por la calidad de la inyección. Las formas de inyección más comunes son:

- "Split" o división de flujo: una vez volatilizada la muestra se desprecia una fracción del volumen inyectado (*Ortega et al., 2001*). Es la inyección más utilizada tradicionalmente en el análisis del aroma y debido a su sencillez, da bandas cromatográficas más estrechas, permite introducir muestras "sucias" y los tiempos de retención que se obtienen son muy reproducibles. Se utiliza para inyección directa de las muestras o en extractos altamente concentrados.

- "Splitless" o flujo sin dividir: toda la muestra una vez volatilizada pasa a la columna (*Grob y Grog, 1969*), por lo que no requiere una concentración tan exhaustiva de la muestra. Sin embargo, difícilmente va a poder aplicarse a extractos aromáticos obtenidos según esquemas clásicos (*Grob, 1988*).

- "On-column" o en columna: la muestra no sufre un paso previo de volatilización, sino que se inyecta directamente en la columna en forma

líquida (*Grob, 1978*). Esta inyección no resulta práctica en muestras "sucias", como son la mayoría de las que se obtienen con las operaciones de rutina en el estudio de los aromas.

Hoy día, cada vez se utiliza con más frecuencia la detección por espectrometría de masas acoplada a la cromatografía de gases para el análisis de aromas, de manera que está sustituyendo al tradicional detector de ionización de llama (*Kourkoutas et al., 2003*).

Una propuesta que proporciona un gran conocimiento sobre los volátiles de determinadas matrices como es el vino o el vinagre, es el caso de la Cromatografía de Gases Multidimensional (CGMD). Aunque la introducción de la columna capilar optimizó la separación de compuestos, la utilización de una sola columna en matrices complejas, como es el vinagre, a menudo no es suficiente para separar determinados analitos. La CGMD permite la conmutación de varias columnas de distinta fase estacionaria, consiguiéndose con ello una alta resolución de los picos cromatográficos. Así, aquellas fracciones de la muestra cuyos analitos no se han separado bien en la primera columna pueden ser desviadas hacia la segunda antes de llegar al detector (*Marriot et al., 2003*). Este dispositivo puede automatizarse, pero tiene la desventaja de que es necesario duplicar la instrumentación y además, sólo un pequeño número de fracciones pueden ser desviadas a la segunda columna en cada análisis. La CGMD se ha usado en el análisis de vinos para el control de la calidad y autenticidad de los mismos (*Herraiz et al., 1990*), identificación de nuevos compuestos aromáticos (*Campo et al., 2007; Darriet el al., 2002*) o para cuantificar odorantes presentes en concentraciones a nivel de trazas (*Martín y Etiévant, 1991*). Esta técnica todavía no se ha aplicado en vinagres.

En los últimos años, la capacidad de separación de analitos se ha mejorado aún más con la aparición de la llamada "two-dimensional comprehensive" cromatografía de gases (*Liu y Phillips, 1991*), en la que toda la muestra una vez que eluye de la primera columna entra en una segunda de naturaleza diferente. Normalmente la primera columna es de naturaleza apolar y la segunda polar. De este modo, todos los analitos de la muestra se van a someter a dos mecanismos de separación, consiguiéndose una completa separación de los mismos (*Marriot et al., 2003, Mondello et al., 2008*). Esta

técnica ya ha sido aplicada en vinos para la determinación de metoxipiracinas (*Ryan et al., 2005*), aminoácidos (*Mayadunne et al., 2005*) y *trans*-resveratrol (*Shao et al., 2003*).

2.5. CARACTERIZACIÓN AROMÁTICA DE LOS COMPUESTOS. DETECCIÓN OLFATOMÉTRICA.

2.5.1. Percepción del aroma

Para que una sustancia produzca sensación de olor debe alcanzar la pituitaria en cantidad suficiente para desencadenar una respuesta que recoja el cerebro. Esto difícilmente puede lograrse si la sustancia no es relativamente volátil por lo que se puede considerar que las sustancias no volátiles son inodoras y que las sustancias responsables del aroma tienen volatilidad contrastada. Como consecuencia inmediata, el estudio del aroma se ha orientado durante muchos años al conocimiento de la composición química de sus compuestos volátiles.

En general, el aroma de cualquier materia 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*). Este umbral de detección se define como la concentración mínima de la sustancia capaz de ser percibida por la media de la población (*Meilgaard, et al., 1999*). 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 ese compuesto a una concentración dada (*Delahunty, et al., 2006*).

Se puede distinguir entre compuestos que participan (aromas activos) y que no participan (aromas inactivos) en el aroma global de un producto, según el valor de actividad aromática de un compuesto (en inglés, OAV) en una matriz determinada, que se define como la relación entre la concentración de la sustancia y su umbral de

olfacción y se expresa en unidades de aroma (*Zeller et al., 2007; Ferreira et al., 2003*). Se considera que una sustancia no participa en el aroma si su valor de aroma es menor que la unidad, y sí que lo hace cuando dicho valor es mayor a uno, siendo la participación tanto mayor cuanto mayor es el valor de aroma (*Grosh, 2001*).

La aplicación de los conceptos y datos de valores de actividad aromática debe hacerse con precaución ya que, si bien permite juzgar de una manera objetiva la contribución de los distintos compuestos aromáticos, no tiene en cuenta ciertas limitaciones: fundamentalmente no considera los efectos sinérgicos y antagónicos de los odorantes, y no tiene en cuenta la ley psicofísica de percepción o ley de Steven (*Stevens, 1971*). El valor de la actividad aromática supone que la percepción es directamente proporcional a la intensidad del estímulo, y eso no es correcto.

La ley de Steven explica la relación entre la intensidad aromática percibida de un compuesto aromático y su concentración:

$$Y = k\Phi^n$$

donde "Y" es la intensidad del aroma, "k" es la constante de proporcionalidad, "Φ"es la concentración del odorante y "n"es un exponente que indica a la velocidad a la que crece la intensidad cuando aumenta la concentración. Esta función psicométrica (concentración-respuesta) se comporta como una curva sigmoidal cuando se representa la intensidad percibida frente al logaritmo de la concentración (Figura 2.18). Como se puede observar, un compuesto aromático empezará a ser percibido cuando presente una concentración igual a su umbral. Es a partir de este punto cuando la intensidad percibida irá aumentando linealmente conforme aumenta la concentración del compuesto hasta que alcanza una concentración a partir de la cual la intensidad no seguirá aumentando con la concentración debido a la saturación de los receptores olfativos.

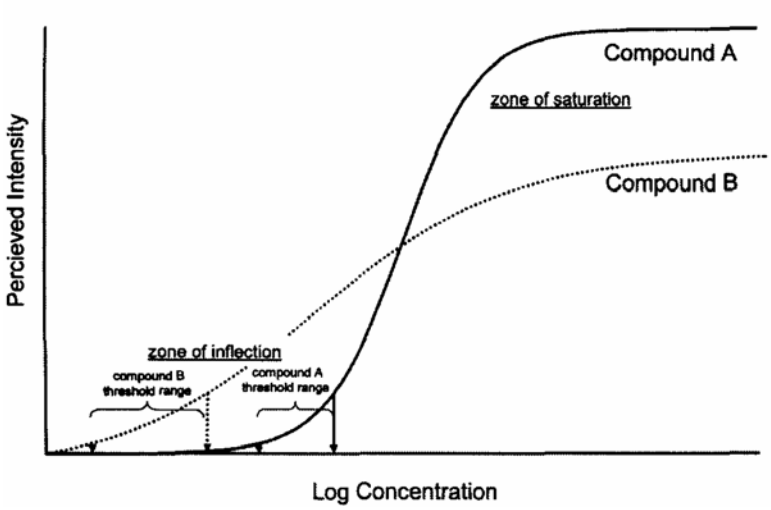


Figura 2.18. *Representación gráfica de la intensidad percibida frente al logaritmo de la concentración de dos compuestos diferentes (Delahunty et al., 2006).*

Por otro lado, si se representa el logaritmo de la intensidad percibida frente al logaritmo de la concentración, el exponente n es la pendiente de la recta e indica cómo se afecta la intensidad percibida cuando aumenta la concentración del estímulo (Figura 2.19). Así, si n < 1, la intensidad del aroma aumentará lentamente con el incremento de concentración; si n = 1, la intensidad será directamente proporcional a la concentración; y si n > 1 la intensidad aumentará más rápidamente con los incrementos de concentración (*Kamadia et al., 2006*).

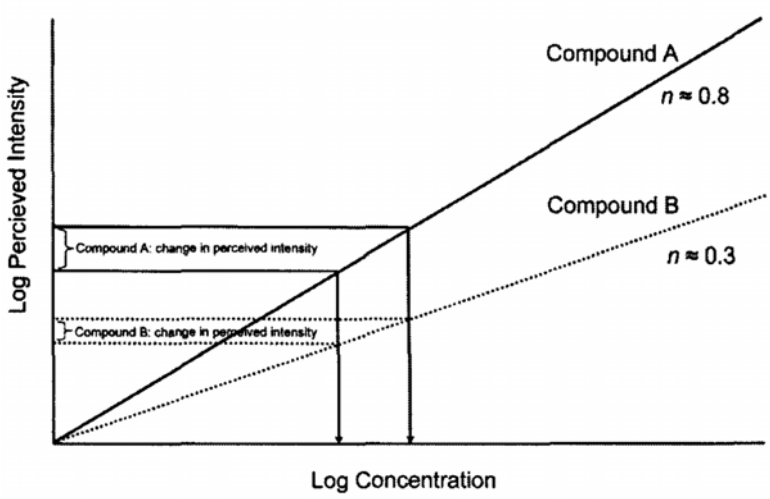


Figura 2.19. *Representación gráfica del logaritmo de la intensidad percibida frente al logaritmo de la concentración de dos compuestos diferentes (Delahunty et al., 2006).*

2.5.2. Cromatografía de gases-olfatometría (CG-O)

La cromatografía de gases acoplada a la detección olfatométrica o "sniffing" (CGO) es la técnica que usa la nariz humana para detectar y evaluar los compuestos volátiles que van eluyendo 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 puede estar conectado en paralelo a detectores convencionales como el detector de ionización de llama (FID) o el espectrómetro de masas (EM) (*Plutowska et al., 2008*), tal como se muestra en la siguiente figura:

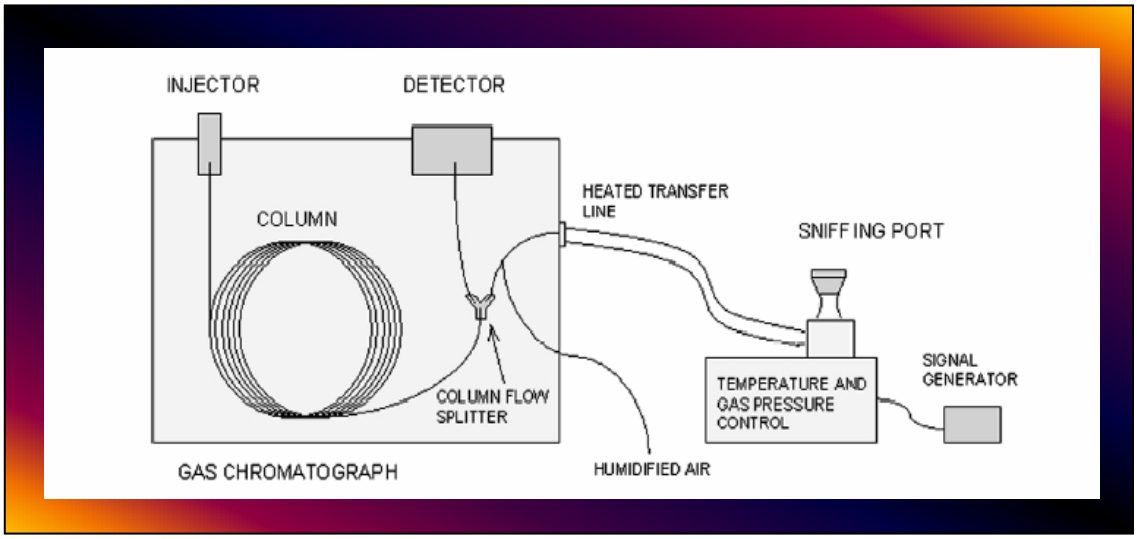


Figura 2.20. *Esquema de un cromatógrafo de gases equipado con un detector olfatométrico.*

Inicialmente, la olfatometría se describió como un método de "cribado" para determinar si un compuesto presente en una muestra tenía actividad aromática o no (*Blank, 1992*). Sin embargo, las aplicaciones de la técnica han ido avanzando, y ahora es común usarla para asignar la importancia relativa de los compuestos volátiles identificados como "aromas activos".

En los últimos años la olfatometría ha sido muy utilizada en el análisis de alimentos y bebidas como la cerveza (*Lermusiau y Collin, 2003; Soares da Costa et al., 2004*), vinos (*Campo et al., 2005; Ferreira et al., 2001*) o whiskies (*Wanikawa et al., 2002*), con el propósito de determinar la relación entre la composición y el contenido en compuestos volátiles y las propiedades organolépticas de dichos productos, así como conocer sus correspondientes perfiles aromáticos (*Plutowska et al., 2008*). Además, la olfatometría también se ha empleado para la identificación de odorantes responsables de aromas desagradables o de posibles contaminaciones (*Darriet et al., 2002; Grosh et al., 1994*).

Por otro lado, esta técnica ha tenido numerosas aplicaciones en el análisis de fragancias y aceites esenciales (*Lan Phi et al., 2006*) y además, varios autores (*Marin et al., 1988*) la han empleado para estudiar la sensibilidad humana a ciertos compuestos ya que la olfatometría permite medir los umbrales y las funciones psicométricas de un amplio número de compuestos.

En vinagres, esta técnica ha sido muy poco utilizada y solamente se ha aplicado a la identificación de compuestos odorantes en vinagres de vino tinto (*Charles et al., 2000*), por lo que puede ser muy interesante ampliar su aplicación en diferentes tipos de vinagres.

Hay una serie de factores que determinan la calidad de los datos obtenidos por olfatometría (*Delahunty et al., 2006*):

- El método usado para la extraer los compuestos volátiles de la muestra de interés determinará la composición del extracto que se analiza por CGO, el cual puede no ser representativo de la muestra en muchos casos.
- Las condiciones cromatográficas del método de análisis determinarán la calidad de la cromatografía, la cual influye en la respuesta del detector humano.
- El comportamiento del detector humano es bastante complejo, y la mayoría de las veces no se tiene en cuenta.

Como cualquier técnica de análisis, la olfatometría presenta ventajas y ciertas limitaciones, tal como se muestra en la tabla siguiente:

Tabla 2.5. *Ventajas e inconvenientes de la olfatometría (Delahunty et al., 2006)*

Ventajas	Inconvenientes
La cantidad de odorante que se presenta al asesor puede controlarse con precisión	Falta de sensibilidad para aquellos compuestos en los que el umbral de percepción es menor que el de detección
Los compuestos que eluyen del cromatografo se evalúan individualmente y por tanto no hay que sintetizarlos o extraerlos y purificarlos previamente	Discriminación de compuestos durante la extracción y concentración de la muestra
Se pueden determinar los umbrales de compuestos no identificados	Co-elución de compuestos
Se pueden determinar las funciones psicométricas de compuestos, relacionando sus concentraciones con sus respectivas intensidades	Incapacidad de interpretar los efectos de sinergia y antagonismo entre los diferentes compuestos aromáticos

Empleando una cromatografía de gases tradicional, los compuestos que tienen características químicas similares tienen una alta probabilidad de co-elución (*Delahunty et al., 2006*). Una reciente y eficaz solución para resolver este problema de co-elución de compuestos ha sido el empleo de una cromatografía de gases multidimensional acoplada a un detector de olfatometría, la cual ya ha sido aplicada para la determinación de aromas activos en kiwi (*Jordán et al., 2002*), aceite de naranja (*Elston et al., 2005*) y whisky de malta (*Wanikawa et al., 2002*).

2.5.3. Técnicas olfatométricas

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 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*). Estas técnicas incluyen un proceso de dilución del extracto con un disolvente. Se realiza la evaluación olfatométrica de cada dilución hasta que los odorantes de interés dejan de ser percibidos. Los panelistas que llevan a cabo el análisis (normalmente 1 ó 2 asesores) anotan cuándo detectan un olor y además dan una descripción del olor, pero no miden la intensidad aromática en ninguna de las concentraciones analizadas.

Dentro de estas técnicas se pueden distinguir el análisis AEDA (acrónimo de "*aroma extract dilution análisis"*) y el análisis CHARM ("c*ombined 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" (FD) (*van Ruth, 2001*). El AEDA ha demostrado ser una técnica muy poderosa para evaluar el impacto de los odorantes que contribuyen en el aroma de diferentes alimentos. Se ha aplicado en diferentes variedades de uva (*Darriet et al., 2002*; *Lopez et al., 1999*), en diferentes tipos de vino como vinos tintos (*Aznar et al., 2001; Escudero et al., 2004; Ferreira et al., 2001; Ferreira et al., 2002*), vinos blancos (*Guth et al., 1997; Silva Ferreira et al., 2003a*) y vinos de Oporto (*Silva Ferreira et al., 2003b*), así como en cerveza (*Gijs et al., 2002*), té (*Schuh y Schieberle, 2006*), salsa de soja (*Steinhaus y Schieberle, 2007*), cacao en polvo (*Frauendorfer y Schieberle, 2006*) o en anises (*Zeller et al., 2007*).

Por otro lado, en el análisis CHARM se anota la duración del aroma para generar picos cromatográficos. Las áreas de pico se expresan en valores adimensionales llamados valores "Charm", los cuales son proporcionales a la cantidad de analito en el extracto e inversamente proporcional al umbral de detección (*Acree et al., 1984*). 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*). Según la literatura, el análisis CHARM se ha aplicado en cervezas (*Kishimoto et al., 2006*) en leches y en productos lácteos (*Mariaca et al., 1997*).

En general, las técnicas de dilución son capaces de discriminar diferentes muestras y además permiten considerar las modificaciones del aroma que se producen en las diferentes concentraciones, pero tienen el inconveniente de que requieren bastante tiempo para completar el análisis y por consiguiente hay más probabilidad de obtener resultados subjetivos y de menor precisión (*Plutowska et al., 2008*). Además, estas técnicas han sido criticadas por asumir que la intensidad aumenta proporcionalmente con la concentración en todos los compuestos aromáticos de una muestra (*Pet´ka et al., 2005*).

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 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. Esta técnica se ha aplicado tanto en vino tintos (*Cullere et al., 2004*) como en blancos (*Lopez et al., 2003*).

Por otro lado, en las otras dos técnicas (Osme y Finger-Span) un panel formado por unos cuatro panelistas va anotando las intensidades de los compuestos continuamente, es decir, desde que empiezan a salir de la columna hasta que se dejan de percibir. Por este motivo se denominan técnicas de tiempo-intensidad dinámicas. Como resultado se obtiene un "aromagrama" de la muestra, donde la altura de pico coincide con la intensidad máxima del compuesto y la anchura con la duración del estímulo. La técnica Osme fue desarrollada por *McDaniel et al. (1990)* y desde entonces ha sido empleada por diferentes investigadores con muy buenos resultados (*Miranda-López et al., 1992*). Años más tarde, *Etiévant et al., 1999* introdujo la técnica Finger-

Span, la cual también ha sido aplicada con éxito por diferentes autores (*Hanaoka et al., 2000; Bernet et al., 2002*).

En general, el principal inconveniente de las técnicas tiempo-intensidad es el intenso entrenamiento que los panelistas requieren para obtener resultados reproducibles. Sin embargo, una vez que el panel esta entrenado, rápidamente podrá caracterizar los perfiles aromáticos de las muestras con una excelente precisión (*Delahunty et al., 2006*).

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 analizan 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 un 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 tienen ventajas e inconvenientes tal como se observa en la tabla siguiente:

Tabla 2.6. *Ventajas e inconvenientes de las diferentes técnicas olfatométricas*

Técnicas GCO	**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

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3. OBJETIVOS

En la presente memoria se pretende caracterizar química y sensorialmente el aroma de diferentes vinagres de vino: vinagres de Jerez, vinagres de vino tinto y blanco.

Para ello se plantean los siguientes objetivos:

1. Estudio de los cambios en la composición de aminoácidos de vinagres de vino :

    1.1. Puesta a punto y aplicación de un método de determinación de aminoácidos mediante Cromatografía de Líquidos empleando el reactivo 6-Aminoquinolil-N Hidroxisuccimidil Carbamato (AQC) en vinagres de vino.

    1.2. Determinación de aminoácidos durante el proceso de acetificación (cultivo superficial y sumergido).

2. Estudio de los compuestos volátiles durante procesos de acetificación y envejecimiento de vinagres de vino:

    2.1. Puesta a punto de un método de determinación de compuestos volátiles por Cromatografía de Gases acoplada a Espectrometría de Masas empleando la técnica de "Extracción por absorción con barras magnéticas agitadoras en espacio de cabeza"

    2.2. Determinación de los compuestos volátiles durante los procesos de acetificación, tanto por cultivo superficial como sumergido, de vinagres de vino tinto.

    2.3. Determinación de los compuestos volátiles durante el envejecimiento de vinagres de vino en barriles de diferentes maderas.

3. Evaluación de la "calidad sensorial" del vinagre de Jerez:

    3.1. Descripción del perfil aromático de las diferentes categorías de vinagre de Jerez, correlacionando los resultados sensoriales con los datos químicos.

    3.2. Identificación de compuestos volátiles con gran impacto sobre el aroma típico del vinagre de Jerez mediante diferentes técnicas olfatométricas (AEDA y frecuencia de detección) para definir sus respectivas capacidades de discriminación.

Los resultados obtenidos en esta Tesis Doctoral han dado lugar a siete artículos científicos: dos publicados, dos aceptados, dos enviados para su publicación y uno en última fase de redacción. Los dos primeros trabajos versan sobre la validación de dos métodos analíticos: el primero para la determinación de aminoácidos y amonio durante la acetificación de vinagres de vino por cromatografía de líquidos usando el reactivo AQC y el segundo para la determinación de compuestos volátiles por cromatografía de gases acoplada a espectrometría de masas empleando la técnica "Extracción por absorción con barras magnéticas agitadoras en espacio de cabeza". En el tercer y cuatro trabajo se estudia la evolución de la composición volátil de vinagres de vino durante los procesos de acetificación (cultivo sumergido y superficial) y durante el envejecimiento en barricas de diferentes maderas. En el quinto trabajo se propone un método estandarizado para el análisis sensorial del vinagre de vino. Este trabajo se ha incluido en esta Tesis para un mejor entendimiento de los dos últimos trabajos en los que se emplea dicho método sensorial. Finalmente, en los dos últimos trabajos se describe el perfil aromático del vinagre de Jerez, correlacionando los resultados sensoriales con los datos químicos con el posterior propósito de identificar odorantes de gran impacto sobre el aroma típico del vinagre de Jerez mediante diferentes técnicas olfatométricas.

Manuscript 1

Raquel M. Callejón; Wendu Tesfaye; M. Jesús Torija; Albert Mas; Ana M. Troncoso; M. Lourdes Morales.

HPLC DETERMINATION OF AMINO ACIDS WITH AQC DERIVATIZATION IN VINEGARS ALONG SUBMERGED AND SURFACE ACETIFICATIONS AND ITS RELATION TO THE MICROBIOTA.

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ORIGINAL PAPER

HPLC determination of amino acids with AQC derivatization in vinegars along submerged and surface acetifications and its relation to the microbiota

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Abstract The present paper reports the determination of 22 amino acids and ammonium along the acetification process by high-performance liquid chromatography (HPLC), employing 6-aminoquinolyl-*N*-hydrosysuccinimidyl carbamate (AQC) as precolumn derivatization reagent. The method was successfully validated obtaining adequate values to selectivity, response linearity, precision and accuracy, as well as low detection and quantification limits. Its utility for routine analysis of amino acids along acetification has been proved. Three red wine substrates were fermented employing two kinds of acetification methods (surfaced and submerged culture) and the requirements of amino acids and ammonium as nitrogen source by acetic acid bacteria were evaluated. The identification of the acetic acid bacteria at species level was also done. At the beginning, the principal amino acid, in terms of abundance, was proline followed by arginine. A different behavior between both the acetification methods was observed. First of all, the consumption of amino acids is much lower in submerged than in surface acetifications. For surface culture acetification, the most consumed was proline, arginine being the main nitrogen source for submerged acetification systems. On the other hand, it was also observed that the nitrogen requirement of the bacteria is proportional to the time spent in the acetification process.

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Keywords Wine vinegar · Amino acid · AQC · Acetification · Wine · Acetic acid bacteria

Introduction

The conversion of wine into vinegar is achieved by acetic acid bacteria which are placed either on the surface or submerged in the liquid to be acetified [1]. From a technological point of view, there are two well-defined methods for vinegar production: traditional processes and submerged methods. The first one is the so-called surface-culture fermentation, where the acetic acid bacteria is placed on the air–liquid interface in direct contact with atmospheric air (oxygen) and, generally, are slow processes [1, 2]. On the contrary, in the submerged culture system, the acetic acid bacteria is suspended in the acetifying liquid in which a strong aeration is applied to ensure the supply of oxygen, resulting in very quick processes [1, 2].

Acetic acid bacteria are aerobic microorganisms which easily grow in slightly acid media, sugar and alcohol being their energy sources [3]. Bacteria requirement of nitrogen is mainly accomplished by amino acids. Because in the case of vinegar the initial substrate comes from a previous alcoholic fermentation, it is important to ensure enough nitrogen is available [4]. Furthermore, certain stabilization techniques applied to wines such as flocculation or filtration may decrease the amino acid concentration depending on the kinds of must and whether amino acids are found in protein or as free amino acids [5]. Amino acids act as precursors of aroma compounds such as higher alcohols, aldehydes, esters and ketonic acids [6] and also of biogenic amines [7].

The consumption of nitrogen and amino acids along alcoholic fermentation has been studied [8–11], but few

works have been performed on this subject, regarding acetic acid fermentation [5]. In previous works [12], we have followed changes in aroma and polyphenolic compounds along acetification but differences between submerged and surface fermentation with regard to amino acids requirements have not been explored.

The analysis of amino acids in wines and wine vinegars must accomplish the determination of primary and secondary amino acids, such as proline and the exact determination of arginine, precursor of compounds such as ethyl carbamate and urea [13]. Furthermore, adequate sensibility and low time performance are advisable.

The determination of these compounds is usually performed by HPLC with precolum derivatization, since a direct detection yields matrix interferences [14]. Hence, the use of fluorescent derivatives such as *O*-phthalaldehyde (OPA), is needed. Ninhydrin, dansyl choride, dabsyl choride, phenylisothiocyanate (PITC) and 9-fluorenylmethylchloroformate (FMOC-Cl) have also been used as derivatization agents to perform this analysis in wine samples [4, 7, 8, 15, 16]. The two reagents more usually employed in wine and vinegar [4, 17], OPA and Dansyl chloride, are not free of substantial drawbacks. In the case of OPA, the main amino acid in grape-derived products, proline does not react in this case [14]. Dansyl chloride derivatives are photosensible and the remaining reagent may cause interferences [14, 18].

We have selected AQC (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) as derivatization agent since it has been successfully used for the determination of amino acids in wine. It reacts with all the amino acids giving stable fluorescent compounds and the excess is hydrolyzed during the derivatization reaction, avoiding interferences [19].

The aim of this study is to use AQC as derivatization agent for the determination of amino acids in wine vinegars and along the acetification process and to explore the different requirements of acetic acid bacteria towards these compounds, also the different pattern of amino acid consumption in surface and submerged methods is reported.

Materials and methods

Reagents and standards

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

Samples

Three submerged acetifications were carried out at laboratory scale fermentor (5 L capacity) and eight surface acetifications in barrels (60 L capacity) of different woods were followed. Three different red wines were employed as raw material in this study; their characteristics are shown in Table 1. Samples were taken at the beginning and at the end of the acetification [the end point was established when an acetic degree (g acetic acid/100 mL vinegar) of 6–7 was reached]. A total of 22 samples have been analyzed along this study.

For validation of the analytical method, sherry wine vinegar and red wine substrate were employed.

Laboratory acetifications

A laboratory scale fermentor (B. Braun Biotech, SA.) was used to produce wine vinegar by a submerged culture acetic fermentation. It is equipped with a cylindrical concave bottom glass culture vessel of 5 L capacity with a height-to-diameter ratio of 2:1; an air supply system with air filters and inlet pipe with sparger ring; a refrigeration system (Frigomix® cooling unit, Sartorius, Goettingen, Germany) with cold water to prevent loss of volatile components; electrical heater jacket 230 V; stirrer with 6-bladed disc impellers; Pt-100 pH-electrode, pO_2-electrode; sensor for temperature measurement Pt-100; measurement and control system micro-DCU 300; stirrer speed control MCU-200 and dosing pump-300.

Optimum conditions for the efficient elaboration of vinegar samples were air flow 150 L h^{-1}, temperature 30 °C, stirring speed 450 rpm, working volume 3.4 L and loading proportion of 1:1 (wine:vinegar) which results in discontinuous cycles with an average duration of 33 h [20].

Derivatization and HPLC analysis of standards and samples

Prior to the derivatization reaction, a standard solution containing 22 amino acids and ammonium (solution 1) was prepared as follows: 125 µL of standard solution or diluted samples (25% with Milli-Q water) and 50 µL of internal

Table 1 Characteristics and codes of wine substrates and vinegar samples

Acetification method	Wine substrate	Cycle or kind of wood	Sampling point	
			Initial	Final
Submerged culture (laboratory fermentor) L	*Substrate K:*Alcohol (%v/v): 15.2 Acidity: 0.6 Glucose + Fructose: 28.76 g L^{-1}+ 61.99 g L^{-1} pH 3.4 Variety: 100% Grenache	1	LK1I	LK1F
		2	LK2I	LK2F
		3	LK3I	LK3F
Surface culture (wooden barrel) B	*Substrate G:* Alcohol (%v/v): 14.5 Acidity: 0.9 Glucose + Fructose: 20.9 g L^{-1}+ 43.4 g L^{-1} pH 3.4 Variety: 100% Grenache	Acacia (A)	BGAI	BGAF
		Chestnut (N)	BGNI	BGNF
		Cherry (C)	BGCI	BGCF
		Oak (O)	BGOI	BGOF
	Substrate H: Alcohol (%v/v): 13.6 Acidity: 0.9 Glucose + Fructose: 0.363 g L^{-1}+ 0.782 g L^{-1} pH 3.3 Variety: Grenache mostly	Acacia (A)	BHAI	BHAF
		Chestnut (N)	BHNI	BHNF
		Cherry (C)	BHCI	BHCF
		Oak (O)	BHOI	BHOF

standard (α-aminobutyric acid 64.45 mg L^{-1}) were mixed and Milli-Q water to a final volume of 2,500 µL was added. Then, 20 µL of this solution was derivatized by "AccQ-Fluor" kit according to the kit's instructions.

HPLC analysis was carried out in a Waters equipment consisting of an autosampler injector Waters 717, a Waters 600E system controller connected to a fluorescence detector, Waters 474. Data treatment was performed in a Waters Millennium[32] data station. The column was a Luna C18, 5 µm, 250 × 4.6 mm and guard column 4.0 × 3.0 mm from Analytical Phenomenex, (Torrance, CA, USA). Detection was carried out by fluorescence with excitation at 250 nm and emission at 395 nm. The injection volume was 20 µL and the separation was obtained at a flow rate of 1 mL min^{-1} at 34 °C with a quaternary gradient program showed in Table 2. The quaternary gradient program employed by Hernández-Orte et al. [19] was modified for the case of vinegar with the purpose of improving resolution in initial and final peaks (Fig. 1).

Microbiological analysis

Identification of the acetic acid bacteria at species level was done in samples described in Table 1. The identification at species level was done by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified fragment of 16S rDNA [21].

Statistical analysis

One-way analysis of variance (ANOVA) was used to test significant differences (significance levels $P < 0.05$).The normality of data was previously verified by Kolmogorov–

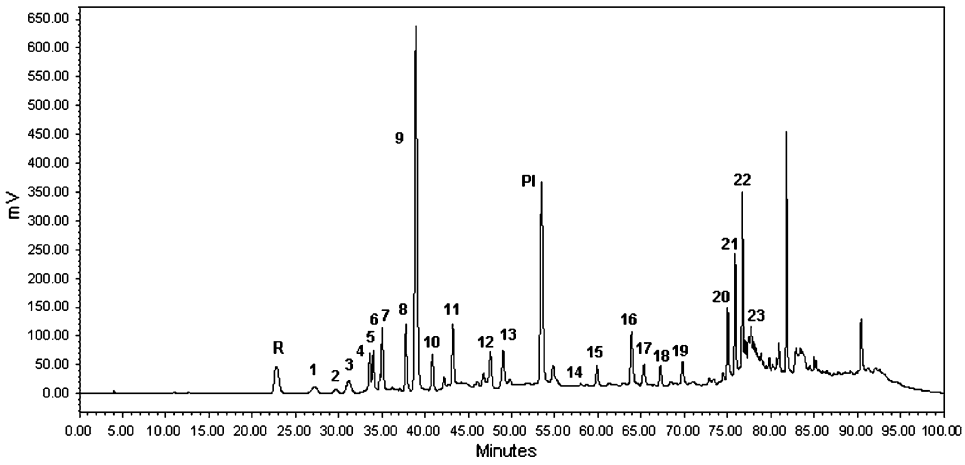

Fig. 1 Chromatogram of standard solution at method optimum conditions. *R* AMQ (hydrolysis product of reactive); *1* Asp; *2* Asn; *3* Ser; *4* Glu; *5* His; *6* Gln; *7* Gly; *8* Arg; *9* Ammonium; *10* Thr; *11* Ala; *12* Pro; *13* GABA; *14* Cys; *15* Tyr; *16* Val; *17* Met; *18* Orn; *19* Lys; *20* Ileu; *21* Leu; *22* Phe; *23* Trp

Table 2 Quaternary gradient program

Time (min)	A (%)	B (%)	C (%)	D (%)
0	0	0	0	100
20	0	0	0	100
22	0	0	100	0
25	0	31	69	0
28	0	34	66	0
44	0	57	43	0
64	0	100	0	0
84	100	0	0	0
94	0	0	0	100

Mobile phases: *A* acetonitrile:water (60:40); *B* sodium acetate pH 5.15:acetronitrile (80:20); *C* sodium acetate pH 5.15:acetronitrile (96:4); *D* sodium acetate pH 4.92:acetronitrile (96:4)

Smirnov test. When variables did not fit normal distribution, the Mann–Whitney test was chosen instead of ANOVA. All statistical analyses were performed by means of Statistica software [22].

Results and discussion

Method validation

The validation parameters studied were selectivity, response linearity, sensitivity (detection and quantification limits), precision (repeatability and intermediate precision) and accuracy (recovery studies).

The selectivity was tested using a vinegar sample and a wine substrate and the selectivity criterion was that resolution between adjacent peaks (*R*) was at least 1.5. In the wine substrate *R* values less than 1.5 were obtained for the following peak pairs: Ser-Glu (1.2), Glu-His (1.1) and NH_4^+-Thr (1.1). For the vinegar sample, *R* values less than 1.5 were obtained for the peak pairs: Glu-His (1.3) and Arg-NH_4^+(1.2). Therefore, acceptable peak resolutions for most of amino acids were achieved.

Linearity over the working range of concentration was verified by regression analyses of the relative peak area as response versus concentration. Table 3 reports regression analysis correlation coefficients (r^2). Figures for all the analytes were above 0.992 showing a linear relation between

Table 3 Results of regression analysis of calibration straight lines, LOD and LOQ

Compound	Maximum concentration of linear range (μg L^{-1})	Intercept	Slope	r^2	LOD (μg L^{-1})	LOQ (μg L^{-1})
Asp	1664	−0.003 ± 0.007	0.00026 ± 0.00001	0.995	25.15	53.99
Asn	1652	0.04 ± 0.01	0.00033 ± 0.00001	0.993	16.5	33.00
Ser	1314	0.07 ± 0.01	0.00041 ± 0.00002	0.992	14.0	28.52
Glu	1839	0.009 ± 0.004	0.000346 ± 0.000005	0.999	13.91	35.84
His	1940	0.033 ± 0.008	0.00053 ± 0.00001	0.997	35.1	70.05
Gln	1827	0.017 ± 0.007	0.00032 ± 0.00001	0.996	26.14	52.27
Gly	938	0.021 ± 0.009	0.00090 ± 0.00002	0.998	6.42	14.74
Arg	2178	−0.001 ± 0.005	0.000464 ± 0.000005	0.999	45.73	62.05
NH_4^+	113	0.757 ± 0.006	0.0035 ± 0.0001	0.997	5.07	16.90
Thr	1489	0.013 ± 0.007	0.00064 ± 0.00001	0.999	0.92	2.11
Ala	1114	0.019 ± 0.006	0.00081 ± 0.00001	0.999	0.20	9.61
Pro	1439	−0.001 ± 0.002	0.000273 ± 0.000003	0.999	36.61	64.40
GABA	1289	0.011 ± 0.009	0.00065 ± 0.00002	0.996	10.21	21.87
Cys	1515	0.0014 ± 0.0008	0.000055 ± 0.000001	0.998	17.4	34.8
Tyr	2265	0.006 ± 0.006	0.000342 ± 0.000005	0.999	33.81	55.96
Val	1464	0.01 ± 0.01	0.00094 ± 0.00002	0.998	17.82	25.92
Met	1865	0.002 ± 0.007	0.00056 ± 0.00001	0.999	26.11	39.75
Orn	1652	0.023 ± 0.006	0.00038 ± 0.00001	0.998	24.24	60.59
Lys	1827	0.0104 ± 0.0003	0.000256 ± 0.000004	0.999	14.35	43.86
Ileu	1640	0.02 ± 0.01	0.001115 ± 0.000002	0.998	7.53	14.35
Leu	1148	−0.01 ± 0.02	0.00127 ± 0.00004	0.995	16.39	31.55
Phe	826	0.02 ± 0.01	0.00132 ± 0.00003	0.996	32.12	83.51
Trp	2553	0.002 ± 0.003	0.000044 ± 0.000002	0.994	110.37	280.78

LOD Limit of detection = 3 signal/noise; *LOQ* Limit of quantification = 10 signal/noise

the standards concentration and the detector response. Moreover, the significance of these correlations was verified by *t* test as Miller and Miller proposed [23]. The results showed that all correlation coefficients were significant for a *P*-value of 0.01.

The limits of detection and quantification were established as the concentration of compound whose peak area gave a signal-to-noise ratio of 3 and 10, respectively. The values obtained (Table 3) suggest that the analytical method is sensitive enough except for tryptophan.

The method is precision was evaluated for the derivatization process, as well as the HPLC method, repeatability and intermediate precision, expressed as relative standard deviation of concentrations (RSD), were calculated employing a vinegar sample diluted with water (15% (v/v)) and spiked with 250 μg $L^{-1}$of each standard. The repeatability of the method was studied from five successive injections of derivatized vinegar and the relative standard deviation ranged from 0.2 for arginine and ammonium to 11.6 for asparagine (Table 4). The study of intermediate precision was performed along a period of 7 days, in which the derivatized vinegar sample was injected in duplicates at five working sessions. The values obtained ranged from 0.3 for leucine to 13.9 for asparagine (Table 4). The repeatability of the derivatization process was tested by means of five derivatization reactions carried out along a working day and intermediate precision employing five derivatization reaction performed over a period of 7 days. The results for repeatability ranged from 0.1 for ammonium to 12.5 for phenylananine (Table 5). The intermediate precision yielded values ranging from 0.1 for ammonium to 12.8 for glycine (Table 5). These results are in agreement with the values proposed by AOAC [24], since the acceptable values of relative standard deviation are 15 and 11% for concentrations of analytes of 100 and 1,000 μg L^{-1}, respectively. Thus, both the processes showed a good precision.

To carry out the recovery studies, a vinegar sample was diluted with water (15% (v/v)) and spiked at three different concentration levels (Table 6). A good degree of accuracy was achieved for most of the compounds, reaching recovery

Table 4 Repeatability and intermediate precision of the analytical method

Compound	Repeatability ($n = 5$)		Intermediate precision ($n = 5$)	
	Mean (μg L^{-1})	RSD[a] (%)	Mean (μg L^{-1})	RSD[a] (%)
Asp	350.1	3.2	402.1	8.3
Asn	142.3	11.6	142.3	13.9
Ser	207.4	1.5	294.1	0.9
Glu	522.0	1.4	532.3	3.3
His	297.8	2.6	283.3	1.4
Gln	270.8	1.8	281.0	0.9
Gly	324.7	0.5	416.9	3.2
Arg	545.6	0.2	526.0	2.4
NH_4^+	222.2	0.2	178.1	0.8
Thr	311.0	2.8	472.9	0.9
Ala	395.3	1.2	467.4	2.3
Pro	639.8	1.3	691.8	2.6
GABA	333.6	6.9	297.1	1.2
Cys	132.0	5.6	188.2	4.8
Tyr	330.0	2.8	375.7	2.4
Val	306.4	2.1	325.7	0.9
Met	240.2	2.8	225.2	3.5
Orn	260.7	0.6	292.2	1.1
Lys	392.3	1.9	361.7	3.3
Ileu	262.7	1.5	266.8	0.6
Leu	315.8	1.7	320.7	0.3
Phe	349.3	4.4	363.9	1.5
Trp	172.1	1.7	170.4	1.1

[a] Relative standard deviation

Table 5 Repeatability and intermediate precision of the derivatization process

Compound	Repeatability ($n = 5$)		Intermediate precision ($n = 5$)	
	Mean (μg L^{-1})	RSD[a] (%)	Mean (μg L^{-1})	RSD[a] (%)
Asp	343.5	0.3	316.0	1.8
Asn	154.1	8.9	145.2	5.3
Ser	217.4	0.6	193.1	2.2
Glu	504.8	0.5	478.1	9.8
His	255.7	3.4	249.3	7.0
Gln	225.0	0.4	212.5	2.1
Gly	307.9	4.9	328.2	12.8
Arg	515.3	0.2	507.0	5.2
NH_4^+	225.6	0.1	225.6	0.1
Thr	313.7	1.7	307.4	5.8
Ala	380.5	1.7	410.8	7.4
Pro	629.8	4.8	653.9	1.4
GABA	368.8	0.6	338.0	0.4
Cys	193.8	8.1	160.5	5.6
Tyr	377.0	2.7	390.7	8.1
Val	378.9	6.8	320.8	0.8
Met	282.7	2.7	278.4	3.3
Orn	267.0	0.7	293.4	8.1
Lys	349.6	1.4	362.7	4.6
Ileu	275.3	2.0	280.8	4.7
Leu	320.2	0.4	322.7	2.4
Phe	324.6	12.5	360.7	1.7
Trp	151.1	6.9	155.2	6.9

[a] Relative standard deviation

Table 6 Results of recovery studies

Compound	Added ($\mu g L^{-1}$)	Recovery (%)	Mean recovery (%)	Analyte	Added ($\mu g\ L^{-1}$)	Recovery (%)	Mean recovery (%)	Analyte	Added ($\mu g\ L^{-1}$)	Recovery (%)	Mean recovery (%)
Asp	250	77.5	76.0 ± 2.2	NH_4^+	250	82.74	87 ± 3	Met	250	91.7	91.3 ± 0.4
	300	73.5			300	86.7			300	91.3	
	350	77.04			350	90.2			350	90.9	
Asn	250	92.2	90 ± 3	Thr	250	92.6	90.1 ± 2.4	Orn	250	69.7	80 ± 9
	300	86.7			300	87.8			300	83.1	
	350	91.5			350	89.7			350	86.0	
Ser	250	52.9	64 ± 10	Ala	250	91.1	87 ± 8	Lys	250	84.5	87 ± 3
	300	68.5			300	78.1			300	85.9	
	350	71.6			350	92.1			350	90.2	
Glu	250	89.7	89 ± 3	Pro	250	86.4	83 ± 8	Ileu	250	88.1	87.6 ± 1.1
	300	84.8			300	73.8			300	86.3	
	350	90.9			350	87.8			350	88.2	
His	250	84.5	88.5 ± 1.2	GABA	250	106.9	106.3 ± 0.9	Leu	250	78.1	82 ± 3
	300	82.4			300	105.3			300	82.5	
	350	82.3			350	106.6			350	84.7	
Gln	250	103.5	102.7 ± 0.7	Cys	250	52.8	53 ± 7	Phe	250	86.2	91 ± 4
	300	102.1			300	46.2			300	92.2	
	350	102.5			350	59.9			350	94.5	
Gly	250	79.0	80.3 ± 1.3	Tyr	250	90.9	91.9 ± 1.4	Trp	250	68.8	80 ± 10
	300	80.2			300	91.4			300	88.3	
	350	81.6			350	93.5			350	82.8	
Arg	250	85.0	81.2 ± 5	Val	250	89.1	86.5 ± 2.3				
	300	75.3			300	85.8					
	350	83.4			350	84.8					

percentages up to 80%, being higher than the acceptable values for AOAC (recovery value of 80% for 1000 $\mu g\ L^{-1}$) [24]. Only two amino acids, cysteine and serine showed low recovery values, 53.0 and 64.3% respectively. Nevertheless, several authors employing other derivatization agents have reported that these amino acids are present in low concentrations in wines and vinegars, even at levels not detectable in the case of cysteine [4, 19, 25].

Sample analysis

The derivatives of amino acid formed by AQC agent are highly fluorescent, hence the samples have to be diluted before the derivatization reaction. A study of sample dilution was carried out, a wine substrate and a vinegar sample were diluted at 5, 15, 25, 35 and 50% with water. Linearity was maintained in this range of dilution except for phenylalanine and tryptophan whose responses were linear between 15 and 50%. The regression coefficients were higher than 0.980 except for ammonium (0.809). In the case of wine substrates, the linear range of dilution was again between 15 and 50%, and most of the regression coefficients were above 0.920, except for histidine (0.874). Aspartic acid ($r^2 = 0.991$) and ammonium ($r^2 = 0.877$) showed linear responses between 25 and 50%. Hence, a dilution of 25% was chosen as the most adequate for vinegar as well as wine.

