Tesis Doctoral

COMPUESTOS BIOACTIVOS EN ALIMENTOS FERMENTADOS OBTENIDOS A PARTIR DE SUSTRATOS NATURALES

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A mis Padres, a la memoria de mi Madre.

"El éxito no es el final, el fracaso no es letal: lo que cuenta es el coraje para continuar".

Winston Churchill

"Success is not final, failure is not fatal: it is the courage to continue that counts".

Winston Churchill

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RESUMEN/SUMMARY

RESUMEN.

La presente Tesis Doctoral se centra en el análisis de compuestos bioactivos en productos fermentados. Una primera parte ha tenido como objeto la determinación de los polifenoles no antocianos en un puré de fresa y en los fermentados alcohólicos y glucónicos obtenidos a partir de este sustrato. También se ha realizado el estudio de estabilidad de una bebida de fresa obtenida a partir de la fermentación glucónica. Dicha bebida fue diseñada como una alternativa viable para el aprovechamiento de los excedentes de producción de una fruta de marcado carácter perecedero y de elevada producción en España.

La segunda parte se ha centrado en la capacidad de las levaduras vínicas como productoras de compuestos bioactivos a partir de determinados aminoácidos aromáticos durante la fermentación alcohólica.

A tal fin, se han identificado y determinado los polifenoles no antocianos mediante el empleo de diferentes técnicas y equipos, como HPLC-DAD, UPLC-QTRAP (QqQ_{LIT}) y UPLC-QExactive. En todos los análisis de espectrometría de masas se utilizó el método MRM (Multiple Reaction Monitoring). Esta tarea identificativa tuvo como objeto el análisis del puré de fresa utilizado posteriormente como sustrato para la elaboración de dos fermentados diferentes (glucónico y alcohólico). El resultado de esta actividad nos permitió identificar un total de 106 compuestos fenólicos, 32 en el puré de fresa, 43 en los fermentados glucónicos, 66 en las fermentaciones alcohólicas y 64 en la bebida. Todo ello facilitó la identificación de 25 compuestos que, a la fecha de las publicaciones, no habían sido aún descritos en la fresa o productos derivados (12 flavonoides, 7 ácidos hidroxicinámicos, 2 taninos hidrolizables, 1 chalcona, 1 estilbeno, 1 ácido hidroxibenzoico, y 1 hidroxifenilacético).

El proceso de fabricación industrial del puré de fresa no comporta cambios sustanciales sobre el contenido de los polifenoles no antocianos, por lo que este proceso es respetuoso con la composición de la fruta, preservando su capacidad antioxidante sin sustanciales cambios.

Otro aspecto tratado en esta Tesis se ha centrado en el empleo del puré de fresa como sustrato para las fermentaciones. El análisis de los fermentados glucónicos constató que los compuestos mayoritarios fueron: la (+)-catequina, el hexósido del ácido *p*-cumárico y el HDDP-galoil glucósido, llegando a alcanzar un 62% del total de polifenoles no antocianos cuantificados. Además, la fermentación glucónica produjo un incremento de la concentración de la mayoría de los polifenoles no antocianos cuantificados (ácido gálico 36%).

En los fermentados alcohólicos se determinó que los compuestos más abundantes fueron: la (+)-catequina, (-)-epicatequin galato, galoil HHDP glucosa y el p-cumaroil glucósido. Por otra parte, se observaron incrementos en compuestos (homovanillico 4% y el monogaloil glucósido 36%) procedentes de la degradación de otros fenoles (quercetina y sus glicósidos y otros taninos en el caso del monogaloil glucósido) y un descenso en la actividad antioxidante (12-18%). Puesto que la fermentación glucónica preserva la actividad antioxidante y mantiene inalterado el contenido de fructosa del sustrato, el fermentado puede ser empleado como el ingrediente principal para el desarrollo de una nueva bebida.

La evaluación de la estabilidad de la bebida a base de fermentados de fresa incluyó la determinación de los polifenoles no antocianos a dos temperaturas de almacenamiento (ambiente y refrigeración). El análisis estadístico demostró que la composición de polifenoles no antocianos permaneciera sin cambios hasta el día 15 a temperatura ambiente (27 – 30ºC) y hasta el día 30 en temperatura de refrigeración (4ºC). Ello unido a la valoración sensorial de la bebida, nos llevó a la conclusión de que el período óptimo de almacenamiento de la bebida de fresa fue de 30 días a temperatura ambiente y de 60 días en condiciones de refrigeración. El análisis de los polifenoles no antocianos mediante UHPLC/MS-MS nos permitió determinar 64 compuestos, revelando que cuatro compuestos experimentaron los cambios más importantes. (+)-Catequina, procianidina B1 y el trímero de la procianidina, disminuyeron mientras que, ácido elágico y *p*-coumárico, aumentaron, especialmente el ácido protocatéquico (13 veces). Estos fenómenos se apreciaron de forma más significativa a temperatura ambiente que en refrigeración.

La segunda parte de esta Tesis aborda la producción de hidroxitirosol (HT) por la acción de las levaduras vínicas a partir de su aminoácido precursor, la tirosina. Mediante el análisis del contenido intracelular, se comprobó que las levaduras *Saccharomyces cerevisiae* (cepas QA23 y RED FRUIT) y no *Saccharomyces* (*Torulaspora delbrueckii)*, producen este compuesto (HT) durante la fermentación alcohólica. Asimismo se realizó, el análisis de cuatro inoculaciones de

levaduras (dos con cultivos puros de QA23 y RED FRUIT que se usaron como control y dos inoculaciones secuenciales) demostrándose que la inoculación con una única cepa de levadura *S. cerevisiae* (QA23) fue la que produjo mayores concentraciones de HT. Por otra parte, se han realizado fermentaciones de seis diferentes variedades de uva blanca (*Corredera, Moscatel, Chardonnay, Palomino fino, Sauvignon Blanc y Vijiriega*) con la levadura *S. cerevisiae* (AROMA WHITE), siendo *Sauvignon Blanc* la que alcanzó mayores concentraciones de HT. A raíz de los resultados obtenidos, se llegó a la conclusión de que tanto la levadura como la composición del mosto son factores que influyen en la producción del HT.

