TESIS DOCTORAL

Facultad de Farmacia
Departamento de Nutrición, Bromatología, Toxicología y Medicina Legal

COMPOSICIÓN POLIFENÓLICA DE VINAGRES DE VINO TINTO: INFLUENCIA DE LA ACETIFICACIÓN Y LA MADERA

ANA BELÉN CEREZO LÓPEZ

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UNIVERSIDAD DE SEVILLA
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INFORMA: Que la Tesis Doctoral titulada "COMPOSICIÓN POLIFENÓLICA DE VINAGRES DE VINO TINTO: INFLUENCIA DE LA ACETIFICACIÓN Y LA MADERA", presentada por la Lda. Dña. ANA BELÉN CEREZO LÓPEZ para optar al grado de Doctor por la Universidad de Sevilla con la mención de "Doctorado Europeo", ha sido realizada en el Área de Nutrición y Bromatología de este Departamento bajo la dirección de la Dra. Ana Mª Troncoso González y la Dra. Mª Carmen García Parrilla, durante el tiempo requerido y reuniendo los requisitos exigidos en este tipo de trabajo.

Y para que así conste, firmo el presente certificado en Sevilla, Septiembre de 2009.

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INFORMAN: Que la Tesis Doctoral titulada "COMPOSICIÓN POLIFENÓLICA DE VINAGRES DE VINO TINTO: INFLUENCIA DE LA ACETIFICACIÓN Y LA MADERA", ha sido realizada por la Lda. Dña. ANA BELÉN CEREZO LÓPEZ en el Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Universidad de Sevilla, bajo su dirección y que reúne, a su juicio, las condiciones requeridas para optar al grado de Doctor por la Universidad de Sevilla con la mención de "Doctorado Europeo".

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A mis padres y hermano
A Manuel

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4. RESULTADOS Y DISCUSIÓN / RESULTS & DISCUSSION

1. RESUMEN / SUMMARY

PHENOLIC COMPOSITION OF RED WINE VINEGARS: INFLUENCE OF THE ACETIFICACION AND WOOD

1. 1. INTRODUCTION

Phenolic composition of wine vinegars has been extensively studied to characterize high quality vinegars as Sherry and Balsamic vinegars. Phenolic compounds has been useful to differentiate Sherry vinegars according to the geographical origin of the wine substrate, the method applied in their elaboration (García-Parrilla et al., 1997) and the ageing process (García-Parrilla et al., 1999; Tesfaye et al., 2002; Tesfaye et al., 2004). Conversely, the phenolic composition of red wine vinegars has scarcely been studied (Andlauer et al., 2000). Anthocyanin compounds, in particular, remain largely unknown.

At present, oak is the most commonly used wood in enology for ageing wines, spirits and vinegars. Its contribution to the quality and chemical composition of the product is well known (Tesfaye et al., 2002; Bosso et al., 2008; Cadahía et al., 2007; Fernández de Simón et al., 2008). Oak wood limits oxygen transfer to avoid excessive wine oxidation. However, vinegar elaboration requires a porous wood to promote the oxygen transfer for the growth of the acetic acid bacteria. Nowadays, other woods such as acacia, cherry, chestnut and mulberry are more and more used to make wines and spirits with different sensory properties. The impact of these woods on chemical composition and sensory properties still needs to be evaluated (Flamini et al., 2007; De Rosso et al., 2009; Fernández de Simón et al., 2009).

Sensory analysis is a powerful tool to evaluate foods and beverages. Indeed, it has been used to discriminate vinegar samples on the basis of the raw material (Gerbi et al., 1997) or the elaboration method (Nieto et al., 1993; González-Viñas et al., 1996). Sensory properties are a quality control criteria thus it is mandatory to assess them in a reproducible way. However, to date, the EU regulates the sensory analysis of oil (Commission Regulation EEC, 1991) but official method for vinegar sensory analysis is still lacking.

On the other hand, condiments made from fruits are being produced, suggesting new alternatives to the largely wine vinegar consumed. Hence as a first step, the characterization based on phenolic composition of the substrate merits attention.

This Doctoral Thesis aims to evaluate the impact of new strategies of vinegar elaboration in their quality, determined by their chemical composition and sensory properties. This involves the characterization of phenolic composition of red wine vinegar during acetification and ageing process, the development of a sensory analysis procedure and the assessment of new fruit substrates.

1.2. EXPERIMENTAL

1.2.1. THE PHENOLIC COMPOSITION OF RED WINE VINEGAR PRODUCED IN BARRELS MADE FROM DIFFERENT WOODS (Cerezo et al., 2008, Food Chemistry, 109, 606-615)

Two red wine vinegars (group F and T) were acetified in barrels made of different woods (oak, chestnut, acacia and cherry) by surface culture. Samples were taken at different points of the acetificacion: O (0.8º acetic, n=24), I (2º acetic, n=48), H (4º acetic, n=48), E (6º acetic, n=46). Phenolic composition of a total of 166 samples was analysed using LC-DAD (Ibern-Gómez et al., 2002), the Total Phenol Index (Watherhouse, 2001) and Total Monomeric Anthocyanins (Giusti & Wrolstad, 2001). Sensory analysis of vinegars (samples E) was performed by an expert sensory panel, making triangle difference and descriptive analyses according to international protocols (ISO 4120, 1983 and ISO 6658, 1985, respectively). Statistical analyses were performed using

One-way analysis of variance (ANOVA) and multivariate analysis of data (cluster analysis and LDA).

1.2.2. ANTHOCYANIN COMPOSITION OF RED WINE VINEGAR: IDENTIFICATION, ANTIOXIDANT ACTIVITY AND CHANGES DURING ACETIFICATION WITH SUBMERGED CULTURE (Cerezo et al., Food Research International, submitted)

A young *Cabernet Sauvignon* monovarietal red wine was acetified by submerged culture under controlled experimental conditions: stirring speed, 450 rpm; the substrate loading proportions of wine/vinegar, 1:1; the loading volume, 3400 mL; air supply, 100-200 L/h; and temperature, 30ºC. The fermentation yield, acetification rate and average time of acetification were 80.79 ± 4.60%, 0.11 ± 0.01º acetic/h and 41 hours, respectively. The resulting vinegar had 8.97º acetic and alcoholic degree of 1.33º (%, v/v). Anthocyanin extract of wine and vinegar were obtained using an Amberlite XAD-7 column (Fluka; 100 x 7cm) and an elution solvent of methanol/acetic acid (19:1, v/v), to further isolate anthocyanin fractions by CCC (solvent system, MTBE/ n-butanol/acetonitrile/water (2/2/1/5, v/v/v/v) with 0.1% TFA; elution mode, head-to-tail; flow rate, 3 mL/min; speed, 850 rpm; and detection, 520 nm). The wine and vinegar and respective extracts and fractions were analysed by: LC-DAD-MS to determine the phenolic compounds; and ORAC (Ou et al., 2001), FRAP (Benzie & Strain, 1996) and DPPH (Sánchez Moreno et al., 1998) to assess the antioxidant activity (AA).

1.2.3. IMPACT OF WOOD ON THE PHENOLIC PROFILE AND SENSORY PROPERTIES OF WINE VINEGARS DURING AGEING (Cerezo et al., Journal of Food Composition and Analysis, Accepted 06/08/2009)

Two red wine vinegars (Grenache variety, groups F and T) and one balsamic vinegar (Trebbiano variety, group M) were aged in different wood barrels (oak, chestnut, acacia and cherry). The samples were taken at 45, 180, 365 and 540 days. Additionally, some vinegars were aged in glass vessels as a control. The phenolic composition of a total of 98 samples were analysed using LC-DAD (Betés-Saura et al., 1996), LC-MS, the Total Phenol Index (Watherhouse, 2001) and Total Monomeric Anthocyanins (Giusti & Wrolstad, 2001). Besides, vinegar samples were tested by sensory analysis (ISO 4120, 1983 and ISO 6658, 1985). Statistical analyses of the data were performed using ANOVA and LDA.

1.2.4. (+)-DIHYDROROBINETIN: A MARKER OF VINEGAR AGEING IN ACACIA (Robinia pseudoacacia) WOOD (Cerezo el al., 2009, Journal of Agriculture and Food Chemistry, 57, 9551-9554)

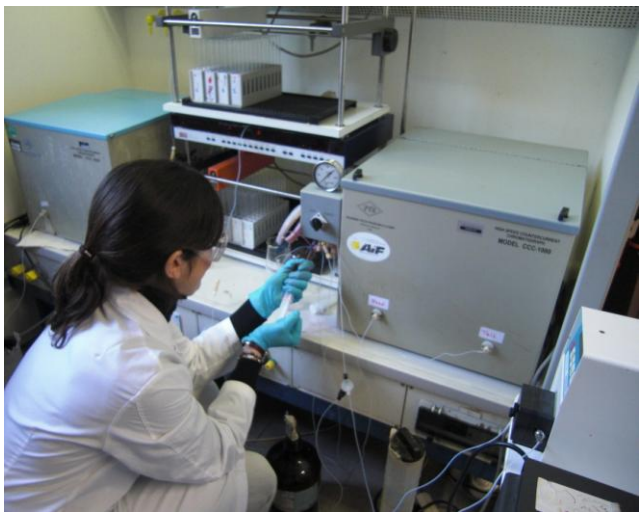
An unknown characteristic peak of vinegars in contact with acacia wood (*Robinia pseudoacacia*) during ageing in nontoasted acacia wood barrels and aged with acacia wood chips (toasted and nontoasted, in different proportions: 0.5% and 1%, w/v) was isolated by semiprepartive-LC and its chemical structure identified by NMR spectroscopy and polarimetric analysis. The quantification was performed by LC-DAD and its antioxidant activity (AA) by ORAC and DPPH assays.

1.2.5. DESCRIPTIVE SENSORY ANALYSIS OF WINE VINEGAR: TASTING PROCEDURE AND RELIABILITY OF NEW ATTRIBUTES (Tesfaye et al., Journal of Sensory Studies, Accepted 12/05/2009)

A sensory analysis procedure and extension of attributes' vocabulary list were developed using standard wine tasting cups (ISO 3591, 1977), already trained panel and a total of 26 different vinegars. The tasting procedure aims to give standard instructions about the olfaction phase, to improve the reliability of the results.

Inclusion (ISO 11035, 1994) and frequency (Torres Hernández, 2000) criteria were used to extend the vocabulary list of attributes, the importance (or discriminant utility) of which was analyzed by using Partial Least Squares Regression related techniques (Martens et al., 2000).

1.2.6. ISOLATION, IDENTIFICATION AND ANTIOXIDANT ACTIVITY OF ANTHOCYANIN COMPOUNDS IN CAMAROSA STRAWBERRY (Cerezo et al., to be submitted to Food Chemistry)



An anthocyanin-enriched strawberry extract was obtained using Amberlite XAD-7 resin and elution solvent of methanol/acetic acid (19:1), to further isolate different anthocyanin fraction by CCC (solvent system: MTBE/n-butanol/acetonitrile/water, 2.75:1.25:1:5). Phenolic compounds were analysed in the extract and the resulted fractions by LC-DAD-MS. After, we submitted both the extract and the purified anthocyanin fractions to gastric and intestinal in vitro digestions (USP23, NF18) to check the effect in anthocyanin compounds and give a more realistic approach

to in vivo situation. Besides, antioxidant activity (ORAC and FRAP) of the extract and fractions before and after simulated digestion was assessed.

1.3. RESULTS

1.3.1. THE PHENOLIC COMPOSITION OF RED WINE VINEGAR PRODUCED IN BARRELS MADE FROM DIFFERENT WOODS (Cerezo et al., 2008, Food Chemistry, 109, 606-615)

A total of twelve phenolic compounds were identified including phenolic acids, flavanols and stilbenes. Most phenolic acids did not significantly change their concentrations in the different acetifications. Conversely, (+)-catechin and *trans*-resveratrol glucoside underwent significant decreases during acetification while gallic acid and gallic ethyl ester increased substantially for those vinegars produced in chestnut wood. Discriminant analysis proved that the phenolic compounds were useful tool to correctly classify the samples (98.6% group F and 100% group T) according to the four type of wood barrels (oak, chestnut, acacia and cherry).

During acetification, a decrease (~50%) in the content of total monomeric anthocyanins was observed.

The results of triangle difference test show that the panel was able to distinguish most of the vinegars according to the different woods they were made in. Vinegars acetified in oak and cherry woods obtained the maximum scores for most of the descriptors.

1.3.2. ANTHOCYANIN COMPOSITION OF RED WINE VINEGAR: IDENTIFICATION, ANTIOXIDANT ACTIVITY AND CHANGES DURING ACETIFICATION WITH SUBMERGED CULTURE (Cerezo et al., Food Research International, submitted)

Anthocyanin-derived pigments (pyranoanthocyanins and ethyl linked compounds) have been identified for the first time in red vinegar in such detail. An

additional original contribution of this study is the identification for the first time of catechyl-pyranocyanidin-3-glucoside and guaiacyl-pyranomalvidin-3-(6-acetyl)-glucoside in vinegar and wine, as well as three anthocyanin compounds not previously reported in vinegar and *Cabernet Sauvignon* wine: carboxy-pyranocyanidin-3-(6-acetyl)-glucoside, carboxy-pyranocyanidin-3-(6-*p*-coumaryl)-glucoside and carboxy-pyranodelfinidin 3-(6-acetyl)-glucoside. After the acetification process vitisin-type and ethyl-linked compounds increased their concentration, while monomeric anthocyanins, phenolic acids (ferulic acid, caffeic acid and caftaric acid) and flavan-3-ol ((+)-catechin) decreased their concentrations.

The AA values decreased from wine to vinegar, by 14.42%, 16.80% and 29.20% according to the FRAP, DPPH and ORAC assays, respectively, but far from the commercial red vinegars (3-7 times lower) (Dávalos et al., 2005; Pellegrini et al., 2003; Alonso et al., 2003). Our data have been obtained from an acetification experiment without substrate dilution and the decrease is due to the changes in chemical composition after the fermentation process. We have also determined for the first time an approximate value of AA for malvidin-3-(6-acetyl)-glucoside isolated from vinegar.

1.3.3. IMPACT OF WOOD ON THE PHENOLIC PROFILE AND SENSORY PROPERTIES OF WINE VINEGARS DURING AGEING (Cerezo et al., Journal of Food Composition and Analysis, Accepted 06/08/2009)

TA decreases during ageing, the highest decrease was observed in vinegars aged in cherry wood in agreement with the decrease in TPI in these samples.

(+)-Taxifolin was identified in samples from red wine vinegars (group F and T) in contact with cherry wood, reaches a maximum concentration (1.69-3.45 mg/L) at 180 days of ageing. Gallic acid and gallic ethyl ester increased their concentration (57-445 and 17-30 mg/L, respectively) in vinegars in contact with chestnut wood. An unknown peak was identified only in samples aged in acacia wood. HMF, 2-furfuraldehyde, protocatechualdehyde and vanillin were affected as a consequence of ageing time. HMF was only identified in vinegar from group M (Balsamic vinegar) and increased its concentration (216-478 mg/L) during ageing process. Vanillin was only identified at 365 days of ageing, 2-furfuraldehyde at 180 days and protocatechualdehyde at 45 days or 180 days and, after that, it disappeared.

Linear discriminant analysis (LDA) proved the validity of phenolic compounds for correctly classifying samples according to the kind of wood (100% for group F and T) and ageing time (100% group M).

The longer the contact in wood, the better the sensory differences were perceived. Woody, sweet and vanilla descriptors presented the highest scores for vinegars aged in oak wood. On the other hand, the ageing in cherry wood increased the mark for red fruit attribute.

1.3.4. (+)-DIHYDROROBINETIN: A MARKER OF VINEGAR AGEING IN ACACIA (Robinia pseudoacacia) WOOD (Cerezo el al., 2009, Journal of Agriculture and Food Chemistry, 57, 9551-9554)

An unknown major compound found only in vinegars in contact with acacia wood was identified. ^{1}H and ^{13}C NMR chemical shifts, and optical rotation revealed its structure to be (+)-dihydrorobinetin, a dihydroflavonol identified for the first time in vinegars as a marker of ageing in this kind of wood. This study also reported for the first time the complete assignment of ^{13}C-NMR data for this compound. Moreover, it revealed a longer contact time with acacia wood results in higher concentrations of (+)-dihydrorobinetin found in vinegars (65-304 mg/L, time 0 to 12 months, respectively). Another finding was that the vinegars aged with nontoasted acacia chips showed significantly higher concentrations of (+)-dihydrorobinetin than with toasted acacia chips (384.8 mg/L and 23.5 mg/L, respectively). The thermal treatment of the wood, then, is crucial in the release of (+)-dihydrorobinetin into the vinegar. The *in vitro* antioxidant activity (AA) of (+)-dihydrorobinetin was also determined, explaining a 13.65% or 8.6% of AA of the final product for the TEAC and ORAC value, respectively.

1.3.5. DESCRIPTIVE SENSORY ANALYSIS OF WINE VINEGAR: TASTING PROCEDURE AND RELIABILITY OF NEW ATTRIBUTES (Tesfaye et al., Journal of Sensory Studies, Accepted 12/05/2009)

Comparing the proposed protocol with *ad libitum* tasting, the standard deviations for aroma intensity, richness in aroma, general impression, coconut odor,

clove odor, vanilla odor, woody odor and wine character were lower when panelists followed the proposed tasting protocol. The panel verified that aromas such as vanilla, coconut and clove were perceived better when the proposed protocol was used probably because acetic acid and ethyl acetate did not mask them.

The list of attributes that describe vinegars was extended to a total of 13 (ethyl acetate odour, alcohol/liquor, pungent sensation, winy character, raisin, woody, citrus, red fruit, vanilla, clove, suit aroma, bitter almond and leather/old) and seven optional (medicinal, apple, coconut, rancid, bacteria, cheese and sawdust/wood shaving) to be included when a particular vinegar is tested. The DPLSR analysis of replications showed a good between-run reproducibility of the selected attributes.

1.3.6. ISOLATION, IDENTIFICATION AND ANTIOXIDANT ACTIVITY OF ANTHOCYANIN COMPOUNDS IN CAMAROSA STRAWBERRY (Cerezo et al., to be submitted to Food Chemistry)

A total of 17 anthocyanin compounds were identified in the strawberry extract including pelargonidin-3-glucoside as major compound followed by pelargonidin-3-rutinoside and eleven pelargonidin and cyanidin derivatives. Additionally, Delphinidin-3-glucoside, peonidin-3-glucoside and cyanidin-3-galactoside were tentatively identified for the first time in strawberry. Another original contribution was de identification of 5-carboxypyranopelargonidin-3-glucoside in *Camarosa* strawberry variety.

A total of 4 fractions were isolated by CCC. Two different fractions contained pelargonidin-3-glucoside and pelargonidin-3-rutinoside at 90% and 92% of purity, respectively. Pelargonidin-3-glucoside was more active than the 3-rutinoside contributing in 32.61% and 17.58% to the overall antioxidant activity (AA) of the extract, for ORAC and FRAP assays, respectively.

Pelargonodin-3-rutinoside fraction and polymeric compounds kept their AA after the digestion processes. However, a remarkable decrease in AA with both methods was observed for pelargonidin-3-glucoside fraction and coil, likely explained by the presence of amyloglucosidase enzyme in the gastric digestion favouring the

deglucosylation and decreasing the AA as a consequence, as the aglycon posses less AA than the corresponding glucoside (Fukumoto & Mazza, 2000; Kähkönen & Heinonen, 2003).

1.4. CONCLUSIONS

One of the original contributions of this Thesis is the identification of anthocyanin derives pigment (pyranoanthocyanins and ethyl-linked compounds) in red wine vinegars. Additionally, catechyl-pyranocyanidin-3-glucoside and guaiacyl-pyranomalvidin-3-(6-acetyl)-glucoside have described in wine and vinegar for the first time, and carboxy-pyranocyanidin-3-(6-acetyl)-glucoside, carboxy-pyranocyanidin-3-(6-p-coumaryl)-glucoside and carboxy-pyranodelfinidin 3-(6-acetyl)-glucoside in vinegar and *Cabernet Sauvignon* wine.

The effect of the submerged acetification process on phenolic composition is as follow: vitisin-type and ethyl-linked compounds increase their concentration, while the concentration of monomeric anthocyanins, phenolic acids (ferulic acid, caffeic acid and caftaric acid) and flavan-3-ol ((+)-catechin) decrease. The concentration of (+)-catechin also decreases during the acetification with surface culture.

The impact of different woods on phenolic composition of red wine vinegar was proved by the release of characteristic phenolic compounds of each wood, as (+)-taxifolin and (+)-dihydrorobinetin in vinegars in contact with cherry and acacia wood, respectively.

An additional original contribution is the tentatively identification of delphinidin-3-glucoside, peonidin-3-glucoside and cyanidin-3-galactoside in strawberry substrate and 5-carboxypyranopelargonidin-3-glucoside in *Camarosa* variety.

Sensory analysis is a useful tool to discriminate vinegars on the basis of the type of wood used in their elaboration. The tasting procedure developed improves the reliability of the results in vinegar sensory analysis.

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2. INTRODUCCIÓN / INTRODUCTION

2.1. EL VINAGRE

2.1.1. DEFINICIÓN

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. El contenido en acidez total de 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."* Se entiende por grado de acidez de los vinagres su acidez total expresada en gramos de ácido acético por 100 mL, a 20ºC.

2.1.2. TIPOS DE VINAGRES

Existe una gran variedad de materias primas de origen agrícola que se emplean para la obtención de vinagres. En los países Mediterráneos la principal materia prima empleada es el vino; en otros países donde no se cultiva la vid (países del norte de Europa, principalmente) o es un cultivo difícil o minoritario, se utilizan otras materias primas como los llamados vinos de frutas, sidra, malta, alcohol de vino, de cereales, de melazas, de suero de leche, de miel o de arroz (Llaguno & Polo, 1991; Peppler & Beaman, 1967).

Los vinagres se pueden clasificar en función del tipo de sustrato empleado o del método usado en su elaboración. En función del sustrato a partir del cual se obtienen (Presidencia del Gobierno, 1993) podemos distinguir:

- *Vinagre de vino*: por fermentación acética de vino.
- *Vinagre de frutas*: obtenido a partir de frutas o bayas.
- *Vinagre de sidra*: a partir de la sidra o sus piquetas.

- *Vinagre de alcohol*: por la fermentación acética de alcohol destilado de origen agrario.
- *Vinagre de cereales*: elaborado 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*: elaborado 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*: obtenido a partir de la miel.
- *Vinagre de suero de leche*: obtenido a partir de suero de leche.

Más recientemente (Comité Europeo de Normalización, UNE-EN 13188, 2000) se reconocen además otros tipos de vinagres:

1. Vinagre de vino de frutas: a partir de vino de frutas mediante el proceso biológico de fermentación acética.
2. Vinagre de vino de bayas: a partir de vino de bayas mediante el proceso biológico de fermentación acética.
3. Vinagre de malta destilado: elaborado mediante la destilación del vinagre de malta, a presión reducida. Contiene solamente los componentes volátiles del vinagre de malta a partir del cual procede.
4. Vinagre de especias y vinagre condimentado: elaborados a partir de algún sustrato anteriormente citado y que además contenga hierbas, condimentos y otros ingredientes, tales como:
    a. Plantas o partes de plantas, incluyendo especias y frutas, que bien deberán utilizarse frescas o en seco y cortadas en rodajas o no, o bien en forma de extractos.
    b. Azúcar
    c. Sal
    d. Miel
    e. Zumos de frutas concentrados o no concentrados.
5. Otros tipos de vinagre: Por ejemplo vinagre de centeno, de cerveza, etc.

2.1.3. MÉTODOS DE ELABORACIÓN DE VINAGRE

La bacteria acética es el agente responsable de la fermentación acética. El aporte de oxígeno del que disponga determina la velocidad de acetificación. Resultan pues, dos métodos fundamentales en la elaboración de vinagre:

1) Acetificación con cultivo superficial

Las bacterias acéticas se encuentran situadas en la superficie del líquido a acetificar libres o bien fijadas a soportes tales como virutas. El crecimiento del cultivo se restringe a una zona reducida del volumen a acetificar con lo cual la velocidad de acetificación es lenta, la producción de escaso volumen y el coste más elevado. La lentitud de la acetificación ocasiona un proceso de envejecimiento simultáneo que favorece el desarrollo de propiedades sensoriales muy apreciadas. Se elaboran así la mayoría de los vinagres tradicionales.

2) Acetificación con cultivo sumergido

Las bacterias acéticas están sumergidas libremente en el seno del líquido a fermentar, en el que constantemente se introduce aire (solo o enriquecido con oxígeno), en condiciones que permitan la máxima transferencia posible desde la fase gaseosa a la fase líquida. Así se obtienen de forma rápida la mayoría de los vinagres que existen en el mercado y cuyo coste de producción es menor.

2.1.3.1. Métodos tradicionales de acetificación con cultivo superficial

a) Método de Orleáns

En el siglo XVII la principal industria vinagrera se hallaba en Orleáns. Allí se desarrolló uno de los métodos más antiguos para la fabricación de vinagre (Mitchell, 1916). Emplea toneles de aproximadamente 250-300 litros de capacidad, que se colocan tumbados en filas horizontales y superpuestas (Figuras 1 y 2). 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. Se mantienen la temperatura entre 20-25ºC. 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.

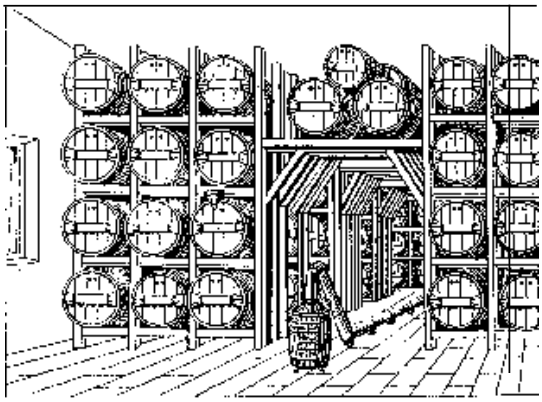


Figura 1. Pilas de botas para la acetificación en una fábrica de vinagre por el método de Orleáns.

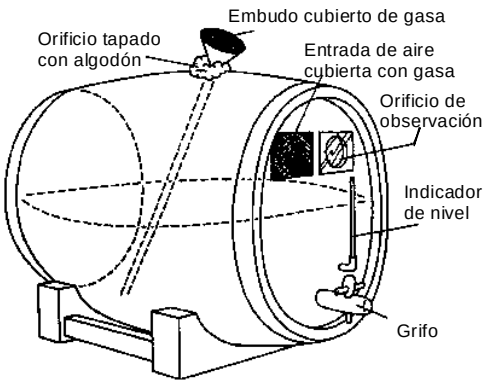


Figura 2. Acetificación por el método de Orleáns (Adams, 1985).

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 (Figura 3). 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.

Este método emplea una cuba giratoria (Figura 3) que dispone de un orificio grande en el centro de uno de los fondos, para procurar la entrada de aire. En uno de los costados, en la parte más alejada de la abertura, se realiza un orificio estrecho para el vaciado del envase, que puede taparse para evitar la salida del líquido. 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 y contiene un termómetro para controlar la temperatura, que es un factor muy importante para todo método rápido o semirrápido.

Mediante este método se puede obtener una cantidad de vinagre igual a la cuarta parte del contenido del tonel cada cuarenta y ocho horas. El vinagre elaborado, que se extrae de la cuba, se sustituye por porciones iguales de vino, continuando la elaboración indefinidamente, salvo accidente (Xandri-Taqueña, 1977).

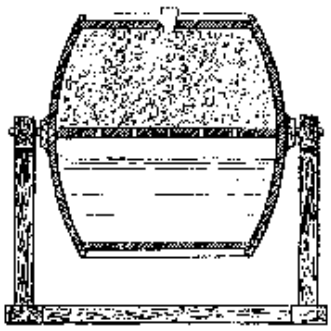


Figura 3. Cuba rotatoria del método Luxemburgués.

c) Método de Schüzenbach o método alemán

Este método emplea toneles o generadores verticales de encina con doble fondo (Figura 4). El primer fondo se encuentra agujereado y sirve como soporte de una serie de capas de virutas de madera de haya, impregnadas de vinagre de buena calidad.

En el borde superior se encuentra un diafragma perforado que permite el paso del vino. El vino se rocía sobre el material poroso que cae por gravedad hasta el fondo del depósito; sobre la superficie del material poroso se encuentran las bacterias acéticas (Peppler & Beaman, 1967). Al pasar el vino por el diafragma, burbujea aire que existe entre las virutas. El vinagre se extrae por la parte inferior y desde allí se vuelve a introducir por la parte superior del depósito mediante el empleo de una bomba de recirculación, hasta alcanzar la concentración de ácido acético deseada. Se descargan aproximadamente las 4/5 partes del volumen líquido y se repone con vino. Esta descarga se realiza cada 7-10 días. Se pueden emplear barriles de roble giratorio, parcialmente lleno de virutas, consiguiéndose así una mejor aireación.

Las ventajas de este proceso son: la regulación de oxígeno y su uso para la producción continua de vinagre. La lentitud de la acetificación implica el simultáneo envejecimiento que favorece el aroma y el gusto del producto (Llaguno & Polo, 1991).

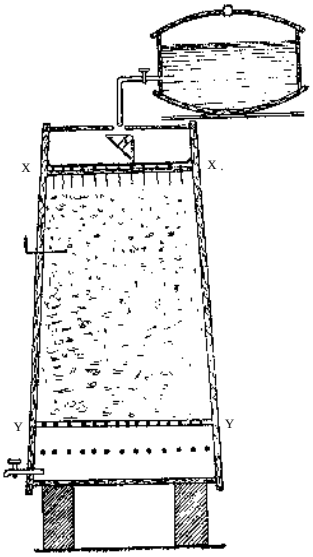


Figura 4. Esquema de un generador vertical, para la producción de vinagres vínicos.

2.1.3.2. Métodos de acetificación con cultivo sumergido

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 por 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, el oxígeno disuelto y los ciclos de carga y descarga.

Las bacterias acéticas son viables entre 28-33ºC, pero la velocidad de fermentación varía en función de la temperatura. La temperatura de la fermentación debe estar comprendida dentro del intervalo entre 30-31ºC (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 las bacterias.

El carácter aerobio de las bacterias acéticas exige oxígeno para su supervivencia. Su carencia, aunque sea por un breve periodo de tiempo (3-5 min), puede paralizar la acetificación. Por otro lado, un elevado suministro de aire puede causar el fenómeno de sobreoxidación y arrastre de los componentes volátiles.

De modo similar, el sustrato a acetificar, por ejemplo el etanol, no debe llegar a agotarse totalmente ya que las bacterias acéticas morirían rápidamente. Normalmente, los acetificadores operan en régimen semicontinuo realizando ciclos de carga-descarga. Así, se impide que las bacterias acéticas metabolicen el ácido acético formado convirtiéndolo en CO_2 y agua. Se descarga aproximadamente la mitad del volumen de líquido, que se repone con nueva materia prima suministrándole sustrato a la bacteria. Al descargar se retiran bacterias reduciendo la población del fermentador de modo que una descarga excesiva ocasionaría un tiempo excesivo en el arranque del siguiente ciclo.

a) Modelos Frings

El acetator Frings fue construido en los años 50 basándose en las investigaciones realizadas por Hromatka y Ebner (1949) y desde ese momento se mantiene funcionando con algunas modificaciones en la mayoría de las industrias vinagreras actuales para elaborar la mayor parte de la producción (Figura 5).

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 30ºC (Ormaechea, 1991).

El éxito de estos equipos se basa en el diseño de las turbinas que emplean. Estas no sólo aspiran de modo continuo aire desde el exterior sino que generan una intensa mezcla del mismo con el medio de cultivo. El resultado de su funcionamiento es una alta eficacia en la transferencia de oxígeno desde el aire.

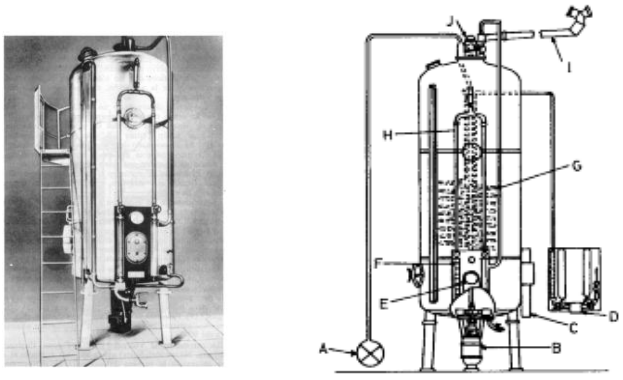


Figura 5. Acetator Frings en acero inoxidable
A, bomba de carga; B, aireador y motor; C, dispositivo para determinación de alcohol residual; D, válvula entrada agua refrigeración; E, termostato regulador; F, rotámetro; G, serpentín de refrigeración; H, entrada de aire; I, salida de gases; J, dispositivo antiespuma

Posteriormente, para evitar las pérdidas de compuestos volátiles se ha desarrollado un sistema de acetificación con cultivo sumergido 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). No obstante, los controles necesarios y el coste del oxígeno puro con respecto al aire encarecen este sistema.

2.1.4. VINAGRES DE CALIDAD: DENOMINACIÓN DE ORIGEN PROTEGIDA

El reconocimiento de la calidad de un producto ha inspirado el desarrollo de normas legislativas para su protección. A lo largo del tiempo los países han desarrollado diferentes regulaciones que se han armonizado en el ámbito de la UE. Existen actualmente distintas figuras de protección: Denominación de Origen Protegida (DOP) e Indicación Geográfica Protegida (IGP). Ésta última asume que los atributos de calidad de un producto dependen de la zona de producción del mismo. En tanto que la DOP asume además que las particularidades del proceso de elaboración contribuyen a su calidad. Actualmente, poseer el reconocimiento de IGP durante 5 años es un requisito previo para la obtención de la DOP.

Hoy día, los vinagres con DOP son: el "Aceto Balsamico Tradizionale di Modena", "Aceto Balsamico Tradizionale di Reggio Emilia" ambos de Italia y los tres españoles el "Vinagre de Jerez", "Vinagre del Condado de Huelva" y el recientemente aprobado "Vinagre de Montilla-Moriles". Así pues, España cuenta con tres de las cinco denominaciones de origen de toda Europa.

La única IGP reconocida hasta ahora es la de "Aceto Balsamico di Modena" (CE, Nº 583/2009).

2.1.4.1. Vinagre Balsámico Tradicional de Módena y Reggio Emilia

En el año 1986, el vinagre balsámico tradicional de la región de Módena y Reggio Emilia fue reconocido como un producto de una Región Específica, terminología equivalente a la actual DOP (CE Nº 93/1986). En el año 2000, obtuvieron

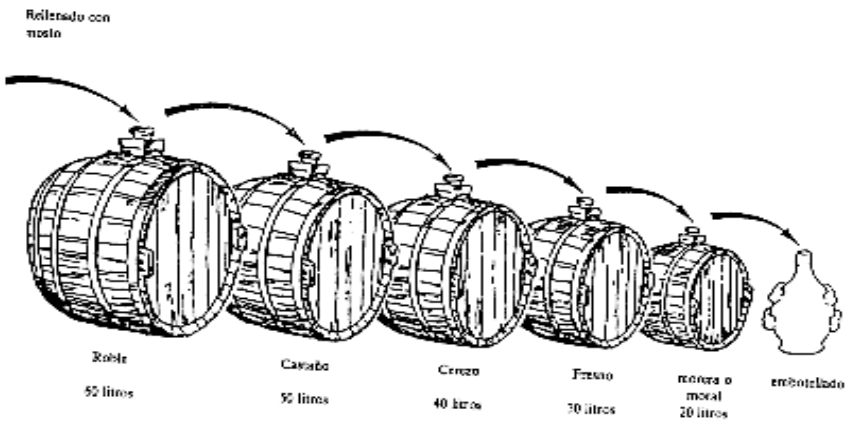
el certificado de Denominación de Origen Protegida los vinagres "Aceto Balsamico Tradizionale di Modena" (ABTM) y "Aceto Balsamico Tradizionale di Reggio Emilia" (ABTRE) por la Comisión Europea debido a sus típicos y únicos procedimientos de producción y a su bien definida área de producción (CE Nº 813/2000).

En ambos casos, el mosto de uva se concentra por calentamiento hasta al menos un tercio de su volumen inicial, mediante ebullición suave, lo que producirá la eliminación de la flora espontánea y un incremento del 30% del contenido en azúcares. La fermentación se lleva a cabo mediante un proceso tradicional en barriles de diferentes maderas y tamaños (Seidemann, 1996). El mosto concentrado es fermentado por levaduras osmófilas pertenecientes al género *Zygosaccharomyces*. Simultáneamente, las bacterias acéticas metabolizan el etanol a ácido acético. La lentitud del proceso ocasiona que tenga lugar un envejecimiento simultáneo.

El vinagre acabado, después de un proceso que dura varios años, se extrae del último barril de la serie de 20 litros de volumen, que se rellena de vinagre procedente del anterior de 30 litros, que a su vez se rellena de otro de 40 litros de capacidad y este de uno de 50 litros que a su vez recibe el vinagre de un barril de 60 litros de capacidad, al que se añade mosto de uva concentrado hasta completar el volumen extraído (Figura 6). La porosidad de cada tipo de madera determina la entrada de oxígeno que influye tanto en el desarrollo de la acetificación como en el aroma y color del vinagre obtenido. Los períodos de tiempo de envejecimiento son de al menos 12 años (Plessi et al., 1988; Stacchini et al., 1990; Turtura & Bortolotti, 1991; Giudici et al., 1992). El producto resultante es un líquido marrón oscuro, de aspecto siruposo, con sabor agridulce.

Las diferencias entre ambas DOP son las variedades de uva utilizadas para elaborar el mosto y los tipos maderas de los barriles utilizados en el proceso de elaboración. Para la elaboración del ABTM se permiten uvas producidas en la provincia de Módena, en particular de *Lambrusco* y *Trebbiano*, mientras que para el ABTRE se usan uvas producidas en el territorio de la provincia de Reggio Emilia como son: *Trebbiano*, *Occhio di Gatto*, *Spergola*, *Berzemino* y *Lambrusco* con las siguientes indicaciones ampelográficas: *Marani*, *Salamino*, *Maestri*, *Montericco*, *Sorbara*, y *Ancellotta*. Por otro lado, las maderas utilizadas en los barriles del ABTM son de: roble,

castaño, cerezo, fresno y moral, en orden decreciente. Mientras que para el ABTRE se usan de castaño, cerezo, moral, enebro y roble, en el mismo orden.



Figur 6. Elaboración del "Aceto Balsamico Tradizionale di Modena".

2.1.4.2. Vinagre de Jerez

Desde 1995, el "Vinagre de Jerez" goza del reconocimiento de producto con Denominación de Origen (DO) por la Consejería de Agricultura y Pesca (BOJA, 09/03/95). Tras diversos cambios, se ha revisado y actualizado a DOP (BOJA, 16/09/08) y modificado su reglamento (BOJA, 29/01/09).

El "Vinagre de Jerez" es el producto resultante de la fermentación acética de vinos aptos elaborados a partir de uvas de la zona de producción de la Denominación de Origen, elaborado por cultivo superficial o sumergido, y envejecido por el sistema dinámico de "criaderas y solera" o por el sistema estático de "añada". Se consideran vinos aptos, los vinos del año procedentes de la zona de producción reconocida por el Consejo Regulador de la Denominación de Origen, o bien los vinos de crianza de las Denominaciones de Origen "Jerez-Xérèx-Sherry" y "Manzanilla-Sanlúcar de Barrameda". La acidez total en acético alcanza como mínimo los 70 g/L, con la excepción de los vinagres al Pedro Ximénez o al Moscatel, que pueden ser de 60 g/L. En el caso de los Vinagres del tipo Gran Reserva, la acidez total mínima en acético es de 80 g/L (BOJA, 16/09/08).

El sistema dinámico de "criaderas y solera" es el más difundido y generalizado. Este sistema está compuesto por una serie indeterminada de botas, usualmente de roble americano, agrupadas en filas horizontales cuyo número puede oscilar entre tres y cinco (Figura 7). La escala que está a ras del suelo recibe el nombre de "solera", sobre ella se encuentra la primera criadera, sobre la cual se sitúa la segunda y así sucesivamente. La edad del vinagre que contienen las botas, aumenta conforme descendemos en el sistema, siendo la solera la de mayor envejecimiento. El vinagre acabado se obtiene de la solera, no pudiendo exceder la cantidad que se saca de la bota en un tercio del contenido de la misma. Esta cantidad se repone con el contenido de la primera criadera. Ésta a su vez se nutre del de la segunda y así sucesivamente, ingresando el sustrato de partida en el sistema por la criadera superior. Estas operaciones se llaman "sacas" y "rocíos", y la periodicidad suele ser entre tres o cuatro veces al año.

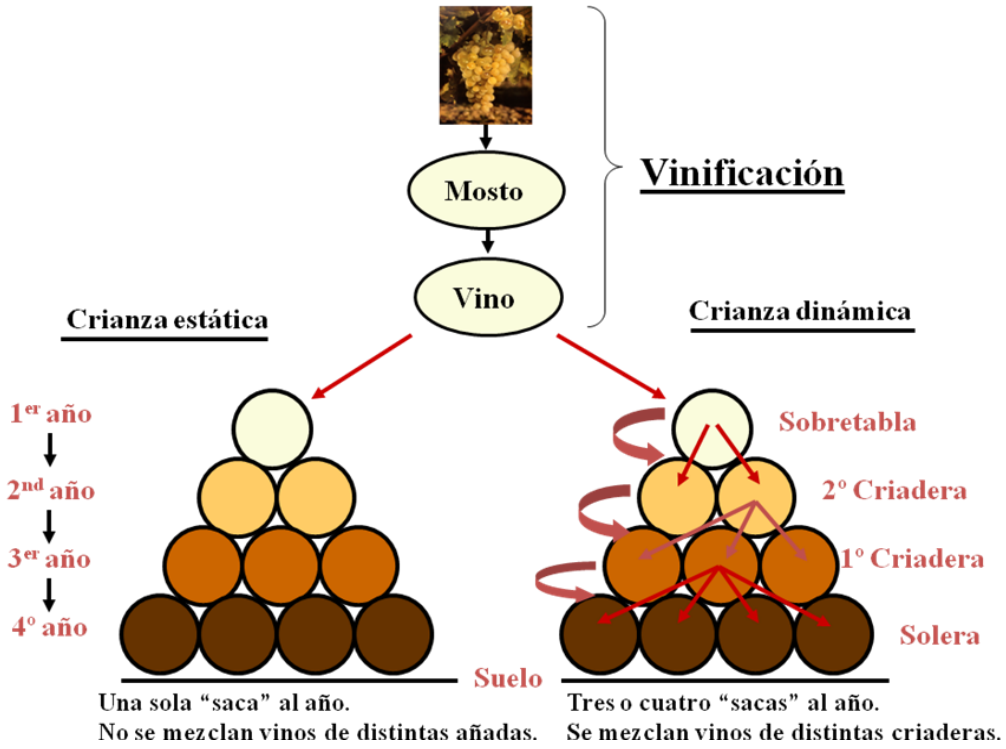


Figura 7. Envejecimiento estático de "añada" y dinámico de "criaderas y solera" de los vinagres de Jerez.

Según los periodos de envejecimiento a los que son sometidos el "Vinagre de Jerez", tanto por sistema estático como dinámico, se distinguen las siguientes categorías (BOJA, 29/01/09):

- "Vinagre de Jerez": envejecimiento mínimo de 6 meses.
- "Vinagre de Jerez Reserva": envejecimiento mínimo de 2 años.
- "Vinagre de Jerez Gran Reserva": envejecimiento mínimo de 10 años.

Adicionalmente, se distinguen los siguientes tipos de "Vinagre de Jerez" semi-dulce, en función de la variedad, los cuales corresponden a cualquiera de las categorías mencionadas anteriormente (BOJA, 29/01/09):

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

2.1.4.3. Vinagre del Condado de Huelva

El "Vinagre del Condado de Huelva" goza del reconocimiento de producto con Denominación de Origen por la Consejería de Agricultura y Pesca desde el año 2002 (BOJA, 24/08/2002). Posteriormente, se ha actualizado a DOP (BOJA, 16/09/08) y modificado su reglamento (BOJA, 30/03/09).

El "Vinagre del Condado de Huelva" se caracteriza por ser un producto obtenido por la acetificación de un vino certificado por el Consejo Regulador de la Denominación de Origen "Condado de Huelva". Debe ser un vino seco, sin resto de azúcares que puedan provocar contaminaciones posteriores con levaduras. La acetificación se produce mediante un sistema de cultivo sumergido. Posteriormente, el vinagre obtenido se puede comercializar como tal o ser sometido a un proceso de envejecimiento. Así pues se pueden distinguir cuatro tipos de vinagre (BOJA, 16/09/08):

- "Vinagre Condado de Huelva": vinagre sin envejecer, con una acidez total mínima en ácido acético de 70 g/L, y un contenido en alcohol residual no superior al 0,5% vol.
- "Vinagre Viejo Condado de Huelva Solera": envejecido mediante el sistema tradicional de "criaderas y solera" durante un tiempo mínimo de seis meses y enriquecido con vino Generoso o Generoso de Licor "Condado de Huelva", no siendo el contenido de alcohol residual superior al 3% vol.
- "Vinagre Viejo Condado de Huelva Reserva": envejecido mediante el tradicional sistema de "criaderas y solera", durante un tiempo mínimo de un año y enriquecido con vino Generoso o Generoso de Licor "Condado de Huelva", no siendo el contenido de alcohol residual superior al 3% vol.
- "Vinagre Viejo Condado de Huelva Añada": envejecido mediante el sistema estático de "añada", durante un tiempo mínimo de tres años y enriquecido con vino Generoso o Generoso de Licor "Condado de Huelva", no siendo el contenido de alcohol residual superior al 3% vol.