Available nitrogen consumption

Available nitrogen is calculated as the sum of that supplied by free amino acids and ammonium. Regarding surface acetification, mean initial nitrogen amounts ranged from 161.7 to 96.0 mg L^{-1} (H and G, respectively) (Tables 8, 9). We observed higher decreases for wine substrate H (41%) as compared to substrate G (27.3%). These considerable difference can be attributed to the unequal time spent in the process, since the acetifications of substrate H lasted around 5–9 months and substrate G around 1.5–2.5 months. For the laboratory submerged culture experience, the mean decreases (along 3 cycles) for available nitrogen was 2.9% (Table 7). Again, a less time-consuming process demands lower nitrogen (Fig. 2). However, the nitrogen consumption difference between submerged and surface acetifica-

Table 7 Concentrations of amino acids and ammonium during submerged culture acetification of substrate K

Compound	Mean concentration (mg L^{-1}) ± standard deviation					
	LK1I	LK1F	LK2I	LK2F	LK3I	LK3F
Asp	$3.63^{a} \pm 0.24$	$3.30^{a} \pm 0.04$	$3.54^{a} \pm 0.09$	$3.45^{a} \pm 0.24$	$3.00^{a} \pm 0.18$	$3.12^{a} \pm 0.20$
Asn	ND	ND	ND	ND	ND	ND
Ser	ND	ND	ND	ND	ND	ND
Glu	$13.4^{a} \pm 0.8$	$12.1^{a} \pm 0.3$	$12.6^{a} \pm 0.5$	$10.2^{b} \pm 0.3$	$9.66^{a} \pm 0.05$	$8.30^{a} \pm 0.60$
His	$4.18^{a} \pm 0.18$	$4.00^{a} \pm 0.80$	$4.60^{a} \pm 0.30$	$4.20^{a} \pm 0.40$	$0.67^{a} \pm 0.04$	$2.00^{a} \pm 0.30$
Gln	ND	ND	ND	ND	ND	ND
Gly	$6.69^{a} \pm 0.12$	$6.24^{a} \pm 0.16$	$7.30^{a} \pm 0.07$	$5.82^{b} \pm 0.23$	$6.40^{a} \pm 0.70$	$5.11^{a} \pm 0.05$
Arg	$12.0^{a} \pm 0.9$	$6.00^{b} \pm 0.60$	$13.8^{a} \pm 0.1$	$11.9^{b} \pm 0.5$	$15.0^{a} \pm 0.0$	$8.64^{b} \pm 0.01$
NH_4^+	ND	ND	ND	ND	ND	ND
Thr	$4.63^{a} \pm 0.16$	$4.00^{a} \pm 0.50$	$4.91^{a} \pm 0.05$	$4.47^{b} \pm 0.09$	$4.32^{a} \pm 0.03$	$3.41^{b} \pm 0.07$
Ala	$9.17^{a} \pm 0.22$	$7.70^{a} \pm 0.50$	$9.81^{a} \pm 0.21$	$5.57^{b} \pm 0.35$	$9.31^{a} \pm 0.19$	$5.14^{b} \pm 0.04$
Pro	$281^{a} \pm 8$	$295^{a} \pm 2$	$324^{a} \pm 0$	$324^{a} \pm 7$	$277^{a} \pm 1$	$290^{b} \pm 2$
GABA	$4.60^{a} \pm 0.60$	$5.79^{a} \pm 0.16$	$6.64^{a} \pm 0.04$	$6.20^{a} \pm 0.40$	$5.29^{a} \pm 0.03$	$5.91^{b} \pm 0.05$
Cys	ND	ND	ND	ND	ND	ND
Tyr	$6.13^{a} \pm 0.05$	$7.60^{a} \pm 1.10$	$9.42^{a} \pm 0.06$	$9.40^{a} \pm 1.00$	$8.50^{a} \pm 0.60$	$6.74^{a} \pm 0.04$
Val	$5.00^{a} \pm 3.00$	$2.44^{a} \pm 0.17$	$3.22^{a} \pm 0.06$	$2.50^{a} \pm 0.40$	$3.21^{a} \pm 0.00$	$2.36^{b} \pm 0.01$
Met	$0.91^{a} \pm 0.13$	$0.27^{b} \pm 0.09$	$0.59^{a} \pm 0.09$	$0.35^{a} \pm 0.01$	$0.58^{a} \pm 0.08$	$0.17^{b} \pm 0.04$
Orn	ND	ND	ND	ND	ND	ND
Lys	$9.17^{a} \pm 0.09$	$11.7^{b} \pm 0.7$	$14.7^{a} \pm 0.1$	$14.4^{a} \pm 0.4$	$12.7^{a} \pm 0.2$	$11.9^{a} \pm 0.3$
Ileu	$1.89^{a} \pm 0.07$	$2.15^{b} \pm 0.00$	$2.35^{a} \pm 0.04$	$1.95^{a} \pm 0.15$	$2.01^{a} \pm 0.01$	$1.75^{b} \pm 0.04$
Leu	$7.90^{a} \pm 0.50$	$7.12^{a} \pm 0.18$	$7.63^{a} \pm 0.12$	$6.59^{b} \pm 0.21$	$7.28^{a} \pm 0.14$	$6.43^{b} \pm 0.10$
Phe	$8.60^{a} \pm 0.40$	$9.20^{a} \pm 0.30$	$9.67^{a} \pm 0.03$	$9.36^{a} \pm 0.22$	$9.97^{a} \pm 0.08$	$10.0^{a} \pm 0.4$
Trp	ND	ND	ND	ND	ND	ND
Available Nitrogen	$49.4^{a} \pm 0.8$	$48.9^{a} \pm 0.7$	$56.9^{a} \pm 0.1$	$54.6^{a} \pm 0.7$	$49.2^{a} \pm 0.3$	$47.5^{a} \pm 1.0$

[a, b] Initial and final concentration within a acetification process with different letter as superscript are significantly different ($P = 0.05$)

ND not detected

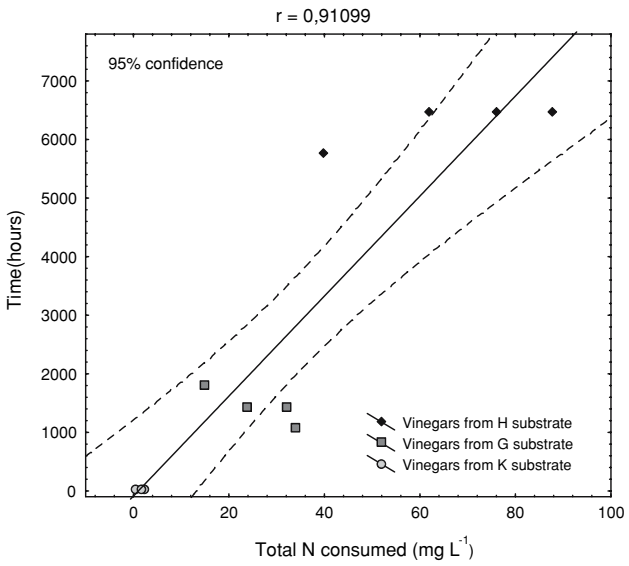

Fig. 2 Correlation between available nitrogen consumption and acetification length

tions can also be due to the differences in biomass growth and the bacteria secondary metabolism, which are more active in the surface acetifications.

When ANOVA was applied, all the decreases of available nitrogen concentration were significant except for the submerged acetification cycles.

Changes in amino acids and ammonium along acetification

At the beginning of acetification, the major amino acid was proline, as it is expected for a grape-derived product [6, 13]. Yeasts have a great difficulty to metabolize proline during alcoholic fermentation [19] due to the need for oxygen equivalents by proline oxidase, which catalyzes the first step in its catabolism [26]. Therefore, some authors [27] have studied the effect of the addition of different amino acids and ammonium concentrations on yeast nitrogen metabolism during alcoholic fermentation, and proline showed to be more consumed in the poorest nitrogen medium. So, yeasts prefer others amino acids, considered

Table 8 Concentrations of amino acids and ammonium during surface culture acetification of substrate G

Compound	Mean concentration (mg L^{-1}) ± standard deviation							
	BGAI	BGAF	BGNI	BGNF	BGCI	BGCF	BGOI	BGOF
Asp	$31.7^{a} \pm 1.4$	$18.5^{b} \pm 1.4$	$35.1^{a} \pm 0.7$	$21.4^{b} \pm 2.0$	$20.4^{a} \pm 1.6$	$17.5^{a} \pm 1.4$	$23.0^{a} \pm 1.5$	$17.5^{a} \pm 1.8$
Asn	ND	ND	ND	ND	ND	ND	ND	ND
Ser	NQ	$7.12^{a} \pm 0.24$	$24.6^{a} \pm 1.7$	$17.9^{a} \pm 0.3$	NQ	$18.0^{a} \pm 0.2$	NQ	$23.3^{a} \pm 0.2$
Glu	$39.4^{a} \pm 0.7$	$40.3^{a} \pm 0.3$	$44.1^{a} \pm .1.1$	$49.0^{a} \pm 3.0$	$28.1^{a} \pm 2.2$	$50.8^{b} \pm 0.6$	$28.0^{a} \pm 3.0$	$42.1^{b} \pm 1.6$
His	$14.3^{a} \pm 0.8$	$14.0^{a} \pm 0.4$	$18.4^{a} \pm 0.7$	$16.1^{a} \pm 1.7$	$16.0^{a} \pm 1.2$	$17.8^{a} \pm 1.6$	$10.7^{a} \pm 0.9$	$17.3^{a} \pm 1.1$
Gln	ND	ND	ND	ND	ND	ND	ND	ND
Gly	$18.5^{a} \pm 1.7$	$13.8^{a} \pm 0.8$	$21.9^{a} \pm 1.1$	$16.6^{a} \pm 1.8$	$14.5^{a} \pm 0.0$	$16.3^{a} \pm 0.3$	$18.1^{a} \pm 1.0$	$19.0^{a} \pm 1.3$
Arg	$61.1^{a} \pm 0.3$	$53.0^{b} \pm 1.0$	$61.0^{a} \pm 6.0$	$55.0^{a} \pm 3.0$	$61.0^{a} \pm 5.0$	$53.4^{a} \pm 0.7$	$54.9^{a} \pm 0.2$	$55.4^{a} \pm 0.7$
NH_4^+	$11.2^{a} \pm 0.9$	$10.6^{a} \pm 1.5$	$13.0^{a} \pm 1.9$	$10.4^{a} \pm 0.6$	$13.8^{a} \pm 0.1$	$9.00^{a} \pm 1.00$	$4.50^{a} \pm 0.50$	$10.3^{a} \pm 0.8$
Thr	$12.8^{a} \pm 0.6$	$10.5^{a} \pm 0.5$	$14.1^{a} \pm 0.6$	$12.3^{a} \pm 0.6$	$9.97^{a} \pm 0.07$	$11.2^{a} \pm 0.7$	$11.7^{a} \pm 0.6$	$12.7^{a} \pm 0.6$
Ala	$33.0^{a} \pm 1.0$	$26.9^{a} \pm 1.8$	$34.0^{a} \pm 0.2$	$28.7^{b} \pm 0.5$	$33.4^{a} \pm 0.6$	$27.5^{a} \pm 2.0$	$24.5^{a} \pm 1.9$	$29.1^{a} \pm 0.8$
Pro	$291^{a} \pm 2$	$94.0^{b} \pm 0.1$	$277^{a} \pm 24$	$71.0^{b} \pm 3.0$	$255^{a} \pm 11$	$68.6^{b} \pm 0.1$	$268^{a} \pm 3$	$44.0^{b} \pm 3.0$
GABA	$11.5^{a} \pm 0.1$	$8.80^{a} \pm 0.06$	ND	NQ	$14.2^{a} \pm 0.0$	$11.3^{a} \pm 0.7$	$12.8^{a} \pm 0.3$	$9.20^{b} \pm 0.30$
Cys	ND	ND	ND	ND	ND	ND	ND	ND
Tyr	$14.6^{a} \pm 0.6$	$12.4^{b} \pm 0.3$	$18.5^{a} \pm 0.3$	$17.5^{a} \pm 0.4$	$10.9^{a} \pm 0.0$	$13.5^{a} \pm 0.7$	$12.0^{a} \pm 0.9$	$16.4^{b} \pm 0.8$
Val	$17.6^{a} \pm 0.8$	$9.10^{b} \pm 0.50$	$9.21^{a} \pm 0.17$	$15.1^{a} \pm 0.8$	$9.04^{a} \pm 0.02$	$11.9^{a} \pm 0.9$	$13.3^{a} \pm 0.9$	$18.9^{b} \pm 0.3$
Met	$2.14^{a} \pm 0.04$	$0.55^{b} \pm 0.06$	$1.49^{a} \pm 0.18$	$0.62^{b} \pm 0.08$	$1.73^{a} \pm 0.03$	$0.50^{b} \pm 0.08$	$1.86^{a} \pm 0.17$	$0.70^{b} + 0.03$
Orn	$5.90^{a} \pm 0.30$	$6.68^{a} \pm 0.00$	$7.79^{a} \pm 0.19$	$6.30^{a} \pm 0.80$	$3.30^{a} \pm 0.50$	$6.92^{a} \pm 0.19$	$5.62^{a} \pm 0.14$	$10.2^{a} \pm 0.3$
Lys	$25.7^{a} \pm 0.5$	$24.8^{a} \pm 0.5$	$25.7^{a} \pm 1.4$	$28.4^{a} \pm 0.7$	$26.6^{a} \pm 1.7$	$25.3^{a} \pm 0.1$	$22.5^{a} \pm 0.5$	$25.0^{b} \pm 0.5$
Ileu	$6.20^{a} \pm 0.30$	$5.01^{b} \pm 0.20$	$6.50^{a} \pm 0.30$	$6.00^{a} \pm 0.50$	$6.20^{a} \pm 0.70$	$5.10^{a} \pm 0.80$	$4.70^{a} \pm 0.30$	$6.10^{b} \pm 0.30$
Leu	$19.0^{a} \pm 0.3$	$14.5^{b} \pm 0.7$	$17.6^{a} \pm 1.0$	$15.6^{a} \pm 1.0$	$14.9^{a} \pm 0.3$	$15.1^{a} \pm 0.5$	$16.0^{a} \pm 0.7$	$16.6^{a} \pm 0.7$
Phe	$18.0^{a} \pm 0.4$	$14.8^{b} \pm 0.8$	$16.0^{a} \pm 1.4$	$16.2^{a} \pm 1.7$	$14.2^{a} \pm 0.1$	$15.9^{a} \pm 1.1$	$14.8^{a} \pm 0.0$	$17.5^{b} \pm 0.9$
Trp	ND	ND	ND	ND	ND	ND	ND	ND
Available Nitrogen	$102^{a} \pm 0$	$68.0^{b} \pm 3.0$	$104^{a} \pm 6$	$72.0^{b} \pm 3.0$	$92.9^{a} \pm 4.7$	$69.0^{b} \pm 3.0$	$85.0^{a} \pm 7.0$	$70.0^{b} \pm 4.0$

[a, b] Initial and final concentrations within a acetification process with different letter as superscript are significantly different ($P = 0.05$)

ND not detected; *NQ* below quantification limit

as good nitrogen sources, instead of proline. Its concentration ranged from 290 mg L^{-1} for substrate G to 750 mg L^{-1} for H (Tables 8, 9). Thus, between 35 and 69% of available nitrogen is supplied by proline. Nevertheless, the consumption of proline by acetic acid bacteria was only important in surface acetifications, accounting 200–300 mg L^{-1}. In the submerged experience, proline concentration remained constant (Table 7). Since Morales et al. [12] observed an uptake of proline during the acetification of sherry wine in a similar experiment; our results may be due to different strains of acetic bacteria involved in process. The fact that proline generally used by acetic acid bacteria was not taken in the submerged acetification can be due to the strain and species diversity, as well as the needs in nitrogen by the acetic acid bacteria in both the acetifications. The surface acetifications were mainly carried out by a population of different strains of *Acetobacter pasterianus* species, with sporadic presence of *Gluconacetobacter europaeus* (Table 10). Instead, the stringent conditions imposed in the submerged acetifications led to an imposition of two strains belonging to *Gluconacetobacter europaeus* species. A limitation in the proline utilization by these two strains would explain the accumulation of this amino acid that is not frequent as most of the acetic acid bacteria can use it [4, 28]. Additionally, in the submerged acetification system, amino acid or nitrogen utilization is rather limited as seen by the differences between surface and submerged systems. This could be due to the lack of acetic acid bacteria growth in the submerged systems, where the bacteria act more as a catalyst, instead of the surface systems when active metabolism and growth of bacteria is observed.

The second amino acid in abundance order, arginine (between 8.5 and 26% of available nitrogen), went down significantly during submerged acetification (Table 7). Besides, this compound decreased in most of the surface processes, but only in half of them, these changes were significant (Table 8, 9).

Glutamic acid and alanine were among the five most abundant amino acids and consumed in the submerged acetification (Table 7). Regarding surface acetifications in

Table 9 Concentrations of amino acids and ammonium during surface culture acetification of substrate H

Compound	Mean concentration (mg L^{-1}) ± standard deviation							
	BHAI	BHAF	BHNI	BHNF	BHCI	BHCF	BHOI	BHOF
Asp	31.0[a] ± 1.2	25.4[b] ± 0.3	32.0[a] ± 0.2	23.1[b] ± 1.2	33.0[a] ± 2.3	22.8[b] ± 0.0	33.0[a] ± 1.3	25.7[b] ± 0.5
Asn	NQ	ND	ND	ND	ND	ND	ND	ND
Ser	9.63[a] ± 0.09	16.5[a] ± 0.0	20.4[a] ± 1.5	19.1[a] ± 0.7	19.5[a] ± 0.6	13.4[b] ± 1.0	NQ	NQ
Glu	49.7[a] ± 2.5	54.8[a] ± 0.1	54.4[a] ± 1.8	50.5[a] ± 1.1	45.2[a] ± 1.2	45.7[a] ± 0.2	49.7[a] ± 2.5	62.3[a] ± 0.1
His	ND	ND	ND	NQ	7.05[a] ± 0.19	NQ	ND	NQ
Gln	ND	ND	ND	ND	ND	ND	ND	ND
Gly	25.9[a] ± 0.5	21.0[a] ± 2.0	31.3[a] ± 0.3	19.6[b] ± 1.1	28.6[a] ± 0.4	20.7[b] ± 1.3	29.4[a] ± 0.2	24.7[a] ± 0.5
Arg	79.2[a] ± 0.3	70.8[b] ± 0.2	82.3[a] ± 1.1	60.2[b] ± 0.6	82.5[a] ± 1.9	60.4[b] ± 0.6	88.7[a] ± 2.3	83.2[a] ± 0.2
NH_4^+	24.5[a] ± 0.6	10.8[b] ± 0.6	29.5[a] ± 0.4	5.40[b] ± 0.60	24.4[a] ± 1.7	7.50[b] ± 0.80	31.0[a] ± 0.7	33.0[a] ± 3.0
Thr	12.1[a] ± 0.3	13.6[a] ± 0.8	14.9[a] ± 0.4	12.8[b] ± 0.2	17.0[a] ± 4.0	11.7[b] ± 0.7	14.6[a] ± 0.6	16.1[a] ± 0.6
Ala	54.1[a] ± 0.8	41.6[b] ± 1.5	60.0[a] ± 1.0	29.8[b] ± 0.3	55.5[a] ± 1.9	31.3[b] ± 1.5	59.6[a] ± 2.3	50.0[b] ± 0.3
Pro	729[a] ± 14	394[b] ± 1	756[a] ± 6	357[b] ± 5	742[a] ± 1	388[b] ± 1	819[a] ± 6	479[b] ± 3
GABA	24.8[a] ± 0.1	24.0[a] ± 1.0	26.8[a] ± 0.6	25.5[a] ± 0.2	25.9[a] ± 0.6	22.8[b] ± 0.0	29.4[a] ± 1.2	31.8[a] ± 1.1
Cys	ND	ND	ND	ND	ND	ND	ND	ND
Tyr	11.4[a] ± 0.4	0.93[b] ± 0.02	11.5[a] ± 0.1	1.80[b] ± 0.12	12.4[a] ± 0.0	1.55[b] ± 0.00	14.1[a] ± 0.9	13.0[a] ± 0.2
Val	20.4[a] ± 0.6	3.00[b] ± 0.30	22.4[a] ± 1.2	2.52[b] ± 0.00	22.2[a] ± 0.5	2.57[b] ± 0.10	23.2[a] ± 0.5	18.8[b] ± 0.6
Met	1.36[a] ± 0.00	0.11[b] ± 0.00	1.52[a] ± 0.03	0.68[b] ± 0.01	1.53[a] ± 0.00	0.80[b] ± 0.06	1.83[a] ± 0.13	0.19[b] ± 0.01
Orn	11.2[a] ± 0.4	ND[b]	17.2[a] ± 0.5	ND[b]	13.1[a] ± 0.9	NDd[b]	10.8[a] ± 0.5	12.1[a] ± 0.2
Lys	22.4[a] ± 0.4	6.90[b] ± 1.05	25.3[a] ± 1.5	ND[b]	24.8[a] ± 0.5	ND[b]	26.1[a] ± 0.8	30.9[b] ± 0.1
Ileu	8.08[a] ± 0.07	5.00[b] ± 0.50	6.90[a] ± 0.40	3.58[b] ± 0.08	6.45[a] ± 0.03	3.38[b] ± 0.17	7.50[a] ± 0.80	8.11[a] ± 0.05
Leu	16.4[a] ± 0.4	2.90[b] ± 0.10	16.1[a] ± 0.1	2.20[b] ± 0.09	15.7[a] ± 0.2	2.14[b] ± 0.02	17.7[a] ± 2.0	16.3[a] ± 0.1
Phe	18.6[a] ± 0.4	8.60[b] ± 0.10	14.7[a] ± 0.6	7.50[b] ± 0.40	14.7[a] ± 0.3	7.61[b] ± 0.12	17.6[a] ± 0.1	16.0[a] ± 0.4
Trp	ND	ND	ND	ND	ND	ND	ND	ND
Available Nitrogen	152[a] ± 1	90.4[b] ± 0.8	165[a] ± 2	77.4[b] ± 1.2	158[a] ± 0	81.5[b] ± 1.3	172[a] ± 8	132[b] ± 2

[a, b] Initial and final concentrations within a acetification process with different letter as superscript are significantly different ($P = 0.05$)

ND not detected

NQ below quantification limit

wood, a common pattern was a significant consumption of alanine with discretes glutamic acid increases.

Other difference between both kinds of processes is related to ammonium. It was not possible to quantify this compound in submerged acetification; however, its relative areas seem to decrease along the process. For surface acetifications, ammonium was only used by acetic bacteria as nitrogen source in some cases.

In those acetifications where amonium is consumed, greater decreases were observed in substrate H than in substrate G, suggesting a correlation with the length of time of fermentation.

Although methionine appears in low concentration, it suffers statistically significant decreases in all cases, around four times greater in surface acetification.

In a Frings turbine fermenter employing cider, white and red wines as substrates, leucine has been reported as the most important amino acid in terms of nitrogen supply and uptake [4]. Nevertheless, in none of our experiences is leucine the most consumed amino acid, neither the principal source of available nitrogen, although, it decreases in all of the cases except in two surface acetifications of substrate G.

Finally, asparagine, glutamine, cysteine and tryptophan were not detected along the acetification process in any of the samples studied. Usually during alcoholic fermentation, these amino acids suffer a considerable decrease reaching even non-detectable amounts, as it occurs for cysteine [19], or they are totally consumed as tryptophan [27]. Moreover, neither serine nor ornithine was detected along submerged acetifications.

Conclusions

A nitrogen consumption was observed in both the types of the acetifications studied, being higher when it is carried out by surface culture. Besides, it was observed that the nitrogen requirement of the bacteria is proportional to the

Table 10 Identification of acetic acid bacteria species in the initial and end points of surface and submerged acetifications

Initial	Species identification	Final	Species identification
LK1I	100% *Ga. europaeus*	LK1F	100% *Ga. europaeus*
LK2I	100% *Ga. europaeus*	LK2F	100% *Ga. europaeus*
LK3I	100% *Ga. europaeus*	LK3F	100% *Ga. europaeus*
BGAI	100% *A. pasteurianus*	BGAF	100% *A. pasteurianus*
BGNI	100% *A. pasteurianus*	BGNF	100% *A. pasteurianus*
BGCI	100% *A. pasteurianus*	BGCF	100% *A. pasteurianus*
BGOI	100% *A. pasteurianus*	BGOF	67% *A. pasteurianus* 33% *Ga. europaeus*
BHAI	100% *A. pasteurianus*	BHAF	100% *A. pasteurianus*
BHNI	100% *A. pasteurianus*	BHNF	100% *A. pasteurianus*
BHCI	100% *A. pasteurianus*	BHCF	100% *A. pasteurianus*
BHOI	100% *A. pasteurianus*	BHOF	100% *A. pasteurianus*

time spent in the acetification process. Although a clear difference was also observed between the two processes that can be attributed to the species diversity as well as the process itself, in the initial substrates, the principal amino acid, in terms of abundance, was proline followed by arginine and a different behaviour between both the acetification methods was observed. Hence, the most consumed amino acid in surface acetification was proline and, on the contrary, arginine in the sumerged one. The different amino acid consumption pattern suggests a possible relation to different acetic acid bacteria strains involved in the process.

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Manuscript 2

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OPTIMIZATION AND VALIDATION OF HEADSPACE SORPTIVE EXTRACTION FOR THE ANALYSIS OF VOLATILE COMPOUNDS IN WINE VINEGARS

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OPTIMIZATION AND VALIDATION OF HEADSPACE SORPTIVE EXTRACTION FOR THE ANALYSIS OF VOLATILE COMPOUNDS IN WINE VINEGARS

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ABSTRACT

Quantification of aroma compounds in wine vinegars is challenging due to the complexity of the matrix and the low concentrations expected. A method for the determination of volatile compounds in wine vinegars employing Headspace Sorptive Extraction-Thermal Desorption-Gas Chromatography-Mass Spectrometry (HSSE-TD-GC-MS) was developed. A central composite design was used to optimize the sampling condition. The proposed method was successfully validated and low detection and quantification limits was obtained. The application of the proposed methodology allows the determination of 53 compounds in different wine vinegars (red, Sherry). Five of them have been detected in wine vinegars for the first time.

KEYWORDS: HSSE-TD-GC-MS, vinegar, stir bar, aroma compounds, validation, central composite design.

1. INTRODUCTION

The volatile composition of wine vinegars is quite complex, with many compounds accounting for low concentrations (ppm to ppb). There are a number of methods used to extract and/or concentrate aroma compounds from foods prior to analysis. Nowadays, solvent free extraction techniques are extensively employed, the best known among them are solid phase microextraction (SPME) [1] and stir bar sorptive extraction (SBSE) [2].

SPME using different type of fibre has been widely applied for the study of aroma in wine [3-6] as well as vinegar [7].Two types of SPME techniques can be used to extract analytes: headspace (HS) when the fibre is exposed in the vapour phase above a liquid or solid sample or direct immersion (DI) when the fibre is directly immersed in the liquid sample [8]. The most important parameters affecting the SPME method are: type of fibre employed, extraction temperature and time, salt concentration and sample volume [9]. The competition between analytes and the saturation of these extraction materials are limiting parameters. In SPME the amount of extraction medium is very small, thus, new strategies are being developed to avoid these limiting aspects of adsorbents [10].

Baltussen et al. [2] developed a new extraction technique known as stir bar sorptive extraction (SBSE), based on the polydimethylsiloxane (PDMS) phase as an extraction medium. The extraction mechanism and advantages are similar to those of SPME. In the technique of SBSE, magnetic stirring rods are incorporated in glass jackets and coated with a layer of polydimethylsiloxane phase. These coated stir bars are commercially known as “Twister”. Several different type of Twisters are available depend on the length (10-20 mm) and phase thickness (0.5-1.0), typically, the 10 mm stir bar are used for 1-50 mL sample volumes. SBSE has shown a much higher sensitivity than SPME by a factor within 100 and 1000 due to the higher content of PDMS (50-300μL). The amount of analyte extracted is proportional to the coating thickness thus increasing the limit of detection of ultra trace compounds [11]. Different comparison studies between the use of headspace-SPME and SBSE [2, 12] or immersion-SPME and SBSE [13, 14] showed the higher capability, greater accuracy and sensitivity of SBSE.

SBSE technique has been successfully applied to the analyses of aroma compounds in wine [15-17]. In a recent work a comparison of SBSE and SPME for the analysis of volatile compounds in vinegar was carried out, SBSE technique was capable of determining a higher amount of compounds, showing a better sensitivity and reproducibility values [18].

The headspace sorptive extraction (HSSE) technique introduced by Bicchi et al [19] and Tienpoint et al [20] is an extension of stir bar sorptive extraction (SBSE) for headspace sampling, which results in a high solute concentration capability. In HSSE, a Twister is placed in an open

glass adapter inside a closed headspace vial. Thus, analytes are extracted from the vapour phase above the sample.

In this case HSSE recovery depends on the overall partition coefficient, K. In turn, K depends on the analyte partition coefficient between PDMS stir bar and sample headspace, K_1, and on the partition coefficient between the headspace and the sample matrix, K_2 [21]. HSSE was successfully applied for the analysis of solid and liquid matrices [21-25]. Although in general direct immersion techniques are more sensible, extraction in the vapour phase generally is preferred [10]. This method presents as advantage to reduce the risk of contamination and to increase the stir bar lifetime [25].

Various parameters affecting the extraction kinetics have to be optimized when developing an extraction method. In similar way as occur with SPME, extraction temperature and time, salt concentration and sample volume are features (or experimental parameters) influencing the analyte equilibrium between the sample and the fibre. These factors have to be optimized to obtain the best extraction condition.

Many independent parameters such as the nature of extraction material, physicochemical characteristics of the matrix (pH, salt content, temperature, etc.), and time can affect the extraction of volatiles and it is likely that the operational variables interact and influence each other's effects on the response. Therefore, it is necessary to use an optimization method that can determine all the factors as well as the possible interactions between these independent variables, so that a set of optimal experimental conditions can be determined [26]. Optimization through factorial design and response surface analysis particularly fulfils this requirement [27].

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing process [28]. The main advantage of RSM is to reduce the number of experimental trials needed to evaluate multiple variables and its interactions; it is less laborious and time-consuming than other approaches [29]. In general, it applies an experimental design such as central composite design to fit a second order polynomial by a least squares technique. An equation is used to describe how the test variables affect the response and determine the interrelationship among the variables [27].

RSM has been extensively used to optimize SPME [8, 30, 31] and SBSE [32] conditions for the analysis of volatile compounds in water, milk and vinegar.

The aim of this work is the optimization of a method for the determination of volatile compounds in wine vinegar employing Headspace Sorptive Extraction-Thermal Desorption-Gas Chromatography-Mass Spectrometry (HSSE-TD-GC-MS). A central composite design was used to optimize the sampling condition: sampling time, temperature and sample volume. The method is

then applied to different wine vinegar samples and compared with previous reported methodologies based on SPME and SBSE.

2. EXPERIMENTAL

2.1. Reagents and Chemicals

The standards of 57 aroma compounds were obtained from the commercial sources as follows: 3, 4, 9, 10, 12, 15, 16, 19, 20, 22, 24-27, 29, 31, 33, 34, 36, 38-40, 42-46, 48-56 (Sigma-Aldrich, Madrid, Spain); 1, 2, 5, 6, 13, 14, 17, 18, 21, 23, 32, 47, 57 (Merck, Darmstadt, Germany); 7, 8, 11, 30, 35, 37, 41 (Fluka, Madrid, Spain). 4-methyl-2-pentanol (Merck) was employed as internal standards (IS). Numbers corresponding to different standards are given in tables. Sodium chloride, acetic acid and ethanol were supplied by Merck, and all of them were of analytical quality. Water was obtained from a Milli-Q purification system (Millipore, USA). A hydroaceticalcoholic solution with a mix of different standards was prepared for the optimization of sampling condition (Table 1). Volatile substances for the optimization were selected on the basis of their previously reported presence in different vinegar samples and their different volatilities. For most of the compounds, concentrations were chose taking into account minimum amounts expected according to published data [7, 33-35].

2.2. Samples

A commercial wine vinegar was used to validate the analytical method. 4 red wine vinegars and 3 Sherry wine vinegars were used to test the suitability of the method and perform the comparative study. Red wine vinegars were produced by traditional fermentation (surface culture) methods in oak wood barrels (VRW1-VRW4). Sherry wine vinegars belong to the three commercial types recognised in the Denomination of Origin, named “Vinagre de Jerez”(VJ), “Vinagre de Jerez Reserva” (VJR) and “Vinagre de Jerez Gran Reserva” (VJGR), accounting for 6 months, 12 months and more than two years ageing in wood barrels, respectively. Each sample was analysed by triplicate.

2.3. HSSE sampling

The HSSE of samples were carried out employing special 20 mL headspace vials with open glass adapters provided by Gerstel (Müllheim an der Ruhr, Germany). 33% ca of NaCl was added to the sample. 10 mm long stir bar coated with 0.5 mm polydimethylsiloxane (PDMS) layer (Twister, Gerstel, Müllheim an der Ruhr, Germany) was put into the glass insert. The vial was tightly capped and was heated in a thermostatic bath. When the vial was at room temperature, the stir bar was removed with tweezers, rinsed with Milli-Q water and dried with a lintfree tissue paper.

Finally, the stir bar was put into a glass tube of 60 mm in length, 6 mm o.d. and 4 mm i.d., which was placed in the autosampler tray of the thermo desorption unit for GC-MS analysis.

2.4. HSSE-TD-GC-MS analysis

Gas chromatography analyses were performed with a 6890 Agilent GC system coupled to a quadrupole mass spectrometer Agilent 5975inert and equipped with a Gerstel Thermo Desorption System (TDS2) and a cryo-focusing CIS-4 PTV injector (Gerstel).

The thermal desorption was performed in splitless mode with a flow rate of 90 mL/min. Desorption temperature program was the following: 35 ºC for 1 min, ramped at 60 ºC/min to 250 ºC held for 5 min. The CIS-4PTV injector, with a Tenax TA inlet liner, was held at –35 ºC with liquid nitrogen for total desorption time and then raised at 10 ºC/s to 290 ºC and held for 4 min. Solvent vent mode was employed for transfer of sample to analytical column.

A CPWax-57CB column (50 m x 0.25 mm, 0.20 μm film thickness, Varian, Middelburg, Holland) was used and the carrier gas was He at a flow rate of 1 mL/min. Oven temperature program: 35 ºC for 5 min, then raised to 220 ºC at 2.5 ºC/min (held 5 min). The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 280 respectively. Electron ionization mass spectra in the full-scan mode were recorded at 70 eV electron energy in the range 35 to 350 amu. All data were recorded using a MS ChemStation. The identity of peaks was assigned using the NIST 98 library and confirmed by retention index of standards when they were available. RIs were calculated in HS-SBSE-GC-MS from the retention times of n-alkanes by linear interpolation, according to the literature [36]. Quantification was performed employing the relative area to internal standard of the target ion of each compound. The samples were analysed by triplicate and blank runs of empty glass tube were done before and after each analysis.

2.5. Experimental Design and Statistical Analysis

In order to perform the optimal values for the experimental variables (called factors in the jargon of experimental design), the response surface methodology (RSM) approach was used [37]. In RSM the experimental response (y) is assumed to be a function of the independent variables or factors. They occupy what is called factor space and a plot of y on this space is the response surface. The aim of optimization is the estimation of the factor coordinates that maximize (or minimize) the objective response y. Because two-level factorial designs are insufficient to adjust the response surface function with quadratic terms, central composite designs (CCD) can be used [37]. CCD are special designs whose building consists of three steps for a generic number of factors (f): (i) A two-level full factorial design 2^f (for $f < 5$), (ii) 2f star points positioned on the coordinate axes of factorial space $(\pm\alpha,0,\ldots0)$, $(0, \pm\alpha,\ldots0)$,.., $(0,0,\ldots \pm\alpha)$ where α is the distance from the centre of

the design to the star point, (iii) A number of n_0 extra points at the centre of the design. Thus, the orthogonal or rotatable design. Rotatable CCD have invariant variance-covariance matrices against orthogonal rotation of coordinates and are featured by a star arm given by $\alpha = 2^{f/4}$.

In our case the controllable factors were: sample volume (x_1), temperature (x_2) and extraction time (x_3). A central composite design (CCD) consisting of a 2^3 factorial design with six star points located at ±1.68 (to ensure rotatability) from the centre of the experimental domain was performed. Therefore, each factor had five levels (+α, +1, 0, -1, -α) that are shown in Table 2. The design was also completed with three replicates of the central point (n_0 = 3). Hence, the complete design consisted of seventeen randomly performed experiments. All the experiments were carried out using the factor coding $x := \frac{x - x^0}{r}\alpha$, where $x^0 = \frac{x^{max} + x^{min}}{2}$ and $r = \frac{x^{max} - x^{min}}{2}$ [37].

The response functions (y) were the total area for compound groups (aldehyde, ketones, acids, esters, alcohols and volatile phenols). These values were related to the controlled factors by a second-degree polynomial equation model:

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2$$

For each set of results the regression coefficients of the polynomial were determined and according to a Student t-test, the corresponding coefficient was tested for significance.

3. RESULTS AND DISCUSSION

3.1. Extraction Condition Optimization

To establish the optimum extraction conditions, we considered the main factors that influence the analytes equilibria between the sample, headspace and the fibre. Hence, the studied variables were: sample volume, temperature and time of extraction. The experimental domain was defined taking into account instrumental and operative limits, as well as the results previously obtained by other authors (Table 2). Sample volume range was established considering the maximum capacity of the vial. Temperature is the variable that mostly influences the headspace composition [21], studying volatile compounds in an essential oil, found that sampling at 75 ºC gave results comparable or even slightly lower than those obtained at 50 ºC. This is probably due to the fact that high temperatures can drastically increase the release of analytes from the PDMS fiber to the headspace. For this reason, the maximum temperature in the experimental design was fixed at 50 ºC. In relation to extraction time, we tried not to enlarge it excessively by selecting a time range resulting from a compromise between minimal time of analysis and good sensitivity.

Finally, ionic strength effect by the addition of NaCl and not stirring of sample were fixed factors. The extraction was performed in a thermostatic bath that allows a more uniform heating of the vial. An amount of NaCl was added until saturation, as proposed by Castro et al [8].

Table 3 shows the values obtained when the Student t-test was applied to the results. *p*-values showed that temperature and time of extraction have a statistically significant effect ($p<0.05$) for most of the groups of compounds, being extraction temperature a significant parameter with $p<0.001$ in four cases and extraction time only in two cases. Sample volume was not significant. The interaction between temperature and extraction time was also significant ($p<0.01$) for lactones and volatile phenols. Summarizing: The outcome of Table 3 shows:

i) The sample volume is not significant always.

ii) The response surface is linear because all coefficients b_{ii} are not significant and b_2 and b_3 are positive values.

Accordingly there is neither maximum nor minimum and thus, the "working optimum" could be selected by hand, because the function is monotonically increasing. The response can be modelled by directly setting the factors coordinates.

For aldehydes, alcohols, ketones, esthers, lactones and volatile phenols maxima areas are reached when temperature and time were high. However, acids can not be suitable modelled because all coefficients are not significant. Thus, a suitable working optimal factor conditions were selected as: 62ºCfor temperature, 60 min for extraction time and 5mL for sample volume.

3.2. Method Validation

The validation study include the determination of response linearity, sensitivity (detection and quantification limits), precision (repeatability and intermediate precision) and accuracy (recovery studies).

Seven levels of concentration for each compound were analysed by triplicate to build the corresponding calibration curves (Table 4).

Linearity over the working range of concentration was verified by regression analyses of the relative peak area of target ion as response versus concentration. The correlation coefficients obtained ranged from 0.996 to 1.000. In addition, the linearity was evaluated graphically calculating response factor (relative area of peaks divided by their respective analyte concentrations) and was plotted as function of analyte concentrations. Two parallel lines are drawn in the graph at 0.95 and 1.05 times the average values of the response factors. The lines obtained were horizontal and had a slope near to zero over the concentration range.

Quantification (LOQ) and detection (LOD) limits were calculated with data generated in the calibration plots according to Miller and Miller [38] as indicated below:

$$\mathrm{LOD} = (3 \times Sa)/b \qquad \mathrm{LOQ} = (10 \times Sa)/b$$

where *S*a is the standard deviation of the interception and *b* is the slope of the regression line. The results obtained, showed in Table 4, pointed out the sensitivity of the method. The values were in

most cases lower that those found by Natera et al [7] employing HS-SPME and in some cases (all the acids, hexanal, 2-methyl-1-butanol, 3-methyl-1-butanol) lower that those found by Guerrero et al [30] employing SBSE.

Repeatability was evaluated by analysing 5 replicates of a spiked vinegar sample using the same method, laboratory and equipment within a working day. Intermediate precision was analysed in a similar way but by replicate analyses on separate days, 5 replicates over a period of 10 days. Results were calculated as the relative standard deviation (RSD) and are presented in Table 5. Most of the values obtained are according to the acceptable RSD percentages from the AOAC Peer Verified Methods [39], except for acetaldehyde diethylacetal and acetoin which showed RSD slightly higher for intermediate precision assays.

Three levels of concentration for each compound were employed to carry out recovery studies (Table 5). Only ethyl benzoate presents recovery values higher than the acceptable recovery percentages from the AOAC Peer Verified Methods [39].

3.4. Sample analysis

The method was applied to the analysis of several vinegars samples (Table 6) and a total of 53 compounds were determined. To our knowledge, among them, 5 had not been previously reported in wine vinegars, although these have been determined in different types of wine such as Sherry [40, 41], red [42, 43], sweet Fiano [44], Madeira [45], Albillo [46], Sauternes [47]. These compounds are principally esters (ethyl 2-methylbutyrate, ethyl heptanoate, ethyl furoate, and ethyl benzoate), as well as acetophenone.

In addition, other volatile compounds such as 2,3-butanediol diacetate and 1,1,6-trimethyl 1,2-dihydro naphthalene (TDN) were detected for the first time as constituents of vinegar aroma. Fig. 1 and 2 show the total ion chromatograms of red and sherry vinegars.

The major volatile compounds in these samples were acetoin, acetaldehyde diethylacetal, isovaleric acid, diacetyl, methyl acetate, isoamyl acetate, ethyl lactate, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol. This is in agreement with the results found by other authors in red and Sherry wine vinegars [18, 48, 49]. Due to their high concentration, a previous dilution of the sample was needed in some cases.

HSSE analytical method, validated in this work and SBSE methodology optimised by Guerrero et al. [15] showed a high sensitivity, reaching LOD and LOQ on the order of $\mu g\ L^{-1}$. Hence, both methods are very useful to analyse volatile compounds in vinegar. Nevertheless, whereas SBSE method is capable of quantifying compounds such as trans-2-hexanal, trans-2-hexen-1-ol, 2-acetyl-5-methylfuran, 2-ethyl hexanoic acid, 4-ethylguaiacol and 5-acetoxymethyl-2-furaldehyde, HSSE allows the determination of a higher amount of compounds which were not

244 determined by SBSE. These aroma compounds are the following: methyl acetate, acetaldehyde
245 diethylacetal, ethyl propanoate, diacetil, ethyl 2-methylbutyrate, amyl acetate, ethyl heptanoate,
246 ethyl furoate, γ-butyrolactone, acetophenone, ethyl benzoate, furfuryl alcohol, guaiacol, cis-trans-β-
247 methyl-γ-octalactones, heptanoic acid, nonanoic acid and vanillin.
248 SBSE method gives lower detection and quantification limits, recovery values are similar for
249 both methods except for isobutanol (lower in HSSE) and best repeatability and reproducibility
250 results are obtained in HSSE methods except for 2-furfuraldehyde, 2-phenylethyl acetate, benzyl
251 alcohol and 2-phenyletanol.
252 As we can see in Table 6, there appears to be very few differences in the qualitative
253 composition of red wine vinegars and Sherry vinegars in general. Hence, diacetyl was only detected
254 in Sherry vinegars, whereas nonanoic acid do not occur in this type of sample. On the other hand,
255 with regard to the quantitative volatile composition of the samples, a lot of compounds reached
256 similar concentrations in both types of vinegars. Nevertheless, Sherry vinegars contained the
257 highest concentrations of some volatile compounds such as ethyl butyrate, ethyl isovalerate, ethyl
258 lactate, isovaleric acid and 4-ethylphenol among others, whereas the red wine vinegars showed
259 quite higher concentrations of, for example, acetaldehyde diethylacetal, ethyl 2-methylbutyrate,
260 benzyl alcohol or cis-trans β-methyl-γ-octalatones.
261
262 **5. CONCLUSIONS**
263 Under the experimental conditions used in this study, HSSE technique has proved to be
264 suitable for the determination of a total of 53 aroma compounds in red wine and Sherry wine
265 vinegars. It is a very simple and solvent-less technique. Low detection and quantification limits
266 were obtained besides good precision and recovery values. Although SBSE method gives lower
267 detection and quantification limits, recovery values are similar for both methods except for
268 isobutanol (lower in HSSE) and best repeatability and reproducibility results are obtained in HSSE
269 methods except for 2-furfuraldehyde, 2-phenylethyl acetate, benzyl alcohol and 2-phenyletanol.
270 In addition this technique is capable to determine a longer number of compounds than SBSE
271 technique in half the time.
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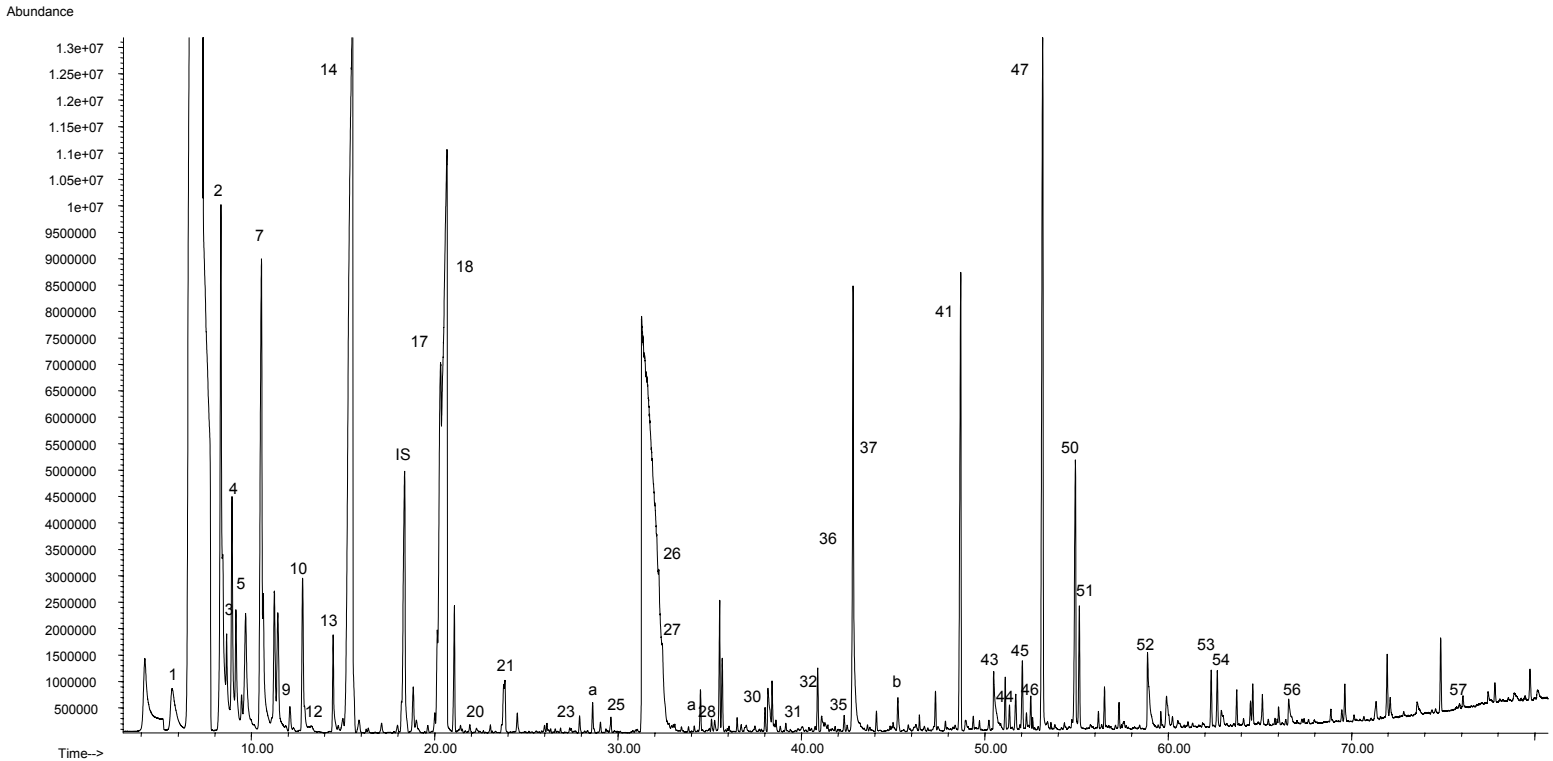
347 **FIGURE CAPTIONS**

348 Fig. 1. Total ion chromatogram of red wine vinegar. Compounds corresponding to each peak
349 number are given in Table 4. Peak a: Isomers of 2,3-butanediol diacetate; peak b:1,1,6-trimethyl
350 1,2-dihydro naphthalene (TDN).

351 Fig. 2. Total ion chromatogram of Sherry wine vinegar. Compounds corresponding to each peak
352 number are given in Table 4. Peak a: Isomers of 2,3-butanediol diacetate; peak b:1,1,6-trimethyl
353 1,2-dihydro naphthalene (TDN).

354


Abundance
1.3e+07
1.25e+07
1.2e+07
1.15e+07
1.1e+07
1.05e+07
1e+07
9500000
9000000
8500000
8000000
7500000
7000000
6500000
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
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Time-->
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20.00
30.00
40.00
50.00
60.00
70.00
1
2
3
4
5
7
9
10
12
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IS
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Figure 1.

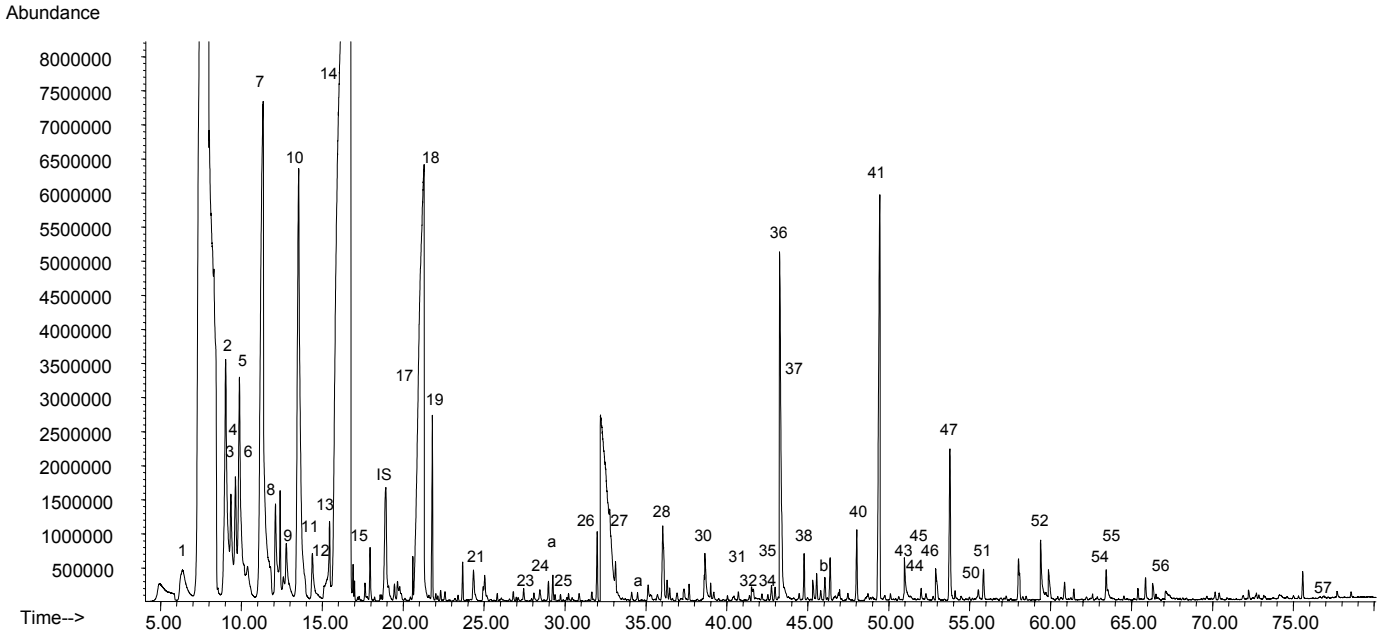
Abundance
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Figure 2.