Con la finalidad de ahondar en el conocimiento de la producción de compuestos bioactivos relacionados con los aminoácidos aromáticos (triptófano, tirosina y fenilalanina), se ha estudiado el contenido intra y extracelular de seis replicados de diferentes fermentaciones efectuadas en mosto sintético utilizando tres cepas de levaduras: dos *Saccharomyces* (QA23· y RED FRUIT) y una no *Saccharomyces* (*Torulaspora delbrueckii*). El resultado de esta tarea nos ha permitido comprobar que dos compuestos, a saber 2-aminoacetofenona (relacionado con un defecto aromático) y triptofol sulfonado están presentes en el medio intracelular, debiéndose su producción a la acción directa de las levaduras. Este hallazgo contrasta con los antecedentes que sostenían que la síntesis se asociaba únicamente a la presencia de sulfitos en el vino. Adicionalmente, hemos podido describir que la de-acetilación de los N-acetil etil ésteres del triptófano y de la tirosina conforma otra vía para la producción de sus correspondientes ésteres etílicos. Y por último, se comprobó que la mayor concentración de melatonina se obtuvo en el día quince de la fermentación, pero éste no es un resultado concluyente.

Para afrontar la dificultad de cuantificar ciertos metabolitos presentes en muy baja concentración (melatonina y serotonina) pertenecientes a la ruta catabólica de los aminoácidos aromáticos, se abordó la optimización de un método de extracción intracelular. Comparándose la eficacia de tres métodos de extracción. Esto nos permitió demostrar que dos métodos de extracción a baja temperatura (con centrifugaciones a -20ºC y 4ºC) fueron más respetuosos con la integridad de los compuestos relacionados con el metabolismo del triptófano, la melatonina y de la tirosina que el efectuado con etanol a ebullición.

En general, los resultados aportados por la presente Tesis Doctoral proporcionan una solución viable para el aprovechamiento de los excedentes de producción de la fresa, profundizando, además, en el conocimiento del comportamiento de las levaduras en relación al potencial bioactivo de los alimentos producidos.

SUMMARY.

This Doctoral Thesis focuses on the analysis of bioactive compounds in fermented foods. The objective of the first part is to determine the non-anthocyanin polyphenols in the strawberry purée and in the gluconic and alcoholic fermented products obtained from this substrate. We also studied the stability of a beverage elaborated from strawberry gluconic fermentation. This beverage was designed as a viable alternative to take advantage of the surplus of the production of this perishable fruit produced in Spain.

The second part focuses on the ability of the winemaking yeast to produce the bioactive compounds from aromatic amino acids along the alcoholic fermentation process.

In order to do so, we have analysed the composition of non-anthocyanin polyphenols, both in the strawberry pureè and in fermented derived products by using different techniques and instruments such as the HPLC-DAD, the UHPLC-QTRAP (QqQ_{LIT}) and the UHPLC-QExactive. MRM (Multiple Reaction Monitoring) was used for all spectrometric mass analysis. The outcome of this activity allowed us to identify 106 phenolic compounds, of which 32 were in the strawberry purée, 43 in the gluconic fermented product, 66 in the alcoholic fermented product and 64 in the drink. As far as we know, 25 compounds had not been reported in the literature, in the strawberry purée or its derivatives (12 flavonoids, 7 hydroxycinnamic acids, 2 hydrolized tannins, 1 chalcone, 1 stilbene, 1 hydroxybenzoic acid and 1 hydroxyphenyl acetic acid).

The industrial process production the strawberry purée does not imply substantial changes in the non-anthocyanin profile, hence preserving the original composition of the fruit and its antioxidant properties.

The strawberry purée was used as a substrate for fermentation processes. The analysis of the gluconic fermented products showed that the compounds with higher concentrations were (+)-catechin, p-coumaric hexoside and HHDP-galloyl glucoside, reaching 62% of the total

non anthocyanin polyphenols. Moreover, gluconic fermentation produced an increase in the concentration of most of the non-anthocyanin quantified polyphenols (36% gallic acid).

Regarding alcoholic fermented products, the most abundant compounds were (+)-catechin, (-)-epicatechin gallate, HHDP-galloyl glucose and *p*-coumaroyl glucoside. Moreover, an increase along alcoholic fermentation was observed for those compounds (homovanillic 4% and monogalloyl glucose 36%) coming from the degradation of other phenolic compounds (quercetin and quercetin glycosides and other tannins in case of monogalloyl glucoside) together with a decrease of the antioxidant activity (12-18%). Since the gluconic fermentation process preserves the antioxidant activity and does not alter the content of the fructose of the substrate, we were able to use the gluconic fermentation product as the main ingredient for the elaboration of a new beverage.

In order to assess the stability of the beverage, we studied the non-anthocyanin polyphenols at two temperatures of storage (room and refrigeration temperature). The statistical analysis showed that the composition of non-anthocyanin polyphenols remains unchanged until day 15 of storage at room temperature (27 – 30ºC) and until day 30 under refrigerated conditions (4ºC). This fact together with the sensorial assessment of the beverage, led us to the conclusion that the optimal storage period was of 30 days at room temperature and 60 days at refrigeration temperature. The analysis of non-anthocyanin polyphenols through UHPLC/MS-MS method was capable to determine 64 compounds revealing important changes in some compounds. Among them, (+)-catechin, procyanidin B1 and procyanidin trimer decreased. On the other hand, ellagic acid and *p*-coumaric acid increased and protocatechuic acid experimented an increase of up to 13 times. These phenomena were more particularly observed at room temperature than in refrigeration.