2.1.4.4. Vinagre de Montilla-Moriles

El "Vinagre de Montilla-Moriles" ha sido reconocido como DOP por la Consejería de Agricultura y Pesca en 2008 (BOJA, 03/10/08). Dicho producto es obtenido exclusivamente de la fermentación acética de vinos de crianza que proceden de la zona amparada por la Denominación de Origen "Montilla-Moriles".

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

- Vinagres de envejecimiento: "Vinagre de Montilla-Moriles Añada" (envejecimiento estático superior a 3 años), "Vinagre de Montilla-Moriles Crianza" (envejecimiento en sistema de criaderas y solera mínimo de 6 meses), "Vinagre de Montilla-Moriles Reserva" (envejecimiento en sistema de criaderas y solera mínimo de 2 años) y "Vinagre de Montilla-Moriles Gran Reserva" (envejecimiento en sistema de criaderas y solera mínimo de 10 años).

- Vinagres Dulces: aquellos a los que se añaden mosto concentrado tanto de uvas pasificadas Pedro Ximénez; "Vinagres Dulces al Pedro Ximénez", como de uvas pasificadas o no de la variedad Moscatel; "Vinagres Dulces al Moscatel". Estos vinagres dulces también se someten a envejecimiento en sistema de "criaderas y solera" obteniéndose vinagres de "Crianza" (mínimo 6 meses), "Reserva" (mínimo 2 años), "Gran Reserva" (superior a 5 años).

En resumen, las DOP de vinagres andaluces difieren en sus consideraciones. Las DOP "Vinagre de Jerez" y "Vinagre del Condado de Huelva" aceptan el uso de vino del año, en tanto que la DOP "Vinagres de Montilla-Moriles" requiere vino de crianza. Todas admiten la acetificación con cultivo sumergido, pero con respecto al envejecimiento se observan discrepancias. La única DOP, que permite la comercialización del producto sin envejecer, es la de "Vinagre del Condado de Huelva". Si bien, la DOP "Vinagre de Jerez" y "Vinagre de Montilla-Moriles" coinciden en el tiempo de envejecimiento de las distintas categorías (6 meses, 2 años y 10 años), el "Vinagre del condado de Huelva" presenta menores requisitos (6 meses, 1 año y 3 años). Puede resultar confuso para el consumidor que las categorías se establezcan con envejecimiento de duración tan dispar.

2.2. CARACTERIZACIÓN DE VINAGRES

La diversidad de los vinagres de vino en el mercado y el incremento de su demanda requiere su caracterización para establecer parámetros de control de calidad. La caracterización del vinagre abarca diferentes objetivos incluyendo la autentificación del producto y la clasificación del mismo basándose en criterios de calidad. Su finalidad es la protección del consumidor frente a la comercialización de productos de calidad inferior a la declarada en la descripción del mismo junto con la defensa de los productores honestos frente a la competencia desleal. Así pues, el vinagre como el resto de productos alimentarios, debe cumplir con las especificaciones de calidad y debe llevar una etiqueta que describa fielmente el producto.

La calidad final del vinagre viene determinada por los materiales de partida utilizados como sustrato fermentativo, el proceso de acetificación usado en la elaboración y el posterior envejecimiento en contacto con la madera.

El primer requisito que debe cumplir el vinagre es poseer la concentración de ácido acético que marca la legislación y que éste haya sido obtenido por fermentación. Los adulterantes más comunes son los derivados del petróleo u obtenidos por pirólisis de la madera (Thomas & Jamin, 2009). Era preciso detectar prácticas fraudulentas como la adicción de ácido acético de síntesis a los vinagres producidos por fermentación (De la Pena et al., 1976; Grau Malonda et al., 1976; Volonterio & Resmini, 1984). Para ello, se llevaron a cabo estudios de estimación del ^{14}C para evidenciar el origen orgánico o sintético del producto (Krueger et al., 1985).

El fraccionamiento de isótopos naturales específicos acoplados a resonancia magnética nuclear (SNIF-RMN) permite detectar la adición del 5% del ácido acético de síntesis. Este límite ocasiona que la práctica deje de ser económicamente rentable para el falsificador (Remaund, et al., 1992; Belton et al., 1996).

La diferenciación entre vinagres fermentados y vinagres sintéticos se logra mediante el análisis de aminoácidos por cromatografía gaseosa-espectrometría de masas. D-Ala, D-Asp y D-Glu demostraron ser marcadores de vinagres producidos por fermentación, ya que están presentes en éstos y ausentes en los vinagres sintéticos (Erbe & Brückner, 1998).

Para diferenciar el origen botánico del sustrato se ha empleado el análisis de isótopos de carbono y oxígeno. Se pueden diferenciar vinagres obtenidos a partir de remolacha, caña o derivados de la uva con los valores de ^{13}C. Pero los diversos sustratos procedentes de la uva como origen botánico: el vino y el alcohol vínico, requieren el análisis de otro parámetro. Para diferenciar entre el origen vínico y el alcohol vínico se usa el contenido de ^{18}O, relacionado con la adicción de agua de la red empleada en el proceso de elaboración (Thomas & Jamin, 2009). En el caso del alcohol vínico es necesaria la dilución para ajustar el grado alcohólico y consecuentemente el acético.

Se ha evaluado el contenido en polialcoholes para distinguir entre los diferentes orígenes: vinagres de vino, alcohol, sidra, malta, manzana y miel, ya que estos compuestos están relacionados con la materia prima y persisten en el producto después de la fermentación alcohólica y acética (Antonelli, et al., 1994; Gerbi, et al., 1995). En particular, el vinagre de manzana posee un alto contenido en sorbitol (3296 mg/L), mientras que el vinagre de miel muestra una alta concentración de manitol (958 mg/L). El vinagre de alcohol fue fácil de diferenciar, ya que mostraba un escaso contenido en polialcoholes (Antonelli, et al., 1996). No obstante, la diferenciación entre distintos vinagres de vino no resulta fácil con este parámetro. Si bien el perfil de polialcoholes permite caracterizar los vinagres de Jerez no pueden discriminar los vinagres de otros orígenes (Italia, Francia y Suiza).

La determinación del origen vínico de los vinagres ha sido otro de los propósitos de la caracterización de vinagres debido a su elevado volumen de producción. La regulación española prohíbe la mezcla de vinagres acabados o de distintos sustratos antes de la fermentación. La práctica fraudulenta más común en la elaboración y comercialización de vinagres, es la adicción de vinagre de alcohol al vinagre de vino.

L-prolina (L-Pro) es un aminoácido característico de la uva y sus derivados, que ha demostrado ser el más abundante en los vinagres de vino (Erbe & Brückner, 1998), llegando a alcanzar concentraciones de hasta 355-2187 mg/L en vinagres andaluces (Troncoso & Guzmán, 1987a); 149-360 mg/L en vinagres vínicos españoles de diversas regiones (Polo et al., 1976) y 307-916 mg/L en vinagres balsámicos (Erbe & Brückner, 1998). Así, contenidos en L-Pro inferiores son sospechosos de adulteración. Existe un método oficial fotométrico para la determinación de L-Pro (Presidencia del Gobierno, 1977) ya que es un parámetro representativo de la calidad y el carácter genuino de un vinagre de vino.

La acetoína es un compuesto que se origina en el vinagre por la acción de las bacterias acéticas sobre distintas sustancias como el 2,3-butilenglicol, ácido aspártico, glicerol, láctico, malónico y fumárico, entre otros. El contenido en acetoína de un vinagre determina su origen, siendo mayor en los vinagres de vino (200-600 mg/L en vinagres de vino andaluces) (Troncoso & Guzmán, 1987a,b). La reglamentación española (Presidencia del Gobierno, 1993) establece que en un vinagre vínico la

concentración de este parámetro no debe ser inferior a 30 mg/L, sin delimitar un máximo.

La caracterización de distintos vinagres de vino, sobre todo aquellos que poseen DOP, como los Vinagres de Jerez y Aceto Balsamico Tradizionale di Modena, se ha llevado a cabo en numerosas ocasiones para garantizar la calidad y protección de estos productos.

Compuestos volátiles como: diacetilo, acetato de isoamilo, ácido isovalérico y acetato de etilo, han demostrado ser buenos marcadores del aroma de los Vinagres de Jerez, encontrándose a altas concentraciones: 13-197, 0,36-11,6, 38-121 y 140-2210 mg/L, respectivamente, muy por encima de su umbral de percepción. Además, el sotolón se identificó sólo en los Vinagres de Jerez, alcanzando las mayores concentraciones en los vinagres más envejecidos (663-939 µg/L) (Callejón et al., 2008a).

Respecto a los vinagres Aceto Balsamico Tradizionale di Modena, su caracterización mediante la determinación de veintitrés aminoácidos en un analizador automático y su posterior análisis estadístico multivariante, ha sido útil para diferenciarlos del resto (Del Signore et al., 2000). Hay que destacar el elevado contenido en 5-hidroximetilfurfural (HMF) como característica de estos vinagres, llegando a tener concentraciones de hasta 5,5 g/Kg (Theobald et al., 1998).

La diferenciación de vinagres de vino elaborados por el método tradicional de acetificación lenta (cultivo superficial) de aquellos acetificados de manera rápida (cultivo sumergido), suscita gran interés por la importancia económica de los primeros.

La acidez, el extracto seco, el contenido en cenizas, el glicerol, el alcohol, los sulfatos (Guerrero et al., 1994), y el contenido mineral (As, Ca, Cu, Fe, K, Mg y Zn) (Guerrero et al., 1997), permite discernir entre producción rápida o artesanal de muestras comerciales de vinagre. Durante la acetificación con cultivo sumergido en sistema abierto tiene lugar una pérdida de compuestos volátiles. Así, disminuyen los alcoholes (metanol, propanol, isobutanol y alcoholes isoamílicos) y el acetato de etilo, en tanto que la acetoína aumenta (Morales et al., 2001). El perfil aromático también fue

una herramienta útil para discriminar entre vinagres de vino tinto elaborados por cultivo superficial y aquellos elaborados por cultivo sumergido (Callejón et al., 2008b).

Los aminoácidos son la fuente de nitrógeno necesaria para el crecimiento de las bacterias acéticas. Su consumo está determinado por el método de acetificación empleado (superficial o sumergido), que a su vez está relacionada con la especie de bacteria acética presente. Así pues, el aminoácido más consumido en la acetificación con cultivo superficial es la prolina, mientras que en el sumergido es la arginina (Callejón et al., 2008c).

El proceso de envejecimiento para mejorar la calidad de los vinagres es una práctica muy extendida en la elaboración de la mayoría de los vinagres de calidad, ya que tiene lugar la formación de nuevos aromas muy apreciados. Los cambios se deben a una concentración de compuestos debido a la pérdida de agua por evaporación a través de los poros de la madera de las barricas. Además, hay que considerar la aparición de nuevos compuestos como el diacetilo y algunos ésteres.

2.2.1. CARACTERIZACIÓN DE VINAGRES DE VINO: COMPUESTOS FENÓLICOS

Los compuestos fenólicos son metabolitos secundarios de las plantas y están presentes en hojas, frutos, semillas, corteza y también en la madera de vegetales. Son un grupo muy amplio de compuestos químicos, mas de 8000, que se caracterizan por presentar todos ellos, el núcleo aromático de benceno, sustituido, como mínimo, con un grupo hidroxilo. Si bien los compuestos fenólicos están presentes en la mayoría de las plantas, existe una enorme diversidad tanto cualitativa como cuantitativa entre las diferentes especies.

Los compuestos fenólicos existentes en los alimentos, se pueden clasificar en libres o polimerizados. Debido al mayor desarrollo de las técnicas analíticas para determinar los de estructuras químicas más sencillas, existe una clasificación establecida en función de la estructura del esqueleto carbonado, pudiéndose distinguir:

- No flavonoides:
  - Ácidos fenólicos:
    - C_6-C_1
    - C_6-C_3
  - Estilbenos: C_6-C_2-C_6

- Flavonoides: C_6-C_3-C_6
  - Flavanoles
  - Flavonoles
  - Flavononoles
  - Flavonas
  - Antocianos

Además, estas estructuras pueden estar esterificadas, por ejemplo, los ácidos cinnamicos con el ácido tartárico (en el caso de la uva), o unidas a glucósidos formando dímeros y polímeros. La enorme complejidad y diversidad estructural no es el objeto del presente resumen, ya que ha sido revisada con detalle por diversos autores (Williams & Grayer, 2004; Rentzsch et al., 2007; Veitch & Grayer, 2007; Guerrero et al., 2009). Si bien, se ha incluido un anexo en el que se detallan las estructuras que pueden facilitar la lectura del presente trabajo (ANEXO).

Están asociados con las características organolépticas de color, flavor y astringencia en los alimentos de origen vegetal (frutas, zumo, vino, etc…). Además, en la última década sus propiedades saludables han sido objeto de numerosos estudios (Rice-Evans et al., 1996; Villano et al., 2005; Fernández-Pachón et al., 2008). Hoy día, se reconocen como un grupo de compuestos bioactivos con propiedades antioxidantes.

Las diferencias de composición fenólica entre las distintas variedades de uvas han puesto de manifiesto su utilidad como marcadores taxonómicos para diferenciar y caracterizar productos, como es el caso de los vinagres.

2.2.1.1. Los compuestos fenólicos en la caracterización de vinagres

La caracterización de los vinagres de vino ha sido dirigida, en su mayoría, a la autentificación de vinagres de calidad como son los Vinagres de Jerez y Aceto Balsamico Tradizionale di Modena por su mayor valor económico.

Actualmente, los vinagres que cuentan con el distintivo de Denominación de Origen Protegida son, en todos los casos, vinagres elaborados a partir de vinos de uvas

blancas (*Trebbiano, Palomino, Zalema, Pedro-Ximénez,*…) y envejecidos en madera. La naturaleza del sustrato, vino blanco, ha determinado que los compuestos fenólicos analizados hayan sido los ácidos fenólicos, estilbenos y flavonoides no-antocianicos. El contacto con madera ha ocasionado el estudio de los aldehídos fenólicos.

La utilidad del perfil fenólico para diferenciar vinagres de vino comerciales según el origen y método de elaboración, se demostró con métodos estadísticos multivariantes. Las diferencias fundamentales fueron la presencia de aldehídos (vainillina, siringaldehído,…) en los vinagres tradicionales y el mayor contenido de flavanoles ((+)-catequina y (-)-epicatequina) en los elaborados por fermentación sumergida. Los porcentajes de clasificación correcta, según el método de acetificación son: del 92% mediante análisis discriminante lineal y del 99,6% en el caso de redes neuronales (García-Parrilla et al., 1997). Los porcentajes de clasificación correcta por el origen fueron menores, pero hay que considerar que las zonas de producción ensayadas son muy próximas geográficamente (Jerez, Condado de Huelva y Montilla-Moriles) y con métodos de elaboración muy similares.

También se ha descrito la composición fenólica de vinagres Balsámicos Tradicionales de Módena (Plessi et al., 2006). Las diferencias fundamentales con los vinagres de Jerez se basan en una menor concentración de ácido gálico; 18 mg/L frente a 27,1 mg/L en los de Jerez. El ácido protocatéquico presenta 18,8 mg/L en el ABTM y está ausente en vinagres de Jerez. También se puede destacar la presencia en cantidades superiores de ácido vainíllico en los ABTM; 8,1 mg/L frente a 0,03 mg/L en los de Jerez. Además, se detectaron en mayores concentraciones: el ácido siríngico y el isoferúlico en ABTM. No obstante, los vinagres de Jerez presentan: ácido cafeoiltartarico, éster glucosídico del ácido *p*-cumaroiltartárico, cafeato de etilo, cumarato de etilo, galato de etilo, protocualdehído, vainillina, siringaldehído (García-Parrilla et al., 1997), que no han sido descritos en el ABTM (Plessi et al, 2006). El mayor número de compuestos fenólicos detectados en vinagres de Jerez, se debe a que es posible realizar el análisis por inyección directa de la muestra. Sin embargo, la naturaleza sirupuosa y el elevado contenido de 5-hidroximetilfurfural y otros derivados furánicos en el ABTM, implica una extracción previa para poder analizar los compuestos minoritarios.

Estos estudios se han realizado analizando muestras comerciales, el resto de los trabajos que se exponen controlan experimentalmente los distintos factores que pueden intervenir en la composición fenólica, como son el sustrato y los métodos empleados en su elaboración (tipo de acetificación y proceso de envejecimiento). Las concentraciones iniciales de estas sustancias en la uva y vino de partida se modifican, en mayor o menor medida, por sucesivas operaciones tecnológicas implicadas en la producción.

Así, la acetificación con cultivo superficial o sumergido juega un importante papel en la evolución posterior de los fenoles. La acetificación es un proceso aerobio en el cual el oxígeno es crucial para el crecimiento de las bacterias. La reactividad de los compuestos fenólicos y el oxígeno está relacionada con el pardeamiento de los vinos blancos y por la evolución de los antocianos, en el caso de los tintos. Es de esperar que el tipo de acetificación, relacionado a su vez con la solubilidad de oxigeno en el medio, resulte determinante en la composición fenólica y que ésta pueda ser útil para discriminar si un producto ha sido elaborado mediante uno u otro método de acetificación. En efecto, la composición fenólica de vinagres comerciales de acetificación rápida es muy diferente de la analizada para los vinagres de acetificación lenta. Cabe destacar, un mayor contenido de flavanoles en los vinagres rápidos, probablemente debido a que se emplean vinos de sobre prensa para su elaboración. Por el contrario, los vinagres de acetificación lenta presentaron mayor contenido en aldehídos fenólicos, ya que normalmente se elaboran en barriles de madera que ceden este tipo de compuestos al producto (García-Parrilla et al., 1998).

La evolución de los compuestos fenólicos en la acetificación con cultivo sumergido se ha estudiado tanto en fermentador de tamaño de laboratorio como en fermentadores Frings. En el caso del fermentador de laboratorio y usando vino de Jerez como sustrato, el perfil fenólico no se modifica significativamente (Morales et al., 2001). Tampoco se observaron disminuciones de los compuestos fenólicos determinados singularmente en el caso de vinagres blancos (Andlauer et al., 2000). Sin embargo, los ciclos del acetificador Frings muestran diferencias entre el inicio y el final de ciclo de carga y descarga. La epicatequina que está en el sustrato en un intervalo de 7,4-27,3 mg/L desaparece tras la acetificación y el ácido gálico y cafeico aumentan presumiblemente por la hidrólisis de sus ésteres (García-Parrilla et al., 1998). En cuanto

a los vinagres de vino tinto es de destacar la disminución del 50% en los compuestos fenólicos fundamentalmente en los antocianos (Andlauer et al., 2000).

El proceso de envejecimiento implica las reacciones de los compuestos a lo largo del tiempo, fundamente polimerizaciones, cesión de compuestos procedentes de la madera y mermas debidas a la evaporación. Las sustancias cedidas por la madera dependerán del tipo de madera y tueste, de la superficie de contacto, y del tiempo de envejecimiento. El proceso de envejecimiento mejora las propiedades del producto, tanto que es un criterio para diferenciar las distintas clases según la normativa. Así pues, se exploró la posibilidad de usar el perfil fenólico para diferenciar entre distintos periodos de envejecimiento. Se observaron diferencias significativas en la composición fenólica entre vinagres de Jerez envejecidos menos y más de 2 años en sistema de criaderas y solera (García-Parrilla et al., 1999).

Tesfaye et al. (2002) siguieron la evolución de los compuestos fenólicos en una experiencia de envejecimiento de vinagres de Jerez en barriles de madera de roble. Constataron que existían diferencias significativas a los 90 días de envejecimiento para los compuestos: vainillina, siringaldehído, coniferaldehído y acido cinámico. A los 180 días, además variaba el ácido vainíllico. Con estos valores se construyó una función, que permitía clasificar correctamente el 97% de las muestras, según su envejecimiento en madera.

El envejecimiento acelerado de vinagres de vino, mediante chips de madera de roble, resulta ser una buena práctica para disminuir el tiempo de envejecimiento. Tesfaye et al. (2004) observaron que a los 15 días de envejecimiento se producían los mayores cambios en la composición fenólica: aumento de la concentración de ácido gálico y sobre todo de los aldehídos aromáticos (siringaldehído, sinapaldehído, coniferaldehído y vainillina).

En resumen, los compuestos fenólicos han servido para diferenciar vinagres de diferente DOP. Se conoce, que la acetificación con cultivo sumergido disminuye la concentración de antocianos pero los compuestos fenólicos simples no se alteran.

2.3. NUEVOS CONDIMENTOS DE FRUTAS

Tal como se recoge en la legislación (Presidencia del Gobierno, 1993) anteriormente mencionada, los vinagres también se pueden elaborar a partir de sustratos de frutas distintos de la uva, a partir del cual se obtienen los sustratos azucarados necesarios para llevar a cabo la doble fermentación alcohólica y acética.

La producción de frutas y otros productos vegetales presenta excedentes con una gran facilidad debido a diversos factores como la estacionalidad, sobreproducción agrícola, modernos sistemas de explotación, existencia de fruta que no presenta salida al mercado por no tener la forma o tamaño adecuado, etc. Esta fruta, se conoce como fruta de segunda calidad y puede suponer hasta un 30% del total de la producción. A pesar de que la tecnología de alimentos ha desarrollado sistemas efectivos de conservación y de producción de nuevos alimentos derivados de dichos productos de muy buena aceptación para el consumidor (mermeladas, frutas desecadas, etc), aún esos sistemas se muestran insuficientes para evitar los excedentes de algunos productos agrícolas. Otra solución es la generación de nuevos alimentos que puedan ser de mayor durabilidad y que conserven al máximo sus características para ser consumidas. Entre los sistemas de conservación de alimentos se pueden destacar las transformaciones fermentativas que permiten el mantenimiento de los productos en alcohol o ácido acético. Para ello se propone nuevas estrategias en el desarrollo de condimentos alimentarios a partir de excedentes de frutas por medio de la acetificación.

Los vinagres y los condimentos de frutas se utilizan en cocina desde hace mucho tiempo tanto por su sabor ácido como por ser un buen conservante alimentario. Hasta el presente, la mayoría de los vinagres denominados de frutas suelen ser productos aromatizados y algunos fermentados y posteriormente destilados, lo que hace que, aunque puedan reconocerse sabores y aromas de las frutas de origen, no presenten diversos componentes, muchos de ellos reconocidos como antioxidantes, en el sentido de que puedan tener acciones favorables para la salud a las concentraciones en las que se encuentran en lo alimentos. Si bien en la bibliografía existen numerosos trabajos sobre la producción de vinos obtenidos a partir de diversas frutas como el kiwi, mango o fresa (Soufleros et al., 2001; Kafkas et al., 2006; Reddy & Reddy, 2005), es muy

escasa en lo referente a vinagres de frutas y circunscrita a países asiáticos, donde estos productos son bien aceptados y consumidos.

La propuesta de utilización de frutas de segunda calidad para la elaboración de condimentos por doble fermentación permite la aparición de nuevos productos que mantengan las cualidades nutricionales de las frutas de partida. De esta manera se desarrolla un sistema para aprovechar una materia prima perecedera, transformándola mediante un proceso biotecnológico en un nuevo producto, característico, diferenciado, de larga duración y que tenga aspectos favorables sobre la salud del consumidor, y a su vez posea nuevas y adecuadas propiedades sensoriales.

Así pues para establecer parámetros de control de calidad de un producto, más aún cuando este es nuevo, es necesaria la caracterización del mismo, además de la de su sustrato de partida. Como ya ha ocurrido antes en el caso de la uva para el vinagre de vino, los compuestos fenólicos jugarán un papel importante en la autentificación y calidad tanto del sustrato como del nuevo producto.

2.4. BIBLIOGRAFÍA

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3. JUSTIFICACIÓN Y OBJETIVOS / SIGNIFICANCE & PURPOSES

3.1. JUSTIFICACIÓN

Los vinagres de calidad han sido elaborados tradicionalmente a partir de vino blanco, muestra de ello son los vinagres de Jerez y Balsámico de Módena. Actualmente, los productos alimenticios se aprecian por su calidad no sólo higiénica y nutritiva sino también por su valor sensorial y comodidad de uso. La alta gastronomía y la restauración afrontan el reto continuo de ofrecer nuevas propuestas organolépticas. Además de los vinagres artesanales ya conocidos, se pretende diseñar otros de características singulares, ya que su elaboración está siendo rentable económicamente.

En los últimos años, hemos asistido a una ampliación de las Denominaciones de Origen Protegidas de vinagres que en su totalidad proceden de vinos blancos. Aunque en el mercado existan vinagres de vino tinto, aún quedan por desarrollar productos de vino tinto mediante métodos artesanales de cultivo superficial y envejecimiento en madera. Es previsible que se desarrollen Denominaciones de Origen de vinagres tintos en un futuro cercano y entonces se requieran parámetros que sirvan para su caracterización. Además de diferentes alternativas de sustrato vínico, se están introduciendo condimentos elaborados con frutas que ofrecen nuevas propuestas para el consumidor. Estas dos estrategias de innovación merecen ser evaluadas.

Tradicionalmente, los vinagres se han elaborado en barriles de madera de roble porque eran los que se utilizaban comúnmente para la producción de vino. Este proceso artesanal está vinculado a la acetificación con cultivo superficial que conlleva un proceso lento en el que el vinagre se acetifica y envejece simultáneamente. Si bien en la elaboración de vinos, la madera de roble es la más idónea por su limitada transferencia de oxígeno, en la elaboración de vinagres se persigue el objetivo contrario: una mayor transferencia de oxígeno a través de la madera para favorecer el crecimiento de las bacterias acéticas. Así pues, surge la oportunidad de estudiar nuevas maderas como: castaño, cerezo y acacia, con mayor porosidad y que den notas sensoriales diferentes a las del roble, a las que el consumidor está más acostumbrado. No hay que olvidar que los barriles de madera no son elementos estancos sino que influyen de manera notable en la calidad final del producto.

El trabajo de esta Tesis está vinculado al proyecto Europeo WINEGAR (Ref. COOP-CT-2005-017269) que pretende comprobar la idoneidad de estas maderas para la elaboración de vinagre, atendiendo a la transferencia de oxígeno y al impacto en las propiedades químicas y sensoriales del producto.

3.2. OBJETIVOS

El propósito de la presente Tesis Doctoral es evaluar la repercusión de nuevas estrategias de elaboración de vinagres en su calidad, determinada en función de su composición química y sus propiedades sensoriales. Se pretende pues, caracterizar la composición fenólica de vinagres de vino tinto y ensayar el posible uso de otros sustratos de frutas como la fresa.

Ya que los vinagres se producen mediante dos tipos de acetificación es preciso valorar la idoneidad del tipo de acetificación para el sustrato de vino tinto, estableciendo las posibles influencias del aporte de oxígeno, particularmente sobre los compuestos antociánicos.

Conviene estimar el empleo de otras maderas como alternativa para el proceso de elaboración, y su repercusión sobre la composición química y sensorial.

Para abordar estos propósitos se plantean los siguientes hitos y tareas:

1. Determinar la composición fenólica en vinagres de vino tinto elaborados tanto con cultivo superficial como con cultivo sumergido.

    A. El diseño experimental de este objetivo consiste en acetificar dos vinos tintos en barricas de acacia, castaño, roble y cerezo, siguiendo la evolución a lo largo del tiempo de los compuestos fenólicos simples no antociánicos por Cromatografía Líquida con detector de haz de diodos (CL-DAD), el Índice de Polifenoles Totales (IPT) y los Antocianos Totales (AT). El tratamiento de los resultados requiere del análisis

estadístico multivariante (este apartado se encuentra descrito en el Capítulo 1).

B. Para estudiar la influencia de la fermentación con cultivo sumergido se plantea la acetificación de un vino tinto en un fermentador de laboratorio con condiciones controladas, y la determinación de los compuestos fenólicos por CL-DAD y Cromatografía Líquida-Espectrometría de Masas (CL-EM), antes y después de la acetificación (Capítulo 2).

C. Describir compuestos antociánicos en vinagre, y consecuente aislamiento de la fracción antociánica por Cromatografía a Contra-Corriente (CCC). Evaluación de su actividad antioxidante, como contribución a las propiedades saludables del producto (Capítulo 2).

2. Verificar la influencia de las maderas de acacia, castaño, roble y cerezo en la composición fenólica de vinagres de calidad durante el proceso de envejecimiento en barricas.

D. Se estudian los compuestos fenólicos no antociánicos, mediante CL-DAD, de dos sustratos de vino tinto y uno balsámico, acetificados con cultivo superficial, durante un periodo de envejecimiento de 12 y 18 meses (Capítulo 3).

3. Proponer marcadores químicos de cada madera.

E. Es preciso identificar los compuestos en primera instancia por CL-DAD-EM. En caso de no ser suficiente se requiere el aislamiento del compuesto mayoritario mediante CL-Semi-Preparativa y posterior determinación de su estructura por Resonancia Magnética Nuclear (RMN). Además, hay que evaluar el impacto de los distintos tratamientos de la madera en su concentración en los vinagres (Capítulo 4).

4. Análisis sensorial de los vinagres de vino.

F. Desarrollar un protocolo de análisis sensorial para la cata de vinagre que incluya nuevos descriptores (Capitulo 5).

G. Evaluar la repercusión de las distintas maderas y periodos de envejecimiento en la características sensoriales de forma que puedan establecerse diferencias significativas entre ellos (Capitulo 1 y 3).

5. Proponer nuevos sustratos, como las fresas, para la innovación de nuevos productos, y la caracterización de su composición fenólica (Capítulo 6).

Esta Tesis Doctoral está estructurada como una recopilación de seis artículos científicos. El trabajo experimental se ha realizado en el Área de Nutrición y Bromatología de la Facultad de Farmacia de la Universidad de Sevilla, y en el Departamento de Química de los Alimentos de la Facultad de Ciencias de la Vida de la Universidad Técnica de Braunschweig. Siguiendo la normativa de la Universidad de Sevilla, el resumen y conclusiones se redactará también en ingles para poder optar a la mención de "Doctorado Europeo".

3.1. SIGNIFICANCE

Traditionally, white wine is the substrate for high quality vinegar as Sherry and Traditional Balsamic Vinegar. Nowadays, food products are appreciated by their safety and nutritional value, by their sensory properties and also for their ready to use commodities. High quality gastronomy faces the continuous challenge of offering products with new organoleptic properties. Hence, food industry searches products with particular sensory characteristics and obviously a profitable elaboration from an economic point of view.

Over last years, new Protection of Designations of Origin (PDO) for vinegars have been recognized, being devoted to white wine. Although we can found red wine vinegar in the market, products obtained by traditional surface culture and ageing in wood, are still lacking. New products development requires chemical characterization and sensory evaluation as quality criteria. Besides, condiments from fruits are being introduced, suggesting new alternatives to the largely wine vinegar consumed. Hence, those strategies of innovation deserve to be evaluated.

Traditionally, wine vinegars have been elaborated in oak wood barrels commonly used in wine production. This handcrafted method involves a slow process in which acetification and ageing occur simultaneously. Oak is the most suitable wood in wine production because it limits oxygen transfer to avoid excessive wine oxidation. However, vinegar elaboration requires a porous wood to promote the growth of the acetic acid bacteria. Hence, the study of new woods as: chestnut, cherry and acacia, to increase aeration and lead different flavours, could be a good strategy. It is necessary to take into account that wood is not just a passive element in the production process but also release some components influencing in the final quality of the product.

This Thesis is within the WINEGAR project scope (Ref. COOP-CT-2005-017269). One of the aims is to test the suitability of different woods to elaborate vinegar encompassing oxygen transfer and the impact on quality.

3.2. PURPOSES

This Thesis aims to evaluate the impact of new strategies of vinegar elaboration in their quality, determined by their chemical composition and sensory properties. This involves the characterization of phenolic composition of red wine vinegar and the assessment of new fruit substrates as strawberry.

Vinegars are produced by two acetification methods; surface and submerged culture. Hence, it is required to test the influence of the process on phenolic composition, particularly, oxygen effect on anthocyanin compounds.

It is necessary to evaluate the use of other woods as alternative to the elaboration process and its effect on chemical composition and sensory properties.

These goals are detailed in these milestones and working plan:

1. Determination of the phenolic composition of red wine vinegar elaborated by surface and submerged culture.

    A. The experimental design includes the red wine acetification in barrels made of chestnut, cherry, acacia and oak wood. The process monitorization consists in the determination of non-anthocyanin phenol compounds by Liquid Chromatography couple to diode detector (LC-DAD), Total Polyphenols (TP) and Total Anthocyanins (TA). Results require statistical multivariate analysis (described in Chapter 1).
    B. Submerged acetification study requires the laboratory scale acetic acid fermentation under controlled conditions and determination of phenolic compounds by LC-DAD and Liquid Chromatography-Mass Spectrometry (LC-MS) (Chapter 2).
    C. Identification of the anthocyanin composition. Isolation of anthocyanin fraction by Counter-Current Chromatography (CCC) and assessment of its antioxidant activity, as contribution of healthy properties of the product (Chapter 2).

2. To test the influence of chestnut, cherry, acacia and oak wood in phenol composition of quality vinegars throughout the ageing process.

   D. Determination of the non-anthocyanin phenol compounds of two red wine vinegars and one balsamic vinegar (both acetified by surface culture) throughout 12 and 18 months of ageing process by LC-DAD (Chapter 3).

3. Proposal of chemical markers of each wood.

   E. Firstly, identification of compounds is attempted by LC-DAD-MS. If it is necessary, isolation of the major compound of wood by semipreparative LC and structure determination by Nuclear Magnetic Resonance (NMR). Evaluation of different wood treatments in its concentration in vinegar (Chapter 4).

4. Sensory analysis of wine vinegars.

   F. Development of a procedure for sensory analysis of vinegar including new descriptors (Chapter 5).
   G. Assessment the influence of different woods and ageing time in sensory characteristic to establish significant differences between them (Chapter 1 and 3).

5. Proposal of new substrates, as strawberries, to develop new products, and describe its phenolic composition (Chapter 6).

This Thesis is presented as a compilation of six scientific publications. The experimental work has been performed in the Area of Nutrition and Food Science of Faculty of Pharmacy of the University of Sevilla, and in the Department of Food Chemistry of Faculty of Science Life of the Technical University of Braunschweig. In order to fulfil University of Sevilla requirements, this Thesis includes an English summary and conclusions to be consider as a "European Doctorate" mention.

4. RESULTADOS Y DISCUSIÓN / RESULTS & DISCUSSION

CAPÍTULO 1 / CHAPTER 1

Ana B. Cerezo; Wendu Tesfaye; M. Jesús Torija; Estíbaliz Mateo; M. Carmen García-Parrilla & Ana M. Troncoso.

THE PHENOLIC COMPOSITION OF RED WINE VINEGAR PRODUCED IN BARRELS MADE FROM DIFFERENT WOODS

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The phenolic composition of red wine vinegar produced in barrels made from different woods

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Abstract

The presence of phenolic compounds has been extensively studied in Sherry and Balsamic vinegars due to their impact on quality but little work has been done on red wine vinegars. Phenolic compounds were monitored during the acetification of red wine vinegars produced by surface culture in different wood barrels (oak, chestnut, acacia and cherry). A total of 166 samples were analysed for phenolic compounds using LC-DAD, the total phenol index (TPI) and the total monomeric anthocyanins (TA). Twelve phenolic compounds were identified corresponding to phenolic acids, flavanols and stilbens. Most phenolic acids did not significantly change their concentrations in the different acetifications. (+)-Catechin and resveratrol glycoside underwent significant decreases during acetification while gallic acid and gallic ethyl ester increased substantially for those vinegars produced in chestnut wood. The concentrations of phenolic compounds were used to build the functions for discriminant analysis. Samples belonging to two wine substrates (groups F and T) were correctly classified with 98.6% (group F) and 100% (group T) for the four types of wood barrels. During acetification a decrease (~50%) in the content of total monomeric anthocyanins was observed. According to the results of triangle difference test the panel was able to distinguish most of the vinegars according to the different woods they were made in. The results of descriptive sensory analysis show that oak and cherry gave the maximum scores for most of the descriptors.

Keywords: Red wine vinegar; Phenolic compounds; Wood barrels; Surface acetification; Sensory analysis

1. Introduction

Wine vinegar is largely produced in Mediterranean countries using different methods which give rise to products of greatly differing quality (Tesfaye, García-Parrilla, & Troncoso, 2002a). There are two methods of production: traditional slow methods in which the acetic acid bacteria is placed on the surface of the acetifying liquid and quick methods where acetic acid bacteria is submerged into the substrate in such a manner that oxygen demand is guaranteed. Traditional methods of production usually include the use of wood barrels and the vinegars obtained are highly valued due to their outstanding sensory properties (González-Viñas, Salvador, & Cabezudo, 1996).

Nowadays, the presence of diverse types of wine vinegars in the market and consumer demand for quality condiments stimulates the characterization and establishment of parameters for quality control. Phenolic compounds have been shown to be good markers of the quality and origin of vinegars. Thus, phenolic compounds of low molecular weight were useful to differentiate both aged vinegars from those which were not aged, and Sherry vinegars from other white wine vinegars (Gálvez, Barroso, & Pérez-Bustamante, 1995). García-Parrilla, González, Heredia, and Troncoso (1997) proved that phenolic compounds are useful for classifying and predicting the membership of samples according

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to the method applied in their elaboration or according to the geographical origin of the substrate wine. Using phenolic composition, the authors were also able to differentiate between Sherry vinegars according to their ageing period in wood (less than two years, more than two years) (García-Parrilla, Heredia, & Troncoso, 1999).

Sherry vinegars aged for two years in small American oak casks resulted in a considerable number of phenolic compounds, mainly aromatic aldehydes, being extracted (Tesfaye, Morales, García-Parrilla, & Troncoso, 2002b).

Accelerated ageing of wine vinegars using oak chips seems to be a good way of decreasing ageing time (Tesfaye, Morales, Benítez, García-Parrilla, & Troncoso, 2004). The concentration of gallic acid and above all aromatic aldehydes (siringaldehyde, coniferaldehyde, sinapaldehyde and vanillin) increased after 15 days.

In general, we can say that the phenolic composition of Sherry and white wine vinegars is well known (Alonso, Castro, Rodríguez, Guillen, & Barroso, 2004; García-Parrilla, León Camacho, Heredia, & Troncoso, 1994; García-Parrilla, Heredia, & Troncoso, 1996; García-Parrilla et al., 1997; García-Parrilla, Heredia, & Troncoso, 1998; García-Parrilla et al., 1999; Morales, Tesfaye, García-Parrilla, Casas, & Troncoso, 2001; Natera, Castro, García-Moreno, Hernández, & García-Barroso, 2003; Tesfaye et al., 2002b, 2004), but little is known about the phenolic composition of red wine vinegars (Andlauer, Stumpf, & Fürst, 2000; Natera et al., 2003).

The aim of this work is to study the different phenolic composition of red wine vinegars when produced by surface acetification in barrels made from four different types of wood: chestnut, acacia, cherry and oak. This is a novel approach since surface acetification is generally done in oak wood barrels. Oak is chosen for the majority of wooden barrels used in wine making because of its limited oxygen transfer and because some compounds (mainly phenols) are extracted into the wine. As for acetification, higher oxygen transfer is needed, therefore, in the present study we use more porous woods in order to test their suitability for wine vinegar production. We also analyze the sensory quality of the vinegars produced.

2. Materials and methods

2.1. Samples

We acetified two red wines (Grenache variety) in two different wineries (group F and T) using a surface culture system. Their characteristics are shown in Table 1. The barrels were constructed specifically for this study. We used a total of 48 barrels, 6 from each different type of wood; oak, chestnut, acacia and cherry. Samples (group F and T) were taken at different points of the acetification process; O (starting point; 0.8° acetic, $n = 24$), I (initial point; 2° acetic, $n = 48$), H (middle point; 4° acetic, $n = 48$) and *E* (finished vinegar; 6° acetic, $n = 46$). A total of 166 samples were analysed.

Table 1
Characteristics of wine substrates

Group	Wine substrate
F	Alcohol (%v/v): 14.5 Acidity: 0.9 g/100 mL Glucose + fructose: 20.9 g/L + 43.4 g/L pH 3.4 Variety: 100% Grenache Acetification length: 45 days
T	Alcohol (%v/v): 13.6 Acidity: 0.9 g/100 mL Glucose + fructose: 0.36 g/L + 0.78 g/L pH 3.4 Variety: Grenache mostly Acetification length: 150 days

Four digital sample codes were used following this order: the first digit corresponds to group (F or T); the second digit corresponds to the point of acetification process (O, I, H, E); the third digit corresponds to the type of wood (A: acacia; C: cherry; S: chestnut; R: oak); and the fourth digit corresponds to the replica number of the barrel (1–6).

2.2. HPLC analysis of phenolic compounds

HPLC analysis of phenols was performed using an Agilent Serie 1100 system equipped with a quaternary pump (Serie 1100 G1311A), automatic injector (Serie 1100 G1313A) and degasser on line (Serie 1100 G1379A). Detection was done using a UV/Vis (Serie 1100 G1315B) coupled to a Chemstation HP A.10.02 (HP/Agilent). The column was a Reverse Phase Zorbax SB C18 particle size 3.5 μm (30 mm × 4.6 mm) protected by a Zorbax SB C18 guard cartridge and kept at 30 °C. Duplicate samples were filtered through a Millex-LCR 13 mm filter before injection. The sample volume injected was 20 μL (Ibern-Gómez, Andrés-Lacueva, Lamuela-Raventós, & Waterhouse, 2002). The flow rate was 4 mL/min couple with a UP microsplitter valve that limited the flow into the detector to 1 mL/min. The following solvents were used: solvent A, water with 0.2% trifluoroacetic acid (TFA); solvent B, acetonitrile with 0.2%TFA. Gradient elution profile was as follows: linear gradients from 0 min to 0.5 min (100% A); 0.5 min to 2 min (98% A), 2 min to 8 min (92% A), 8 min to 15 min (85% A), 15 min to 18 min (77% A). Identification was based both on retention time and on UV–Visible spectra matching of the corresponding standards. Quantification was performed by external calibration at 280 and 320 nm.

The standards of 28 phenolic compounds were purchased from Fluka, Sigma, Merck and Chromadex.

2.3. Other parameters

The total phenols index (TPI) was determined by the Folin–Ciocalteu micro-method proposed by Waterhouse (2001). Results were expressed as gallic acid equivalent (GAE).

The total monomeric anthocyanins (TA) were estimated by a pH differential method (Giusti & Wrolstad, 2001). Absorbance (A) was measured at 520 nm and at 700 nm in buffers at pH 1.0 and 4.5 using the following equation: $A = [(A_{\lambda vis\text{-}max} - A_{700})_{pH\,1.0} - (A_{\lambda vis\text{-}max} - A_{700})_{pH\,4.5}]$, with a molar extinction coefficient of malvidin-3-glucoside (M3G) of 20,200 L cm^{-1} mol^{-1}. Results were expressed as mg M3G/L.

2.4. Sensory analysis

An expert sensory panel composed of eight members previously trained in the sensory analysis of vinegar (Tesfaye et al., 2002a) made triangle difference and descriptive analyses according to international protocols (ISO 4120, 1983 and ISO 6658, 1985, respectively). Vinegar samples were tasted by direct olfaction as described in a previous paper (Tesfaye et al., 2002a).

Following methodology for descriptive analysis (Stonem & Sidel, 2004), ten attributes (ethyl acetate, pungent sensation, wine character, woody flavour, red fruit, sweet aroma, bitter almond, vanilla, raisin qualities and general impression) were formally selected by consensus to describe the wine vinegar samples.

2.5. Statistical analysis

Statistical analyses were performed by means of Statistica software (Statsoft, 2001). One-way analysis of variance (ANOVA) was used to test significant differences. Multivariate analysis of data included cluster analysis and standard discriminant analysis.

3. Results and discussion

3.1. Total polyphenol index

Generally the TPI did not show significant changes ($p < 0.05$) during the different acetifications, as seen in Table 2. Two exceptions to this tendency were seen. The first was a slight decrease of no more than 13% (group F: acacia and cherry; and group T: cherry). The second was a slight increase of no more than 20% in the group T samples acetified in chestnut. In fact, submerged culture acetification of red wine vinegars resulted in a 13% reduction in polyphenols (Andlauer et al., 2000).

Data reported in previous literature referred either to traditional surface culture or submerged culture. In general, traditional methods take longer with simultaneous extraction from wood also taking place. Evaporation and concentration is also considerable (Tesfaye et al., 2002b). Our results reveal that in our study these phenomena are unlikely to occur since acetification length is only 45 and 150 days for both groups (F and T, respectively).

ANOVA analysis was also done to explore differences between finished vinegars. In most cases there were significant differences according to the type of wood used (Table 3).