Table 1. Chemical composition of the solution employed in optimisation experiments.

Compound	Concentration (μg/L)
Benzaldehyde	621
Diacetyl (mg/L)	80
α-ionone	50
Isovaleric acid	225
Octanoic acid	120
Propanoic acid	112
Ethyl acetate	1000
Methyl acetate	3000
Isoamyl acetate	153
Ethyl lactate	4210
Eugenol	109
Guaiacol	113
Isobutanol	2000
2-Methyl-1-butanol	193
3-Methyl-1-butanol	374
2-phenylethanol	300
γ-Butyrolactone	221
trans-β-Methyl-γ-octalactone	89
cis-β-Methyl-γ-octalactone	80
Vanillin (mg/L)	30
2-furfuraldehyde	50
Diethyl succinate	76.4
Acetoin (mg/L)	150
Acetic acid (g/L)	70
Ethanol (mL/L)	20

Table 2. Central composite design.

Factors	Axial (-α)	Low (-1)	Centre (0)	High (+1)	Axial (+α)
Sample Volume (V) [a]	5.0	6.01	7.5	8.99	10
Temperature (T) [b]	28.2	35	45	55	61.8
Time (t) [c]	9.8	20	35	50	60.2
[a] mL; [b] Celsius degree; [c] min					

Table 3. Regression coefficient for dependent variables studied in central composite design.

Regression coefficient	Response							
	Aldehydes	Ketones	Acids	Esters	Alcohols	Lactones	Volatile Phenols	Total area
b_0	318938000	119393000	6176610	240926000	107477000	16515400	23295104	1146082443
b_1	-8003690	3761920	-1877940	-7035840	-8688740	-1706450	-2496380	-45042700
b_2	89550100**	52977055***	1632055	56891524	33599210**	9403158***	15701849***	296011575*
b_3	74566499**	25084480*	2992492	74450641*	20548158	5044477***	8208737***	255079252*
b_{11}	-4266380	-6119190	2997180	2078320	833879	96707	-82390	52066700
b_{22}	-29430900	-1381810	832105	-5542530	-11637400	2059850	2128050	-62908600
b_{33}	-14888200	-11102100	1757900	-576009	-10307500	-446646	-1902230	-53899700
b_{12}	-8785976	1165384	-925449	11803205	-777933	-2808942*	-3153928	-17994111
b_{13}	-6410107	2456265	-815306	-6958353	-653391	-1333388	-1054480	-17753039
b_{23}	26894636	18626959	-1270074	29813691	6347596	4508174**	7801118**	91607873

Subscripts: 1= volume; 2= temperature; 3= time.
Level of significance: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

Table 4. Results of regression analysis of calibration straight lines, LOD and LOQ.

No.	Compound	Retention Time (min)	RI	m/z	Linear range (µg L^{-1})	Linear Regression	r^2	LOD (µg L^{-1})	LOQ (µg L^{-1})
1	Methyl acetate*	6.40	894	74	5 – 58	$y = 6 \cdot 10^{-3}x - 0.0006$	0.9990	1.8	5.9
2	Acetaldehyde diethylacetal*	8.02	928	103	22 - 152	$y = 5 \cdot 10^{-7}x - 0.0004$	0.9992	4.4	15
3	Ethyl propanoate	9.37	956	57	11 - 1091	y = 0.0004x - 0.0019	0.9983	38	127
4	Ethyl isobutyrate	9.64	962	71	10 - 1413	y = 0.0006x - 0.014	0.9994	29	98
5	Propyl acetate	9.89	967	61	10 - 3018	y = 0.0002x - 0.0034	0.9999	18	58
6	Diacetyl*	10.00	969	86	10 – 250	$y = 2 \cdot 10^{-3}x + 0.015$	0.9996	4	14
7	Isobutyl acetate	11.35	998	56	10 - 3032	y = 0.0006x - 0.014	0.9995	33	111
8	Ethyl butyrate	12.11	1014	71	10 - 1008	y = 0.0012x - 0.012	0.9991	24	80
9	Ethyl 2-methylbutyrate	12.78	1028	57	10 - 1413	y = 0.0018x - 0.013	0.9996	20	67
10	Ethyl isovalerate	13.55	1044	88	10 - 1410	y = 0.0017x - 0.006	0.9997	16	52
11	Butyl acetate	13.63	1046	56	0.5 -1413	y = 0.0006x + 0.0219	0.9993	20	66
12	Hexanal	13.96	1053	44	0.1 – 80	y = 0.0003x + 0.0012	0.9998	1.6	5.2
13	Isobutanol*	15.46	1084	43	0.7 – 51	$y = 2 \cdot 10^{-2}x - 0.010$	0.9998	0.5	1.6
14	Isoamyl acetate	16.76	1112	55	41 - 6139	y = 0.0009x + 0.07	1.000	37	125
15	Ethyl valerate	16.90	1115	88	3.3 - 81	y = 0.0019x - 0.006	0.9987	2.8	9.4
16	Amyl acetate	18.57	1150	43	10 - 1000	y = 0.0009x - 0.008	0.9996	17	56
17	2-Methyl-1-butanol	20.9	1199	57	26 - 2018	$y = 8 \cdot 10^{-5}x + 0.0019$	1.000	7.3	24
18	3-Methyl-1-butanol	21.28	1207	55	26 - 5011	$y = 7 \cdot 10^{-5}x + 0.005$	0.9998	45	150
19	Ethyl hexanoate	21.80	1208	88	10 - 1014	y = 0.0034x + 0.015	0.9997	13	42
20	Hexyl acetate	23.67	1257	43	1.7 - 1425	y = 0.0031x + 0.033	0.9996	15	51
21	Acetoin*	24.33	1271	45	5.9 - 603	$y = 5 \cdot 10^{-4}x + 0.006$	0.9999	7.4	25
22	Ethyl heptanoate	26.80	1323	88	0.1 - 100	y = 0.0042x - 0.0024	0.9993	1.9	6.3
23	Ethyl lactate*	27.44	1337	45	3.1- 159	$y = 9 \cdot 10^{-3}x + 0.008$	1.000	0.8	2.5
24	1-Hexanol	28.45	1358	56	10 - 1418	y = 0.0005x - 0.003	0.9994	23	76
25	*Cis*-3-hexen-1-ol	29.89	1388	67	10 - 101	y = 0.0001x - 0.0004	0.9994	2.7	9
26	Ethyl octanoate	31.98	1432	88	10 - 1516	y = 0.0032x + 0.05	0.9995	23	78
27	2-Furfuraldehyde	33.12	1456	96	10 - 726	$y = 6 \cdot 10^{-5}x + 0.017$	0.9993	14	48
28	Benzaldehyde	36.03	1518	77	10 - 725	y = 0.0006x + 0.004	0.9996	11	38
29	Ethyl nonanoate	36.89	1536	88	0.1 - 101	y = 0.0025x + 0.003	0.9989	2.4	8.1
30	5-Methyl-2-furfuraldehyde	38.61	1572	110	4.2 - 101	y = 0.0001x + 0.003	0.9962	7	23

31	Ethyl furoate	40.71	1617	95	4.2– 126	$y = 0.0001x + 7 \cdot 10^{-5}$	0.9986	4.1	14
32	γ-Butyrolactone	41.40	1631	42	10 - 1420	$y = 3 \cdot 10^{-6}x + 0.003$	0.9987	54	181
33	Acetophenone	42.17	1647	105	10 - 722	$y = 0.0001x - 0.005$	0.9994	14	47
34	Ethyl benzoate	42.76	1659	105	0.6 - 1425	$y = 0.0041x - 4 \cdot 10^{-5}$	0.9994	23	77
35	Furfuryl alcohol	42.97	1664	98	10 - 501	$y = 2 \cdot 10^{-5}x + 0.006$	0.9995	14	47
36	Isovaleric acid	43.25	1670	60	77 - 10256	$y = 3 \cdot 10^{-5}x + 0.004$	1.000	21	70
37	Diethyl succinate	43.40	1673	101	5 - 10323	$y = 0.0002x + 0.0007$	1.000	13	44
38	α-Terpineol	44.77	1702	59	10 - 723	$y = 0.0005x - 0.001$	0.9993	16	52
39	Benzyl acetate	45.55	1718	108	10 - 1418	$y = 0.0011x - 0.003$	0.9991	29	98
40	Ethyl phenylacetate	48.03	1770	91	10 - 1409	$y = 0.0028x + 0.05$	0.9997	20	68
41	2-Phenylethyl acetate	49.45	1800	104	250 - 5002	$y = 0.0013x + 0.4274$	0.9992	135	451
42	α-Ionone	51.04	1834	121	0.1 - 100	$y = 0.0029x - 0.0005$	1.000	0.6	1.9
43	Hexanoic acid	50.97	1832	60	10 - 1412	$y = 6 \cdot 10^{-5}x + 0.019$	0.999	43	142
44	Guaiacol	51.31	1840	109	0.1 - 120	$y = 0.0001x + 0.0008$	0.9998	1.3	4.3
45	Benzyl alcohol	52.29	1860	79	10 - 725	$y = 2 \cdot 10^{-5}x + 0.0004$	0.9994	14	45
46	*Trans*-β-methyl-γ-octalactone	52.73	1869	99	11 - 804	$y = 0.0004x - 0.024$	0.9994	19	62
47	2-Phenylethanol	53.77	1891	91	5 - 5010	$y = 6 \cdot 10^{-5}x + 0.0004$	0.9999	45	149
48	β-Ionone	54.65	1910	177	0.1 - 100	$y = 0.0039x - 0.005$	0.999	2.8	9.1
49	4-Methylguaiacol	55.17	1921	138	3.3 - 110	$y = 0.0002x - 0.0002$	0.9995	2.5	8.3
50	Heptanoic acid	55.48	1927	60	5 - 698	$y = 0.0001x + 0.007$	0.9995	13	42
51	*Cis*-β-methyl-γ-octalactone	55.53	1928	99	10 - 722	$y = 0.0003x - 0.01$	0.9995	22	75
52	Octanoic acid	59.38	2009	60	10 - 1413	$y = 0.0002x + 0.011$	0.9996	24	80
53	Eugenol	63.06	2087	164	80 - 722	$y = 0.0004x - 0.022$	0.9982	25	85
54	4-Ethylphenol	63.54	2097	107	12 - 121	$y = 3 \cdot 10^{-5}x + 0.0001$	0.9998	2.3	7.6
55	Nonanoic acid	63.6	2098	60	5 - 682	$y = 0.0002x + 0.012$	0.9995	16	53
56	Decanoic acid	67.10	2172	73	5 – 704	$y = 0.0003x + 0.005$	1.000	3.3	11
57	Vanillin	76.86	2378	151	56 - 5600	$y = 2 \cdot 10^{-7}x + 0.0005$	0.9995	162	539
-	4-methyl-2-pentanol (IS)	19.00	1159	45	-	-	-	-	-

* mg L^{-1}

LOD: Limit of detection = 3 signal/noise

LOQ: Limit of quantification = 10 signal/noise

Table 5. Results of Recovery, Repeatability and Intermediate Precision studies.

No.	Compound	Added (μg L^{-1})	Recovery (%)	Mean Recovery (%)	Repeatability (%RSD)	Intermediate Precision (%RSD)
1	Methyl acetate*	10040 16119 34541	101.8 92.1 97.6	97.2 ± 4.9	2.4	3.6
2	Acetaldehyde diethyl acetal*	21638 43277 70541	99.0 103.0 107.2	103.1 ± 4.1	3.9	7.5
3	Ethyl propanoate	160.4 426.8 1091.4	89.7 103.1 107.1	100.0 ± 9.1	4.0	7.3
4	Ethyl isobutyrate	75.7 148.4 690.5	99.2 99.7 102.6	100.5 ± 1.9	1.3	1.7
5	Propyl acetate	157 418 740	99.7 101.2 100.0	100.3 ± 0.8	2.1	3.0
6	Diacetyl*	25.5 50.3 124.2	101.3 96.3 93.2	96.9 ± 4.1	2.5	4.4
7	Isobutyl acetate	75.8 148.6 701.4	100.7 100.8 102.2	101.2 ± 0.9	1.4	2.1
8	Ethyl butyrate	150.3 400.7 699.2	99.6 99.4 100.8	99.9 ± 0.8	1.3	1.8
9	Ethyl 2-methylbutyrate	75.7 395.7 690.5	103.1 102.0 105.0	103.3 ± 1.5	2.7	3.5
10	Ethyl isovalerate	75.9 148.7 691.9	100.6 100.7 99.4	100.2 ± 0.7	1.0	1.1
11	Butyl acetate	148 395 690	106 95 92	97.5± 7.1	6.3	6.5
12	Hexanal	10.0 25.1 50.1	100.0 101.2 100.5	100.6 ± 0.6	2.9	3.3
13	Isobutanol*	6.01 15.0 25.41	102.1 100.0 98.4	100.2 ± 1.9	0.8	1.2
14	Isoamyl acetate	614 1207 2455	99.7 103.2 97.4	100.1 ± 2.9	1.8	2.2
15	Ethyl valerate	10.1 25.2 50.5	102.5 98.0 95.3	98.6 ± 3.6	2.3	3.7
16	Amyl acetate	147.1 392.2 684.3	103.1 103.9 97.7	101.6 ± 3.4	2.5	3.2
17	2-Methyl-1-butanol	126 757 2008	100.2 95.4 94.1	96.6 ± 3.2	0.6	0.8
18	3-Methyl-1-butanol	125 752	99.3 102.2	100.7 ± 2.1	2.2	3.8

		1994	99.9			
19	Ethyl hexanoate	149.1 397.5 693.6	89.6 95.5 87.6	90.9 ± 4.1	1.4	2.2
20	Hexyl acetate	50.4 75.4 100.8	100.2 107.0 107.5	104.9 ± 4.1	3.8	4.8
21	Acetoin*	25.1 74.7 149.3	99.3 86.7 91.4	92.5 ± 6.4	3.4	6.2
22	Ethyl heptanoate	10.0 25.1 50.2	85.1 98.7 90.1	91.3 ± 6.9	1.9	2.8
23	Ethyl lactate*	3.06 7.65 89.3	93.9 104.3 103.3	100.5 ± 5.7	6.0	6.9
24	1-Hexanol	148.8 396.9 692.6	99.2 99.8 100.4	99.8 ± 0.6	2.2	2.8
25	*Cis*-3-hexen-1-ol	3.3 10.1 50.5	99.3 98.6 103.4	100.4 ± 2.6	3.5	3.9
26	Ethyl octanoate	148.6 396.2 691.3	97.9 107.7 94.3	100.0 ± 6.9	3.8	6.7
27	2-Furfuraldehyde	80.8 130.9 503.2	99.8 101.8 95.0	98.9 ± 3.5	10.9	14.7
28	Benzaldehyde	76.6 150.1 698.2	105.5 94.9 93.1	97.8 ± 6.7	0.9	1.9
29	Ethyl nonanoate	10.10 25.25 50.49	94.2 98.3 114.6	102.3 ± 10.8	2.6	3.5
30	5-Methyl-2-furfuraldehyde	4.3 32.9 65.7	97.5 100.3 95.9	99.5 ± 1.7	3.7	7.9
31	Ethyl furoate	4.2 31.6 63.1	98.3 92.2 100.7	97.1± 4.4	5.1	9.3
32	γ-Butyrolactone	301 693.8 1420.0	104.3 99.1 99.4	100.9 ± 2.9	4.8	7.0
33	Acetophenone	81 130 319	93.3 87.0 95.7	92.0 ± 4.5	2.6	4.1
34	Ethyl benzoate	82 131 181	130.3 116.3 121.1	122.6 ± 7.1	4.4	6.5
35	Furfuryl alcohol	75.4 320 501	101.0 107 108	105.4 ± 3.9	7.9	8.1
36	Isovaleric acid	1612 4884 12210	101.7 99.3 97.4	99.4 ± 2.2	4.0	5.0
37	Diethyl succinate	543 3206 6520	87.3 97.3 102.0	95.5 ± 7.5	3.6	9.5
38	α-Terpineol	130 180 320	85.6 100.9 101.6	96.0 ± 9.1	4.2	8.0
39	Benzyl acetate	148.9	96.3	97.0 ± 2.7	2.7	3.0

		397.0 692.8	94.6 100.0			
40	Ethyl phenylacetate	147.9 394.4 688.2	103.6 105.0 103.5	104.0 ± 0.9	2.8	3.2
41	2-Phenylethyl acetate	250 750 2000	99.3 99.3 98.0	98.9 ± 0.8	6.9	7.7
42	α-Ionone	10.1 25.2 50.4	92.7 95.3 102.6	96.9 ± 5.1	4.2	5.8
43	Hexanoic acid	148.2 395.3 689.8	99.5 100.4 99.3	99.7 ± 0.6	3.7	6.2
44	Guaiacol	4.0 12.0 60.2	99.9 103.2 99.4	100.8 ± 2.0	1.9	3.2
45	Benzyl alcohol	103 606 1232	102.5 99.5 95.5	99.1 ± 3.5	7.6	9.0
46	*Trans*-β-methyl-γ-octalactone	90.6 145.0 200.5	112.3 100.9 100.7	104.6 ± 6.6	7.2	52.73
47	2-Phenylethanol	501 1503 10021	100.7 107.6 100.8	103.0 ± 3.9	7.8	8.0
48	β-Ionone	3.3 10.0 50.1	104.0 88.2 100.1	97.4 ± 8.2	3.9	6.7
49	4-Methylguaiacol	3.3 25.2 50.4	78.3 84.8 80.5	81.2 ± 3.3	6.7	9.9
50	Heptanoic acid	70 186 325	96.2 102.6 99.8	99.5 ± 3.2	4.8	5.0
51	*Cis*-β-methyl-γ-octalactone	81 130 319	99.8 99.5 99.6	99.6 ± 0.1	2.4	2.5
52	Octanoic acid	76 148 396	97.6 100.0 99.9	99.2 ± 1.4	4.4	4.7
53	Eugenol	130.2 182.0 319.3	90.6 94.2 102.9	95.9 ± 6.3	2.9	2.2
54	4-Ethylphenol	29 58 115	82.8 104.9 86.1	91.3 ± 11.9	7.4	8.5
55	Nonanoic acid	37 72 190	93.5 100.6 103.3	99.1 ± 5.07	6.7	9.7
56	Decanoic acid	75 201 350	96.0 82.1 94.4	90.8 ± 7.6	3.4	5.7
57	Vanillin	1000 3000 5200	95.1 107.5 99.4	100.6 ± 6.3	5.9	6.8

* mg L^{-1}

Table 6. Concentrations of Volatile Compounds in the Seven wine vinegars

No.	Compounds	Mean concentration (µg L^{-1}) ± Standard deviation						
		VJ	**VR**	**VJGR**	**VRW1**	**VRW2**	**VRW3**	**VRW4**
1	Methyl acetate*	22.2 ± 0.7	11.6 ± 1.5	22.4 ± 0.8	15.5 ± 1.1	15.3 ± 0.7	34 ± 3	40.1 ± 0.6
2	Acetaldehyde diethylacetal*	9.59 ± 0.17	61.7 ± 4.1	42.9 ± 1.3	193 ± 10	91± 2.7	126 ± 9	90.7 ± 0.7
3	Ethyl propanoate	665 ± 14	1264 ± 92	1213 ± 51	224 ± 18	568 ± 16.1	343 ± 21	1142 ± 38
4	Ethyl isobutyrate	361 ± 13	545 ± 19	578 ± 30	289 ± 43	484 ± 15.8	465 ± 44	1033 ± 31
5	Propyl acetate	727 ± 10	1274 ± 76	3665 ± 155	1041 ± 103	2242± 199	691 ± 27	2078 ± 72
6	Diacetyl*	17.1 ± 0.4	33 ± 1	42.5 ± 0.6	-	-	-	-
7	Isobutyl acetate	1457.7 ± 0.4	1840 ± 35	4330 ± 259	1572 ± 14	2059 ± 33.6	1425 ± 140	2284 ± 17
8	Ethyl butyrate	100 ± 0.3	209 ± 14	335 ± 8	n.q.	78 ± 4	n.q.	143 ± 1
9	Ethyl 2-methylbutyrate	n.q.	109 ± 4	104 ± 4	289 ± 43	484 ± 15.8	465 ± 44	1033 ± 31
10	Ethyl isovalerate	558 ± 10	1015 ± 16	1305 ± 65	n.q.	n.q.	150 ± 6	491 ± 21
11	Butyl acetate	n.d.	n.d.	155 ± 8	n.q.	106 ± 4	n.d.	n.q.
12	Hexanal	9.38 ± 0.17	-	17.5 ± 0.1	n.d.	n.d.	13 ± 1	47 ± 6
13	Isobutanol*	5.6 ± 0.3	3.55 ± 0.04	8.53 ± 0.09	7.2 ± 1.1	7.61 ± 0.06	7.9 ± 0.5	12.9 ± 0.3
14	Isoamyl acetate*	3.72 ± 0.04	4.3 ± 0.1	11.6 ± 0.5	4.1 ± 0.2	6.6 ± 0.1	2.5 ± 0.3	5.87 ± 0.08
15	Ethyl valerate	n.q.	n.q.	19.0 ± 0.9	n.q.	n.q.	n.q.	11.4 ± 0.5
16	Amyl acetate	n.d.	n.d.	n.d.	n.d.	nd	n.d.	n.q.
17	2-Methyl-1-butanol*	9.88 ± 0.06	13.5 ± 1.6	6.1 ± 0.6	5.7 ± 0.5	5.09 ± 0.08	11.26 ± 0.05	14 ± 1.0

*: concentration in mg L^{-1} n.d.: below detection limit
n.q.: below quantification limit -: no peak

Table 6. (Continued)

No.	Compounds	Mean concentration (μg L^{-1}) ± Standard deviation						
		VJ	VR	VJGR	VRW1	VRW2	VRW3	VRW4
18	3-Methyl-1-butanol*	18.1 ± 0.6	27 ± 3	48.2 ± 2.3	33.6 ± 0.8	49.3 ± 0.7	29.3 ± 1.6	53.7 ± 0.6
19	Ethyl hexanoate	n.q.	49.3 ± 3.7	81 ± 5	n.q.	54.1 ± 2.6	n.q.	121 ± 9
20	Hexyl acetate	n.d.	n.d.	n.d.	n.q.	74.1± 2.2	n.d.	82 ± 3
21	Acetoin*	518 ± 28	569 ± 24	358 ± 6	209 ± 15	364 ± 17	496 ± 21	740 ± 33
22	Ethyl heptanoate	n.d.	n.d.	n.q.	n.d	n.d	n.d	n.d
23	Ethyl lactate*	9.2 ± 0.8	9.2 ± 0.5	7.4 ± 0.6	2.5 ± 0.2	5.23 ± 0.21	1.23 ± 0.02	2.09 ± 0.05
24	1-Hexanol	n.q.	n.q.	83 ± 3	227 ± 6	362 ± 6	n.q.	n.q.
25	Cis-3-hexen-1-ol	52.85 ± 1.17	51.8 ± 0.3	43.7 ± 0.5	30.5 ± 0.7	44.4 ± 2.1	31.7 ± 0.6	45.2 ± 0.4
26	Ethyl octanoate	n.d.	n.d.	n.q.	n.d.	n.q.	n.d.	n.d.
27	2-Furfuraldehyde	329 ± 23	878 ± 58	1292 ± 102	71 ±	61.7 ±	201 ± 30	598 ± 87
28	Benzaldehyde	99.4 ± 0.6	121 ± 5	254 ± 6	81 ± 3	54.5 ± 1.7	89 ± 3	n.q.
30	5-methyl-2-furfuraldehyde	59 ± 2	n.q.	229 ± 15	n.d.	n.d.	n.d.	n.d.
31	Ethyl furoate	88 ± 6	231.1 ± 21.8	303 ± 4	29.9 ± 1.4	76.3 ± 1.8	77 ± 6	122 ± 8
32	γ-Butyrolactone	1655 ± 156	924 ± 70	2760 ± 233	1373 ± 140	1006 ± 132	1461 ± 35	2238 ± 6
33	Acetophenone	n.q.	n.q.	n.q.	n.d.	n.d.	n.q.	n.q.
34	Ethyl benzoate	n.d.	6.7 ± 0.5	13.5 ± 0.4	n.d.	n.d.	n.d.	n.d.
35	Furfuryl alcohol	323 ± 38	390 ± 8	885 ± 96	357 ± 36	80 ± 10	393 ± 6	1044 ± 58
36	Isovaleric acid*	49.0 ± 3.9	54 ± 4	59.4 ± 1.8	1.82 ± 0.024	1.82 ± 0.08	11.2 ± 0.1	16.3 ± 1.2

*: concentration in mg L^{-1} n.d.: below detection limit
n.q.: below quantification limit -: no peak

Table 6. (Continued)

No.	Compounds	Mean concentration (µg L^{-1}) ± Standard deviation						
		VJ	**VR**	**VJGR**	**VRW1**	**VRW2**	**VRW3**	**VRW4**
37	Diethyl succinate	1720 ± 155	218 ± 21	281 ± 6	1455 ± 175	12029 ± 775	2259 ± 133	6507 ± 494
38	α-Terpineol	n.q.	n.d.	121 ± 2	n.d.	n.d.	n.q.	n.d.
39	Benzyl acetate	n.d.	n.q.	n.q.	n.d.	152 ± 4	n.d.	n.q.
40	Ethyl phenylacetate	n.q.	n.q.	109 ± 3	n.d.	n.d.	n.d.	n.q.
42	2-Phenylethyl acetate	984 ± 102	1134 ± 29	2090 ± 42	1135 ± 119	1618 ± 11	765 ± 90	1027 ± 132
43	Hexanoic acid	1325 ± 154	2063 ± 47	2269 ± 134	167 ± 24	1029 ± 65	2008 ± 71	3322 ± 164
44	Guaiacol	n.d.	n.q.	11.3 ± 0.1	14.40 ± 0.16	11.3 ± 0.4	23.0 ± 0.3	30.6 ± 0.8
45	Benzyl alcohol	350 ± 41	737 ± 25	646 ± 18	143 ± 3	3375 ± 213	787 ± 29	630 ± 37
46	*Trans*-β-methyl-γ-octalactone	77 ± 7	64.8 ± 2.3	74 ± 1.69	74.5 ± 1.3	137 ± 16	313 ± 15	161 ± 13
47	2-Phenylethanol*	11.5 ± 1.1	9.4 ± 0.9	12.7 ± 0.3	13.9 ± 1.4	20.3 ± 0.5	30.3±2.2	24.7 ± 0.7
50	Heptanoic acid	n.d.	n.q.	151 ± 14	nd	nq	133 ± 16	153 ± 8
51	*Cis*-β-methyl-γ-octalactone	107 ± 9	n.q.	125.0 ± 1.5	183 ± 13	446 ± 58	1534 ± 197	1424 ± 210
52	Octanoic acid	531 ± 61	368 ± 4	627 ± 42	n.d.	273 ± 41	497 ± 63	732 ± 70
53	Eugenol	n.d.	n.q.	n.d.	n.q.	n.q.	118 ± 16	n.q.
54	4-Ethylphenol	1652 ± 96	1191 ± 95	901 ± 8	22 ± 1	514 ± 74	102 ± 8	395 ± 55
55	Nonanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
56	Decanoic acid	73 ± 6	37.2 ± 0.7	68.1 ± 0.2	n.d.	n.d.	45.7 ± 0.9	136 ± 19
57	Vanillin	n.d.	4438 ± 355	2572 ± 253	n.d.	n.d.	2663 ± 15	3587 ± 400

*: concentration in mg L^{-1} n.d.: below detection limit
n.q.: below quantification limit -: no peak

Manuscript 3

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VOLATILE COMPOUNDS IN RED WINE VINEGARS OBTAINED BY SUBMERGED AND SURFACE ACETIFICATION IN DIFFERENT WOODS

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Abstract: Changes in volatile components were investigated during controlled acetifications. The substrates used to perform traditional surface acetifications were two red wines. Barrels of four different woods (oak, chestnut, acacia and cherry) were used. Submerged acetifications were performed at the laboratory scale. Volatile compounds were analysed by Headspace Sorptive Extraction and Gas Chromatography-Mass Spectrometry (HSSE-TD-GC-MS). Out of 57 compounds identified in the samples, 38 were quantified. Of these, ethyl furoate, ethyl benzoate and limonene had never been described in wine vinegars. Acetifications led to an increase in the total quantity of volatile components, which were higher in the surface processes. Acetic esters were predominant in surface culture vinegars, whereas acids were predominant in submerged culture vinegars. Oak-lactones were quantified only in vinegars produced in oak barrels. Ethyl furoate and ethyl benzoate increased in cherry wood barrels. Multivariate statistical analysis supported the influence of the process on the volatile composition of the final vinegars.

TITLE: VOLATILE COMPOUNDS IN RED WINE VINEGARS OBTAINED BY SUBMERGED AND SURFACE ACETIFICATION IN DIFFERENT WOODS

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ABSTRACT

Changes in volatile components were investigated during controlled acetifications. The substrates used to perform traditional surface acetifications were two red wines. Barrels of four different woods (oak, chestnut, acacia and cherry) were used. Submerged acetifications were performed at the laboratory scale. Volatile compounds were analysed by Headspace Sorptive Extraction and Gas Chromatography–Mass Spectrometry (HSSE-TD-GC-MS). Out of 57 compounds identified in the samples, 38 were quantified. Of these, ethyl furoate, ethyl benzoate and limonene had never been described in wine vinegars. Acetifications led to an increase in the total quantity of volatile components, which were higher in the surface processes. Acetic esters were predominant in surface culture vinegars, whereas acids were predominant in submerged culture vinegars. Oak-lactones were quantified only in vinegars produced in oak barrels. Ethyl furoate and ethyl benzoate increased in cherry wood barrels. Multivariate

statistical analysis supported the influence of the process on the volatile composition of the final vinegars.

KEYWORDS

Volatile compounds; acetification; vinegar; wine; wood; acetic acid bacteria; HSSE-GC-MS

1. Introduction

Vinegar is derived from the conversion by bacteria of ethanol to acetic acid. It can therefore be produced from any alcoholic material, ranging from alcohol-water mixtures to wines. Its color and aroma depend greatly on the initial substrate (Tesfaye, Morales, García-Parrilla, & Troncoso, 2002).

Wine vinegar is the result of two fermentation processes (the conversion of sugars in must into ethanol by yeasts and the oxidation of the ethanol by acetic acid bacteria). Usually, wine producing countries are also major wine vinegar producers. This is especially true of countries with warmer temperatures since acetic acid bacteria require high temperatures to develop (De Ory, Romero & Cantero, 1998). In Mediterranean countries vinegar is produced by various methods that produce vinegars of greatly different qualities (Tesfaye, Garcıía-Parrilla & Troncoso, 2002).

Vinegar production methods range from traditional methods that employ wooden casks (the Orleans Process) and surface culture to submerged industrial fermentation (Morales, Tesfaye, García-Parrilla, Casas, & Troncoso, 2001b). Large quantities of vinegar demand industrial fermentation systems capable of producing volumes that are reliably controlled (De Ory, Romero, & Cantero, 1999). Many technical devices have been developed to improve the industrial production of vinegar. Nowadays these are generally based on submerged acetification systems, improvements

which have increased the speed of transformation of ethanol into acetic acid in the presence of acetic acid bacteria (Tesfaye *et al.,* 2002). Vinegars produced by slow traditional surface methods generally fetch higher prices because their better sensory quality is recognized by the consumer (Tesfaye, Morales, García-Parrilla & Troncoso, 2002).

Vinegar characterization comprises a wide range of values obtained from physicochemical and sensory parameters (Carnacini & Gerbi, 1992). Although several major volatile compounds, such as acetic acid, ethyl acetate and acetaldehyde, account for a vinegar's final aroma, many other minor compounds could help to explain the complexity of the overall sensation, especially if the vinegars are produced in wooden barrels. These minoritary aroma components determine the quality of the vinegar. It is difficult to quantify these minor aroma compounds in wine vinegar because of the complexity of the matrix and the low concentrations expected. Over seventy aroma components have been identified in the volatile fraction of wine vinegars. These include alcohols, esters, carbonyls, acids, phenols, lactones, and acetals, etc. Aroma is therefore the result of a combination of various chemical characteristics covering a wide range of polarity, solubility and volatility. Moreover, several compounds are expected to be present at very low concentrations (< mg/L).

The volatil profile of Sherry and white vinegars is generally well known (Morales, González, Casas, & Troncoso, 2001a; Natera, Castro, García, García, & Barroso, 2002) but little work has been done with red wine vinegars. Charles, Martin, Ginies, Etievant, Coste, and Guichard, (2000) applied GC coupled to olfactometric detection to ascertain potent aroma compounds in red wine vinegars.

There are few studies of the changes in volatile compounds during the conversion of wine into vinegar (Morales et al., 2001b; Valero, Berlanga, Roldán,

Jiménez, García, & Mauricio, 2005; Guerrero, Natera, Castro, & Barroso, 2006). The relative importance of the quality of the substrate wine and the acetification method has therefore not been fully addressed.

Stir bar sorptive extraction (SBSE) techniques were developed to obtain higher sampling capacity than with solid phase microextraction (SPME) (Guerrero, Natera, Castro, & Barroso, 2007). We use SBSE extraction made in the headspace, which is better at reducing the risk of contamination. This method has been used to succesfully analyse volatile compounds in wine (Weldegergis, Tredoux, & Crouch, 2007).

In this paper we examine the changes in volatile compounds during acetification in submerged and surface cultures of acetic acid bacteria in order to evaluate whether the volatile profiles of the vinegars depend on the procedure used. We also determine how the type of wood used for the acetification of red wines affects the volatile profile of the final products. To do so we study the different compositions of red wine vinegars when produced by surface acetification in barrels made from four types of wood—chestnut, acacia, cherry, and oak. This is a novel approach since surface acetification is generally done in oak barrels. This is because of the limited oxygen transfer and because some compounds are extracted into the wine. Since higher oxygen transfer is needed for acetification, we use more porous woods in order to test their suitability for wine.

2. Experimental

2.1. Samples

A total of 22 samples were analysed in this study. We acetified two red wines. Their main physicochemical characteristics are shown in Table 1. Both substrate wines (G and H) were subjected to traditional surface acetification in two different wineries,

101 one in Banyuls (France) and one in Priorat (Spain). We also used substrate G to perform
102 submerged acetifications in a laboratory fermentor (5 L capacity). The total number of
103 acetifications was eleven.

104 For surface acetification we used barrels (60 L capacity) made from four
105 different woods (oak, chestnut, acacia and cherry). Samples (n = 16) were taken at the
106 beginning and at the end of the acetification process. The end point was established
107 when the acetic acid degree (g acetic acid/100 mL vinegar) reached 6-7º. In the initial
108 samples (n = 11, Table 1), the mixture of substrate wine plus acetic acid bacteria starter
109 had a ratio of 50:50 and the acetic acid degree was ca. 0.8º. Acetifications lasted ca. 2
110 months for substrate G, and ca. 9 months for substrate H, mainly depending on room
111 temperature (Table 1).

112 For submerged acetification, three successive cycles were performed in the
113 laboratory. Samples (n = 6) were also taken at the beginning and at the end of the
114 process. The acetification lasted ca. 33 hours.

115 *2.2. Apparatus*

116 A laboratory scale fermentor (B. Braun Biotech, S.A.) was used to produce wine
117 vinegar by a submerged culture. This was equipped with: a cylindrical concave bottom
118 glass culture vessel with a capacity of 5 litres and a height-to-diameter ratio of 2:1; an
119 air supply system with air filters and inlet pipe with sparger ring; a refrigeration system
120 (Frigomix® cooling unit; Sartorius, Goettingen, Germany) with cold water to prevent
121 loss of volatile components; an electrical heater jacket 230V; a stirrer with 6-bladed disc
122 impellers; a Pt-100 pH-electrode, a pO_2-electrode; a sensor for temperature
123 measurement Pt-100; a micro-DCU 300 measurement and control system; a MCU-200
124 stirrer speed control; and a dosing pump-300.

Optimum conditions for the efficient production of vinegar samples were: an air flow of 150 litres h^{-1}, a temperature of 30 ºC, a stirring speed of 450 rpm, a working volume of 3.4 litres, a loading proportion of 1:1 (wine:vinegar), which results in discontinuous cycles with an average duration of 33 h (Tesfaye, García-Parrilla & Troncoso, 2000).

2.3. Reagents

The standards of 41 volatile compounds, given in Tables 2–4, were obtained from commercial sources as follows: 2, 9, 10, 12, 13, 15, 16, 18-20, 26, 27, 29, 31-35, 37-41 (Sigma-Aldrich, Madrid, Spain); 1, 3, 4, 5, 7, 8, 17, 22-25, 30, 36 (Merck, Darmstadt, Germany); 6, 11, 14, 21, 28 (Fluka, Madrid, Spain). 4-methyl-2-pentanol (Merck) was used as internal standard (IS). Sodium chloride, ethanol and acetic acid, all of analytical quality, were from Merck. Water was obtained from a Milli-Q purification system (Millipore, USA).

2.4. Headspace Sorptive Extraction (HSSE) GC-MS Analysis

Gas chromatography analyses were performed with a 6890 Agilent GC system coupled to a quadrupole mass spectrometer Agilent 5975inert and equipped with a Gerstel Thermo Desorption System (TDS2) and a cryo-focusing CIS-4 PTV injector (Gerstel).

Conditions for the HSSE have been optimised (Callejón, Troncoso & Morales, submitted): 5 mL of sample with 1.67 g of NaCl were placed into 20 mL headspace vials. The special open glass adapter (Gerstel, Müllheim an der Ruhr, Germany) was then introduced into the vial. A 10 mm long stir bar coated with 0.5 mm polydimethylsiloxane (PDMS) layer (Twister, Gerstel, Müllheim and der Ruhr, Germany) was put into the glass insert. The vial was tightly capped and heated at 62 ºC in a thermostatic bath for 60 minutes. When the vial was at room temperature, the stir

bar was removed with tweezers, rinsed with Milli-Q water and dried with lint-free tissue paper. Finally, the stir bar was put into a glass tube, which was placed on the autosampler tray of the thermo desorption unit.

Thermal desorption was performed in splitless mode with a flow rate of 90 mL/min. The desorption temperature program was as follows: 35 ºC for 1 min, ramped at 60 ºC/min to 250 ºC and held for 5 min. The CIS-4PTV injector, with a Tenax TA inlet liner, was held at –35 ºC with liquid nitrogen for total desorption time, raised at 10 ºC/s to 290 ºC and then held for 4 min. Solvent vent mode was used to transfer the sample to the analytical column.

A CPWax-57CB column (50 m x 0.25 mm, 0.20 μm film thickness, Varian, Middelburg, Holland) was used. The carrier gas was He at a flow rate of 1 mL/min. The oven temperature program was: 35 ºC for 5 min, which was then raised to 220 ºC at 2.5 ºC/min (held for 5 min). The quadrupole, source and transfer line temperatures were kept at 150, 230 and 280 ºC, respectively. Electron ionization mass spectra in the full-scan mode were recorded at 70 eV electron energy in the 35 to 350 amu range. All data were recorded using a MS ChemStation. The identity of the peaks was assigned using the NIST 98 library and confirmed by the retention index of standards when available. Quantification was performed using the relative area to internal standard of the target ion of each compound. The samples were analysed in triplicate and blank runs of empty glass tube were done before and after each analysis.

2.5. Statistical analysis

All statistical analyses were performed using Statistica software (StatSoft, 2001). One-way ANOVA was used to evaluate significant differences (significance levels $p<0.05$).The normality of data was previously verified by the Kolmogorov-Smirnov test. When variables did not fit the normal distribution, the Mann Whitney test

was chosen instead of ANOVA. Principal Component Analysis (PCA) was carried out as an unsupervised method in order to ascertain the latent structure of the data.

3. Results and Discussion

Minor volatile compounds (n= 57) were analysed by the HSSE-GS-MS method. Of these, 38 were present at quantities above their respective quantification limits in the samples analysed (Tables 2-4). Some of them (ethyl furoate, ethyl benzoate and limonene) had never been described in wine vinegars.

3.1. General changes in volatile compounds during acetification

Initial samples had high concentrations for alcohols, representing ca. 40% of total volatile compounds (Fig. 1). 3-Methyl-1-butanol, 2-methyl-1-butanol, isobutanol and 2-phenylethanol had the highest concentrations. These alcohols, produced during alcoholic fermentation, are related to the yeast amino acid metabolism (Bayonove, Baumes, Crouzet & Günata, 2000).

As we can see in Tables 2-4, substrate H contains a higher amount of volatile compounds (especially ethyl esters, Fig. 1) than substrate G.

Total volatile compounds increased in all acetifications. This was statistically significant in samples from substrate G acetified in wooden barrels (mean increases of 42%). As expected, however, samples obtained by submerged acetification had the lowest increases (only 6%). This result agrees with those of previous studies (Morales et al., 2001b), where losses of highly volatile compounds in the submerged acetification were due to use of an open acetification system with forced aeration.

During the acetification process, the acetic acid bacteria can metabolise other alcohols in a similar way to ethanol and produce their respective fatty acids (Nieto, González-Viñas, Barba, Martín-Álvarez, Aldalve, García-Moreno, & Cabezudo, 1993).

The decreases in 2-methyl-1-butanol, 3-methyl-1-butanol, 1-hexenol and *cis*-3-hexen-1-ol were concomitant with increases in isovaleric, hexanoic and octanoic acids in all the acetifications studied. These changes were statistically significant ($p<0.05$) for these compounds except for 2-methyl-1-butanol in the laboratory submerged acetifications (Tables 2-4).

The hydrolysis of most ethyl esters, such as ethyl propionate, ethyl butyrate, ethyl hexanoate and ethyl lactate, took place throughout acetification due to the active consumption of ethanol by acetic acid bacteria. One exception was ethyl isobutyrate, which increased in both acetification methods.

3.2. Volatile compounds as affected by submerged and surface acetification

We examined the differences in the volatile profiles of samples obtained by surface and submerged acetification of the same wine substrate. In general, from a qualitative point of view these differences were not relevant.

Acetic esters were predominant for vinegars obtained by surface acetification in wooden barrels (Fig. 1). These compounds are related to fruity aroma (Du Plessis, 1983). On the other hand, vinegars from submerged culture were rich in acids, especially isovaleric acid. This compound smells of cheese or foot (Aznar, Lopez, Cacho & Ferreira, 2001). Its threshold for vinegar is 150 μg/L (Callejón, Morales, Silva Ferreira, Troncoso, 2007) and in high concentrations it could result in an off-flavour. We should stress that submerged acetifications are highly selective for acetic acid bacteria and in this condition the main species was *Gluconacetobacter europaeus.* On the other hand, *Acetobacter pasteurianus* was predominant in superficial acetifications (Callejon et al., 2008). The differences between submerged and superficial acetification therefore involve a combination of species and metabolism.

With regard to alcohols, several authors report a different rate of consumption of 3-methyl-1-butanol and 2-methyl-1-butanol in submerged acetification processes (2-methyl-1-butanol is consumed slowly by acetic acid bacteria (Nieto et al., 1993)). Our results agree with this observation as the ratio between 3-methyl-1-butanol and 2-methyl-1-butanol fell sharply during submerged acetification. Another alcohol—isobutanol—behaved differently in submerged and superficial acetifications. The concentration of isobutanol decreased in all the surface acetifications but increased in the three submerged acetifications, as was also observed by Guerrero, Castro, Natera, Palma, Gómez Beser and Barroso (2006).

Another characteristic compound of acetification is acetoin (Morales et al., 2001a). Acetoin is produced during alcoholic fermentation by the action of several microorganisms and is present in fermented foods and beverages such as wine, vinegar and several dairy products (Caligiani, Silva, & Palla, 2007). Although acetoin generally increased during acetification, this increase was only significant in the surface processes. Acetoin therefore reached very different concentrations at the end of acetification and was higher in the surface culture (209–339 mg/L) than in the submerged culture (72–92 mg/L) (Tables 2 and 3). Some authors postulate that the amount of acetoin found in vinegars depends on the composition of the wine substrate (Pizarro, Esteban-Díez, Sáenz-González & González-Sáiz, 2008). Although this may be partially true, our results clearly show that the amount of this compound was more related to the type of acetification than to the substrate (Fig. 1).

Ethyl isovalerate and diethyl succinate increased in submerged acetifications but decreased in surface acetifications. This suggests that the hydrolysis of ethyl esters may be favoured by the length of the acetification or by the acetic acid bacteria strains involved in the process (Callejón, Tesfaye, Torija, Mas, Troncoso & Morales, 2007).

Another well-known phenomenon is the formation of acetic esters from the reaction of alcohols with acetic acid (Morales, Tesfaye, García-Parrilla, Casas & Troncoso, 2002; Ribéreau-Gayon, Glories, Maujean & Dubourdieu, 2006). As Tables 2 and 3 show, the concentration of methyl acetate significantly increased during both the submerged and the surface processes. Increases in this compound in red wine vinegars are expected since they contain considerable amounts of methanol and the formation of methyl esters is favoured by the acidic environment (Morales et al., 2002). Nevertheless, the differences in the concentration of methyl acetate from the two methods of acetification were statistically significant. The concentration was higher in surface cultures probably because of the duration of the processes.

The levels of 2-phenylethyl acetate were consistently higher at the end of all the processes studied (1.1–1.8 mg/L). This result agrees with those of other authors (Blanch, Tabera, Sanz, Herraiz & Reglero, 1992; Guerrero et al., 2007; Charles et al., 2000). These increases were statistically significant in the surface acetifications. 2-phenylethanol and 2-phenylethyl acetate are rose-like aroma compounds produced form L-phenylalanine by yeast (Etschmann, Sell & Schrader, 2005).

During surface acetifications, propyl, isobutyl and isoamyl acetates suffered significant changes in their concentrations. Similarly as it occurs for wine (Ribéreau-Gayon et al., 2006) these acetates of high alcohols could contribute to the aromatic complexity of these vinegars. In the submerged process, on the other hand, their concentrations decreased significantly, possibly due to the losses of these compounds, with low boiling points, which are favoured by the strong aeration of the laboratory fermentor (Morales et al., 2001a).

The concentration of acetaldehyde diethylacetal decreased during all acetifications. In surface acetification this decrease was significant. The hydrolysis of

acetaldehyde diethylacetal is probably favoured during acetification since this yields acetaldehyde, which is an intermediary metabolite in the transformation of ethanol to acetic acid and is therefore converted into acetic acid by the same metabolic pathway.

The levels of γ-butyrolactone were significantly lower in most acetifications, including all submerged acetifications. These findings do not agree with those observed during the submerged acetification of sherry wine (Morales et al., 2001b).

3.3. Influence of different woods on the volatile profile of vinegars

Oak is usually used to manufacture barrels for wine storage. Only occasionally is chestnut used, while the use of acacia, cherry and mulberry is even rarer. The effect of wood on the acetification process and the quality of vinegar has not yet been studied. In this study, we compared the use of chestnut, cherry and acacia with the use of oak. Tables 2–4 show that only for certain compounds does the type of wood used influence the final volatile composition of wine vinegars. As expected, isomers of oak-lactones were only quantified in vinegar produced in oak barrels (Tables 2 and 3). We also observed increases of ethyl furoate and ethyl benzoate in cherry wood barrels. Ethyl furoate is a furane-derived compound present in various wines, such as red (Escudero, Campo, Fariña, Cacho & Ferreira 2007), Sherry (Zea, Moyano, Moreno, Cortes & Medina, 2001; Moreno, Zea, Moyano & Medina, 2005) and Madeira (Campo, Ferreira, Escudero, Marqués & Cacho, 2006). However, it had never been described in wine vinegars. As Tables 3 and 4 show, although ethyl furoate is present in all the initial samples, its concentrations are highest in the final samples acetified in cherry wood barrels (increases of ca. 43%). We can therefore consider ethyl furoate and ethyl benzoate as specific characteristics of this type of wood.