The second part of the Thesis focuses on the production of hydroxytyrosol (HT) resulting from the action of the winemaking yeast over its precursor amino acid, tyrosine. The analysis of the intracellular media confirmed that the *Saccharomyces cerevisiae* strains of yeast (QA23 and RED FRUIT) and the non-*Saccharomyces* strain (*Torulaspora delbrueckii*) produced this compound (HT) during the alcoholic fermentation process. Likewise, we studied four inoculations of yeast cultures (two pure cultures with strains QA23 and RED FRUIT as controls and two sequential inoculations). We found that inoculations with the single strain of yeast S. *cerevisiae* (QA23) yielded the highest concentrations for HT. Besides, samples coming from the fermentation of 6 white winegrapes musts were analysed (*Corredera, Moscatel, Chardonnay,*

Palomino fino, *Sauvignon Blanc y Vijiriega*). The yeast culture used for these experiments was *S. cerevisiae* (AROMA WHITE). It was observed that the fermentation of *Sauvignon Blanc* grape must produced the highest concentration for HT. As a consequence, it was concluded that both the yeast strain and the must composition are factors that influence HT production.

With the aim to get a better knowledge of the bioactive synthesis related to aromatic amino acids (tryptophan, tyrosine and phenylalanine), we studied the intra and extra cellular media of six replicates of different fermentations of synthetic must. Three winemaking yeasts, two strains of *Saccharomyces* (QA23 and RED FRUIT) and one of the non-*Saccharomyces* (*Torulaspora delbrueckii*) were selected for these experiments. The result of this task helped us to realize that two compounds, 2-aminoacetophenone (that gives a bad flavour to fermented foods) and tryptophol sulfonated are present in the intracellular media, and hence whose production is due to the result of the direct action of the yeast. This finding proves that their synthesis is not exclusively associated with the presence of sulphites in wine, as it is referenced in the literature. Additionally, we observed the de-acetylation of N-acetyl-ethyl esters of tryptophan and tyrosine as an alternative way to produce their own ethyl esters. And finally, the highest concentrations of melatonin were measured at the fiftieth day of the fermentation by QA23 not being a conclusive result

To tackle the difficulty to quantify certain metabolites whose concentration is very low (melatonin and serotonin, belonging to the aromatic amino acids catabolism pathway), an optimisation of the intracellular extraction method was performed. Hence, we compared the efficiency of three extraction methods. We found that the two methods at low temperature (with centrifugations at -20ºC and 4ºC) were more respectful towards the integrity of the compounds (Tryptophan and tyrosine related compounds) than the extraction with boiling ethanol.

Overall, the results brought in this Doctoral Thesis bring a viable solution to optimize the excess of the strawberry production, and it deepens in knowledge of the performance of the yeast in relation to the bioactive potential of the produced food.

ABREVIATURAS

Abreviaturas.

2AA: 2-amino acetofenona.

5-HT: Serotonina.

5-HTOL: 5-Metoxitriptofol.

5H-IAA: ácido hydroxi indol -3-acético.

5HIAA: ácido 5-hidroxi indol acético.

5HTP: 5-hidroxi triptófano.

5MIAA: ácido 5-methoxi indol acético.

5MOT: 5-Metoxitriptamina.

6-aMTs: sulfatoxi melatonina.

AAPH: 2,2'-diazo-bis (amidine-propane-dihydrochloride).

ANOVA: Análisis de la varianza.

ANT: ácido antranílico.

APCI: ionización química a presión atmosférica.

CABD: Centro Andaluz de Biología del Desarrollo.

CH_3-IND: 3-metil-indol.

CH_3O-IAA: ácido 5-metoxi-3-indol acético.

CH_3O-TRP: 5-Metoxi triptófano.

CID: Disociación inducida por colisión.

DOPA: dopamina.

DPPH: 2,2-difenil-1-picrilhidracilo.

E-I Ca: ácido 3-etil-indol carboxílico.

ESI: ionización por electrospray.

EUROSTAT: Oficina de Estadística de la Unión Europea.

FAOSTAT: Base de datos de estadística alimentaria y agrícola de la Organización de Alimentos y Agricultura de las Naciones Unidas.

HEPES: solución Tampón del ácido N-(2-hidroxietil) piperazina-N'-(2-etanosulfonico) a 70 mM.

HHDP: grupo hexahidroxidifenil.

HPLC: Cromatografía líquida de alta resolución.

HRMS: Espectrometría de masas de alta resolución.

HT: hidroxitirosol.

I_3C: indol carbinol.

IAA-EE: éster etílico del ácido 3-indol acético.

IAA: ácido 3-Indol acético.

IAAME: éster metílico de ácido indol acético.

IBA: ácido 3-indol butírico.

ICA: indol carboxaldehido:

IFAPA: Instituto de Investigación y Formación Agraria y Pesquera – Junta de Andalucía.

ILA: ácido 3-indole láctico.

IND: indol.

IPA: ácido 3-indol propiónico.

IPy: ácido 3-indol pirúvico.

IS: estándar interno.

KYN: DL-kinurenine.

KYNA: ácido kinurenico.

LC-DAD: cromatografía líquida asociada a un detector de diodos.

LC-MS: Asociación de cromatografía líquida con espectrometría de masas.

LDA: análisis discriminante lineal.

M-IAA: ácido metil indol acético.

m/z: relación masa- carga.

MEL: melatonina.

MLT: Melatonina.

MRM: Monitorización de Reacción Múltiple.

MS/MS: espectrometría de masas en tándem.

N-SER: N-acetil serotonina.

N-TEE: N-acetil etil éster del triptófano.

N-TRP-EE: N-acetil etil éster del tritófano.

N-TYR-EE: N-acetil etil éster de L-tirosina.

Nano-ESI: ionización nano-electrospray.

NIC: nicotinamida.

OH-ANT: ácido 3-hidroxi-antranílico.

OH-KYN: 3-hidroxi kynurenine.

OH-MEL: 6-Hidroximelatonina.

OH-Ph-AA: ácido 4-hidroxi-fenil acético.