3.2. Phenolic compounds

In our samples we identified twelve phenolic compounds derived from benzoic and cinnamic acids, together with the tartaric acid esters of these acids, and flavanols, stilbens and metabolic products of yeast, such as tyrosol. Caftaric

Table 2
Means and standard deviations (SD) for total phenol index (TPI) and total anthocyanins (TA) (in mg/L)

		Group F				Group T			
		TPI		TA		TPI		TA	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
A	O	1923.6^{a,c}	42.5^{a}	1.30^{a,c}	0.00^{a}	1393.3^{a}	130.8^{a}	0.42^{a,c}	0.00^{a}
	I	1853.9^{b}	38.6^{b}	1.05^{b}	0.08^{b}	1426.67^{b}	75.18^{b}	0.34^{b}	0.04^{b}
	H	1787.3^{b}	32.4^{b}	0.73^{b}	0.08^{b}	1447.9^{b}	111.3^{b}	0.33^{b}	0.02^{b}
	E	1817.58^{b,d}	47.14^{b}	0.71^{b,d}	0.10^{b}	1480.00^{b}	68.21^{b}	0.22^{b,d}	0.04^{b}
C	O	1781.2^{a,c}	20.4^{a}	1.23^{a,c}	0.00^{a}	1393.33^{a,c}	39.03^{a}	0.42^{a,c}	0.00^{a}
	I	1743.8^{b}	93.4^{b}	0.86^{b}	0.13^{b}	1357.0^{b}	30.0^{b}	0.35^{b}	0.02^{b}
	H	1744.85^{b}	64.11^{b}	0.69^{b}	0.07^{b}	1366.1^{b}	98.4^{b}	0.33^{b}	0.03^{b}
	E	1587.3^{b,d}	72.9^{b}	0.39^{b,d}	0.09^{b}	1006.19^{b,d}	20.20^{b}	0.16^{b,d}	0.02^{b}
S	O	1911.5^{a}	74.9^{a}	1.23^{a,c}	0.00^{a}	1338.8^{a,c}	31.7^{a}	0.49^{a,c}	0.00^{a}
	I	1817.6^{b}	55.7^{b}	0.90^{b}	0.08^{b}	1535.8^{b}	32.4^{b}	0.38^{b}	0.03^{b}
	H	1849.4^{b}	24.7^{b}	0.70^{b}	0.09^{b}	1596.4^{b}	51.4^{b}	0.35^{b}	0.05^{b}
	E	1882.7^{b}	70.7^{b}	0.49^{b,d}	0.06^{b}	1618.1^{b,d}	58.9^{b}	0.24^{b,d}	0.04^{b}
R	O	1847.9^{a}	32.4^{a}	1.21^{a,c}	0.06^{a}	1387.3^{a}	45.7^{a}	0.42^{a,c}	0.00^{a}
	I	1796.4^{b}	75.7^{b}	0.97^{b}	0.17^{b}	1417.58^{b}	47.14^{b}	0.36^{b}	0.03^{b}
	H	1775.2^{b}	60.5^{b}	0.80^{b}	0.21^{b}	1417.6^{b}	45.4^{b}	0.37^{b}	0.03^{b}
	E	1714.6^{b}	38.6^{b}	0.63^{b,d}	0.03^{b}	1411.0^{b}	36.7^{b}	0.23^{b,d}	0.04^{b}

[a] Means values for two barrels.
[b] Means values for six barrels.
[c,d] Starting and final concentration within acetification process with different letter as superscript are significantly different ($p < 0.05$).

Table 3
ANOVA

	P-level					
	A–C	A–S	A–R	C–S	C–R	S–R
Group F	0.0041	0.0729	0.1075	0.0069	0.2058	0.0298
Group T	0.0112	0.0231	0.0875	0.0045	0.0176	0.0026

A = acacia; C = cherry; S = chestnut; R = oak.
Significant differences (p-value < 0.05) in the TPI of the finished vinegars depending on type of wood barrel.

acid was a major phenolic, followed by gallic acid and tyrosol, which agreed with previously reported data for Sherry vinegars obtained by submerged culture (Morales et al., 2001). Moreover, resveratrol glucoside was only identified in samples from group F. Tables 4 and 5 show the phenolic composition of the finished vinegars.

We searched for natural groupings among the samples using unsupervised pattern recognition methods. Thus, the data matrix was subjected to a hierarchical agglomerative cluster analysis of cases. Taking the euclidean distance as metric and the Ward's method as amalgamation rule (Ward, 1963), we obtained the dendrogram. A simple inspection allows some observations to be easily made (Figs. 1 and 2):

- The first subcluster grouped almost all samples from chestnut barrels in both groups.
- A second subcluster grouped 83% of the samples from starting point (O) and 33% of initial point (I) in group F. This means that the samples are very similar between different woods during the first stages of the acetification process when using the same starting substrate.
- A second subcluster appeared for group T, which grouped 72% of the samples obtained from oak barrels.
- Finally, a third subcluster grouped almost all the finished vinegars for both groups F and T.

Discriminant analysis was done to check the validity of phenolic compounds in order to classify samples according to the kind of wood used in their elaboration. Samples of starting point (O) were excluded because they had not been in contact with the wood. The classification function was created using the standard method plus eight variables (gallic acid, protocatechuic acid, tyrosol, caftaric acid, vanillic acid, (+)-catechin, syringic acid and gallic ethyl ester) which showed significant differences ($p < 0.05$) when pairs of woods were compared. With this function the samples were correctly classified at 98.6% (group F) and 100% (group T). The functions' roots in the discriminant space

Table 4
Means and standard deviations of the concentration (mg/L) of phenolic compounds in the different finished vinegars

Compounds	Acacia		Cherry	
	Group F	Group T	Group F	Group T
Gallic acid	28.51 ± 0.20	32.65 ± 0.06	28.59 ± 0.15	29.52 ± 0.04
Protocatechuic acid	6.7 ± 0.4	11.29 ± 0.08	5.10 ± 0.25	5.80 ± 0.02
Tyrosol	16.1 ± 0.7	19.7 ± 0.3	16.8 ± 0.5	20.08 ± 0.17
Caftaric acid	263.1 ± 3.3	156.8 ± 0.5	264.52 ± 5.03	165.15 ± 0.23
Vanillic acid	1.08 ± 0.08	1.31 ± 0.02	1.22 ± 0.05	1.78 ± 0.01
(+)-Catechin	–	–	2.35 ± 0.00	–
Caffeic acid	–	5.68 ± 0.00	5.40 ± 0.15	6.00 ± 0.01
Syringic acid	3.0 ± 0.3	2.66 ± 0.04	4.49 ± 0.19	4.31 ± 0.01
Gallic ethyl ester	12.98 ± 0.06	–	7.8 ± 0.5	–
(−)-Epicatechin	–	–	–	–
Resveratrol glucoside	3.44 ± 0.13	–	3.31 ± 0.22	–
Ellagic acid	2.8 ± 0.3	5.3 ± 0.6	1.54 ± 0.10	4.08 ± 0.03

Table 5
Means and standard deviations of the concentration (mg/L) of phenolic compounds in the different finished vinegars

Compounds	Chestnut		Oak	
	Group F	Group T	Group F	Group T
Gallic acid	77.9 ± 0.9	162.72 ± 0.07	30.25 ± 0.10	33.97 ± 0.06
Protocatechuic acid	6.5 ± 0.8	7.91 ± 0.07	5.78 ± 0.13	5.79 ± 0.01
Tyrosol	16.5 ± 0.3	14.18 ± 0.17	16.10 ± 0.07	13.30 ± 0.16
Caftaric acid	268.8 ± 0.6	186.17 ± 0.11	273.53 ± 2.22	176.61 ± 0.24
Vanillic acid	1.26 ± 0.04	1.55 ± 0.04	1.36 ± 0.05	1.67 ± 0.05
(+)-Catechin	2.80 ± 0.23	1.72 ± 0.02	7.61 ± 0.12	2.03 ± 0.07
Caffeic acid	5.79 ± 0.09	5.76 ± 0.03	6.14 ± 0.06	5.67 ± 0.02
Syringic acid	4.4 ± 0.6	4.14 ± 0.01	4.95 ± 0.10	4.21 ± 0.01
Gallic ethyl ester	10.74 ± 1.07	17.87 ± 0.11	9.3 ± 0.5	–
(−)-Epicatechin	–	3.53 ± 0.07	–	–
Resveratrol glucoside	3.39 ± 0.13	–	3.73 ± 0.06	–
Ellagic acid	3.86 ± 0.20	3.7 ± 0.6	4.44 ± 0.11	4.13 ± 0.00

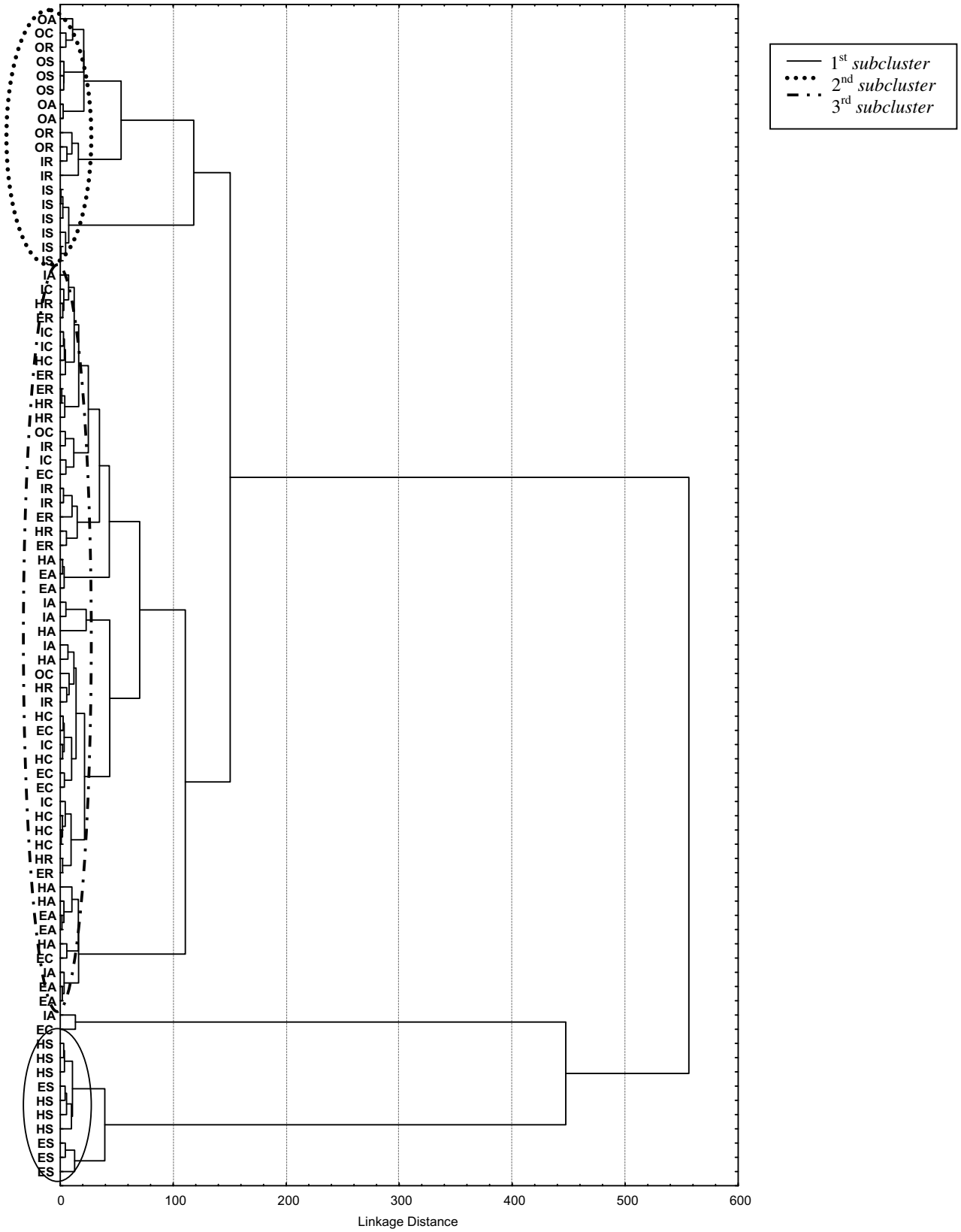

Fig. 1. Dendrogram obtained (Cluster analysis, Ward's method) with phenolic compounds as variables for samples from winery F ($n = 83$).

are shown in Figs. 3 and 4. As can be seen, samples are grouped according to the kind of wood.

Changes in phenolic compounds during eight acetifications seem to be discrete when analysed using LC-DAD

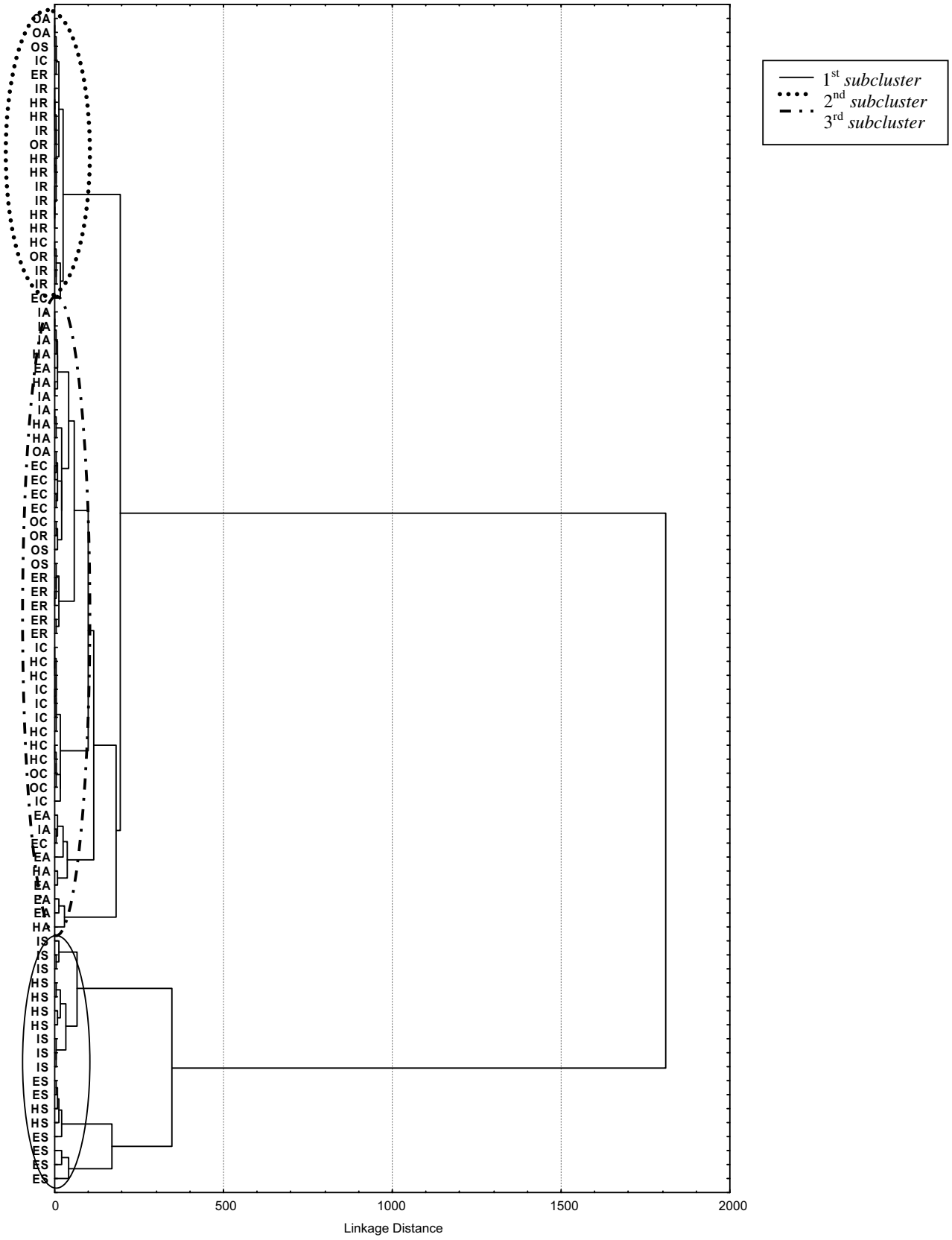

Fig. 2. Dendrogram obtained (Cluster analysis, Ward's method) with phenolic compounds as variables for samples from winery T ($n = 85$).

(data not shown), as reported above for the TPI. Gallic acid increased significantly in chestnut barrels in both groups F (30–78 mg/L) and T (31–163 mg/L), as can be seen in Fig. 5 In the other barrels it remained invariable

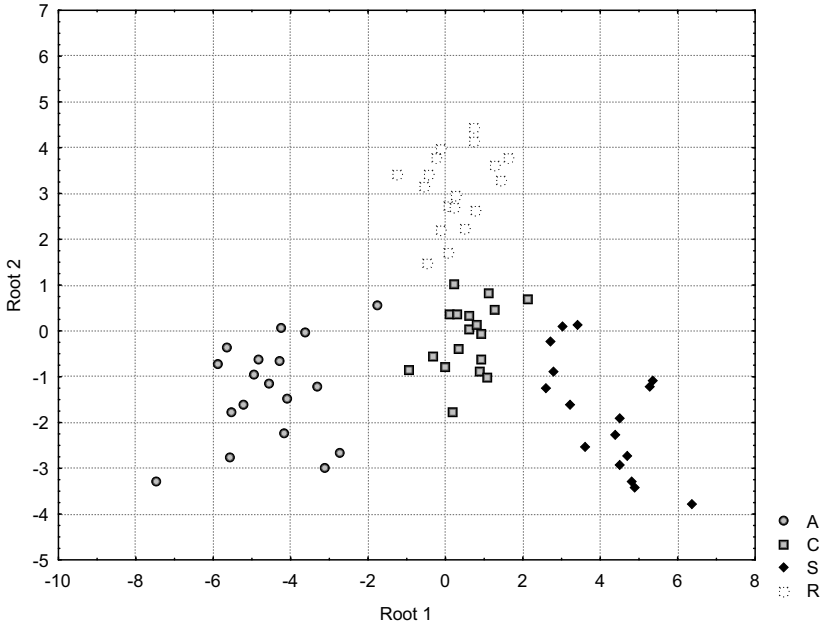

Fig. 3. Plot of the two first roots issued from discriminant analysis for group F of samples.

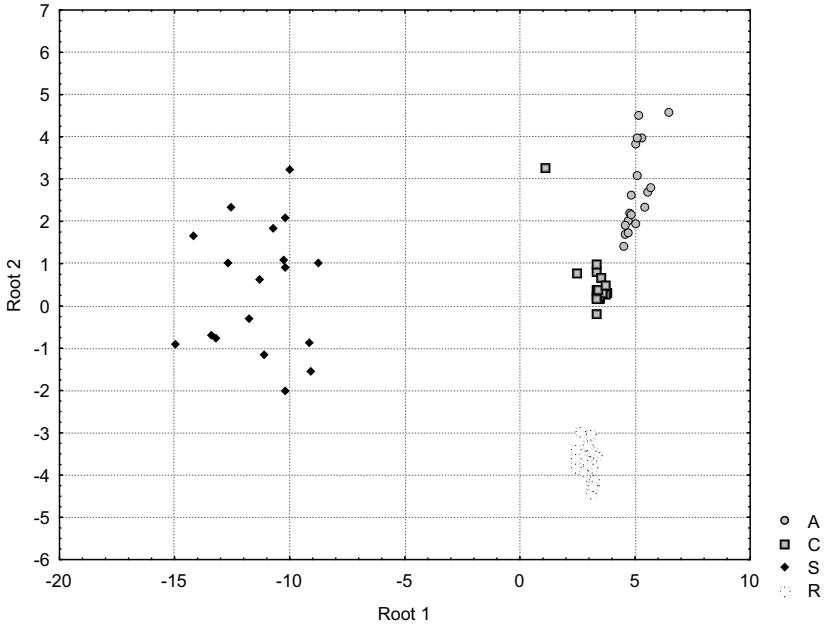

Fig. 4. Plot of the two first roots issued from discriminant analysis for group T of samples.

during acetification (29–30 mg/L). This result agrees with that obtained by other authors (Salagoity-Auguste, Tricard, Marsal, & Sudraud, 1986) who observed a greater ratio of gallic acid extraction from commercial chestnut than from oak. Chestnut releases a higher concentration of gallic acid and, as a consequence, the formation of gallic ethyl ester is more likely in chestnut barrels (Fig. 6).

Most phenolic acids did not significantly change their concentrations in the different acetifications. This is in agreement with previous studies on submerged culture acetifications of Sherry wine (Morales et al., 2001). However, a significant decrease of (+)-catechin concentration (group F and T) and resveratrol glucoside (group F) was observed during the acetification process in each wood we studied. This could be due either to polimerization, precipitation or oxidation phenomena (Escribano-Bailón, Dangles, & Brouillard, 1996; Saucier, Bourgeois, Vitry, Roux, & Glories, 1997).

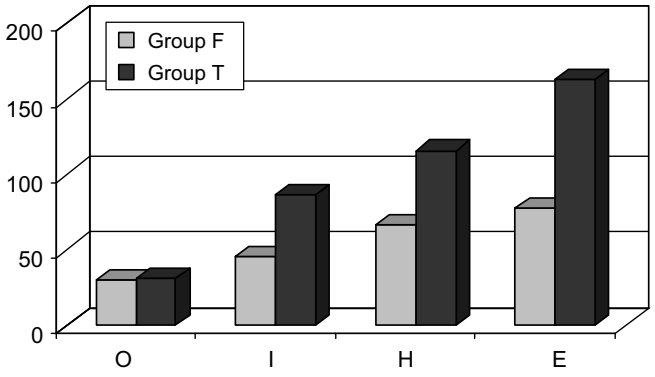

Fig. 5. Evolution of gallic acid concentration (mg/L) during the acetification in chestnut barrels.

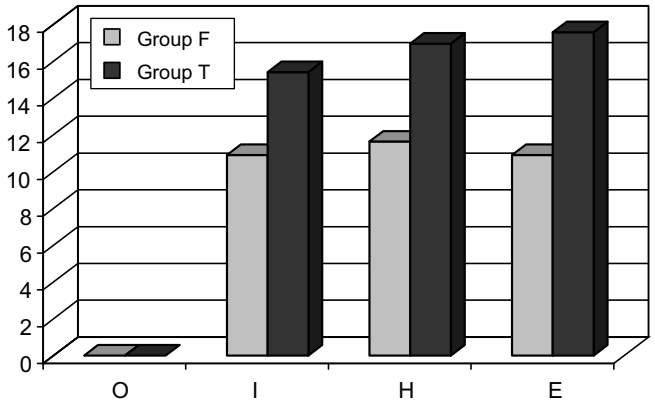

Fig. 6. Evolution of gallic ethyl ester concentration (mg/L) during the acetification in chestnut barrels.

3.3. Total anthocyanins

The total anthocyanins content in the starting red wines ranged between 1.20 mg of M3G/L (group F) and 0.45 mg M3G/L (group T). These values seem very low when compared with the usual figures for these parameters in red wine, which range between 50 mg/L and 170 mg/L (Sánchez-Moreno, Cao, Ou, & Prior, 2003). It can probably be assumed that these red wines had a degree of high evolution and warm-up. The vinegar winery usually employs them because they favour acetification.

Table 6
Probability levels of triangle difference tests for finished vinegars acetified in different woods (group F; A = acacia; C = cherry; R = oak; S = chestnut)

	FEA	FEC (%)	FER (%)	FES (%)
FEA	–	5	0.1	0.1
FEC		–	0.1	0.1
FER			–	0.1
FES				–

Table 7
Probability levels of triangle difference tests for finished vinegars acetified in different woods (group T; A = acacia; C = cherry; R = oak; S = chestnut)

	TEA	TEC	TER (%)	TES (%)
TEA	–	n.s.	0.1	0.1
TEC		–	0.1	1
TER			–	0.1
TES				–

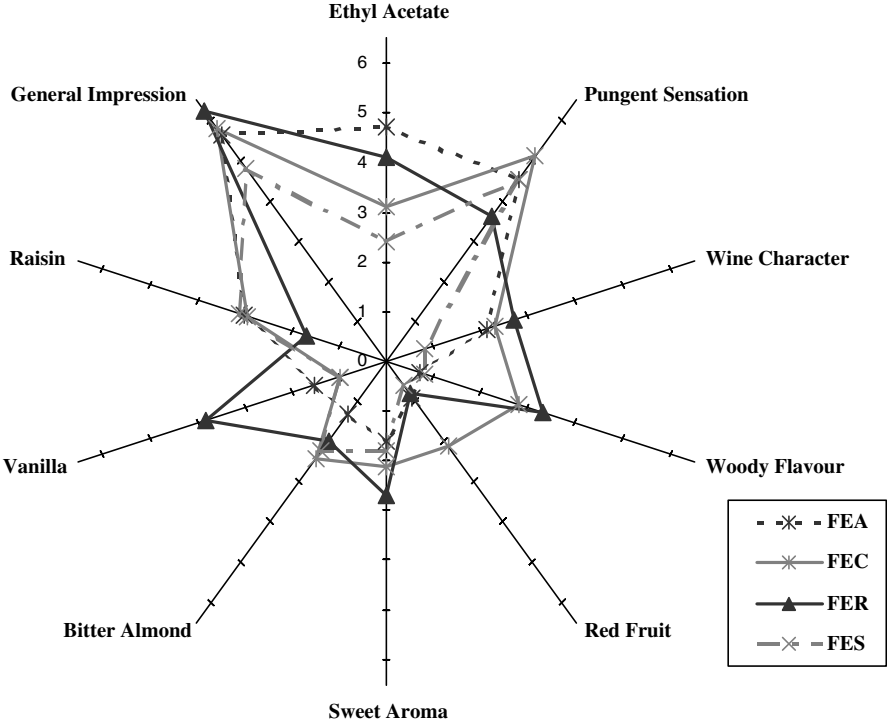

Fig. 7. Sensory analysis. Spider chart of finished vinegars from group F elaborated in the different woods (A = acacia, R = oak, S = chestnut, C = cherry).

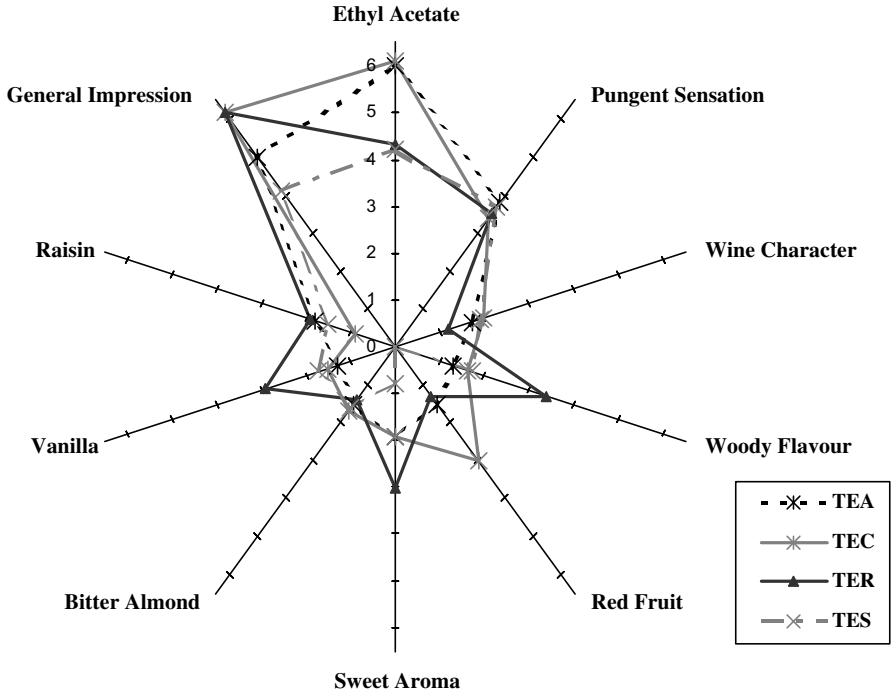

Fig. 8. Sensory analysis. Spider chart of finished vinegars from group T elaborated in different woods (A = acacia, R = oak, S = chestnut, C = cherry).

Analysis of variance showed significant decreases during the acetification process in each wood for both groups F and T ($p < 0.05$). The average decrease was 56% in group F and 51.6% in group T as reported by Andlauer et al. (2000) for red wine vinegars obtained by submerged culture.

3.4. Sensory analysis

The panel carried out triangle tests in order to differentiate which vinegars had been made in which wood. The results showed that the panel was able to differentiate most of the vinegars with different significance levels (Tables 6 and 7).

In order to obtain more information, the vinegars' sensory profile was built up according to the marks given for each attribute by the whole panel. Figs. 7 and 8 show the spider charts for vinegars from different woods. As can be seen, vinegars' sensory profiles were similar within either group but different between groups F and T. Nevertheless, woody aroma and vanilla perception accounts for higher marks in vinegars from oak wood. The red fruit attribute note was higher for vinegars obtained from cherry woods. In addition, higher scores for general impression were given to the vinegars from cherry and oak woods.

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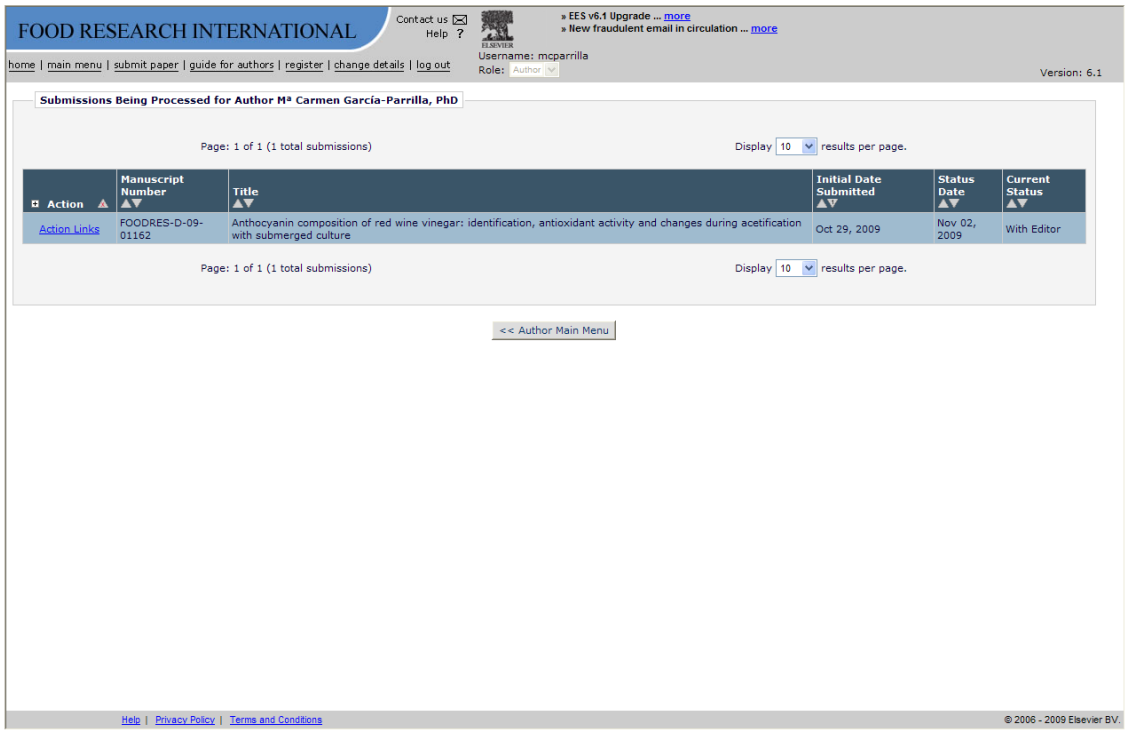
CAPÍTULO 2 / CHAPTER 2

Ana B. Cerezo; Elyana Cuevas; Peter Winterhalter; M. Carmen Garcia-Parrilla & Ana M. Troncoso.

ANTHOCYANIN COMPOSITION OF RED WINE VINEGAR: IDENTIFICATION, ANTIOXIDANT ACTIVITY AND CHANGES DURING ACETIFICATION WITH SUBMERGED CULTURE

Food Research International
Submitted
FOODRES-D-09-01162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Anthocyanin composition of red wine vinegar: identification, antioxidant activity and changes during acetification with submerged culture

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Abstract

Vinegars elaborated from white wine can be characterized by their phenolic composition. Indeed, for authenticity purpose, phenolic composition can be used to identify Sherry and Balsamic vinegars. However, the phenolic composition of red wine vinegars has scarcely been studied. Anthocyanin compounds in particular remain largely unknown. This study focuses on the analysis of anthocyanin compounds in red wine vinegar and the effect of acetification with submerged culture on such vinegars.

The vinegar used in this study was produced from a young *Cabernet Sauvignon* wine in a laboratory-scale fermenter. Subsequent analyses of both wine and vinegar included their anthocyanin profile (by LC-DAD-MS), and their non-anthocyanin phenolic profile (by LC-DAD). In addition, wine and vinegar anthocyanin extracts were fractionated by CCC to determine the contribution of the fractions to overall antioxidant activity (AA), using ORAC, FRAP and DPPH assays.

A total of 20 anthocyanin compounds were identified in the vinegar. As far as we know, this is the first time that anthocyanin-derived pigments (pyranoanthocyanins and ethyl-linked compounds) have been identified in red vinegar in such detail. Moreover, an original contribution of this study is the identification for the first time of catechyl-pyranocyanidin-3-glucoside and guaiacyl-pyranomalvidin-3-(6-acetyl)-glucoside in vinegar and wine, as well as three anthocyanin compounds not previously reported in vinegar or *Cabernet Sauvignon* wine: carboxy-pyranocyanidin-3-(6-acetyl)-glucoside, carboxy-pyranocyanidin-3-(6-p-coumaryl)-glucoside and carboxy-pyranodelfinidin 3-(6-acetyl)-glucoside. After the acetification process, vitisin-type and ethyl-linked compounds increased and monomeric anthocyanins, phenolic acids (ferulic acid, caffeic acid and caftaric acid) and flavan-3-ol ((+)-catechin) decreased.

Although the proportion by weight of the polymeric compound fraction is similar in wine and vinegar, the AA of these polymers in vinegar is significantly greater ($p < 0.05$). We have also determined for the first time an approximate value of AA for malvidin-3-(6-acetyl)-glucoside isolated from vinegar.

Key words: anthocyanin, vinegar, acetification, antioxidant, Cabernet Sauvignon, malvidin-3-(6-acetyl)-glucoside, wine

1. Introduction

Interest in high quality culinary products is on the rise. Among vinegars, Balsamic and Sherry vinegar can command higher prices than the wines they are produced from. Producers are developing new products for use in cooking, such as red wine vinegars which are proving successful on the market.

The existing literature devoted to the chemical composition of vinegars focuses on Sherry and Balsamic vinegars. Red wine vinegars, however, have scarcely been studied. Chemical characterization is required to guarantee the authenticity and quality of final products such as these vinegars. Phenolic compounds have proved to be useful substrate markers for the characterization of vinegars according to origin (Galvez, Barroso & Pérez-Bustamante, 1995), acetification methods (Garcia-Parrilla, González, Heredia & Troncoso, 1997) and type of ageing process (Tesfaye, Morales, Garcia-Parrilla & Troncoso, 2002; Tesfaye, Morales, Benítez, Garcia-Parrilla & Troncoso, 2004). As most high-quality vinegars are produced from white wine, phenolic acids and aldehydes are useful markers for the characterization of this type of vinegar. However, the anthocyanin compounds present in red wine vinegar have not yet been studied in depth (Andlauer, Stumpf & Fürst, 2000).

The production conditions (oxygen effect and final pH) of vinegars and wines can affect the anthocyanin fraction. Indeed, the role oxygen plays in anthocyanin chemistry is currently a topic of particular interest in this field. The acetification process requires a high air flow (100-200 L/h) meaning 21-42 L/h of oxygen flow is needed to promote acetic bacteria metabolism and the oxidation of ethanol to acetic acid. In the micro-oxygenation process, the wine is treated with closely-controlled sub-saturation doses of oxygen (5-10 mL/Lmonth). This technique has been proved to facilitate the formation of anthocyanin-derived compounds (Cano-López, Pardo-Minguez & López-Roca, 2006) which can help to stabilize the color. It is also used to improve the structure and body of wines, to remove sulphides and reduce unwanted aromas, and to soften the astringency caused by tannins (Parish, Wollan & Paul, 2000). Nevertheless, little is known about the effect of the acetification process on the anthocyanin composition of vinegars, and this needs to be studied.

In order to fully describe the antioxidant properties as related to the chemical composition of drinks and foods, anthocyanin must be determined. The complexity of the wine matrix and the lack of anthocyanin standards make it difficult to assess their antioxidant activity (AA). To explore the relevance of these compounds in the AA of a product, a preliminary step consisting of solid phase extraction (Fernandez-Pachón, Villaño, Garcia-Parrilla & Troncoso, 2004), ultra-filtration (De Beer, Joubert, Marais & Manley, 2006) or CCC (Noguer, Cerezo, Rentzsch & Winterhalter, 2008) is needed to separate the compounds from the matrix. Although several authors have reported the AA of both Traditional Balsamic Vinegar (Verzelloni, Tagliazucchi & Conte, 2007; Tagliazucchi, Verzelloni & Conte, 2008) and commercial vinegar (Dávalos, Bartolomé & Gómez-Cordovés, 2005), more research into their chemical composition needs to be done.

The aims of this paper are to analyze anthocyanin compounds in red wine vinegar and to assess the changes in these compounds due to the acetification process. In addition, the anthocyanin fractions have been isolated by CCC to determine their contribution to overall AA, since this is considered a criterion of the product's quality.

2. Material and Methods

2.1. Samples

The substrate used to produce vinegar was a young *Cabernet Sauvignon* monovarietal red wine elaborated under controlled experimental conditions by the "Rancho La Merced" research center (Jerez de la Frontera, Spain). The wine has an alcoholic degree of 12.5º (%, v/v), tartaric acid content of 0.9 g 100 m/L and sugar content of 1.23 g/L.

2.2. Acetification process

The wine was acetified in a laboratory scale fermenter (B. Braun Biotech, S.A.). Optimum conditions for acetification have previously been described (Tesfaye, Garcia-Parrilla & Troncoso, 2000), and briefly are as follows: stirring speed, 450 rpm; substrate loading proportions of wine/vinegar, 1:1; loading volume, 3400 mL. Air supply and temperature were maintained constant, within values of 100-200 L/h and 30ºC, respectively.

The culture of acetic acid bacteria came from a white wine vinegar and was mixed with the substrate 1:1. Four acetification cycles were rejected until we considered the product to be sufficiently homogeneous (i.e. when the culture of the acetic acid bacteria

represented < 2 % v/v, and had a fermentation yield of 80.79 ± 4.60%) and the acetification cycles were reproducible (acetification rate 0.11 ± 0.01 ºacetic/h).

The resulting vinegar had 8.97 g/100mL of acetic acid and an alcoholic degree of 1.33º (%, v/v). A total of eight cycles were required to obtain 15.30 L of vinegar. The average time taken for acetification was 41 hours. The fermentation yield and acetification rate for the eight cycles were 80.8 ± 4.68% and 0.11 ± 0.01 ºacetic/h, respectively.

2.3. Isolation of fractions

2.3.1. Sample preparation for CCC

An Amberlite XAD-7 column (Fluka; 100 x 7cm) was first conditioned with 2 L of methanol, and then with 2 L of Milli-Q water. A total of 6 L of red wine or vinegar were diluted with water (1:1), loaded onto the column and cleaned with 3 L of water to remove sugars, proteins, organic acids, and minerals. Elution was performed with 2 L of methanol/acetic acid (19:1, v/v). The flow rate was 1 drop/second. The eluate was concentrated with a rotary evaporator under vacuum, frozen and freeze-dried to obtain 3.09 and 2.92 g of anthocyanin-enriched extract from 1 L of wine and vinegar, respectively.

2.3.2. CCC

The wine and vinegar XAD-7 extracts were fractioned with a high-speed model CCC-1000 instrument, manufactured by Pharma-Tech Research Corp. (Baltimore, MD), equipped with three preparative coils, connected in series (tubing diameter of 2.6 mm

and total volume of 850 mL). The solvent system consisted of MTBE/n-butanol/acetonitrile/water (2:2:1:5, v/v/v/v) acidified with 0.1 % trifluoroacetic acid. The elution mode was head-to-tail with the lighter (organic) phase acting as the stationary phase and the aqueous phase as the mobile phase. The flow rate was set at 3 mL/min and delivered by a BT 3020 HPLC pump (Jasco, Gross-Umstadt, Germany). The separation was run at a speed of 850 rpm. One gram of wine or vinegar extract, maximum, was dissolved in 25 mL of a mixture of the upper (organic) and lower (aqueous) phases (50:50; v/v) and injected into the system by a loop injector. Fractions of 12 mL were collected with a fraction collector. Elution was monitored with a K-2501 UV/vis detector (Knauer, Berlin, Germany) at 520 nm. The fractions were collected according to the profile of the chromatogram. After the evaporation of organic solvents, the fractions were frozen and freeze-dried again.

2.4. Identification and quantification of anthocyanin compounds

2.4.1. LC-MS

The anthocyanin compounds were identified using an LC system consisting of a model G1328A binary pump (Agilent, Palo Alto, CA) equipped with an autosampler (Agilent Technologies, 1200 Series, G 1329), coupled to a Bruker Esquire mass spectrometer with electrospray ionization. Data were processed using Esquire NT 4.0 software (Bruker). The MS/MS parameters were as follows: positive mode; capillary voltage, -2500 V; end plate offset, -500 V; capillary exit, 1, 10 V; capillary exit offset 70 V, skimmer 1, 20 V; skimmer 2, 10 V; dry gas (N2) temperature, 300 °C; flow, 11 L/min; nebulizer, 10 psi; and scan range *m*/*z*, 50-2500. The separations were performed on a

250 x 4.6 mm, 5 µm, C18 Luna column (Phenomenex, Germany). Samples were filtered through a Chromatofil® PET 0.45 µm membrane filter before injection. The sample volume injected was 50 µl. Two different solvents were used as the mobile phase: solvent A (water/acetonitrile/formic acid, 87:3:10 v/v/v) and solvent B (water/acetonitrile/formic acid, 40:50:10 v/v/v), at a flow rate of 0.5 mL/min and a linear gradient as follows: 0 min 6% B, 20 min 20% B, 35 min 40% B, 40 min 60% B, 45 min 90% B, 55 min 6% B.

2.4.2. LC-DAD

The anthocyanin compounds were quantified using an LC system equipped with a binary pump (Jasco PU-980), automatic injector (Jasco AS-950) and degasser (Jasco DG-980-50-3). Detection was carried out using a UV/Vis diode detector (Jasco MD-1510), and data were processed using Borwin-PDA Version 1.0 software. The chromatographic conditions were as described above. Anthocyanins were detected at 520 nm. Quantification was performed by external calibration expressed as malvidin-3-glucoside equivalents.

2.5. Identification and quantification of non-anthocyanin phenolic compounds

LC analyses of *non-anthocyanin* phenolic compounds were performed using an Agilent Series 1100 system equipped with a quaternary pump (Series 1100 G1311A), automatic injector (Series 1100 G1313A) and degasser (Series 1100 G1379A). Detection was carried out using an UV/Vis diode detector (Series 1100 G1315B) coupled to a Chemstation HP A.10.02 (HP/Agilent). The column was an Agilent Zorbax SB-C18,

4.6 x 250 mm and 3.5 µm. Duplicate samples were filtered through a Millex-LCR 13 mm filter before injection. The chromatographic conditions had previously been used for vinegar analysis (Tesfaye et al., 2004). The method uses a binary gradient: A (glacial acetic acid/water pH 2.65), B (20% A + 80% acetonitrile) programmed for the following gradient: 0 min 0% B; 5 min 2% B; 10 min 4% B; 15 min 10% B; 30 min 20% B; 35 min 30% B; 40 min 100% B; 45 min 0% B. The sample volume injected was 50 µl. The flow rate was 1.5 mL/min, and the temperature was set at 40 ºC. Quantification was performed by external calibration at their maximum absorbance.

The standards of non-anthocyanin phenolic compounds were purchased from Fluka, Sigma, Merk, Safc and Chromadex.

2.6. Antioxidant activity of the samples

2.6.1. ORAC assay

The ORAC assay is based on a previously reported method with slight modifications (Ou, Hampsch-Woodill & Prior, 2001). Briefly, it is as follows: 50 µl of sample or Trolox are mixed with 100 µl of Fluorescein (45 nM) and 50 µl of AAPH (15 mM). Fluorescence is recorded for 60 min (the excitation wavelength is set at 485 nm; the emission wavelength at 528 nm). Measurements were taken in triplicate in a multi-detector microplate reader (Synergy HT, Biotek®). Trolox was used as a calibration standard (0.5 – 9.5 µM).

The results were calculated as ORAC values from the differences between the blank and the sample areas under the fluorescein decay curve. They are expressed as mmol Trolox equivalents (TE)/g of fraction or extract.

226 *2.6.2. FRAP method*

227

228 The FRAP reagent consists of acetate buffer (300 mM, pH 3.6), TPTZ (10 Mm in HCl
229 40 Mm) and $FeCl_3$ $6H_20$ (20 mM) (10:1:1, v/v/v). A total of 3 mL of FRAP reagent was
230 mixed with 300µL Mili-Q water and 100 µL of sample. Absorbance was measured after
231 8 minutes at 593 nm. An aqueous solution of $FeSO_4x7H_2O$ in the 0-1 mM range was
232 used for calibration. Results are expressed as mmol of Fe^{+2}/g of fraction or extract
233 (Benzie & Strain, 1996).

234

235 *2.6.3. DPPH method*

236

237 A total of 0.1 mL of sample or Trolox (0.000 – 1.000 mM) was added to 3.9 mL of
238 $DPPH^{\bullet}$ (0.063 mM), all in methanolic solution. Absorbance was measured at 515 nm
239 after 60 min (when the reaction reached equilibrium). The blank reference cuvette
240 contained methanol. Initial absorbance was close to 0.700 in all cases (Sánchez-Moreno,
241 Larrauri, & Saura-Calixto, 1998). All measurements were performed in triplicate.
242 Results are expressed as mmol Trolox equivalents (TE)/g of fraction or extract.