Finally, the concentration of guaiacol grew significantly above its quantification limits in all woods (3.5–33.4 μg/L) (see Tables 3 and 4). Eugenol, another volatile

compound reported in vinegars as extracted from wood by lignin degradation (Morales, Benitez, and Troncoso, 2004; Pizarro et al., 2008) was poorly quantified in this study (Tables 3 and 4). This difference may be due to wood toasting. In a previous study (Morales, Benitez, and Troncoso, 2004), medium or heavy toasted barrels were used. In this study, however, the barrels had only the minimum toasting needed for manufacture.

3.4. Principal Component Analysis (PCA)

Data on the final volatile composition of vinegars were submitted to principal component analysis (PCA) to identify any groupings among the samples studied and the variables related with them. In this multivariate analysis these correlated variables were not considered (González, 2007). The first four PCs explained 89.8% of total variance and were chosen on the basis of Kaiser's criterion (eigenvalues of over 1.0) (Morales et al., 2001a). Figure 2 shows the score plot of the first two PCs. The first factor, which explains 40.40% of total variance, is mainly influenced (factor loading > 0.5) by variables 3-methyl-1-butanol, isobutyl acetate, benzaldehyde, ethyl propionate, acetaldehyde diethylacetal, and γ-butyrolactone. The second factor is closely related to octanoic acid, guaiacol, methyl acetate, and 2-methyl-1-butanol variables. The last two of these had negative values.

Regarding sample distribution into the plan made up the firt two PCs (Fig.2), the separation of the vinegar samples into three groups is evident. PC1 divides the samples into two groups depending on the acetification method (surface or submerged). PC2, on the other hand, divides the vinegars from the surface process according to the substrate used, however it groups vinegars LG and BH. These results show the importance of the process in the final volatile composition of the vinegars.

4. Conclusion

Acetification conditions clearly influence the final volatile composition of wine vinegars. Changes were significant for several compounds in surface culture, probably because of the longer time and the microorganisms involved. New volatile compounds such as ethyl furoate, ethyl benzoate and limonene have been identified for the first time in wine vinegars. Oak and cherry give the vinegars specific volatile compounds such as oak lactones in oak and ethyl furoate and ethyl benzoate in cherry wood. In general, the final quantitative volatile composition of vinegars was not affected by the type of wood used. Finally, multivariate statistical analysis supports the influence of the process on the volatile composition of the final vinegars.

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453

454 **Figure Captions**

455 Fig. 1. Contribution (%) of different groups of volatile compounds to the total content
456 of vinegar samples.

457 Fig. 2. Score plot of volatile compound variables and vinegar samples into the plan
458 made up of the first two principal components (PC1 against PC2).

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Figure(s)

Figure 1

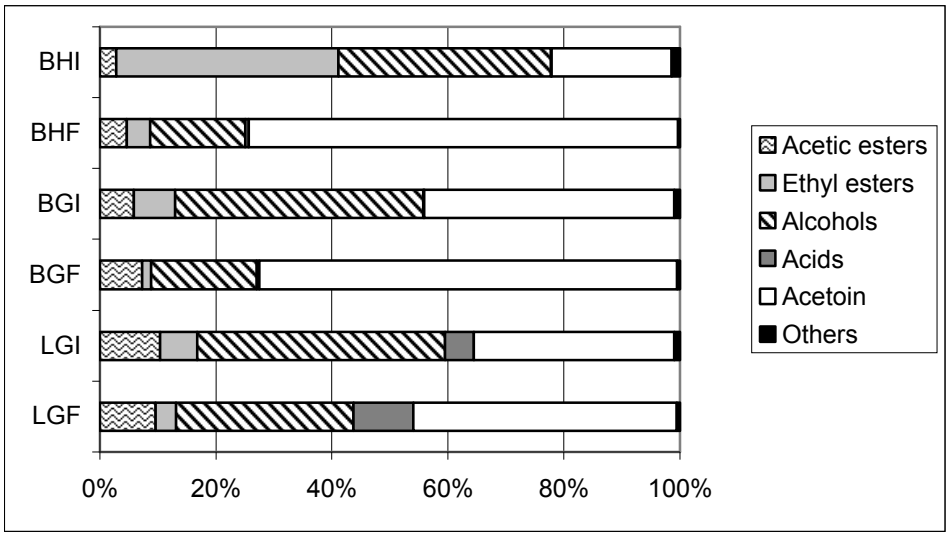
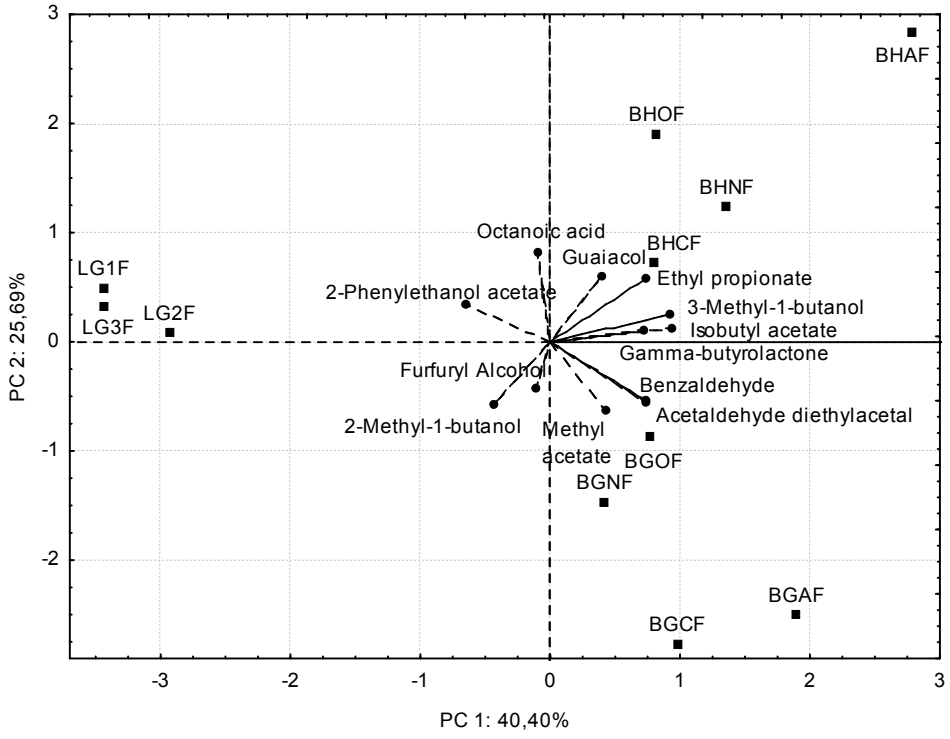


Figure 2



Table(s)

Table 1. Characteristics and codes of wine substrates and vinegar samples

Wine substrate	Acetification Method	Cycle or Kind of wood	Duration	Sampling point	
				Initial	Final
Substrate G: Alcohol (%v/v): 14.5 Glucose+Fructose: 20.9 g/ L + 43.4 g/ L pH: 3.4 Variety: 100% Grenache	Submerged culture (Laboratory fermentor) L	1	36 Hours	LG1I	LG1F
		2	30 Hours	LG2I	LG2F
		3	30 Hours	LG3I	LG3F
	Surface culture B Winery Banyuls (France)	Acacia (A)	1.5 Months	BGAI	BGAF
		Chestnut (N)	2 Months	BGNI	BGNF
		Cherry (C)	2 Months	BGCI	BGCF
		Oak (O)	2.5 Months	BGOI	BGOF
Substrate H: Alcohol (%v/v): 13.6 Glucose+Fructose: 0.36 g/ L+ 0.78 g/ L pH: 3.3 Variety: Grenache mostly	Surface culture B Winery Priorat (Spain)	Acacia (A)	9 Months	BHAI	BHAF
		Chestnut (N)	9 Months	BHNI	BHNF
		Cherry (C)	9 Months	BHCI	BHCF
		Oak (O)	8 Months	BHOI	BHOF

Table 2. Mean concentrations of volatile compounds at the beginning and at the end of the three cycles of submerged acetification

Nº	Compound	Concentration (µg/L) ± SD		Nº	Compound	Concentration (µg/L) ± SD	
		LGI	LGF			LGI	LGF
Acetals				Alcohols			
1	Acetaldehyde diethylacetal*	388 ± 214[a]	32 ± 15[a]	23	Isobutanol*	7.8 ± 1.7[a]	7.8 ± 2.0[a]
Aldehydes				24	2-Methyl-1-butanol*	10.7 ± 2.8 [a]	8.2 ± 0.9[a]
2	2-Furfuraldehyde	132 ± 75[a]	44 ± 76[a]	25	3-Methyl-1-butanol*	33 ± 7[a]	16.3 ± 1[b]
3	Benzaldehyde	26 ± 9[a]	32 ± 6[a]	26	1-Hexanol	215 ± 49[a]	nd [b]
	Total aldehydes	158[a]	76[a]	27	*Cis*-3-hexen-1-ol	29 ± 7[a]	19 ± 2[b]
Acetic esters				28	Furfuryl alcohol	359 ± 371[a]	291 ± 328[a]
4	Methyl acetate*	10.3 ± 3.0[a]	12.1 ± 2.9[b]	29	Benzyl alcohol	157 ± 36[a]	156 ± 16[a]
5	Propyl acetate	505 ± 135[a]	331 ± 116.4[b]	30	2-Phenylethanol*	21 ± 7[a]	23 ± 8[a]
6	Isobutyl acetate*	1.6 ± 0.2[a]	1.1 ± 0.1[b]		Total Alcohols*	74.0[a]	56.4[a]
8	Isoamyl acetate*	4.2 ± 0.9[a]	2.4 ± 0.6[b]	Acids			
11	2-Phenylethyl acetate*	1.4 ± 0.4[a]	1.8 ± 0.7[a]	31	Isovaleric acid*	8.6 ± 2.9[a]	18.4 ± 2.4[b]
	Total Acetic Esters*	17.9[a]	17.7[a]	32	Hexanoic acid	nd[a]	394 ± 142[b]
Ethylic esters				34	Octanoic acid	30 ± 52[a]	220 ± 83[b]
12	Ethyl propanoate	195 ± 33[a]			Total Acids*	8.64[a]	19.1[b]
13	Ethyl isobutyrate	161± 16[a]	151 ± 48[a]	Terpenes			
14	Ethyl butyrate	nd	nq	35	Limonene	81 ± 9[a]	76 ± 14[a]
15	Ethyl isovalerate	83 ± 19[a]	93 ± 34[a]	Lactones			
16	Ethyl hexanoate	nq	nd	36	γ-Butyrolactone	1182 ± 264[a]	811 ± 75[a]
17	Ethyl lactate*	9.2 ± 3.9[a]	4.5 ± 0.7[a]		Total lactones	1182[a]	811[a]
19	Ethyl Furoate	12.3 ± 11[a]	5.4 ± 9.3[a]	Phenols			
21	Diethyl succinate*	1.2 ± 0.4[a]	1.6 ± 0.7[a]	39	Guaiacol	5.7 ± 0.6[a]	7.9 ± 2.3[a]
	Total Ethylic Esters*	11.0[a]	6.5[a]	40	Eugenol	nq	nq
Ketones					Total Phenols	5.7[a]	7.9[a]
22	Acetoin*	60 ± 23[a]	84 ± 10[a]		Total Anounts*	173.2[a]	184.4[a]

* Concentration in mg/L
nd: not detected
nq: below quantification limit
[a, b] Initial and final concentration with different letter as superscript are significatively different (p<0.05)

Table 3. Change of volatile compounds in surface acetification of wine substrate G

Nº	Compound	Mean concentration (µg/L) ± SD							
		BGAI	**BGAF**	**BGNI**	**BGNF**	**BGCI**	**BGCF**	**BGOI**	**BGOF**
Acetals									
1	Acetaldehyde diethylacetal*	685 ± 30	255 ± 43	357 ± 10	128 ± 11	385 ± 30	274 ± 32	383 ± 26	193 ± 10
Aldehydes									
2	2-Furfuraldehyde	92 ± 7	76 ± 8	159 ± 15	58 ± 7	177 ± 27	94± 14	129 ± 19	71 ± 7
3	Benzaldehyde	nd	106 ± 1	nd	98 ± 4	56.2 ± 5.7	134 ± 4	61.6 ± 2.7	81 ± 3
	Total Aldehydes	92	182	148	156	223	228	191	152
Acetic Esters									
4	Methyl acetate*	9.48 ± 1.23	22.5 ± 2.8	9.2 ± 0.4	20.5 ± 2.3	9.1 ± 1.0	20.90 ± 2.2	13.9 ± 1.4	15.5 ± 1.1
5	Propyl acetate	570 ± 71	1032 ± 30	322 ± 30	951 ± 30	334 ± 38	741 ± 58	551 ± 56	1041 ± 103
6	Isobutyl acetate*	0.70 ± 0.04	2.0 ± 0.3	0.61 ± 0.08	1.65 ± 0.06	0.53 ± 0.06	1.9 ± 0.1	0.72 ± 0.05	1.57 ± 0.01
8	Isoamyl acetate*	1.6 ± 0.2	5.0 ± 0.7	1.8 ± 0.3	3.8 ± 0.2	1.5 ± 0.2	5.2 ± 0.5	1.9 ± 0.1	4.1 ± 0.2
9	Hexyl acetate	nd	56 ± 6	nd	nq	nd	nq	nd	nq
11	2-Phenylethyl acetate*	nd	1.0 ± 0.1	nd	1.20 ± 0.02	nd	1.6 ± 0.2	nd	1.1 ± 0.1
	Total Acetic Esters*	12.4	31.6	11.9	28.0	11.6	30.3	17.0	23.3
Ethylic esters									
12	Ethyl propanoate	263 ± 33	332 ± 34	240 ± 20	228 ± 4	235 ± 14	192 ± 1	243 ± 23	224 ± 18
13	Ethyl isobutyrate	287 ± 22	521 ± 79	291 ± 32	297 ± 9	246 ± 17	332 ± 28	261 ± 18	361 ± 33
14	Ethyl butyrate	125 ± 7	nq	136 ± 15	nd	118.1 ± 0.4	nq	129 ± 13	nq
15	Ethyl isovalerate	nq	nq	nq	nq	nq	nq	nq	nq
16	Ethyl hexanoate	105 ± 5	44 ± 7	111 ± 6	nq	97 ± 6	nq	104 ± 12	nq
17	Ethyl lactate*	15.2 ± 1.9	7.0 ± 1.0	13.4 ± 0.5	2.9 ± 0.4	13.5 ± 0.1	2.2 ± 0.2	10.3 ± 1.6	2.5 ± 0.2
18	Ethyl octanoate	nq	nq	107 ± 14	nd	nq	nd	nq	nd
19	Ethyl furoate	28.4 ± 1.7	28.6 ± 0.5	27 ± 3	28.9 ± 0.7	35 ± 3	75 ± 7	28 ± 3	29.9 ± 1.4
20	Ethyl benzoate	nd	nd	nd	nd	nd	nq	nd	nd
21	Diethyl succinate*	2.4 ± 0.2	1.5 ± 0.1	2.1 ± 0.3	1.8 ± 0.2	2.74 ± 0.01	2.1 ± 0.3	1.5 ± 0.2	1.5 ± 0.2
	Total Ethylic Esters*	18.3	9.42	16.5	5.27	16.9	4.91	12.5	4.54
Ketones									
22	Acetoin*	107 ± 11	339 ±43	86 ± 13	297 ± 49	73.7 ± 8.8	280 ± 37	124 ± 16	209 ± 15
Alcohols									
23	Isobutanol*	22.0 ± 1.6	10.1 ± 0.2	22.4 ± 1.7	5.8 ± 0.4	19.8 ± 1.5	10.2 ± 0.9	19.8 ± 0.3	7.2 ± 1.1
24	2-Methyl-1-butanol*	9.5 ± 0.5	9.7 ± 1.3	9.7 ± 0.4	5.8 ± 0.7	8.4 ± 1.1	8.4 ± 1.1	8.72 ± 0.04	5.7 ± 0.5
25	3-Methyl-1-butanol*	50.2 ± 0.3	49 ± 7	51 ± 5	30.8 ± 0.4	47.9 ± 1.5	36.9 ± 1.7	48.7 ± 0.5	33.6 ± 0.8
26	1-Hexanol	427 ± 5	307 ± 34	465 ± 28	180 ± 4	474 ± 16.7	227 ± 14.3	439 ± 43	227 ± 6
27	*Cis*-3-hexen-1-ol	47 ± 2	47.1 ± 3.1	52.9 ± 2.3	30.0 ± 0.1	52.5 ± 3.3	38.5 ± 1.6	48.1 ± 4.5	30.5 ± 0.7
28	Furfuryl alcohol	389 ± 48	350 ± 37	792 ± 110	551 ± 82	703 ± 86	197 ± 18	324 ± 44	357 ± 45

* Concentration in mg/L
nd: not detected
nq: below quantification limit

Table 3. (Continued)

Nº	Compound	Mean concentration (µg/L) ± SD							
		BGAI	**BGAF**	**BGNI**	**BGNF**	**BGCI**	**BGCF**	**BGOI**	**BGOF**
Alcohols									
29	Benzyl alcohol	216 ± 14	287 ± 40	143 ± 14	158 ± 3	143 ± 10	137 ± 13	148 ± 7	143 ± 3
30	2-Phenylethanol*	17.5 ± 2.2	17.6 ± 2.4	16.8 ± 2.2	17.5 ± 0.5	17.5 ± 0.4	19.2 ± 2.4	13.1 ± 1.5	13.9 ± 1.4
	Total Alcohols*	100.3	87.6	101.0	60.6	94.9	75.3	91.3	61.1
Acids									
31	Isovaleric acid*	0.40 ± 0.05	1.07 ± 0.05	0.46 ± 0.06	1.5 ± 0.1	0.53 ± 0.03	0.69 ± 0.08	0.29 ± 0.03	1.82 ± 0.02
32	Hexanoic acid	nd	504 ± 59	nd	506 ± 22	nd	nd	nd	167 ± 24
34	Octanoic acid	nd	100 ± 14.9	nd	162 ± 4	nd	nd	nd	92 ± 1
	Total Acids*	0.40	1.68	0.46	2.15	0.53	0.69	0.41	1.39
Terpenes									
35	Limonene	74.8 ± 2.4	166 ± 22	93.7 ± 1.5	140 ± 4.2	75.3 ± 0.7	142 ± 15	78.4 ± 1.5	118 ± 4.2
Lactones									
36	γ-Butyrolactone	1509 ± 14	1343 ± 184	1955 ± 188	1299 ± 187	1648 ± 123	957 ± 85	1193 ± 182	1373 ± 140
37	*Trans*-β-methyl-γ-octalactone	nd	nd	nd	nd	nd	nd	nd	74.5 ± 1.3
38	*Cis*-β-methyl-γ-octalactone	nd	nd	nd	nd	nd	nd	nd	183 ± 13
	Total Lactones	1509	1343	1955	1299	1648	957	1193	1631
Phenols									
39	Guaiacol	nd	5.8 ± 0.7	nq	3.45 ± 0.12	nd	7.88 ± 0.22	nq	14.40 ± 0.16
40	Eugenol	nq	nq	nq	nq	nq	nq	nq	nq
41	4-Ethylphenol	13.8 ± 0.6	31.4 ± 0.5	22.1 ± 2.1	139 ± 11	13.7 ± 1.1	54 ± 6	nq	22.8 ± 0.9
	Total phenols	348	625	176	556	225	381	451	612
	Total amounts*	240.8	471.5	217.0	395.3	200.2	393.1	247.3	302.4

* Concentration in mg/L
nd: not detected
nq: below quantification limit

Table 4. Change of volatile compounds in surface acetification of wine substrate H

Nº	Compound	Mean concentration (µg/L) ± SD							
		BGAI	BGAF	BGNI	BGNF	BGCI	BGCF	BGOI	BGOF
Acetals									
1	Acetaldehyde diethylacetal*	2116± 266	116± 5	2602± 261	161± 8	1043± 63	210± 15	3518± 153	91± 3
Aldehydes									
2	2-Furfuraldehyde	548 ± 74	nq	490 ± 52	68 ± 7	294 ± 38	nd	251 ± 30	62 ± 7
3	Benzaldehyde	nq	81 ± 3	nd	nq	370 ± 1	78 ± 4	41 ± 3	54.5 ± 1.7
	Total Aldehydes	548	81	490	112	663	78	292	116
Acetic esters									
4	Methyl acetate*	9.4 ± 0.8	17.2 ± 0.9	8.9 ± 0.3	10.1 ± 0.4	7.12 ± 0.134	8.33 ± 0.6	7.1 ± 0.7	13.4 ± 0.6
5	Propyl acetate	666 ± 7	274 ± 227	480 ± 56	2034 ± 48	342 ± 36	2083 ± 97	501 ± 24	2242 ± 199
6	Isobutyl acetate*	0.54 ± 0.07	2.3 ± 0.1	0.43 ± 0.02	1.7 ± 0.09	0.45 ± 0.07	1.7 ± 0.1	0.5 ± 0.01	2.06 ± 0.03
7	Butyl acetate	nq	99± 6	nq	106 ± 11	nq	109 ± 12	nq	106 ± 4
8	Isoamyl acetate*	2.1 ± 0.3	7.1 ± 0.4	1.83 ± 0.04	5.5 ± 0.2	1.64 ± 0.15	5.2 ± 0.4	2.16 ± 0.13	6.58 ± 0.13
9	Hexyl acetate	nd	101.9 ± 2.4	nd	68.9± 2.2	nd	66.0 ± 5	nd	74.1± 2.2
10	Benzyl acetate	nd	190 ± 19	nd	135 ± 10	nd	nq	nd	152 ± 4
11	2-Phenylethyl acetate*	nd	1.70 ± 0.14	nd	1.26 ± 0.19	nd	1.17 ± 0.11	nd	1.62 ± 0.01
	Total Acetic Esters*	12.7	31.4	11.7	20.9	9.56	18.6	10.3	26.2
Ethylic esters									
12	Ethyl propanoate	687 ± 59	614 ± 17	660 ± 95	645 ± 56	318 ± 27	544 ± 44	584.9 ± 0.1	568 ± 16
13	Ethyl isobutyrate	443 ± 68	383 ± 48	415 ± 24	420 ± 21	202 ± 23	345 ± 32	491 ± 21	484 ± 16
14	Ethyl butyrate	310 ± 48	69 ± 6	284 ± 18	59 ± 4	171 ± 7	56 ± 6	305 ± 20	78 ± 4
15	Ethyl isovalerate	151± 21	nq	133 ± 3	nq	63 ± 3	nq	159 ± 18	nq
16	Ethyl hexanoate	272 ± 31	57 ± 3	243 ± 6	55.1 ± 1.6	142 ± 4	44 ± 7	280 ± 24	54 ± 3
17	Ethyl lactate*	164 ± 25	7.2 ± 0.7	140.1 ± 14.9	11.0 ± 1.5	96.2 ± 2.1	7.86± 0.25	126.4 ± 1.1	5.23 ± 0.21
18	Ethyl octanoate	370 ± 37	nd	334 ± 37	nq	nq	nd	410 ± 15	nq
19	Ethyl furoate	112 ± 7	79 ± 13	107 ± 12	84.2± 0.5	98 ± 8	150 ± 17	115 ± 14	76.3 ± 1.8
20	Ethyl benzoate	nd	nd	nd	nd	nd	107 ± 3	nd	nd
21	Diethyl succinate*	17.7 ± 2.0	13.6 ± 1.7	13.7 ± 1.8	11.9 ± 1.4	15.0 ± 2.0	10.7 ± 1.3	23.5 ± 0.17	12.0 ± 0.8
	Total Ethylic Esters*	183.9	22.0	156.1	24.1	112.2	19.8	152.2	18.4
Ketones									
22	Acetoin*	117± 20	419.7 ± 49.2	84 ± 15	495± 56	51 ± 4	271.1± 0.2	74.0 ± 10.7	364 ± 17
Alcohols									
23	Isobutanol*	27.2 ± 0.4	7.1 ± 0.2	31 ± 4	6.9 ± 0.3	28.8 ± 1.6	6.6 ± 0.5	31.5 ± 2.4	7.61 ± 0.06
24	2-Methyl-1-butanol*	8.4 ± 0.9	5.55 ± 0.17	8.9 ± 0.9	4.88 ± 0.11	8.8 ± 0.6	4.66 ± 0.24	9.9 ± 0.9	5.09 ± 0.09
25	3-Methyl-1-butanol*	84.1 ± 1.3	54.4 ± 1.7	93.1 ± 12.2	54± 3	80 ± 3	49.5 ± 0.3	90.0± 0.1	49.3 ± 0.7
26	1-Hexanol	875 ± 6	384 ± 29	824 ± 9	456 ± 29	781 ± 37	345 ± 3	895 ± 4	362 ± 6
27	*Cis*-3-hexen-1-ol	69 ± 7	39.8 ± 1.5	71 ± 8	52 ± 5	58.9 ± 2.2	37.2 ± 1.1	58 ± 4	44.4 ± 2.1
28	Furfuryl alcohol	1403 ± 160	80 ± 8	1242 ± 146	348 ± 37	682 ± 76	88 ± 7	613 ± 37	80 ± 7

*Concentration in mg/L
nd: not detected
nq: below quantification limit

Table 4. (Continued)

Nº	Compound	Mean concentration (µg/L) ± SD							
		BGAI	**BGAF**	**BGNI**	**BGNF**	**BGCI**	**BGCF**	**BGOI**	**BGOF**
Alcohols									
29	Benzyl alcohol	2935 ± 322	3727 ± 583	1655 ± 210	3137 ± 120	1850 ± 207	1159 ± 66	3073 ± 213	3375 ± 213
30	2-Phenylethanol*	17 ± 2	22 ± 3	9.3 ± 1.3	17.9 ± 1.7	11.8 ± 1.5	13.7 ± 1.0	19.8 ± 1.7	20.2 ± 0.5
	Total Alcohols*	141.8	93.7	146.0	87.3	133.1	76.1	155.9	86.2
Acids									
31	Isovaleric acid*	1.20 ± 0.05	1.89 ± 0.04	0.24 ± 0.02	2.64 ± 0.01	0.37 ± 0.04	2.4 ± 0.3	0.36 ± 0.06	1.82 ± 0.09
32	Hexanoic acid	nd	1078 ± 161	nd	662± 83	nd	553 ± 67	nd	1029 ± 65
33	Heptanoic acid	nd	57.7 ± 5.4	nd	nd	nd	nq	nd	nq
34	Octanoic acid	nd	342 ± 40	nd	196 ± 21	nd	183 ± 20	nd	273 ± 41
	Total Acids*	1.20	3.31	0.24	3.50	0.37	3.18	0.36	3.12
Terpenes									
35	Limonene	nq	119 ± 3	nq	106± 3	nq	97 ± 7	nq	122 ± 2
Lactones									
36	γ-Butyrolactone	4070 ± 407	1710 ± 107	2842 ± 368	1427 ± 207	2166 ± 27	709 ± 83	2286 ± 296	1006 ± 81
37	*Trans*-β-methyl-γ-octalactone	nd	nd	nd	nd	nd	nd	nd	137 ± 16
38	*Cis*-β-methyl-γ-octalactone	nd	nd	nd	nd	nd	nd	nd	446 ± 54
	Total Lactones	4070	1710	2842	1427	2166	709	2286	1589
Phenols									
39	Guaiacol	6.38 ± 0.12	33.43 ± 0.24	7.4 ± 0.2	9.0 ± 0.9	10.0 ± 0.2	6.0 ± 0.7	4.14 ± 0.03	11.3 ± 0.4
40	Eugenol	nd	nd	nd	106 ± 13	nd	nd	nd	nd
41	4-Ethylphenol	342 ± 37	592 ± 81	169 ± 13	441 ± 51	215 ± 18	375 ± 47	447 ± 61	515 ± 67
	Total Phenols	348	625	176	556	225	381	451	612
	Total amounts*	463.8	572.7	404.4	633.6	310.1	390.2	399.4	500.7

* Concentration in mg/L
nd: not detected
nq: below quantification limit

Manuscript 4

Raquel M. Callejón; M. Jesús Torija; Albert Mas; M. Lourdes Morales;

Ana M. Troncoso.

CHANGES OF VOLATILE COMPOUNDS IN WINE VINEGARS DURING AGING IN BARRELS MADE FROM DIFFERENT WOODS

Manuscript in the final stage of drafting

TITLE: CHANGES OF VOLATILE COMPOUNDS IN WINE VINEGARS DURING AGING IN BARRELS MADE FROM DIFFERENT WOODS

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ABSTRACT

Changes in volatile components of red wine vinegars and balsamic vinegars were investigated during aging in wood barrels and bottles. Samples were taken at the beginning (t = 0) and at the end (t = 1 year) of aging. Barrels of four different woods (oak, chestnut, acacia and cherry) were used. A total of 57 volatile compounds were analysed by Gas Chromatography-Flame Ionization Detector (GC-FID) and Headspace Sorptive Extraction GC–Mass Spectrometry (HSSE-TD-GC-MS). We only observed significant increases in the total content of volatile compounds for balsamic vinegars. When we compared aging in bottle versus wood barrels, we found that the overall content of volatile compounds was superior in vinegars aged in bottle, probably due to sorption phenomena on new wood barrels. Oak-lactones presented the highest increases for those vinegars aged in oak barrels. Ethyl furoate, ethyl benzoate, benzaldehyde and acetophenone reached the highest concentrations in cherry wood barrels hence, these

compounds seems to be characteristic of this wood. Eugenol was only present in vinegars aged in chestnut and oak barrels.

KEYWORDS

Volatile compounds; aging; wine vinegar; wood; HSSE-GC-MS

1. Introduction

Aging in barrels is a very old tradition extensively used to store and age wine and other beverages for centuries (Waterhouse & Towey, 1994). Most casks used to age wine, spirits and another wine derived products are made of oak wood (Pérez-Coello, Sanz & Cabezudo, 1997). The oak wood used in wine-making comes mainly from two sources: American oak (*Quercus alba*) and French oak (*Quercus robur* and *Quercus petraea*). Their chemical compositions have been carefully studied and are quantitatively different (Singleton, 1995; Díaz-Maroto, Guchu, Castro-Vazquez, de Torres, & Pérez-Coello, 2008; Bozalongo, Carrillo, Fernández Torroba, & Tena, 2007).

The production of quality wine vinegars involves a maturation step in wood barrels, this happens in the case of Jerez-Xérès-Sherry DO (denomination of origin), where the oak used is *Quercus alba* (American oak) and the minimum time required for a vinegar to be considered Sherry vinegar is six months (Consejería de Agricultura y Pesca, 2000). Another traditional vinegar marketed all around the world is the aceto balsamico tradizionale of Modena, where the fermented cooked must is aged for a long time (at least 12 years) in a set of barrels of different woods and volumes (Giudici, Altieri, & Cavalli, 1992; Cocchi, Durante, Grandi, Manzini, & Marchetti, 2008).

During vinegar aging a series of processes that cause important improvements in the sensorial complexity take place. Aging in wood involves changes in colour, polyphenolic profile (Tesfaye, Morales, García-Parrilla, & Troncoso, 2002) and aroma of wine vinegars (Morales, Tesfaye, García-Parrilla, Casas & Troncoso, 2002). It is well

known that while vinegars are aged in barrels, the volatile compounds are enriched mainly as a result of two important processes: they are concentrated because water is lost through the wood pores and new compounds are formed, such as esters (Morales et al., 2002). In addition, oak wood release numerous specific compounds to wine, many of which are formed during the toasting of the wood (Garde-Cerdán, & Ancín-Azpilicueta, 2006). In addition, barrel oak wood is a porous material that allows occurring processes associated with the so-called "low oxidation conditions" (Vivas, & Glories 1993).

Up to date, oak is the wood usually employed in the aging of wines or vinegars although occasionally chestnut and more rarely acacia, cherry or mulberry are also used. However, the influence of the type of wood on the aroma of the aged vinegars has not been studied yet. The porosity and the chemical composition of each type of wood are different and they may affect to the final aroma of vinegars. Effective methods for evaluating the transfer are being employed (Vivas, Debeda, Menil, de Gaulejac, & Nonier, 2003). Empirical data collected suggest that mulberry has a higher porosity than other woods such as acacia, cherry or chesnut, although the porosity of these last three is superior to oak wood.

The goals of the present work are, first to evaluate the changes in the aroma profile that take place during the aging of red and white wine vinegars in barrels made from four different types of wood (chestnut, acacia, cherry and oak); second to study the influence of each type of wood in the aroma of the aged vinegars; and third to compare final aroma of vinegars aged in wood with vinegars aged in glass bottles.

2. Experimental

2.1. Samples.

Twelve wine vinegars produced from three wines (two red wines and one white wine) were submitted to aging in four types of 60 L wood barrels (oak, chestnut, acacia and cherry). Each of the three series was aged in a different winery (Table 1), placed in Banyuls (France), Priorat (Spain) and Reggio Emilia (Italy). The balsamic vinegar was elaborated mixing cooked grape must and white wine vinegar. Vinegars were aged during one year in the same type of barrels were they had been previously acetified. The 60 L barrels were manufactured by the barrel-making Torner (Vilafranca del Penedes, Spain). In addition, four simultaneous aging controls in 8 L glass bottles closed by cork stoppers were carried out in winery 2 (Priorat). Hence, a total of sixteen aging processes were studied taking samples at the beginning and at the end of aging (Table 1), 0 and 12 months, respectively.

2.2. Reagents and Chemicals.

The standards of 57 aroma compounds, given in Tables 2-5 were obtained from the commercial sources as follows: 2, 3, 14, 15, 19-21, 23-27, 29-32, 40-42, 45-51, 53-57 (Sigma-Aldrich, Madrid, Spain); 1, 4, 6-10, 13, 17, 18, 28, 34-39, 44, 52 (Merck, Darmstadt, Germany); 5, 11, 12, 16, 22, 33, 43 (Fluka, Madrid, Spain). 4-methyl-2-pentanol (Merck) was employed as internal standard (IS). Water was obtained from a Milli-Q purification system (Millipore, USA).

2.3. Volatile compounds analysis.

We used two methods of analysis since expected volatile compounds have different volatilities and wide range of concentrations. Hence, major volatile compounds were determined by direct injection using Gas Chromatography-Flame Ionization Detector (GC-FID). Minor compounds were previously extracted by Headspace Sorptive Extraction (HSSE) and determined by Gas Chomatography-Mass Spectrometry (HSSE-GC-MS).

GC-FID Analysis. Ethyl acetate, acetaldehyde, metanol, ethanol and propanol were quantified by GC-FID using the method proposed by Morales, Benitez, & Troncoso (2004). 1 mL sample was filtered through Millex-GV_{13} filters of 0.22 μm and 1 μL of 4-methyl-2-pentanol at 102.14 mg/L was added as internal standard (IS). Filtered samples were analyzed using a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector (FID). 1 μL was injected in the split mode (1:60) into a column CP-Wax 57 CB, 50 m x 0.25 mm DI x 0.2 μm film thickness (Varian, Middelburg, Netherlands). The carrier gas was H_2 at 1 mL/min. The program temperature was: 35 ºC for 5 min, ramped at 4 ºC/min to 150 ºC held for 17.5 min. The injector was set to 220 ºC and the detector to 250 ºC. Data acquisition software was HPChemstation data processing system (Agilent Technologies).

HSSE-GC-MS Analysis. The HSSE sampling conditions were as follows (Callejón, Troncoso, & Morales, 2008): 5 mL of sample (wine vinegar) and 10 μL of 4-methyl-2-pentanol (IS) at 1045 mg/L was placed into a 20-mL headspace vial with 1.67 g of NaCl. A 10 mm long stir bar coated with 0.5 mm polydimethylsiloxane (PDMS) layer (Twister, Gerstel, Müllheim an der Ruhr, Germany) was put in an open glass insert and placed into the vial to achieve the extraction in the headspace. Then, the vial was tightly capped and heated for 60 minutes at 62 ºC in a thermostatic bath. The stir bar was removed with tweezers, rinsed with Milli-Q water and dried with a lintfree tissue paper. Finally, for the thermal desorption (TD), the stir bar was placed into a glass tube of 60 mm in length, 6 mm o.d. and 4 mm i.d., which was placed in the autosampler tray of the thermo desorption unit for GC-MS analysis.

Gas Chromatography analysis was carried out with a 6890 Agilent GC system coupled to a quadrupole mass spectrometer Agilent 5975inert and equipped with a Gerstel, Thermo Desorption System (TDS2) and a cryo-focusing CIS-4 PTV injector (Gerstel).

The thermal desorption was performed in splitless mode and with a flow rate of 90 mL/min. Desorption temperature program was the following: 35 ºC for 1 min, ramped at 60 ºC/min to 250 ºC held for 5 min. The CIS-4 PTV injector, with a Tenax TA inlet liner, was held at –35 ºC with liquid nitrogen for total desorption time and then raised at 10 ºC/s to 290 ºC and held for 4 min. Solvent vent mode was employed for transfer of sample to analytical column. A CPWax-57CB column, 50 m x 0.25 mm, 0.20 μm film thickness (Varian, Middelburg, Netherlands) was used and the carrier gas was He at a flow rate of 1 mL/min. Oven temperature program: 35 ºC for 5 min, then raised to 220 ºC at 2.5 ºC/min (held 5 min). The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 280 respectively. Electron ionization mass spectra in the full-scan mode were recorded at 70 eV electron energy in the range 35 to 350 amu.

All data were recorded using a MS ChemStation. The identity of peaks was assigned using the NIST 98 library and confirmed by retention index of standards when they were available. Quantification was performed employing the relative area to internal standard of the target ion of each compound. The samples were analyzed by triplicate and blank runs of empty glass tube were done before and after each analysis. RIs were calculated from the retention times of n-alkanes by linear interpolation, according to the literature (Silva Ferreira, Hogg, & Guedes de Pinho, 2003).

2.4. Statistical analysis.

One-way analysis of variance (for the 57 volatile compounds determined) were performed by means of Statistica, version 7.0 software (Statsoft, Tulsa, USA).

3. Results and Discussion

A total of 28 samples, belonging to wine vinegars aged in wood and in bottles, were analysed for 57 volatile compounds. Their respective concentration appears in Tables 2-4: Data are collected in chemical families.

Alcohols is the major group of compounds in finished vinegars due to the high amounts of residual ethanol (19-42 g/L). The next group of volatile compounds in order of abundance is acetic esters being the principal compound of this fraction ethyl acetate.

The main phenomenon that takes place during aging is the enrichment of the aroma due to the loss of water through the wood pores (Singleton, 1995). However, in this experiment we only observed significant increases of the total volatile concentration during the aging of samples from winery 3, while vinegars aged in winery 2 suffered significant decreases. Vinegars from the winery 3 showed enrichment in all the groups except ketones.

Regarding the evolution of the different group of compounds, volatile acids were the only group that significantly increased in all the aging processes. In wines fermented in wood, these compounds also increase, hence they seem to be extracted from wood and specially favoured by the use of new barrels (González-Marco, Jiménez-Moreno, & Ancín-Azpilicueta, 2008). However, we observed that acids also increased in samples stored in bottle, the average augment was similar to the observed in vinegars from winery 1 and 3. Hexanoic and heptanoic acids suffered significant increases in all aging experiences and in most of cases for octanoic and isovaleric acid. This last acid was the most abundant according with previous findings in wine vinegar (Guerrero, Natera, Castro, & Barroso, 2007). Isovaleric acid has an odor described like cheese or foot (Aznar, Lopez, Cacho & Ferreira, 2001), its threshold for vinegar is 150 μg/L (Callejón,

Morales, Silva Ferreira, Troncoso, 2008a) and in high concentrations could result an off-flavour.

Esters are continuously formed and hydrolysed and their oscillations are independent of wood contact. Equilibrium is governed by relative abundance of acids and alcohols, pH and temperature, among others.

Different authors have reported that acetic esters are formed during the aging of vinegars in outstanding quantities (Morales et al., 2002; Morales, González, Casas, & Troncoso, 2001), mainly ethyl acetate. As can be seen in Tables 2-5, ethyl acetate was the most abundant acetic ester followed by methyl acetate, both compounds make a different contribution to vinegar aroma due to the fact that they do not present fruity aromas as the rest of acetic esters do. Ethyl acetate suffered significant changes in all the aging processes, increasing in vinegars from winery 1 and 3 and decreasing in samples from 2 (bottle and wood aging). By the contrary, methyl acetate increased significantly in all aging process. The formation of methyl acetate is favoured by the acidic environment (Morales et al., 2002) and high amounts of methanol. Red wines have a higher concentration of this alcohol (152 mg/L) than white wines (63mg/L) due to hydrolysis of methoxyl group of the skin grape pectins (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). According with this, initial concentrations of methanol in red vinegars ranged 79-193 mg/L while white vinegars were 20-23 mg/L, and consequently methyl acetate reached higher concentrations in red vinegars (21-42 mg/L) than in white vinegars (13-23 mg/L). For the remaining acetic esters, significant increases are observed for white wine vinegar (group 3) during aging. This fact will probably contribute to a higher fruity character as result of wood aging.

In wines, ethyl esters of fatty acids are produced during yeast fermentation and they decrease during aging to reach a chemical equilibrium. However, in vinegars these

compounds decrease during surface acetification process due to the consumption of ethanol by acetic bacteria (Callejón, Tesfaye, Torija, Mas, Troncoso, & Morales, 2008b). As can be observed in Tables 2-5, they increased during aging, probably to reach the chemical equilibrium. These increases were statistically significant for ethyl isobutyrate and ethyl isovalerate in all the processes and in most of the cases for propanoate and ethyl hexanoate.

Aldehydes and lactones reached higher concentrations at the end of the aging in all the cases, being the changes statistically significant for samples from winery 2.

3.1. Aging in barrels versus bottles

A comparative study of two kinds of aging, in wood barrel and in glass bottles, was carried out in one of the wineries (group 2).

The overall content of volatile compounds was superior in vinegars aged in bottle, accounting statistically significant differences with respect to the vinegars from wood barrels at the end of one year aging. This result could be explained because, as occur in wine (Jarauta, Cacho, & Ferreira, 2005), several compounds disappear in the vinegar stored in wood as a likely consequence of sorption processes on the wood. The phenomenon is more probably to occur when new barrels are employed as is our case.

Aldehydes and alcohols reach significantly higher amounts in vinegars from bottles. The samples aged in wood barrels showed important increases of acetic acid content (medium increases in acetic acid degree ranging from 1.8 to 2.5) together with a corresponding decrease in ethanol concentration. This fact points out that acetification is probably taking place simultaneously with aging. Acetic acid degree of samples stored in bottles also increased although in a lesser extent (increases ranging from 0.1 to 0.7), displaying a possible microbiological activity except for the sample 2ABF where ethanol content did not decrease (Figure 1). In both aging experiments (wood and

bottle), significant decreases of ethyl acetate were produced in most cases as a consequence of ethanol requirement of acetic acid bacteria that shift the chemical equilibrium.

Tesfaye, Morales, Benitez, Garcia-Parrilla, & Troncoso (2004) reported increases of acetaldehyde during an experimental aging in bottle of wine vinegar due to the oxidation of ethanol. We have observed in our aging bottle assay (Tables 3-4) increases of acetaldehyde in all the cases. This compound is an intermediary metabolite of acetification and it can be transformed in acetic acid by bacteria or lost from the barrel due to its high volatility.

Diacetyl is a compound formed from the oxidation of acetoin and it is related to aging in oak barrels of sherry vinegars (Morales et al., 2002; Callejón, Morales, Troncoso, Silva Ferreira, 2008c). Nevertheless we found this compound in all our bottle experiments and it only was found in wood when higher increase of acetoin was observed.

Limonene trends to augment in bottle aging whilst in wood disappear.

These facts could be explained by the sorption phenomenon in wood already hypothesized for other terpenes (linalool) in wine aging experiments (Garde-Cerdán et al., 2006).

3.2. Influence of the different woods in the aromatic profile of aged vinegars

In this study we have used four types of wood to acetify and subsequently maturate wine vinegars to ascertain if the kind of wood has any influence on the final quality of aroma. Significant differences in the total volatile content of final samples (1 year aging) have been found. If we remove ethanol and ethyl acetate as major aroma compounds whose concentrations are too high to analyse differences among samples, and consider the total amount of the rest of volatile compounds they are similar in all

the aged vinegars independently of the kind of wood employed and there are not significant differences among them.

We observed differences in certain aroma compounds which showed different trends depending on the kind of wood, as follows.

As expected, isomers of oak-lactones presented higher concentrations in vinegars aged in oak wood, being the *cis* isomer which suffered major increases. The cis/trans ratios obtained (ranging 5-8), are in accordance with the values found in wines aged in American oak barrels (Towey & Waterhouse, 1996). These compounds reached concentrations ca. twenty times higher than its detection threshold in vinegar (78 μg/L) (Callejón et al., 2008a), hence vinegars aged in oak wood will present the typical "wood" aroma.

In samples from winery 3 (balsamic vinegars, Italy), small amounts of oak lactones appear in all vinegars at the beginning of aging due to the fact that cooked grape musts employed were stored in oak wood barrels. Important increases for both isomers were only found for samples aged in oak wood.

Ethyl benzoate was only detected in initial samples acetified in cherry wood and during aging, this compound increased. We also observed important increases of ethyl furoate during the aging in cherry barrels, reaching the highest concentrations. Besides, acetophenone, which presented concentrations under its quantification limit (LOQ) at the beginning of aging of all the vinegars, could be only quantified in those vinegars aged in cherry wood. Benzaldehyde content seems to be related to this kind of wood according with results of Flamini, Dalla Vedova, Cancian, Panighel, & De Rosso (2007). These compounds, have been determined in different types of wine, some of them aged in oak barrels, such as Sherry (Zea, Moyano, Moreno, Cortes, & Medina, 2001; Moreno, Zea, Moyano, & Medina, 2005), red (Escudero, Campo, Fariña, Cacho,

& Ferreira, 2007; Zhang, Xu, Duan, Qu, & Wu, 2007), sweet Fiano (Genovese, Gambuti, Piombino, & Moio, 2007), Madeira (Campo, Ferreira, Escudero, Marqués, & Cacho, 2006), Albillo (Sánchez-Palomo, González-Viñas, Díaz-Maroto, Soriano-Pérez, & Pérez-Coello, 2007) or Sauternes (Bailly, Jerkovic, Marchand-Brynaert, & Collin, 2006). However their concentrations were lower than those observed in our vinegars aged in cherry (Tables 2-5). Hence, these compounds may be characteristic of this specific type of wood.

During the aging in bottle of vinegar previously acetified in cherry wood we also observed increase in ethyl benzoate, ethyl furoate and benzaldehyde. Probably, cherry wood released a number of precursors and during aging they are transformed in the compounds before mentioned.

There are some compounds considered as oak wood extractable that have been extensively studied in wines and contribute to wine flavour (Jarauta et al., 2005).

Eugenol was not detected in vinegar aged in either acacia or cherry barrels. However it is present in all final vinegars from chestnut and in most of oak barrels reaching a mean concentration of ca. 111µg/L. These results were similar to those of Flamini et al. (2007). These authors, analysed extracts from acacia, cherry, mulberry, chestnut and oak wood, and found that only the latter two woods presented a noteworthy concentration of eugenol.

Vanillin is a compound whose aroma is related to oak barrels. In general, as time of contact between wine and oak wood increases, the levels of vanillin increase (Ortega-Heras, González-Sanjosé, & González-Huerta, 2007). However, Flamini et al. (2007) pointed out that chestnut wood provides higher concentration of this aromatic aldehyde than oak wood. In our case, we observed the highest concentration increases in the

sample aged in chestnut from winery 3. In the other two wineries, vanillin showed the major increases in samples aged in oak and chestnut wood.

Guaiacol also presented high concentrations in the initial samples from winery 3, much higher than those found in wines (Escudero et al., 2007; Ortega-Heras et al., 2007), supporting the idea, above mentioned, that cooked grape musts employed was probably stored in oak wood barrels. In these cases, the content of this compounds suffered statistically significant increases.

4-ethylphenol is produced by non-*Saccharomyces* yeasts and it is undesirable for aroma, being an organoleptic defect (Martorell, Martí, Mestres, Busto, & Guasch, 2002). Initial vinegars from winery 2 and 3 accounted concentrations above 200 µg/L. In both cases the final concentration were superior to those found in wine aged in oak wood by Jarauta et al. (2005). The formation of ethylphenols is favoured in wines with lower alcohol concentration (Garde-Cerdán et al., 2006) for this reason it is not surprising to find higher content of 4-ethylphenol in vinegars than in wine.

Hundreds of volatile compounds have been identified in untoasted oak wood (Chatonnet, 1991; Maga, 1984; Nishimura, Ohnishi, Masuda, Koga, & Matsuyama, 1983; Díaz-Maroto et al., 2008) among them vanillin, eugenol, guaiacyl derivatives and whisky–lactones (Sefton, Francis, Pocock, Williams, 1993). Toasting increases the levels of those compounds found in untoasted oak (Chatonnet, Boidron, Dubourdieu, & Pons, 1989; Díaz-Maroto et al., 2008), such as guaiacyl derivates, vanillin or eugenol as well as leads to the formation of other new compounds like furanic aldehydes (Guchu, Díaz-Maroto, Pérez-Coello, González-Viñas, & Cabezudo Ibáñez, 2006).