OH-Ph-Py: ácido 4-hidroxi-fenil pirúvico.

OH-TRP: 5-hidroxi-L-triptófano.

ORAC: Capacidad de absorción de radicales oxígeno.

PANOPA: nitrógeno amino primario.

PCA: análisis de componentes principales.

Ph-AA: ácido fenil acético.

Ph-LA: ácido fenil láctico.

Ph-Py: ácido fenil pirúvico.

PHE: fenil alanina.

QLIT: Analizador de cuadrupolo-Analizador de trampa de iones lineal.

SERO: serotonina.

SPE: Extracción en fase sólida.

TEE: éster etílico del triptófano.

TOF: analizador de tiempo de vuelo.

TOL: Triptofol.

Trólox: ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico.

TRP-EE: éster etílico del triptófano.

TRP-ME: éster metílico del DL-triptófano.

TRP:triptófano.

TRYPT: triptamina.

TYL: tirosol.

TYR-EE: éster etílico de la tirosina.

TYR-ME: éster metílico de L-tirosina.

TYR: L-tirosina.

TYRA: tiramina.

TYRME: éster metílico de la tirosina.

UHPLC: Cromatografía líquida de ultra resolución.

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1. INTRODUCCIÓN

1

1.-INTRODUCCIÓN

EL total de la producción de fresa en Europa representó en 2015 un volumen de 1.764.391 toneladas. Los primeros productores europeos de fresa, por volumen de producción, fueron: España, Polonia, Federación Rusa, Alemania e Italia (FAOSTAT página web consultada 12/2017). España produjo en el año 2015 un total de 397.369 toneladas (FAOSTAT página web consultada 12/2017) con un valor económico de 457,982 millones de euros (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente, página web consultada 12/2017).

La provincia de Huelva (Andalucía- España) es la cuarta zona de mayor producción de fresa (*Fragaria X ananassa*) del mundo (FAOSTAT, 2012). Esta fruta se caracteriza por ser cultivada en un período muy corto de tiempo ofreciendo una gran abundancia de cosechas. Esta sobreproducción produce un decrecimiento en los precios y, al existir más oferta que demanda, los excedentes de producción son desperdiciados. A esta circunstancia se une el marcado carácter perecedero de la fresa que impide un almacenamiento prolongado, produciéndose relevantes pérdidas económicas en el sector.

A. COMPOSICIÓN DE LA FRESA (*Fragaria X ananassa*).

1. Características de la fruta.

La fresa es el fruto de la planta rastrera perteneciente a la familia de las Rosáceas, la subfamilia Rosoideae y al género *Fragaria*.

La fresa silvestre más abundante es la *Fragaria vesca*, mientras que la especie más cultivada en el mundo se denomina *Fragaria ananassa*. Las otras especies frecuentemente cultivadas son la *Fragaria virginiana* que es procedente del norte de América y la *Fragaria chiloensis* que, como su nombre indica, es originaria de Chile (Jianghao Sun, Liu, Yang, Slovin, & Chen, 2014).

1. INTRODUCCIÓN

Los frutos de la especie *Fragaria ananassa* se caracterizan por su mayor tamaño y excelente sabor, siendo estas cualidades las que han hecho que el cultivo de esta variedad sea el más extendido a nivel mundial.

El fruto se define como un agregado porque proviene de una sola flor que posee carpelos separados. De cada ovario sale un fruto que está formado por varios aquenios. Estos se conocen como falsas semillas que aparecen adosadas en la pulpa del fruto. Cada aquenio es un fruto monocarpo seco y de una sola semilla. En el proceso de formación del fruto, cuando los óvulos se transforman en aquenios, éstos estimulan el engrosamiento del receptáculo que posteriormente forma el fruto carnoso.

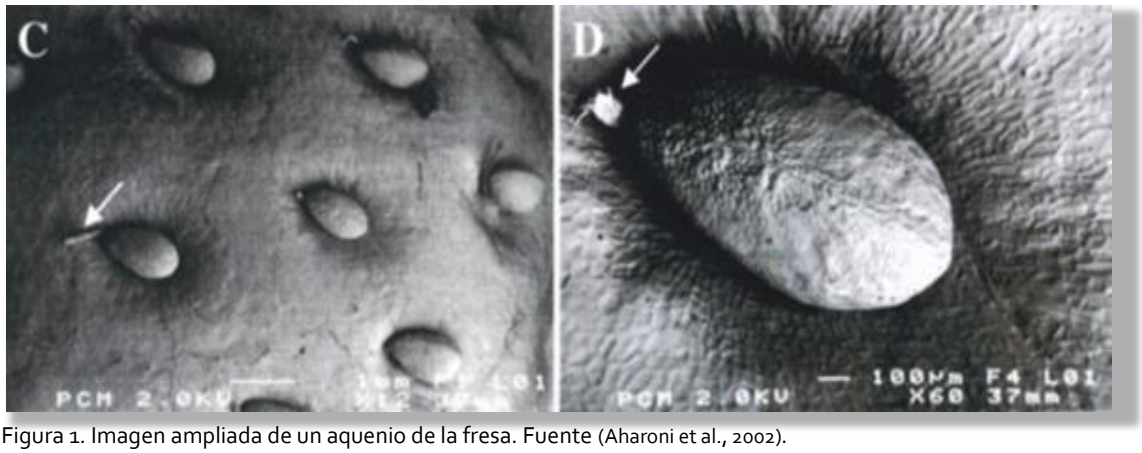

Figura 1. Imagen ampliada de un aquenio de la fresa. Fuente (Aharoni et al., 2002).

Tabla 1. Composición de los macronutrientes y micronutrientes de la fresa. Fuente (Universidad Técnica de Dinamarca ,página web consultada (www.frida.fooddate.dk 12-2017) y Base de datos Española de Composición de Alimentos (BEDCA) (www.bedca.net 12-2017).