243

244 *2.7. Statistical analysis*

245

246 Statistical software (Statsoft, 2001) was used to test significant differences (T-test;
247 $p<0.05$).

248

249

250

3. Results and discussion

3.1. Anthocyanin profile of wine and vinegar

Table 1 lists a total of 20 anthocyanin compounds detected, identified by their elution order, UV/Vis and mass spectrometric characteristics and comparison with data from the literature (Cano-López et al., 2006; Flamini, 2003; Atanasova, Fulcrand, Cheynier & Moutounet, 2002; Revilla, Pérez-Magariño, González-San José & Beltrán, 1999; Monagas, Núñez, Bartolomé y Gómez-Cordovés, 2003; Heier, Blaas, Droβ & Wittkowski, 2002; Hillebrand, Schwarz & Winterhalter, 2004), expressed as mg/L of malvidin 3-glucoside.

Wine and vinegar presented a large variety of anthocyanin-derived pigments (pyranoanthocyanins and ethyl-linked compounds) and acetylated anthocyanins (Table 1). This composition is characteristic of *Cabernet Sauvignon* wine (Monagas et al., 2003; Pérez-Magariño & González-San José, 2004). As expected, malvidin-3-glucoside was the main anthocyanin in both wine and vinegar, followed by malvidin-3-(6-acetyl)-glucoside (Pérez-Magariño et al., 2004; Schwarz, Quast, von Baer & Winterhalter, 2003a; Noriega & Casp, 2007). Malvidin-3-glucoside-ethyl-catechin (ethyl-linked compound) was observed in three different peaks of the wine and vinegar chromatograms corresponding to different stereo isomers, as reported by other authors for *Cabernet Sauvignon* (Cano-López et al., 2006; Atanasova et al., 2002; Monagas et al., 2003).

As far as we know, this is the first time that anthocyanin-derived pigments (pyranoanthocyanins and ethyl-linked compounds) have been identified in red wine vinegar in such detail (Table 1). An additional original contribution of our work is the

276 identification of catechyl-pyranocyanidin-3-glucoside and guaiacyl-pyranomalvidin-3-
277 (6-acetyl)-glucoside in vinegar and wine (Figure 1). Furthermore, carboxy-
278 pyranocyanidin-3-(6-acetyl)-glucoside, carboxy-pyranocyanidin-3-(6-p-coumaryl)-
279 glucoside and carboxy-pyranodelfinidin-3-(6-acetyl)-glucoside (Figure 2) were
280 identified for the first time in vinegar and *Cabernet Sauvignon* wine.
281 The acetification conditions were set for an aeration flow of 100-200 L/h for 41 hours,
282 which is considerable oxygen exposure (Jimenez-Hornero, Santos-Dueñas & García-
283 García, 2009). The effect of oxygen exposure on the anthocyanin profile of the wine is
284 studied under micro-oxygenation conditions. This technique is usually performed at 5-
285 10 mL/L·mont·h of oxygen flow for 4-7 months (Cano-López et al., 2006; Atanasova et
286 al., 2002), which is at least 10^6 times less flow than that used during the acetification
287 process. Micro-oxygenation has been proven to enhance the formation of vitisin-type
288 compounds (vitisin A, acetyl vitisin A, carboxy-pyranopetunidin-3-glucoside, etc)
289 (Cano-López et al., 2006) and ethyl-linked compounds (malvidin-3-glucoside-ethyl-
290 catechin) (Cano-López et al., 2006; Atanasova et al., 2002). Conversely, monoglucoside
291 anthocyanins and their acylated derivatives diminish with time and even more under
292 micro-oxygenation conditions, due to the formation of anthocyanin-derived compounds
293 and degradation reactions (Cano-López et al., 2006; Atanasova et al., 2002). Table 1
294 shows that after the acetification process, most of the vitisin-type compounds increased
295 significantly ($p<0.05$). In addition, one of the isomers of malvidin-3-glucoside-ethyl-
296 catechin increased as well. Accordingly, the concentration of the main monomeric
297 anthocyanin decreased significantly ($p<0.05$) from wine to vinegar (Table 1). To sum
298 up, vitisin-type and ethyl-linked compounds increase and monomeric anthocyanins
299 decrease, in accordance with the reactivity of these compounds in the presence of
300 oxygen.

301 *3.2. Non-anthocyanin phenolic profile of wine and vinegar*

302

303 Table 2 gives the concentration of non-anthocyanin phenolic compounds determined in
304 wine and vinegar. (+)-Catechin decreased by around 50%, which is consistent with
305 previously reported data on red wine vinegar acetification by submerged culture
306 (Cerezo, Tesfaye, Torija, Mateo, Garcia-Parrilla & Troncoso, 2008). In fact, malvidin-
307 3-glucoside-ethyl-catechin increased (Table 1) and polymerization reactions are likely
308 to occur. Caffeic and caftaric acids decreased significantly ($p<0.05$), probably due to the
309 formation of anthocyanin derivatives, as a direct reaction of anthocyanin 3-glucoside
310 and caffeic acid has been described (Hillebrand et al., 2004; Schwarz, Wabnitz &
311 Winterhalter, 2003b; Schwarz, Hofmann & Winterhalter, 2004). Considering both
312 compounds, a total decrease of $4.37*10^{-5}$ mol/L of caffeic acid was found after the
313 acetification process. However, the catechyl-pyranocyanidin-3-glucoside increased by
314 just $3.25*10^{-6}$ mol/L. Ferulic acid also decreased significantly ($p<0.05$) after the
315 acetification process (Table 2). It is known that 4-vinylguaiacol is a derivative of ferulic
316 acid which can react to form anthocyanin derivates (Sarni-Manchado, Fulcrand,
317 Souquet, Cheynier & Moutounet, 1996; Fulcrand, Cameira dos Santos, Sarni-
318 Manchado, Cheynier & Favre-Bonvin, 1996). Additionally, a direct reaction between
319 ferulic acid and malvidin-3-glucoside has been reported (Schwarz et al., 2003b). Indeed,
320 ferulic acid concentration decreased by $2.05*10^{-6}$ mol/L, while guaiacyl-
321 pyranomalvidin-3-(6-acetyl)-glucoside increased by $4.85*10^{-7}$ mol/L and the other
322 derivative (guaiacyl-pyranomalvidin-3-glucoside) was found only in trace amounts.
323 Previous studies involving white wine acetification in identical fermentation conditions
324 proved these hydroxycinnamic acids did not undergo statistically significant changes
325 during the acetification process (Morales, Tesfaye, Garcia-Parrilla, Casas & Troncoso,

2001). It is clear that a chemical reaction with anthocyanic compounds is responsible for this decrease rather than a microbiological effect due to acetic acid bacteria.

3.3. Phenolic composition of the extracts and fractions obtained after CCC isolation

A total of three fractions were isolated from wine extract and five fractions from vinegar extract by CCC. Those samples were analyzed by LC/DAD (non-anthocyanin phenolic compounds) and LC/DAD/MS (anthocyanin compounds). Non-anthocyanin phenolic compounds were removed while the extracts were being obtained (section 2.3.1). The fractions consisted of the following:

Fraction 1 contained polymeric compounds from both wine and vinegar extracts.

Fraction 2 from both wine and vinegar extract presented a mix of different anthocyanins.

Fraction 3 consisted mainly of malvidin-3-glucoside from 58-88% in fractions from vinegar extract and 58-59% in fractions from wine extract.

A total of two fractions more were isolated from vinegar extract. Fraction 4 contained a mixture of different anthocyanin compounds. Fraction 5 consisted mostly of malvidin-3-(6-acetyl)-glucoside at 81% (Figure 3).

In both cases the coil fraction is the remaining anthocyanins that could not be isolated.

Detailed descriptions of the fractions compositions are displayed in Table 3.

3.4. Antioxidant activity

Tables 4 and 5 give the values for the AA of wine and vinegar, respectively, and the extracts obtained and fractions isolated. The AA values decreased from wine to vinegar,

351 by 14.42%, 16.80% and 29.20% according to the FRAP, DPPH and ORAC assays,
352 respectively. The decrease in AA is larger according to the ORAC methods, which
353 involves peroxyl radicals. The AA presented by commercial red wine is between 3 and
354 7.1 times higher than that presented by red wine vinegars available on the market
355 (Dávalos et al., 2005; Pellegrini, Serafini, Colombini, Del Rio & Salvatore, 2003;
356 Alonso, Guillén & Barroso, 2003). During industrial vinegar production the wine
357 substrate is diluted to reduce the alcoholic degree and promote the growth of acetic
358 bacteria. This practice, therefore, may explain the lower AA found in commercial
359 vinegars compared to wines. Our data were obtained by means of an acetification
360 experiment without substrate dilution. Hence, the decrease in AA found in our study is
361 due to the changes in chemical composition after the fermentation process, rather than
362 the dilution effect. Phenolic acid compounds (caftaric acid, caffeic acid and ferulic acid)
363 and flavan-3-ol compound ((+)-catechin) have been found to have high antioxidant
364 activity (Villano, Fernández-Pachón, Troncoso & Garcia-Parrilla, 2005). Their decrease
365 after acetification process can explain the decrease in AA of the vinegar.
366 Results are expressed in AA per gram of extract and AA per gram of fraction, to show
367 the efficacy of each. Although the weight of the polymeric compounds fraction (fraction
368 1) is similar in wine and vinegar, the AA of polymers in vinegar is significantly higher
369 ($p< 0.05$). No relevant conclusions concerning AA can be drawn from the data for
370 fractions 2 in wine and vinegar or fraction 4 in vinegar, as they are a mixture of
371 compounds, or for fraction 3 in wine and vinegar (consisting of malvidin-3-glucoside)
372 as has been already reported (Aaby, Hvattum & Skrede, 2004; Kähkönen & Heinonen,
373 2003; Muselík, García-Alonso, Martín-López, Zemlieka & Rivas-Gonzalo, 2007).
374 However, fraction 5 of vinegar contains more than 80% malvidin-3-(6-acetyl)-glucoside
375 (Figure 3) and has an antioxidant value of 10.34 ± 0.18, 3.49 ± 0.07 and 5.18 ± 0.02

mmol Trolox/g fraction by ORAC, FRAP and DPPH methods, respectively. The AA of malvidin 3-acetylglucoside has never before been reported. Hence, this is the first time that an approximate value of AA is reported for malvidin-3-acetylglucoside.

4. Conclusions

This study makes new contributions to describing the anthocyanin profile of vinegar and wine. Pyranoanthocyanins and ethyl linked compounds have been identified for the first time in red wine vinegar. Moreover, Catechyl-pyranocyanidin-3-glucoside and guaiacyl-pyranomalvidin-3-(6-acetyl)-glucoside have been found for the first time in vinegar and wine, and carboxy-pyranocyanidin-3-(6-acetyl)-glucoside, carboxy-pyranocyanidin-3-(6-p-coumaryl)-glucoside and carboxy-pyranodelfinidin 3-(6-acetyl)-glucoside in vinegar and *Cabernet Sauvignon* wine.

The main changes in chemical composition are increases in vitisin-type and ethyl-linked compounds and decreases in monomeric anthocyanins, phenolic acids (ferulic acid, caffeic acid and caftaric acid) and flavan-3-ol ((+)-catechin).

Furthermore, this paper includes a tentative description of the AA of malvidin-3-(6-acetyl)-glucoside.

Abbreviations

AA, antioxidant activity; CCC, countercurrent chromatography; MTBE, methyl *tert*-butyl ether; ORAC, Oxygen Radical Absorbance Capacity; TROLOX, 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; AAPH, 2,2´-Diazo bis amidine propane dihydrochloride; DPPH$^{\bullet}$, 1,1diphenyl-2-picrylhydrazyl; TEAC, Trolox Equivalent

Antioxidant Capacity; FRAP, Ferric Reducing Ability; TPTZ, 2,4,6-Tripyridyl-*s*-triazine.

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Figure captions

Figure 1. Mass spectra and fragmentation pattern of catechyl-pyranocyanidin-3-glucoside (**a,b**) and guaiacyl-pyranomalvidin-3-(6-acetyl)-glucoside (**c,d**) in wine and vinegar samples.

Figure 2. Mass spectra and fragmentation pattern of carboxy-pyranocyanidin-3-(6-acetyl)-glucoside (**a,b**), carboxy-pyranocyanidin-3-(6-p-coumaryl)-glucoside (**c,d**) and carboxy-pyranodelfinidin 3-(6-acetyl)-glucoside (**e,f**) in wine and vinegar samples.

Figure 3. LC-DAD chromatogram of fraction 5 1st inj. at 520 nm.

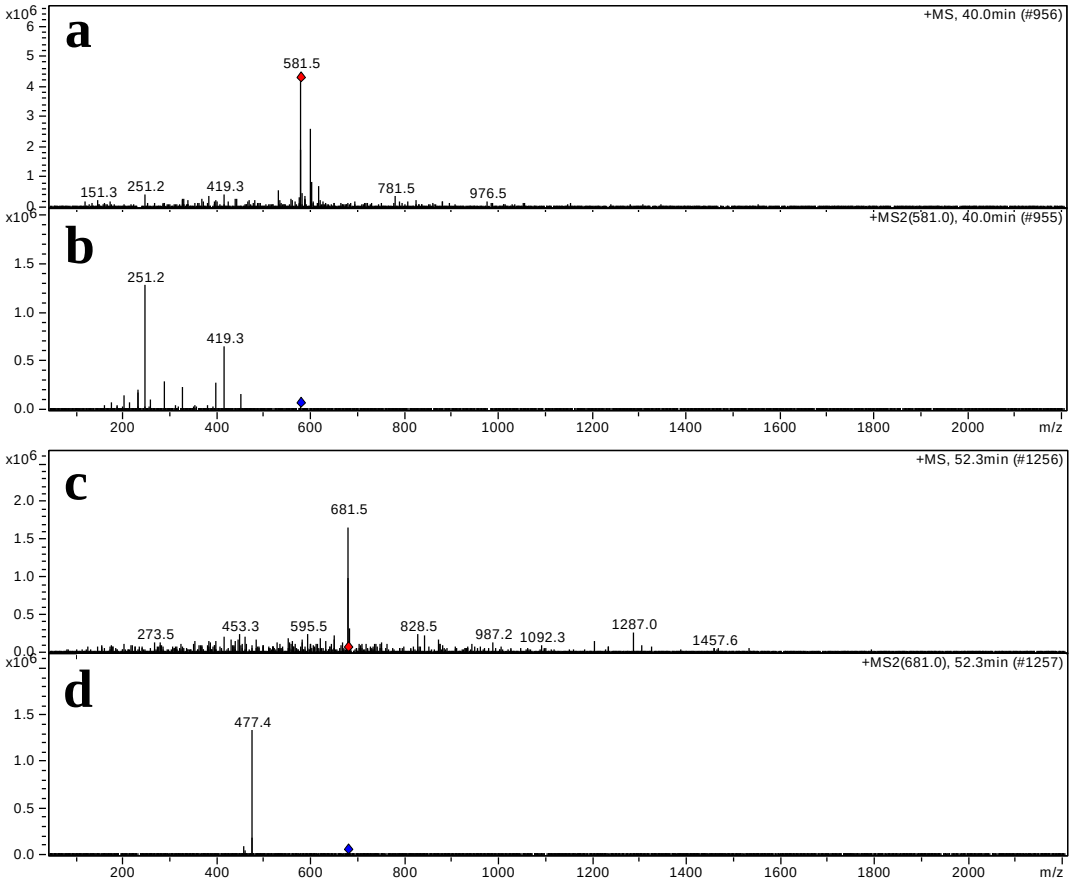
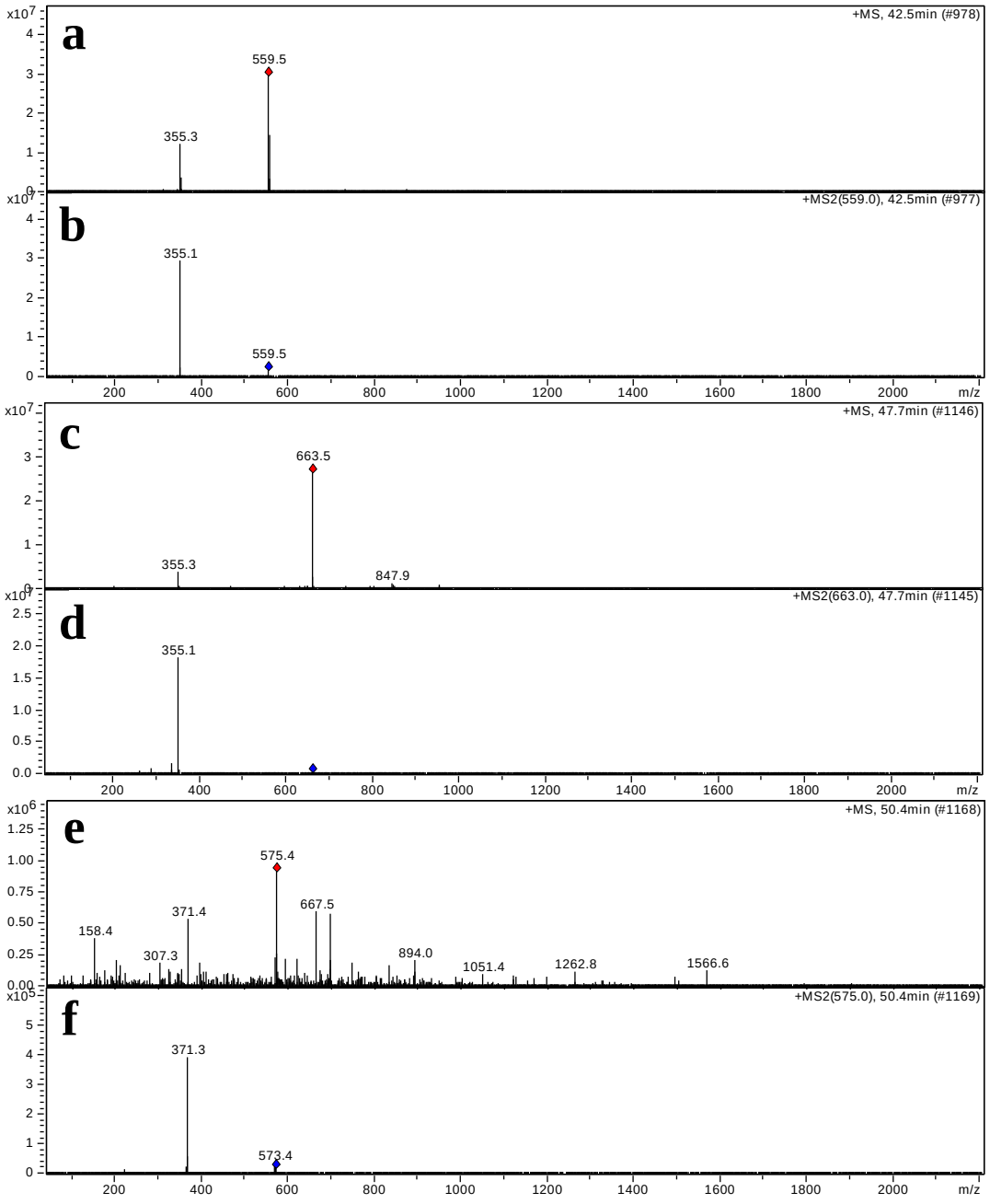

a
+MS, 40.0min (#956)
581.5
151.3
251.2
419.3
781.5
976.5
b
+MS2(581.0), 40.0min (#955)
251.2
419.3
c
+MS, 52.3min (#1256)
681.5
273.5
453.3
595.5
828.5
987.2
1092.3
1287.0
1457.6
d
+MS2(681.0), 52.3min (#1257)
477.4
m/z

Figure 1.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+MS, 42.5min (#978)
559.5
355.3
b
+MS2(559.0), 42.5min (#977)
355.1
559.5
c
+MS, 47.7min (#1146)
663.5
355.3
847.9
d
+MS2(663.0), 47.7min (#1145)
355.1
e
+MS, 50.4min (#1168)
575.4
667.5
371.4
158.4
307.3
894.0
1051.4
1262.8
1566.6
f
+MS2(575.0), 50.4min (#1169)
371.3
573.4
200
400
600
800
1000
1200
1400
1600
1800
2000
m/z

Figure 2.

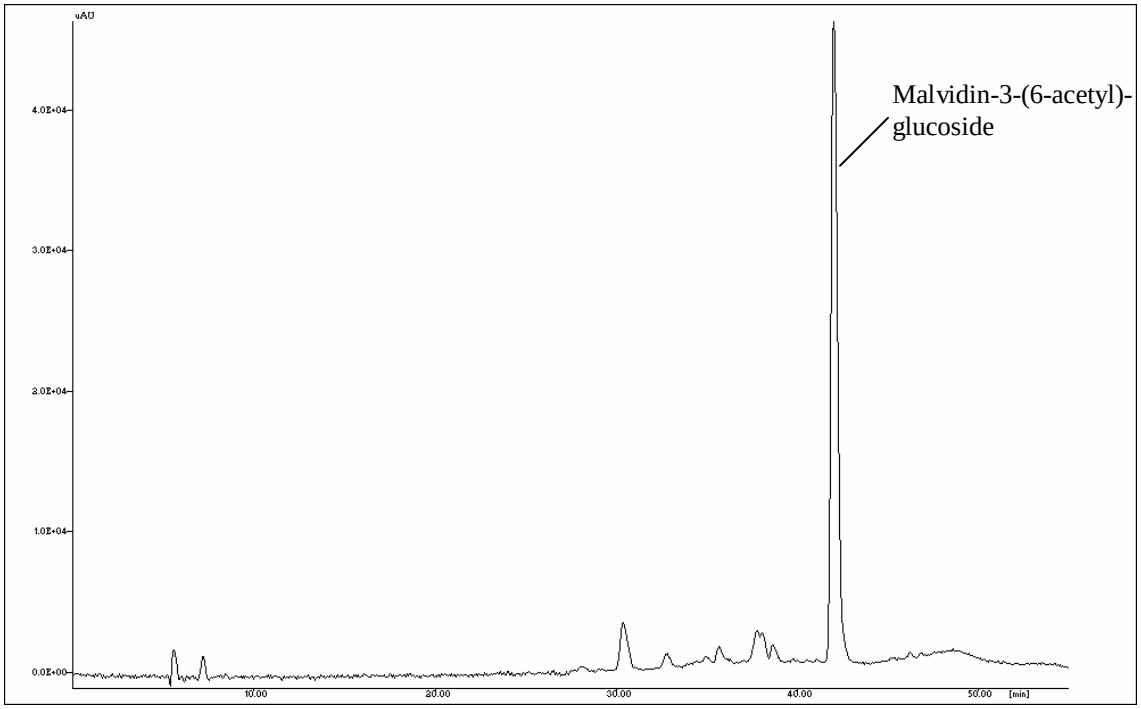
uAU
4.0E+04
3.0E+04
2.0E+04
1.0E+04
0.0E+00
10.00
20.00
30.00
40.00
50.00 [min]
Malvidin-3-(6-acetyl)-
glucoside

Figure 3.

Anthocyanin compounds	t_R (min)	$[M^+]$ m/z	MS^2 m/z	Concentration (mg/L)	
				Wine	Vinegar
Malvidin-3-glucoside	30.4	493	331	57.1 ± 0.5[a]	53.04 ± 0.4[b]
Carboxy-pyranomalvidin-3-glucoside (vitisin A)	36.3*	561	399	8.36 ± 0.04[a]	9.03 ± 0.07[b]
Malvidin-3-glucoside-ethyl-(epi)catechin	36.7*	809		*tr*	t*r*
Malvidin-3-glucoside-4-vinyl (Vitisin B)	37.7*	517	355	12.69 ± 0.13[a]	14.25 ± 0.13[b]
Malvidin-3-glucoside-ethyl-(epi)catechin	38.4*	809		6.66 ± 0.01[a]	7.76 ± 0.07[b]
Acetyl vitisin A	39.7*	603	399	3.47 ± 0.11	3.17 ± 0.03
Catechyl-pyranocyanidin-3-glucoside	40.0*	581	419	3.74 ± 0.02[a]	5.63 ± 0.01[b]
Malvidin-3-glucoside-ethyl-(epi)catechin	40.4*	809		2.25 ± 0.03[a]	1.73 ± 0.05[b]
Carboxy-pyranocyanidin-3-(6-acetyl)-glucoside	41.8*	559	355	11.63 ± 0.05	11.77 ± 0.13
Carboxy-pyranopeonidin-3-glucoside	42.9*	531	369	2.13 ± 0.04[a]	1,71 ± 0.02[b]
Peonidin-3-(6-acetyl)-glucoside	45.0*	505	301	2.71 ± 0.04[a]	3.01 ± 0.04[b]
Malvidin-3-(6-acetyl)-glucoside	45.8	535	331	29.6 ± 0.5[a]	26.3 ± 0.6[b]
Carboxy-pyranocyanidin-3-(6-p-coumaryl)-glucoside	47.7*	663	355	2.66 ± 0.10[a]	3.36 ± 0.12[b]
Malvidin 3-(6-p-coumaryl)-glucoside	49.3	639	331	6.10 ± 0,12[a]	8.2 ± 0.6[b]
Carboxy-pyranodelphinidin-3-(6-acetyl)-glucoside	50.3*	575	371	ni[a]	2.12 ± 0.05[b]
Phenyl-pyranomalvidin-3-glucoside	50.7*	609	447	2.45 ± 0.09	2.65 ± 0.06
Guaiacyl-pyranomalvidin-3-glucoside	51.2*	639	477	*tr*	*tr*
Phenyl-pyranomalvidin-3-(6-acetyl)-glucoside	51.9*	651	447	5.74 ± 0.07[a]	5.14 ± 0.09[b]
Guaiacyl-pyranomalvidin-3-(6-acetyl)-glucoside	52.4*	681	477	2.4 ± 0.5	2.73 ± 0.20
Phenyl-pyranomalvidin-3-(6-p-coumaroyl)-glucoside	52.9*	755	447	1.58 ± 0.01[a]	*tr*[b]

[ab] superscript letters indicate significant difference ($p < 0.05$) in wine and vinegar, respectively; *tr*: trace; ni: non identified; t_R: retention time by LC/MS method; *identified in vinegar for the first time.

Table 1. Means and Standard Deviations of the concentration (mg/L) of anthocyanin compounds identified from wine and vinegar expressed as malvidin-3-glucoside equivalents and determined by LC-DAD. Parameters used for anthocyanin identification by LC-MS are also shown.

Non-anthocyanin phenolic compounds	Concentration (mg/L)	
	Wine	**Vinegar**
Gallic acid	40.38 ± 0.13	41.2 ± 0.3
(+)-Catechin	21.3 ± 0.6[a]	10.61 ± 0.04[b]
Syringic acid	13.73 ± 0.06[a]	17.58 ± 0.02[b]
Caftaric acid	149.2 ± 1.7[a]	137.1 ± 0.9[b]
Caffeic acid	5.80 ± 0.03[a]	4.89 ± 0.00[b]
p-Coumaric acid	1.33 ± 0.01[a]	1.45 ± 0.00[b]
Ferulic acid	2.75 ± 0.01[a]	2.35 ± 0.01[b]

[ab] different letter as superscript indicate significant difference ($p < 0.05$) in wine and vinegar, respectively

Table 2. Means and Standard Deviations of the concentration (mg/L) of non-anthocyanin phenolic compounds in wine and vinegar.

	Extracts	
	Wine	Vinegar
Fraction 1 1st inj.	Polymer compounds	Polymer compounds
Fraction 1 2nd inj.	Polymer compounds	Polymer compounds
Fraction 1' 1st inj.	Vitisin B (14%); acetyl vitisin A (12%); malvidin-3-glucoside (7%); malvidin-3-(6-acetil)-glucoside (6%)	-
Fraction 2 1st inj.	Malvidin-3-glucoside (19%); Vitisin B (17%)	Malvidin-3-glucoside (27%); carboxy-pyranocyanidin-3-(6-acetyl)-glucoside (14%); malvidin 3-(6-acetil)-glucoside (14%); vitisin B (13%)
Fraction 2 2nd inj.	Malvidin-3-glucoside (55%); Vitisin B (45%)	Vitisin B (25%); acetyl vitisin A (21%); malvidin-3-glucoside (13%); malvidin 3-(6-acetyl)-glucoside (10%)
Fraction 3 1st inj.	Malvidin-3-glucoside (58%); carboxy-pyranocyanidin-3-(6-acetyl)-glucoside (14%); malvidin-3-(6-acetil)-glucoside (7%)	Malvidin-3-glucoside (88%); carboxy-pyranocyanidin-3-(6-acetyl)-glucoside (12%)
Fraction 3 2nd inj.	Malvidin-3-glucoside (59%); carboxy-pyranocyanidin-3-(6-acetyl)-glucoside (14%); malvidin-3-(6-acetyl)-glucoside (11%); vitisin B (6%)	Malvidin-3-glucoside (58%); carboxy-pyranocyanidin-3-(6-acetyl)-glucoside (6%)
Fraction 4 1st inj.	-	Malvidin-3-glucoside-ethyl-(epi)catechin (19%); catechyl-pyranocyanidin 3-glucoside (19%); malvidin-3-glucoside (17%); malvidin-3-(6-acetyl)-glucoside (16%)
Fraction 5 1st inj.	-	Malvidin-3-(6-acetyl)-glucoside (81%); malvidin-3-glucoside (8%)
Coil fraction 1st inj.	Malvidin-3-(6-acetyl)-glucoside (15%); malvidin-3-(6-p-coumaryl)-glucoside (7%); carboxy-pyranocyanidin-3-(6-p-coumaryl)-glucoside (5%); malvidi-3-glucoside (5%); carboxy-pyranocyanidin-3-(6-acetyl)-glucoside (5%)	Malvidin-3-glucoside (6%); catechyl-pyranocyanidin 3-glucoside (6%)
Coil fraction 2nd inj	Malvidin-3-(6-acetyl)-glucoside (12%); malvidin-3-glucoside (9%); malvidin-3-(6-p-coumaryl)-glucoside (6%); carboxy-pyranocyanidin-3-(6-acetyl)-glucoside (5%); carboxy-pyranocyanidin-3-(6-p-coumaryl)-glucoside (3%)	Catechyl-pyranocyanidin-3-glucoside (20%); malvidin-3-glucoside-ethyl-(epi)catechin (18%); carboxy-pyranocyanidin-3-(6-acetyl)-glucoside (12%); malvidin-3-(6-acetyl)-glucoside (11%); malvidin-3-glucoside (11%);

-: fraction not isolated in this extract

Table 3. Chemical composition of fractions obtained after CCC isolation from wine and vinegar extract.

Samples	ORAC		FRAP		DPPH	
	mmol Trolox/g fraction	mmol Trolox/g extract	mmol Fe^{2+}/g fraction	mmol Fe^{2+}/g extract	mmol Trolox/g fraction	mmol Trolox/g extract
Wine extract		8.7 ± 0.6		2.68 ±0.00		3.88 ± 0.12
Fraction 1 1st inj.	4.28 ± 0.09	1.3 ± 0.03	6.26 ± 0.02	1.83 ±0.01	4.77 ± 0.02	1.40 ± 0.01
Fraction 1 2nd inj.	6.19 ± 0.21	1.90 ± 0.06	7.81 ± 0.07	2.40 ± 0.02	5.70 ± 0.13	1.73 ± 0.01
Fraction 1' 1st inj.	7.3 ± 0.4	0.77 ± 0.04	4.7 ± 0.3	0.50 ± 0.03	5.77 ±0.16	0.61 ± 0.02
Fraction 2 1st inj.	5.53 ± 0.05	0.18 ± 0.00	2.53 ± 0.00	0.09 ± 0.00	5.3 ± 0.4	0.17 ± 0.02
Fraction 2 2nd inj.	10.7 ± 0.6	0.41 ± 0.02	7.4 ± 0.3	0.28 ± 0.01	6.9 ± 0.4	0.26 ± 0.01
Fraction 3 1st inj.	2.78 ± 0.24	0.16 ± 0.02	1.15 ± 0.02	0.07 ± 0.00	3.48 ± 0.02	0.21 ± 0.00
Fraction 3 2nd inj.	11.26 ± 0.3	0.94 ± 0.03	4.14 ±0.09	0.35 ± 0.01	6.10 ± 0.16	0.51 ± 0.01
Coil fraction 1st inj.	6.1 ± 0.3	1.85 ± 0.09	1.99 ± 0.04	0.61 ± 0.01	4.2 ± 0.3	1.41 ± 0.09
Coil fraction 2nd inj	8.56 ± 0.21	2.88 ± 0.07	4.81 ± 0.18	1.62 ± 0.06	5.58 ± 0.05	1.88 ± 0.02
Wine	6.54 ± 0.21[a]		11.23 ± 0.11[a]		12.26 ± 0.18[a]	

Expressed as [a] mM Trolox equivalents

Table 4. Antioxidant activity of wine, anthocyanin extract and fractions isolated.

Samples	ORAC		FRAP		DPPH	
	mmol Trolox/g fraction	mmol Trolox/g extract	mmol Fe^{2+}/g fraction	mmol Fe^{2+}/g extract	mmol Trolox/g fraction	mmol Trolox/g extract
Vinegar extract		8.53 ± 0.04		3.11 ± 0.02		4.77 ± 0.16
Fraction 1 1st inj.	18.0 ± 1.3	5.0 ± 0.4	9.94 ± 0.18	2.74 ± 0.05	10.27 ± 0.08	2.83 ± 0.02
Fraction 1 2nd inj.	6.1 ± 0.3	1.42 ± 0.06	4.40 ± 0.04	0.51 ± 0.01	5.28 ± 0.05	0.61 ± 0.01
Fraction 2 1st inj.	12.2 ± 0.3	0.88 ± 0.02	7.89 ± 0.18	0.57 ± 0.01	6.56 ± 0.02	0.48 ± 0.01
Fraction 2 2nd inj.	9.0 ± 0.4	1.02 ± 0.04	2.15 ± 0.00	0.13 ± 0.01	3.99 ± 0.00	0.23 ± 0.01
Fraction 3 1st inj.	6.0 ± 0.5	0.39 ± 0.03	3.94 ± 0.25	0.26 ± 0.02	4.32 ± 0.00	0.29 ± 0.01
Fraction 3 2nd inj.	3.69 ± 0.16	0.24 ± 0.01	1.61 ± 0.09	0.05 ± 0.00	3.83 ± 0.05	0.13 ± 0.01
Fraction 4 1st inj.	6.56 ± 0.52	0.42 ± 0.03	1.38 ± 0.18	0.09 ± 0.01	2.85 ± 0.07	0.19 ± 0.01
Fraction 5 1st inj.	10.34 ± 0.18	0.38 ± 0.01	3.49 ± 0.07	0.13 ± 0.00	5.18 ± 0.02	0.19 ± 0.00
Coil fraction 1st inj.	10.3 ± 0.7	1.50 ± 0.10	5.29 ± 0.00	0.78 ± 0.00	6.33 ± 0.16	0.93 ± 0.02
Coil fraction 2nd inj	9.7 ± 0.5	2.21 ± 0.11	2.57 ± 0.11	0.29 ± 0.01	3.73 ± 0.19	0.42 ± 0.02
Vinegar	4.63 ± 0.09[a]		9.61 ± 0.20[a]		10.2 ± 0.3[a]	

Expressed as [a] mM Trolox equivalents

Table 5. Antioxidant activity of vinegar, anthocyanin extract and fractions isolated.

CAPÍTULO 3 / CHAPTER 3

Ana B. Cerezo; Wendu Tesfaye; M. E. Soria-Díaz; M. Jesús Torija; Estíbaliz Mateo; M. Carmen Garcia-Parrilla & Ana M. Troncoso.

IMPACT OF WOOD ON THE PHENOLIC PROFILE AND SENSORY PROPERTIES OF WINE VINEGARS DURING AGEING

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Title: Impact of wood on the phenolic profile and sensory properties of wine vinegars during ageing
Journal of Food Composition and Analysis

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Impact of wood on the phenolic profile and sensory properties of wine vinegars during ageing

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Abstract

A crucial step in the production of high quality wine vinegars is ageing in wood. This study aims to determine how wood barrels affect vinegar quality. For this purpose, vinegars were aged in barrels made from acacia, cherry, chestnut, and oak wood. Two kinds of vinegars were studied; balsamic and red-wine vinegars. Analysis of samples included: the total phenol index (TPI), the total monomeric anthocyanins (TA), the dry extract and the phenolic and furanic compounds (by LC-DAD), in a total of 98 samples. The concentration of HMF, 2-furfuraldehyde, protocatechualdehyde and vanillin significantly increased during ageing. Taxifolin is present in vinegars aged in cherry wood confirmed by LC-MS/MS. Hence, it can be a marker of vinegars aged in cherry wood. This is the first time that this compound has been identified in wine vinegars. On the other hand, ellagic acid is a characteristic compound for samples aged in chestnut and oak barrels. An unknown peak was found in all vinegar samples (group F, T and M) which were aged in acacia wood.

Linear discriminant analysis was used to classify samples as a function of the type of wood and ageing time, with phenolic compounds as variables. Obtained linear functions classified correctly at 100% red vinegars according to the type of wood. If ageing time (0-365 days) is considered, 62.5% and 100% of the red and balsamic vinegar samples, respectively, were well classified.

The impact of wood on vinegar quality is large enough to be perceived by the senses in triangle tests. Woody, sweet and vanilla descriptors present highest scores for vinegars aged in oak wood. On the other hand, the ageing in cherry wood increases the mark for red fruit attribute.

These results prove that wood used in vinegar ageing determines both the chemical composition and sensorial properties.

Keywords: Phenolic compounds, wood barrels, sensory analysis, aging, oak, chestnut, cherry, acacia.

1. Introduction

Overall quality of wine vinegars is determined by the substrate, the acetification conditions and ageing. For vinegar to be high quality it must be aged in wooden butts. The organoleptic properties that develop during ageing make the finished product highly valued (Tesfaye et al., 2003). Good examples of high-quality vinegars are Sherry vinegar and Traditional balsamic vinegar (ABT). Sherry wine vinegar is produced in a series of wooden oak butts during which acetification and ageing take place simultaneously. ABT is obtained by a traditional method. The cooked must is fermented and the product is aged in a set of wooden barrels of decreasing sizes and of different woods (Sanarico et al., 2002; Plessi et al., 2006).

The phenolic composition of aged vinegars has been considered as a parameter for evaluating quality (García-Parrilla et al., 1999). Previous research (Carrero et al., 1999; García-Parrilla et al., 1999) has found that the levels of aromatic aldehydes and their derivatives, produced by hydroalcoholysis of the lignin from wood, are higher in aged vinegars. In addition, 5-hydroxymethylfurfuraldehyde (HMF), protocatechualdehyde, 2-furfuraldehyde, *p*-hydroxybenzaldehyde, vanillin and syringaldehyde have been identified in most of the aged Sherry vinegars. A considerable number of phenolic compounds, mainly aldehydes, are released from wood. Considerable increases were observed for syringaldehyde, coniferaldehyde and vanillin when Sherry vinegar was

aged in oak wood (Tesfaye et al., 2002b). Moreover, syringaldehyde, coniferaldehyde and sinapaldehyde, and vanillic and gallic acids are found in larger concentrations in Sherry vinegar aged with oak chips (Tesfaye et al., 2004).

The purpose of this paper is to study the different phenolic compositions of wine vinegars when they are aged in barrels made from four different types of wood: chestnut, acacia, cherry and oak. The paper includes different substrate: balsamic and red wine vinegars. Woods with large pores and high oxygen transfer suitable for acetification process and therefore vinegar making were tested. The impact of these woods on vinegar quality has not been studied before. Besides, the experiment with woods intends to produce sensory innovations in vinegar production. Thus, cherry and chestnut oaks were tested as they are commonly used in the elaboration of aerated wines (sweet wines). Oak was included as it is the most common to aged vinegar and acacia as its use has not been reported before. Phenolic compounds and sensory analysis were tested throughout the ageing process.

2. Materials and methods

2.1. Samples

Two red wine vinegars (Grenache variety, groups F and T) and one balsamic vinegar (Trebbiano variety, group M) acetified in different wood barrels (oak, chestnut, acacia and cherry) were used as substrates for the ageing process. Table 1 shows the characteristics of the vinegars.

New barrels with a capacity of 60 L made of oak, chestnut, acacia and cherry wood were used for the ageing process. The wood was not toasted. Samples were taken at

different ageing time periods; the zero point was the vinegar substrate (acetic degree: 6 g/100 mL), and subsequently 45, 180 and 365. For group M, samples aged at 540 days were also included.

The experimental design planned in the funded project included all the possible combinations of wood used during acetification and ageing. Results for samples acetified and aged in different woods did not allow to extract further conclusion except for cherry wood. For the sake of simplicity, we present in this paper the samples with relevant results: vinegars acetified and aged with the same wood (n = 52) and vinegars in contact with cherry wood either during acetification (n = 18) or ageing process (n = 18).

Finally, for the sake of comparison, we analysed some vinegars that had been acetified in different woods and aged in glass vessels as a control (n= 10).

A total of 98 samples of wine vinegar were analyzed in this study.

Four digital sample codes were used in this order: the first digit corresponds to the substrate (F, T or M); the second digit corresponds to the type of barrel in which samples were acetified (A: acacia; C: cherry; S: chestnut; R: oak); the third digit is the type of barrel used for ageing (A: acacia; C: cherry; S: chestnut; R: oak) or bottle ageing (B); and the fourth digit is the time of sampling (0, 45, 180, 365, 545 days).

2.2. LC-DAD analysis of phenolic compounds

The LC of non-anthocyanin phenols was analysed using an Agilent Series 1100 system equipped with a quaternary pump (Series 1100 G1311A), automatic injector (Series 1100 G1313A) and degasser on line (Series 1100 G1379A). A UV/Vis diode detector (Series 1100 G1315B) coupled to a Chemstation HP A.10.02 (HP/Agilent) was used for

125 detection. The column was a Merck LiChroCART 250-4 Superspher 100 RP-18.
126 Duplicate samples were filtered through a Millex-LCR 13mm filter before injection.
127 The chromatographic conditions have been described by Betés-Saura et al. (1996). The
128 method uses a binary gradient: A (glacial acetic acid/water pH 2.65), B (20% A + 80%
129 acetonitrile) programmed in a gradient as follows: 0 min (100% A); 5 min (98% A +
130 2% B); 10 min (96% A + 4% B); 15 min (90% A + 10% B); 30 min (80% A + 20% B);
131 35 min (70% A + 30% B); 40 min (100% B); 45 min (100% A); 60 min (100% A). The
132 sample volume injected was 50 µl. The flow rate was 1.5 $mL \cdot min^{-1}$ and the temperature
133 was set at 40 ºC. Identification was based on both retention time and matching the UV-
134 Visible spectra to the corresponding standards. Quantification was performed by
135 external calibration with respective standards at 280 and 320 nm in accordance with the
136 maximum absorbance of each compound. Once the spectral matching succeeded results
137 were confirmed by spiking with respective standards to achieve a complete
138 identification by means of the peak purity test. The peaks that did not fulfill these
139 requirements were not quantified.
140 The standards of 49 phenolic compounds were purchased from Fluka [3-(4-
141 hydroxyphenyl) propionic acid, 5-methylfurfural, acacetin, acetosiringone, benzoic acid,
142 caffeic acid, cinnamic acid, gallic acid, *p*-coumaric acid, coniferyl alcohol, (-)-
143 epicatechin, furfuryl alcohol, gallic ethyl ester, quercetin glucoside, homovanillic acid,
144 isovanillin, *m*-hydroxybenzoic acid, methyl gallate, *p*-hydroxybenzoic acid, quercetin,
145 tyrosol and vanillyl alcohol], Sigma [(+)-taxifolin, (-)-epicatechin gallate, vanillic acid,
146 2-furfuraldehyde, ellagic acid, ferulic acid, gentisic acid, sinapic acid, syringic acid, (-)-
147 catechin, coniferaldehyde, (-)-epigallocatechin, ethyl vanillate, protocatechualdehyde,
148 resveratrol, sinapaldehyde and syringaldehyde], Merck [vanillin], Safc [ethyl vanillin
149 and 5-hydroxymethylfurfural] and Chromadex [3-o-methylgallic acid, 5-

hydroxyisovanillic acid, acetovanillone, caftaric acid, procyanidin-B1, procianydin-B2 and procyanidin-B3].

2.3. LC-MS/MS analysis of taxifolin

Chromatographic separation was performed using a PelkinElmer Series 200 LC system (Wellesley, USA) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, USA) consisting of a hybrid triple quadrupole linear ion trap (QqQ_{LIT}) mass spectrometer equipped with an electrospray ion source. LC analyses were performed on a 250 x 4.6 mm Zorbax SB-C18 reversed-phase column with a particle size of 3.5 µm (Agilent). The flow rate was 0.4 ml min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water, and (B) acetonitrile. Both components contained 0.1% formic acid (v/v). The elution profile was: 50% B (2 min), a linear gradient to 80% B (15 min). The injection volume was 20 µl.

Multiple Reaction Monitoring (MRM) was applied where the parent ions and fragment ions were monitored at Q1 and Q3, respectively. The transitions for the taxifolin are: 303.0/285.0 and 303.0/124.9; the collision energies (CE) were -10 V and – 25 V, respectively; and the peak retention time (t_R) was 7.30 min.

For LC-ESI-MS/MS analyses, the mass spectrometer was set to the following optimised tune parameters: curtain gas 35 psi, ion spray voltage –4500 V, source temperature 350ºC, source gas 30 psi, declustering potential −60 V, and entrance potential −10 V. A dwell time of 300 ms was set for each transition.