Furanic compounds like 2-furfuraldehyde and 5-methyl-2-furfuraldehyde are originated by the thermal degradation of hemicellulose and cellulose respectively (Bozalongo et al., 2007).

In initial samples, 5-methyl-2-furfuraldehyde was only quantified in samples from winery 3, ranging between 615-953 μg/L. On the other hand, although 2-furfuraldehyde appears in most of initial samples, it presented the highest concentrations in samples from winery 3 (Table 5). This can be due to the fact that must cooking generates an important amount of furanic compounds as a consequence of sugar degradation (Antonelli, Chinnici, & Masino, 2004). 5-methyl-2-furfuraldehyde and 2-furfualdehyde increased in these samples during aging, showing statistical significant changes for both compounds. The threshold perception of furanic compounds is high, being 6.2 mg/L for 2-furfuladehyde (Tesfaye, Morales, Callejón, Cerezo, García-Parrilla, & Troncoso, 2008), hence its concentration in final vinegars (1.33 - 4.53 mg/L) did not surpass its threshold. It is believed that furanic compounds do not have an important role in the aroma of products aged in oak wood, although they enhance the perception of oak lactones (Reazin, 1981).

In this study, the barrels were toasted the minimum needed for manufacture. For this reason, the content of furanic aldehydes compounds was lower than expected (Tesfaye et al. 2002). 2-furfuraldehyde trends to augment even during aging in bottles, this last fact was also observed in wine aged in oak barrels during nine moths and then in bottles during one year (Pérez-Prieto, López-Roca, Martínez-Cutillas, Pardo-Mínguez, & Gómez-Plaza, 2003).

4. Conclusions

There was not a common pattern for changes of volatile composition during aging. In general, we observed that the only group of volatile compounds that increased during the 16 aging experiments were acids. When we compared aging in bottle versus wood, we found that the overall increase of volatile compounds was superior in

vinegars aged in bottle. This fact is probably due to sorption phenomena that may take place on new wood barrels. In general, the final quantitative volatile composition of vinegars was not affected by the type of wood used, however, certain compounds can be related to the kind of wood used. Hence, oak-lactones reached the highest concentrations in vinegars aged in oak wood. Ethyl benzoate, ethyl furoate, acetophenone and benzaldehyde seem to be related to Cherry wood since, excepting ethyl furoate, they only could be quantified in this kind of wood. Lastly, eugenol was only present in all vinegars aged in chestnut and in most of vinegars aged in oak barrels.

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Figure Caption

Figure 1. Changes in alcohol and acetic degree during aging process.

Figure 1.

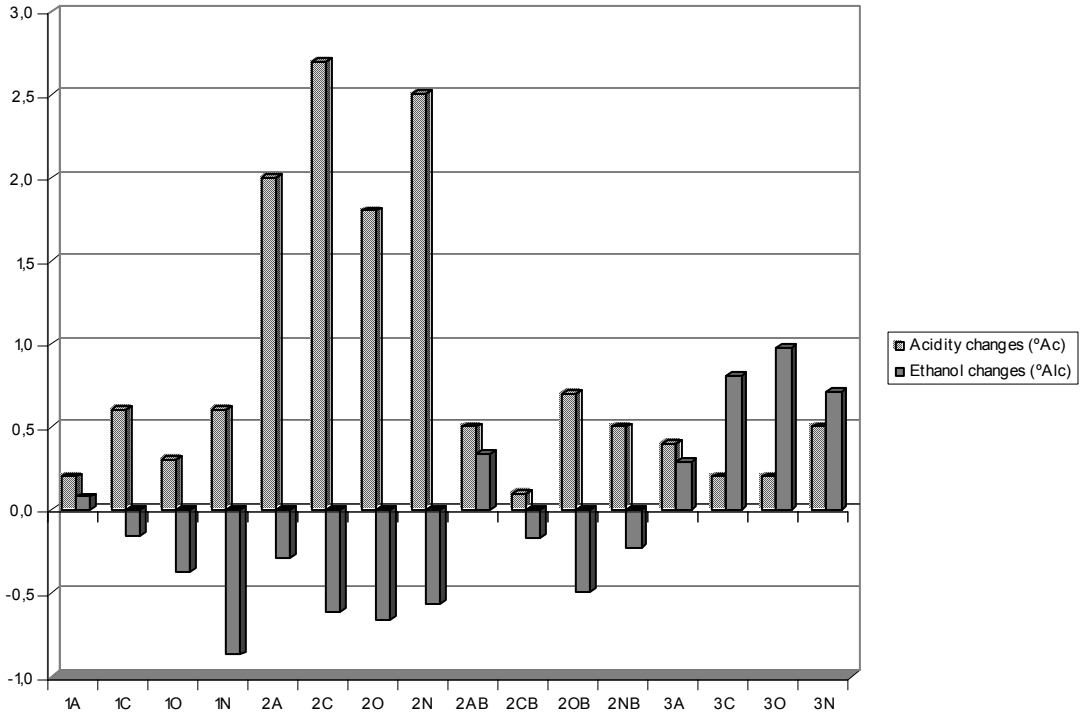

Table 1. Characteristics and codes of wineries and vinegar samples.

Winery and Vinegars	**Acetification (Kind of wood)**	**Aging**	**Sampling point**		
			Initial (0 days)	**Final (365 days)**	**Final Bottle**
Banyuls (1) (France) *Red Wine Substrate: Grenache var. Initial acetic degree: 6.5º ± 0.2*	Acacia (A)	Acacia (A)	1AI	1AF	-
	Cherry (C)	Cherry (C)	1CI	1CF	-
	Oak (O)	Oak (O)	1OI	1OF	-
	Chestnut (N)	Chestnut (N)	1NI	1NF	-
Priorat (2) (Spain) *Red Wine Substrate: Grenache var. Initial acetic degree: 6.4º ± 0.3*	Acacia (A)	Acacia (A)	2AI	2AF	2ABF
	Cherry (C)	Cherry (C)	2CI	2CF	2CBF
	Oak (O)	Oak (O)	2OI	2OF	2OBF
	Chestnut (N)	Chestnut (N)	2NI	2NF	2NBF
Reggio Emilia (3) (Italy) *White Wine Substrate: Trebbiano var. Initial acetic degree: 5.75º ± 0.06*	Acacia (A)	Acacia (A)	3AI	3AF	-
	Cherry (C)	Cherry (C)	3CI	3CF	-
	Oak (O)	Oak (O)	3OI	3OF	-
	Chestnut (N)	Chestnut (N)	3NI	3NF	-

Table 2. Volatile compounds of red wine vinegar aged in winery 1.

Nº	Compound	Mean concentration (µg/L) ± SD							
		Acacia		Cherry		Oak		Chesnut	
		1AI	1AF	1CI	1CF	1OI	1OF	1NI	1NF
Aldehydes									
1	Acetaldehyde[*1]	14.2 ± 0.3	34 ± 5	25 ± 4	67 ± 8	5.25 ± 0.01	70 ± 8	24.7 ± 0.2	95 ± 6
2	Hexanal	9.5 ± 1.2	84 ± 3	37.3 ± 1.6	10.8 ± 1.4	44 ± 1	13 ± 1	10 ± 1	27 ± 3
3	2-Furfuraldehyde	841 ± 81	925 ± 84	127 ± 16	104 ± 6	418 ± 54	201 ± 23	294 ± 10	396 ± 54
4	Benzaldehyde	49 ± 3	nq	69 ± 5	nq	89 ± 3	nq	52.5 ± 1.1	nq
5	5-Methyl-2-furfuraldehyde	nd	184 ± 9	nd	nd	nd	nd	nd	nd
6	Vanillin	nq	2367 ± 266	1170 ± 112	3301 ± 266	nq	2663 ± 15	876 ± 76	3607 ± 193
	Total aldehydes[*]	15	38	27	71	6.3	73	26	100
Acetals									
7	Acetaldehyde diethylacetal[*]	105.9 ± 1.5	130 ± 14	122.4 ± 1.1	176 ± 8	47.86 ± 0.17	126 ± 9	571.6 ± 1.5	194.6 ± 1.5
Acetic esters									
8	Methyl acetate[*]	11.5 ± 0.5	42 ± 3	15 ± 1	25± 1	10.08 ± 0.23	34 ± 3	5.99 ± 0.16	38.2 ± 2.3
9	Ethyl acetate[*1]	1315 ± 170	2888 ± 369	1157 ± 75	1618 ± 216	132 ± 7	1993 ± 85	221 ± 1	3064 ± 55
10	Propyl acetate	516 ± 17	1380 ± 23	566 ± 25	948 ± 14	385 ± 3	691 ± 27	121 ± 5	1421 ± 14
11	Isobutyl acetate[*]	1.17 ± 0.07	2.9 ± 0.1	1.28 ± 0.04	1.80 ± 0.01	0.97 ± 0.06	1.4 ± 0.01	0.33 ± 0.04	2.8 ± 0.1
12	Butyl acetate	nd	nq	nd	nd	nd	nd	nd	nq
13	Isoamyl acetate[*]	3.1 ± 0.2	8.0 ± 0.4	3.6 ± 0.2	3.4 ± 0.06	2.94 ± 0.02	2.5 ± 0.3	1.2 ± 0.2	6.6 ± 0.01
15	Hexyl acetate	nd	nq	nd	nd	nd	nd	nd	nq
16	Phenylmethyl acetate	nd	nd	nd	nd	nd	nd	nd	nd
17	Benzyl acetate	nd	nd	nd	nd	nd	nd	nd	nd
18	2-Phenylethyl acetate[*]	0.81 ± 0.07	1.94 ± 0.04	0.92 ± 0.12	0.79 ± 0.12	1.0 ± 0.1	0.77 ± 0.09	0.69 ± 0.04	1.88 ± 0.20
	Total acetic esters[*]	1332	2945	1178	1650	147	2033	229	3114
Ethyl esters									
19	Ethyl propanoate	156 ± 7	537 ± 27	155 ± 5	480 ± 16	118 ± 3	343 ± 21	159 ± 4	628 ± 6
20	Ethyl isobutyrate	160 ± 4	803 ± 34	175 ± 3	547 ± 24	176 ± 7	465 ± 44	168 ± 12	869 ± 31
21	Ethyl butyrate	nq	nq	nq	nq	nq	nq	134 ± 13	nq
22	Ethyl isovalerate	nd	97 ± 7	nq	155 ± 9	nd	150 ± 6	nq	178 ± 7
23	Ethyl valerate	12 ± 0.2	nq	nq	nq	nd	nq	nq	nq
24	Ethyl hexanoate	nd	59 ± 2	nd	nq	nd	nq	174 ± 26	68 ± 3
26	Ethyl lactate[*]	3.7 ± 0.3	1.33 ± 0.05	4.1 ± 0.3	2.38 ± 0.22	2.75 ± 0.09	1.23 ± 0.02	19.1 ± 1.5	2.2 ± 0.3
27	Ethyl octanoate	nd	nd	nd	nd	nd	nd	nd	nd
28	Ethyl furoate	26.5 ± 1.9	51.0 ± 0.7	72 ± 5	140 ± 1	34 ± 1	77 ± 6	21.4 ± 1.3	80 ± 4
29	Ethyl benzoate	nd	nd	nq	nq	nd	nd	nd	nd
30	Diethyl succinate[*]	1.65 ± 0.06	1.54 ± 0.03	2.24 ± 0.32	1.40 ± 0.01	2.16 ± 0.19	2.26 ± 0.13	1.65 ± 0.04	2.29 ± 0.25
	Total ethylic esters[*]	5.8	4.4	6.7	5.1	5.2	4.5	21	6.3

[*] Concentration in mg/L
[1] GC-FID
nd: not detected
nq: below quantification limit

Table 2. (Continued)

Nº	Compound	Mean concentration (µg/L) ± SD							
		Acacia		Cherry		Oak		Chesnut	
		1AI	1AF	1CI	1CF	1OI	1OF	1NI	1NF
Alcohols									
31	Metanol*[1]	79 ± 1	84 ± 11	82 ± 5	63 ± 3	130.3 ± 0.8	70 ± 5	112 ± 13	136 ± 19
32	Ethanol*[1]	3510 ± 22	4143 ± 573	455 ± 653	3339 ± 10	6287 ± 704	3284± 14	15093 ± 227	8125 ± 1297
33	1-Propanol*[1]	3.4 ± 0.2	4.04 ± 0.40	4.3 ± 0.4	3.2 ± 0.3	6.57± 0.24	1.19 ± 0.03	5.4 ± 0.2	8.63 ± 0.05
34	Isobutanol*	6.89 ± 0.13	9.2 ± 0.7	10.9 ± 0.9	6.55 ± 0.12	11.65 ± 0.04	7.9 ± 0.5	11.4 ± 1.5	9.5 ± 0.3
35	2-Methyl-1-butanol*	7.5 ± 0.8	15.0 ± 0.4	7.6 ± 0.4	13.5 ± 0.3	6.9 ± 0.5	11.26 ± 0.05	15.68 ± 0.02	15.6 ± 0.6
36	3-Methyl-1-butanol*	41 ± 3	46.2 ± 0.5	42.3 ± 1.8	46.5 ± 0.8	34.9 ± 0.8	29.3 ± 1.6	75 ± 4	46.5 ± 0.8
37	1-Hexanol	194 ± 10	209 ± 3	157 ± 8	nq	167 ± 4	nq	519 ± 20	168 ± 3
38	*Cis*-3-hexen-1-ol	36.9 ± 1.6	43.3 ± 0.8	41 ± 4	35.0 ± 0.2	35 ± 1	31.7 ± 0.6	60.7 ± 9	46.2 ± 0.4
39	Benzyl alcohol	160 ± 13	441 ± 63	132 ± 8	388 ± 4	184 ± 10	787 ± 29	164 ± 5	756 ± 45
40	Furfuryl alcohol	nd	758 ± 93	nq	200 ± 11	nq	393 ± 6	488 ± 19	490 ± 50
41	2-Phenylethanol*	17.5 ± 1.6	17.5 ± 3	20 ± 3	14.7± 0.0	23 ± 7	30.3±2.2	20.9 ± 1.3	28.7 ± 1.5
	Total alcohols*	3667	4320	4714	3487	6501	3435	15335	8372
Ketones									
42	Diacetyl*	nd	nd	nd	nd	nd	nd	nd	1.24 ± 0.08
43	Acetoin*	362 ± 39	334.2 ± 0.2	304 ± 32	498 ± 64	194 ± 16	496 ± 21	107 ± 16	501 ± 54
44	Acetophenone	nd	nq	nq	57.2 ± 1.6	nq	nq	nq	nq
	Total ketones*	362	334	304	498	194	496	107	502
Acids									
45	Isovaleric acid*	2.2 ± 0.3	2.5 ± 0.1	2.9 ± 0.3	6.4 ± 0.3	1.16 ± 0.01	11.2 ± 0.1	0.78 ± 0.092	5.0 ± 0.3
46	Hexanoic acid	553 ± 30	1905 ± 173	634 ± 35	783 ± 14	437 ± 53	2008 ± 71	602 ± 83	2096 ± 97
47	Heptanoic acid	nq	267 ± 35	98 ± 7	430 ± 49	nd	133 ± 16	nq	279 ± 5
48	Octanoic acid	189 ± 7	292 ± 30	204 ± 15	274 ± 17	160 ± 14	497 ± 63	231 ± 33	296 ± 22
49	Nonanoic acid	nd	nq	nd	nq	nd	nq	nd	53 ± 1
50	Decanoic acid	nd	20.4 ± 0.6	31 ± 2	35.5 ± 1.5	27.0 ± 1.1	46 ± 1	38 ± 3	15 ± 1
	Total acids*	2.9	5.0	3.9	7.8	1.8	14	1.7	7.7
Terpenes									
51	Limonene	91.3 ± 2.4	nd	93 ± 7	nd	89.4 ± 2.3	nd	nq	nd
Lactones									
52	γ-Butyrolactone*	0.6 ± 0.1	2.6 ± 0.3	1.05 ± 0.06	1.77 ± 0.15	1.15 ± 0.01	1.46 ± 0.04	1.58 ± 0.14	2.58 ± 0.02
53	*Trans*-β-methyl-γ-octalactone	nd	nd	nd	nd	102 ± 6	313 ± 15	nd	nd
54	*Cis*-β-methyl-γ-octalactone	nd	nd	nd	nd	363 ± 35	1534 ± 197	nd	nd
	Total lactones*	0.6	2.6	1.05	1.77	1.15	1.6	3.3	2.58
Phenols									
55	Guaiacol	14.4 ± 1.7	22 ± 2	6.2 ± 0.6	6.3 ± 0.0	8.4 ± 1.1	23.0 ± 0.3	5.2 ± 0.5	8.7 ± 0.1
56	Eugenol	nd	nd	nd	nd	nq	118 ± 16	nd	87 ± 10
57	4-Ethylphenol	49 ± 6	52.1 ± 0.5	88 ± 10	65 ± 2	94 ± 5	102 ± 8	149 ± 16	162 ± 22
	Total phenols	63	74	94	71	103	243	154	258
	Total amounts*	5491	7779	6357	5896	6905	6185	16293	12299

*Concentration in mg/L
[1]GC-FID
nd: not detected
nq: below quantification limit

Table 3. Volatile compounds of red wine vinegars from winery 2 aged in wood (acacia and cherry) and bottle.

Nº	Compound	Mean concentration (µg/L) ± SD					
		Acacia			Cherry		
		2AI	2AF	2ABF	2CI	2CF	2CBF
Aldehydes							
1	Acetaldehyde[*1]	35.5 ± 0.5	60 ± 5	116 ± 6	66 ± 4	40 ± 2	134 ± 15
2	Hexanal	nq	11.8 ± 1.5	19.6 ± 2.3	15.9 ± 1.6	11 ± 1	18.7 ± 0.3
3	2-Furfuraldehyde	426 ± 57	495 ± 26	584 ± 59	nd	198 ± 20	740 ± 83
4	Benzaldehyde	nq	nq	nq	89.0 ± 1.8	295 ± 31	387 ± 14
5	5-Methyl-2-furfuraldehyde	nd	nq	43 ± 5	nd	nd	35 ± 4
6	Vanillin	nq	1831 ± 129	nq	nq	1687 ± 176	nq
	Total aldehydes[*]	36	63	123	66	42	138
Acetals							
7	Acetaldehyde diethylacetal[*]	154 ± 5	133 ± 5	208 ± 9	270 ± 7	272 ± 24	266 ± 12
Acetic esters							
8	Methyl acetate[*]	17.7 ± 0.3	45.62 ± 0.16	30.5 ± 3.4	10.28 ± 0.15	26.9 ± 2.6	28.226 ± 3.627
9	Ethyl acetate[*1]	4476 ± 378	3276 ± 125	3348 ± 517	3892 ± 572	918 ± 27	2472 ± 347
10	Propyl acetate	3227 ± 17	2760 ± 188	3603 ± 264	2141 ± 143	2898 ± 44	4440 ± 263
11	Isobutyl acetate[*]	3.12 ± 0.13	2.9 ± 0.3	3.2 ± 0.2	2.05 ± 0.06	2.79 ± 0.08	3.7 ± 0.3
12	Butyl acetate	131 ± 7	71 ± 10	170 ± 19	88.3 ± 0.1	133 ± 12	363 ± 43
13	Isoamyl acetate[*]	10.23 ± 0.42	5.6 ± 0.3	8.64 ± 0.08	7.07 ± 0.23	9.3 ± 0.5	14 ± 2
14	Amyl acetate	nd	nd	nq	nd	nd	63 ± 8
15	Hexyl acetate	69 ± 9	nd	nq	63 ± 5	61 ± 8	159 ± 23
16	Phenylmethyl acetate	187 ± 19	nq	115 ± 4	nq	nq	198 ± 29
17	Benzyl acetate	nd	nd	nq	nd	nq	nq
18	2-Phenylethyl acetate[*]	1.89 ± 0.23	0.80 ± 0.03	1.61 ± 0.14	1.10 ± 0.16	2.69 ± 0.35	3.8 ± 0.4
	Total esters[*]	4513	3333	3396	3915	962	2527
Ethylic esters							
19	Ethyl propanoate	661 ± 16	1343 ± 118	1352 ±120.8	539 ± 16	1278 ± 16	1921 ± 228
20	Ethyl isobutyrate	458 ± 56	1267 ± 173	1107 ± 126	268 ± 10	793 ± 49	1552 ± 197
21	Ethyl butyrate	nq	nq	137 ± 11	nq	99 ± 1	2045 ± 21
22	Ethyl isovalerate	nq	397 ± 33	288 ± 37	nq	145 ± 11	288 ± 33
23	Ethyl valerate	nq	9.8 ± 0.9	10.0 ± 0.5	nq	10 ± 1	13.9 ± 1.6
24	Ethyl hexanoate	52 ± 6	142 ± 20	107 ± 16	43 ± 4	104 ± 9	217 ± 28
25	Ethyl heptanoate	nd	nd	8 ± 1	nd	nd	8 ± 1
26	Ethyl lactate[*]	17.6 ± 0.4	2.0 ± 0.3	2.5 ± 0.3	14.2 ± 1.7	5.0 ± 0.4	5.5 ± 0.3
27	Ethyl octanoate	nd	nd	nd	nd	nd	169 ± 25
28	Ethyl furoate	97 ± 9	99 ± 6	148 ± 17	191 ± 13	426 ± 30	412 ± 56
29	Ethyl benzoate	nd	nd	nd	113 ± 7	245 ± 15	233 ± 4
30	Diethyl succinate[*]	22.5 ± 1.6	5.3 ± 0.7	15 ± 10	8.6 ± 1.2	16.6 ± 2.3	15 ± 2
	Total ethylic esters[*]	41	11	21	24	25	26

[*] Concentration in mg/L
[1] GC-FID
nd: not detected
nq: below quantification limit

Table 3. (Continued)

Nº	Compound	Mean concentration (µg/L) ± SD					
		Acacia			Cherry		
		2AI	2AF	2ABF	2CI	2CF	2CBF
Alcohols							
31	Metanol[*1]	118 ± 18	107 ± 7	187 ± 4	170 ± 26	119 ± 9	213 ± 3
32	Ethanol[*1]	6522 ± 826	4240 ± 281	9223 ± 114	10784 ± 116	5863 ± 379	9421 ± 544
33	1-Propanol[*1]	22 ± 3	16.77± 0.02	37.0 ± 0.3	36 ± 1	27 ± 3	54 ± 3
34	Isobutanol[*]	7.87 ± 0.17	13 ± 0.5	73.5 ± 0.2	8.43 ± 0.13	10.34 ± 0.08	10.270 ± 0.786
35	2-Methyl-1-butanol[*]	13.62 ± 0.25	17.8 ± 0.6	13.9 ± 1.6	8.7 ± 0.4	12.0 ± 0.6	13.5 ± 1.8
36	3-Methyl-1-butanol[*]	89 ± 6	67 ± 7	75 ± 3	75 ± 5	74 ± 4	80 ± 7
37	1-Hexanol	569 ± 20	nq	369 ± 42	438 ± 26	462 ± 23	732 ± 28
38	*Cis*-3-hexen-1-ol	58.9 ± 0.4	51.2 ± 2.5	60 ± 1	44 ± 1	55 ± 4	81 ± 3
39	Benzyl alcohol	5670 ± 608	518 ± 33	1402 ± 187	1657 ± 381	1022 ± 114	865 ± 109
40	Furfuryl alcohol	604 ± 66	589 ± 76	1492 ± 73	54 ± 4	519 ± 70	1401 ± 190
41	2-Phenylethanol[*]	47 ± 6	20 ± 2	30 ± 5	14.8 ± 1.1	39 ± 5	33 ± 5
	Total alcohols[*]	6826	4483	9576	11099	6146	9828
Ketones							
42	Diacetyl[*]	nd	nd	4.6 ± 0.5	nd	2.9 ± 0.3	14 ± 1
43	Acetoin[*]	1437 ± 148	821 ± 116	699 ± 1	743 ± 53	1022 ± 115	713 ± 421
44	Acetophenone	nd	nd	nq	nq	139 ± 15	79 ± 10
	Total ketones[*]	1437	821	704	743	1025	727
Acids							
45	Isovaleric acid[*]	3.1 ± 0.2	18.5 ± 0.3	8040 ± 713	2.0 ± 0.1	5.5 ± 0.6	3868 ± 297
46	Hexanoic acid	1636 ± 127	2152 ± 260	4129 ± 214	647 ± 10	3029 ± 289	2813 ± 383
47	Heptanoic acid	nq	100 ± 1	227 ± 12	nq	166 ± 15	301 ± 42
48	Octanoic acid	975 ± 127	454 ± 44	1274 ± 41	222 ± 14	912 ± 130	935 ± 136
49	Nonanoic acid	nd	62 ± 7	119 ± 5	nd	81 ± 10	nq
50	Decanoic acid	163 ± 21	37 ± 5	nq	222 ± 14	101 ± 13	nq
	Total acids[*]	5.9	21	14	3.1	9.8	8
Terpenes							
51	Limonene	112 ± 4	nd	79.2 ± 9.6	80 ± 3	nd	234 ± 14.6
Lactones							
52	γ-Butyrolactone[*]	3.90 ± 0.33	1.92 ± 0.08	4.7 ± 0.3	2.2 ± 0.1	2.29 ± 0.34	2.97 ± 0.21
53	*Trans*-β-methyl-γ-octalactone	nd	nd	nd	nd	nd	nd
54	*Cis*-β-methyl-γ-octalactone	nd	nd	nd	nd	nd	nd
	Total lactones[*]	3.9	1.92	4.7	2.2	2.29	2.97
Phenols							
55	Guaiacol	22.3 ± 0.1	22.5 ± 2.0	47 ± 6	8.0 ± 0.6	25.2 ± 0.6	36 ± 4
56	Eugenol	nd	nd	nd	nd	nd	nd
57	4-Ethylphenol	871 ± 37	329 ± 37	1583	364 ± 47	1101 ± 148	967
	Total phenols	893	351	1630	372	1126	1003
	Total amounts[*]	13018	8867	14048	16123	8487	13523

[*] Concentration in mg/L
[1] GC-FID
nd: not detected
nq: below quantification limit

Table 4. Volatile compounds of red wine vinegars from winery 2 aged in wood (oak and chesnut) and bottle.

Nº	Compound	Mean concentration (µg/L) ± SD					
		Oak			Chesnut		
		2OI	2OF	2OBF	2NI	2NF	2NBF
Aldehydes							
1	Acetaldehyde[*1]	52 ± 6	44 ± 5	71 ± 7	32 ± 4	41 ± 4	49 ± 5
2	Hexanal	24 ± 1	47 ± 6	16.2 ± 1.4	39 ± 2	38 ± 4	24.4 ± 3.4
3	2-Furfuraldehyde	nq	598 ± 87	195 ± 15	nd	66 ± 3	76 ± 1
4	Benzaldehyde	nq	nq	nq	nq	nq	nq
5	5-Methyl-2-furfuraldehyde	nd	nd	nd	nd	nd	nd
6	Vanillin	nd	3587 ± 460	897 ± 83	nq	2250 ± 3	nd
	Total aldehydes[*]	52	49	72	32	43	49
Acetals							
7	Acetaldehyde diethylacetal[*]	193 ± 7	90.7 ± 0.7	116 ± 13	184 ± 8	141.5 ± 0.3	101 ± 5
Acetic esters							
8	Methyl acetate[*]	16.9 ± 0.5	40.1 ± 0.6	24.713 ± 0.462	14.1 ± 0.3	25.2 ± 0.6	21.8 ± 1.6
9	Ethyl acetate[*1]	3955 ± 266	1739 ± 1	1614 ± 227	2905 ± 363	2660 ± 326	2089 ± 281
10	Propyl acetate	2207 ± 118	2078 ± 72	2584 ± 26	2681 ± 41	2466 ± 135	2876 ± 79
11	Isobutyl acetate[*]	2.17 ± 0.08	2.28 ± 0.02	2.15 ± 0.04	2.16 ± 0.13	2.57 ± 0.22	2.21 ± 0.19
12	Butyl acetate	83.8 ± 0.6	nq	106 ± 1	93 ± 2	89 ± 12	135 ± 8
13	Isoamyl acetate[*]	7.26 ± 0.05	5.87 ± 0.08	5.51 ± 0.13	7.61 ± 0.22	6.1 ± 0.5	6.5 ± 0.3
14	Amyl acetate	nd	nd	nq	nd	nd	nq
15	Hexyl acetate	62 ± 2	82 ± 3	nq	nd	nd	nq
16	Phenylmethyl acetate	142 ± 6	nq	122 ± 18	137 ± 6	nq	nq
17	Benzyl acetate	nd	nq	nq	nd	nq	nq
18	2-Phenylethyl acetate[*]	2.05 ± 0.28	1.03 ± 0.11	1.6 ± 0.2	1.49 ± 0.09	1.62 ± 0.22	1.6 ± 0.22
	Total esters[*]	399	1790	1651	2933	2698	2124
Ethylic esters							
19	Ethyl propanoate	527 ± 20	1142 ± 38	1166 ± 19	644 ± 20	1143 ± 123	1248 ± 41
20	Ethyl isobutyrate	284 ± 7	1033 ± 31	798 ± 8	394 ± 11	940 ± 42	1006 ± 67
21	Ethyl butyrate	nq	143 ± 1	174 ± 4	nq	87 ± 7	131 ± 6
22	Ethyl isovalerate	nq	491 ± 21	200 ± 7	nq	462 ± 25	203 ±19
23	Ethyl valerate	nq	11.4 ± 0.5	11.4 ±0.2	nq	9.5 ± 0.7	10.3 ± 0.4
24	Ethyl hexanoate	nq	121 ± 9	121 ± 7	48.6 ± 1.4	122 ± 3	132 ± 9
25	Ethyl heptanoate	nd	nd	nq	nd	nd	nq
26	Ethyl lactate[*]	10.9 ± 0.1	2.09 ± 0.05	1.5 ± 0.2	14.3 ± 0.2	1.99 ± 0.13	1.7 ± 0.1
27	Ethyl octanoate	nd	nd	nd	nd	nd	103 ± 11
28	Ethyl furoate	101 ± 5	122 ± 8	140 ± 16	94 ± 5	130 ± 1	141 ± 20
29	Ethyl benzoate	nd	nd	nd	nd	nd	nd
30	Diethyl succinate[*]	22 ± 3	6.5 ± 0.5	8.5 ± 1.3	15.3 ± 1.3	12.1 ± 1.6	7.5 ± 0.8
	Total ethylic esters[*]	34	12	13	31	17	12

[*] Concentration in mg/L
[1]GC-FID
nd: not detected
nq: below quantification limit

Table 4. (Continued)

Nº	Compound	Mean concentration (µg/L) ± SD					
		Oak			Chesnut		
		2OI	2OF	2OBF	2NI	2NF	2NBF
Alcohols							
31	Metanol[*1]	193 ± 25	126 ± 19	155 ± 9	145 ± 20	111 ± 2	168 ± 22
32	Ethanol[*1]	9480 ± 1450	4204 ± 623	5553 ± 242	9471 ± 959	4927 ± 570	7673 ± 71
33	1-Propanol[*1]	35 ± 4	16.1 ± 1.7	22.6 ± 0.7	33 ± 3	18.4 ± 1.2	40 ± 6
34	Isobutanol[*]	9.2 ± 0.6	12.9 ± 0.3	7.2 ± 0.2	9.2 ± 0.6	9.3 ± 0.6	7.1 ± 0.3
35	2-Methyl-1-butanol[*]	10.6 ± 0.4	14 ± 1	9.1 ± 0.3	8.9 ± 0.4	14.00 ± 0.09	9.2 ± 0.6
36	3-Methyl-1-butanol[*]	78.3 ± 0.3	53.7 ± 0.6	51 ± 1	82 ± 3	55.9 ± 1.7	56 ± 2
37	1-Hexanol	449.1 ± 0.6	nq	282 ± 13	503 ± 15	nq	469 ± 23
38	*Cis*-3-hexen-1-ol	55.0 ± 1.2	45.2 ± 0.4	54 ± 2	58 ± 1	45 ± 4	59 ± 3
39	Benzyl alcohol	4407 ± 466	630 ± 37	721 ± 102	3869 ± 421	868 ± 114	545 ± 61
40	Furfuryl alcohol	nd	1044 ± 58	304 ± 43	nq	342 ± 20	157 ± 24
41	2-Phenylethanol[*]	29 ± 5	24.7 ± 0.7	32 ± 1	24 ± 3	33 ± 4	21 ± 3
	Total alcohols[*]	9840	4453	5831	9777	5171	7976
Ketones							
42	Diacetyl[*]	nd	nd	5.0 ± 0.6	nd	nd	3.49 ± 0.07
43	Acetoin[*]	488 ± 43	740 ± 33	809 ± 96	499 ± 6	686 ± 51	594 ± 49
44	Acetophenone	nd	nq	nd	nq	nq	nq
	Total ketones[*]	488	740	814	499	686	598
Acids							
45	Isovaleric acid[*]	2.0 ± 0.2	16.3 ± 1.2	8891 ± 37	1.78 ± 0.05	17.6 ± 0.2	3374 ± 218
46	Hexanoic acid	1077 ± 45	3322 ± 164	2433 ± 198	1067 ± 55	3790 ± 505	1844 ± 199
47	Heptanoic acid	nq	153 ± 8	103 ± 4	114 ± 5	242 ± 28	103 ± 2
48	Octanoic acid	284 ± 14	732 ± 70	1098 ± 28	301 ± 29	994 ± 118	434 ± 62
49	Nonanoic acid	nd	86.3 ± 0.7	57 ± 3	nd	89 ± 12	nd
50	Decanoic acid	48 ± 6	136 ± 18	157 ± 4	17 ± 2	141 ± 19	nq
	Total acids[*]	3.4	21	13	3.3	23	6
Terpenes							
51	Limonene	76.6 ± 1.2	nd	104 ± 1.8	91.1 ± 1.7	nd	104 ± 0.9
Lactones							
52	γ-Butyrolactone[*]	1.23 ± 0.17	2.24 ± 0.06	1.75 ± 0.16	1.56 ± 0.19	2.10 ± 0.26	1.74 ± 0.15
53	*Trans*-β-methyl-γ-octalactone	126 ± 13	161 ± 21	nd	nd	nd	nd
54	*Cis*-β-methyl-γ-octalactone	422 ± 54	1424 ± 198	nd	nd	nd	nd
	Total lactones[*]	1.8	3.8	1.75	1.56	2.1	1.74
Phenols							
55	Guaiacol	13.9 ± 1.2	30.6 ± 0.8	22 ± 3	10.2 ± 0.1	10.7 ± 1.3	nq
56	Eugenol	nq	nq	nq	nq	162 ± 17	nd
57	4-Ethylphenol	427 ± 54	395 ± 55	1002	518 ± 49	932 ± 125	577
	Total phenols	441	426	1024	528	1105	577
	Total amounts[*]	14598	7159	8512	13461	8783	10868

[*] Concentration in mg/L
[1] GC-FID
nd: not detected
nq: below quantification limit

Table 5. Volatile compounds of white wine vinegars aged in winery 3.

Nº	Compound	Mean concentration (µg/L) ± SD							
		Acacia		Cherry		Oak		Chesnut	
		3AI	3AF	3CI	3CF	3OI	3OF	3NI	3NF
Aldehydes									
1	Acetaldehyde[*1]	38 ± 3	80 ± 12	27.9 ± 0.5	203 ± 26	35 ± 2	56 ± 4	33 ± 3	38 ± 5
2	Hexanal	5.0 ± 0.2	28 ± 4	11.5 ± 0.6	43 ± 5	10.7 ± 0.8	61 ± 4	15.8 ± 1.9	49 ± 6
3	2-Furfuraldehyde	1096 ± 130	1330 ± 178	1286 ± 48	4527 ± 583	1056 ± 106	3703 ± 469	1338 ± 163	2494 ± 309
4	Benzaldehyde	nd	58.3 ± 1.1	36 ± 4	988 ± 48	nq	115 ± 3	30 ± 2	145 ± 19
5	5-Methyl-2-furfuraldehyde	615 ± 49	1451 ± 198	953 ± 56	2979 ± 455	729 ± 42	2282 ± 47	685 ± 99	2160 ± 141
6	Vanillin	1499 ± 19	2603 ± 358	593 ± 28	1747 ± 236	2994 ± 444	2272 ± 75	738 ± 47	5320 ± 729
	Total aldehydes[*]	42	85	31	213	41	64	36	48
Acetals									
7	Acetaldehyde diethylacetal[*]	164 ± 10	183 ± 15	198 ± 2	545 ± 32	193 ± 5	223 ± 6	204 ± 8	291 ± 3
Acetic esters									
8	Methyl acetate[*]	8.6 ± 0.4	13.1 ± 1.1	13.0 ± 1.8	23 ± 3	10.2 ± 1.2	16.6 ± 1.4	11.7 ± 1.2	20 ± 2
9	Ethyl acetate[*1]	2893 ± 20	5141 ± 273	2256 ± 281	4857 ± 239	1929 ± 34	3751 ± 170	2193 ± 236	1714 ± 106
10	Propyl acetate	3473 ± 380	3928 ± 281	3949 ± 145	4816 ± 614	3923 ± 517	4605 ± 456	4230 ± 295	5154 ± 593
11	Isobutyl acetate[*]	2.14 ± 0.15	2.81 ± 0.02	2.42 ± 0.12	3.2 ± 0.3	2.4 ± 0.3	3.1 ± 0.4	2.8 ± 0.3	3.4 ± 0.3
12	Butyl acetate	61 ± 5	68 ± 3	78 ± 7	136 ± 16	77 ± 6	108 ± 13	85 ± 8	123 ± 15
13	Isoamyl acetate[*]	3.6 ± 0.5	5.1 ± 0.3	3.87 ± 0.13	5.9 ± 0.8	4.0 ± 0.4	5.3 ± 0.7	4.5 ± 0.5	5.9 ± 0.8
15	Hexyl acetate	nd	nd	nd	nd	nd	nd	nd	nd
16	Phenylmethyl acetate	nd	nq	nq	nq	nq	nq	nq	nq
17	Benzyl acetate	239 ± 15	373 ± 31	264 ± 3	442 ± 25	328 ± 20	512 ± 54	387 ± 25	597 ± 50
18	2-Phenylethyl acetate[*]	0.68 ± 0.06	1.52 ± 0.16	0.91 ± 0.01	1.93 ± 0.14	1.04 ± 0.09	2.2 ± 0.2	1.13 ± 0.11	2.4 ± 0.1
	Total acetic esters[*]	2912	5168	2281	4897	1951	3783	2218	1751
Ethylic esters									
19	Ethyl propanoate	3531 ± 327	3839 ± 306	3831 ± 95	4397 ± 534	3969 ± 268	4547 ± 636	4311 ± 404	4857 ± 415
20	Ethyl isobutyrate	1022 ± 109	1339 ± 91	1161 ± 54	1531 ± 65	1202 ± 116	1653 ± 120	1388 ± 106	1826 ± 151
21	Ethyl butyrate	324 ± 22	366 ± 3	367 ± 12	501 ± 69	387 ± 22	770 ± 88	426 ± 26	510 ± 53
22	Ethyl isovalerate	831 ± 74	1318 ± 76	883 ± 49	1427 ± 179	969 ± 81	1492 ± 171	1139 ± 112	1663 ± 212
23	Ethyl valerate	13.8 ± 1.5	16.6 ± 0.1	19.6 ± 0.9	33 ± 4	18.1 ± 0.8	26 ± 4	19.1 ± 1.2	24 ± 3
24	Ethyl hexanoate	84 ± 10	109 ± 7	90 ± 4	131.9 ± 0.1	98 ± 8	143 ± 2	115 ± 12	166 ± 20
26	Ethyl lactate[*]	50 ± 5	55 ± 7	44.2 ± 2.2	60 ± 4	41.4 ± 1.5	49 ± 6	43 ± 5	47.7 ± 2.5
27	Ethyl octanoate	nd	nd	nd	nd	nd	nd	nd	nd
28	Ethyl furoate	174 ± 10	247 ± 7	228 ± 32	522 ± 61	220 ± 3	313 ± 4	243 ± 20	348 ± 23
29	Ethyl benzoate	nd	nd	nd	nq	nd	nd	nd	nd
30	Diethyl succinate[*]	3475 ± 228	5926 ± 711	4290 ± 275	5524 ± 188	4925 ± 430	8199 ± 355	5825 ± 861	10329 ± 1042
	Total ethylic esters[*]	60	69	55	75	53	66	57	68

[*] Concentration in mg/L
[1] GC-FID
nd: not detected
nq: below quantification limit

Table 5. (Continued)

Nº	Compound	Mean concentration (µg/L) ± SD							
		Acacia		Cherry		Oak		Chesnut	
		3AI	3AF	3CI	3CF	3OI	3OF	3NI	3NF
Alcohols									
31	Metanol[*1]	20 ± 1	69 ± 10	23 ± 1	98 ± 12	22 ± 2	79 ± 13	20.1 ± 0.7	48 ± 7
32	Ethanol[*1]	5302 ± 99	7620 ± 15	4841 ± 131	11283 ± 468	4616 ± 140	12387 ± 106	5189 ± 468	10839 ± 108
33	1-Propanol[*1]	18 ± 0.5	39.5 ± 0.8	23.5 ± 0.8	60 ± 2	19.6± 1.8	63 ± 9	26 ± 2	52 ± 7
34	Isobutanol[*]	8.7 ± 0.3	8.7 ± 1.2	8.79 ± 0.07	10.1 ± 1.4	9.9 ± 0.8	10.8 ± 1.3	10.6 ± 1.2	13.1 ± 1.6
35	2-Methyl-1-butanol[*]	7.66 ± 0.19	8.9 ± 0.3	8.15 ± 0.18	9.3 ± 0.4	8.6 ± 0.4	9.7 ± 0.8	9.5 ± 1	11 ± 1
36	3-Methyl-1-butanol[*]	28.9 ± 1.9	33.7 ± 1.6	30.5 ± 0.4	34.4 ± 0.4	32 ± 2	35.7 ± 0.8	35 ± 3	39 ± 3
37	1-Hexanol	nq	nq	nq	nq	nq	nq	nq	nq
38	*Cis*-3-hexen-1-ol	16 ± 1	19.4 ± 1.6	18.7 ± 0.3	21.4 ± 0.4	18.3 ± 0.4	20.4 ± 1.6	18.9 ± 0.2	23.7 ± 1.6
39	Benzyl alcohol	319 ± 43	437 ± 25	380 ± 2	432 ± 11	529 ± 45	563 ± 79	407 ± 58	610 ± 11
40	Furfuryl alcohol	907 ± 68	460 ± 53	448 ± 52	1217 ± 55	635 ± 69	1147 ± 59	1088 ± 148	625 ± 65
41	2-Phenylethanol[*]	12.6 ± 1.6	18.2 ± 0.5	14.9 ± 0.1	17.9 ± 0.8	21 ± 3	22.9 ± 0.1	16 ± 2	23.1 ± 0.7
	Total alcohols[*]	5399	7799	4951	11515	4725	12609	5308	11027
Ketones									
42	Diacetyl[*]	21.1 ± 0.7	50 ± 5	29 ± 1	50 ± 6	19 ± 2	56 ± 3	22 ± 2	49 ± 4
43	Acetoin[*]	1432 ± 197	696 ± 94	1117 ± 94	1427 ± 87	931 ± 16	1020 ± 132	924 ± 128	899 ± 68
44	Acetophenone	nq	nq	nq	118.6 ± 0.6	nq	nq	nq	nq
	Total ketones[*]	1453	747	1146	1477	949	1076	946	949
Acids									
45	Isovaleric acid[*]	24.7 ± 0.4	28 ± 2	22.7 ± 0.3	23.7 ± 1.4	24.4 ± 0.2	33.2 ± 0.8	22.1 ± 0.3	24.8 ± 1.6
46	Hexanoic acid	974 ± 112	3861 ± 532	1231 ± 113	1861 ± 258	1424 ± 164	2296 ± 214	1594 ± 38	3811 ± 209
47	Heptanoic acid	nq	451 ± 65	nq	83 ± 10	nq	237 ± 29	77 ± 8	422 ± 35
48	Octanoic acid	194 ± 17	717 ± 108	223 ± 8	446 ± 55	299 ± 42	546 ± 24	314 ± 27	751 ± 51
49	Nonanoic acid	nq	124 ± 14	nd	nq	152 ± 20	52.8 ± 0.3	34 ± 4	149 ± 20
50	Decanoic acid	44 ± 4	128 ± 4	45 ± 5	86 ± 13	66 ± 7	81 ± 6	52 ± 6	151 ± 8
	Total acids[*]	26	33	24	26	26	36	24	30
Terpenes									
51	Limonene	nd	nd	nd	nd	nd	nd	nd	nd
Lactones									
52	γ-Butyrolactone[*]	2.6 ± 0.4	3.07 ± 0.05	2.5 ± 0.3	2.8 ± 0.4	1.67 ± 0.12	3.34 ± 0.02	2.2 ± 0.3	2.28 ± 0.02
53	*Trans*-β-methyl-γ-octalactone	65.3 ± 0.9	73 ± 3	66.0 ± 0.5	66.7 ± 0.8	85 ± 6	237 ± 30	74.4 ± 2.8	87 ± 9
54	*Cis*-β-methyl-γ-octalactone	77.8 ± 1.7	109 ± 11	82.1 ± 1.7	82.2 ± 0.6	204 ± 25	1179 ± 167	123 ± 14	191 ± 13
	Total lactones[*]	2.8	3.3	2.8	3.0	2.0	4.8	2.3	2.5
Phenols									
55	Guaiacol	120 ± 12	232 ± 7	152 ± 4	224 ± 16	149 ± 16	301 ± 43	157 ± 13	286 ± 7
56	Eugenol	nd	nd	nd	nd	nd	81 ± 10	nd	108 ± 9
57	4-Ethylphenol	286 ± 16	509 ± 64	352 ± 14	428 ± 18	405 ± 52	509 ± 51	420 ± 61	641 ± 60
	Total phenols	406	741	504	652	554	891	578	982
	Total amounts[*]	10058	14088	8689	19149	7942	17863	8794	14168

[*] Concentration in mg/L
[1] GC-FID
nd: not detected
nq: below quantification limit

Manuscript 5

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OPTIMIZATION OF SENSORY ANALYSIS OF WINE VINEGAR

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[Optimization of Sensory Analysis of Wine Vinegar]

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ABSTRACT:

A procedure for sensory analysis of vinegar has been optimized to obtain reproducible results and minimize standard deviation of attributes. A new tasting protocol was proposed, obtaining low dispersions in panelists answers. The sensory panel (n=9) evaluated the impact of five chemical compounds (acetaldehyde, benzaldehyde, ethyl acetate, 2-furaldehyde, and vainillin) whose concentrations are known in vinegars and applied the Ascending Method of Limits Test (ASTM) to determine their thresholds in an 6% v/v acetic acid solution. The list of attributes to describe vinegars was broadened up to a total 13, whose relevance (or discriminant utility) is analyzed by using Partial Least Squares Regression related techniques. The proposed methodology proved to be useful to taste very different types of wine vinegars, ie. Sherry , red wine and whit wine vinegars.

Key words: vinegar; sensory analysis protocol; odor threshold; sensory profiling, reliability

1. Introduction

Wine vinegar is used as a condiment to preserve and improve sensory characteristics of food. It can be produced by traditional methods of acetification in which the acetic bacteria are placed on the surface of the alcoholic liquid to be acetified. Oxygen transfer is then very limited. As a consequence, the process takes a long time to achieve the required acetic degree and the product is, therefore, aged simultaneously (Tesfaye and others 2002a). These vinegars are very appreciated in gastronomy and they reach high prices. However, most commercial vinegars are produced by submerged culture of the acetic acid bacteria and an extraordinary oxygen supply that allows reaching the acetic degree in 36 hours. Large volumes are produced in short times and prices can be more competitive. Most of the volatile compounds are lost in the process because of the strong aeration and in general, sensory quality is lower (Morales and others 2001; Romero and Cantero 1998). Nevertheless, vinegar is more than an acidulant agent and those components different from acetic acid can be perceived by senses (González-Viñas and others 1996). Recently, the interest for high quality vinegars has increased and there is a wider diversity of raw materials used in their elaboration (Natera 2003).

Sensory analysis has been used to discriminate vinegar samples either based on the raw material used (Gerbi and others 1997) or depending on the elaboration method followed (Nieto and others 1993; González-Viñas and others 1996).

As price is related to their sensory properties, there is a need of evaluating them in an objective way. Up to date there is not a standardized method to perform sensory analysis

of vinegars. For other products such as olive oil a systematic approach to organoleptic evaluation of the product has been described (Commission Regulation EEC 1991).

The main difficulty to taste this product is the pungent sensation produced by acetic acid, its major component. Acetic acid masks the perception of other aromas, especially for untrained panelists. In addition, it produces fatigue of receptors in a shorter time than alcohol derived products. One of the purposes of this research was to design a procedure of tasting vinegars that could overcome these drawbacks. On the other hand, more reliable results are expected if all the steps of tasting that could have an influence on results are described and followed in detail. Scientific literature regarding sensory studies on wine vinegars is very scarce (Nieto and others 1993; González-Viñas and others 1996; Tesfaye and others 2002).