	Cont./100g	Unidades	Vitaminas	Cont./100g	Unidades
Energía, KJ	146,0	KJ	Vit. A	1,0	RE
Grasa total	0,5	g	beta-caroteno	40,0	µg
Proteínas totales	0,7	g	Vit. E	2,0	α-TE
Carbohidratos	7,1	g	Vit B2, Riboflavina	0,04	Mg
Fibra	2,2	g	Niacina	0,434	Mg
Aqua	86,6	g	Vit. B6 total	0,06	Mg
Minerales	**Cont./100g**	**Unidades**	Pantoténico	0,129	Mg
Sodium, Na	2,0	mg	Biotina	0,701	µg
Potasio, K	190,0	mg	Folato	20,0	µg
Calcio, Ca	25,0	mg	Vit. C	60,0	Mg
Magnesio, Mg	12,0	mg	**Carbohidratos**	**Cont./100g**	**Unidades**
Fósforo, P	26,0	mg	Fructosa	3,16	g
Hierro Fe	0,8	mg	Glucosa	2,85	g
Cobre, Cu	0,038	mg	Sacarosa	0,056	g
Zinc, Zn	0,104	mg	Azúcares totales	6,07	g
Iodo (ioduro)	8,0	µg			
Selenio, Se	traza				
Chromo, Cr	0,069	µg			

RE (equivalentes de retinol); α-TE (equivalentes de α tocoferol)

En Huelva (España) se cultivan principalmente las variedades Candonga, Fortuna y Camarosa (datos procedentes de la cosecha de 2012), aunque con posterioridad la variedad Camarosa ha dejado de emplearse intensivamente, favoreciendo los cultivos de variedades más tempranas como Fortuna, Sabrina y Splendor (Agencia de Gestión Agraria y Pesquera de Andalucía , 2013).

La fresa contiene altos niveles de micronutrientes como vitamina C, folato y además compuestos bioactivos como polifenoles y melatonina entre otros, considerados beneficiosos para la salud. En la tabla 1 se detallan los principales macronutrientes y micronutrientes de la fresa.

2. Compuestos polifenólicos de la fresa.

La fresa es una fuente muy rica en compuestos bioactivos (Oszmianski & Wojdylo, 2009; Stürtz, Cerezo, Cantos-Villar, & Garcia-Parrilla, 2011) y, en particular, en compuestos polifenólicos con alto poder antioxidante (Hannum, 2004), llegándose a considerar que el consumo regular de esta fruta puede ser beneficioso para la salud humana (Hannum, 2004) .

Los polifenoles son metabolitos secundarios de las plantas ubicuos en el reino vegetal que generan gran interés por sus numerosas propiedades. Contribuyen en gran medida, a las propiedades organolépticas de la frutas.

Los referidos compuestos son sintetizados durante el desarrollo natural de la planta así como en situaciones de estrés derivadas de la exposición a radiaciones ultravioleta, actuando como mecanismo de respuesta. Los polifenoles basan su poder antioxidante en la donación de un electrón al radical libre para convertirle en una molécula inofensiva (Charles W. I. Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012).

2.1. Clasificación de los polifenoles.

Los polifenoles presentan una gran diversidad de estructuras químicas, desde moléculas simples como monómeros y oligómeros a polímeros. Todos tienen en común el poseer un anillo aromático que porta al menos un grupo hidroxilo. En general, por su estructura química, se clasifican en dos grandes grupos: flavonoides y no flavonoides. Los primeros comprenden el grupo con la estructura C6-C_3-C6. Dentro de este grupo, se encuentran las flavanonas, los dihidroflavonoles, los flavonoles, los flavan 3-oles, los

antocianos, las isoflavonas y las procianidinas, también llamadas proantocianidinas o taninos condensados.

Los no flavonoides se clasifican y se subdividen en ácidos fenólicos y estilbenos y taninos hidrolizables (Figura 2).

Siguiendo la estructura de la figura 2, los polifenoles se clasifican en flavonoides y no-flavonoides

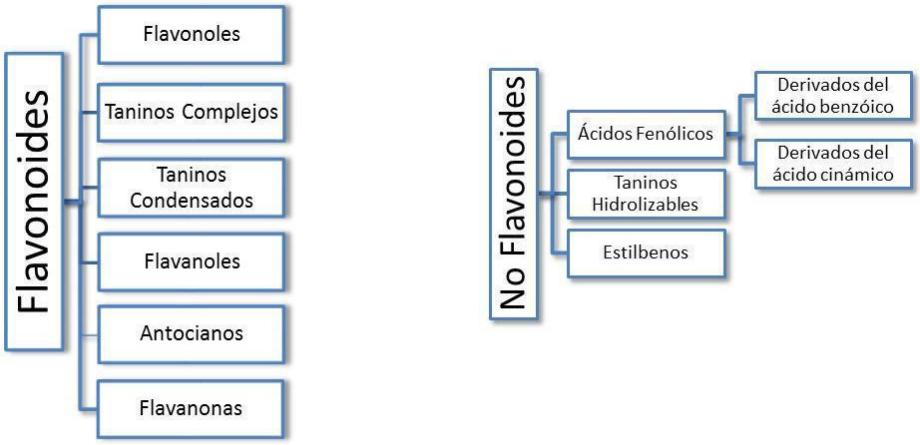

Figura 2. Clasificación de los polifenoles.

Los elagitaninos, conjuntamente con los antocianos y los flavan 3-oles, son los compuestos fenólicos más abundantes de la fresa (Aaby, Mazur, Nes, & Skrede, 2012; Määttä-Riihinen, Kamal-Eldin, & Törrönen, 2004), y en concreto los ácidos *p*-cumáricos en forma de diglicosilados y los ésteres. Los ésteres del ácido gálico son propios de la fresa y no se han descrito en otras especies de Rosáceas (Määttä-Riihinen et al., 2004). En la Figura 3 se representa la distribución de las principales clases de polifenoles descritas en la fresa.