175 *2.4. ESI-MS and MS/MS analysis*

176

177 Negative mode ESI-MS and MS/MS were performed using a QTRAP LC/MS/MS
178 system equipped with an electrospray ion source. Sample dissolved in acetonitrile:
179 water (50:50 v/v) containing 0.1% formic acid was infused at 10 µl*min^{-1}. The capillary
180 voltage was -4500 V, the declustering potential was -30 V, and the collision energy was
181 set at -30 V.

182

183 *2.5. Other parameters*

184

185 The total phenol index (TPI) was determined by the Folin-Ciocalteu micro-method
186 proposed by Waterhouse (2001). Results were expressed as gallic acid equivalents
187 (GAE).
188 Total monomeric anthocyanins (TA) were estimated by a pH differential method (Giusti
189 & Wrolstad, 2001). Absorbance ($A_{\lambda vis\text{-}max}$) was measured at 520 nm and the molar
190 extinction coefficient of malvidin-3-glucoside (M3G) was 20200 L cm^{-1} mol^{-1}. Results
191 were expressed as mg M3G/L.
192 Dry extract was determined gravimetrically according to the Spanish Official Methods
193 (Presidencia del Gobierno, 1977) and expressed as g/100 mL vinegar.

194

195 *2.6. Sensory analysis*

196

197 The sensory panel was made up of seven experts previously trained in the sensory
198 analysis of vinegar (Tesfaye et al., 2002a). Duplicate triangle and descriptive analyses

were made according to international protocols (ISO 4120:1983 and ISO 6658:1985, respectively). Vinegar samples were tasted by direct olfaction (Tesfaye et al., 2002a). Nine attributes (ethyl acetate, pungent sensation, wine character, woody aroma, red fruit, sweet aroma, bitter almond, vanilla and raisin qualities) were formally selected by consensus following the methodology for the descriptive analysis (Stonem & Sidel, 2004).

2.7. Statistical analysis

Statistical analyses were performed by means of Statistica software (Statsoft, 2001). One-way analysis of variance (ANOVA) was used to test significant differences. Multivariate analysis of data included standard linear discriminant analysis.

3. Results and discussion

3.1. Total phenol index (TPI)

Tables 2-4 show the TPI values in the 12 ageing experiments (3 vinegar substrates x 4 types of wood) in different woods. The kind of wood clearly influences the evolution of the TPI.

For balsamic vinegars, increases (30-50%) were significant for samples aged in cherry, chestnut and oak woods. These results agree with those obtained by Tesfaye et al. (2002b) for Sherry vinegars aged in oak barrels. Samples aged in acacia wood did not show significant changes in the TPI during ageing (Figure 1) whilst those aged in chestnut wood increased significantly.

224 *3.2. Total anthocyanins (TA)*

225

226 As is known, TA decreases during the ageing of red wines probably due to
227 polymerisation. Moreover, TA values were significantly lower in wines aged in
228 American oak than in Spanish oak (Hernandez et al., 2007). In our case, TA also
229 decreases during ageing. The highest decrease was observed in vinegars aged in cherry
230 wood (Figure 2), in agreement with the decrease in TPI in these samples.

231

232 *3.3. Dry extract*

233

234 The mean initial and final values for dry extract, expressed in g/100mL, were as
235 follows: group F (9.02-10.62); group T (2.60-3.25) and group M (14.20-17.86). Dry
236 extract increased during ageing for the three groups F, T and M. The average increase
237 was 15%, 20% and 20% at 365, 365 and 545 days, respectively. We did not find
238 significant differences between the types of wood. This increase is favoured because
239 water is lost through the pores of the barrel (diffusion and evaporation), which should
240 be taken into account when the results are interpreted.

241

242 *3.4. Phenolic compounds*

243

244 We determined a total of 20 phenolic compounds in the samples analysed. Tables 2 and
245 3 show the phenolic composition and furanic derivatives of red wine vinegars. Table 4
246 provides the same information for balsamic vinegars. The following compounds were
247 only present in red wine vinegars with minor changes after one year of ageing: caftaric
248 acid (247.73-226.15 mg/L), tyrosol (23.38-24.38 mg/L), syringic acid (9.11-8.94 mg/L),

2-furfuraldehyde (0.36-0.78 mg/L) and ferulic acid (0.71-0.93 mg/L). As they do not change significantly during ageing, they are not included in tables. Below the results and the variables under study—influence of wood type and ageing time—are discussed.

3.4.1. Wood type

Taxifolin has not been previously reported in wine vinegars. Taxifolin is a dihydroflavonol (dihydroquercetin), a secondary metabolite of plants. This dihydroflavonol had been previously described in different *Prunus sp* (Shimomura et al., 1989; Bilia, Cecchini et al., 1993). Recently, it has been identified in *Prunus serotina* (Mayer et al., 2006) and for first time in *Prunus dulcis* (almond) (Monagas et al., 2007). On the other hand, taxifolin has been found in wines (Baderschneider & Winterhalter, 2001; Pozo-Bayón et al., 2003), aromatic bitter (4.82 mg/L) and cherry liqueur (5.25 mg/L) (Rodtjer et al., 2006) but it has never been described in vinegars before. The high price of cherry wood probably limits its use to high quality products such as traditional balsamic vinegar.

We only identified taxifolin in samples from red wine vinegars (group F and T) which had been in contact with cherry wood (Tables 2, 3 and 5). We confirmed its identity by LC-MRM MS/MS analysis. The vinegars in this study are in contact with cherry wood during the acetification process, during the ageing process or during both of them. The acetification lasted 1.5 months for group F and 5 months for group T (Table 1). The end of acetification is the starting point of ageing (point zero). Thus the longer the contact with cherry wood during acetification is, the higher the concentration of taxifolin. During ageing in cherry wood, taxifolin reaches a maximum concentration at 180 days (in both F and T) (Figure 3) and then it decreases (Tables 2 and 3). In the vinegars

274 acetified in cherry woods and subsequently aged in bottles, taxifolin concentration
275 decreases significantly ($p< 0.05$) over 365 days of ageing. If vinegars are acetified in
276 cherry wood and aged in barrels made of a different wood (acacia, oak or chestnut),
277 taxifolin disappears at 180 days (Table 5). Conversely, in those vinegars acetified in
278 other woods (acacia, oak or chestnut) and aged in cherry, the concentration of taxifolin
279 increases during ageing. Thus, taxifolin seems to be a marker for vinegar samples aged
280 in cherry wood.
281 Chestnut wood (*Castanea sativa*) was formerly used to transport wine and to produce
282 ABT. Chestnut has also been studied because it is of interest for the tannin industry
283 (Peng et al., 1991; Viriot et al., 1994; Lampire et al., 1998), and the ageing of wine
284 (Climaco & Borralho, 1995; Vivas et al., 1996) and brandy (Canas et al., 1999; Caldeira
285 et al., 2002; Caldeira et al., 2006; Caldeira et al., 2006). The gallic acid concentration of
286 the three vinegar groups acetified and aged in chestnut wood increases considerably
287 during ageing (Tables 2-4) in agreement with the results obtained by Cerezo et al.,
288 (2008). This favours the formation of gallic ethyl ester (Tables 2 and 3).
289 We were able to quantify ellagic acid only in those vinegars aged in chestnut and oak
290 wood (Tables 2-4). The presence of ellagic acid has been explained by the hydrolysis of
291 numerous chestnut and oak wood ellagitannins (Peng et all., 1991; Klumpers et al.,
292 1994; Viriot et all., 1994). Thus, ellagic acid is found at autstanding levels in brandies
293 aged in chestnut wood (16 - 61 mg/L) (Canas et all., 1999).
294 An unknown peak was recorded in all vinegar samples (group F, T and M) which had
295 been in contact with acacia wood during ageing or acetification. During ageing its area
296 increased. It presents a spectrum like-flavonol (λ_{max} 280-320 nm) (Figure 4). In order
297 for it to be identified, vinegars were enriched with the flavonoid acacetin, but it did not
298 match this identity. The peak was isolated and ESI-MS and MS/MS experiments were

299 performed. The MS spectrum showed a pseudomolecular ion $[M-H]^-$ at *m/z* 303 and the
300 MS/MS spectrum showed a different fragmentation pattern from that of taxifolin (data
301 not shown). The t_R determined by LC-DAD analysis was also different: the unknown
302 peak elutes at 14.4 min and taxifolin at 23.7 min. Barry et al. (2005) found a peak with
303 the same MS spectrum in extracts of acacia.
304 To summarize, wood type is associated with major changes in the following phenolic
305 compounds: taxifolin, gallic acid, gallic ethyl ester and ellagic acid.
306
307 *3.4.2. Ageing time*
308
309 Ageing time favours the release of phenolic compounds, especially aldehydes, in red
310 vinegars. Vanillin was only identified at 365 days of ageing, 2-furfuraldehyde at 180
311 days and protocatechualdehyde at 45 days or 180 days and, after that, it disappeared.
312 This compound is present in white (Natera et al., 2003; Tesfaye et al., 2002b; Alonso et
313 al., 2004; respectively) and red wine vinegars (Natera et al., 2003) with 1, 2 or even 5
314 years of ageing in wood.
315 Wood toasting favours the release of phenolic aldehydes in wines or macerates (Cadahía
316 et al., 2001; Doussot et al., 2002; Matejicek et al., 2005). Tesfaye et al. (2002b)
317 determined a number of aldehyde compounds (protocatechualdehyde, syringaldehyde,
318 vanillin, 2-furfuraldehyde, *p*-hydroxybenzaldehyde and coniferaldehyde) in white
319 vinegars aged in toasted wood barrels. Since the wood barrels in the present study are
320 not toasted, the aldehydes are released later and in lower amounts.
321 HMF was only identified in balsamic vinegars (group M) and together with 2-
322 furfuraldehyde its concentration increased during ageing in balsamic vinegars (Table 4).
323 In fact, this vinegar was produced from cooked must and as a consequence of sugar

degradation, a higher amount of furanic compounds were produced (Antonelli et al., 2004). Thus, at the starting point of ageing (0 days) the concentration of HMF is high (212-237 mg/L). There are at least three processes to explain the increase in furanic compounds during ageing. First, the process of concentration as a consequence of water loss during ageing. Total acidity remained practically constant (5.7-6.3 g/100 mL) and the dry extract increased by 20%. As furanic compounds double their concentration (Table 4) another explanation must be considered. Furanic derivatives have been ascribed to hemicellulose degradation (Abad et al., 1997) and the autohydrolysis of wood (Garrote et al., 2007) as a consequence of wood toasting (120-160º C). Conversely, Canas et al. (1999) detected no furanic derivates in untreated chestnut wood as in our case. In fact, in red vinegar no HMF was detected and 2-furaldehyde did not increase during ageing. Therefore, the vinegar substrate composition must explain the differences. Masino et al. (2005) observed that sugar degradation in traditional balsamic vinegar may also occur during ageing and that HMF formation is not limited to the step of must cooking. The results suggest that the degradation of the remaining sugars may explain the increase in HMF and 2-furfuraldehyde in balsamic vinegars obtained from cooked must.

In summary, 4 compounds (HMF, 2-furfuraldehyde, protocatechualdehyde and vanillin) were affected as a consequence of ageing time.

3.4.3. Statistical analysis

This study contained samples acetified and aged in the same type of wood barrel (group F; n= 16, T; n= 16 and M; n= 20). As variables, we selected those phenolic compounds

348 which accounted for significant differences (p<0.05) when pairs of woods or ageing
349 times (0-365 days) were compared.
350 We performed linear discriminant analysis (LDA) to check the validity of phenolic
351 compounds for classifying samples according to the kind of wood and ageing time. The
352 classification function was created using the standard method. The classification
353 function for group F included the variables previously proposed as wood markers (gallic
354 acid, gallic ethyl ester, taxifolin and ellagic acid) and vanillic and caffeic acid proved
355 useful for discriminant purposes. With this function the samples from group F were
356 100% correctly classified. As can be seen in Figure 5, the samples are grouped
357 according to the kind of wood. The classification function created for group T included
358 all the above variables except caffeic acid. With this function the samples were 100%
359 correctly classified (Figure 6). Multivariate analysis was performed for group M with
360 only two variables (gallic acid and ellagic acid) that showed significant differences
361 between the pairs of woods. With this function the samples were 95% correctly
362 classified. Despite the high probability, the Wilks´ Lambda (λ_W) of the discriminant
363 function is higher (λ_W = 0.06296) than of the groups F (λ_W = 0.00132) and T (λ_W =
364 0.00061) (Ramis & García, 2001). As a consequence, the samples in group M are not
365 well separated according to the kind of wood.
366 The variables used to create the function for classifying the samples according to ageing
367 time in group F were protocatechualdehyde, *p*-hydroxybenzoic acid, vanillic acid and
368 vanillin. In group T, the variables were all the ones mentioned above except vanillic
369 acid. And in group M, the variables were 5-hydroxymethylfurfural, 2-furfuraldehyde,
370 caffeic acid and *p*-coumaric acid. The results were discrete for red vinegars (62.5 %) but
371 excellent for balsamic vinegars (100 %).
372

373 *3.5. Sensory analysis*

374

375 The panel carried out 54 triangle tests to differentiate between woods and 36 triangular
376 tests to differentiate between vinegars at different ageing times (see tables 6 and 7,
377 respectively). In general, differences between woods are better perceived in red vinegars
378 than in balsamic vinegars (Table 6) in agreement with results from LDA. The longer the
379 contact in wood, the better the sensorial differences are perceived (Table 6).
380 The panel was not able to differentiate between samples aged in chestnut wood during
381 ageing (Table 7). Thus, chestnut wood is inert from a sensorial point of view for one
382 year of ageing.
383 The vinegars` sensory profiles were built using the marks given for each attribute by the
384 panel. Figures 7-9 show the spider charts for vinegars aged for one year. As can be seen,
385 woody aroma, sweet aroma and vanilla perception have the highest marks (p<0.05) in
386 vinegars aged in oak wood. The red fruit note was highest (p<0.05) for vinegars aged in
387 cherry woods and increased as ageing progressed in vinegars aged in cherry wood
388 (Figure 10). On the other hand, the scores for ethyl acetate, pungent sensation, woody
389 flavour and general impression increased during ageing in the different woods. These
390 results agree with those obtained in a previous study on acetification in different woods
391 (Cerezo et al., 2008). Finally, the highest scores for general impression were given to
392 the vinegars aged in cherry and oak woods.

393

394 **4. Conclusions**

395

396 Impact of wood on the phenolic composition of vinegar is clearly evidenced during
397 ageing. TPI, TA, phenol compounds and sensory profile were influenced by the kind of

wood during ageing. In fact, taxifolin has been identified for the first time in vinegar as a cherry wood marker. Different vinegars´ sensory profile were developed to each wood and evolved along ageing.

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Figure caption

Figure 1. Evolution of TPI during the ageing in acacia and chestnut wood barrels from different groups (F, T and M).

Figure 2. Evolution of TA in aged vinegars in cherry wood barrels (group F and T).

Figure 3. LC chromatogram of vinegar (group T) acetified in cherry wood and 0 days of ageing (top), and vinegar acetified and aged in cherry wood along 180 days (bottom).

Figure 4. Chromatogram and spectrum of the unknown peak from vinegars in contact with acacia woods.

Figure 5. Plot of the two first roots issued from Discriminant analysis for group F of samples.

Figure 6. Plot of the two first roots issued from Discriminant analysis for group T of samples.

Figure 7. Sensory analysis. Spider chart of aged vinegars (365 days) in the different woods from group F (A= acacia, R= oak, S=chestnut, C=cherry).

Figure 8. Sensory analysis. Spider chart of aged vinegars (365 days) in the different woods from group T (A= acacia, R= oak, S=chestnut, C=cherry).

Figure 9. Sensory analysis. Spider chart of aged vinegars (365 days) in the different woods from group M (A= acacia, R= oak, S=chestnut, C=cherry).

Figure 10. Sensory analysis. Spider chart of vinegars from group F aged in cherry wood at different ageing time.

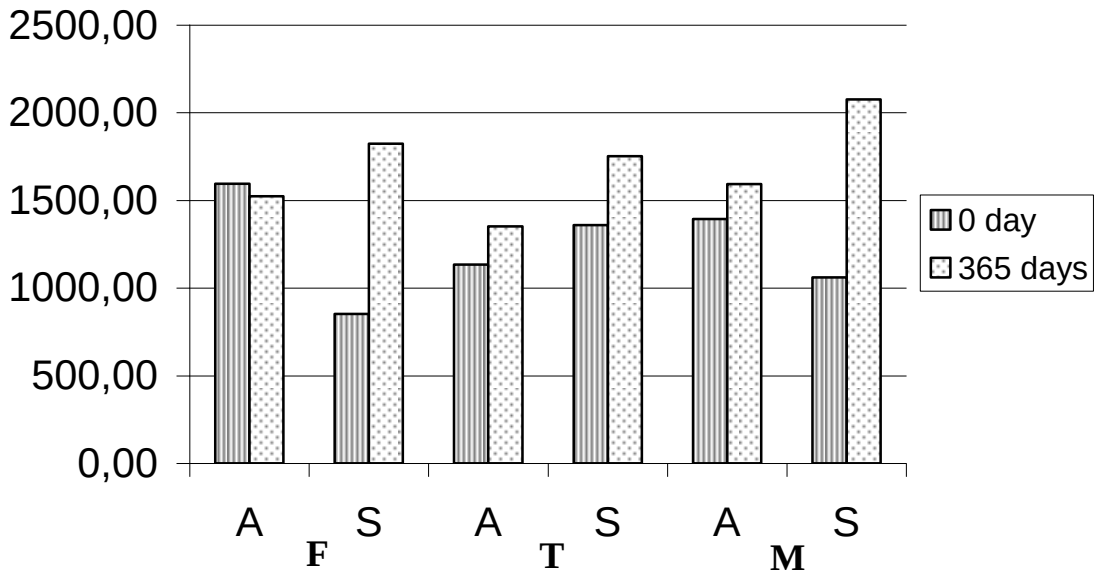

Figure 1. Ana B. Cerezo.

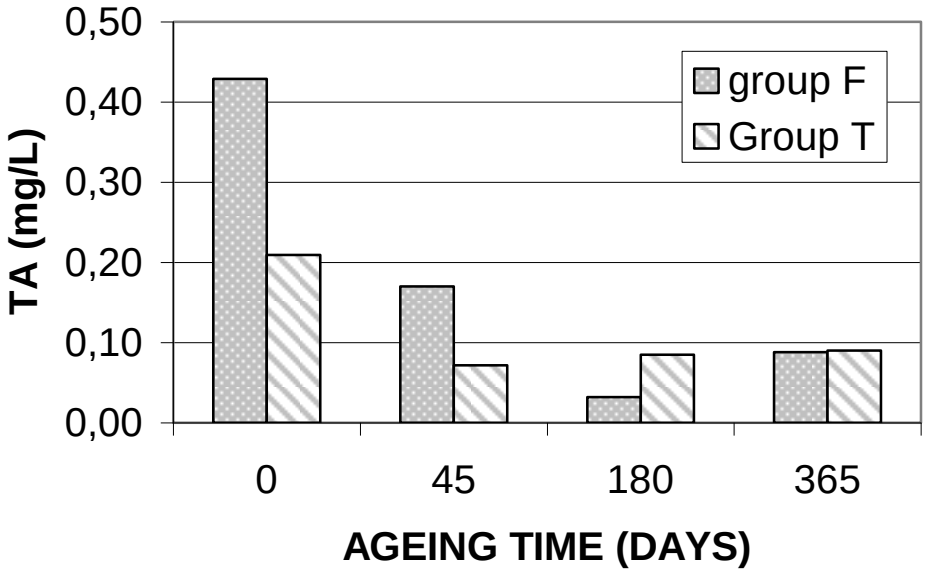

Figure 2. Ana B. Cerezo.

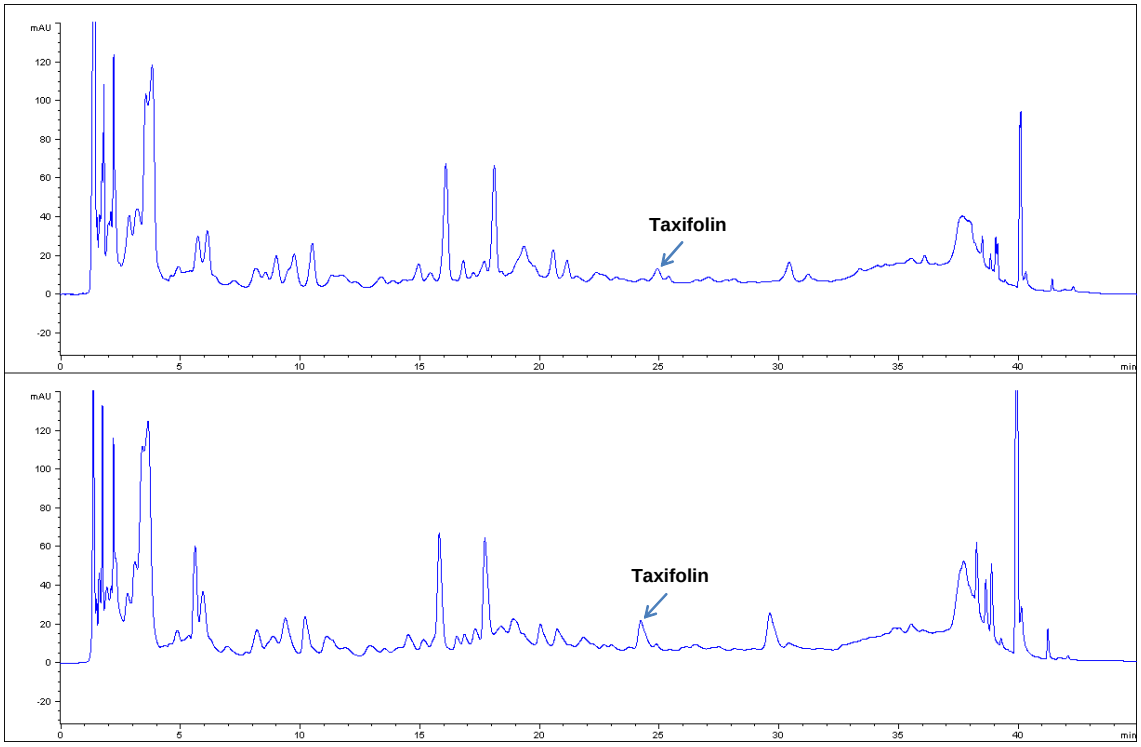

Figure 3. Ana B. Cerezo

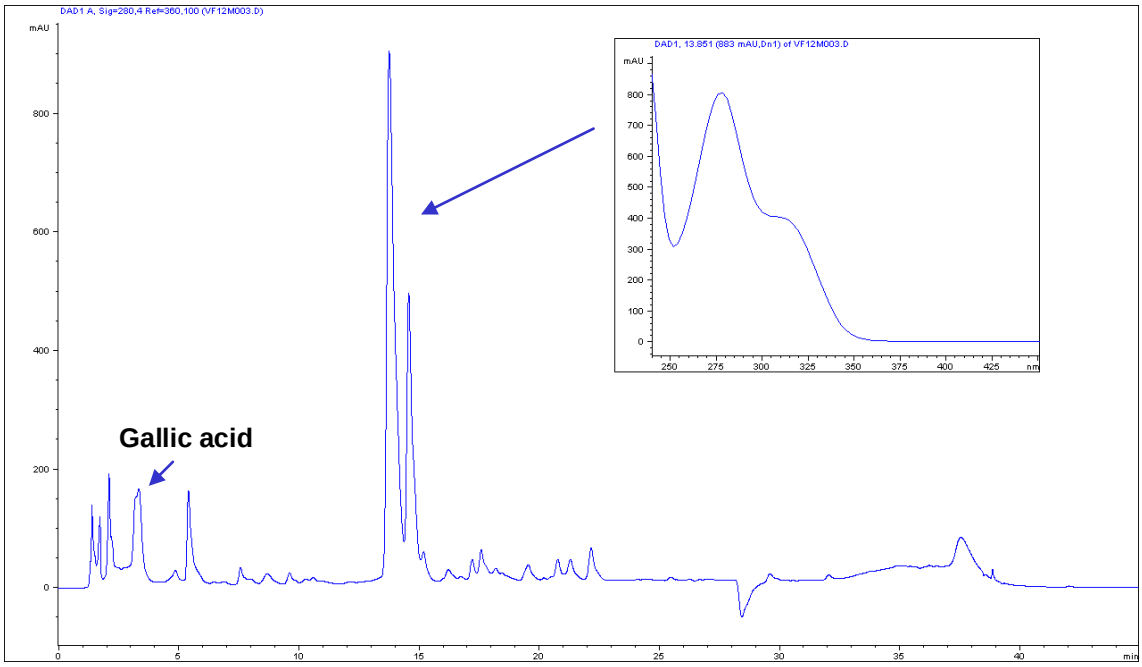

Figure 4. Ana B. Cerezo.

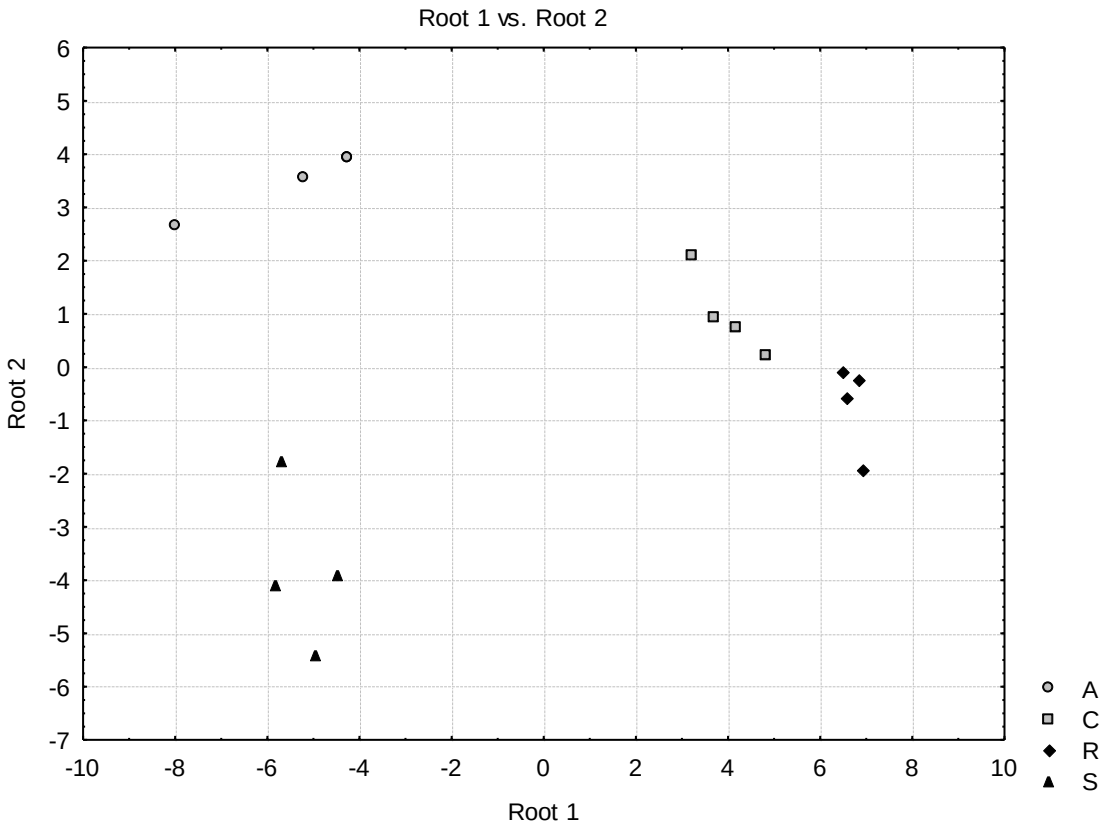

Figure 5. Ana B. Cerezo.

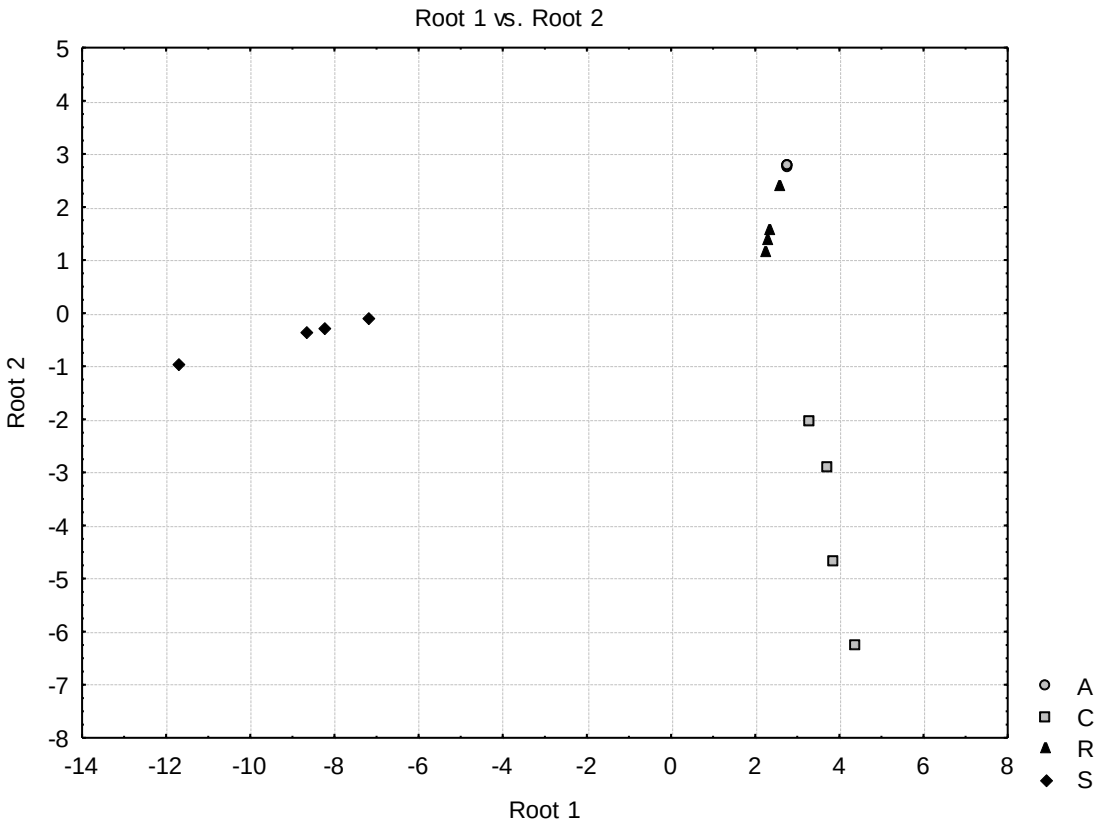

Figure 6. Ana B. Cerezo.

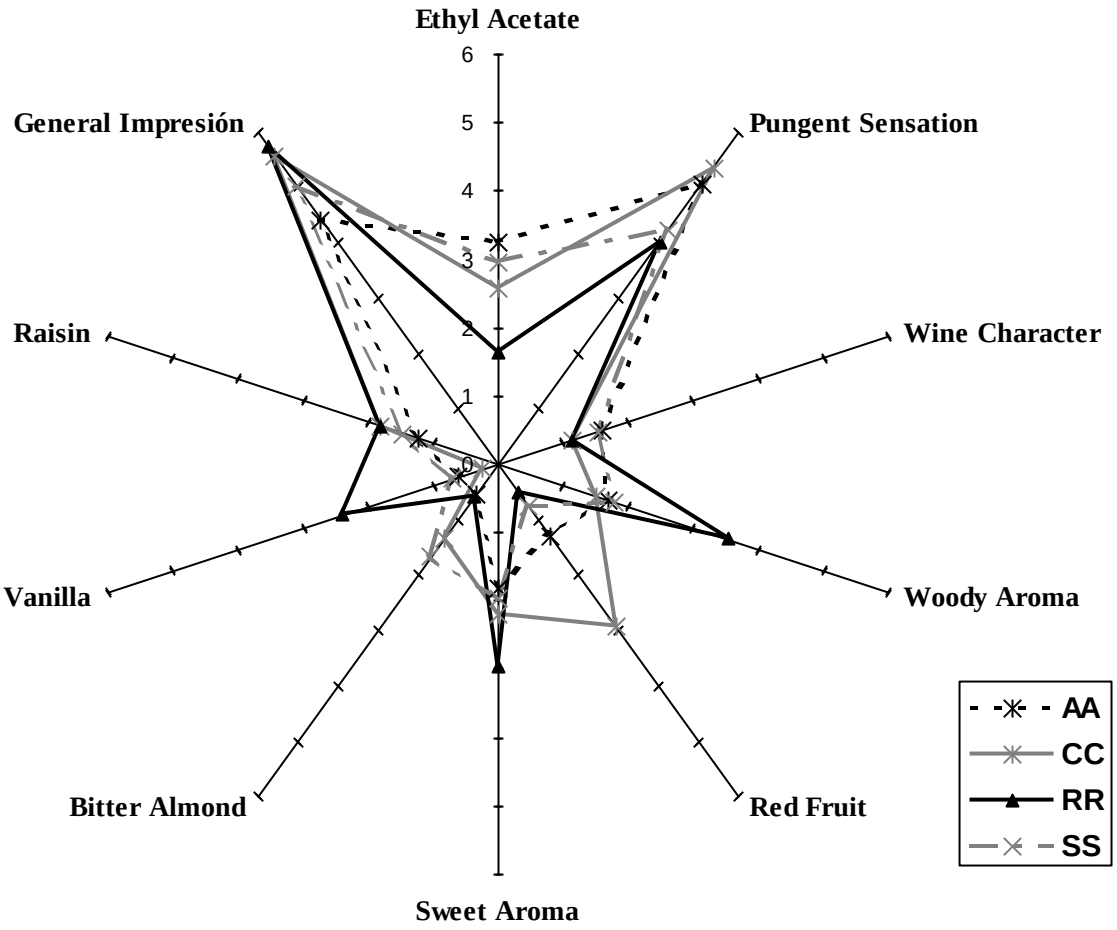

Figure 7. Ana B. Cerezo.

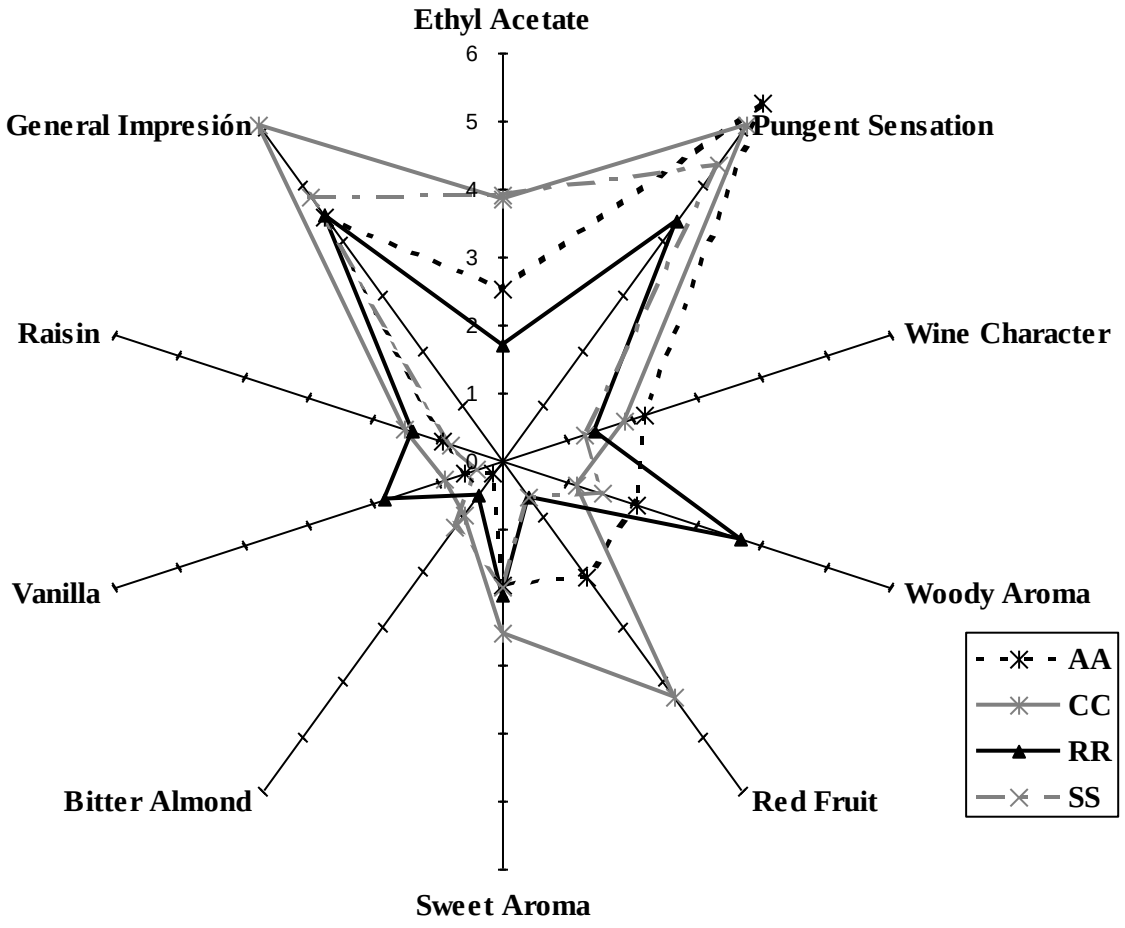

Figure 8. Ana B. Cerezo.

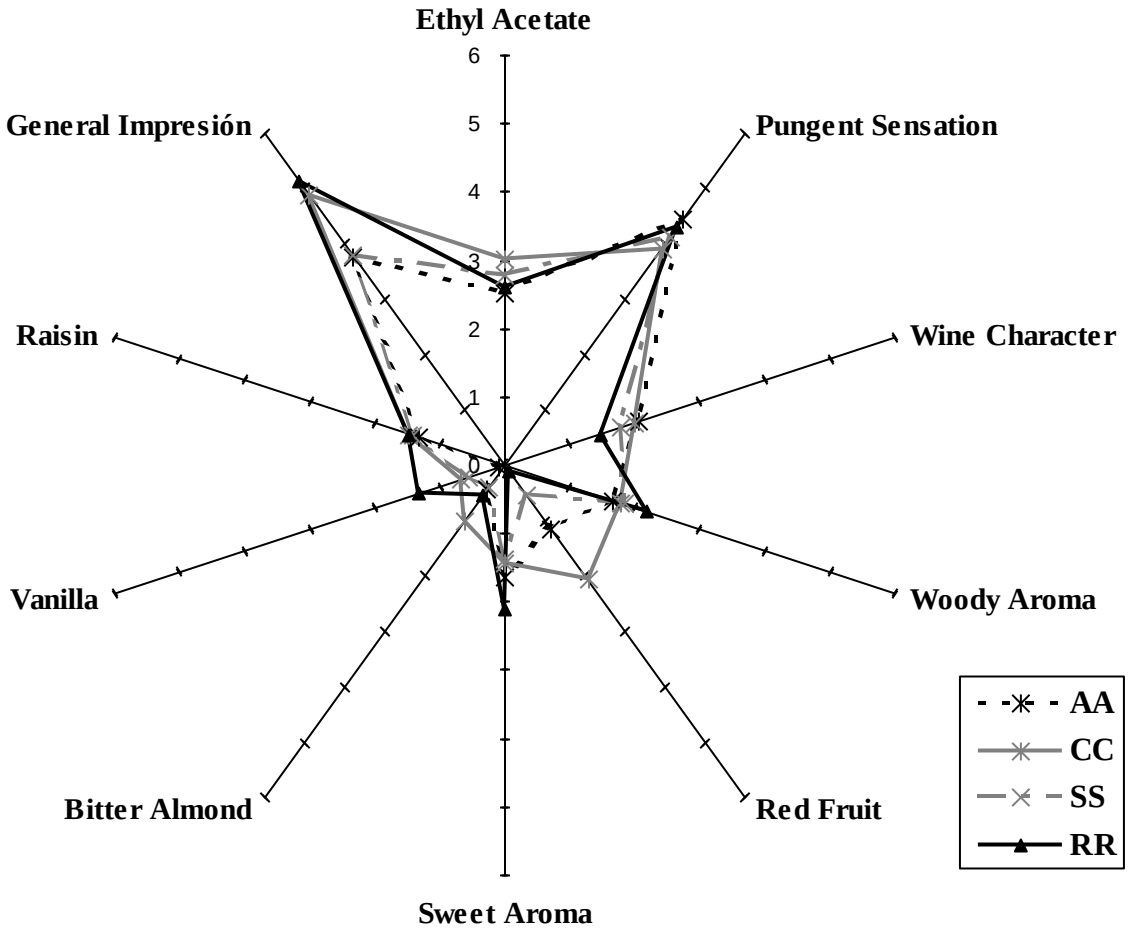

Figure 9. Ana B. Cerezo.

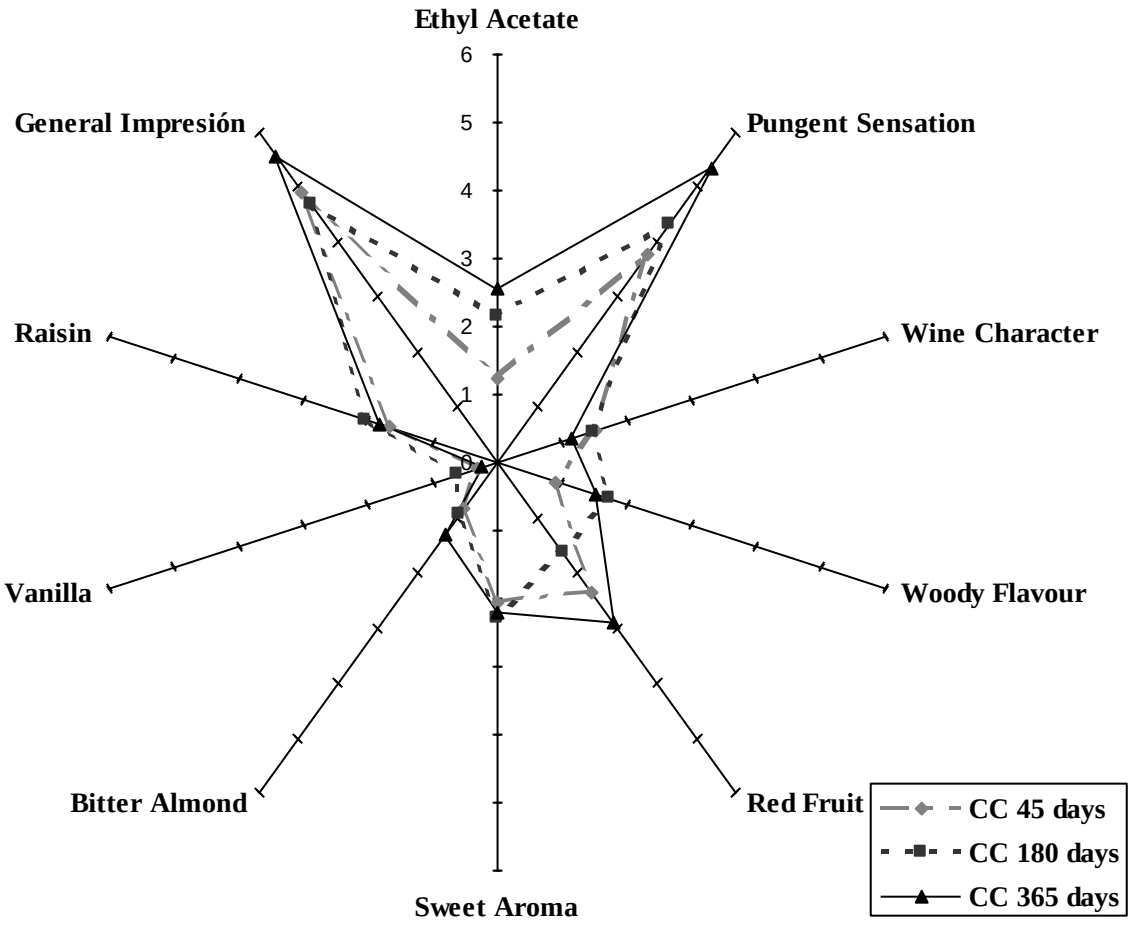

Figure 10. Ana B. Cerezo.

Group	Vinegar substrates
F	Alcohol (%v/v): 2.9 Acidity: 6.5 g/100 mL pH 2.8 Variety: 100% Grenache Dry extract: 9.02 – 10.62 g/100 mL Time of acetification: 1.5 months
T	Alcohol (%v/v): 4.1 Acidity: 6.4 g/100 mL pH 2.8 Variety: Grenache mostly Dry extract: 2.60 – 3.25 g/100 mL Time of acetification: 5 months
M	Alcohol (%v/v): 3.6 Acidity: 6.2 g/100 mL pH: 2.8 White wine vinegar + acetified cooked must (45:55) (var. Trebbiano) Dry extract: 14.20 – 17.86 g/100 mL

Table 1. Characteristics of vinegar substrates (mean values).