The aim of this work is to improve the sensory analysis of wine vinegar, seeking for new attributes and minimizing deviations among tasters. In order to accomplish this purpose we have performed the following steps. First, we have detailed a tasting protocol and asked the panelist to fulfill it carefully. We compared results with those obtained from those of panelist tasting *ad libitum*. Second, we have compared two cups: the standardized cup and the cup of commonly used in wine vinegar cellars. Third, we explored the impact of five chemical compounds present in vinegar for its sensory properties and established the detection limits both in water and in 6º acetic degrees solutions. These thresholds are used to determine the potential of these substances at low concentrations to impart odor (ASTM International E679 2004). Finally, we propose and test a group of terms as descriptors and the vocabulary list used to describe vinegars was

broadened. These new attributes have been evaluated for its reliability and validity for sample featuring.

2. Materials and Methods

2.1. Tasting Panel

Nine panelists aged between 25 and 45 years old, non smokers took part in this study. All members have received theoretical and practical training in sensory analysis. Six of them were expert tasters and had more than five years experience in tasting vinegars and had taken part in other studies already published. The selection and training had been already described elsewhere (Tesfaye and others 2002). Basically, judges proved their capacity of recognizing the basic tastes as a requirement to become members of the panel (ISO 3972: 1991). Additionally, they get used to ranking solutions of acetic acid, ethyl acetate, wood extracts or wine dilutions. None of the panelist received economical remuneration for participating in this study.

2.2. Tasting cups

Two kinds of cups were used in this study: the wine tasting standardized cup (ISO 3591:1977) and a cup of common use in Sherry wine vinegar cellars. The main difference among them is the diameter at the top, 46 mm for the first one; 41 mm for the second and the total capacity is 215 mL and 165 mL respectively. Certain members of our panel proposed that the smaller the diameter is the better perception of minor aromas they have. Both cups were of dark glass to avoid color's influence in tasting answer (Tesfaye and others 2002).

2.3. Tasting Samples

131 **2.3.1. Samples used for the comparison of tasting cups and optimization of tasting**
132 **procedure**

133 Three wine vinegar samples were used in this study: OA (vinegar obtained from red wine
134 by surface culture), OB (commercial white wine vinegar obtained form submerged
135 culture) and OC (Sherry wine vinegar provided by the Regulatory Council of
136 Denomination of Origin). They were chosen as being representative of the most
137 consumed wine vinegars.

138 **2.3.2. Solutions for the determination of the individual impact of five odorant**
139 **compounds present in vinegar**

140 Solutions of pure standards of acetaldehyde (Sigma-Aldrich, ≥ 99.5%), benzaldehyde
141 (Aldrich, ≥ 99.5%), 2-furaldehyde (Aldrich ≥ 99.5%), ethyl acetate (Sigma-Aldrich, ≥
142 99.5%) and vanillin (Merck ≥ 99%) are used in this study.

143 Stock solutions of the pure standards of each odorant were prepared both in Milli-Q water
144 and in a 6% v/v acetic acid solution (the standard acetic degree for vinegar). From these
145 solutions six different concentrations were prepared for each compound. Material exempt
146 of odors was used throughout. Table 1 summarizes the concentration of each standard in
147 the two series (water and water acetic acid solutions) used. The series of dilutions of
148 water or acetic acid were tasted in different sessions. Only one session was performed per
149 day and the duplicates of each test were done in different days. This study was carried out
150 during 2 months. The judges were asked if they perceive an aroma, if they could
151 recognize it and if they could associate it to any other (ISO 5496: 1992).

152 **2.3.3. Samples for broadening the vocabulary list**

153 Twelve different representative vinegar samples and one acetic acid solution were tested
154 two Sherry wine vinegars, four red wine vinegars from Grenache and Cabernet Sauvignon
155 varieties, one white wine vinegar elaborated by submerged culture acetification, one
156 commercial apple vinegar, one spirit vinegar, one commercial honey vinegar, one
157 commercial balsamic vinegar from Modena and a 6% acetic acid water solution
158 containing 2-furaldehyde (30 mg/L), acetaldehyde (4mg/L), benzaldehyde (4 mg/L), ethyl
159 acetate (600 mg/L), vanillin (10 mg/L) and ethanol 2% v/v ethanol.

160 **2.3.4. Samples to study the reliability of attributes**

161 A total 12 wine vinegar representative samples were selected for this study, as follows:
162 four Sherry vinegars (VJ1, VJ2, VJ3), one of them Very old (named *Gran Reserva*)
163 Sherry vinegar (VJ4), six red wine vinegars made in barrels by surface culture (VR1,
164 VR2, VR3, VR4, VR5 and VR6) one red wine vinegar made by submerged acetification
165 (VS1), one commercial white wine vinegar (VS2).

166 **2.4. Sensory tests**

167 **2.4.1. Tasting procedure**

168 In our previous experiences judges were allowed to taste vinegars as they prefer. No
169 recommendations were given. (Tesfaye and others 2002). However, in the present work
170 we designed a sensory analysis procedure and asked the judges to follow it carefully.
171 After the organizer of the panel had instructed the judges orally, the written procedure
172 was provided to them in all tasting session as follows:

173 The olfaction phase

174 - Take off the lid covering the cup.

- Shake the cup with circular movements for 10 seconds. Incline the cup to get the inner surface of the cup wet with the sample.

- Smell the sample on the border of the cup. Please, avoid the centre where pungent sensation is more intense. The cup must be inclined 45º with regard the nose and can be turned smoothly. Inspirations must be slow, short, and not intense till you have your own criteria.

- Olfaction time can not be longer than 15 seconds. If it is not possible to take a decision, you can make a pause before trying again.

- Then, the sample is thrown away and the empty cup is smelled.

2.4.2. Attributes selection

Samples described in section 2.3.1. were presented to the panel. Each panelist gave a maximum list of odors perceived from each sample based on the unguided free selection technique (Guerrero 1996).

2.4.3. Descriptive sensory analysis

We used the profile method to analyze vinegar samples. The intensity of each attribute was marked on an unstructured 10 cm straight line labeled with not noticeable to very strong on the left and right extremes respectively (ISO 4121: 1987).

2.4.4. Determination of odor threshold by a forced choice ascending concentration series method of limits

Group thresholds were calculated following the procedures established for determination of odor and taste threshold by a forced choice ascending concentration series method of limits (ASTM International E679 2004).

2.4.5 Relevance and reliability of attributes

To perform this study, twelve wine vinegar samples were tasted by triplicate using the selected attributes on an unstructured scale. Their relevance and validity for characterizing vinegar samples is studied by preparing two special data sets (Martens and others 2000). The mean data set, composed by n = 12×3 objects (12 vinegar samples for triplicate) and p = 13 sensory variables (the mean values given by the assessors) and the Design data set, consisting of n = 16×3 objects as above and p = 15 design variables for products and replicates (12 samples and 3 replications). Each vinegar sample or replication was represented by a categorical indicator variable (with values 0 or 1). For example, the three replicates of the same vinegar sample are labeled as 1 and the rest as 0. Replications made at the same run are labeled as 1 (for instance, the first replication of each vinegar) and the remainder as 0. Accordingly, partial least squares techniques can be applied by using these data matrices.

2.5. Statistical Analysis

Statistical analysis was performed thanks to Statistica 7 software (Statsoft 1994) and the Unscrambler 9.1 (CAMO ASA 2004).

3. Results and Discussion

3.1. Optimization of tasting procedure and cup

The hypothesis is that more reliable results can be obtained if samples are tasted following a standardized protocol than if each panelist performed the sensory analysis *ad libitum*. Samples were tasted using previously selected attributes for sensory analysis of wine vinegars (Morales and others 2006). The panel followed the procedure described in section 2.4.1 and results were compared with those obtained from *ad libitum* tasting in

terms of standard deviations of scores given to each attribute (Table 2). As can be noticed the standard deviation for the following attributes: aroma intensity, richness in aroma, general impression, coconut odor, clove odor, vanilla odor, woody odor and wine character are lower being results more precise when the samples were analyzed following the newly proposed procedure.

We obtained higher standard deviations for the attributes ethyl acetate (glue odor) and pungent sensation when using the proposed protocol. The two compounds responsible of these attributes: acetic acid and ethyl acetate are major volatile compounds in this matrix. By the other hand, ethyl acetate, can reach high concentrations in wine vinegars produced by traditional surface method. As a consequence of our results, we propose to evaluate the ethyl acetate odor and pungent sensation before shaking the cup and the rest of attributes after shaking, obtaining by these means lower standard deviations. The panel verified that aromas as vanilla, coconut and clove were perceived better by using the proposed protocol as acetic acid and ethyl acetate did not mask them.

The effect of shaking on pungent sensation and ethyl acetate raised certain concerns with the type of cup. Thus, the ISO standardized cup and the cup normally employed in Sherry wineries were compared. This last has a smaller diameter that could avoid this effect. Table 2 shows that the standard deviations of the following attributes: aroma intensity, richness in aroma, ethyl acetate, woody odor, wine character, pungent sensation and general impression were lower in standardized cups than in common cellar cups either the panelists are allowed to taste vinegars with *ad libitum* or with the proposed procedure.. Smelling the cup after emptying the tasting cup did not improve the precision of the selected attributes (Table 3). However, all the judges agree that other aromas not

perceived previously are then discerned. Its utility will be evaluated during new attributes selection phase.

3.2. Determination of the impact of five common odorants in vinegars

To improve the descriptive analysis we studied the impact that certain compounds have on sensory characteristics of vinegars. Hence we selected a number of aroma compounds that are common constituents of wine vinegar and whose occurrence and concentrations had been previously determined in wine vinegars (Natera-Martin and others 2002; Tesfaye and others 2002b) as follows: acetaldehyde, benzaldehyde, ethyl acetate, 2-furaldehyde and vanilla (Table 1). Acetaldehyde is formed as the result of the acetic fermentation, whereas ethyl acetate as a condensation reaction between ethanol and acetic acid, vanillin, 2-furaldehyde and benzaldehyde are wood extractables mainly associated with the aging process. The Ascending Method of Limits test of the American Society for Testing and Materials (ASTM) was applied in order to establish the thresholds (Meilgaard 1991, 1993). The concentration scale steps during the preparation of dilutions were biased by a factor 1.3-2. The thresholds for each compound were determined both in Milli-Q water and 6% v/v acetic acid solutions. Table 4 displays the results obtained and the following thresholds were established for each compound. As can be observed the acetic acid effect raises the threshold up to 30 folds, depending on the compound, thus illustrating the difficulty of tasting vinegars.

3.3. Broadening the vocabulary list

The task of generating vocabulary should focus on the difference between the products, rather than simply compiling a dictionary of adjectives (Murray and others 2001). Thus, eleven representative vinegars (different in their elaboration method, raw material used,

and geographical origin) and a synthetic vinegar were used to select new attributes. An initial list of 64 different attributes was suggested. After the lexicon generation, the panel discussed all together in order to discard repeated and synonym words and then to select appropriate terms as follows. The inclusion criteria were that terms should have relevance to the product, discriminate clearly between samples, clearly known by each panelist and simply detectable (ISO 11035: 1994). Those attribute which were described by more than 50% of the panel for the same vinegar and for the same tasting session. A definition (Civille and Lyon 1996) was given to each attribute included (Table 5). Thus, in this step 20 attributes were selected (Table 5) (ethyl acetate, alcohol/liquor, pungent sensation, winy, resinous, woody, citrus, red fruits, vanillin, sweet aroma, bitter almond, leather/old, general impression, medicinal/acetaldehyde, apple, coconut, rancid, bacteria, cheese, sawdust/wood shaving). For simplicity purposes the panel reduced the list of describing vocabulary to 13 consensually (Table 6) and the remaining seven (medicinal/acetaldehyde, apple, coconut, rancid, bacteria, cheese, sawdust/wood shaving) are considered as optional attributes to include when particular vinegar is tested. Furthermore, the panel decided that odors like bacteria and sawdust/wood shaving were negative odors to vinegar.

3.2.3 Study of the reliability of attributes

Finally the newly developed attributes were evaluated different wine vinegar samples Previously a triangle test was performed to confirm that the panel was able to differentiate the six vinegar samples. Each sample was submitted to the descriptive test using the following 13 newly established attributes. The spider chart of a pair of samples corresponding to Sherry wine vinegars are represented in Figure 1, the intensity of woody

odor, vanilla, leather/old odor and ethyl acetate odor are more evident in vinegar VJ2 that is older than vinegar VJ1.

Different analytical approaches have been reported for studying the reliability of sensory data (Nielsen and others 2004). For instance, most used approaches are Generalized Procrustes Analysis (Byrne and others 2001), one way ANOVA combined with Principal Component Analysis (PCA) (Couronne 1997), ANOVA with egg-shell plots (Naes 1988), ANOVA, cluster analysis, consonance analysis and PCA (King and others 2001), agreement coefficient and reliability coefficient (Bi 2003) and techniques based on Discriminant Partial Least Squares Regression (DPLSR) (Thybo and Martens 2000).

In the present work, we use typical descriptive statistical analysis and unsupervised learning display methods like PCA for a preliminary study and techniques based on partial least squares for deeper study of variable reliability. DPLSR on the mean and design data sets using sensory variables (descriptors) as X-matrix and design variables as Y-matrix can be used to find which sensory attributes discriminate between the vinegars in a reliable way.

Basic statistics, plots of percentiles and histograms of the mean data set give a first look into which attributes describe sensory differences in a reliable way. Several sensory variables show a non normal distribution and accordingly, an initial data analysis of the selected attributes has been performed according to the box-and-whisker plot (Figure 2), which is a graphical representation of the called 'five number summary' of the data set, described by its extremes, its lower and upper quartiles and its median value. As can be observed in Figure 2, several attributes show asymmetry (skewness), but the variables

were not transformed (by using logarithmic or squared data transformation) because this did not lead to results improvement.

After a PCA of the mean data matrix, six principal components (PCs) arose according to ensure communalities close to 0.80. The explained variance is of about 89%. Attributes contributions to PCs based on covariance indicates that there is no noisy variables to be removed. However, according to the plot of the projection of attributes on the factor plane of PC1 and PC2, as can be observed in Figure 3, some of them are redundant. For instance, EA (ethyl acetate) is positively correlated with RED (red fruits) and BALM (balsamic) and negatively with OLD.

The sensory differences among the products were studied by using the DPLSR approach. Accordingly, three significant PLS components were obtained that explain on average 85% of the systematic variation in sensory data. This ensures that the selected attributes are relevant to product variation. Figure 4 shows the correlation loadings (a PLS biplot) for the two first PLS components. As can be seen, the first component spans a wide flavor variation (EA, BALM, RED, VAN and SWEET against OLD and PUNG). Most attributes are displayed at positive loadings of the first component, except CIT, PUNG, WIN and OLD. OLD exhibits highest negative contribution to the first component, whereas CIT has a loading very close to 0. The second component shows a spanning ability especially for attributes WIN, RES and LIQ. Vinegar samples are grouped by forming a cluster belonging to VR category (VR1-VR6), the remaining being more dispersed. Anyway VR samples are located at positive scores of the first component and the rest of samples at negative scores. It is remarkable that replications (REP1-REP3) cluster together that implies a good between-run reproducibility.

4. Conclusion

A new standardized protocol for vinegar sensory analysis was proposed. The lack of standardized protocol was among the main factors which contributed for response differences among panelists. This protocol guaranteed the steps to follow by each sensory analysis panelist and reduce the differences of responses generated by the panel. The thresholds of five common components of vinegar (acetaldehyde, benzaldehyde, ethyl acetate, 2-furaldehydw and vanilla) were established. Thirteen sensory attributes and seven optional attributes were pointed out for descriptive analysis of vinegar.

The selected attributes shown a good between-run reproducibility as shown by DPLSR analysis on replications.

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Figure Captions

Figure 1. Sensory profile plot of mean attribute values for two Sherry wine vinegars (VJ1 and VJ2)

Figure 2. Box and whisker plots for the 13 attributes considering the studied samples (n=12) by triplicate.

Figure 3. Projection of attributes onto the plane formed by the first PCs

Figure 4. Coorrelation loadings with PLS1 versus PLS2 from a DPLSR model relating sensory data (X) and design variables (Y).

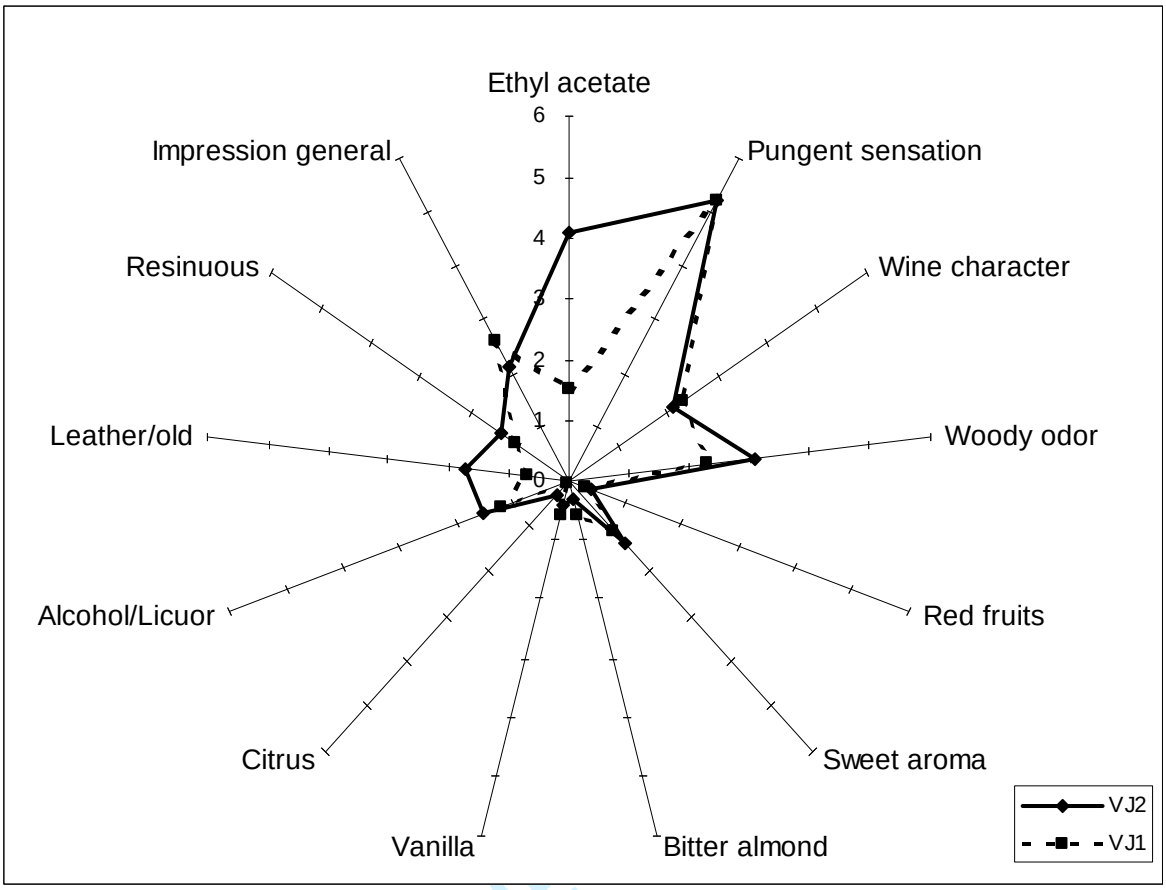

Figure 1.

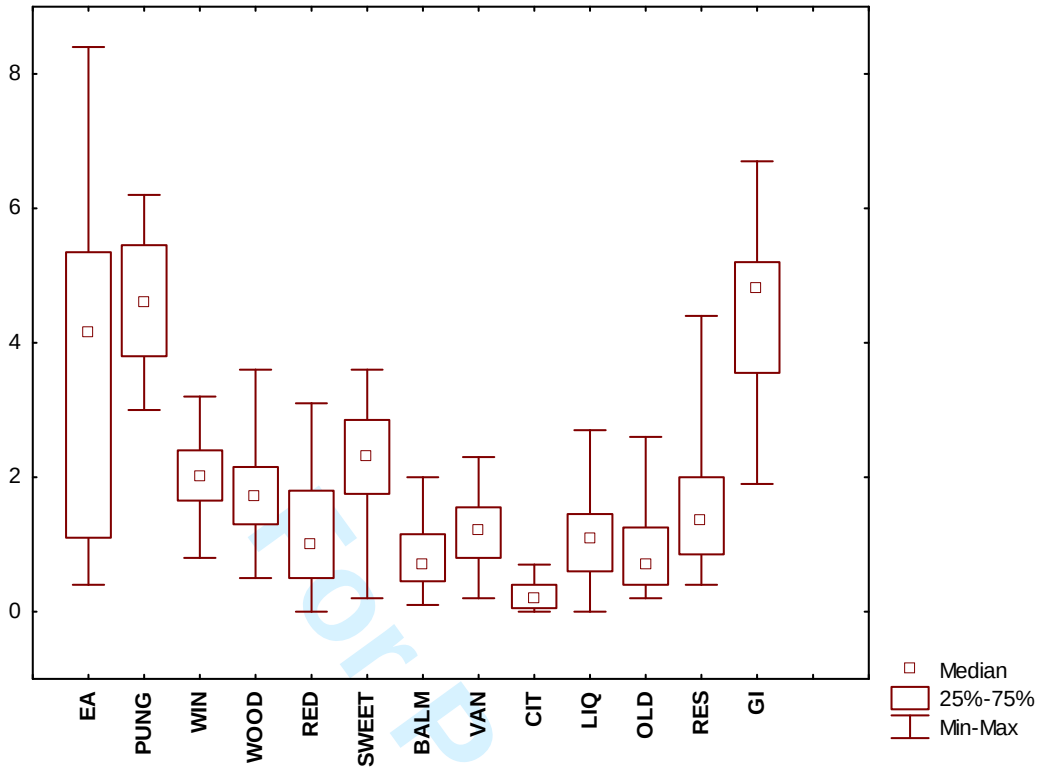

Figure 2.

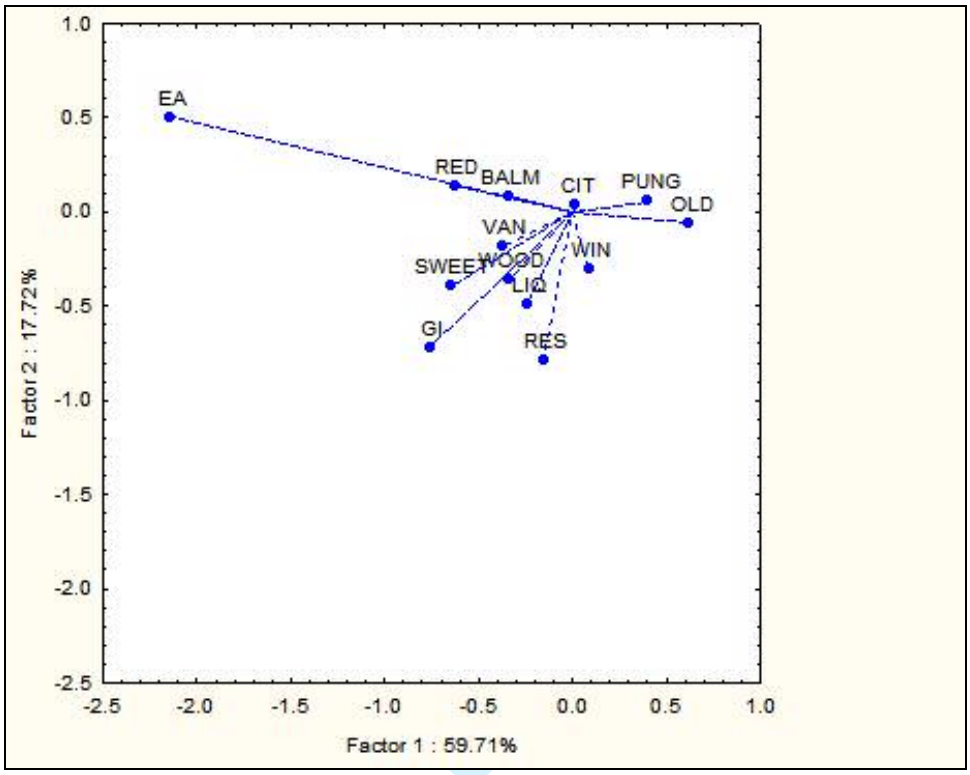

Figure 3.

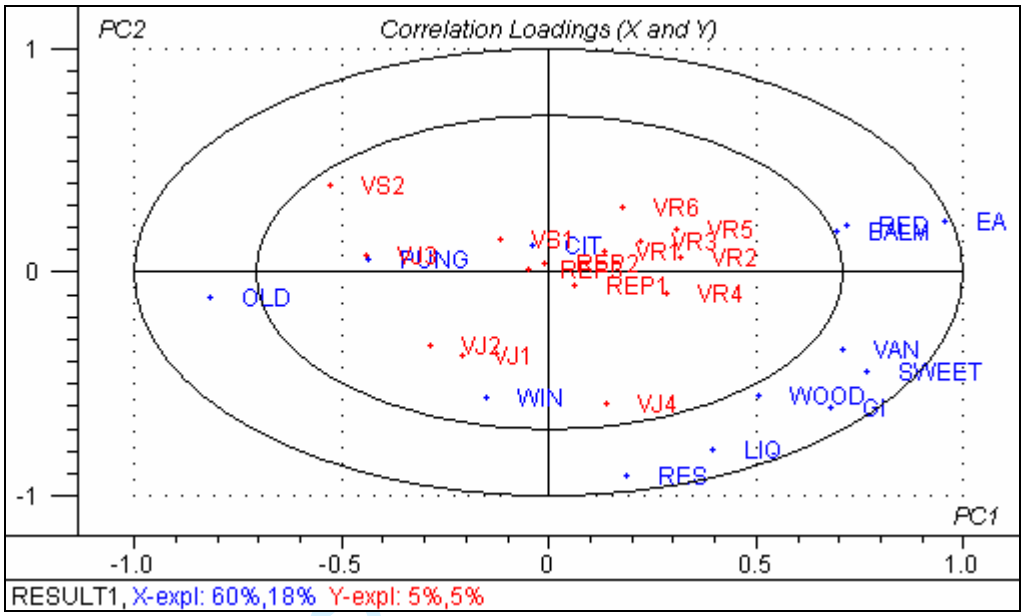

Figure 4.

Table 1.

Pure standard odorant solutions used for the determination of threshold concentrations

Odorants (pure standards)	Stock solution concentration		Taste solution concentrations		Number of taste solutions (Milli-Q water or 6% v/v acetic acid solutions at a time)
	Milli-Q water (mg/L)	6% v/v acetic acid solution (mg/L)	Milli-Q water (µg/L)	6% v/v acetic acid solution	
Acetaldehyde	2	2	50-270	250-1350 µg/L	6
Benzaldehyde	2	2	25-200	100-280 µg/L	6
Ethyl acetate	100	500	10-75	50-200 mg/L	6
2-furaldehyde	2	30	100-2000	5-15 mg/L	6
Vanillin	2	2	10-60	30-960 µg/L	6

Table 2
Mean and Standard deviation values of scores given to attributes of three vinegar samples analysed either ad *libitum* (WOP) or with the newly proposed protocol (WP) using ISO standardized cups (S) or commonly used cellar cups (C)

Type of tasting glass	Samples	Tasting procedure	Aroma Intensity	Richness in Aroma	Ethyl acetate odour	Woody odour	Wine character	Pungent sensation	General impression	Coconut odour	Clove odour	Vanilla odour
[4]S	OA	[6]WP	0.60	1.31	3.20	2.23	2.44	2.05	1.46	1.57	0.97	1.59
		[7]WOP	1.43	1.81	2.40	1.31	1.93	1.77	1.60	2.78	2.60	2.95
	OB	[6]WP	1.50	1.14	1.14	0.72	1.85	2.36	1.35	0.26	2.16	0.30
		[7]WOP	2.23	2.06	1.41	1.59	1.86	1.87	1.53	1.22	3.72	1.34
	OC	[6]WP	1.58	1.53	2.10	2.34	2.83	1.90	1.82	1.01	2.79	1.77
		[7]WOP	1.20	1.97	2.07	2.47	2.67	1,62	2.10	1.30	3.07	2.49
[5]C	OA	[6]WP	2.56	2.71	3.28	2.63	2.55	2.20	2.42	2.96	1.03	3.73
		[7]WOP	2.10	2.60	2.26	2.70	3.00	1.91	2.00	1.80	2.80	3.00
	OB	[6]WP	2.61	1.56	1.94	1.47	2.05	2.51	1.67	0.42	1.27	0.49
		[7]WOP	2.75	2.23	1.09	1.25	2.21	1.75	2.71	0.49	1.78	0.73
	OC	[6]WP	1.77	1.88	2.48	2.66	2.65	2.08	2.11	0.75	2.99	2.66
		[7]WOP	2.24	2.19	1.87	2.57	2.46	1.28	2.49	1.88	2.60	2.64
[4]S	Mean standard deviation (±σ)	[6]WP	1.22	1.32	2.15	1.76	2.37	2.1	1.54	0.94	1.94	1.22
		[7]WOP	2.62	1.95	1.96	1.79	2.15	1.75	1.74	1.77	3.13	2.26
[5]C	Mean standard deviation (±σ)	[6]WP	2.31	2.05	2.57	2.25	2.42	2.26	2.07	1.38	1.76	2.29
		[7]WOP	2.36	2.34	1.74	2.17	2.56	1.65	2.4	1.39	2.39	2.12

[4]Standardized cup, [5]Sherry wine cellar cup , [6]WP = Following the established Protocol, [7]WOP = *ad libitum*
For samples code see section 2.3.1.

Table 3

Mean Standard deviation values of the tasting panel after emptying the tasting glass and following the proposed procedure

Tasting Procedure	Samples	Type of tasting glass	Aromatic Intensity	Richness in Aroma	Ethyl acetate odor	Woody odor	Wine character	Pungent sensation	General impression	Coconut odor	Clove odor	Vanilla odor
[6]WP	OA	[4]S	1.92	2.49	1.76	2.74	2.21	1.35	2.23	1.21	1.65	1.15
		[5]C	2.40	2.54	3.37	3.63	2.79	1.76	2.28	2.58	0.87	3.19
	OB	[4]S	1.31	1.39	2.05	1.42	1.33	1.85	1.33	1.91	1.79	1.08
		[5]C	1.87	1.53	2.07	2.61	2.23	2.87	1.,98	1.11	1.45	0.87
	OC	[4]S	1.68	1.94	1.27	2.63	2.87	1.73	2.,01	1.25	2.72	2.00
		[5]C	2.36	2.17	1.78	3.08	2.80	1.73	1.97	1.41	2.14	3.08

[4]Standardized cup, [5]Sherry wine vinegar cellar cup, [6]WP = Following the established Protocol

Table 4

Threshold Log Standard Deviation and observed Range for 5 aroma compounds added to Milli-Q water and 6% v/v acetic acid solution

Sample	Group Threshold	Log Standard deviation	Observed range of thresholds	Number of tasters
1. Acetaldehyde in Milli-Q water	80.2 µg/L	0.086	50 -270 µg/L	7
2. Acetaldehyde in 6% acetic acid solution	401.8 µg/L	0.086	250-1350 µg/L	7
3. Benzaldehyde in Milli-Q water	104.2 µg/L	0.241	25-200 µg/L	8
4. Benzaldehyde in 6% acetic acid solution	157.8 µg/L	0.090	100-280 µg/L	8
5. Ethyl acetate in Milli-Q water	22.3 mg/L	0.100	10-75 mg/L	8
6. Ethyl acetate in 6% acetic acid solution	90.8 mg/L	0.065	50-200 mg/L	8
7. 2-Furaldehyde in Milli-Q water	208 µg/L	0.222	100-2000 µg/L	8
8. 2-Furaldehyde in 6% acetic acid solution	6.2 mg/L	0.235	5-15 mg/L	8
9. Vanillin in Milli-Q water	32.2 µg/L	0.056	10-60 µg/L	8
10. Vanillin in 6% acetic acid solution	94.4 µg/L	0.300	30-960 µg/L	8

Table 5.

Sensory attributes definitions, Standard references and evaluation technique used

Attributes	Definition	References	Evaluation technique
Ethyl acetate	Odours associated with glue, nail polish remover odour	Ethyl acetate solution	90 ppm of ethyl acetate in 6%v/v acetic acid solution
Alcohol/Liquor	Odours associated with spirits aged in oak barrels like Brandy or aged wine	Brandy/Sherry wine	80 ml Sherry wine mixed with 6.7 ml acetic acid and 13.4 ml water
Pungent sensation	Sensation produced by aqueous solution of acetic acid	Acetic acid solution	7% v/v of acetic acid solution
Medicinal	Odours associated with medicines, Pharmacy	Acetaldehyde solution	0.5 ppm of acetaldehyde in 6%v/v acetic acid solution
Winy characteristics	Describes an olfactory complex sensation, characteristic of vinegars elaborated from wine	Young white wine	80 ml young white wine mixed with 6.7 ml acetic acid and 13.4 ml water
Raisins	Odour associated with grape resins	Grape raisins	Sun dried grape resin
Woody	Odour associated with toasted oak shaving at 180ºC for 3 hours	Toasted American oak shaving macerated in 6% acetic acid solution	2% w/v toasted American oak shaving in 6% acetic acid solution for 15 days
Citrus	Sensation produced by aqueous solution of citric acid	Citric acid solutions	0.5 ppm citral in 6%v/v acetic acid solution
Apples	Odours associated with apple fruit, characteristic of cider vinegars	Apple Juice	80 ml apple juice in 6%v/v acetic acid solution
Coconut	Odour associated with coconut	β-methyl-γ-octalactone solution	7.14 ppm in 6%v/v acetic acid
Red fruits	Odours associated with strawberry, black berry	Strawberry juice	80 ml strawberry juice in 6%v/v acetic acid solution
Vanillin	Sensation produced by aqueous solution of vanillin	Vanillin solution	0.25 ppm of vanillin in 6%v/v acetic acid solution

Sweet aroma	Odour associated with honey, characteristic of honey vinegar	Honey	2 g of honey in 6% v/v acetic acid solution
Rancid	Sensation produced by aqueous solution of 2-furaldehyde	2-furaldehyde solution	0.7 ppm 2-furaldehyde ppm in 6%v/v acetic acid solution
Bitter almond	Sensation produced by aqueous solution of Benzaldehyde	Benzaldehyde solution	0,27 ppm of benzaldehyde in 6%v/v acetic acid solution
Bacteria	Odour associated with acetic acid bacteria on the culture media, fermented vinegar before sterilization	Recently fermented vinegar	Recently fermented vinegar without sterilization
Cheese	Odour associated with aged cheese,	Aged cheese, milk derived products	Aged cheese (negative quality aspect)
Sawdust/Wood shavings	Odour associated with fresh wood, to our case this odour is considered as negative quality aspect	Woody odour during sharpening a pencil, carpenters workshop	2% w/v fresh American oak wood shaving (without toasting) macerated in 6% v/v acetic acid solution

Table 6.

List of selected attributes for descriptive sensory analysis

Grouping characteristics	Selected Attributes
1. Chemicals	Ethyl acetate odour Alcohol/Liquor Pungent sensation/Acetic acid **Medicinal/Acetaldehyde (optional)**
2. Wine	Winy character Raisin
3. Woody	Woody
4. Fruity	Citrus **Apples (optional)** **Coconut (optional)** Red fruits
5. Spicy	Vanillin
6. Others	Sweet aroma **Rancid/2-furaldehyde (optional)** Bitter almond/Benzaldehyde Leather/old
7. Negative odours	**Bacteria (optional)** **Cheese (optional)** **Sawdust/Wood shavings (optional)**

Table 7. 13 Attributes selected for descriptive analysis of wine vinegar samples

1. Ethyl acetate odour (EA)
2. Pungent sensation (PUNG)
3.Winy character (WIN)
4. Woody (WOOD)
5. Alcohol/liquor (LIQ)
6. Raisin (RES)
7. Citrus (CIT)
8. Red fruits (RED)
9. Vanillin (VAN)
10. Sweet aroma (SWEET)
11. Bitter almond (BALM)
12. Leather/old (OLD)
13. General impression (GI)

Manuscript 6

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Defining the Typical Aroma of Sherry Vinegar: Sensory and Chemical Approach

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The aroma of the three different classes of Sherry vinegar was evaluated by gas chromatography/mass spectrometry (GC-MS) and gas chromatography/olfactometry (GC-O). GC-O was employed to identify substances responsible for aromatic notes associated with the selected descriptors of the typical aroma of Sherry vinegar and odor activity values (OAV) calculated to measure the single impact effect of different compounds selected by GC-O. Diacetyl, isoamyl acetate, ethyl isobutyrate, isovaleric acid, sotolon, and ethyl acetate reached high OAVs, turning out to be characteristic odor active compounds in Sherry vinegars. A total of 58 compounds were quantified, among them, 7 had not been previously reported in Sherry wine vinegars: ethyl 2-methylbutyrate, ethyl heptanoate, ethyl furoate, and ethyl benzoate, acetophenone, nonanoic acid, and sotolon. Linear discriminant analysis (LDA) reveals that using aroma compounds as variables, we can classify Sherry vinegars with 100% correct scores as different from red wine vinegars.

KEYWORDS: Aroma; Sherry vinegar; sensory analysis; GC-olfactometry; OAV; sotolon

INTRODUCTION

The quality of food products is a multivariate notion in which sensory properties play a crucial role. There is a need for the characterization of the typical sensory properties of traditional products (*1*) not only for the industrialization of food production, but also for laws on food safety and even for the development of innovative products.

Sherry vinegar can be considered a traditional food product used as seasoning and as a condiment. Wine vinegar is a grape-derived product obtained by a double-fermentative process (alcoholic and acetic). From a technological point of view, there are two well defined methods for its production: traditional processes and submerged methods (*2*). The first one is the so-called surface culture fermentation, where the acetic acid bacteria is placed on the air−liquid interface in direct contact with atmospheric air. Thus, oxygen availability to the acetic acid bacteria is not boundless, and a long period of time is required to obtain a high acetic degree. This process usually takes place in wood barrels. As a consequence, chemical modifications related with aging occur at the same time, and a highly appreciated product is obtained. Nowadays, traditional and selected vinegars (Sherry vinegars and traditional balsamic vinegars from Modena, among others) are produced following this method. Their sensory complexity is the consequence of chemical composition of the product, and the extraordinary organoleptic properties are acquired thanks to the method of production followed, the so-called "criaderas y solera" system (*3*). This particular method of production consists of a dynamic aging system in contrast with the not so usual static method, in which vinegar is produced and aged in a single butt (*4, 5*).

Therefore, Sherry vinegar regulation also allows the production by submerged culture acetification followed by aging in wood (dynamic or static system). Three qualities for Sherry vinegar are considered according to aging time in oak barrels: "Vinagre de Jerez" (minimum of 6 months), "Reserva" (at least 2 years), and finally, the new category "Gran Reserva" (at least 10 years) (*4*).

The aroma is one of the most important indicators of vinegar quality. For this reason, manufacturers choose the best raw materials as well as the optimum acetification conditions to increase the aromatic quality of wine vinegar and to present new products to the consumers (*2*). Although most of the volatile constituents are already present in wine, the final content is closely related to the genuine characteristics of the vinegar itself (*6*). The flavor of wine vinegars is determined by a series of volatile constituents with three different origins: wine substrate, acetification, and aging. During acetification, volatile compounds from wine may suffer important transformations. The acetic acid bacteria can metabolize high alcohols, in a way similar to that of ethanol, producing an increase in acid concentration. Moreover, ethylic esters are hydrolyzed, and at the same time, acetic esters such as isoamyl and methyl acetates are formed. Acetoin

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Table 1. List of Vinegar Samples

vinegar type	origin	time of aging	samples	acetic degree
red wine vinegar	Winery Banyuls (France)	0 months	VT1	6
		12 months	VT2	6
	Winery Priorat (Spain)	0 months	VT3	6
		12 months	VT4	6
white wine vinegar	Winery Modena (Italy)	0 months	VB1	6
		12 months	VB2	6
		6 months ("Vinagre Jerez")	VJ1	7
			VJ2	7
			VJ3	7
			VR1	7
			VR2	8
Sherry vinegar	Commercial samples (Market)	24 months ("Reserva")	VR3	8
			VR4	8
			VR5	9
			VGR1	7
		120 months ("Gran Reserva")	VGR2	10
			VGR3	10

also increases during acetification, being higher in traditional vinegars (*3, 7*).

During the aging of vinegar in wood barrels, there are several phenomena taking place as follows: (i) Loss of water through the pores of the casks and, consequently, a concentration of the rest of compounds; (ii) extraction of some compounds from wood (wood-extractables), mainly aromatic aldehydes; (iii) condensation (esterification); and (iv) oxidation (acetoin, diacetyl formation). Ultimately, these processes are responsible for the increase in the aromatic complexity of the vinegar.

Although the aroma composition of Sherry vinegars has been studied by several authors (*6, 8, 9*) as well as changes along aging in wood (*5, 10*), the contribution of individual compounds to the characteristic aroma of Sherry vinegar has not been considered up to now. Hence, the aim of the present work is to describe the aroma profile of the different categories of Sherry vinegar correlating the sensory results with the chemical data by measuring the single impact effect determined by the OAV (odor activity value) of different compounds selected by gas chromatography-olfactometry (GC-O). The use of GC-O allows one to screen and identify substances responsible for aromatic notes associated with the selected descriptors of the typical aroma of Sherry vinegar.

MATERIALS AND METHODS

Samples. We used for this study a total of 17 vinegars. The samples were divided into three categories (red wine, balsamic, and Sherry vinegars) according to the raw material and origin (**Table 1**). Four red and 2 balsamic vinegars were obtained from different wineries. All samples were elaborated by the traditional method (surface culture) in oak wood barrels. Eleven representative Sherry vinegars were purchased in the market (commercial samples), and they belong to the three categories established by Sherry vinegar regulation in accordance with aging time in oak barrels: 3 "Vinagre Jerez", (6 months old), 5 "Reserva" vinegars (2 years old), and 3 "Gran Reserva" vinegars (at least 10 years old).

Reagents and Chemicals. The standards of 58 aroma compounds, given in **Table 3**, were obtained from the commercial sources as follows: 2, 3, 14, 15, 19−21, 23−27, 29−32, 40−42, 45−51, and 53−58 (Sigma-Aldrich, Madrid, Spain); 1, 4, 6−10, 13, 17, 18, 28, 34−39, 44, and 52 (Merck, Darmstadt, Germany); 5, 11, 12, 16, 22, 33, and 43 (Fluka, Madrid, Spain). 3,4-Dimethylphenol (Sigma-Aldrich) and 4-methyl-2-pentanol (Merck) were employed as internal standards (IS). Dichloromethane, hexane, ether, anhydrous sodium sulfate, sodium chloride, and acetic acid were obtained from Merck, and all of them were of analytical quality. Water was obtained from a Milli-Q purification system (Millipore, USA).

Table 2. Similarity of the Odors of the Four Extracts to Vinegar Reference (VR1): Scaling and Rank[a]

extracts	similarity values (SV) and SD	rank first	rank second	rank third
dichloromethane	7.80a ± 0.91	5	0	0
hexane	5.3b ± 1.82	0	4	1
ether	3.4bc ± 2.36	0	1	4

[a] Five panelists, discontinuous scale (0−10); SV with the same letter were not significantly different at a level of 5%.

Sensory Analysis. *Sensory Panel.* The expert sensory panel that carried out the different experiments described in this work was composed of seven tasters (five females and two males), all of them belonging to a laboratory and with a lot of experience in wine vinegar sensory analysis (*11*).

Training was performed according to international protocols (ISO 4120:1983 and ISO 6658: 1985) (*12, 13*).

Descriptors Selection. Following methodology for descriptive analysis in wine vinegars (*11, 14, 15*), 10 attributes were chosen by consensus to describe wine vinegar samples as follows: ethyl acetate, pungent sensation, wine character, woody odor, red fruit, sweet aroma, bitter almond, vanilla, raisin, alcohol/liquor, and general impression. This last descriptor can be considered as a hedonic attribute since the sensory panel cannot be trained in it. The selected attributes were compiled in a tasting-card, and panelists were asked to rank each descriptor on a 10-cm unstructured scale (from not noticeable to very strong).

Threshold Determination. There are several published methodologies to calculate thresholds for flavor volatiles (*16, 17*), and we decided to use the method approved by the American Society for Testing and Materials (ASTM) (*18, 19*).

First, an ascending order test was carried out to delimit the proper concentration range to study and familiarize panelists with the odor of the compounds. Five 3-fold dilutions (3*x*, *x*, *x*/3, *x*/9, and *x*/27) were prepared by dispersing the substance whose threshold was to be determined in the medium of interest (acetic acid 7% w/v). Panelists were asked to indicate in which solution they perceived any odor. We fixed the *x* value (concentration of aroma compound) as a concentration 5-fold higher than the correspondent threshold values referenced in literature for wine (*20–22*) due to the marked interference of acetic acid.

Second, according to Plotto et al. (*23*), the three-alternative forced choice (3-AFC) test was used for threshold determination (*19*) (ASTM Designation: E-679, 2004). Four 3-ACFs a day were performed. Thus, three samples were given to panelists: two controls (7% acetic acid solution) and one test dilution (standard in 7% acetic acid solution). The test dilutions differed from the preceding one by a factor of 2 (2*x*, *x*, *x*/2, *x*/4...), and successive dilutions were tested until the lowest was consistently missed. The amount of aroma compound 2*x* corresponds to the minimum concentration of the substance that was perceived by at least 80% of the panel in the ascending order test. In this last case, we employed a factor of 2 since the threshold value was close to the concentration tested.

Then, the best-estimate criterion (*19, 23*) was used to calculate individual thresholds as follows: the threshold for each individual (best-estimate threshold) was an interpolated value determined as the geometric mean between the last concentration missed and the first concentration detected. Finally, the panel threshold was calculated as the geometric mean of the best-estimate thresholds of every individual panelist for each compound.