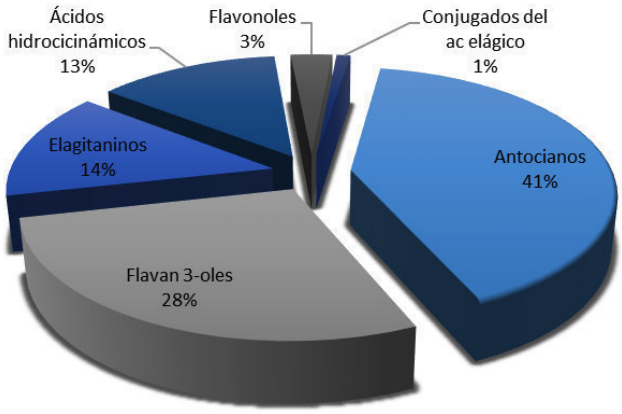

Figura 3. Distribución de las principales clases de polifenoles en la fresa.
Adaptado de (Aaby et al., 2012).

2.1.1. Flavonoides.

Es el nombre genérico de una clase de más de 6.500 moléculas cuya clasificación se basa en la presencia de un esqueleto de 15 átomos de carbono (Corradini et al., 2011). Se clasifican en subclases de compuestos: flavonoles, flavanonas, flavan-3-oles o flavanoles, antocianos, taninos complejos y taninos condensados.

Se caracterizan por tener una estructura C6-C3-C6 (dos anillos aromáticos bencénicos) A y B unidos entre sí por una cadena de tres átomos de carbono conformando un anillo heterocíclico oxigenado unido al anillo A) tal y como muestra la Figura 4. Constituyen la mayoría de los compuestos responsables de la coloración amarilla, roja y azul de las frutas. En la Tabla 2 se muestran los flavonoides más comunes en las frutas y vegetales.

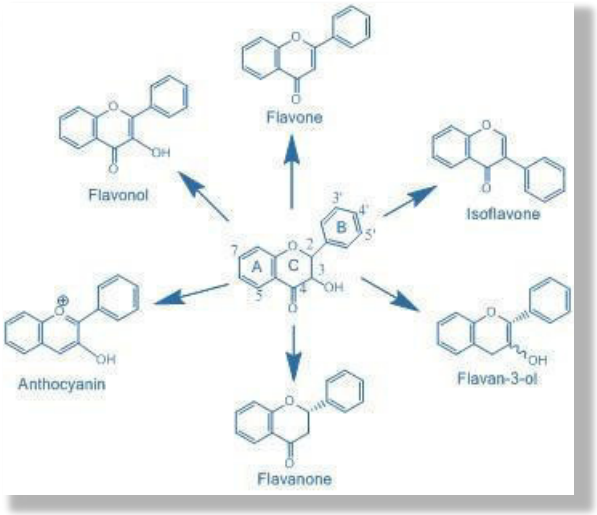

Figura 4. Estructura química de los flavonoides (Nishiumi et al., 2011).

Están ampliamente distribuidos en frutas y vegetales. Son reconocidos antioxidantes naturales por su habilidad de quelar metales y atrapar radicales libres, como lo demuestran estudios in vitro (Charles W. I. Haminiuk et al., 2012).

Los flavonoides constituyen aproximadamente las dos terceras partes de los fenoles de la dieta, presentándose más en forma de glicósidos que en forma de agliconas libres. La glicosilación hace que los flavonoides sean menos reactivos, más polares y, por tanto, más solubles en agua. Se puede considerar que esta modificación es una forma de protección de la planta para prevenir daños citoplasmáticos y como una forma segura de almacenar a estos compuestos en las vacuolas celulares (Corradini y col., 2011). En los *O*-glicósidos de los flavonoides, uno o más grupos hidroxilos de la aglicona se unen al azúcar mediante la

formación de un enlace O-C. En principio, cada grupo hidroxilo puede ser glicosilado pero ciertas posiciones se ven favorecidas. Así, por ejemplo: el grupo 7-hidroxilo en flavonas y flavanonas; el 3 y 7-hidroxilo en flavonoles y flavanonas y el 3 y el 5-hidroxilo en antocianos son los sitios más comunes de glicosilación (Corradini y col., 2011).

Tabla 2. Flavonoides más comunes y la fuente natural en donde se han descrito (De la Rosa, Álvarez-Parrilla & González-Aguilar, 2002

Subgrupo de Flavonoides	Favonoides más comunes	Frutas/vegetales
Flavonas	Apigenina, luteonina	Remolacha, pimientos, coles de Bruselas, repollo, coliflor, cebollino, col rizada, lechuga, espinacas, tomates, berro
Flavonoles	Quercetina, kaempferol, miricetina, isorhamnetin	Manzanas, frutos rojos, brócoli, repollo, coliflor, arándanos, uvas, col rizada, cebolla, pimientos , espinacas, acelgas, tomates , berros
Flavanonas	Taxifolin	Frutas cítricas
Antocianos	Cianidina, delfinidina, malvidina, pelargonidina, peonidina, petunidina	Frutos rojos, berenjena, granada, ciruelas, cebolla morada, patatas rojas, uva roja, rábano rojo, fresas, cualquier fruta o vegetal con color rojo o púrpura
Flavanoles / Procianidinas	Catequina, epicatequina, galocatequina y galto de epigalocatequina	Manzanas, uvas, ciruelas, peras, mangos, melocotones, frutos rojos y vegetales en general
	Monómeros,dímeros (procianidinas B1, B2), y oligómeros	Manzanas, cerezas, frutos rojos, uvas, melocotones y peras
Chalconas	Xantohumol Floretin	Lúpulo Manzanas
Isoflavonas	Daidzeína, genisteína	Brotes de soja

Basado en: (Tsao y col. 2005)

La glucosa es el azúcar que más comúnmente se ha hallado glicosilando a los flavonoides, seguido de la galactosa, la ramnosa, la xilosa y la arabinosa. Por el contrario, es menos común encontrar a los ácidos glucurónicos y galacturónidos asociados a los flavonoides. Los disacáridos también se encuentran asociados con estos compuestos; los más comunes son los rutósidos (6-*O*-α-L-ramnosil-D-glucosa) y el neohesperidosido (2-*O*-α-L-ramnosil-D-glucosa)(Corradini y col, 2011).