Wood	Days	TPI	Compounds										
			1	5	6	8	10	11	13	14	15	19	20
Acacia	0	1594±121	40.90±0.04	-	5.1±1.3	-	-	-	-	-	-	-	-
	45	1723±61	40.9±0.3	3.04±0.09	2.9±0.8	-	-	-	-	-	1.72±0.03	-	-
	180	1690±141	38.32±0.021	-	3.67±0.01	-	-	-	-	5.72±0.02	-	-	-
	365	1523±20	42.05±0.03	-	-	-	-	-	0.90±0.01	1.56±0.03	-	-	-
Cherry	0	1066±20	33.0±0.4	-	4.54±0.01	-	3.64±0.06	-	-	-	-	-	-
	45	837.14±20.20	33.21±0.14	-	4.37±0.06	2.35±0.06	3.86±0.06	-	-	-	-	-	-
	180	265.1±60.6	34.43±0.03	1.47±0.03	4.83±0.01	2.52±0.04	3.98±0.14	3.61±0.01	-	4.92±0.05	-	1.94±0.01	-
	365	465.50±20.20	36.01±0.00	-	-	-	2.77±0.06	2.47±0.00	0.73±0.01	1.40±0.02	-	1.69±0.03	-
Oak	0	1409±61	38.66±0.08	-	4.48±0.02	-	4.95±0.00	-	-	-	-	-	-
	45	1394±202	40.08±0.09	-	4.48±0.05	2.69±0.00	4.78±0.04	-	-	-	-	-	-
	180	437.9±60.6	46.23±0.03	-	4.82±0.03	-	4.23±0.04	4.85±0.04	-	5.64±0.05	-	-	-
	365	879.5±40.4	49.48±0.04	-	-	-	2.87±0.03	4.79±0.02	0.90±0.05	2.36±0.05	-	-	4.65±0.03
Chestnut	0	851.4±40.4	120.81±0.10	-	3.34±0.04	-	3.48±0.09	-	-	17.82±0.19	-	-	3.18±0.04
	45	1966±81	163.0±0.4	5.71±0.01	-	3.28±0.04	-	4.85±0.02	-	17.07±0.06	-	-	-
	180	1960±0	334.79±0.12	5.06±0.02	-	-	-	5.29±0.01	-	26.65±0.04	-	-	1.93±0.00
	365	1823±40	384.0±0.5	-	-	-	-	-	-	22.6±0.3	-	-	4.77±0.13

1: gallic acid; 5: protocatechualdehyde; 6: p-hydroxybenzoic acid; 8: Methyl gallate; 10: vanillic acid; 11: caffeic acid; 13: vanillin; 14: gallic ethyl ester; 15: isovanillin; 19: taxifolin; 20: ellagic acid. (-): n.d.

Table 2. Means and Standard Deviations of the concentration (mg/L) of the selected phenolic compounds and TPI (mg/L) in vinegars acetified and aged in the same wood (group F).

Wood	Days	TPI	Compounds									
			1	5	6	10	11	13	14	17	19	20
Acacia	0	1133±212	38.90±0.09	-	4.30±0.00	-	-	-	-	-	-	-
	45	1368±687	36.81±0.13	5.46±0.05	-	-	-	-	-	-	-	-
	180	1011±20	36.02±0.20	5.36±0.00	-	-	-	-	-	-	-	-
	365	1351±20	35.24±0.12	-	-	-	-	-	-	-	-	-
Cherry	0	559.01±47.14	34.7±4.7	-	5.22±0.10	4.00±0.06	-	-	-	-	1.59±0.03	-
	45	365.71±0.00	33.30±0.03	-	-	5.08±0.06	3.32±0.01	-	-	-	2.66±0.01	-
	180	411.18±20.20	31.81±0.03	-	-	4.80±0.08	3.63±0.03	-	-	-	3.45±0.00	-
	365	379.73±20.20	35.33±0.09	-	-	3.18±0.05	1.85±0.05	-	-	-	2.13±0.01	-
Oak	0	576.4±23.6	38.03±0.14	-	5.31±0.07	4.53±0.11	-	-	-	2.38±0.00	-	-
	45	636.98±20.20	42.43±0.10	-	-	1.31±0.00	3.59±0.01	-	-	-	-	5.1±0.3
	180	466.6±60.6	45.39±0.06	-	-	4.14±0.00	4.10±0.03	-	-	-	-	5.56±0.04
	365	722.72±20.20	54.79±0.04	-	-	3.46±0.10	2.36±0.03	0.53±0.05	-	-	-	3.27±0.12
Chestnut	0	1360±47	211.1±0.5	-	5.56±0.02	5.20±0.12	-	-	26.86±0.23	2.41±0.01	-	-
	45	1251±40	292.0±0.4	5.06±0.19	-	5.76±0.03	-	-	30.47±0.04	-	-	10.04±0.11
	180	1439±61	384.4±.03	4.74±0.00	-	6.02±0.03	4.19±0.04	-	38.9±0.3	-	-	2.78±0.08
	365	1751±20	445.3±0.9	-	-	4.57±0.01	-	1.40±0.11	28.66±0.01	-	-	1.88±0.00

1: gallic acid; 5: protocatechualdehyde; 6: p-hydroxybenzoic acid 10: vanillic acid; 13: vanillin; 14: gallic ethyl ester; 19: taxifolin; 20: ellagic acid; 11: caffeic acid; 17: syringaldehyde. (-): n.d.

Table 3. Means and Standard Deviations of the concentration (mg/L) of the selected important phenolic compounds and TPI (mg/L) in vinegars acetified and aged in the same wood (group T).

Wood	Days	TPI	Compounds					
			1	2	4	11	16	20
Acacia	0	1393±24	33.72±0.23	230.98±0.05	1.7±0.5	2.02±0.16	1.90±0.02	-
	45	1577±0	34.50±0.15	231.51±1.06	2.89±0.11	-	2.12±0.00	-
	180	1359±71	36.65±0.09	285.3±0.3	2.56±0.05	-	2.04±0.01	-
	365	1927±24	38.82±0.23	370.66±0.02	3.91±0.01	-	2.06±0.03	-
	540	1593±71	43.5±0.7	440.95±0.15	4.15±0.06	-	2.57±0.02	-
Cherry	0	809.31±47.14	26.97±0.13	216.1±0.3	2.15±0.00	2.14±0.01	1.88±0.01	-
	45	1106±141	27.22±0.03	212.08±0.04	4.5±0.4	-	1.98±0.01	-
	180	925.3±70.7	24.87±0.13	253.71±0.14	4.92±0.13	-	1.88±0.01	-
	365	1092±71	25.65±0.18	356.48±0.05	6.98±0.00	-	2.03±0.02	-
	540	1293±24	24.0±0.7	421.3±0.3	7.07±0.01	-	2.44±0.03	-
Oak	0	842.67±47.14	30.33±0.04	237.17±0.08	1.94±0.22	2.35±0.12	2.05±0.01	2.90±0.03
	45	925.3±70.7	30.47±0.04	255.88±0.08	5.45±0.01	-	2.02±0.02	3.66±0.01
	180	1026±24	30.85±0.11	286.07±0.21	5.98±0.01	-	1.93±0.01	4.9±0.3
	365	1092±71	32.58±0.14	382.78±0.20	6.87±0.05	-	1.92±0.02	5.2±0.3
	540	1210±0	34.55±0.23	438.6±0.5	7.05±0.03	-	2.26±0.05	4.44±0.03
Chestnut	0	1060±24	57.11±0.06	219.8±0.4	1.73±0.07	2.35±0.12	2.01±0.01	4.2±0.3
	45	1275±94	87.66±0.03	241.0±0.6	4.80±0.13	-	2.42±0.02	4.07±0.07
	180	1475±94	123.9±0.8	290.38±0.21	5.61±0.03	-	2.00±0.01	9.4±0.3
	365	1693±71	158.1±0.9	407.51±0.02	7.88±0.07	-	2.06±0.03	9.70±0.18
	540	2076±94	199.6±0.6	478.4±0.5	8.56±0.08	-	2.51±0.01	7.3±0.3

1: gallic acid; 2: 5-HMF; 4: 2-furfuraldehyde; 11: caffeic acid; 16: p-coumaric acid; 20: ellagic acid. (-): n.d.

Table 4. Means and Standard Deviations of the concentration (mg/L) of the selected important phenolic compounds and TPI (mg/L) in vinegars acetified and aged in the same wood (group M).

Group	Acetification process	Aging process	Aging time (days)	Taxifolin	
				Mean	SD
T	C	-	0	1.59	0.03
T	C	Bottle	365	0.94	0.02
F	A	-	0	-	-
F	A	C	45	-	-
F	A	C	180	1.55	0.00
F	A	C	365	1.39	0.08
F	R	-	0	-	-
F	R	C	45	-	-
F	R	C	180	1.63	0.03
F	R	C	365	1.03	0.06
F	S	-	0	-	-
F	S	C	45	1.29	0.03
F	S	C	180	1.67	0.05
F	S	C	365	2.01	0.01
F	C	-	0	-	-
F	C	S	45	-	-
F	C	S	180	-	-
F	C	S	365	-	-
T	A	-	0	-	-
T	A	C	45	1.82	0.03
T	A	C	180	2.48	0.00
T	A	C	365	2.60	0.02
T	R	-	0	-	-
T	R	C	45	1.96	0.02
T	R	C	180	2.81	0.01
T	R	C	365	3.09	0.06
T	S	-	0	-	-
T	S	C	45	1.79	0.07
T	S	C	180	2.36	0.05
T	S	C	365	1.62	0.01
T	C	-	0	1.59	0.03
T	C	A	45	-	-
T	C	A	180	-	-
T	C	A	365	-	-
T	C	-	0	1.59	0.03
T	C	R	45	1.76	0.04
T	C	R	180	-	-
T	C	R	365	-	-
T	C	-	0	1.59	0.03
T	C	S	45	1.53	0.04
T	C	S	180	-	-
T	C	S	365	-	-

A: acacia; C: cherry; R: oak; S: chestnut.

Table 5. Means and Standard Deviations of the concentration (mg/L) of taxifolin and gallic acid in differents aged vinegars.

Comparisons		Probability level		
		Group F	Group T	Group M
45 days	AA-CC	n.s.	n.s.	n.s.
	AA-SS	5 %	n.s.	5 %
	AA-RR	1 %	n.s.	5 %
	CC-SS	1 %	5 %	n.s.
	CC-RR	5 %	5 %	n.s.
	SS-RR	5 %	0.1 %	n.s.
180 days	AA-CC	n.s.	5 %	n.s.
	AA-SS	5 %	5 %	n.s.
	AA-RR	0.1 %	0.1 %	5 %
	CC-SS	0.1 %	0.1 %	0.1 %
	CC-RR	5 %	0.1 %	0.1 %
	SS-RR	0.1 %	0.1 %	n.s.
365 days	AA-CC	n.s.	1 %	1 %
	AA-SS	1 %	1 %	n.s.
	AA-RR	0.1 %	1 %	5 %
	CC-SS	1 %	n.s.	0.1 %
	CC-RR	1 %	0.1 %	n.s.
	SS-RR	0.1 %	0.1 %	n.s.

n.s.; no significant

Table 6. Results of triangle tests of the different vinegars at the same aging time and different woods (A= acacia; C=cherry; R=oak; S=chestnut).

Comparison	Probability level		
	Group F	Group T	Group M
$AA_{45} - AA_{180}$	n.s.	1 %	n.s.
$AA_{180} - AA_{365}$	n.s.	0.1 %	n.s.
$AA_{45} - AA_{365}$	n.s.	0.1 %	1 %
$CC_{45} - CC_{180}$	5 %	n.s.	0.1 %
$CC_{180} - CC_{365}$	n.s.	5 %	n.s.
$CC_{45} - CC_{365}$	5 %	0.1 %	0.1%
$SS_{45} - SS_{180}$	n.s.	n.s.	n.s.
$SS_{180} - SS_{365}$	n.s.	n.s.	n.s.
$SS_{45} - SS_{365}$	n.s.	n.s.	5 %
$RR_{45} - RR_{180}$	5 %	n.s.	5 %
$RR_{180} - RR_{365}$	n.s.	0.1 %	n.s.
$RR_{45} - RR_{365}$	1 %	0.1 %	n.s.

n.s.; no significant

Table 7. Results of triangle tests of the different vinegars in the same wood and different aging period (A= acacia; C=cherry; R=oak; S=chestnut).

CAPÍTULO 4 / CHAPTER 4

Ana B. Cerezo; José L. Espartero; Peter Winterhalter; M. Carmen Garcia-Parrilla & Ana M. Troncoso.

(+)-DIHYDROROBINETIN: A MARKER OF VINEGAR AGING IN ACACIA (*Robinia pseudoacacia*) WOOD

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(+)-Dihydrorobinetin: a Marker of Vinegar Aging in Acacia (*Robinia pseudoacacia*) Wood

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The use of acacia wood for the aging of vinegars is increasing because the efficient air transfer through the pores permits a good acetification rate. In this study, vinegars aged in acacia (*Robinia pseudoacacia*) wood barrels were analyzed and found to contain a characteristic compound, which increased during the aging process. This so far unknown compound was isolated by semipreparative LC and structurally identified by NMR spectroscopy. ^{1}H and ^{13}C NMR chemical shifts and optical rotation revealed its structure to be (+)-dihydrorobinetin, a dihydroflavonol identified for the first time in vinegars as a marker of aging in this kind of wood. This study also reports for the first time the complete assignment of ^{13}C NMR data for this compound. Moreover, it revealed a longer contact time with acacia wood results in higher concentrations of (+)-dihydrorobinetin found in vinegars. Another finding was that the vinegars aged with nontoasted acacia chips showed significantly higher concentrations of (+)-dihydrorobinetin than found in vinegars aged with toasted acacia chips (384.8 and 23.5 mg/L, respectively). The in vitro antioxidant activity (DPPH$^{\bullet}$ and ORAC assays) of (+)-dihydrorobinetin was also determined. (+)-Dihydrorobinetin is reported here for the first time as a chemical marker of vinegars aged in acacia wood and can be used for authenticity purposes.

KEYWORDS: Acacia; wood; vinegar; antioxidant; NMR; (+)-dihydrorobinetin; *Robinia pseudoacacia*.

INTRODUCTION

Sherry vinegar and traditional balsamic vinegar (ABT) are high-quality vinegars that are famous all over the world. Sherry wine vinegar is produced in a series of wooden oak butts in which acetification and aging take place simultaneously. ABT is obtained by a traditional method that ferments the cooked must and ages the product in a set of wooden barrels. The barrels are made in decreasing sizes (from 60 to 20 L) and of different woods (oak, chestnut, cherry, ash, juniper, and mulberry) (*1*−*4*). Both elaboration processes of high-quality vinegars require an aging period in wooden butts. During this period, the finished product develops the desired organoleptic properties that make it highly appreciated (*5*).

At present, oak is the most commonly used wood in enology for aging wines, spirits, and vinegars. Its contribution to the quality and chemical composition of the product is well-known (*6*−*9*). Nowadays, other woods such as acacia, cherry, chestnut, and mulberry are increasingly being used to make products with other sensory properties. The impact that these woods have on chemical composition and sensorial properties still needs to be evaluated (*10*−*12*).

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The EU-funded WINEGAR project (ref. COOP-CT-2005-017269) proposed using four different kinds of woods (acacia, chestnut, cherry, and oak) for wine vinegar production. The two main purposes of the project were to choose the wood with the air transfer that best fulfilled the acetification oxygen requirements and to obtain a high-quality product. Thus, the vinegars were produced by traditional means in barrels especially made for the project, and the quality and chemical composition were checked throughout the process (*13*). The chemical characterization of vinegar can be used for a variety of purposes including authentication and product classification according to quality criteria. The analysis of the vinegars aged in acacia (*Robinia pseudoacacia*) revealed a compound that was not present in vinegars aged in the other woods. This compound was likely to serve as a chemical marker of acacia wood for authenticity purposes.

The aims of the study described herein have been, first, to isolate and identify this compound; second, to evaluate its release from acacia wood; and, finally, to explore the influence of wood treatments on its concentration in vinegars. Last but not least, the antioxidant activity of this compound has been determined by DPPH and ORAC assays. To the best of our knowledge, this is the first time that this compound has been described in products aged in wood and its antioxidant activity presented.

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MATERIAL AND METHODS

Samples. Three wines (two red Garnacha variety wines, groups G1 and G2; and one white Trebbiano variety wine, group T) were acetified and subsequently aged in acacia barrels (*R. pseudoacacia*) of 60 L capacity. The barrels were made especially for this experiment and were not toasted. Surface culture acetification took place until the acetic degree reached 6°. After filtration to remove the acetic acid bacteria, the aging period started. Samples were taken at 0, 1.5, 6, and 12 months.

One red wine vinegar (Cabernet sauvignon variety, group C), fermented with submerged culture in a laboratory acetator, was aged with acacia wood chips (*R. pseudoacacia*). Toasted and nontoasted chips (5−10 mm) were tested in different proportions (0.5 and 1%, w/v). The samples were analyzed at 0, 15, and 30 days of aging with chips.

Isolation. *Sample Preparation for Semipreparative LC.* An Amberlite XAD7 column (Fluka; 43 cm × 22 mm) was first conditioned with 200 mL of methanol and then with 500 mL of Milli-Q water. A total of 700 mL of diluted vinegar sample with water (1:1) was loaded onto the column and cleaned with 500 mL of water to remove sugars, proteins, organic acids, and minerals. Elution was performed with 300 mL of methanol/water (70:30). The flow rate was 1 drop/s. The eluate was concentrated with a rotary evaporator under vacuum, frozen, and freeze-dried to obtain 1.6 g of extract from 1 L of vinegar.

Semipreparative LC. The unknown compound was isolated using a preparative LC system equipped with a binary pump (Knauer K-1001) and a Knauer injection valve with a 500 μL loop. It was detected with an UV detector (Knauer K-2600), and data were processed by Eurochrom 2000 V.2.05. The column was a Luna 5 μm C18 (2), 250 × 10 mm (Phenomenex). Samples were filtered through a Chromatofil PET 0.45 μm membrane filter before injection. Two different solvents were used as the mobile phase: solvent A (glacial acetic acid in water at pH 2.65) and solvent B (20% solvent A mixed with 80% acetonitrile), at a flow rate of 4 mL/min and a the following linear gradient: 0 min, 10% B; 32 min, 10% B; 36 min, 100% B; 41 min, 10% B.

Identification. *ESI-MS and MS/MS Analysis.* Negative mode ESI-MS and MS/MS were performed using a QTRAP LC-MS/MS system equipped with an electrospray ion source. A sample dissolved in acetonitrile/water (50:50 v/v) containing 0.1% formic acid was infused at 10 μL min^{-1}. The capillary voltage was −4500 V, the declustering potential was −30 V, and the collision energy was set at −30 V.

NMR Analysis. NMR spectroscopy was used to examine the isolated compound in a solution (10 mg/mL) of 99.6% DMSO-d_6. Spectra were recorded at 303 K on a Bruker Avance-500 spectrometer operating at 500.13 MHz (^{1}H) and 125.75 MHz (^{13}C), respectively. Chemical shifts are given in parts per million (ppm), using the DMSO signals (2.49 and 39.5 ppm for ^{1}H and ^{13}C, respectively) as references. The 2D homonuclear proton double-quantum filtered correlation experiment (DQF-COSY) (*14*) was performed in the phase-sensitive mode using the Bruker standard pulse sequence. The 2D heteronuclear one-bond proton−carbon correlation experiment (*15*) was recorded in the ^{1}H detection mode (inverse detection) via single-quantum coherence (HSQC). ^{13}C decoupling was achieved by the GARP scheme. This experiment was slightly modified by implementing an editing block in the sequence. The long-range proton−carbon correlation experiment (HMBC) (*16*) was collected in the ^{1}H detection mode. The delay time was 80 ms between the first and second pulses, and there were 96 scans per increment. The pure absorption 2D NOESY experiment was performed using a mixing time of 400 ms.

Polarimetric Analysis. Polarimetric analysis was performed to check the enantiomeric nature of the compound. Optical rotation ($[\alpha]_D$) was determined with a Perkin 341 polarimeter at 25 °C. The dihydrorobinetin concentration was 0.45 g/100 mL; Me_2CO/H_2O (1:1) was used as solvent.

Vinegar Samples Analysis. *LC-DAD.* LC analysis of the unknown phenolic compound was performed using an Agilent series 1100 system equipped with a quaternary pump (series 1100 G1311A), automatic injector (series 1100 G1313A), and degasser (series 1100 G1379A). Detection was accomplished using an UV−vis diode detector (series 1100 G1315B) coupled to a Chemstation HP A.10.02 (HP/Agilent). The column was a Merck LiChroCART 250-4 Superspher 100 RP-18 1.16056.0001. Duplicate samples were filtered through a Millex-LCR 13 mm filter before injection. The chromatographic conditions had previously been used for vinegar analysis (*17*). The method uses a binary gradient, A (glacial acetic acid/water pH 2.65) and B (20% A + 80% acetonitrile), programmed in the following gradient: 0 min, 100% A; 5 min, 98% A + 2% B; 10 min, 96% A + 4% B; 15 min, 90% A + 10% B; 30 min, 80% A + 20% B; 35 min, 70% A + 30% B; 40 min, 100% B; 45 min, 100% A; 60 min, 100% A. The sample volume injected was 50 μL. The flow rate was 1.5 mL min^{-1}, and the temperature was set at 40 °C. Quantification was performed by external calibration at 280 nm.

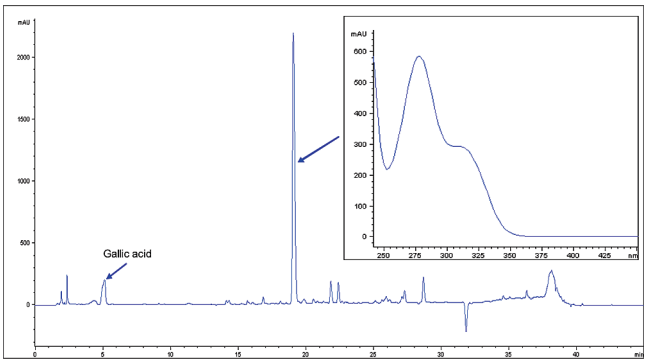

Figure 1. LC-DAD chromatogram at 280 nm of red wine vinegar (Garnacha variety) aged in an acacia wood barrel for 12 months.

Antioxidant Activity of (+)-Dihydrorobinetin. *ORAC Assay.* The ORAC assay is based on a previously reported method with slight modifications (*18*). Briefly, it is as follows: 50 μL of sample or Trolox is mixed with 100 μL of fluorescein (45 nM) and 50 μL of AAPH (15 mM). Fluorescence is recorded for 60 min (the excitation wavelength is set at 485 nm and the emission wavelength at 528 nm). Measurements were taken in triplicate in a multidetector microplate reader (Synergy HT, Biotek). Trolox was used as a calibration standard (0.5−9.5 μM).

The results were calculated as ORAC values from the differences between the blank and the sample areas under the fluorescein decay curve. They are expressed as Trolox equivalents.

DPPH Method. A total of 0.1 mL of (+)-dihydrorobinetin (0.328−0.164 mM) or sample (1:50, v/v) or Trolox (0.000−1.000 mM) was added to 3.9 mL of DPPH$^\bullet$ (0.063 mM), all in methanolic solution. Absorbance was measured at 515 nm after 60 min (when the reaction reached equilibrium). The blank reference cuvette contained methanol. The initial absorbance was close to 0.700 in all cases. All measurements were performed in triplicate. A linear curve was obtained by plotting four concentrations of (+)-dihydrorobinetin against the respective Trolox concentrations. The Trolox value corresponding to a 1 mM concentration of (+)-dihydrorobinetin is, by definition, its TEAC value.

RESULTS AND DISCUSSION

Peak Isolation and Identification. Figure 1 shows the LC-DAD chromatogram of a vinegar aged in acacia wood for 12 months. As can be seen, there is a major peak at t_R = 14.4 min and 280 nm, which was previously reported in vinegars aged in acacia wood, and its area increases with aging (*19*).

To isolate this still unknown compound from the vinegar extract, semiprepartive LC was applied. After concentration and freeze-drying, 264.1 mg of compound/L vinegar was obtained. Its MS spectrum showed a molecular ion $[M]^{\bullet+}$ at m/z 304, and the MS/MS spectrum showed the following fragmentation pattern of m/z: 275, 168, 149, 139, 137, 121.

NMR analysis was carried out to make a complete structural determination. Hence, ^{1}H and ^{13}C NMR chemical shifts were unequivocally assigned by 2D DQF-COSY, NOESY, HSQC, and HMBC experiments (**Table 1**). A relative 2,3-trans configuration was evident from the homonuclear coupling constant for protons in these positions ($J_{2,3}$ = 11.1 Hz), which indicated a trans-diaxial disposition between them. Moreover, this fact was further confirmed by the existence of an intense NOE effect between H-2′ and H-3, as observed in the 2D-NOESY spectrum

Table 1. ^{1}H and ^{13}C NMR Data of Isolated Compound [a]

position	δ_H	δ_C [b]	HMBC [b,c]
2	4.89 (d, 11.1)	83.7	3, 4, *1′*, *2′*
3	4.31 (d, 11.1)	72.6	2
4		192.1	
5	7.61 (d, 8.6)	128.6	*4*, 7
6	6.51 (dd, 1.9)	110.8	8, 10
7		164.9	
8	6.28 (d, 1.9)	102.3	6, 10
9		162.7	
10		112.0	
1′		127.4	
2′	6.40 (s)	106.9	*2*, 3′, 4′
3′		145.6	
4′		133.3	
OH in 3	5.44 (bs)		
phenolics OH	8.89 (bs)		

[a] Recorded in DMSO-d_6; chemical shifts are expressed as δ values in ppm; signal multiplicities and coupling constants (Hz) are shown in parentheses. [b] Carbons showing long-range couplings to proton, $^{n}J_{CH}$ ($n \geq 2$). [c] Inter-ring couplings are shown in *italics*.

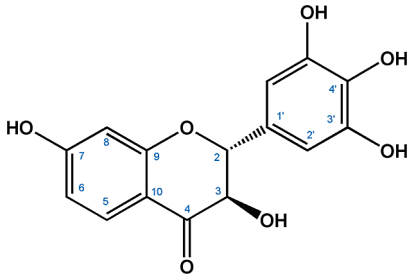

Figure 2. Molecular structure of (+)-dihydrorobinetin.

Table 2. (+)-Dihydrorobinetin Concentration (Milligrams per Liter) in Vinegar Samples Aged in Nontoasted Acacia Wood Barrels

	substrates[a]		
aging time (months)	G1	G2	T
0	65.34 ± 0.20	129.7 ± 0.3	36.7 ± 1.9
1.5	124.7 ± 4.8	325.86 ± 0.17	109.1 ± 0.7
6	300.64 ± 0.18	412.10 ± 0.11	214.4 ± 2.7
12	304.7 ± 0.6	438.58 ± 2.16	266.3 ± 0.9

[a] G1 and G2, different substrates of Garnacha variety; T, Trebbiano variety.

(not shown). Our ^{1}H NMR data agreed with data obtained previously by Saleh et al. (*20*) for dihydrorobinetin (**Figure 2**). Nevertheless, as far as we know, this is the first study to include the complete assignment of ^{13}C NMR data for this compound. The confirmation of these assignments (including quaternary carbon) was obtained from the $^{1}H-^{13}C$ long-range correlation NMR experiment (HMBC) (**Table 1**). Of significance was the appearance of cross-peaks corresponding to the long-range couplings of H_2 (C ring) with $C_{1'}$ (B ring), of $H_{2'}$ (B ring) with C_2 (C ring), and of H_5 (A ring) with C_4 (C ring). Dihydrorobinetin has been previously described in the stemwood of *R. pseudoacacia* L. (*21*) and in the leaf extracts of some *Cordia* spp. (*22*).

Optical rotation analysis revealed an $[\alpha]_D$ value of +12.6° (C 0.45, 1:1 Me_2CO/H_2O), which is in agreement with the data obtained by Weinges for (+)-dihydrorobinetin (*23*). Our work reveals that (+)-dihydrorobinetin can be released from the acacia wood to vinegar during aging.

Quantification of (+)-Dihydrorobinetin. Table 2 shows the concentration of (+)-dihydrorobinetin in vinegars throughout the aging in acacia barrels. As aging is considered to start after the acetification in acacia wood was complete, there must be a period of 1.5−5 months of wood contact before aging begins. It was found that the longer the contact with acacia wood was, the higher the concentration of (+)-dihydrorobinetin. The concentration of (+)-dihydrorobinetin after 12 months of aging was far higher (266.3−438.58 mg/L) than concentrations reported during vinegar aging for other phenolic compounds such as aldehydes, which can reach concentrations of up to 39 mg/L (*9*, *24*). The concentration of (+)-dihydrorobinetin was higher in red vinegars than white ones because the contact period with acacia wood was longer during the acetification process (**Table 2**).

Table 3. (+)-Dihydrorobinetin Concentration (Milligrams per Liter) in Vinegar Samples (Group C) Aged with Different Percentages (w/v) and Thermal Treatment of Acacia Wood Chips for Different Aging Times

		treatment	
% (w/v)	aging time (days)	nontoasted	toasted
0.5	15	234.6 ± 0.9	11.1 ± 0.4
	30	246.8 ± 1.4	11.6 ± 0.5
1	15	388.5 ± 4.4	20.06 ± 0.04
	30	384.8 ± 2.5	23.50 ± 0.13

The concentration of (+)-dihydrorobinetin in vinegar aged with nontoasted acacia chips was 20 times higher the concentration in vinegar aged with toasted acacia chips (**Table 3**). The thermal treatment of the wood, then, is crucial for the release of (+)-dihydrorobinetin into the vinegar. As expected, the higher the content of wood chips was, the greater the release of (+)-dihydrorobinetin (**Table 3**). Also, 15 days of aging is enough to obtain the highest increases of (+)-dihydrorobinetin (84−100%), and the delay of 15 days more has no significant effects (**Table 3**). This agrees with the data obtained by Tesfaye et al. (*17*) about the release of other phenolic compounds in vinegars aged with chips.

Antioxidant activity of (+)-Dihydrorobinetin. The TEAC and ORAC values of (+)-dihydrorobinetin were 1.57 ± 0.03 mM and 0.51 ± 0.06 μmol of Trolox/μmol of (+)-dihydrorobinetin. The main characteristics of the molecular structure influencing the antioxidant activity are the number of −OH groups in the B ring and the double bond in the C ring, which increase electron delocalization. (+)-Dihydrorobinetin is a dihydroflavonol with three −OH groups in the B ring but no conjugated double bond in the C ring. As a consequence, its antioxidant activity was slightly lower than that of myricetin (*25*). However, it should be taken into account as its concentration in the finished product is high. Indeed, we assessed the antioxidant value of the vinegar without (+)-dihydrorobinetin (group C) and this vinegar aged for 30 days with a (+)-dihydrorobinetin concentration at 384.8 mg/L (**Table 3**). The TEAC values were 11.64 ± 0.41 mM Trolox equivalents for vinegar and 14.65 ± 0.35 mM Trolox equivalents for aged vinegar. The ORAC results were 5912.6 ± 707.8 and 7466.8 ± 1281.7 μM Trolox equivalents. If we consider the concentration of (+)-dihydrorobinetin in the final product and the antioxidant activity values for this compound, the (+)-dihydrorobinetin explains 13.65 or 8.6% for the TEAC or ORAC value. This contribution to overall antioxidant activity of the product is higher than that of other phenolic compounds described in wines (*26*).

Conclusions. (+)-Dihydrorobinetin is released into products aged in barrels made of acacia. Therefore, it might be useful as a chemical marker of products aged in acacia. Nontoasted wood releases a higher amount of (+)-dihydrorobinetin than toasted wood. The in vitro antioxidant activity of (+)-dihydrorobinetin is

also reported because the high amounts of the compound in the product mean that aging in acacia wood increases its overall antioxidant activity, contributing to the functional properties of vinegars.

ABBREVIATIONS USED

ABT, traditional balsamic vinegar; EU, European Union; NMR, nuclear magnetic resonance; ORAC, oxygen radical absorbance capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; AAPH, 2,2′-diazobis(amidinopropane) dihydrochloride; DPPH•, 1,1-diphenyl-2-picrylhydrazyl; TEAC, Trolox equivalent antioxidant capacity.

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CAPÍTULO 5 / CHAPTER 5

W. Tesfaye; M. L. Morales; R. M. Callejón; **Ana B. Cerezo**; A.G. González; M. C. García-Parrilla & A. M. Troncoso.

DESCRIPTIVE SENSORY ANALYSIS OF WINE VINEGAR: TASTING PROCEDURE AND RELIABILITY OF NEW ATTRIBUTES

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This is to confirm that the paper in which you are a co-author,

Descriptive sensory analysis of wine vinegar: tasting procedure and reliability of new attributes

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is to be published in *Journal of Sensory Studies, likely in Issue 1, Volume 25, 2010*.

Best regards,

Maximo Gacula Jr.

Descriptive sensory analysis of wine vinegar: tasting procedure and reliability of new attributes

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Abstract

A procedure for the sensory analysis of vinegar was developed that provides reproducible results and minimizes the standard deviation of attributes. A new tasting protocol was proposed that leads to low dispersions of panelists' answers. The list of attributes that describe vinegars was extended to a total of 13, the importance (or discriminant utility) of which was analyzed by using Partial Least Squares Regression related techniques. The attributes selected showed a good between-run reproducibility as was shown by the DPLSR analysis on replications. The proposed methodology proved to be useful for tasting very different types of wine vinegars (ie. Sherry and red and white wine vinegars).

Practical applications

As the vinegar market is now making products from different raw materials we believed that it would be interesting to improve sensory analysis. The EU regulates the sensory analysis of oil but not of vinegar. Our manuscript describes a tasting protocol that aims to provide more reproducible results, proposes a broader vocabulary for describing vinegars and uses statistical tools to verify the reliability of new attributes.

Key words: vinegar; sensory analysis protocol; sensory profiling; PLS; descriptive and sensory analysis

1. Introduction

Vinegar can be obtained from different sugar and starch rich materials such as malt, cider, honey, grape must or fruits by a double fermentation process: alcoholic and acetic fermentations. Wine vinegar is used as a condiment to preserve and improve sensory characteristics of food, mainly in Mediterranean countries. It can be produced by traditional methods of acetification in which acetic acid bacteria are placed on the surface of the alcoholic liquid to be acetified. Oxygen transfer is limited and, as a consequence, the process is slow. However, acetification and aging occur simultaneously (Tesfaye, Morales, Garcia-Parrilla, & Troncoso, 2002a). These vinegars are expensive and appreciated in gastronomy. Nevertheless, most commercial vinegars are produced by submerged culture of acetic acid bacteria and an oxygen supply that enables the acetic degree to be reached 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 aeration is strong and, in general, sensory quality is lower (Morales, Gonzalez, Casas, & Troncoso, 2001; Romero & Cantero, 1998). Nevertheless, vinegar is more than an acidulant and those components other than acetic acid can be perceived by the senses (González-Viñas, Salvador, & Cabezudo, 1996). Recently, interest in high quality vinegars has increased and a wider variety of raw materials are now being used in their elaboration (Natera, Castro, Garcia-Moreno, Hernandez, & Garcia-Barroso, 2003).

Sensory analysis has been used to discriminate vinegar samples on the basis of the raw material (Gerbi, Zeppa, Antonelli, Natali, & Carnacini, 1997) or the elaboration method (Nieto et al., 1993; González-Viñas et al., 1996).

As sensory properties are related to price, they need to be evaluated objectively. To date there is no standardized method of performing sensory analysis of vinegars. For other products such as olive oil a systematic approach to organoleptic evaluation of the product has been described and regulated (Commission Regulation EEC, 1991).

The main difficulty of tasting 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. It also produces fatigue of the receptors in a shorter time than alcohol-derived products. One of the purposes of this research was to design a procedure for tasting vinegars that could overcome these drawbacks. What is more, if all the steps of tasting are described and followed in detail, then it is only to be expected tat the results will be more reliable. There is very little scientific literature on sensory studies of wine vinegars (Nieto et al., 1993; González-Viñas et al., 1996; Tesfaye, García-Parrilla, & Troncoso, 2002).

A variety of analytical approaches have been reported for studying the reliability of sensory data (Nielsen, Hyldig, & Sörensen, 2004). For instance, the most commonly used approaches are Generalized Procrustes Analysis (Byrne, O'Sullivan, Dijksterhuis, Bredie, & Martens, 2001), one way ANOVA combined with Principal Component Analysis (PCA) (Couronne, 1997), ANOVA with egg-shell plots (Naes, 1998), ANOVA, cluster analysis, consonance analysis and PCA (King, Hall, & Cliff, 2001), agreement coefficients and reliability coefficients (Bi, 2003) and techniques based on Discriminant Partial Least Squares Regression (DPLSR) (Thybo & Martens, 2000). In the present paper, we use typical descriptive statistical analysis and unsupervised learning display methods such as PCA for a preliminary study and techniques based on partial least squares (PLS) for futher study of variable reliability.

The aim of this paper is to improve the sensory analysis of wine vinegar by evaluating the effects of tasting protocol (structured and *ad libitum*) on panelist performance. We also aim to expand the sensory vocabulary used to evaluate vinegars and the reliability of new attributes.

2. Materials and Methods

2.1. Tasting panel

Nine non smokers between 25 and 45 years old took part in this study. They had all had theoretical and practical training in sensory analysis. Six were expert tasters with more than five years experience in tasting vinegars. Panel selection and training was described by Tesfaye et al. (2002). Basically, the judges had to prove their ability to recognize the basic tastes as required by ISO 3972: 1991. The panelists were not remunerated for participating.

2.2. Tasting cup

We used standard wine tasting cups (ISO 3591:1977) to perform this study. They were of dark glass so that colour would not influence the panelist's response (Tesfaye et al., 2002).

2.3. Vinegar Samples

Three wine vinegar samples were used to check the tasting procedure in this study: OA (vinegar obtained from red wine by surface culture), OB (commercial white wine vinegar

obtained from submerged culture) and OC (Sherry wine vinegar provided by the Regulatory Council of the Denomination of Origin).

To extend the vocabulary list, eleven different representative vinegar samples and one synthetic vinegar were tested: two Sherry wine vinegars, four red wine vinegars of the Grenache and Cabernet Sauvignon varieties, one white wine vinegar elaborated by submerged culture acetification, one commercial apple vinegar, one spirit vinegar, one commercial honey vinegar, one commercial balsamic vinegar from Modena and a 6% acetic acid water solution containing 2-furaldehyde (30 mg/L), acetaldehyde (4mg/L), benzaldehyde (4 mg/L), ethyl acetate (600 mg/L), vanillin (10 mg/L) and ethanol 2% v/v (synthetic vinegar).

To study the reliability of the attributes, a total of 12 representative wine vinegar samples were selected: four Sherry vinegars (VJ1 - VJ3), one of which was the very old Sherry vinegar called *Gran Reserva* (VJ4), six red wine vinegars made in barrels by surface culture (VR1 - VR6), one red wine vinegar made by submerged acetification (VS1), and one commercial white wine vinegar (VS2) made by submerged acetification.

2.4. Sensory tests

2.4.1. Tasting procedure

In our previous experiments judges were allowed to taste vinegars in their own way. They were given no recommendations (Tesfaye et al., 2002). However, in the present study we designed a sensory analysis procedure and asked the judges to follow it carefully. After the

organizer of the panel had instructed the judges orally, they were given the following written procedure:

The olfaction phase

- Take off the lid covering the cup.

- Swirl the liquid in the cup for 10 seconds. Incline the cup to moisten the inner surface of the cup with the sample.

- Smell the sample at the brim of the cup. Please avoid the centre where the pungent sensation is more intense. The cup must be held so that it is at an angle of 45º to the nose and can be turned smoothly. Inspirations must be slow, short, and not intense till you have your own criteria.

- Olfaction time must not be longer than 15 seconds. If you cannot decide, pause before trying again.

- Then, throw the sample away and smell the empty cup.

Then a previously developed sensory descriptive analysis for white vine vinegar was used by the panel (Morales et al., 2006).

2.4.2. Extending the vocabulary list

The task of generating vocabulary should focus on differentiating between products, rather than simply compiling a dictionary of adjectives (Murray, Delahunty, & Baxter, 2001). Thus, an initial list of 64 terms was put forward by the panel. The panel members then discussed the terms proposed in an attempt to discard repeated and synonymous words and to select the most appropriate terms. The inclusion criteria were that terms should be relevant to the product, clearly discriminate between samples, be clearly understood by each panelist

and easily detectable (ISO 11035:1994). The frequency criteria described previously (Torres Hernández, 2000) were used to select the attributes.

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 "not noticeable" and "very strong" at the left and right end points, respectively (ISO 4121: 1987).

2.4.4. Relevance and reliability of attributes

Twelve wine vinegar samples (described above) were tasted in triplicate by the sensory panel on different days, using the selected attributes on an unstructured scale (descriptive analysis). A total of 36 descriptive analyses were performed by the panel.

Two special data sets were prepared so that their relevance and validity for characterizing vinegar samples could be studied by PLS statistical analysis (Martens, Bredie, & Martens, 2000). The mean data set consisted of n = 12×3 objects (12 vinegar samples in triplicate) and p = 13 sensory variables (the mean values given by the assessors) and the Design data set consisted of n = 12×3 objects as above and p = 15 design variables for products and replicates (12 samples and 3 replications). Each vinegar sample or replication was represented in the matrix by a categorical indicator variable (with values 0 or 1). For example, the three replicates of the same vinegar sample are labeled 1 and the rest 0. Replications made for 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 using the Statistica 7 software (Statsoft, 1995) and Unscrambler 9.1 (CAMO ASA, 2004).

3. Results and Discussion

3.1. Tasting procedure

The hypothesis was that results would be more reliable if samples were tasted following a standardized protocol rather than *ad libitum*. The comparison of the proposed protocol with *ad libitum* tasting are presented in Table 1. The standard deviations for aroma intensity, richness in aroma, general impression, coconut odor, clove odor, vanilla odor, woody odor and wine character were lower when panelists followed the proposed tasting protocol. Standard deviations were higher for ethyl acetate (glue odor) and the pungent sensation when this protocol was used. Acetic acid and ethyl acetate are major volatile compounds in this matrix. Ethyl acetate can reach high concentrations in wine vinegars produced by traditional surface methods. As a consequence of all our results, we propose that further research be made to evaluate the ethyl acetate odor and pungent sensation before the cup is shaken and the other of attributes afterwards.

The panel verified that aromas such as vanilla, coconut and clove were perceived better when the proposed protocol was used probably because acetic acid and ethyl acetate did not mask them (Table 1).

Smelling the tasting cup after it had been emptied did not improve the precision of the attributes selected (Table 2). However, the panel agreed that other aromas not previously perceived were discerned. The usefulness of this procedure will be evaluated during the selection of new attributes.

3.2. Extending the vocabulary list

A further 20 attributes were selected and defined in accordance with the procedure described in section 2.4.2 (Table 3) as follows: ethyl acetate, alcohol/liquor, pungent sensation, winy, raisin, woody, clove, citrus, red fruits, vanilla, sweet aroma, bitter almond, old/leather, medicinal, apple, coconut, rancid, bacteria, cheese and sawdust/wood shaving. As some of the attributes were used to test just one particular vinegar, the panel decided to remove them from the list for purposes of simplicity. Besides, other attributes for describing negative odors like bacteria and sawdust/wood shaving were not considered suitable for descriptive analysis. If they are perceived in a vinegar, then the product should be rejected regardless of their intensity. Therefore, it was considered more practical to mark their presence/absence rather than their intensities. Hence, the list of descriptors was finally reduced to 13 attributes (Table 4) and the remaining seven (medicinal, apple, coconut, rancid, bacteria, cheese, sawdust/wood shaving) were considered as optional attributes to be included when a particular vinegar is tested.

236 *3.3. Study of the reliability of the attributes*

237

238 Finally the newly developed attributes were evaluated by using 12 wine vinegar samples.
239 Each sample was submitted to the descriptive test using the 13 new descriptors (Table 5).
240 The spider chart for a pair of Sherry wine vinegars samples can be seen in Figure 1. The
241 intensity of woody odor, vanilla, leather/old odor and ethyl acetate odor is more evident in
242 vinegar VJ2, which is older than vinegar VJ1.

243 DPLSR can be used on the mean and design data sets with sensory variables (descriptors) as
244 the X-matrix and design variables as the Y-matrix to find which sensory attributes can
245 reliably discriminate between the vinegars.

246 Basic statistics, plots of percentiles and histograms of the mean data set provide us with a
247 preliminary idea about which attributes reliably describe sensory differences. Several sensory
248 variables show a non normal distribution so an initial data analysis of the selected attributes
249 has been performed according to the box-and-whisker plot (Figure 2). This plot is a
250 graphical representation of the so-called 'five number summary' of the data set, described by
251 its end points, its lower and upper quartiles and its median value. As can be observed in
252 Figure 2, several attributes show asymmetry (skewness), but the variables were not
253 transformed (with logarithmic or squared data transformation) because this did not improve
254 results.

255 After a PCA of the mean data matrix, six principal components (PCs) arose according to
256 ensure communalities close to 0.80. The explained variance is of about 89%. The
257 contributions of attributes to PCs based on covariance indicate that no noisy variables need
258 to be removed. However, according to the plot in Figure 3, some of the attributes projected

on the factor plane of PC1 and PC2 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 with the DPLSR approach. 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 first two PLS components. As can be seen, the first component spans a wide variation in flavor (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 makes the 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, red wine vinegars), the remaining being more dispersed. Anyway, red wine vinegar samples are located at positive scores of the first component and the remaining samples at negative scores. It should be pointed out 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 because the lack of a standardized protocol was one of the main factors for response differences among panelists. This protocol guaranteed each panelist would follow certain steps and reduced the differences in the responses given by the panel. Thirteen sensory attributes were selected for

the descriptive analysis of wine vinegars. The DPLSR analysis of replications showed that the selected attributes had a good between-run reproducibility.