Selection of a Representative Extract for GC-O. A representative 2 year-old Sherry vinegar (VR1) was extracted with different organic solvents: hexane, ether, and dichloromethane. For each solvent, 50 mL of vinegar was extracted twice with 5 mL. Similarity tests were performed between the aroma of the obtained extracts and the vinegar

Table 3. Range of Volatile Compound Concentrations in Different Groups of Vinegar Samples

No	compound	mean concentration (μg/L) VJ ($n = 3$)	VR ($n = 5$)	GR ($n = 3$)	VT ($n = 4$)	VB ($n = 2$)	previously reported in Sherry wine vinegar
				Aldehydes			
1	acetaldehyde[c,a]	7.9 − 23.3	14.2 − 98.4	18.7 − 51.2	5.2 − 70.3	24.8 − 56.3	*5, 8*
2	hexanal[d]	n.d. − 9.41	n.d. − 10.7	17.0 − 35.6	12.5 − 46.7	10.7 − 60.6	*9*
3	2-furfuraldehyde	329 − 1358	336 − 1701	1189 − 7841	0.0 − 598	1056 − 3703	*9, 10, 59*
4	benzaldehyde	0.0 − 99.4	58.2 − 160	148 − 1561	0.0 − 89.0	0.0 − 115	*9, 46, 59, 60*
5	5-methyl-2-furfuraldehyde[d]	59 − 248	n.d. − 133	133 − 458	n.d.	729 − 2282	*9, 10, 59*
6	vanillin	n.d. − 1271	n.d. − 8875	2572 − 3926	n.q. − 3587	1494 − 4368	*4, 10*
	total aldehydes[c]	10.8 − 23.8	24.9 − 99.4	22.8 − 65.0	5.8 − 73.2	41.0 −64.0	
				Acetal			
7	acetaldehyde diethylacetal[c,d]	2.0 − 9.6	n.d. − 61.7	3.5 − 115	47.8 − 193	194 − 223	*46*
				Acetic Esters			
8	methyl acetate[c]	12.0 − 19.1	8.7 − 23.9	19.2 − 44.5	10.1 − 40.1	10.2 − 16.6	*5, 46*
9	ethyl acetate[c,a,d]	289 − 712	140 − 2210	351 − 452	132 − 3955	1929 − 3751	*7, 46, 59*
10	propyl acetate[d]	193 − 727	61 − 2354	886 − 3665	385 − 2207	3923 − 4605	*8, 9, 46*
11	isobutyl acetate	662 − 1458	290 − 2513	1241 − 4719	967 − 2284	2394 − 3083	*9, 46*
12	butyl acetate	n.d.	n.d. − 119	21.1 − 350	0.0 − 83.8	77 − 108	*9, 46*
13	isoamyl acetate[c]	1.02 − 3.72	0.36 − 5.59	2.03 − 11.6	2.47 − 7.26	3.97 − 5.30	*7, 9, 46, 60*
14	hexyl acetate	n.d.	n.d.	n.d.	n.d. −82	n.d.	*9, 46*
15	benzyl acetate	n.d.	n.d.	n.d. −190	n.d. − 142	n.d.	*9, 46, 60*
16	2-phenylethyl acetate	309 − 984	527 − 1491	1343 − 2090	765 − 2051	1035 − 2241	*9, 46, 60*
	total acetic esters[c]	304 − 737	159 − 2229	392 − 524	147 − 3986	1951 − 3783	
				Ketones			
17	diacetyl[c]	13.1 − 23.9	14.9 − 32.5	42.5 − 197	n.d.	18.5 − 55.7	*5, 6*
18	acetoin[c,d]	276 − 597	270 − 979	358 − 601	194 − 740	930 − 1020	*9, 46, 60*
19	acetophenone	n.d. − n.q.	n.d. − n.q.	n.q. − 62.1	n.d. − n.q.	n.q.	
	total ketones[c]	289 − 621	294 − 1007	401 − 798	194 − 496	949 − 1076	
				Ethylic Esters			
20	ethyl propanoate[d]	214 − 665	n.q. − 1493	700 − 6396	118 − 1142	3969 − 4547	*46*
21	ethyl isobutyrate[d]	269 − 361	n.q. − 671	330 − 1379	176 −1033	1202 − 1653	*9*
22	ethyl butyrate	50.7 − 209	n.q. − 338	98.6 − 1061	n.q. − 143	387 − 770	*9, 46*
23	ethyl 2-methylbutyrate	n.q. − 71.1	n.q. − 156	49.9 − 401	n.d.	n.d.	
24	ethyl isovalerate	371 − 788	n.q. − 1015	466 − 3317	n.d. − 491	969 − 1492	*9, 46*
25	ethyl valerate	n.d.	n.d. − 13.3	n.d. − 42.5	n.d. − 11.4	18.1 − 25.7	*9, 60*
26	ethyl hexanoate	n.d.	n.d. − 63	n.d. − 248	n.d. −121	98.5 − 143	*9, 46, 60*
27	ethyl heptanoate	n.d	n.d	n.d. − 7.46	n.d.	n.d.	
28	ethyl lactate[c,d]	1.24 − 9.19	0.0 − 9.23	2.04 − 30.4	1.23 − 10.8	41.4 − 48.9	*5, 9*
29	ethyl octanoate	n.d.	n.d.	n.q. −91.4	n.d.	n.d.	*9, 46*
30	ethyl furoate	25.1 − 88.0	36.6 − 255	251 − 422	34.1 − 122	220 − 313	
31	ethyl benzoate	n.d.	n.d. − 7.6	n.d. − 41.6	n.d.	n.d.	
32	ethyl phenylacetate	n.d.	n.q. −136	86.6 − 200	n.d.	328 − 512	*9*
33	diethyl succinate[c,d]	0.08 − 1.7	0.09 − 0.53	0.1 − 0.53	2.15 − 21.8	4.9 − 8.2	*5, 7, 9, 46, 60*
	total ethylic esters[c]	2.86 − 12.7	0.35 − 12.9	4.21 − 44.8	5.24 − 33.6	53.2 − 66.0	
				Alcohols			
34	methanol[c,a,d]	15.9 − 30.4	30.8 − 53.1	19.7 − 68.5	69.9 − 193	22.1 − 78.6	*5, 8*
35	ethanol[c,a,d]	425 − 1135	1002 − 3022	944 − 3412	3284 − 9479	4616 − 12387	*5, 8*
36	1-propanol[c,a,d]	n.d. − 0.97	n.d. − 14.4	0.30 − 19.2	1.19 − 34.7	19.6 − 62.6	*5, 8*
37	isobutanol[c,d]	3.16 − 5.56	2.27 − 5.85	3.45 − 8.53	7.98 − 12.9	9.97 − 10.8	*9*
38	2-methyl-1-butanol[c]	7.64 − 9.88	2.24 − 13.5	6.13 − 12.5	6.91 − 14.4	8.54 − 9.70	*5, 7–9, 46, 60*
39	3-methyl-1-butanol[c,d]	4.77 − 18.1	1.49 − 26.6	7.58 − 48.2	29.3 − 78.3	31.7 − 35.7	*5, 7, 8, 46, 60*
40	1-hexanol	n.d. −88	n.d.	n.d. − 88	n.q. − 449	n.q.	*9, 46*
41	*cis*-3-hexen-1-ol	14.2 − 52.5	15.9 − 51.8	27.0 − 43.7	31.7 − 55.0	18.3 − 20.4	*9, 46*
42	benzyl alcohol	137 − 624	133 − 737	378 − 1236	184 − 4407	529 − 563	*9, 46, 60*
43	furfuryl alcohol	289 − 413	134 − 1142	255 − 1124	0.0 − 1004	635 − 1147	*10, 46*
44	2-phenylethanol[c,d]	5.93 − 11.5	4.99 − 11.2	12.7 − 18.9	23.4 − 30.3	20.7 − 22.9	*5, 7–9, 46, 60*
	total alcohols[c]	475 − 1212	1063 − 3134	997 − 3560	3435 − 9840	4730 − 12609	
				Terpene			
45	α-terpineol	n.d. − n.q.	n.d. − 69.6	n.d. − 121	n.q.	n.d.	*9*
				Acids			
46	isovaleric acid[c,d]	38.4 − 57.2	39.6 − 55.0	58.7 − 121	1.16 − 11.2	24.5 − 33.2	*9, 60*
47	hexanoic acid	784 − 1325	683 − 2185	1860 − 2269	437 − 3322	1424 − 2296	*9, 46, 60*
48	heptanoic acid	n.d.	n.d. − 150	114 − 302	n.d. − 153	n.d. − 237	(*46*)
49	octanoic acid	144 − 531	182 − 704	350 − 774	160 − 732	299 − 546	*9, 46, 60*
50	nonanoic acid	n.d.	n.d.	n.d.	n.d. − 86.3	n.d. −52.8	
51	decanoic acid	24.4 − 113	21.6 − 92.0	68.1 − 106	27.0 − 136	66.3 − 80.8	*9, 46, 60*
	total acids[c]	39.9 − 58.2	42.3 − 58.1	61.1 − 62.6	1.79 − 20.8	26.2 − 36.4	
				Lactones			
52	γ-butyrolactone	682 − 1655	924 − 6583	2693 − 5385	1154 − 2238	1674 − 3337	*7, 46*
53	*trans*-β-methyl-γ-octalactone [d]	64 − 77	65 − 88	75 − 117	102 − 313	85 − 237	*10*

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Table 3. Continued

No	compound	mean concentration (μg/L) VJ ($n = 3$)	VR ($n = 5$)	GR ($n = 3$)	VT ($n = 4$)	VB ($n = 2$)	previously reported in Sherry wine vinegar
54	*cis*-β-methyl-γ-octalactone[d]	n.q. − 136	n.q. − 152	125 − 155	363 − 1534	204 − 1179	*10*
55	sotolon[b,d]	n.d.	n.d. − 748	663 − 939	n.d.	n.d.	
	total lactones	894 − 1829	1167 − 7478	3628 − 6536	1619 − 3823	1963 − 4753	
				Phenols			
56	guaiacol[d]	n.d. − 9.8	n.q. − 16.1	11.3 − 21.3	8.4 − 30.6	149 − 301	*10*
57	eugenol	n.d.	n.d.	n.d.	n.q. − 118	n.q. − 81.0	*9, 10*
58	4-Ethylphenol[d]	290 − 1652	427 − 1516	901 − 2382	94.3 − 427	405 − 509	*9, 60*
	total phenols	290 − 1652	427 − 1530	912 − 2397	103 − 441	554 − 891	
	total amounts[c]	1155 − 2576	2305 − 6105	2211 − 5127	6059 − 14405	7754 − 17640	
				Relative Area (Abundance)			
	2-acetylfuran	0.006 − 0.017	0.008 − 0.016	0.019 − 0.039	0.002 −0.006	0.087 − 0.114	
	2,3-butanediol diacetate	0.085 − 0.163	0.115 − 0.315	0.189 − 0.529	0.027 − 0.140	0.199 −0.209	
	TDN	0.044 − 0.105	0.011 − 0.049	0.016 − 0.030	0.008 − 0.415	0.008 − 0.014	

[a] GC-FID n.d.: below detection limit. [b] LLE-GC-MS n.q.: below quantification limit. [c] Concentration in mg/L. [d] Significant differences ($p > 0.05$) among Sherry, red, and Balsamic vinegars.

(*24*). A drop of extract was placed on a perfume sampling paper, and the aroma was compared with the original vinegar as a pair. Five members of our sensory panel were asked to rate the similarity on a discontinuous scale from 0 (no similarity) to 10 (equal) of each extract with the VR1 vinegar.

Gas-Chromatography (GC) Analysis. We used three different methods to determine the volatile compounds of interest in Sherry vinegar samples. A total of 52 compounds were determined by headspace sorptive extraction (HSSE) gas chomatography-mass spectrometry (HSSE-GC-MS). This method was not adequate for the determination of some major compounds such as ethyl acetate, ethanol, methanol, acetaldehyde, and propanol because of their high concentrations, among others. Hence, these 5 compounds were quantified by GC-flame ionization detector (GC-FID). For the special case of sotolon (polar compound), the HSSE-GC-MS method was not suitable because of the apolar nature of the sorbent in the stir bar, polydimethylsiloxane (PDMS). For this reason, sotolon was determined by liquid−liquid extraction GC-MS (LLE-GC-MS).

GC-FID Analysis. Ethyl acetate, acetaldehyde, methanol, ethanol, and propanol were quantified by GC-FID using the method proposed by Morales et al. (*7*). A 1 mL sample was filtered through Millex-GV_{13} filters of 0.22 μm, and 1 μL of 4-methyl-2-pentanol at 102.14 mg/L was added as internal standard (IS). Filtered samples were analyzed using a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector (FID). One microliter was injected in the split mode (1:60) into a CP-Wax 57 CB column, 50 m × 0.25 mm DI × 0.2 μm film thickness (Varian, Middelburg, Netherlands). The carrier gas was H_2 at 1 mL/min. The program temperature was 35 °C for 5 min, ramped at 4 °C/min to 150 °C held for 17.5 min. The injector was set to 220 °C and the detector to 250 °C. Data acquisition software was HPChemstation data processing system (Agilent Technologies).

Liquid−Liquid Extraction GC-MS (LLE-GC-MS). 4,5-Dimethyl-3-hydroxy-2(5*H*)-furanone (Sotolon) was quantified by LLE-GC-MS using the method proposed and validated by Silva Ferreira et al. (*24, 25*). To 50 mL of the samples, 5 g of anhydrous sodium sulfate was added and extracted twice with 5 mL of dichloromethane. The two organic phases obtained were blended and dried over anhydrous sodium sulfate. Then, 2.5 mL of the organic extract was concentrated 5 times under a nitrogen stream, and 5 μL of 3,4-dimethylphenol in dichloromethane at 0.55 mg/L was added as internal standard (IS). Four microliters of extracts were analyzed by GC-MS, using the conditions described elsewhere with minimum changes (*24*). The column employed was a CPWax- 57CB, with 50 m × 0.25 mm and 0.20 μm film thickness (Varian, Middelburg, Netherlands). The injector port was heated to 220 °C in splitless mode for 1 min, with a total flow rate of 53.5 mL. The carrier gas was He at a flow rate of 1 mL/min. The oven temperature was 40° (for 1 min), which was then increased at 2 °C/min to 220 °C and held for 30 min. The quadrupole, source, and transfer line temperatures were maintained at 150, 230, and 280 °C, respectively. The analysis was performed in SIM mode, and the ions selected for each compound studied were *m/z* 83 (sotolon) and *m/z* 107 (IS).

Headspace Sorptive Extraction GC-MS Analysis (HSSE-GC-MS). The HSSE sampling conditions were as follows (*26*): 5 mL of sample (wine vinegar) and 10 μL of 4-methyl-2-pentanol (IS) at 1045 mg/L was placed into a 20-mL headspace vial with 1.67 g of NaCl. A 10 mm long stir bar coated with 0.5 mm polydimethylsiloxane (PDMS) layer (Twister, Gerstel, Müllheim an der Ruhr, Germany) was placed in an open glass insert and placed into the vial to achieve the extraction in the headspace. Then, the vial was tightly capped and heated for 60 min at 62 °C in a thermostatic bath. The stir bar was removed with tweezers, rinsed with Milli-Q water, and dried with lintfree tissue paper. Finally, for the thermal desorption (TD), the stir bar was placed into a glass tube of 60 mm length, 6 mm o.d., and 4 mm i.d., which was placed in the autosampler tray of the thermo desorption unit for GC-MS analysis.

Gas chromatography analysis was carried out with a 6890 Agilent GC system coupled to a quadrupole mass spectrometer Agilent 5975inert and equipped with a Gerstel, Thermo Desorption System (TDS2) and a cryo-focusing CIS-4 PTV injector (Gerstel). The thermal desorption was performed in splitless mode and with a flow rate of 90 mL/min. The desorption temperature program was the following: 35 °C for 1 min, ramped at 60 °C/min to 250 °C, and held for 5 min. The CIS-4 PTV injector, with a Tenax TA inlet liner, was held at −35 °C with liquid nitrogen for total desorption time and then raised at 10 °C/s to 290 °C, and held for 4 min. Solvent vent mode was employed for the transfer of sample to the analytical column. A CPWax-57CB column, 50 m × 0.25 mm and 0.20 μm film thickness (Varian, Middelburg, Netherlands), was used, and the carrier gas was He at a flow rate of 1 mL/min. Oven temperature program was 35 °C for 5 min, then raised to 220 at 2.5 °C/min (held 5 min). The quadrupole, source, and transfer line temperatures were maintained at 150, 230, and 280, respectively. Electron ionization mass spectra in the full-scan mode were recorded at 70 eV electron energy in the range 35 to 350 amu.

All data were recorded using a MS ChemStation. The identity of 64 peaks (52 of them quantified) was assigned using the NIST 98 library and confirmed by retention index of standards when they were available. Quantification was performed employing the relative area to internal standard of the target ion of each compound. We built the respective calibration curves for each compound, plotting concentration versus relative areas. The samples were analyzed by triplicate, and blank runs of empty glass tubes were done before and after each analysis.

Gas Chromatography−Olfactometry. To identify substances responsible for the aromatic notes associated with the selected descriptors of the typical aroma of Sherry vinegar, GC olfactometric analysis was applied to three representative samples corresponding to the three different qualities of Sherry vinegars: VJ2, VR1, and GR2. Extraction was performed according to the methodology previously described for

ohio2/yjf-yjf/yjf-yjf/yjf99907/yjf5156d07z | 23:ver.3 | 7/12/08 | 13:27 | Msc: jf-2008-00903n | TEID: ajk00 | BATID: jf9a18

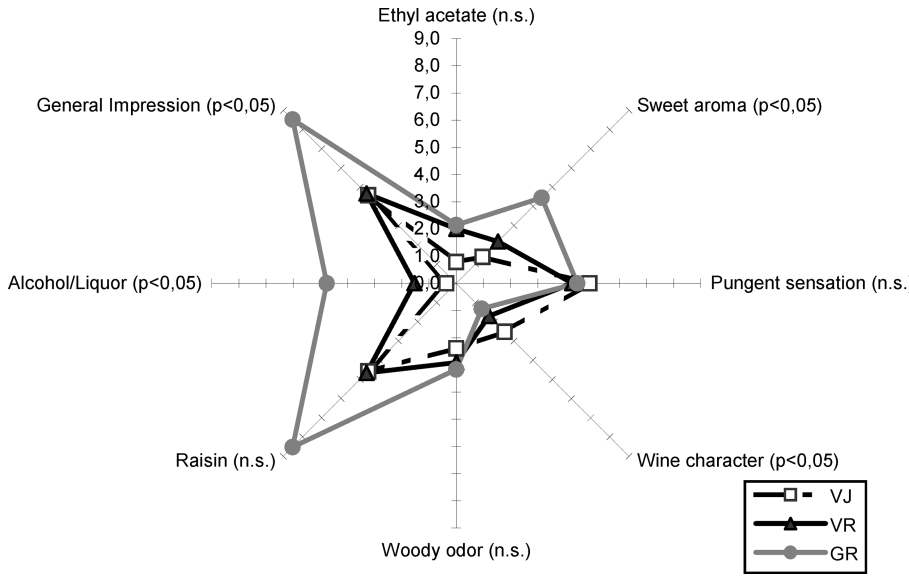

Figure 1. Aroma profile of three representative Sherry vinegars, one from each category ("Vinagre Jerez", "Reserva", and "Gran Reserva").

LLE-GC-MS. Then, 2 mL of this organic phase was concentrated 5 times under a nitrogen stream. Several dichloromethane extracts from different vinegars were submitted to GC-O. Chromatographic conditions were the following: Hewlett-Packard HP 5890 gas chromatograph; BP-21 column (50 m × 0.25 μm), fused silica (SGE, France); hydrogen (5.0, Air−liquid, France); flow, 1.2 mL/min; injector temperature, 220 °C; oven temperature, 40 °C for 1 min programmed at the rate of 2 °C/min to 220 °C, maintained during 30 min. Extract aliquots of 1 μL were injected into the GC in splitless mode (0.5 min); split flow, 30 mL/min. The makeup gas employed on the olfactometric device (SGE, France) was air (80% N_2; 20% O_2) (Air−liquid, France). Two streams were used: one was bubbled in water, nose moistener, the other was applied at the exit of the GC column to lower the temperature of the effluent.

Compound Identification. Identification of odorants was performed by comparison of MS spectra, chromatographic retention indices (RIs), and odor description with experimental and literature data. RIs were calculated in GC-FID-O and HS-SBSE-GC-MS from the retention times of *n*-alkanes by linear interpolation, according to the literature (*25*).

Statistical Analysis. All statistical analysis were performed by means of Statistica software, version 7.0 (Statsoft, Tulsa, USA).

RESULTS AND DISCUSSION

Sensory Descriptive Analysis of Sherry Vinegars. The 11 Sherry vinegar samples considered in this study were described by the expert sensory panel (*15*). The selected descriptors were ethyl acetate, sweet aroma, pungent sensation, wine character, woody odor, raisin, alcohol/liquor, and general impression. The attributes bitter almond, red fruit, and vanilla were not considered as they reached very low scores in all of the samples. Representative spider charts are shown in **Figure 1**. Pungent sensation and general impression reached the highest scores, while ethyl acetate and wine character accounted for the lowest marks. The scores obtained in the three categories for ethyl acetate, woody odor, and pungent sensation were similar, which suggests that these are generic characteristics of Sherry vinegars. Raisin reached similar marks in "Vinagre Jerez", and "Reserva" vinegars; however, "Gran Reserva" samples accounted higher marks to this attribute. On the contrary, the scores given to the descriptors general impression, alcohol/liquor, sweet aroma, and wine character were significantly different among the three different qualities (according to the $p \leq 0.05$ value obtained in the ANOVA). General impression, alcohol/liquor, and sweet aroma reached the highest values for "Gran Reserva" (the oldest vinegars). Hence, these three sensory descriptors are related with the time spent by vinegars in wood, that is to say, with the aging of vinegars. Conversely, wine character reached the highest scores for "Vinagre Jerez" (the youngest vinegars), being inversely correlated with aging.

Selection of a Representative Extract for GC-O. A total of five panelists were asked to score the similarity between the odor of three extracts (dichloromethane, hexane, and ether extracts) and the odor of the VR1 vinegar itself. Results indicate that among the four extracts tested, the dichloromethane extract is the most representative since it reached the highest similarity values and was ranked by all the panelists in the first place (**Table 2**). In addition, the dichloromethane extract showed significant differences ($p < 0.05$) with the other extracts. Hence, this solvent was chosen to perform the GC-Olfactometry analysis.

Quantification and Identification of Aroma Compounds. **Table 3** shows ranges of aroma compounds determined in 11 Sherry vinegars, 4 red, and 2 balsamic vinegars, and as can be seen, a total of 58 compounds were quantified. To our knowledge, among them, 7 had not been previously reported in wine vinegars. These new compounds are principally esters (ethyl 2-methylbutyrate, ethyl heptanoate, ethyl furoate, and ethyl benzoate), ketones (acetophenone), acids (nonanoic acid), and lactones (sotolon). Some of them have been previously identified and quantified in Sherry wines (ethyl heptanoate, ethyl furoate, ethyl benzoate, and sotolon) (*27, 28*) and red wines (ethyl 2-methylbutyrate) (*29, 30*). Sotolon was identified as a key aroma compound in flor wines (*20, 31–33*). This compound is a very powerful odorant, which contributes to the characteristic sensory impression of several foods, and it is also present in other types of wines such as the Botrytised wines, Jura wines, ("vins jaunes" and "vins doux naturelles"), Port, and Tokay (*34*). The odor of sotolon is described as nutty at low concentrations and curry at higher levels. Therefore, the presence of this molecule is closely related with aging in Port wines (*24, 35*), and according to Moreno et al. (*28*), sotolon along with acetaldehyde diethylacetal can be used as markers of the changes in "fino" Sherry wine during its biological aging. The formation mechanism of sotolon is not totally clarified in wines (*33, 36–41*). Nevertheless, some studies have demonstrated that oxygen has an important role on the rate of formation of this key odorant (*22, 24, 25, 35, 42*). In addition, it seems that sotolon is originated in biologically aged wines by chemical reaction

between α-ketobutyric acid and the acetaldehyde produced by flor yeasts (*20*), as proposed by Pham et al. (*33*).

In this work, sotolon was detected in 7 of the 11 Sherry vinegars (all the "Reserva" samples, except for VR2, and the three "Gran Reserva" vinegars) with a concentration ranging 663−939 μg/L. This compound is found at levels from only few dozen μg/L in young wines to about 100 μg/L in 10-year-old wines and up to 200 μg/L after 10 additional oxidative aging years (*20, 24*). In agreement with this, except for VR2, sotolon was detected and quantified in the most aged Sherry vinegar categories ("Reserva" and "Gran Reserva"). As can be seen in **Table 3**, most aged vinegars (GR) account for the highest concentrations. Hence, the formation of this compound is favored by time in an oxidative medium. Sotolon was not quantified in any "Jerez Vinegar" class samples. This is in agreement with Zea et al. (*43*) since "fino" Sherry wines aged for less than 2.5 years showed very low concentrations of sotolon, below its odor threshold in wine (5 μg/L). After this time, concentrations of sotolon were higher, ca. 700 μg/L, and were increasing with aging. However, it is remarkable that sotolon was not quantified in any of the red and balsamic vinegars (**Table 3**).

Being present in concentrations between 132 and 3955 mg/L, ethyl acetate was by far the major volatile compound in all of the samples, followed by considerable amounts of acetoin (194−1020 mg/L). For Sherry wine vinegars, outstanding concentrations for diacetyl (13.7−197 mg/L) and isovaleric acid (38.4−121 mg/L) were also determined. Among these compounds, ethyl acetate has a particular relevance due to its great influence on the final sensory profile of Sherry vinegars. In addition, we observed a correlation between amounts of ethyl acetate and ethanol ($r = 0.8$). Other authors also observed that acetoin and isovaleric acid were two of the major volatile compounds quantified in commercial Sherry vinegars (*8, 9*). Diacetyl (produced by the oxidation of acetoin) has been reported to increase with aging and is proposed as an indicator of the age of Sherry vinegars (*5*). This finding has been confirmed in our samples. This compound was not present in red wine vinegars (**Table 3**).

Acetaldehyde diethylacetal is formed in Sherry wine from the acetaldehyde produced by flor yeast and exhibits a strong odor impact on wines under biological aging, to which it contributes with green fruit and liquorice aroma notes (*20*). This compound was quantified in most of the studied Sherry vinegars, with concentrations ranging between 2.0 and 223 mg/L.

Hexyl acetate and eugenol could not be quantified in the Sherry vinegars since their concentrations were under their limits of detection (LOD). Vanillin, eugenol, guaiacol, and *cis*- and *trans*-β-methyl-γ-octalactone, also named oak lactones, could be effectively extracted from oak wood and oak chips during experimental aging of wine vinegars (*10*). Eugenol has a clove-like aroma, and its concentration increases when barrels are heated at medium or heavy toast levels. This compound is present at very low concentrations in Sherry vinegars (*9*), usually under detection limits (LOD). Vanillin was quantified in 7 of the 11 Sherry vinegars in a concentration ranging between 1271 and 8875 μg/L. This compound is considered an important contributor to the quality of barrel-aged wines, and its content in wood barrels depends on differences in heat penetration, rather than the intensity of toasting. *cis*-β-Methyl-γ-octalactone was quantified in eight Sherry vinegars and in all of the red and balsamic vinegars. The *cis*/*trans* ratios were higher than 5 for red and balsamic samples aged in new barrels and lower than 2 for Sherry vinegars, as opposed to wine aged in American oak barrels, whose ratios are always greater than 5 (*44*). This is probable because of the fact that Sherry vinegars, in general, are produced in very old wood barrels.

Besides sotolon (previously mentioned), ethyl-2-methylbutyrate, ethyl heptanoate, ethyl octanoate, ethyl benzoate, acetophenone, and α-terpineol were only quantified in Sherry vinegars since their concentrations were under the limits of quantification (LOQ) in red and balsamic samples. In addition, ethyl heptanoate, ethyl octanoate, α-terpineol, and acetophenone were only determined in "Gran Reserva" vinegars.

Concentrations found for hexanal, 2-phenylethyl acetate, ethyl furoate, 2-phenylethanol, and sotolon were significantly different ($p \leq 0.05$) among the three different Sherry qualities, reaching the highest concentrations for "Gran Reserva" vinegars.

However, as was expected, red vinegars accounted for the highest amounts of methanol since red wines have higher concentration (152 mg/L) than rosés (91 mg/L), while white wines have even less (63 mg/L) (*45*). Consequently, the concentration of methyl acetate in red vinegars was also proportionally higher in relation to the time of aging.

Other volatile compounds identified in the samples and confirmed with their corresponding mass spectra of respective standards were acids such as propanoic (RI 1544), isobutyric (RI 1572), butyric (RI 1648), and pentanoic (RI 1751) acids, and *cis*-3-hexen-1-ol acetate (RI 1304), isomers of linalool oxide (RI 1355 and 1377), methyl salicylate (RI 1766), and 5-hydroximethyl-2-furfulraldehyde (RI 2357). Most of them had been previously reported as constituents of Sherry vinegar aroma (*9, 46*). Methyl salicylate and isomers of linalool oxide have been identified in wines, but to our knowledge, it is the first time they are described in wine vinegars. 5-Hydroximethyl-2-furfulraldehyde (5-HMF), which is primarily a Maillard reaction product, increased in the oldest samples according to other authors (*4*). This compound can be extracted from oak wood, although its presence in vinegars has been traditionally attributed to the legal practice of must caramel addition.

Other compounds were tentatively identified with the aid of the NIST library and RIs, since their corresponding standards were not available: isomers of 2,3-butanediol diacetate (RI 1380 and 1488), acetylfuran (RI 1504), and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (RI 1734). TDN if present at concentrations above 20 μg/L causes an unpleasant kerosene or petrol-like note, contributing to the off-flavor of wine. Nevertheless, several authors pointed out a positive influence in the wine aroma complexity when TDN is present at concentrations lower than the threshold limit (*47*). This compound seems to be a genuine compound of long aged cavas (*48*) and Riesling wines (*49*), in which it increases with maturation. Nevertheless, as can be seen in **Table 3**, we obtain relative areas of TDN decreasing with time of aging in Sherry vinegars. This finding is similar to that in Madeira wines in which this compound decreases with oxidative aging (*47*).

Correlations between sensory descriptors and aroma compounds (data not shown) demostrate that sweet aroma is the descriptor better correlated with a major number of volatile compounds, a total of 16, with most of them being esters, such as isoamyl acetate, ethyl furoate, and isobutyl acetate ($r > 0.7$). On the contrary, raisin and wine character are not correlated with any single compounds. It is also remarkable that *trans*-β-methyl-γ-octalactone is correlated with woody odor ($r = 0.74$) as was expected since it is responsible for the oak wood odorant note present in barrel-aged alcoholic beverages (*50, 51*). Besides, the sensory attribute alcohol/liquor is correlated with sotolon ($r = 0.7$).

Table 4. Classification and Cross-Validation Results of LDA

	predicted group membership (%)	
	Sherry vinegar	red vinegar
Original Model		
Sherry vinegar	100	0
red vinegar	0	100
Cross-Validation Model		
Sherry vinegar	100	0
red vinegar	0	100

Multivariate Statistical Analysis. To perform multivariate statistical analysis, we made a substantial reduction of variables. First, those compounds accounting for a high number of no detected or no quantified scores in the samples were eliminated. Moreover, redundant variables with high correlation coefficients ($r > 0.7$) were eliminated. Finally, we used 14 variables: acetaldehyde, ethanol, methyl acetate, ethyl isobutyrate, diacetyl, hexanal, 2-methyl-1-butanol, acetoin, γ-butirolactone, *trans*-β-methyl-γ-octalactone, 4-ethylphenol, decanoic acid, and sotolon.

Linear discriminant analysis (LDA) was performed considering two groups of samples: Sherry vinegars and red vinegars. Balsamic vinegars ($n = 2$) were excluded because of a very low number of samples ($n = 2$). LDA is a supervised chemometric method widely used for classification purposes. This method minimizes the variance within categories and maximizes the variance between categories. LDA renders a number of orthogonal linear discriminant functions equal to the number of categories minus one; when two classes are considered, one linear discriminant function is obtained.

When LDA is applied to a set of samples, the samples are usually divided into a training set and a test set, the first one to find discriminant functions and the second one to check the utility of those discriminant functions to correctly classify new samples. In our case, we have used the so-called leave-one-out method (*52*) consisting in dividing the whole set of samples into two groups: a training set holding all the samples except one which is used then as a test set. Thus, LDA was applied as many times as the number of samples.

One discriminant function that includes the variables ethanol, diacetyl, hexanal, *trans*-β-methyl-γ-octalactone, 4-ethylphenol, and sotolon was obtained when the LDA forward stepwise method was applied. We have obtained 100% of correct classifications of samples in the cross-validation analysis by the leave-one-out method, and the results are reported in **Table 4**.

GC-O. GC-O experiments were conducted with dichloromethane extracts obtained from three Sherry vinegars, representative of each quality (VJ2 "Vinagre Jerez", VR1 "Reserva", and GR2 "Gran Reserva"). This olfactometry study as screening procedure was performed by a panel of four individuals, and sniffing of samples were carried out in triplicate to increase the robustness of data. Results of the screening are summarized in **Table 5**. We attempted to correlate the chemical molecules identified by MS with the aroma perceived with the same RI. The descriptors were selected according to their frequency of citations. Hedonic terms (good/bad) and their analogues were not considered and were replaced by the most cited.

A total of 80 odors were obtained in the sniffing of the three samples. Among them, 25 were found in the three vinegars, and only 8 of them were detected by all of the panelists: glue (RI 1063), butter (RI 1084), cherry/strawberry (RI 1118), banana/mulberry/strawberry (RI 1123), strawberry/banana (RI 1414), pungent (RI 1422), cheese (RI 1705), and curry/liquorice (RI 2201). These odor-active regions were identified as ethyl acetate, diacetyl, butyl acetate, isoamyl acetate, ethyl octanoate, acetic acid, isovaleric acid, and sotolon, respectively. In addition, other 9 odors perceived in all of the samples reached a frequency $\geq 50\%$: strawberry (RI 1080, ethyl isobutyrate), river water/lake/vapor (RI 1532, unknown), cheese/feet (RI 1595, isobutyric acid), burned/burned hair (RI 1655, unknown), cheese/vomit (RI 1811, unknown), boiled vegetable (RI 1875, unknown), clove (RI 2054, eugenol), sweet/vanilla (RI 2076, unknown), and flower/fruit/banana (RI 2151, unknown).

By comparison of the three vinegars, we can see that sample GR2 revealed 64 odor-active areas with 18 of them reaching the maximum frequency (100%). VR1 presented 62 odors, 14 of them being perceived by all the assessors, and VJ2 was the sample with a minor number of odor-active regions up to 46, and only 10 of them obtained the maximum frequency. Hence, a greater aromatic complexity is observed when the time of aging in wood increases.

Odor Activity Values. After screening by detection frequency in GC-O, calculation of odor activities values (OAVs) enables a more reliable evaluation of potent odorants for a given product, despite its limitations. OAVs are obtained by dividing the concentration of the compound by its recognition threshold in a suitable matrix (*53*). Hence, OAV is linearly proportional to concentration and threshold (*54*). However, it is known that the slope of the psychometric function of a compound varies markedly between different compounds (*55*). So, the intensity of some volatile compounds will rise rapidly after exceeding their odor threshold (OT), while the intensity increment of other volatile compounds can be very small over many orders of concentration magnitude (*56*).

In relation to the OAV concept, although it is not a psychophysical measure for perceived odor intensity, it is assumed that the odorants showing high OAVs contribute strongly to the overall aroma (*54, 57*). However, because of masking, a compound showing an OAV > 1 can still be insignificant in a mixture and has to be examined further by sensory analysis (*53*).

First of all, we had to calculate our own odor thresholds for the special case of vinegar matrices. For that, we selected those odorants which either reached high detection frequency in GC-O, or high concentrations in Sherry vinegar, or even those with important impact in wines. To estimate the odor contributions of the selected odorants, their OAVs were calculated on the basis of their nasal thresholds in a 7% (w/v) acetic acid solution (**Table 6**). Compounds in the table are ranked according to the maximum odor activity values (OAV max) reached in the three Sherry wine vinegars under study in GC-O experiments (VJ2, VR1, and GR2). Altogether, 20 of the 27 odorants showed in **Table 6** reached concentrations above their odor thresholds in this set of Sherry vinegars. Data in the table confirm the results obtained in the olfactometry study to almost all of the selected odorants and in fact support the usefulness and validity of the GC-O approach in this work. Hence, it can be seen that nearly all of the compounds with high GC-O scores also had high OAV. The single exception to this observation is butyl acetate, with OAV < 1.

The highest odor activity value of 4899 was calculated for diacetyl, followed by isoamyl acetate, which was the second in rank. In addition, an increase in OAV for diacetyl and isoamyl acetate was observed in vinegars with longer aging in wood. Hence, GR2 displayed the highest OAVs. Other compounds such as acetaldehyde diethylacetal, ethyl isovalerate, ethyl

Table 5. Detection Frequency (%) of the Odors of VJ2, VR1, and GR2 Sherry Vinegars Detected and Described by the Sniffing Panel

RI[b]	RI[c]	odor quality	odorant (tentative identification)	VJ2	VR1	GR2
1063		glue	ethyl acetate	100	100	100
1070		alcohol	ethanol	75	50	50
1072		rancid	unknown	25	50	75
1076	928	chemical, alcohol, grass, plastic	acetaldehyde diethylacetal	25	50	0
1080	962	strawberry	ethyl isobutyrate	50	100	100
1084	969	butter	diacetyl	100	100	100
1089	998	plastic, medicinal, chemical	isobutyl acetate	0	100	50
1097	1014	strawberry	ethyl butyrate	0	50	75
1105	1028	fruit, banana	ethyl 2-methylbutyrate	0	75	50
1110	1044	strawberry	ethyl isovalerate	75	0	25
1118	1046	cherry, strawberry	butyl acetate	100	100	100
1123	1112	banana, mulberry, strawberry	isoamyl acetate	100	100	100
1173	1150	fruit, banana	amyl acetate	0	75	50
1181		banana	unknown	0	50	0
1220	1207	rancid	3-methylbutanol	25	50	0
1239	1208	banana, fruit, mulberry	ethyl hexanoate	0	75	50
1254	1257	mulberry, banana	hexyl acetate	0	0	75
1277		rancid	unknown	0	0	25
1297		boiled potato	unknown	50	50	0
1327	1271	sweet, yogurt, dairy product	acetoin	0	50	25
1360		toasted maize	3-hydroxy-2-pentanone	0	25	50
1414	1432	strawberry, banana	ethyl octanoate	100	100	100
1418		boiled potato	unknown	75	0	75
1422		pungent	acetic acid	100	100	100
1438		fruit, flower, strawberry	linalool oxide (isomer)	50	0	100
1439		feet	unknown	0	75	50
1461		strawberry, sweet, mulberry	unknown	0	25	75
1484		boiled potato	methional	0	75	100
1496		strawberry, sweet	unknown	0	100	50
1510		toasted maize, fried chicken, burned	2,3-butanediol diacetate	50	25	25
1513		boiled potato	unknown	0	0	25
1517		strawberry	unknown	0	0	50
1520		plastic	unknown	25	0	25
1532		river water	unknown	75	100	100
1537		strawberry, alcohol, roses, sweet	unknown	0	50	50
1545		banana, mulberry	ethyl 3-hydroxybutanoate	25	50	0
1553		flower, roses, sweet	unknown	50	25	75
1557	1518	aspirin, mulberry, fruit	benzaldehyde	100	75	0
1563	1536	aspirin, mulberry, cherry	ethyl nonanoate	100	100	0
1586		rancid, cheese, feet	propanoic acid	0	50	75
1595		cheese, feet	isobutyric acid	75	75	100
1655		burned, burned hair	unknown	75	75	50
1659		strawberry	unknown	50	0	25
1661		cheese, vomit	butyric acid	0	100	50
1671	1664	burned, burned hair	furfuryl alcohol	0	25	25
1679	1659	sweet	ethyl benzoate	50	0	0
1685		roses, talcum power, perfume	unknown	0	0	50
1705	1670	cheese	isovaleric acid	100	100	100
1722		strawberry	unknown	0	0	25
1747		rancid, cheese	pentanoic acid	25	50	50
1762		boiled vegetable or potatoes	methionol	50	50	0
1763		strawberry, fruit	unknown	75	0	25
1780	1770	plasticine, wax pencil	ethyl phenylacetate	0	50	25
1786		urine	ethyl salicylate	0	25	0
1789	1800	grass, feet, humidity	2-phenylethyl acetate	0	25	25
1796		roses, floral perfume	unknown	50	0	100
1802		metallic	unknown	0	25	0
1809		boiled vegetable	unknown	0	50	0
1811		cheese, vomit	unknown	75	50	100
1842		sweet, fruit, fruit preserve	unknown	25	25	0
1858		stewed apples, apple juice	β-damascenone	0	50	75
1875		boiled vegetable	unknown	50	50	50
1878		cheese, feet	unknown	25	25	75
1880		fruit, fruit preserve	unknown	0	25	75
1889		sweet, vanilla	2-methyl-3-hydroxy-4-pyrone	75	50	0
1896		metallic	unknown	50	0	25
1932	1927	cheese	heptanoic acid	0	50	50
2017		flower (daisy), chamomile tea	4-ethylguaiacol	0	25	25
2028	2009	urine, chamomile tea, chemical	octanoic acid	0	0	25
2033		flower, honey, roses	unknown	75	0	100
2051		coconut, sweet	γ-decalactone[a]	0	50	100
2054	2087	clove	eugenol	50	50	75
2076		sweet, vanilla	unknown	75	75	100
2098		clove, vanilla, pepper	4-vinylguaiacol	25	50	75
2105		toasted, dried fruit	unknown	25	0	25
2113		liquor, “oloroso sherry wine”, sweet	unknown	50	50	0
2137	2098	sweet, vanilla	nonanoic acid	25	25	75
2149	2097	cardboard, metallic, meta	4- ethylphenol[a]	0	25	0
2151		flower, fruit, banana	unknown	75	75	75
2201		curry, liquorice, “oloroso sherry wine”, toffee, syrupy sugar	sotolon	100	100	100

[a] Possibly identified compounds. [b] BP-21 column. [c] CPWax- 57CB column.

Table 6. Odour-Activity Values (OAV) and Odor Thresholds

odorant	odor threshold (μg/L)	OAV max	VJ2	VR1	GR2
diacetyl	40	4899	595	807	4899
isoamyl acetate	12	1146	118	365	1146
acetaldehyde diethylacetal	133	865	15	464	865
isovaleric acid	150	807	380	359	807
ethyl isovalerate	4.4	754	84	321	754
ethyl isobutyrate	3.66	377	73.5	149	377
4-ethylphenol	4	326	72	297	326
acetaldehyde[a]	402	155	43	155	130
acetoin	8800	68	68	65	68
2-phenylethyl acetate	88	65	4.7	13	65
ethyl octanoate	1.5	62			62
sotolon	16	59		47	59
vanillin[a]	94	47		47	42
isobutyl acetate	177	27	4.7	10	27
ethyl acetate[a]	91000	14	1.5	10	14
ethyl propanoate	516	12.3	0.5	2.5	12.3
benzaldehyde[a]	158	9.9		0.77	9.9
trans/cis-oaklactones	78	2.7	1.5	1.6	2.7
hexanoic acid	2600	1.8	0.3	0.8	1.8
furfural[a]	6200	1.26	0.22	0.14	1.26
butyl acetate	453	0.8			0.8
octanoic acid	987	0.8	0.15	0.37	0.8
furfuryl alcohol	1415	0.79	0.29	0.28	0.79
propyl acetate	6708	0.5	0.03	0.19	0.5
bencyl alcohol	16900	0.3	0.04	0.04	0.3
ethyl benzoate	210	0.20		0.03	0.20
eugenol	0.17				

[a] Threshold reported in a previous work (*15*).

isobutyrate, and isobutyl acetate also reached high OAVs in the three samples, and their values increased with time.

However, OAV of ethyl acetate was >1 in the three samples but only reached an OAV max of 14 because of its high odor threshold. In spite of this, this compound has a great influence on the final sensory profile (*58*). In addition, ethyl acetate presents a characteristic (glue) aroma, very easy to recognize, and it is one of the selected sensory descriptors for vinegars.

Ethyl propanoate and hexanoic acid showed OAV <1 in VJ2 samples (the youngest vinegar). However, these OAVs increased with aging reaching values major than 1 in the oldest vinegars.

Finally, according to the results of OAV, GC-O, and GC-MS, diacetyl, isoamyl acetate, isovaleric acid, sotolon, and ethyl acetate are characteristic odor active compounds in Sherry vinegars since they showed concentrations far above their odor thresholds and were detected in the three samples analyzed by all panelists. These results confirmed the active role of the five compounds in the tipicity of Sherry vinegars.

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Manuscript 7

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Targeting Key Aromatic Substances on the Typical Aroma of Sherry Vinegar

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Two gas chromatography−olfactometry (GC−O) techniques were used to screen targeting compounds with an impact on the perceived quality of Sherry vinegar: detection frequency and aroma extract dilution analysis (AEDA). The GC−O study revealed the presence of 108 aromatic notes, of which 64 were identified. Diacetyl, isoamyl acetate, acetic acid, and sotolon reached the highest frequency and flavor dilution (FD) factors. Ethyl acetate accounted for the maximum frequency but only a FD factor of 4. To test the sensory impact of these odorants, they were added to a 7% (w/v) acetic acid solution. We determined similarity values (SV) between solutions and the Sherry vinegar. The highest value from the similarity test was observed when diacetyl, ethyl acetate, and sotolon were added simultaneously. The profile of this model solution and a representative Sherry vinegar showed good similarity in the general impression descriptor, which emphasizes the important contribution of these three compounds to the global aroma of this vinegar.

KEYWORDS: Aroma; Sherry vinegar; GC−olfactometry; detection frequency; aroma extract dilution analysis; aroma recombination; sotolon

INTRODUCTION

Sherry vinegar is a very appreciated commodity produced in the Sherry wine region and has its own protected denomination of origin (*1, 2*). A minimum period of 6 months of aging in wood barrels is mandatory for these products. Its main characteristics are a high acetic degree (legally minimum than 7°) and a special flavor, which resembles that of Sherry wine. Although its composition and sensory characteristics have been studied by different authors, very few studies deal with its aroma composition (*3–6*). To date, 96 aroma compounds have been identified in Sherry vinegars (*4–11*): 23 carbonyl compounds, 2 ethers, 1 acetal, 26 esters, 3 lactones, 20 alcohols, 6 volatile phenols, 1 terpene, and 14 acids. However, systematic studies to indicate the odorants responsible for the characteristic bouquet of Sherry vinegar have not been reported up to now. Among these volatile compounds, ethyl acetate accounts for the highest concentrations ranging from 107−1247 mg/L (*7, 9, 12*). Recently, we have identified sotolon (*12*) for the first time in Sherry vinegar.

Targeting substances with a large impact on the perceived quality of a food product constitutes one of the most challenging tasks in flavor research. The main difficulty is found on the fusion between sensory and chemical data. Despite the controversy concerning the best-suited technique for a given matrix, several methods using gas chromatography coupled with olfactometry (GC−O) procedures have been applied to the purpose of ranking substances by their respective impact on the overall aroma of foodstuff (*13–23*). They can be divided into three main categories: (i) dilution procedures, such as CHARM analysis, also called dilution to threshold, which was developed by Acree et al. (*24*) or aroma extract dilution analysis (AEDA) (*25*); (ii) direct intensity methods, which include posterior intensity methods (*26*), OSME (*27*), and finger span method (*28*); and (iii) frequency counting with scoring attribution (*29*).

AEDA measures the maximum dilution of an extract that an odor is perceived and reports this as the flavor dilution factor (*30*). The AEDA technique proved to be very powerful for screening the impact of odor contributors to an aroma and identifying molecules in several foodstuffs (*31*). Moreover, it allows considering odor modifications because of different concentrations. The major drawbacks are that AEDA only reports the maximum dilution value and the length of time required to complete the analysis on each dilution for a single extract (*30*). This fact results in the use of only one or two assessors and the limitations concerning variation because of individuals. These last disadvantages could be overcome by the use of multiple sniff ports. In addition, the results obtained are based on detection threshold and not real intensities (*28*).

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The frequency detection method has also been widely used to screen odorants with a large impact in several matrices, such as wines (*22, 32*), oil (*33*), and red vinegars (*13*). In this method, a panel of assessors carries out GC−O on the same extract and the number of panelists that detect an odor active compound at the olfactory detector outlet is considered as a measure for the odor intensity of the compound (*33*). Hence, compounds that are detected more frequently are concluded to have a greater relative importance on the odor of the given sample (*34*). The fundamental benefits of detection frequency is it simplicity. In addition, it is the least time-consuming and the easiest to conduct, and panelists do not require much training (*34*). However, the main limitation of this method relates to the scale of measurement. Hence, at a particular concentration, a compound may be perceived by all assessors reaching the maximum frequency, but if the concentration is increased, the odor intensity will also increase and, however, the detection frequency cannot (*30*). Nevertheless, the discriminative capabilities of the detection frequency may be improved by taking into account intensity, although this procedure requires intensive training of the panel. For this reason, some authors, to quantify the results for each odor region, employ the "adjusted frequency" or "modified frequency", in which frequency and intensity average of the odor region are taken into account (*22, 32, 35*).

The aim of this work was to target volatiles with a large impact on the perceived tipicity of Sherry vinegar using AEDA and frequency methods, and they were compared to define their respective discrimination ability.

MATERIALS AND METHODS

Vinegar Sample. A representative 2 year old vinegar ("Vinagre Reserva", VR1) was selected by the sensory panel as being a Sherry vinegar "type", with the methodology described in a previous work (*12*). Its acetic degree was 7% (w/v).

Chemicals and Reagents. The standards of 58 aroma compounds studied, given in **Table 1**, were obtained from the commercial sources as follows: 2, 3, 14, 15, 19−21, 23−27, 29−32, 40−42, 45−51, and 53−58 (Sigma-Aldrich, Madrid, Spain); 1, 4, 6−10, 13, 17, 18, 28, 34−39, 44, and 52 (Merck, Darmstadt, Germany); 5, 11, 12, 16, 22, 33, and 43 (Fluka, Madrid, Spain). 4-Methyl-2-pentanol (Merck) and 3,4-dimethylphenol (Sigma-Aldrich) were employed as internal standards (IS). Dichloromethane, anhydrous sodium sulfate, sodium chloride, and acetic acid were obtained from Merck, and all of them were of analytical quality. Water was obtained from a Milli-Q purification system (Millipore, Billerica, MA).

Chemical Analysis. We used three different methods to determine the volatile compounds of our interest in Sherry vinegar samples, showed in **Table 1**. A total of 52 compounds were determined by headspace sorptive extraction gas chomatography−mass spectrometry (HSSE−GC−MS). This method was not adequate for the determination of some major compounds, such as ethyl acetate, ethanol, methanol, acetaldehyde, and propanol, because of their high concentrations, among others reasons. Hence, these five compounds were quantified by a direct GC−flame ionization detector (GC−FID). For the special case of sotolon (polar compound), the HSSE−GC−MS method was not suitable because of the apolar nature of the polydimethylsiloxane (PDMS) sorbent in the stir bar. For this reason, sotolon was determined by liquid−liquid extraction GC−MS (LLE−GC−MS).