2.1.1.1. Flavonoles y flavanonas.

Los flavonoles tienen con un grupo hidroxilo en posición 3, que puede estar glicosilado (Tsao y col., 2010), además de tener un doble enlace entre los carbonos 2 y 3 (C. Andrés-Lacueva y col., 2009). Se han identificado aproximadamente unas 450 flavonoles en forma de agliconas

en las plantas. Sin embargo, solo cuatro (quercetina, kaempferol, miricetina e isorhamnetina) se han encontrado en las frutas (Corradini y col., 2011) (Figura 5).

En la fresa, los flavonoles están concentrados en las semillas (aquenos), cuyo contenido es cuatro veces mayor que en la pulpa. Los derivados más comunes son el 3-glucósido y el 3-glucurónido de quercetina (Strik, R. Howard, Hager, & Talcott, 2007).

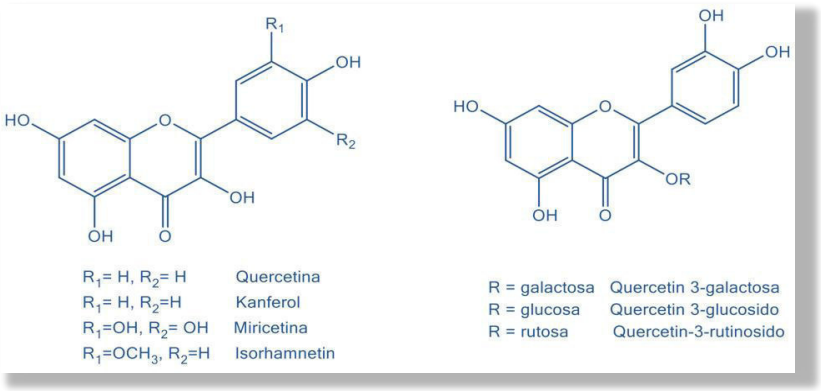

Figura 5. Principales flavonoles agliconas (izquierda) y estructura de los principales glicósidos de quercetina (derecha).

La mayoría de los flavonoles se presenta en forma de *O*-glicósidos (hidroxilo en posición 3 y 7) y, muy raramente, como C-glicósidos. Aproximadamente 900 flavonoles han sido identificados en forma de 3-, 7-, 4´-glicosidos y sus combinaciones. Las esterificaciones en posición 5-*O*- son raras. Los azúcares que más frecuentemente se encuentran esterificando éstas moléculas son la glucosa (el más común), la galactosa y la ramnosa. Menos frecuentes son la xilosa, la arabinosa y el ácido glucurónico (Corradini y col., 2012).

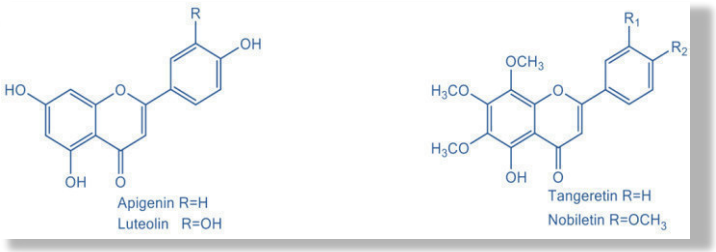

Figura 6. Principales flavonas.

La estructura de las flavanonas es, básicamente, la misma que de los flavonoles, excepto por el grupo hidroxilo en posición 3. También se conocen como dihidroflavonas, siendo el grupo menos numeroso de los flavonoides. En los últimos años, el número de estos compuestos descritos se ha duplicado. Sus representantes más comunes son la naringenina,

la hesperetina y el eriodictiol. En la fresa se han descrito recientemente el derivado 3-O-arabinósido del taxifolin (J Sun, Liu, Yang, Slovin, & Chen, 2014), y la hexosa de eriodictiol (Fait et al., 2008).

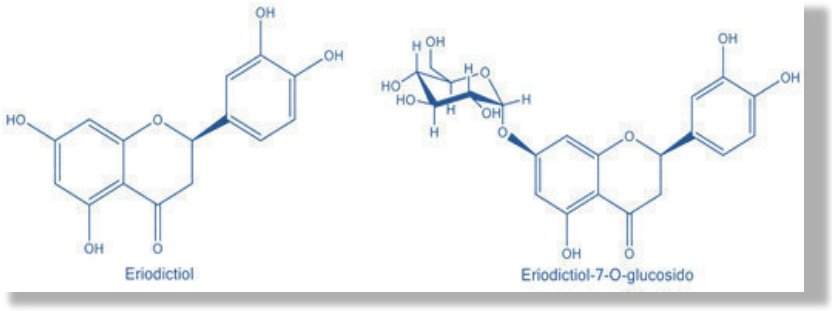

Figura 7. Estructura química del eriodictiol y su principal glicósido.

Los flavanonoles o dihidroflavonoles son flavanonas con un grupo hidroxilo en posición 3. Los representantes más conocidos de este grupo son el taxifolin (dihidroquercetina) y la aromadendrina o dihidrokaempferol

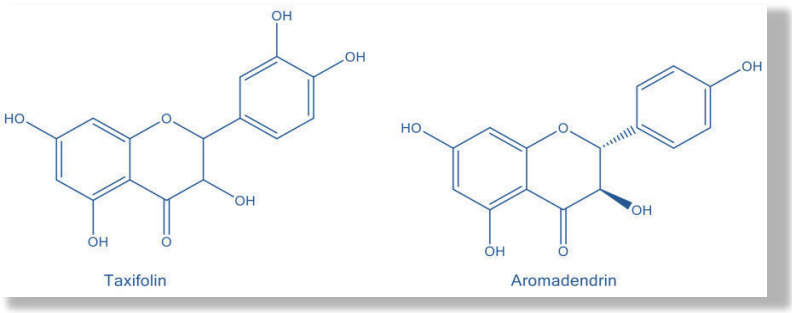

Figura 8. Estructura química de los principales flavanonoles.