Acknowledgments

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355

Figures and tables

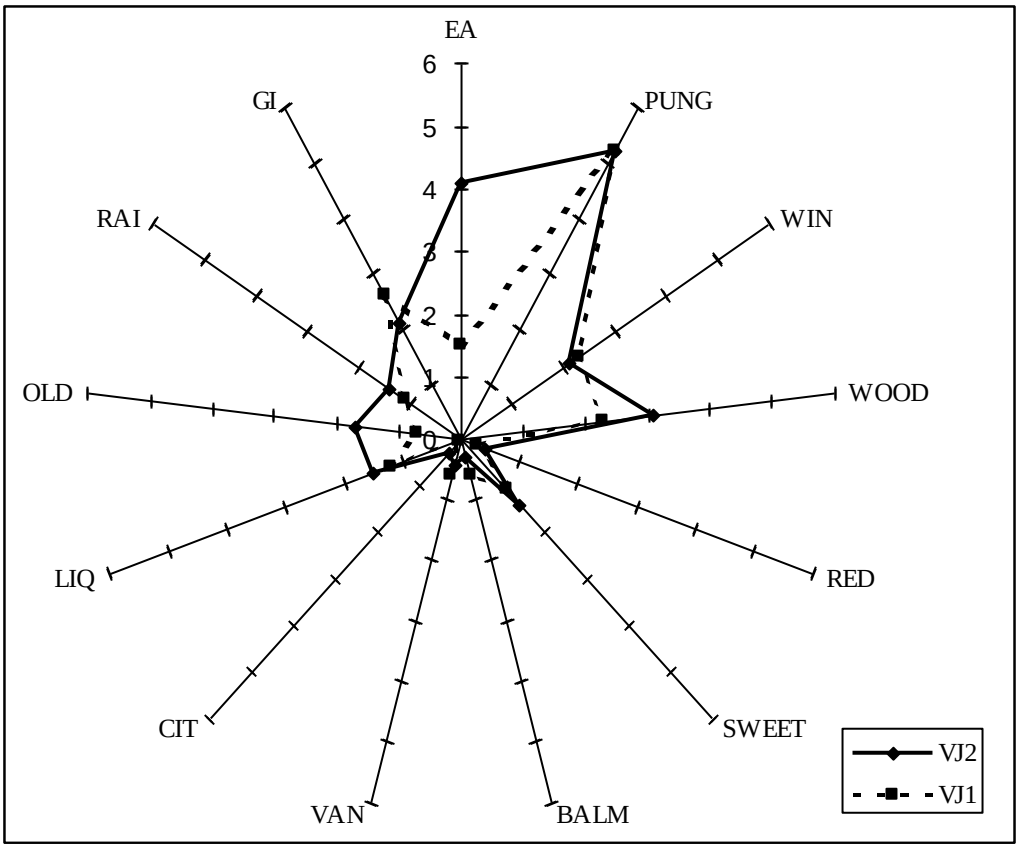
EA
PUNG
WIN
WOOD
RED
SWEET
BALM
VAN
CIT
LIQ
OLD
RAI
GI
0
1
2
3
4
5
6
VJ2
VJ1

Figure 1.

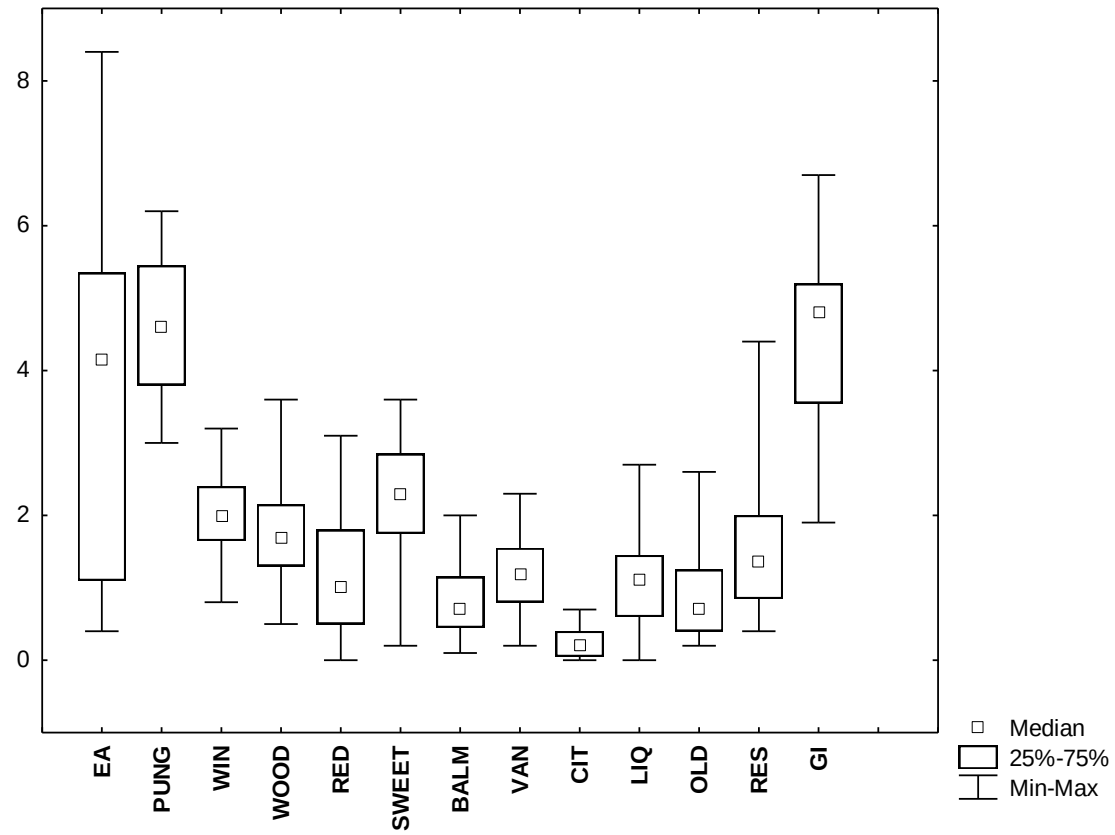
8
6
4
2
0
EA
PUNG
WIN
WOOD
RED
SWEET
BALM
VAN
CIT
LIQ
OLD
RES
GI
Median
25%-75%
Min-Max

Figure 2.

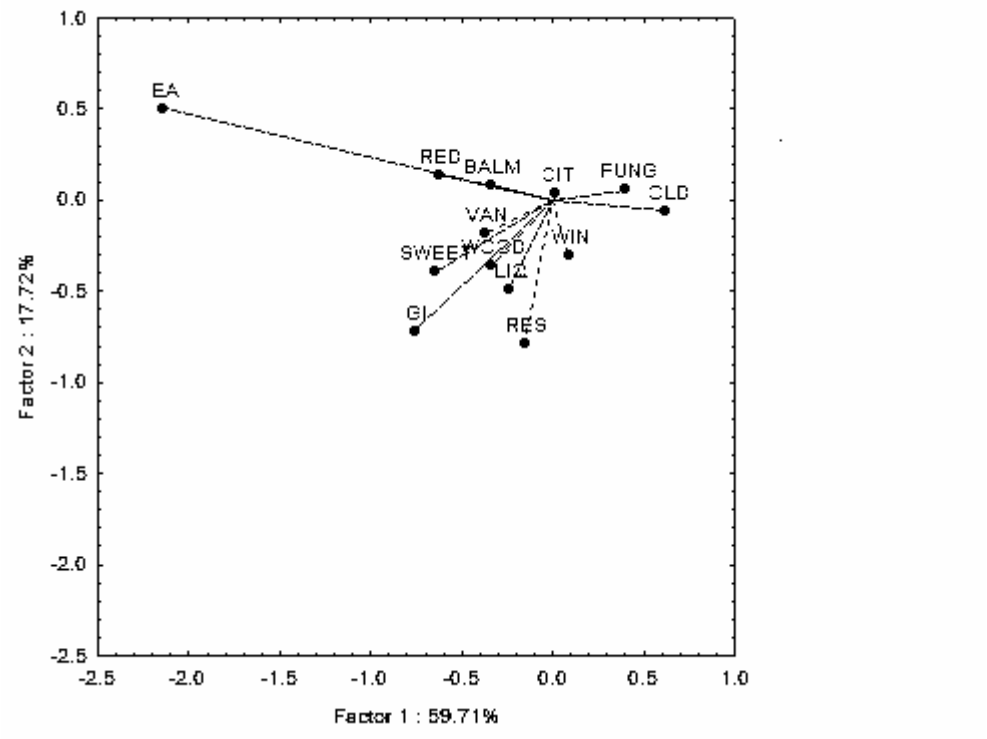
EA
RED
BALM
CIT
FUNG
CLD
VAN
SWEET
WOOD
WIN
LIZ
GI
RES
Factor 2 : 17.72%
Factor 1 : 59.71%
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0

Figure 3.

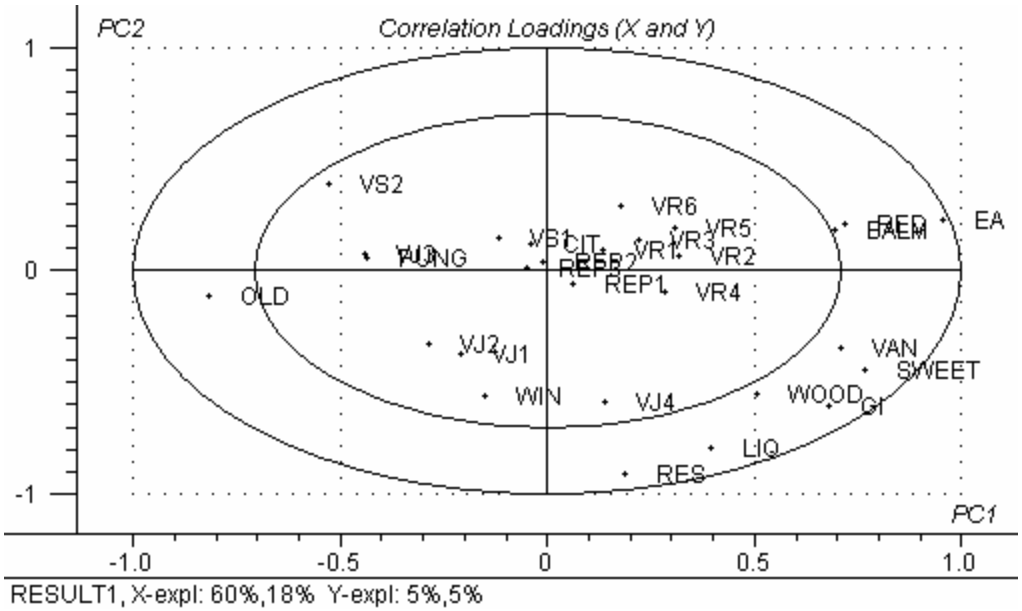
PC2
Correlation Loadings (X and Y)
1
0
-1
VS2
VR6
VR5
VR3
VR1
VR2
VR4
EA
OLD
VJ2
VJ1
VAN
SWEET
WIN
VJ4
WOOD
LIQ
RES
PC1
-1.0
-0.5
0
0.5
1.0
RESULT1, X-expl: 60%,18% Y-expl: 5%,5%

Figure 4.

Samples	Tasting procedure	Aroma Intensity	Richness in Aroma	Ethyl acetate odor	Woody odor	Wine character	Pungent sensation	General impression	Coconut odor	Clove odor	Vanilla odor
OA	PP	0.60	1.31	3.20	2.23	2.44	2.05	1.46	1.57	0.97	1.59
	AL	1.43	1.81	2.40	1.31	1.93	1.77	1.60	2.78	2.60	2.95
OB	PP	1.50	1.14	1.14	0.72	1.85	2.36	1.35	0.26	2.16	0.30
	AL	2.23	2.06	1.41	1.59	1.86	1.87	1.53	1.22	3.72	1.34
OC	PP	1.58	1.53	2.10	2.34	2.83	1.90	1.82	1.01	2.79	1.77
	AL	1.20	1.97	2.07	2.47	2.67	1.62	2.10	1.30	3.07	2.49
Mean standard deviation (±σ)	PP	1.22	1.32	2.15	1.76	2.37	2.1	1.54	0.94	1.94	1.22
	AL	2.62	1.95	1.96	1.79	2.15	1.75	1.74	1.77	3.13	2.26

PP= Following the proposed protocol, AL = *ad libitum*
For sample codes see section 2.3.

Table 1.
Standard deviation values of scores given to attributes of three vinegar samples analysed either ad *libitum* (AL) or with the newly proposed protocol (PP) using ISO standardized cups.

Tasting Procedure	Samples	Aromatic Intensity	Richness in Aroma	Ethyl acetate odor	Woody odor	Wine character	Pungent sensation	General impression	Coconut odor	Clove odor	Vanilla odor
PP	OA	1.92	2.49	1.76	2.74	2.21	1.35	2.23	1.21	1.65	1.15
	OB	1.31	1.39	2.05	1.42	1.33	1.85	1.33	1.91	1.79	1.08
	OC	1.68	1.94	1.27	2.63	2.87	1.73	2.01	1.25	2.72	2.00

PP= Following the proposed protocol

Table 2.
Mean Standard deviation values of the tasting panel after the tasting glass has been emptied and the proposed procedure followed

Attributes	Definition	References	Evaluation technique
Ethyl acetate	Odours associated with glue and nail polish remover	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 such as 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 an 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	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
Raisin	Odour associated with grape raisins	Grape raisin	Sun dried grape raisin
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
Clove	Sensation produced by aqueous solution of eugenol	Eugenol solution	10 ppm of eugenol in 6% v/v acetic acid solution
Red fruits	Odours associated with strawberry, black berry	Strawberry juice	80 ml strawberry juice in 6%v/v acetic acid solution
Vanilla	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. In our case, this odour is considered to be negative	Woody odour produced by sharpening a pencil, carpenter's workshop	2% w/v fresh American oak wood shaving (without toasting) macerated in 6% v/v acetic acid solution
Old/leather	Odor associated with leather	Leather	Leather pieces in 6% v/v acetic acid solution

Table 3.
Definition of sensory attributes, standard references and evaluation technique used.

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

Table 4.
List of selected attributes for descriptive sensory analysis

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

Table 5.
Thirteen attributes selected for descriptive analysis of wine vinegar samples

CAPÍTULO 6 / CHAPTER 6

Ana B. Cerezo; Elyana Cuevas; Peter Winterhalter; M. Carmen Garcia-Parrilla & Ana M. Troncoso.

ISOLATION, IDENTIFICATION AND ANTIOXIDANT ACTIVITY OF ANTHOCYANIN COMPOUNDS IN *CAMAROSA* STRAWBERRY

To be submitted to Food Chemistry

Isolation, identification and antioxidant activity of anthocyanin compounds in *Camarosa* strawberry

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Abstract

This paper explores the bioactive composition of strawberry (*Camarosa* variety). For this purpose, anthocyanin composition is described by LC-DAD-MS. Pelargonidin-3-glucoside was the major compound followed by pelargonidin-3-rutinoside and eleven pelargonidin and cyanidin derivatives. Additionally, delphinidin-3-glucoside, peonidin-3-glucoside and cyanidin-3-galactoside were tentatively identified for the first time in strawberry. Another original contribution was de identification of 5-carboxypyranopelargonidin-3-glucoside in *Camarosa* strawberry variety.

Bioactivity of foods depends on the concentrations in bioactive compounds and their activity related to their chemical structure. We isolated two different fractions with pelargonidin-3-glucoside and pelargonidin-3-rutinoside at 90% and 92% of purity, respectively by CCC. Pelargonidin-3-glucoside is more active than the 3-rutinoside as determined by ORAC and FRAP assays. The sugar substituent determines the antioxidant activity, presenting the monosaccharide anthocyanin more antioxidant activity than the rutinoside. Pelargonidin-3-glucoside contributed in 32.61% and 17.58% to the overall AA of the extract, for ORAC and FRAP assays, respectively.

Recent studies reveal that enzymatic digestion can affect anthocyanin pigments and their value after digestion can be considered a more realistic approach to in vivo situation. Therefore, we submitted both the food itself (strawberry) and the purified anthocyanin fractions to simulated digestion. Pelargonodin-3-rutinoside fraction and polymeric compounds kept their antioxidant activity after the digestion processes, as no rutinoside enzyme is involved.

Key words: anthocyanin, strawberry, Camarosa, antioxidant, digestion, CCC

1. Introduction

Strawberry is widely consumed, both fresh and as ingredient of processed products. It can be considered a very rich source of bioactive compounds including: vitamin C, E, β-carotene and phenolic compounds (phenol acids, flavan-3-ols, flavonols and anthocyanins) (Oszmianski & Wojdylo, 2009).

Phenolic compounds have proved to be useful as markers of strawberry cultivars (Meyer, Watkins, Pritts, & Liu, 2003), types of processing (Oszmianski et al., 2009; Hartmann, Patz, Andlauer, Dietrich, & Ludwig, 2008; Wojdylo, Figiel, & Oszmianski, 2009) and day/night growing temperatures (Wang & Zheng, 2001). The major phenol compounds are procyanidins (137-179 mg/Kg of fresh weight), ellagitanins (87-117 mg/Kg of fresh weight), (+)-catechin (24-90 mg/Kg of fresh weight) and *p*-coumaroyl esters (23 mg/Kg of fresh weight) (Määttä-Riihinen, Kamal-Eldin, & Torronen, 2004; Aaby, Skrede, & Wrolstad, 2005).

It is well known that pelargonidin-3-glucoside is the major anthocyanin in strawberry (153-652 mg/Kg of fresh weight) (Lopes-da-Silva, Escribano-Bailón, Pérez Alonso, Rivas-Gonzalo and Santos-Buelga, 2007; García-Vigera, Zafrilla, & Tomas-Barberan, 1998). Those authors analyzed different cultivars (cv. *Eris, Oso Grande, Carisma, Tudnew, Camarosa, Chandler and Tudla*) and found differences among varieties. Camarosa is one of the variety with highest concentrations on anthocyanin compounds (García-Vigera et al., 1998; Lopes-da-Silva et al., 2007), explaining by the more intense pigmentation of the inner tissues of the fruit. Glucose is the most usual substituting sugar, but rutinose, arabinose and rhamnose have been tentatively identified. Other minor anthocyanins are acylated derivatives with the following organic acids: malic,

malonic, succinic or acetic acids (Lopes-da-Silva et al., 2007). Despite these very recent results, the strawberry anthocyanin composition is far from being completely described.

Another interest for determining anthocyanins is their antioxidant properties and contribution to the overall antioxidant activity (AA) of drinks and foods. AA of strawberry fruits has been analyzed and related with its phenolic composition (Wang, et al., 2001; Meyer, et al., 2003; Aaby, et al., 2005; Hartmann, et al., 2008; Wojdyo, et al., 2009; Oszmianski, et al., 2009). Nevertheless, to explore the relevance of a certain anthocyanin to the AA of a product, it is necessary to assess the AA of the compound as well as its concentration. If the standard is not available, a prior step of isolation is required. Zhang, Seeram, Lee, Feng and Heber (2008) used an extensive sample preparation procedure including solid phase extraction and subsequently medium-pressure liquid chromatography (MPLC) to separate the anthocyanin compounds. Conversely, Countercurrent chromatography (CCC) is a separation technique that in few steps can produce significant amounts of more than 95% pure compounds used for identification and/or property studies. The two main advantages of CCC compared to classical liquid chromatography (LC) are the solute loading capacity and the absence of adsorptive matrix. It prevents the irreversible solute adsorption, contamination, size exclusion, residual silanols and pH limitations common to LC technique (Degenhardt, Knapp, & Winterhalter, 2000). The main use of CCC is the isolation and fractionation of many bioactive compounds extracted from natural products as flavonoids, betalains, stilbenes….

Once isolated, antioxidant activities can be assessed in vitro with radical scavenging methods. If the antioxidant is meant to exert its action after its consumption, changes due to digestion should be taken into account. Indeed, the effect of digestion with simulated conditions has already been tested in foods as wine (Martínez-Ortega, García-

Parrilla, & Troncoso, 2001) orange juice (Gil-Izquierdo, Gil, Ferresres, & Tomas-Barberán, 2001) and chokeberry (Bermúdez-Soto, Tomás-Barberán, & García-Conesa, 2007). Noguer, Cerezo, Rentzsch, Winterhalter, Troncoso and García-Parrilla (2008) verified many simple phenolic acids appeared after gastric and intestinal digestion. Presumably, they were released from the polymeric fraction of the aged wine under study. This approach extends the interpretation of in vitro antioxidant values for nutritional purposes.

The aims of this paper are to determine the anthocyanin compounds in strawberry in order to screen the antioxidant properties of isolated fraction both before and after simulated digestion.

2. Material and Methods

2.1. Samples

The sample used for the present study was a strawberry puree seedless (variety Camarosa) obtained from Hudisa company (Lepe, Spain) in 2008. The strawberry puree was a ºBrix 8.5.

2.2. Isolation of fractions

2.2.1. Sample preparation for CCC

An amberlite XAD-7 column (Fluka; 100 x 7cm) was aconditionated with 2 litres of methanol, and then 2 litres of water. Six litres of seedless strawberry purée diluted with

water (1:1) and filtered were loaded onto the column and cleaned with 9 litres of water to remove sugars, proteins, organic acids, and ions and then eluted with 2 litres of mixture (methanol/acetic acid, 19:1). The flow rate was 1 drop/second. The eluated was concentrated with a rotary evaporator under vacuum, frozen and freeze-dried to obtain 1.3 g from L of strawberry purée.

2.2.2. CCC

This strawberry XAD-7 extract was fractioned with a high-speed model CCC-1000 manufactured by Pharma-Tech Research Corp. (Baltimore, MD) equipped with three preparative coils, connected in series (tubing diameter of 2.6 mm and total volume of 850 mL). The solvent system consisted of MTBE/n-butanol/acetonitrile/water (2.75:1.25:1:5) acidified with 0.1% trifluoroacetic acid. The elution mode was head to tail with the lighter (organic) phase acting as the stationary phase and the aqueous phase acting as the mobile phase. The flow rate was set at 3 mL/min and delivered by a BT 3020 HPLC pump (Jasco, Gross-Umstadt, Germany). The separation was run at a speed of 850 rpm. One gram of the strawberry extract was dissolved in 25 mL of a mixture of the upper (organic) and lower (aqueous) phases (50:50, v/v) and injected into the system by a loop injection. Fractions of 12 mL were collected with a fraction collector. Elution was monitored with a K-2501 UV/vis detector (Knauer, Berlin, Germany) at 520 nm. The fractions were collected according to the profile of the chromatogram. After the evaporation of organic solvents, the fractions were frozen and freeze-dried again.

150 *2.3. Identification and quantification of anthocyanin compounds*

151

152 *2.3.1. LC-MS*

153

154 The anthocyanin compounds were identified using an LC system consisting of a model
155 G1328A binary pump (Agilent, Palo Alto, CA) equipped with an autosampler (Agilent
156 Technologies, 1200 Series, G 1329), coupled to a Bruker Esquire mass spectrometer
157 with electrospray ionization. Data were processed using Esquire NT 4.0 software
158 (Bruker). The MS/MS parameters were as follows: positive mode; capillary voltage, -
159 2500 V; end plate offset, -500 V; capillary exit, 1, 10 V; capillary exit offset 70 V,
160 skimmer 1, 20 V; skimmer 2, 10 V; dry gas (N2) temperature, 300 °C; flow, 11 L/min;
161 nebulizer, 10 psi; and scan range *m*/*z*, 50-2500. The separations were performed on a
162 250 x 4.6 mm, 5 µm, C18 Luna column (Phenomenex, Germany). Samples were filtered
163 through a Chromatofil® PET 0.45 µm membrane filter before injection. The sample
164 volume injected was 50 µl. Two different solvents were used as the mobile phase:
165 solvent A (water/acetonitrile/formic acid, 87:3:10 v/v/v) and solvent B
166 (water/acetonitrile/formic acid, 40:50:10 v/v/v), at a flow rate of 0.5 mL/min and a
167 linear gradient as follows: 0 min 6% B, 20 min 20% B, 35 min 40% B, 40 min 60% B,
168 45 min 90% B, 55 min 6% B.

169

170 *2.3.2. HPLC*

171

172 The anthocyanin compounds were quantified using an LC system equipped with a
173 binary pump (Jasco PU-980), automatic injector (Jasco AS-950) and degasser (Jasco
174 DG-980-50-3). Detection was carried out using a UV/Vis diode detector (Jasco MD-

1510), and data were processed using Borwin-PDA Version 1.0 software. The chromatographic conditions were as described above. Anthocyanins were detected at 520 nm. Quantification was performed by external calibration expressed as cyanidin-3-glucoside equivalents.

2.4. Identification and quantification of non-anthocyanin phenolic compounds

LC analyses of *non-anthocyanin* phenolic compounds were performed using an Agilent Series 1100 system equipped with a quaternary pump (Series 1100 G1311A), automatic injector (Series 1100 G1313A) and degasser (Series 1100 G1379A). Detection was carried out using an UV/Vis diode detector (Series 1100 G1315B) coupled to a Chemstation HP A.10.02 (HP/Agilent). The column was an Agilent Zorbax SB-C18, 4.6 x 250 mm and 3.5 µm. Duplicate samples were filtered through a Millex-LCR 13 mm filter before injection. The method uses a binary gradient: A (glacial acetic acid/water pH 2.65), B (20% A + 80% acetonitrile) programmed for the following gradient: 0 min 0% B; 5 min 2% B; 10 min 4% B; 15 min 10% B; 30 min 20% B; 35 min 30% B; 40 min 100% B; 45 min 0% B. The sample volume injected was 50 µl. The flow rate was 1.5 mL/min, and the temperature was set at 40 ºC. Quantification was performed by external calibration at their maximum absorbance.

The standards of non-anthocyanin phenolic compounds were purchased from Fluka, Sigma, Merk, Safc and Chromadex.

200 *2.5. Gastric e Intestinal in vitro digestion*

201

202 Gastric-simulated fluid was made according to USP (USP23, NF18): 2 g NaCl, 3.2 g of
203 pepsin, 7.0 mL HCl and enough distilled water to make 1 L. This test solution had a pH
204 of 1.2. We followed the USP (USP23, NF18) recipe to prepare intestinal simulated
205 fluid: dilute 6.8 g potassium phosphate monobasic in 250 mL of water; mix with 190
206 mL NaOH 0.2 N; add 400 mL of water and 10 g pancreatin; adjust to pH=7.5 ± 0.1;
207 and dilute up to 1000 mL with water. α-Amylase dilution (120 mg/mL) was made in
208 Trizma-maleate buffer (0.2 M, pH 5.8 - 8.2). The digestion procedure (USP23, NF18),
209 which required shaking throughout at 37 ºC, was as follows: treat samples (0.5 g of each
210 fraction) with 28.7 mL of gastric simulated fluid during 30 min; adjust pH to 4.5 ± 0.2
211 with NaOH 0.5M; add 0.15 mL of amyloglucosidase dilution (120 mg/mL) and wait for
212 30 min.; adjust pH to 6.9 ± 0.2 with NaOH 0.5N and add 1.66 mL of α-amylase
213 dilution; let this act for 45 minutes and then centrifuge (10 min, 3000 rpm); after that
214 take a 1 mL aliquot to keep it in the freezer (-80 ºC) until further antioxidant analysis;
215 submit the rest of the gastric digested sample to 28.7 mL of intestinal simulated fluid for
216 30 minutes; add a solution containing Lipase (0.023 g) and bile extract (0.058 g) in 3.61
217 mL of phosphate buffer (pH = 7.5); after 30 min and centrifugation (10 min, 3000 rpm),
218 take the supernatant and freeze it for further analysis. Simulated digestion solutions
219 (gastric and intestinal) without the CCC fractions were assessed as blank for the both
220 digestion samples.
221 Simulated digestion (gastric and intestinal) was performed to each fraction by
222 duplicated except to fraction 3 because of the lack of this fraction.

223

224

225 *2.6. Antioxidant activity*

226

227 *2.6.1. ORAC test*

228

229 The procedure is based on a previously reported method with slight modifications (Ou,
230 Hampsch-Woodill, & Prior, 2001). Briefly, it is described as follows: 50 µl of sample or
231 Trolox with 100 µl of Florescein (45 nM) and 50µl of AAPH (15 mM). Fluorescence is
232 recorded for 60 min (excitation wavelength is set at 485nm; emission wavelength at
233 528nm). Measurements were taken in triplicate in a multi-detector microplate reader
234 (Synergy HT, Biotek®). Trolox was used as a calibration standard (0.5 – 9.5 µM).
235 The results were calculated as ORAC values using the differences between the blank
236 and the sample areas under the fluorescein decay curve. They are expressed as mmol
237 Trolox equivalents (TE)/g of fraction or extract.

238

239 *2.6.2. FRAP method*

240

241 The FRAP reagent consists of acetate buffer (300 mM, pH 3.6), TPTZ (10 Mm in HCl
242 40 Mm) and $FeCl_3$ $6H_20$ (20 mM) (10:1:1, v/v/v). A total of 3 mL of FRAP reagent was
243 mixed with 300µL Mili-Q water and 100 µL of sample. Absorbance was measured after
244 8 min at 593 nm. An aqueous solution of $FeSO_4x7H_2O$ in the 0-1 mM range was used
245 for calibration. All determinations were performed in triplicates. Results are expressed
246 as mmol of Fe^{+2}/g of fraction or extract (Benzie & Strain, 1996).

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248

249

3. Results and discussion

3.1. Identification of anthocyanins

Figure 1 shows the anthocyanin profile of strawberry extract obtained as described in 2.2.1 by the LC-DAD chromatogram at 520 nm. A total of seventeen anthocyanin compounds were identified by their elution order, UV/Vis, and mass spectrometric characteristics as compared with reported data in the literature (Table 1). Pg-3-glu (peak 9) was the major compound followed by Pg-3-rut (peak 10), in agreement with previous reports (Lopes-da-Silva, de Pascual Teresa, Rivas-Gonzalo, & Santos-Buelga, 2002; Wu & Prior, 2005). Peak 16 shows identical molecular ion at *m/z* 535 and MS^2 fragment at *m/z* 287 than Cy-3-(6-malonyl)-3-glu (Figure 2), proposed as a variety marker of *Camarosa* strawberry (Lopes-da-Silva et al., 2007), but different retention time. Hence, we can propose it as a Cy derivative but not further information could be obtained that allowed speculation about its structure.

Other compounds previously identified by other authors are as follow: Peaks 1, 2 and 5 correspond to condensed pigments containing C-C linked anthocyanin (Pg) and flavanol ((epi)catechin and (epi)afzelechin) residues; Peak 13 was associated to Pg-dissacharide (hexose + pentose) acylated with acetic acid. Peak 17 (*m/z* at 503) lost 232 amu (malonyldeoxyhexose or succinylpentose) suggesting the presence either Pg-3-(6-succinyl)-ara or -3-(6-malonyl)-rham.

One of the original contribution of our work to the anthocyanin profile of *Camarosa* strawberry was the identification of peak 12 as 5-carboxypyranopelargonidin-3-glucoside (Figure 3) previously isolated from strawberry (Andersen, Fossen, Torskangerpoll, Fossen, & Hauge, 2004) and identified in *Carisma, Oso Grande* and

Tudnew strawberry variesties (Lopes-de-Silva et al., 2007) but not reported in *Camarosa* variety. Anthocyanin-derived pigments (pyranoanthocyanin) have been extensively studied in aged red wines (Schwarz, Hofmann, & Winterhalter, 2004). Their detection in plant extracts suggest that they are a natural pigments not exclusively formed during ageing process in a beverage (González-Paramás et al., 2006). Besides, we detected peak 14 that showed identical aglycone cation at *m/z* 271 than Pg but different molecular ions at *m/z* 461, showing that they derive from Pg. We have not found this fragmentation pattern in the literature. In addition, peaks 4 and 11 were assigned as Dp-3-glc (Figure 4) and Pn-3-glu (Figure 5) matching both to their mass spectrometric characteristics and elution order as has previously been reported in wine, blood orange or açai (Schwarz, Hillebrand, Habben, Degenhardt, & Winterhalter, 2003; Hillebrand, Schwarz, & Winterhalter, 2004; Vera de Rosso, Hillebrand, Cuevas, Obbio, Winterhalter, & Mercadante, 2008). Moreover, peak 3 presented similar molecular ion at *m/z* 449 and MS^2 fragment at *m/z* 287 (Figure 6) than Cy-3-glu (peak 4), but different retention time: 14 and 19 min, peak 3 and 6, respectively. Its elution order and mass characteristics could suggest it as Cy-3-gal.

Dp-3-glu, Pn-3-glu and Cy-3-gal have been previously described in blueberry, grape, cranberry, blackberry, sweet cherry, apple, black plum or plum fruits (Wu et al., 2005), but not in strawberry. As far as we know, Dp-3-glu, Pn-3-glu and Cy-3-gal are tentatively proposed for the first time in strawberry.

3.2. Antioxidant activity of anthocyanin compounds from strawberry fractions

The lack of available anthocyanin standars makes necessary their isolation to determine the antioxidant activity of most of the anthocyanin compounds.

300 The suitable solvent system and volume ratio described for CCC isolation of
301 anthocyanin compounds is as follows: MTBE/n-butanol/acetonitrile/water; 2:2:1:5,
302 v/v/v/v, 0.1% TFA (Degenhardt et al., 2000). Quast (2008) modified the solvents´ ratio
303 to achieve a good separation for strawberry anthocyanins consisting in MTBE/n-
304 butanol/acetonitrile/water; 2.75:1.25:1:5, v/v/v/v.
305 Four fractions were suitable isolated with this solvent system (Figure 7) and analyzed
306 by LC-DAD-MS afterwards.
307 Fraction 1 consisted mostly of polymeric pigments.
308 Fraction 2 contained Pg-3-rut. Its area represents the 92% of total chromatogram
309 (Figure 8).
310 Fraction 3 consisted in a mix of different anthocyanin mostly 50% was Cy-3-glu, 5% of
311 catechi-Pg-3-glu and remains of epicatechin-Pg-3-glu and (epi)azfelechin-Pg-3-glu.
312 Fraction 4 included Pg-3-glu (90%), Cy-3-glu and 3-gal and Pg-3-(6-acetyl)-glu at low
313 proportions (Figure 9).
314 The coil kept residual amounts of Pg-3-glu and Pg-3-(6-acetyl)-glu.
315 The solvent system used for CCC is useful to isolate high purity fractions especially in
316 the case of fraction 2 (Pg-3-rut at 92%) and fraction 4 (Pg-3-glu at 90%).
317 Besides, Table 2 displays phenolic acids and flavonoids concentration determined in the
318 strawberry extract and coil.
319 Table 3 shows the AA of isolated fractions. As can be seen, the AA ranks as follows
320 Fraction 4 > fraction 2 similar to fraction 3. Pg-3-glu is more active than the
321 corresponding 3-rut. Note that differences in AA between Pg-3-glu and Pg-3-rut
322 fractions with ORAC method (pH 7.5) are remarkable (> 50%). If the FRAP method
323 (pH 1.2) is considered the differences in AA are just a 14%. Previously, the relation
324 between aglycone chemical structures and AA has been related to the number of OH in

325 the B ring (Kähkönen & Heinonen, 2003). As both compounds present the same
326 aglycone and they are soluble enough in water, the differences in sugar moiety and pH
327 in the reaction medium must be considered. Glucose and rutinose are reducing sugars
328 and the extent of these properties at pH 7.4 related to the hemiacetal structure and the
329 stability of the Pg derivatives explains the difference in AA_{ORAC} value. On the other
330 hand, acid pH (FRAP method, pH 1.2) shows similar AA between Pg-3-glu and Pg-3-
331 rut fractions.
332 Our results support that the sugar substituent determines the antioxidant activity,
333 depending on the pH medium, presenting the monoglucoside anthocyanin more
334 antioxidant activity than the rutinoside at neutral pH.
335 The contribution of the compounds to the total AA depends of the AA power, as well as
336 the relative abundance of the compounds. If we consider the concentration of Pg-3-glu
337 in the strawberry extract and the tentatively AA values for this compound, Pg-3-glu
338 explain a 32.61% and 17.58% for ORAC and FRAP value, respectively. Taking into
339 account the Pg-3-rut concentration in the extract and its AA, this compound explains
340 1.64 and 0.11% for ORAC and FRAP values.
341
342 *3.3. Antioxidant activity of strawberry fractions after in vitro digestions*
343
344 Gastric and intestinal digestion with simulated conditions was tested in fractions to
345 assay the influence of the physiological conditions on the AA of fractions.
346 As can be seen in Table 3, fraction 2 consisting in Pg-3-rut does not vary in terms of
347 AA_{FRAP} after both treatments, likely explained by the lack of enzyme deglycosidase for
348 rutinoside sugar. On the other hand, a remarkable decrease in AA with both methods
349 was observed for fraction 4 and the coil rich in Pg-3-glu. This fact could be explained

350 by the presence of amyloglucosidase enzyme in the simulated digestion favouring the
351 deglucosylation and decreasing the AA as a consequence, as the aglycon posses less AA
352 than the corresponding glucoside (Fukumoto & Mazza, 2000; Kähkönen et al., 2003).
353 The results also show that AA of fraction containing polymeric compounds remains
354 invariable after digestions. Conversely, AA of aged red wine fractions presented
355 remarkably higher values after digestions, as the high amount of polymeric pigment
356 released simple phenolic compounds with higher AA (Noguer et al. 2008).
357 (+)-Catechin and quercetin decreased their concentration after gastric digestion as
358 reported in Table 2 in agreement with previously published data (Martinez-Ortega et al,
359 2001). Formation of (+)-catechin was observed after gastric digestion of fraction 3
360 containing (epi)catechin-Pg-3-glu (Table 2). This result suggests that hydrolysis of the
361 Pg derivative occurred. *p*-Coumaric acid was also detected as a metabolite of intestinal
362 digestion of fraction 4. Various benzoic acids (protocatechuic and gallic acid) and
363 hydroxycinnamic (*p*-coumaric and caffeic acid) acid derivatives have been previously
364 identified as metabolites from chemical and enzymatic digestion of anthocyanin
365 fractions from wine (Noguer et al., 2008).
366 Overall AA decrease after simulated digestion, however (+)-catechin and phenolic acids
367 as *p*-coumaric acid are released. These results imply that AA compiled in food tables for
368 nutritional purpose should take into account the effects due to gastric and intestinal
369 digestions on phenolic compounds in their composition.
370
371 **4. Conclusions**
372
373 This study makes new contributions to the anthocyanin profile of strawberry as follows:
374 Dp-3-glu, Pn-3-glu and Cy-3-gal. Additionally, 5-carboxypyranopelargonidin-3-

glucoside has been identified for the first time in *Camarosa* strawberry variety. Two fractions have been isolated with high purity; Pg-3-glu and Pg-3-rut, and their tentatively AA have been achieved showing the 3-gluc higher AA than 3-rut. Influence of the physiological conditions on the AA of fractions remains invariable for Pg-3-rut and polymeric pigments and decrease for Pg-3-glu, (+)-catechin and quercetin.

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Abbreviations

AA, antioxidant activity; CCC, countercurrent chromatography; MTBE, methyl *tert*-butyl ether; ORAC, Oxygen Radical Absorbance Capacity; TROLOX, 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; AAPH, 2,2´-Diazo bis amidine propane dihydrochloride; FRAP, Ferric Reducing Ability; TPTZ, 2,4,6-Tripyridyl-*s*-triazine.

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Figure caption

Figure 1. LC Chromatogram of strawberry extract at 520nm.

Figure 2. MS data of cyanidin derivative.

Figure 3. MS data of 5-carboxypyranopelargonidin-3-glucoside

Figure 4. MS data of delphinidin-3-glucoside

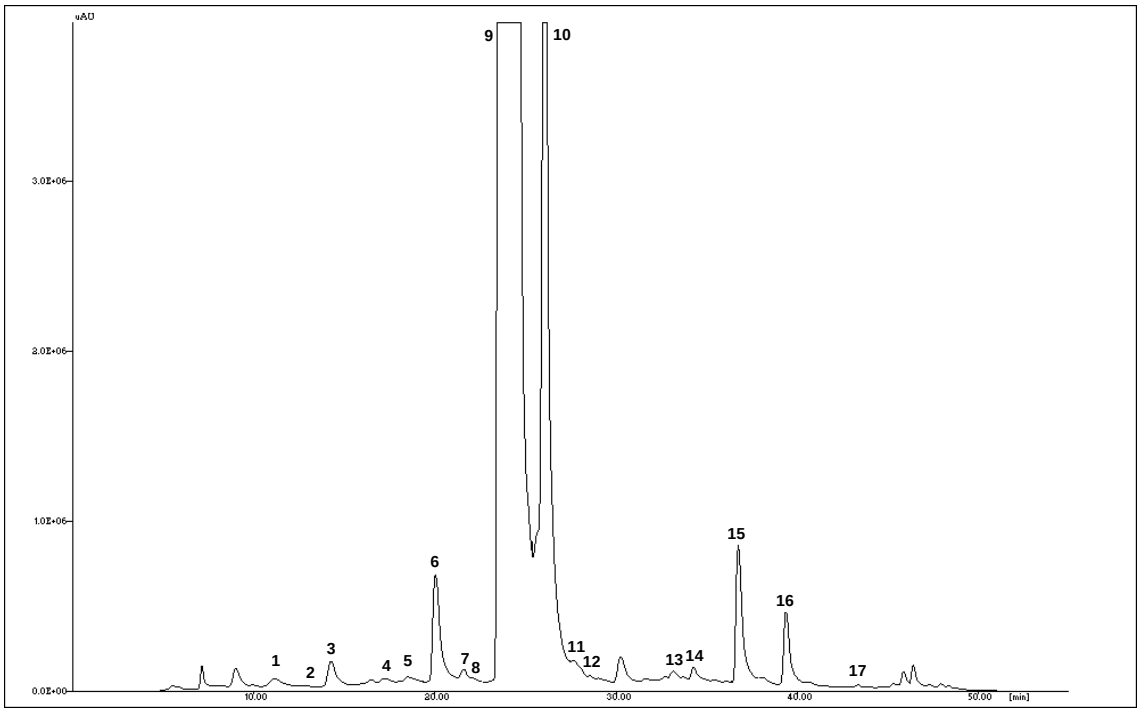
Figure 5. MS data of peonidin-3-glucoside.

Figure 6. MS data of cyanidin-3-galactoside.

Figure 7. CCC chromatogram of strawberry anthocyanin extract at 520nm.

Figure 8. LC chromatogram of fraction 2 at 520nm.

Figure 9. LC chromatogram of fraction 4 at 520nm.


uAU
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2.0E+06
1.0E+06
0.0E+00
10.00
20.00
30.00
40.00
50.00
[min]
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Figure 1.

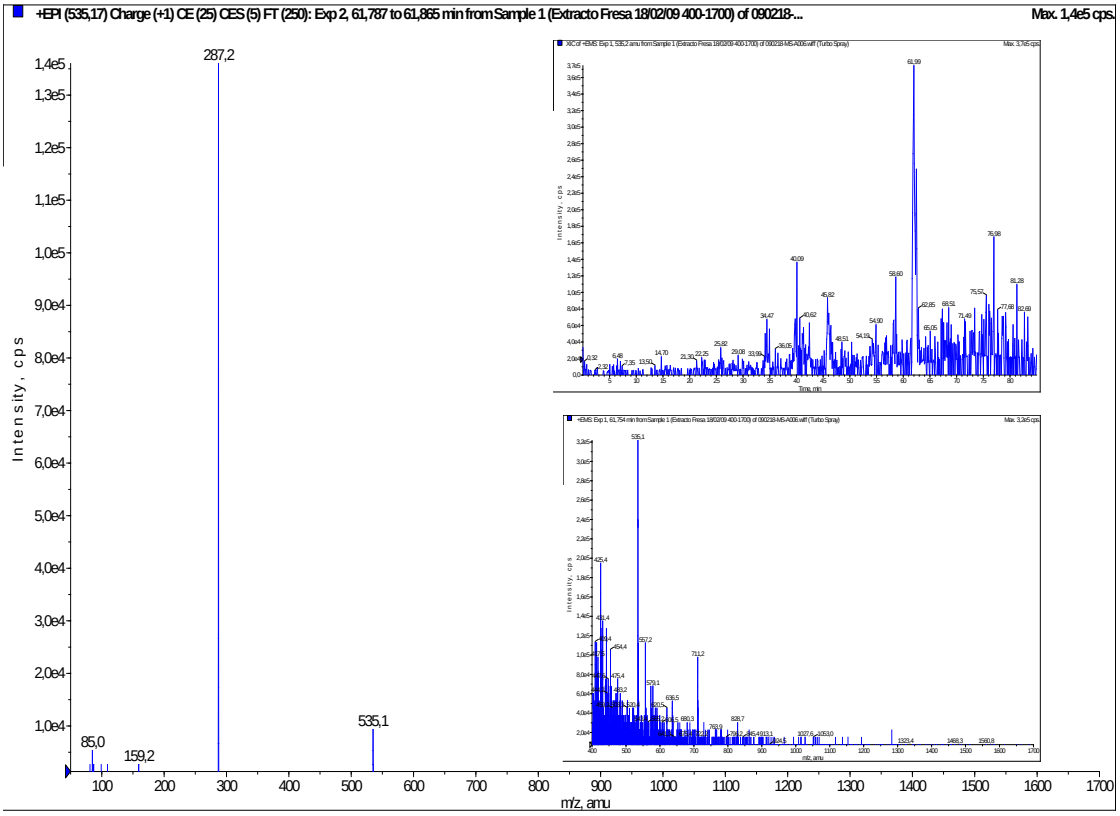
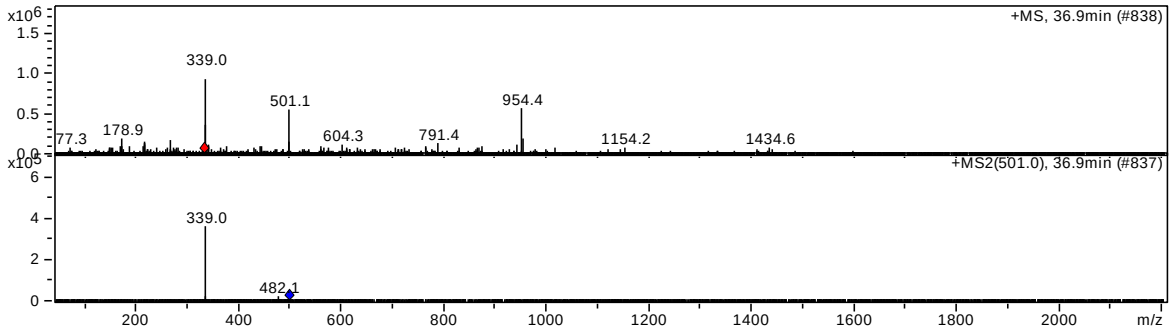

+EPI (535,17) Charge (+1) CE (25) CES (5) FT (250): Exp 2, 61,787 to 61,865 min from Sample 1 (Extracto Fresa 18/02/09 400-1700) of 090218-...
Max. 1,4e5 cps.
Intensity, cps
m/z, amu
287,2
535,1
85,0
159,2

Figure 2.