Gas Chromatography−Flame Ionization Detector (GC−FID). Ethyl acetate, acetaldehyde, methanol, ethanol, and propanol were quantified by GC−FID using the method proposed by Morales et al. (*9*). A total of 1 mL of samples was filtered through Millex-GV_{13} filters of 0.22 μm, and 1 μL of 4-methyl-1-pentanol at 102.14 mg/L was added as an internal standard (IS). Filtered samples were analyzed using a Hewlett-Packard 6890 gas chromatograph equipped with a FID. A total of 1 μL was injected in the split mode (1:60) into a CP-Wax 57 CB, 50 m × 0.25 mm DI × 0.2 μm film thickness (Varian, Middelburg, The Netherlands). The carrier gas was H_2 at 1 mL/min. The program temperature was 35 °C for 5 min, ramped at 4 °C/min to 150 °C, and held for 17.5 min. The injector was set to 220 °C, and the detector was set to 250 °C. Data acquisition software was a HPChemstation data processing system (Agilent Technologies, Santa Clara, CA).

Liquid−Liquid Extraction GC−MS (LLE−GC−MS). 4,5-Dimethyl-3-hydroxy-2(5*H*)-furanone (sotolon) was quantified by LLE−GC−MS using the method proposed and validated by Ferreira et al. (*36*). The

Table 1. Volatile Compounds of VR1 Sherry Vinegar Sample

number	compound	mean concentration (μg/L) ± SD
	Aldehydes	
1	acetaldehyde[a,b]	63 ± 2
2	hexanal	
3	2-furfuraldehyde	878 ± 58
4	benzaldehyde	121 ± 5
5	5-methyl-2-furfuraldehyde	nq[c]
6	vanillin	4438 ± 355
	total aldehydes[a]	68
	Acetal	
7	acetaldehyde diethylacetal[a]	61.7 ± 4.1
	Acetic Esters	
8	methyl acetate[a]	11.6 ± 1.5
9	ethyl acetate[a,b]	884 ± 24
10	propyl acetate	1274 ± 76
11	isobutyl acetate	1840 ± 35
12	butyl acetate	nd[d]
13	isoamyl acetate[a]	4.3 ± 0.1
14	hexyl acetate	nd[d]
15	benzyl acetate	nq[c]
16	2-phenylethyl acetate	1134 ± 29
	total acetic esters[a]	904
	Ketones	
17	diacetyl[a]	33 ± 1
18	acetoin[a]	569 ± 24
19	acetophenone	nq[c]
	total ketones[a]	602
	Ethylic Esters	
20	ethyl propanoate	1264 ± 92
21	ethyl isobutyrate	545 ± 19
22	ethyl butyrate	209 ± 14
23	ethyl 2-methylbutyrate	109 ± 4
24	ethyl isovalerate	1015 ± 16
25	ethyl valerate	nq[c]
26	ethyl hexanoate	49.3 ± 3.7
27	ethyl heptanoate	nd[d]
28	ethyl lactate[a]	9.2 ± 0.5
29	ethyl octanoate	nd[d]
30	ethyl furoate	231.1 ± 21.8
31	ethyl benzoate	6.7 ± 0.5
32	ethyl phenylacetate	nq[c]
33	diethyl succinate[a]	218 ± 21
	total ethylic esters[a]	221
	Alcohols	
34	metanol[a,b]	53 ± 4
35	etanol[a,b]	3022 ± 192
36	1-propanol[a,b]	6.7 ± 1.6
37	isobutanol[a]	3551 ± 44
38	2-methyl-1-butanol[a]	13.5 ± 1.6
39	3-methyl-1-butanol[a]	27 ± 3
40	1-hexanol	nq[c]
41	*cis*-3-hexen-1-ol	51.8 ± 0.3
42	benzyl alcohol	737 ± 25
43	furfuryl alcohol	390 ± 8
44	2-phenylethanol[a]	9.4 ± 0.9
	total alcohols[a]	6684
	Terpene	
45	α-terpineol	nd[d]
	Acids	
46	isovaleric acid[a]	54 ± 4
47	hexanoic acid	2063 ± 47
48	heptanoic acid	nq[c]
49	octanoic acid	368 ± 4
50	nonanoic acid	nd[d]
51	decanoic acid	37.2 ± 0.7
	total acids[a]	57
	Lactones	
52	γ-butyrolactone	924 ± 70
53	*trans*-β-methyl-γ-octalactone	64.8 ± 2.3
54	*cis*-β-methyl-γ-octalactone	nq[c]
55	sotolon[e]	748 ± 11
	total lactones	1737
	Phenols	
56	guaiacol	nq[c]
57	eugenol	nq[c]
58	4-ethylphenol	1191 ± 95
	total phenols	1191
	total amounts[a]	8601

[a] Concentration in mg/L. [b] GC−FID. [c] nq = below the quantification limit. [d] nd = below the detection limit. [e] LLE−GC−MS.

Table 2. Detection Frequency and AEDA of the Odors of VR1 Sherry Wine Vinegar

number	RI	odor quality	odorant (tentative identification)	detection frequency	FD1	FD2
1	1063	glue	ethyl acetate	9	2	4
2	1070	alcohol	ethanol	5	16	8
3	1072	rancid	unknown	3	2	1
4	1076	chemical, alcohol, grass, plastic	acetaldehyde diethylacetal	5	16	64
5	1080	strawberry	ethyl isobutyrate	7	512	1024
6	1084	butter	diacetyl	9	4096	4096
7	1089	plastic, medicinal, chemical	isobutyl acetate	8	1	2
8	1097	strawberry	ethyl butyrate	4	1	1
9	1105	fruit, banana	ethyl 2-methylbutyrate	6	32	32
10	1110	strawberry	ethyl 3-methylbutyrate	6	2	2
11	1118	cherry, strawberry	butyl acetate	6	128	1024
12	1123	banana, mulberry, strawberry	isoamyl acetate	9	4096	4096
13	1156	aspirin, banana	ethyl valerate	6	64	64
14	1173	fruit, banana	amyl acetate	3	8	8
15	1181	banana	unknown	3	1	1
16	1220	rancid	3-methylbutanol	3	32	32
17	1239	banana, fruit, mulberry	ethyl hexanoate	5	1	1
18	1254	mulberry, banana	hexyl acetate	3	4	4
19	1277	rancid	unknown	4	1	1
20	1297	boiled potato	unknown	3	16	16
21	1327	sweet, yogurt, dairy product	acetoin	5	128	128
22	1360	toasted maize	3-hydroxy-2-pentanone	3	2	2
23	1407	metallic	unknown	3	1	1
24	1414	strawberry, banana	ethyl octanoate	6	32	256
25	1422	pungent	acetic acid	9	1024	1024
26	1438	fruit, flower, strawberry	linalool oxide (isomer)	3	4	4
27	1439	feet	unknown	6	2	2
28	1441	alcoholic, sweet	2-furfuraldehyde	4	1	1
29	1443	flower, grass, eau-decologne	1-heptanol	4	1	1
30	1447	metallic	unknown	4	1	1
31	1455	aspirin, mulberry, fruit, strawberry	unknown	6	2	2
32	1461	strawberry, sweet, mulberry	unknown	3	1	2
33	1468	mulberry, fruit, banana, strawberry	unknown	8	1	2
34	1479	alcohol, strawberry, sweet	linalool oxide (isomer)	6	8	4
35	1484	boiled potato	methional	7	4	4
36	1496	strawberry, sweet	unknown	8	8	16
37	1505	humidity, ground, vapor	unknown	5	4	2
38	1510	toasted maize, fried chicken	2,3-butanediol diacetate	3	4	4
40	1515	metallic, iron	unknown	4	1	2
41	1532	river water, vapor	unknown	9	1	2
42	1537	strawberry, alcohol, roses, sweet	unknown	5	64	256
43	1545	banana, mulberry	ethyl 3-hydroxybutanoate	5	64	256
44	1553	flower, roses, sweet	unknown	4	1	1
45	1557	mulberry, fruit	benzaldehyde	7	2	4
46	1563	aspirin, mulberry	ethyl nonanoate	8	128	256
47	1586	rancid, acid, cheese, feet	propanoic acid	4	2	2
48	1595	cheese, feet	isobutyric acid	8	64	128
49	1655	burned, burned hair	unknown	7	8	16
50	1661	cheese, vomit	butyric acid	9	256	256
51	1671	burned, burned hair	furfuryl alcohol	3	2	4
52	1679	Sweet	ethyl benzoate	3	2	2
53	1685	roses, talcum power, perfume	unknown	3	2	2
54	1705	cheese	isovaleric acid	9	128	128
55	1747	rancid, cheese	pentanoic acid	4	4	2
56	1762	boiled vegetable or potatoes	methionol	5	4	8
57	1765	plastic	methyl salicylate	6	4	4
58	1780	plasticine, wax pencil	ethyl phenylacetate	4	1	1
59	1786	urine	ethyl Salicylate	4	4	2
60	1789	grass, feet, humidity	ethyl phenylacetate	3	1	4
61	1802	metallic	unknown	3	1	1
62	1809	boiled vegetable	unknown	4	2	4
63	1811	cheese, vomit	unknown	4	2	2
64	1842	sweet, fruit, fruit preserve	unknown	3	32	128
65	1845	boiled vegetable or potato	hexanoic acid	3	2	4
66	1858	stewed apples, apple juice	β-damascenone	5	256	256
67	1875	boiled vegetable	unknown	4	2	1
68	1878	cheese, feet	unknown	3	4	4
69	1880	fruit, fruit preserve	unknown	3	2	2
70	1889	sweet, vanilla	2-methyl-3-hydroxy-4-pyrone	5	256	256
71	1897	plastic, medicinal	guaiacol	3	1	2
72	1904	grass, lemon, mint	unknown	5	16	256
73	1909	metallic, alcohol	benzyl alcohol	3	4	4
74	1932	cheese	heptanoic acid	4	1	1
75	2017	flower (daisy), chamomile tea	4-ethylguaiacol	4	4	16

Table 2. Continued

number	RI	odor quality	odorant (tentative identification)	detection frequency	FD1	FD2
76	2019	oxide, sweet, eau-decologne	pantolactone	3	16	64
77	2028	urine, chamomile tea, chemical	octanoic acid	5	256	4
78	2051	coconut, sweet	γ-decalactone[a]	5	16	256
79	2054	clove	eugenol	8	2	2
80	2068	flower, men perfume, lemon	unknown	3	64	64
81	2076	sweet, vanilla	unknown	6	8	256
82	2098	clove, vanilla, pepper	4-vinylguaiacol	5	4	256
83	2113	liquor, "oloroso sherry wine", sweet	unknown	5	64	256
84	2137	cheese	nonanoic acid	3	4	2
85	2149	metallic	4-ethylphenol[a]	3	1	1
86	2151	flower, fruit, banana	unknown	7	128	512
87	2201	curry, liquorice, "oloroso sherry wine", toffee, syrupy sugar	sotolon	9	512	512
88	2241	chamomile tea, sweet, flower	decanoic acid	4	4	64
89	2247	syrupy sugar, liquor, sweet	unknown	3	4	4
90	2255	sweet-rancid, wood, liquor, raisin	unknown	7	512	512
91	2273	toffee, liquorice, sweet wine	benzoic acid	4	64	64
92	2280	syrupy sugar, toasted wood, sweet	unknown	4	64	64
93	2306	toasted, metal-oxide, port wine	unknown	4	4	4
94	2313	sweet wine, liquor, toasted	unknown	6	512	512
95	2319	oloroso sherry wine, sweet, liquorice	methoxyeugenol	4	32	128
96	2343	syrupy sugar, sweet, coffee	5-hydroxy-2- methylfurfural	6	16	128
97	2360	liquor, liquorice, sweet, raisin	unknown	7	512	512
98	2369	alcohol, raisin, liquor	ethyl vanillate	3	8	16
99	2391	raisin, oloroso sherry wine, liquorice	acetovanillone	5	256	256
100	2451	raisin-metal, oloroso sherry wine	unknown	3	1	1
101	2471	honey, liquorice, wood, liquor	phenylacetic acid	6	64	128
102	2489	raisin, chamomile tea	unknown	3	128	128
103	2494	liquor, oxide, oloroso sherry wine	unknown	4	128	128
104	2514	chocolate	syringaldehyde[a]	6	512	256
105	2524	alcohol, sweet	homovallinic alcohol	3	4	4
106	2535	smoke of cigarettes, toasted chicken, metallic	unknown	3	16	8
107	2598	grass, vinegar	unknown	4	512	512
108	2607	chocolate liquor, ripe fruit,	homovanillinic acid	5	2	2

[a] Possibly identified compounds.

extraction procedure was carried out as follows: to 50 mL of Sherry wine vinegar "type" (VR1) was added 5 g of anhydrous sodium sulfate and extracted twice with 5 mL of dichloromethane. The two organic phases obtained were blended and dried over anhydrous sodium sulfate. Then, 2.5 mL of the organic extract was concentrated 5 times under a nitrogen stream and 5 μL of 3,4-dimethylphenol in dichloromethane at 0.55 mg/L was added as an IS. A total of 4 μL of extracts was analyzed by GC−MS, using the conditions described elsewhere with minimum changes (*21*). The column employed was a CPWax-57CB 50 m × 0.25 mm, 0.20 μm film thickness (Varian, Middlelburg, The Netherlands). The injector port was heated to 220 °C in splitless mode for 1 min, with a total flow rate of 53.5 mL. The carrier gas was He at a flow rate of 1 mL/min. The oven temperature was 40 °C (for 1 min), which was then increased at 2 °C/min to 220 °C and held for 30 min. The quadrupole, source, and transfer line temperatures were maintained at 150, 230, and 280 °C, respectively. The analysis was performed in SIM mode, and the ions selected were *m/z* 83 (sotolon) and *m/z* 107 (IS).

Headspace Sorptive Extraction GC−MS (HSSE−GC−MS) Analysis. The HSSE sampling conditions were as follows (*37*): 5 mL of sample (wine vinegar) and 10 μL of 4-methyl-2-pentanol (IS) at 1045 mg/L was placed into a 20 mL headspace vial with 1.67 g of NaCl. A 10 mm long stir bar coated with 0.5 mm polydimethylsiloxane (PDMS) layer (Twister, Gerstel, Müllheim an der Ruhr, Germany) was put in an open glass insert and placed into the vial to achieve the extraction in the headspace. Then, the vial was tightly capped and heated for 60 min at 62 °C in a thermostatic bath. The stir bar was removed with tweezers, rinsed with Milli-Q water, and dried with a lintfree tissue paper. Finally, for the thermal desorption (TD), the stir bar was placed into a glass tube of 60 mm in length, 6 mm o.d., and 4 mm i.d., which was placed in the autosampler tray of the thermo desorption unit for GC−MS analysis.

GC analysis was carried out with a 6890 Agilent GC system coupled to a quadrupole mass spectrometer Agilent 5975inert and equipped with a Gerstel, thermo desorption system (TDS2) and a cryo-focusing CIS-4 PTV injector (Gerstel). The thermal desorption was performed in splitless mode and with a flow rate of 90 mL/min. The desorption temperature program was the following: 35 °C for 1 min, ramped at 60 °C/min to 250 °C, and held for 5 min. The CIS-4 PTV injector, with a Tenax TA inlet liner, was held at −35 °C with liquid nitrogen for total desorption time, then raised at 10 °C/s to 290 °C, and held for 4 min. The solvent vent mode was employed for transfer of the sample to the analytical column. A CPWax-57CB column 50 m × 0.25 mm, 0.20 μm film thickness (Varian, Middlelburg, The Netherlands) was used, and the carrier gas was He, at a flow rate of 1 mL/min. Oven temperature program: 35 °C for 5 min and then raised to 220 °C at 2.5 °C/min (held 5 min). The quadrupole, source, and transfer line temperatures were maintained at 150, 230, and 280 °C, respectively. Electron ionization mass spectra in the full-scan mode were recorded at 70 eV electron energy in the range of 35−350 amu.

All data were recorded using a MS ChemStation. The identity of peaks ($n = 53$) was assigned using the NIST 98 library and confirmed by the retention index of standards when they were available. Quantification was performed employing the relative area to the internal standard of the target ion of each compound. The samples were analyzed by triplicate, and blank runs of empty glass tube were performed before and after each analysis.

Sensory Studies. *Sensory Panel.* The test panel that carried out the different sensory experiments described in this work was composed of eight tasters (six females and two males), all of them belonging to the laboratory staff and with a long experience in wine vinegar sensory analysis (*12, 38, 39*).

Descriptive Sensory Analysis. The profile method was used to describe vinegars by a set of attributes that were previously selected and checked by the test panel (*39*). The selected attributes were as follows: ethyl acetate, pungent sensation, wine character, woody odor, sweet aroma, raisin, alcohol/liquor, and general impression. This last descriptor can be considered as a hedonic attribute because the sensory panel can not be trained in it. The intensity of each attribute was marked

Table 3. Results Obtained from Sensorial Analysis by a Comparison Test[a]

aroma model disolutions	similarity value (SV)	standard deviation (SD)
as + [3]	1.54	0.58
as + [1]	1.70	0.55
as + [1] + [3]	1.79	0.62
as + [2]	1.83	0.62
as + [4]	2.76	0.58
as + [1] + [2] + [3]	3.15	0.58
as + [1] + [2]	3.25	0.62
as + [1] + [4]	3.55	0.55
as + [2] + [3]	3.57	0.55
as + [3] + [4]	4.08	0.61
as + [2] + [4]	4.11	0.58
as + [2] + [3] + [4]	4.35	0.62
as + [1] + [3] + [4]	4.57	0.58
as + [1] + [2] + [3] + [4]	4.59	0.58
as + [1] + [2] + [4]	5.38	0.55

[a] as, acetic acid solution (7% v/v); [1], diacetyl; [2], ethyl acetate; [3], isoamyl acetate; and [4], sotolon.

on an unstructured 10 cm straight line labeled with not noticeable to very strong on the left and right extremes, respectively.

Similarity Test. Aroma model solutions were prepared in a 7% (w/v) acetic acid solution by diluting the compounds that reached the highest scores in GC−O, in the same concentrations found for the control sample VR1 (**Table 1**): diacetyl (33 mg/L), ethyl acetate (884 mg/L), isoamyl acetate (4.3 mg/L), and sotolon (748 μg/L). As result of all possible combinations of these four compounds (**Table 3**), we prepared 15 model solutions.

The control sample (VR1) and all of the models were presented to the panel for similarity tests (15 mL of the control sample or models in black coded glasses covered with a Petri box). The order of sample presentation was random for all of the subjects. The panel was asked to rate the similarity on a discontinuous scale from 0 (no similarity) to 9 (equal) of each model with the control vinegar (*36*). The obtained data were processed according to analysis of variation (ANOVA) to establish differences among all of the models and VR1 sample (*36*). Finally, a descriptive analysis of the more similar model was performed. The panel used our previously established tasting card (*39*), and they were asked to rank each descriptor on a 10 cm unstructured scale (from not noticeable to very strong).

Gas Chromatography−Olfactometry (GC−O). To identify substances responsible for aromatic notes associated with the selected descriptors of the typical aroma of aged Sherry vinegar (*12*), GC olfactometric analysis was employed in a representative Sherry vinegar (VR1). Extraction was performed according to the methodology previously described for LLE−GC−MS. Then, 2 mL of this organic phase was concentrated 5 times under a nitrogen stream. Several dichloromethane extracts from the sample VR1 were submitted to the GC, which was equipped with an olfactometric detector ODO II (SGE, France) customized by Dr. Silva Ferreira's group with two olfactory outlets, to obtain simultaneous odor evaluation from multiple panelist.

Chromatographic conditions were the following: VARIAN 3800 gas chromatograph; column BP-21 (50 m × 0.25 mm × 0.22 μm) fused silica (SGE, France); hydrogen (5.0, Gasin, Portugal); flow (1.0 mL/min); injector temperature, 220 °C; oven temperature, 40 °C for 1 min programmed at the rate of 2 °C/min to 220 °C, maintained during 30 min. Extract aliquots of 1 μL were injected into the GC in a splitless mode during 0.5 min; split flow, 30 mL/min.

Inside the oven, the column flow is split between the two olfactory ports using an 20 cm inactive column, the flow was measured at the end and adjusted to 1 mL/min. Each independent heated transfer tube was kept at isothermal conditions.

The make-up gas employed on the olfactometric device was air (80% N_2; 20% O_2) (air−liquid, France). Two streams were used; one was bubbled in water, nose moister, at ca. 150 mL/min and the other was applied at the exit of the GC column to lower the temperature of the effluent at 15 mL/min.

Odor Detection Frequency. A panel of two individuals carried out simultaneously a total of nine sniffings of the sample in duplicate, using the same operational conditions and the same chromatograph, to increase the robustness of data. Assessors were asked to smell the effluent of the column and to give a verbal description of each perceived odor, even if they did not recognize the odor. The odor zones reported by each panel member were compared for each retention index. The descriptors were selected according to their frequency of citations. Hedonic terms were not considered (good/bad) nor those considered to be analogues, which were replaced by the most cited.

Aroma Extract Dilution Analysis (AEDA). The extract was stepwise diluted with dichloromethane (1 + 1 by vol.), and aliquots of the dilutions (1 μL) were evaluated (*21*). The process stopped when no aromas were detected by assessors. The result is expressed as the flavor dilution (FD) factor, which is the ratio of the concentration of the odorant in the initial extract to its concentration in the most diluted extract in which the odor is still detectable by GC−O (*15, 16*).

Compound Identification. Identification of odorants was performed by comparison of mass spectra, chromatographic retention indexes (RIs), and odor description with experimental and literature data. Chromatographic RIs were calculated in GC−O and HSSE−GC−MS from the retention times of *n*-alkanes by linear interpolation, according to the literature (*21*).

Statistical Analysis. All statistical analysis were performed by means of Statistica, version 7.0 software (Statsoft, Tulsa, OK).

RESULTS AND DISCUSSION

GC−O. The GC−O experiments were performed on extracts obtained in dichloromethane, because they shown to be the most representative (*12*). The results derived from the olfactometry study carried out in the VR1 sample are summarized in **Table 2**.

Odor Detection Frequency. As result of nine sniffings, more than 400 odors were detected during the GC−O experiments, but for the sake of simplicity, those not reaching a maximum frequency of 3 were arbitrarily considered as noise. After this operation, the number of odors detected was reduced to 108, as shown in **Table 2**, and 64 of them have been positively identified (retention index, odor quality, and MS similar to those of pure reference standards). Among them, a variety of different odor qualities, such as glue (RI 1063), butter (RI 1084), banana/mulberry/strawberry (RI 1123), pungent (RI 1422), cheese/vomit (RI 1661), cheese/unpleasant (RI 1705), and curry/liquorice (RI 2201) reached the highest frequencies (100%). These odor-active regions were identified as ethyl acetate, diacetyl, isoamyl acetate, acetic acid, butyric acid, isovaleric acid, and sotolon, respectively. Besides, 16 identified odorant compounds were detected with high frequency, between 67 and 89% of the sniffings: ethyl isobutyrate (RI 1080), isobutyl acetate (RI 1089), ethyl 2-methylbutyrate (RI 1105), ethyl 3-methylbutyrate (RI 1110), butyl acetate (RI 1118), ethyl valerate (RI 1156), ethyl octanoate (RI 1414), linalool oxide (RI 1479), methional (RI 1484), benzaldehyde (RI 1557), ethyl nonanoate (RI 1563), isobutyric acid (RI 1595), methyl salicylate (RI 1765), eugenol (RI 2054), 5-hydroxy-2-methylfurfural (RI 2343), and phenylacetic acid (RI 2471). The rest of the identified odorants presented a low frequency (<67%). These odorants were closely related to alcohol, chemical, plastic, fruit, cheese/rancid, sweet, dairy product, boiled potato/toasted maize/burned, flowers, chamomile tea, spicy, and liquor/sweet wine/raisin descriptors.

Regarding non-identified odorants (n = 40), only seven accounted for high frequencies (>67%): mulberry/fruit/banana (RI 1468), strawberry/sweet (RI 1496), river water/vapor (RI 1532), burned hair (RI 1655), flower/fruit/banana (RI 2151), sweet-rancid/wood/liquor (RI 2255), and sweet wine/liquor/toasted (RI 2313).

AEDA. FD factors as defined by Grosch (*15*) have been calculated and are displayed in **Table 2**. AEDA was carried

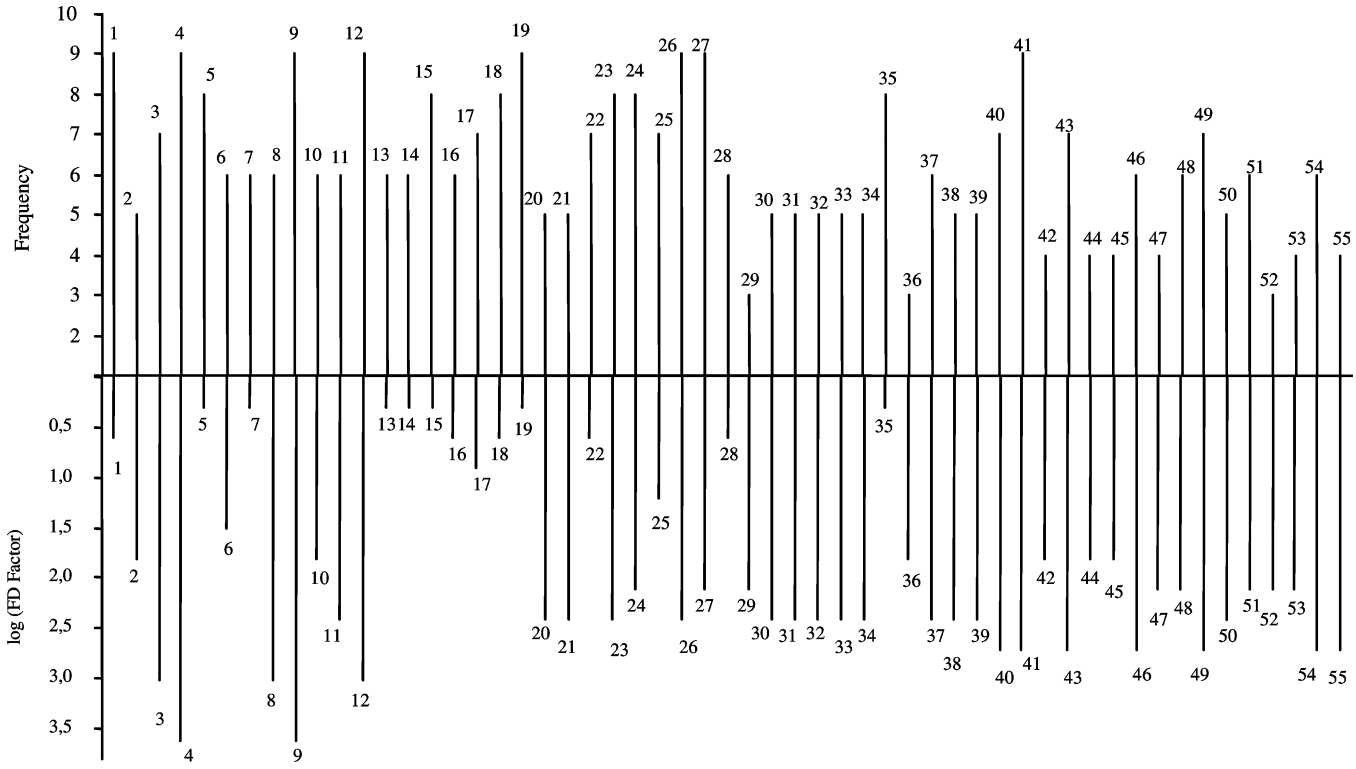

Figure 1. Frequency and log (FD factor) of odorants that reached frequency ≥50% and/or FD factor ≥64: 1, ethyl acetate; 2, acetaldehyde diethylacetal; 3, ethyl isobutyrate; 4, diacetyl; 5, isobutyl acetate; 6, ethyl 2-methylbutyrate; 7, ethyl 3-methylbutyrate; 8, butyl acetate; 9, isoamyl acetate; 10, ethyl valerate; 11, ethyl octanoate; 12, acetic acid; 13, unknown; 14, unknown; 15, unknown; 16, linalool oxide; 17, methional; 18, unknown; 19, unknown; 20, unknown; 21, ethyl 3-hydroxybutanoate; 22, benzaldehyde; 23, ethyl nonanoate; 24, isobutyric acid; 25, unknown; 26, butyric acid; 27, isovaleric acid; 28, methyl salicylate; 29, unknown; 30, β-damascenone; 31, 2-methyl-3-hydroxy-4-pyrone; 32, unknown; 33, octanoic acid; 34, γ-decalactone; 35, eugenol; 36, unknown; 37, unknown; 38, 4-vinylguaiacol; 39, unknown; 40, unknown; 41, sotolon; 42, decanoic acid; 43, unknown; 44, benzoic acid; 45, unknown; 46, unknown; 47, methoxyeugenol; 48, 5-hydroxy-2-methylfurfural; 49, unknown; 50, acetovanillone; 51, phenylacetic acid; 52, unknown; 53, unknown; 54, syringaldehyde; and 55, unknown.

out by two assessors in a GC equipped with two sniff ports, and hence the table shows two FD factors corresponding to each port (FD_1 and FD_2). The use of a multiple sniff port is very useful because it decreases the analysis time required, which is one of the disadvantages of dilution methods, such as AEDA (*30*). Therefore, it is possible to know if one assessor presents anosmia to any odorant by comparison of results, being the specific anosmia an important danger of this technique because it has a serious impact for underestimating the importance of an odor (*30*) and the consensus about descriptors is easier.

As can be seen in **Table 2**, FD_1 and FD_2 values agree in most of the odorants and they only present differences of more than one dilution factor in 19 odor compounds. For these situations, we have considered the higher FD factor.

By sniffing of serial dilutions, 26 odor active compounds account FD factors ≥256. Among them, nine are non-identified compounds. Identified compounds correspond to different families (esters, acids, carbonyl, and Maillard compounds) being the most powerful diacetyl and isoamyl acetate (FD factor of 4096), followed by ethyl isobutyrate, butyl acetate, and acetic acid (FD factor of 1026). They have all been previously described in Sherry wine vinegar (*4, 5, 7, 11*); in fact, diacetyl concentration has been related to the age of vinegars (*4*), and esters formation is favored along time, such as isoamyl acetate, which is one of the major esters of Sherry vinegars. Despite being the most characteristic aroma in wine vinegars and having been detected in all of the GC−O previous experiments, ethyl acetate accounts for a FD factor of only 4. This can be due to its high volatility and its high odor threshold (90.8 mg/L). Hence, assessors can detect it in all of the sniffings of the extract, reaching the maximum frequency (100%), because its concentration in this vinegar sample (884 mg/L) largely exceeds its threshold. In addition, ethyl acetate presents a characteristic aroma (glue) easy to recognize, and because of its high volatility, it is the first compound to be detected by the human senses of smell; therefore, there are no problems of saturation. Nevertheless, when the second dilution is performed, ethyl acetate disappears because of the two abovementioned reasons. Sotolon (4,5-dimethyl-3-hydroxy-2(5*H*)-furanone), a characteristic compound in oxidized wines (*36*), reaches a high FD factor of 512, and to our knowledge, it was identified for the first time in wine vinegar in our previous work (*12*). This is not surprising because of the fact that this compound is closely related to oxidative aging and Sherry flor wines (*40, 41*).

Nine odorants present FD factors of 128, among them acetoin, isobutyric acid, isovaleric acid, methoxyeugenol, 5-hydroxy-2-methylfurfural, and phenylacetate. Acetoin increases during acetification (*4*), hence it is a characteristic compound in vinegars (*5–7*). Its odor is described as sweet, yogurt, and dairy products; however, its FD factor is 128, conversely to its oxidation product, diacetyl, which accounts for a very high FD factor.

On the other hand, there is a number of non-identified compounds accounting for high FD factors. These odorants are closely related to sweet, liquor, wood, and raisins descriptors.

By comparison of results obtained with the two techniques used in this study, detection frequency and AEDA, we can see that they agree in many cases (**Figure 1**). Hence, diacetyl, isoamyl acetate, acetic acid, and sotolon reached the maximum frequency and the highest FD factors, therefore being potent

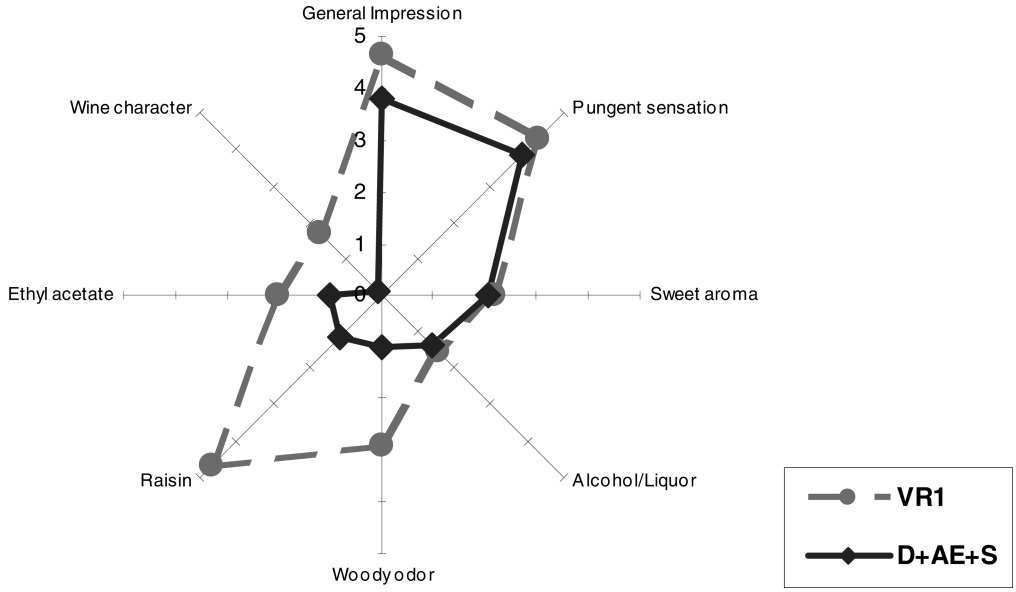

Figure 2. Orthonasal flavor profiles of the VR1 sample (gray lines) and the aroma model (black lines).

odorants of Sherry vinegar. However, results are not in agreement for certain odorants. In fact, some of them accounted for high frequencies and low FD factors, such as RI 1063 (ethyl acetate), RI 1089 (isobutyl acetate), RI 2054 (eugenol), RI 1532 (unknown), among others. The explanation of this fact could be compounds with time intensity, concentrations above their thresholds, familiarity with descriptors, and smaller coelutions, which yield high frequencies. However, FD factors will vary according to the concentrations of compounds. In addition, it is known that the slope of the psychometric function of a compound varies markedly between different compounds. Therefore, odorants with steep psychometric functions and a higher odor threshold will demonstrate high detection frequencies but low FD factors (*30*). On the contrary, other compounds reached low frequencies and high FD factors, for example, RI 1327 (acetoin), RI 1842 (unknown), RI 2391 (acetovanillone), or RI 2598 (unknown). This fact can be explained because these compounds are examples of those species with very flat dose−response curve. In addition, compounds with concentrations largely above their odor thresholds are expected to reach high FD factors; however, sometimes they can coelute with other compounds when the sample is not diluted, reaching low frequencies because of ambiguous descriptors given by the panel.

Various authors have critically compared the different GC−O methodologies, using either mixtures of references standards or real systems (*42, 43*), and discrepancies in results existed because they are based on different principles. As was mentioned in the Introduction, each of the GC−O methodologies have their own advantages and limitations. For this reason, we can say there is not a universal standard method or technique to determine the relative importance of the volatile compounds identified as being odor-active. Hence, the use of both techniques allows for the obtaining of more information and reduces the errors associated to the use of only one of them. Further research will be directed to calculate the "modified frequency" (MF), which takes into account the frequency and intensity average of the odor region, to improve the discriminative power of the detection frequency.

According to several authors (*18, 44, 45*), dilution to threshold methods, such as AEDA, are valuable tools for the screening of odor-active compounds in a given food. However, AEDA does not provide information on the aroma contribution of single compounds, because the matrix significantly influences the volatility of an odorant and, thus, its concentration in the headspace above the food.

In relation to the OAV concept, odorants should contribute to the overall aroma if they exceed their odor threshold in a given matrix (*46*). In a previous work (*12*), we obtained the odor activity values of those odorants that accounted for either high detection frequencies in GC−O, high concentrations in Sherry vinegar, or even those with reported impact in wines. Among them, the highest OAV, equal to 807, was calculated for diacetyl. Besides, isoamyl acetate, ethyl isobutyrate, and sotolon, compounds that reached high scores for frequency and AEDA (**Table 2**) also showed high OAVs (365, 149, and 47, respectively). Hence, these compounds should contribute to the aroma of this sample (VR1), because their concentrations clearly exceeded their odor thresholds. On the other hand, because of its high volatility and its high odor threshold, ethyl acetate showed a low FD factor in the AEDA, but it contributes to the overall aroma because its OAV is >1, specifically 9.7. This result is in agreement with the detection frequency, where ethyl acetate was detected in all of the sniffings. Therefore, this compound should also be considered as a potent odorant.

Similarity Test. OAVs indicate whether a single compound is present above a threshold in a given matrix and should, therefore, contribute to a given aroma. However, it is difficult to explain how interactions of single key odorants showing a broad range of different odor qualities can finally lead to the overall aroma of the food itself (*44, 46*).

Therefore, to further investigate the contribution of the odorants selected to the Sherry wine vinegar aroma, they were added alone or in combination with a 7% (w/v) acetic acid solution, at concentrations found in the VR1 sample (**Table 1**).

For this purpose, we selected those compounds with a FD factor ≥512 and detected in all of the sniffings: diacetyl, isoamyl acetate, and sotolon. In addition, ethyl acetate was also selected despite its low FD factor because it was detected in all of the cases and has an OAV >1. Moreover, it is one of the typical sensory attributes of Sherry vinegar used as a descriptor in the descriptive sensory analysis chart for Sherry vinegars (*38*).

A simple comparison test was carried out to rate the degree of similarity between each of the spiked solutions and the VR1

Sherry vinegar. The average of the similarity values (SV) as well as the standard deviation calculated for each pair is given in **Table 3**.

ANOVA showed significant differences between samples and no significant differences between panelists at the 95% level. The highest SV was observed when diacetyl, ethyl acetate, and sotolon were added simultaneously to the 7% acetic acid solution immediately followed by the solution containing the four abovementioned compounds (**Table 3**). Theoretically, this last solution should be the most similar sample because it contains all of the selected odorants.

Comparing the SVs obtained (**Table 3**) by simple additions of isoamyl acetate (1.5), diethyl (1.7), ethyl acetate (1.8), and sotolon (2.8) reveals the highest impact of this last compound in the typical aroma of Sherry vinegar. Furthermore, it is important to point out that all combinations that contained sotolon rated with the highest similarity values. Only the solution spiked with ethyl acetate and isoamyl acetate reached SVs slightly superior to one of the combinations with sotolon (**Table 3**).

Hence, these results suggest that sotolon is an important contributor to the typical aroma of VR1 Sherry vinegar sample. Besides, in our previous work (*12*), it was observed that sotolon was only present in those Sherry vinegars aged in wood for more than 2 years, "Reserva" and "Gran Reserva", increasing its concentration with the time of aging. Thus, this compound could be related to the oxidative aging of Sherry vinegars and could be a key odorant of this kind of vinegar, in the same way that it happens in oxidative aged port wines (*36*).

The aroma of the more similar sample was evaluated by our sensory panel using a descriptive chart in comparison to VR1 Sherry vinegar, and the results are presented as a spider chart diagram (**Figure 2**). As we can see, the intensities of the odor qualities "wine character", "woody odor", and "raisin" were rated higher in the sample than in the model dilution. These results suggest that none of the added compounds is very related to these attributes. According to the previous work (*12*), "raisin" and "wine character" did not display a good correlation with any compounds. However *trans*-β-methyl-γ-octalactones were related to "woody odor" ($r = 0.74$).

On the other hand, the profile of the model solution and sample VR1 show a good similarity in the general impression descriptor, which emphasizes the important contribution of these three molecules (diacetyl, ethyl acetate, and sotolon) to the global aroma of this Sherry vinegar. Hence, they can be considered as key odorants for Sherry vinegar. In addition, the descriptors "sweet aroma", "pungent sensation", and "alcohol/liquor" displayed almost the same intensities in both samples. According to descriptors of sotolon showed in **Table 2**, this compound could be one of the odorants responsible of the sensory attribute "alcohol/liquor". In addition, this descriptor was correlated with sotolon ($r = 0.7$) in our previous work (*12*). Obviously, acid acetic is accountable for the "pungent sensation", and the "sweet aroma" descriptor could be related to diacetyl, ethyl acetate, and sotolon.

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5. CONCLUSIONES

De los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral se obtienen las siguientes conclusiones:

1. Se han seguido los cambios en el contenido de aminoácidos y nitrógeno total durante 8 acetificaciones con cultivo superficial en diferentes maderas y 3 con cultivo sumergido. El consumo de nitrógeno fue mayor en las acetificaciones con cultivo superficial. El requerimiento de nitrógeno por las bacterias acéticas fue proporcional a la duración del proceso de acetificación. En la acetificación superficial se consumió prolina en mayor proporción y arginina en la sumergida. El patrón de consumo de los diferentes aminoácidos es diferente en los dos tipos de acetificaciones y sugiere una posible relación con las diferentes cepas de bacterias acéticas implicadas en el proceso.

2. Se ha validado un método de "Extracción por absorción con barras magnéticas agitadoras en espacio de cabeza" para la determinación de compuestos volátiles por Cromatografía de Gases y Espectrometría de Masas. Esta técnica permite determinar un mayor número de compuestos que la técnica de "Extracción por absorción con barras magnéticas en inmersión" en la mitad de tiempo. Se han determinado un total de 53 compuestos volátiles en vinagres de vino. Entre ellos, 2-metilbutirato de etilo, heptanoato de etilo, furoato de etilo, benzoato de etilo, acetofenona, limoneno, ácido nonanoico y sotolón se han determinado por primera vez en vinagres de vino.

3. Durante la acetificación (ya sea con cultivo superficial o sumergido) tiene lugar a un aumento en la cantidad total de compuestos volátiles, siendo mayor en los procesos superficiales. Los acetatos fueron los compuestos más abundantes en los vinagres obtenidos por cultivo superficial, mientras que en los producidos por cultivo sumergido fueron los ácidos. En los vinagres acetificados en madera de roble se determinaron los isómeros de whisky-lactonas mientras que para las muestras acetificadas en madera de cerezo fue característico el incremento en furoato de etilo y benzoato de etilo. Para las maderas de acacia y castaño no se

encontraró ningún compuesto volátil distintivo. Mediante estadística multivariante se demuestra que el tipo de acetificación es determinante en la composición volátil de los vinagres acabados.

4. Durante el envejecimiento de vinagres de vino tinto y balsámicos en barricas y en botella, no hubo una tendencia común en los cambios de la composición volátil. En general, los ácidos fueron el único grupo de compuestos volátiles que incrementó durante los 16 procesos de envejecimiento. Cuando se comparó el envejecimiento en botella con el envejecimiento en madera, se observó un incremento superior en la composición volátil en los vinagres envejecidos en botella, probablemente debido a los fenómenos de absorción que tienen lugar en barriles nuevos. Las whisky-lactonas alcanzaron las mayores concentraciones en los vinagres envejecidos en madera de roble. Los compuestos benzoato de etilo, furoato de etilo, acetofenona y benzaldehído parecen estar relacionados con la madera de cerezo. Por último, el eugenol sólo estaba presente en los vinagres envejecidos en castaño y en la mayoría de los vinagres envejecidos en roble.

5. El estudio olfatométrico reveló que los vinagres de Jerez pertenecientes a la categoría "Gran Reserva" mostraron un mayor número de zonas aromáticamente activas, seguida por los "Reservas" y "Vinagres de Jerez". Por tanto, el perfil aromático es más complejo conforme aumenta la edad.

   Diacetilo, acetato de isoamilo, ácido isovalérico, sotolón y acetato de etilo alcanzaron las mayores frecuencias de detección en el estudio olfatométrico y valores de actividad aromática (OAV) superiores a 1. De los 3 tipos de vinagres de vino estudiados (vinagres de vino tinto, blanco y vinagres de Jerez), el sotolón sólo está presente en los vinagres de Jerez, alcanzando las mayores concentraciones en los vinagres más envejecidos. Este compuesto mostró una alta correlación con el descriptor sensorial "alcohol/licor", pudiendo ser uno de los compuestos responsables de este atributo sensorial.

6. Los resultados obtenidos con las dos técnicas olfatométricas empleadas, AEDA y Frecuencia de Detección coincidieron en muchos casos. Sin embargo, para ciertos odorantes, como el acetato de etilo, se observaron discrepancias en los resultados debido a los diferentes principios en los que se basan estas técnicas.

Los valores de similaridad más altos se obtuvieron cuando se añadieron simultáneamente diacetilo, acetato de etilo y sotolón a una solución de ácido acético al 7%. El perfil aromático de esta disolución modelo y el vinagre de Jerez representativo mostró unas intensidades similares en los descriptores "dulce", "sensación punzante" y "alcohol/licor" además de una buena similaridad en la "impresión general". Este resultado resalta la importante contribución de estos tres compuestos (diacetilo, acetato de etilo y sotolón) al aroma global del vinagre de Jerez, pudiéndose considerar como odorantes típicos para el vinagre de Jerez.

5. CONCLUSIONS

The main conclusions that can be drawn based on the results obtained during the development of this Doctoral Thesis are as follows:

1. Changes on amino acids and total Nitrogen have been monitored along 8 surface culture acetifications performed in different kind of wood barrels and 3 submerged acetifications carried out at a laboratory scale. Nitrogen consumption was higher for surface culture acetifications and proportional to the time spent in the fermentation process. In surface culture acetifications proline was the amino acid most consumed and arginine for submerged acetifications. The pattern of amino acid consumption is different in the two kinds of acetifications, suggesting a possible relation with the different acetic acid bacteria strains involved.

2. A Headspace Sorptive Extraction (HSSE) method for the determination of volatile compounds by Gas Chromatography and Mass Spectrometry has been validated. This technique allows the determination of a higher number of compounds and in half the time than the Immersion Stir Bar Sorptive Extraction (SBSE) technique. A total of 53 aroma compounds present in wine vinegars have been determined. Among them, ethyl 2-methylbutyrate, ethyl heptanoate, ethyl furoate, ethyl benzoate, acetophenone, limonene**,** nonanoic acid and sotolon had not been previously described in wine vinegars.

3. Acetifications (either by submerged or surface culture) led to an increase in the total quantity of aroma components, which were higher in the surface processes. Acetic esters were predominant in surface culture vinegars, whereas acids were predominant in submerged culture vinegars. Whisky-lactones were only quantified in those vinegars produced in oak barrels. Ethyl furoate and ethyl benzoate increased in cherry wood barrels. Multivariate statistical analysis supported the influence of process on the volatile composition of the finished vinegars.

4. During the aging of red wine vinegar and balsamic vinegars in barrels and bottles, there was not a common pattern for changes of volatile composition. In general, we observed that the only group of volatile compounds that increased during the 16 aging experiments were organic acids. When we compared aging in bottle versus wood, we found that the overall increase of volatile compounds was superior in vinegars aged in bottle, probably due to sorption phenomena that may take place on new wood barrels. Oak-lactones reached the highest concentrations in vinegars aged in oak wood. Ethyl benzoate, ethyl furoate, acetophenone and benzaldehyde seem to be related to Cherry wood. Lastly, eugenol was only present in all vinegars aged in chestnut and in most of vinegars aged in oak barrels.

5. The aroma profile of Sherry vinegar has been described, correlating the sensory results with the chemical data, and volatile compounds with large impact on the perceived tipicity of Sherry vinegar have been identified by using different olfactometric techniques. Regarding GC-Olfactometry, Sherry vinegar samples belonging to the "Gran Reserva" class revealed a higher number of odor active zones, followed by "Reserva" and "Vinagre de Jerez", thus yielding a more complex profile as age increases.

   Diacetyl, isoamyl acetate, isovaleric acid, sotolon and ethyl acetate reached the highest frequencies in the GC-Olfactometry and odor activity values (OAV) higher than 1. From the GC-MS analysis of three types of vinegars (red, white and Sherry vinegars), we conclude that sotolon is only present in Sherry vinegars, reaching the highest concentrations in the most aged vinegars. This compound displayed a high correlation with the sensory descriptor "alcohol/liquor", hence, it could be responsible of this sensory attribute.

6. Results obtained with the two olfactometric techniques used, AEDA and Detection Frequency, are substantially equivalent. .with the exception of certain odorants such as ethyl acetate, due to the fact that both techniques are based on different principles.The highest similarity values (SVs) were observed when diacetyl, ethyl acetate and sotolon were added simultaneously to the 7% acetic acid solution. The profile of this model solution and the representative Sherry vinegar showed similar intensities in the "sweet aroma", "pungent sensation"

and “alcohol/liquor” descriptors, in addition to a good similarity in the “general impression”. This result emphasizes the important contribution of these 3 molecules (diacetyl, ethyl acetate and sotolon) to the global aroma of this Sherry vinegar. Hence they can be considered as key odorants for Sherry vinegar.