+MS, 36.9min (#838)
339.0
501.1
954.4
178.9
77.3
604.3
791.4
1154.2
1434.6
+MS2(501.0), 36.9min (#837)
339.0
482.1
m/z
Figure 3.

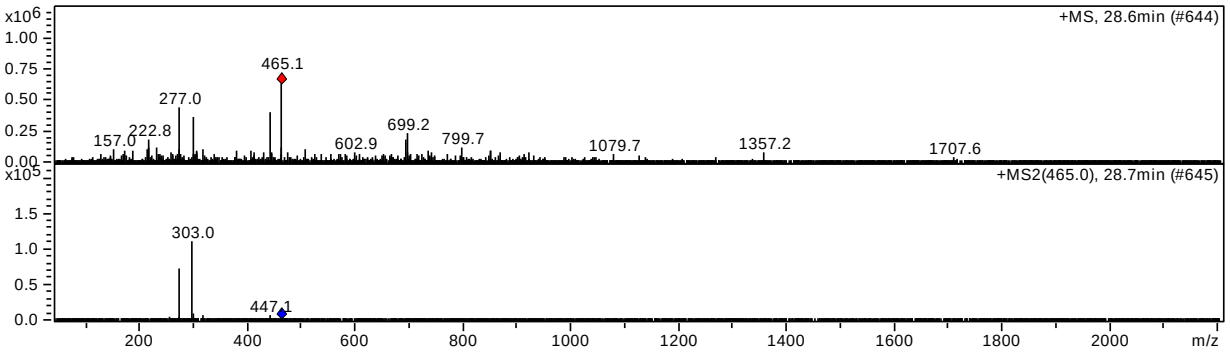
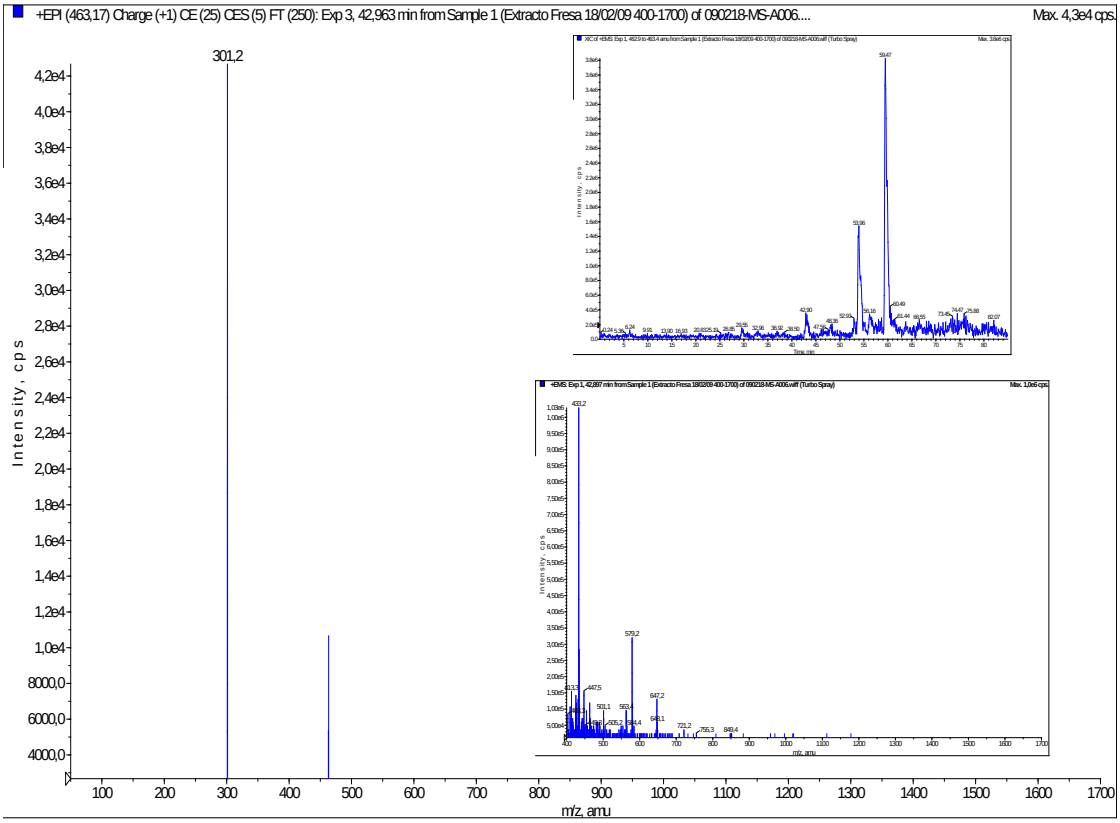

x10^6
1.00
0.75
0.50
0.25
0.00
+MS, 28.6min (#644)
465.1
277.0
222.8
157.0
699.2
602.9
799.7
1079.7
1357.2
1707.6
x10^5
1.5
1.0
0.5
0.0
+MS2(465.0), 28.7min (#645)
303.0
447.1
200
400
600
800
1000
1200
1400
1600
1800
2000
m/z

Figure 4.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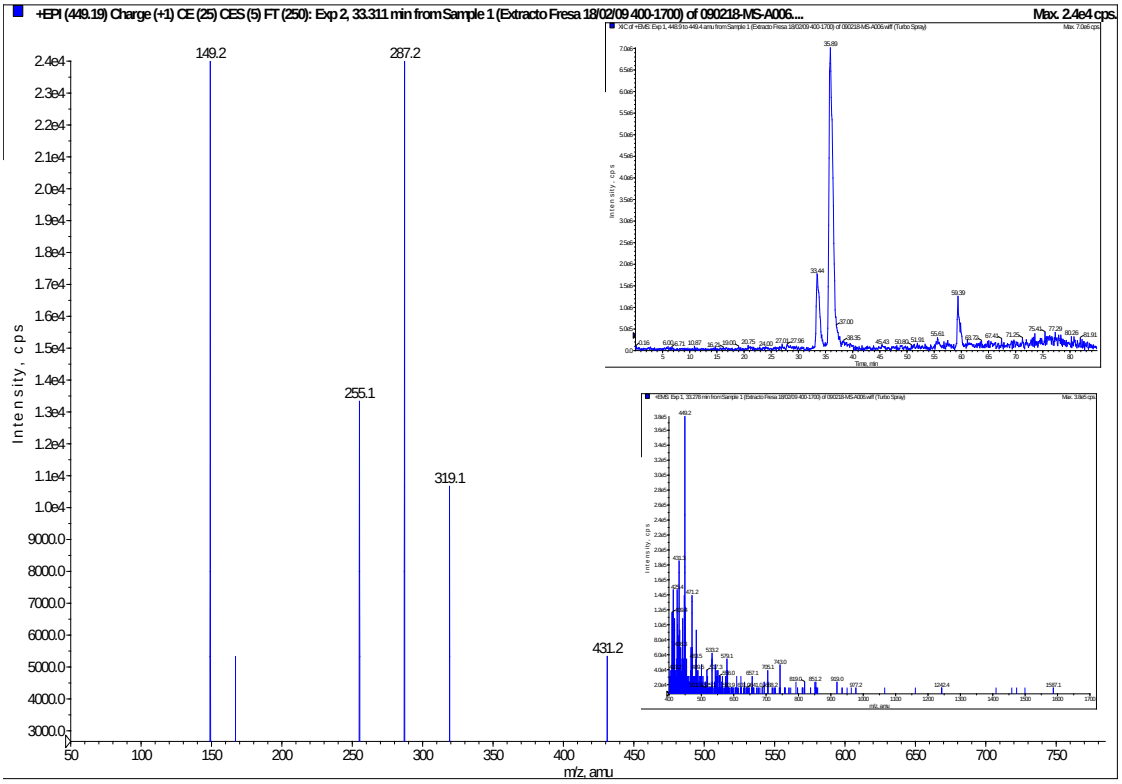
Max. 4,3e4 cps.
301,2
Intensity, cps
m/z, amu

Figure 5.


+EPI (449.19) Charge (+1) CE (25) CES (5) FT (250): Exp 2, 33.311 min from Sample 1 (Extracto Fresa 18/02/09 400-1700) of 090218-MS-A006....
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255.1
319.1
431.2
Intensity, cps
m/z, amu

Figure 6.

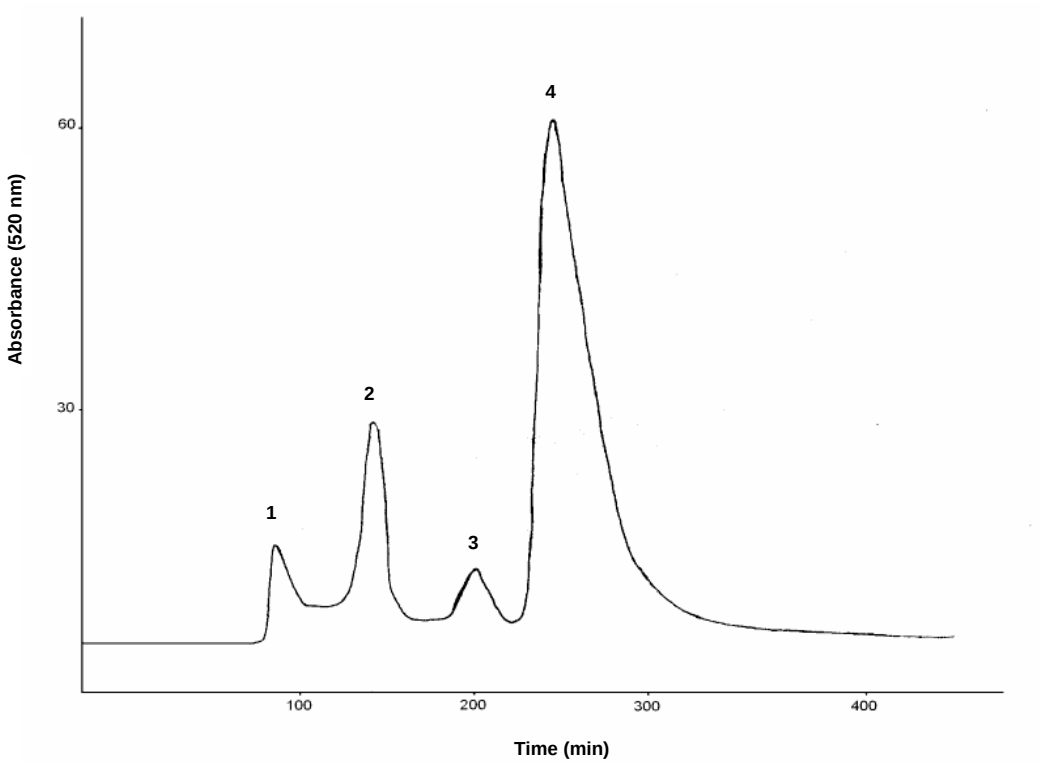


Figure 7.

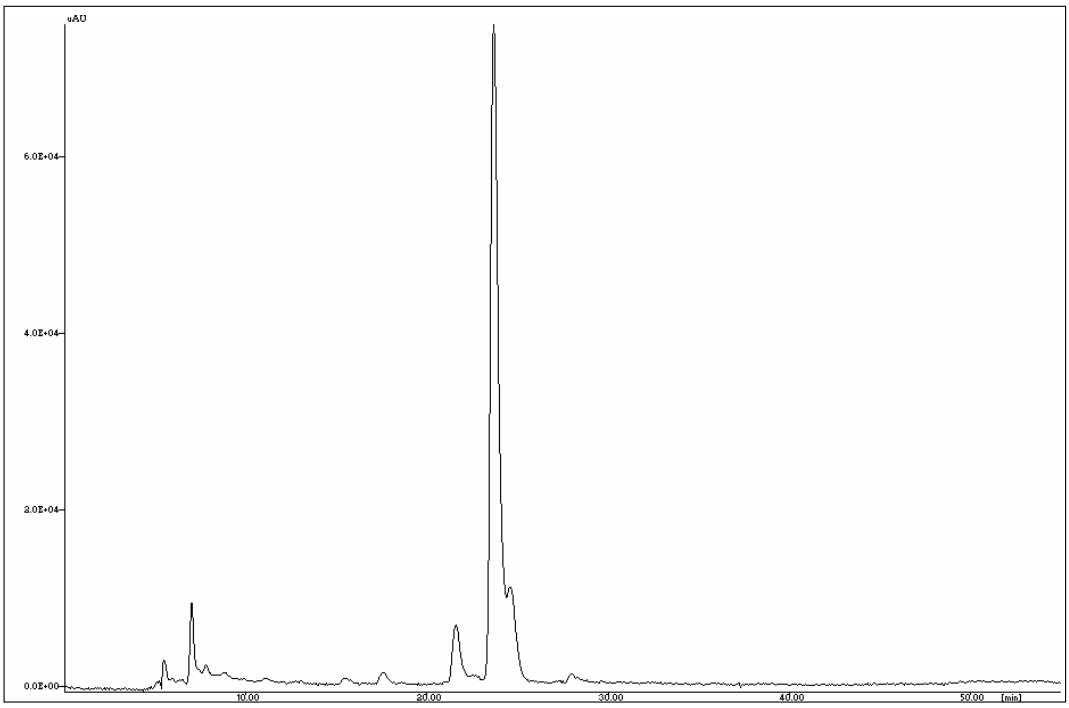


Figure 8.

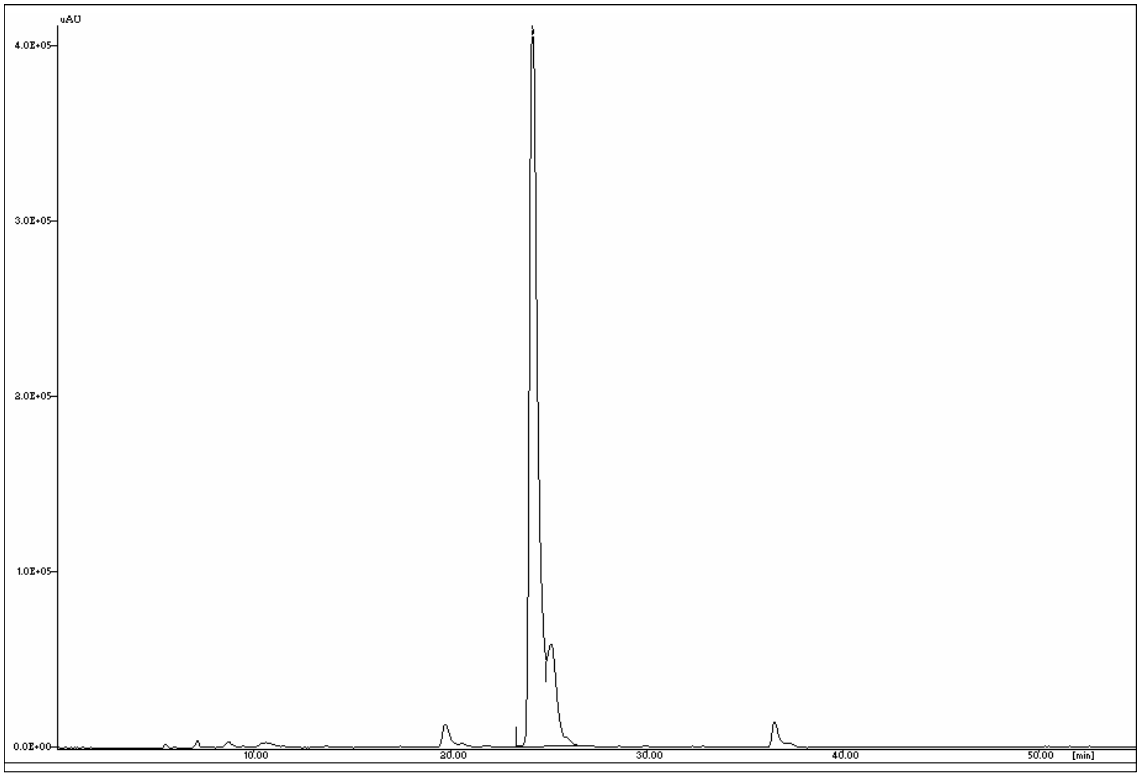

uAU
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
10.00
20.00
30.00
40.00
50.00
[min]

Figure 9.

Peak number	t_R (min)	$[M^+]$ *m/z*	MS^2 *m/z*	Compounds	Concentration (mg/Kg strawberry)	References
1	11.08	721	559/407/313	Catechin-(4,8)-Pg-3-glu	1.16 ± 0.03	Fossen, Rayyan & Andersen, 2004
2	13.0	721	559/407/313	Epicatechin-(4,8)-Pg-3-glu	T_r	
3	14.18	449	287	Cy-3-gal	2.14 ± 0.02	-
4	17.1	465	303	Dp-3-glu	0.70 ± 0.00	-
5	18.45	705	543/407/313	(epi)Afzelechin-Pg-3-glu	0.77 ± 0.04	Fossen et al., 2004
6	19.96	449	287	Cy-3-glu	6.84 ± 0.02	
7	21.5	595	433/271	Pg-3,5-diglu	0.77 ± 0.00	Lopes-da-Silva, de Pascual Teresa, Rivas-Gonzalo, & Santos-Buelga, 2002; Wu & Prior, 2005
8	22.0	595	449/287	Cy-3-rut	T_r	
9	23.4	433	271	Pg-3-glu	250.66 ± 0.02	
10	25.8	579	433/271	Pg-3-rut	27.60 ± 0.20	
11	27.5	463	301	Pn-3-glu	0.55 ± 0.01	-
12	28.9	501	339	5-Carboxy-pyranopelargonidin-3-glucoside	0.46 ± 0.00	-
13	33.1	607	271	Pg dissacharide (hexose + pentose) acylated with acetic acid	0.70 ± 0.01	Lopes-da-Silva et al., 2002
14	34.22	461	271	Pg derivative	0.98 ± 0.00	-
15	36.7	475	271	Pg-3-(6-acetyl)-glu	8.21 ± 0.07	Lopes-de-Silva et al., 2007
16	39.33	535	287	Cy derivative	4.34 ± 0.04	-
17	43.3	503	271	Pg-3-(6-succinyl)-ara/ pg-3-(6-malonyl)-rham	0.49 ± 0.00	Lopes da Silva, et al., 2007; Aaby et al., 2007

Pg: pelarginidin; Cy: cyanidin; Pn: peonidin; Dp: delphinidin; glu: glucoside; gal: galactoside; rut: rutinoside; ara: arabinoside; rham: rhamnoside; t_R: retention time by LC-DAD; t_r: trace; -: non previously reported in strawberry or *Camarosa* variety

Table 1. Concentration and LC-MS and LC-DAD dates of anthocyanin compounds identified in strawberry extract.

	Concentration (mg/Kg strawberry)				
Samples	**1**	**2**	**3**	**4**	**5**
SE	5.62 ± 0.04	31.39 ± 0.12	23.91 ± 0.11	7.78 ± 0.01	12.5 ± 0.3
GD SE	3.41 ± 0.03	21.2 ± 0.3	19.19 ± 0.22	7.53 ± 0.01	9.04 ± 0.08
	3.61 ± 0.03	20.5 ± 0.5	19.31 ± 0.09	7.36 ± 0.03	8.77 ± 0.15
ID SE	3.70 ± 0.05	23.24 ± 0.03	19.8 ± 0.3	7.89 ± 0.06	9.64 ± 0.08
	3.57 ± 0.02	22.64 ± 0.00	19.7 ± 0.3	7.90 ± 0.03	9.52 ± 0.09
F1	-	-	-	-	-
GD F1	-	-	-	-	-
	-	-	-	-	-
ID F1	-	-	-	-	-
	-	-	-	-	-
F2	-	-	-	-	-
GD F2	-	-	-	-	-
	-	-	-	-	-
ID F2	-	-	-	-	-
	-	-	-	-	-
F3	-	-	-	-	-
GD F3	-	0.31 ± 0.01	-	-	-
ID F3	-	0.27 ± 0.01	-	-	-
F4	-	3.05 ± 0.05	-	-	-
GD F4	-	2.73 ± 0.11	4.60 ± 0.07	-	-
	-	2.9 ± 0.3	4.87 ± 0.24	-	-
ID F4	-	2.69 ± 0.17	5.48 ± 0.19	0.52 ± 0.00	-
	-	2.67 ± 0.20	5.15 ± 0.17	0.49 ± 0.01	-
Coil	2.54 ± 0.02	24.62 ± 0.05	7.07 ± 0.08	4.92 ± 0.04	7.66 ± 0.13
GD coil	1.66 ± 0.13	15.1 ± 0.8	4.72 ± 0.09	3.31 ± 0.20	4.70 ± 0.23
	1.48 ± 0.08	13.9 ± 0.7	4.61 ± 0.03	3.06 ± 0.12	4.33 ± 0.16
ID coil	1.57 ± 0.03	14.1 ± 0.8	4.61 ± 0.06	3.14 ± 0.16	4.60 ± 0.16
	1.03 ± 0.03	9.4 ± 0.5	-	-	-

1: p-hidroxybenzoic acid; 2: (+)-catechin; 3: ellagic acid; 4: p-coumaric acid; 5: quercetin glucoside; GD: after gastric digestion and amiloglucosidase funtion; ID: after intestinal digestion; SE: strawberry extract; F: fraction;

Table 2. Means and Standard Deviations of the concentration (mg/Kg) of non-anthocyanin phenolic compounds in strawberry extract and fractions before and after simulated digestion.

Samples	ORAC			FRAP		
	BD (mmol Trolox/g fraction)	GD (mmol Trolox/g fraction)	ID (mmol Trolox/g fraction)	BD (mmol Fe^{+2}/g fraction)	GD (mmol Fe^{+2}/g fraction)	ID (mmol Fe^{+2}/g fraction)
F1	2.7 ± 1.5	3.7 ± 0.4 4.2 ± 0.3	5.1 ± 0.9 2.8 ± 0.6	2.75 ± 0.05	2.56 ± 0.11 2.22 ± 0.12	2.17 ± 0.10 2.21 ± 0.01
F2	10.7 ± 0.9	3.9 ± 0.3 3.0 ± 2.5	15.6 ± 3.9 15.74 ± 1.23	5.27 ± 0.06	5.33 ± 0.04 3.9 ± 0.3	4.16 ± 0.06 4.62 ± 0.19
F3	11.9 ± 0.3	11.6 ± 0.7	9.6 ± 0.3	7.67 ± 0.39	3.45 ± 0.17	3.45 ± 0.25
F4	22.93 ± 0.06	14.74 ± 1.15 12.63 ± 4.16	13.11 ± 0.10 9.3 ± 1.4	6.15 ± 0.14	4.15 ± 0.17 4.3 ± 0.3	3.43 ± 0.00 3.60 ± 0.24
Coil	24.46 ± 2.12	18.3 ± 1.4 17.5 ± 0.9	14.79 ± 1.11 13.45 ± 1.01	12.5 ± 0.5	6.31 ± 0.07 7.5 ± 0.3	5.75 ± 0.07 5.46 ± 0.11

BD: before digestion; GD: after gastric digestion and amiloglucosidase function; ID: after intestinal digestion

Table 3. Antioxidant activity of strawberry fractions before and after simulated digestion.

5. CONCLUSIONES / CONCLUSIONS

De los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral se obtienen las siguientes conclusiones:

1. Si bien el Índice de Polifenoles Totales, en general, no varía significativamente a lo largo de la acetificación con cultivo superficial, los Antocianos Totales experimentan una disminución del 50%. Los únicos compuestos fenólicos no antociánicos que disminuyen significativamente a lo largo de la acetificación son (+)-catequina, que incluso llega a desaparecer, y glucósido de *trans*-resveratrol.

2. Los principales cambios en la composición fenólica de vinagres de vino tinto debidos al proceso de acetificación sumergida son: el incremento de la concentración de compuestos del tipo vitisina y etil derivados y una disminución de la concentración de los antocianos monoméricos (malvidina-3-glucósido y malvidina-3-(6-acetil)-glucósido), de los ácidos fenólicos (ferúlico, cafeico y caftárico) y de los flavan-3-ol ((+)-catequina).

3. Se han identificado por primera vez en vinagre de vino derivados antociánicos del tipo piranoantocianos y etil derivados. Asimismo, catequil-piranocianidina-3-glucosido y guaiaquil-piranomalvidina-3-(6-acetil)-glucósido se han identificado por primera vez en vinagre y vino, y carboxi-piranocianidina-3-(6-acetil)-glucósido, carboxi-piranocianidina-3-(6-p-coumaril)-glucósido y carboxi-piranodelfinidina-3-(6-acetil)-glucósido en vino de *Cabernet Sauvignon* y en vinagre. Además, se ha descrito la actividad antioxidante de malvidina-3-(6-acetil)-glucósido aislada en una fracción al 81% de pureza.

4. El tipo de madera con el que el vinagre ha estado en contacto determina su composición fenólica. Los compuestos marcadores de cada tipo de madera son: (+)-taxifolin para cerezo, altas concentraciones de ácido gálico (galato de etilo) para castaño y (+)-dihidrorobinetina para acacia. De hecho, se pueden construir funciones discriminantes que clasifican los vinagres de vino tinto con un 98-100% de aciertos.

5. (+)-Dihydrorobinetina, aislada de vinagres en contacto con madera de acacia, presenta actividad antioxidante. Su alta concentración en el vinagre envejecido en acacia (266-348 mg/L) hace que contribuya entre 8,6-13,6% al valor de actividad antioxidante total del vinagre. Este compuesto se degrada durante el tostado de la madera, disminuyendo la cantidad cedida al vinagre (23,5 mg/L).

6. El protocolo de cata desarrollado para el análisis de vinagres aumenta la precisión de los resultados del panel. Los trece atributos seleccionados para el análisis descriptivo mostraron una buena reproducibilidad, demostrada por el análisis DPLSR.

7. El impacto de la madera en el aroma de los vinagres es percibido por los miembros del panel, tanto al final de la acetificación como después del envejecimiento. Los resultados obtenidos de las pruebas triangulares en el análisis sensorial, indican que los miembros del panel de cata son capaces de discriminar la mayoría de las muestras elaboradas en las distintas maderas. Las muestras más valoradas en el análisis descriptivo son aquellas que están en contacto con las maderas de roble y cerezo. Los vinagres en contacto con madera de cerezo poseen notas más elevadas del descriptor frutos rojos, y este aumenta con el envejecimiento. Por el contrario, las notas a madera, dulzor y vainilla destacan más en los vinagres en contacto con madera de roble.

8. La identificación de delfinidina-3-glucósido, peonidina-3-glucósido y cianidina-3-galactósido ha contribuido a la descripción de la composición antociánica del sustrato de fresa, además de la identificación de 5-carboxipiranopelargonidina-3-glucósido en la variedad *Camarosa*. La actividad antioxidante de dos fracciones antociánicas aisladas con un alta pureza mostró que la pelargonidina-3-glucóside posee mayor actividad que la pelargonidina-3-rutinósido.

The main conclusions that can be drawn on the basis of the obtained results are:

1. The Total Phenols Index does not significantly change through the acetification by surface culture while the Total Anthocyanin decreases around 50%. The concentration of phenolic compounds that decrease significantly due to the acetification process are *trans*-resveratrol glucoside and (+)-catechin. The latest one even disappears.

2. After the acetification process of red wine vinegar by submerged culture the concentration of vitisin-type and ethyl-linked compounds increase, while monomeric anthocyanins (malvidin-3-glucoside and malvidin-3-(6-acetyl)-glucoside), phenolic acids (ferulic acid, caffeic acid and caftaric acid) and flavan-3-ol compound ((+)-catechin) decrease.

3. Pyranoanthocyanins and ethyl linked compounds have been identified for the first time in red wine vinegar. Furthermore, catechyl-pyranocyanidin-3-glucoside and guaiacyl-pyranomalvidin-3-(6-acetyl)-glucoside have been described in wine and vinegar for the first time, and carboxy-pyranocyanidin-3-(6-acetyl)-glucoside, carboxy-pyranocyanidin-3-(6-p-coumaryl)-glucoside and carboxy-pyranodelfinidin-3-(6-acetyl)-glucoside in vinegar and *Cabernet Sauvignon* wine. Antioxidant activity has been approximately determined for malvidin-3-(6-acetyl)-glucoside isolated from vinegar.

4. Wood used in vinegar elaboration determines its phenolic composition. (+)-Taxifolin, high concentration of gallic acid and (+)-dihydrorobinetin are markers of cherry, chestnut and acacia wood, respectively. In fact, linear function obtained with phenolic compounds as variables, correctly classify at 98-100% of red wine vinegars.

5. The *in vitro* antioxidant activity of (+)-dihydrorobinetin is reported. The high amounts of this compound in vinegars aged in acacia wood (266-348 mg/L) increase its overall antioxidant activity at 8.6-13.6%, contributing to the

functional properties of vinegars. The thermal treatment of the wood decreases the release of (+)-dihydrorobinetin into the vinegar (23.5 mg/L).

6. The tasting protocol proposed provides reproducible results and increases the accuracy in panelists' answers. The selected attributes for descriptive analysis showed a good between-run reproducibility as shown by the DPLSR analysis of replications.

7. The impact of wood on vinegar aroma is perceived by the panel, at the end of acetification and after ageing. According to the results of triangle test, the panel is able to distinguish most of the vinegars according to the different woods they were made in. The highest scores for general impression are given to the vinegars elaborated using cherry or oak woods. The red fruit note is highest for vinegars in contact with cherry woods and increased throughout the ageing process. On the other hand, woody aroma, sweet aroma and vanilla perception have the highest marks in vinegars elaborated in oak wood.

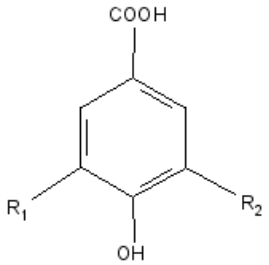
8. This study makes new contributions to the anthocyanin profile of strawberry as follows: delphinidin-3-glucoside, peonidin-3-glucoside and cyanidin-3-galactoside. Additionally, 5-carboxypyranopelargonidin-3-glucoside has been identified for the first time in *Camarosa* strawberry variety. The antioxidant activity of two fractions isolated with high purity showed that pelargonidin-3-glucoside is more active than pelargonidin-3-rutinoside fraction.

6. ANEXO / APPENDIX

NO FLAVONOIDES

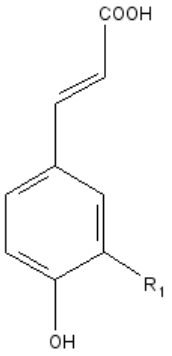
Ácidos fenólicos

Ácidos benzoicos C_6-C_1



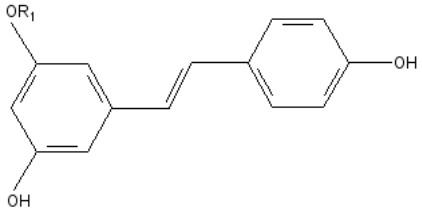
R1=OH;R2=OH	Ácido gálico
R1=H;R2= OCH_3	Ácido vainíllico
R1=OH;R2=H	Ácido Protocatéquico
R1= OCH_3;R2=OCH_3	Ácido siríngico
R1=H;R2=H	Ácido p-hidroxibenzoico

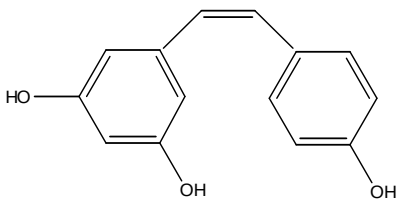
Ácidos hidroxicinnámicos C_6-C_3



R1=OH	Ácido cafeico
R1=H	Ácido p-cumárico
R1= OCH_3	Ácido ferúlico

Estilbenos C_6-C_2-C_6



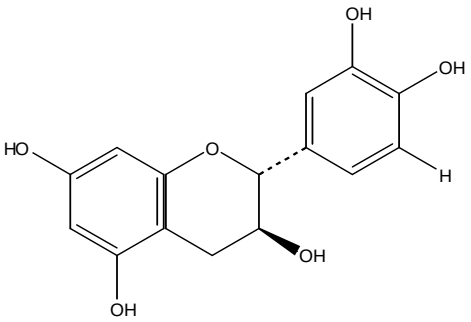


R=H *trans*-Resveratrol *cis*-Resveratrol

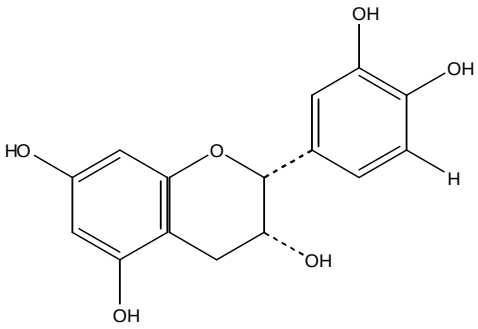
R=D-Glucosa Glucosido de *trans*-resveratrol

FLAVONOIDES C_6-C_3-C_6

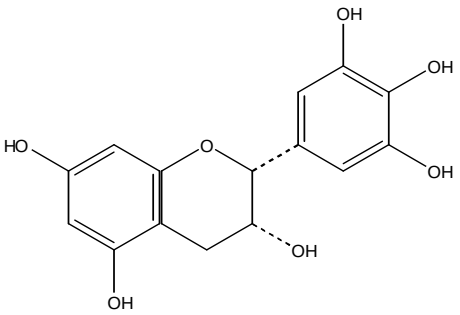
Flavanoles



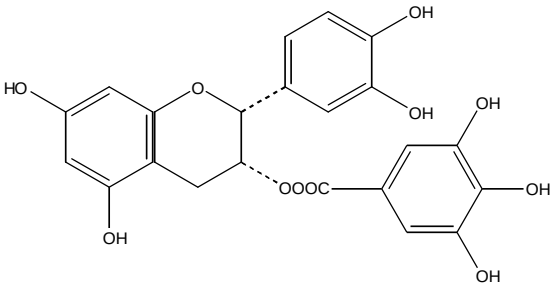
(+)-Catequina



(-)-Epicatequina

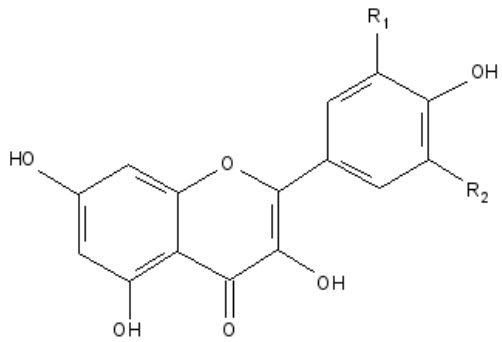


(-)-Epigalocatequina



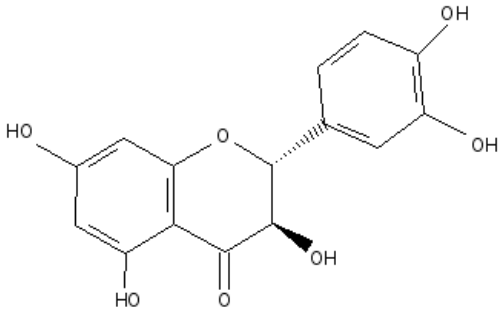
Galato de (-)-epicatequina

Flavonoles

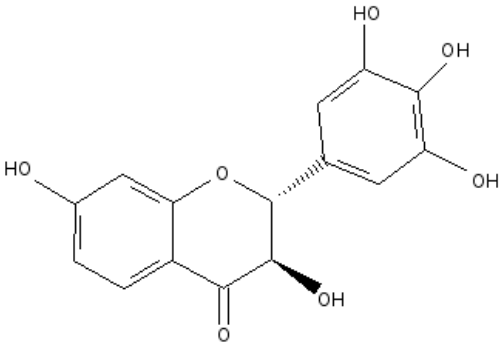


R1=H;R2=H	Kaempferol
R1=OH;R2=H	Quercetina
R1=OH;R2=OH	Miricetina

Flavononoles o dihidroflavonoles

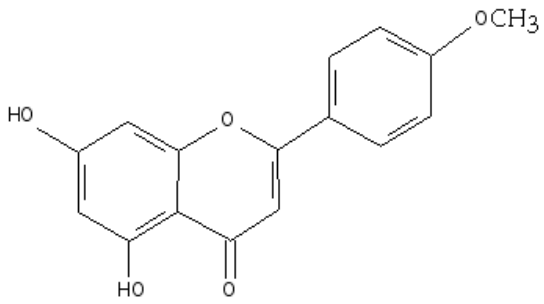


(+)-Taxifolin



(+)-Dihidrorobinetina

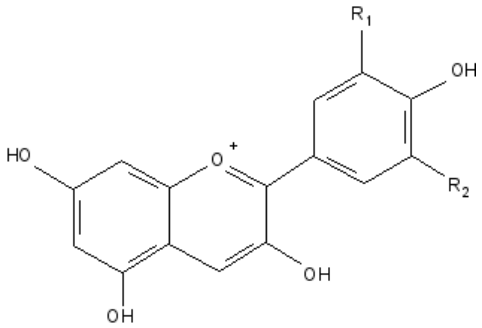
Flavonas



Acacetina

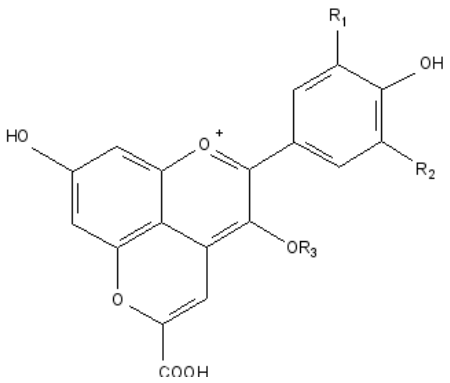
Antocianos

Antocianidinas



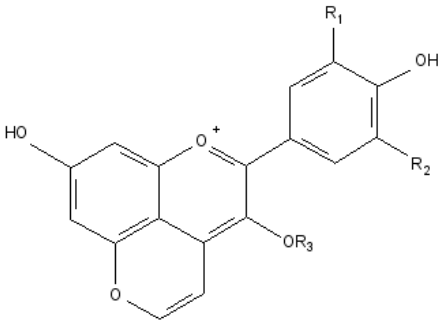
R1=OH;R2=H	Cianidina
R1=OH;R2=OH	Delfinidina
R1= OCH_3;R2=H	Peonidina
R1= OCH_3;R2=OH	Petunidina
R1= OCH_3;R2= OCH_3	Malvidina
R1=H;R2=H	Pelargonidina

Piranoantocianos



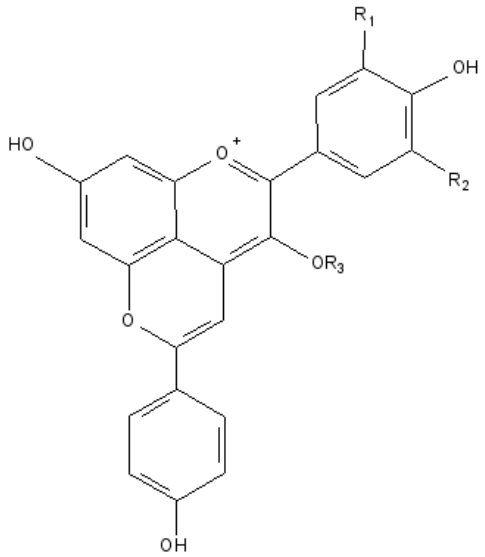
5-Carboxipiranoantociano

(Tipo Vitisina A)

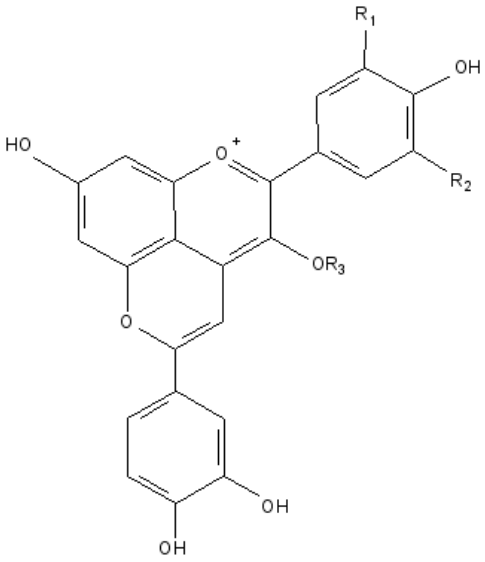


Piranoantociano

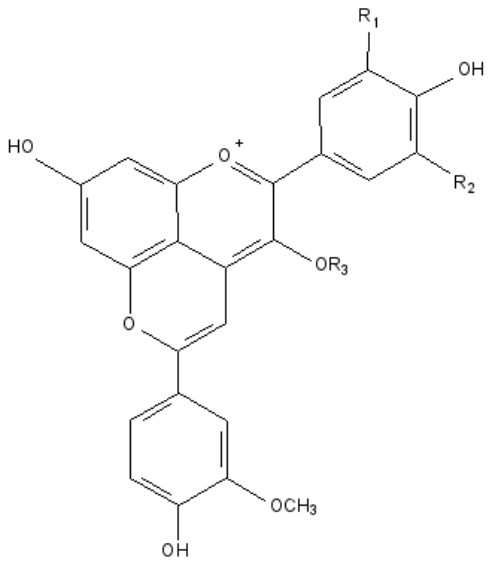
(Tipo Vitisina B)



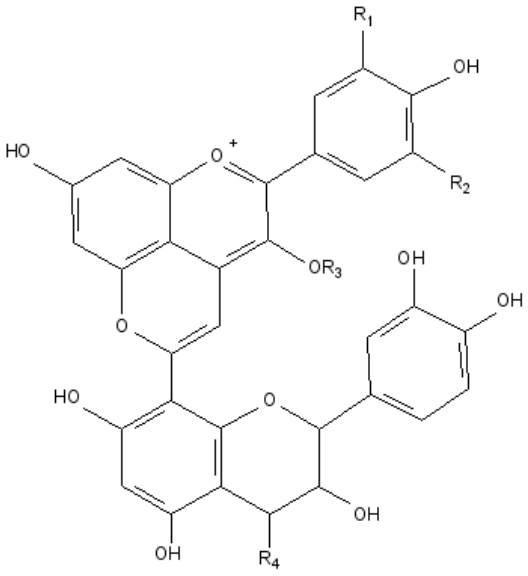
Vinilfenol aducto



Vinilcatecol aducto

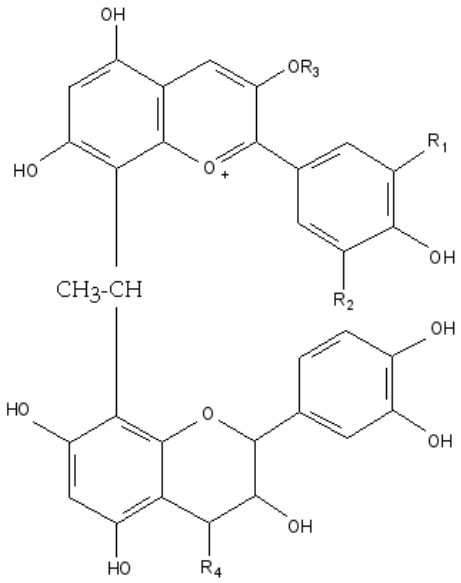


Vinilguaiacol aducto

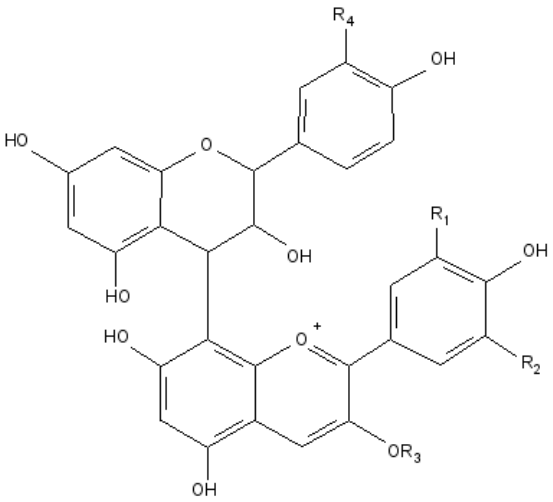


Vinilflavanol-piranoantociano

Otros derivados antociánicos



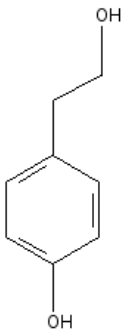
Etil derivado



Flavanol-(4,8)-antociano

OTROS FENOLES

Alcohol fenólico



Tirosol

Facultad de Farmacia
Departamento de Nutrición, Bromatología, Toxicología y Medicina Legal

COMPOSICIÓN POLIFENÓLICA DE VINAGRES DE VINO TINTO: INFLUENCIA DE LA ACETIFICACIÓN Y LA MADERA

ANA BELÉN CEREZO LÓPEZ