2.1.1.2. Flavanoles, taninos condensados y taninos complejos.

Los flavanoles son también conocidos como flavan-3-oles porque poseen un grupo hidroxilo en posición 3 del anillo C. Tienen dos isómeros dependiendo de la disposición espacial de la unión entre el anillo B, la posición 2 del átomo de carbono y el grupo hidroxilo en la posición 3. Los flavanoles predominantes son la (+)-catequina y (-)-epicatequina, la (+)-galocatequina y su (-)-epigalocatequina. En la Figura 9 se muestran las estructuras de (+)-

Los flavanoles se presentan comúnmente en la naturaleza en forma de agliconas y no formando glicósidos (USDA Flavonoids Database Release 3.1, 2014).

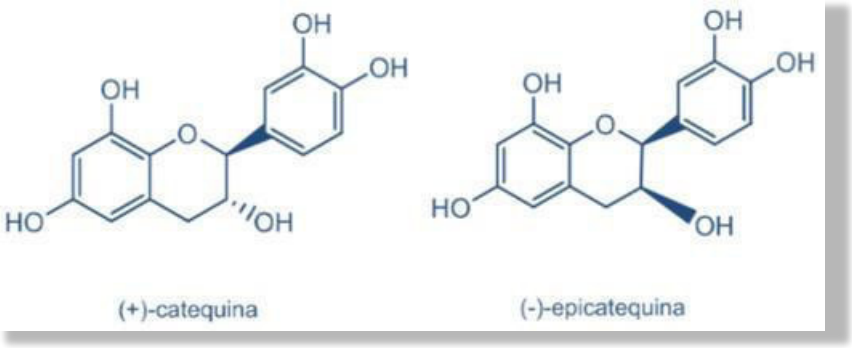

Figura 9. Estructura química de los dos isómeros (+)-catequina y (-)-epicatequina.

Los taninos condensados son las formas poliméricas de los flavanoles. También se les conoce como proantocianinas o procianidinas. Se pueden dividir en procianidina y prodelfinidina en función de sus unidades constitutivas más comunes como son la (-)-epicatequina, (+)-catequina en la procianidina y la (-)-epigalocatequina y (+)-galocatequina en la prodelfinidina (Corradini et al., 2011).

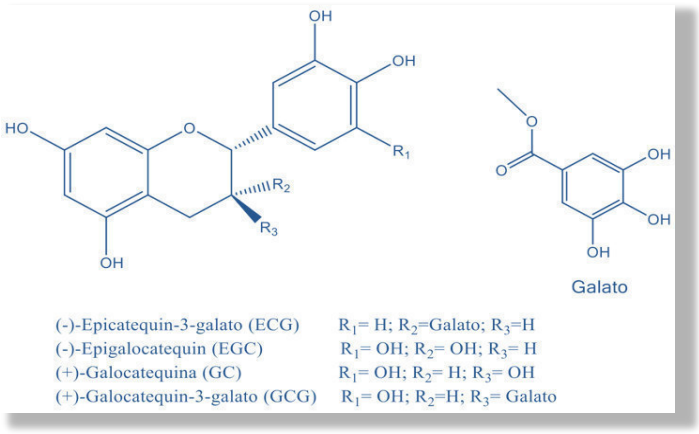

Figura 10.Estructura de los principales ésteres de (+)-catequina y (-)-epicatequina.

Se constituyen, además, como amplio grupo de compuestos, cuyos pesos moleculares están comprendidos entre 500 a 2.800 Da. Han sido identificadas aproximadamente 50 procianidinas desde dímeros a hexámeros. Las unidades consecutivas de los taninos condensados se unen entre sí a través de uniones interflavonoides entre C-4 y C-8 o C-4 y C-6 (Shahidi & Naczk, 2011).

condensados se unen entre sí a través de uniones interflavonoides entre C-4 y C-8 o C-4 y C-6 (Shahidi & Naczk, 2011).

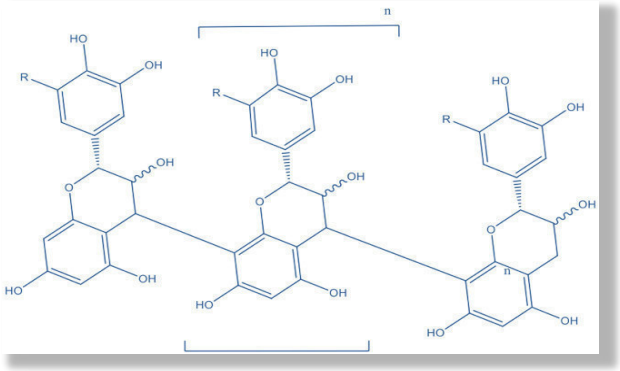

Figura 11. Estructura de tipo –B más común para los taninos condensados (R=H) procianidina y (R=OH) prodelfinidina.

Los taninos complejos son los que resultan de la unión de (+)-catequinas o (-)-epicatequinas con el ácido gálico o el ácido elágico. Un ejemplo de este tipo es el galato de (+)-catequina que contiene unidades hidrolizables y condensadas.

En la fresa se han descrito proantocianidinas (taninos condensados) y los flavanoles: (+)-catequina, (-)-epicatequina y (-)- epiafzalechina (K Hanhineva, 2011).

2.1.1.3. Antocianos.

Conforman el único grupo de los flavonoides que provee a la planta de un color característico. El color rojo, azul o púrpura de las pequeñas bayas, manzanas rojas, cerezas, lechugas rojas y muchas otras frutas y vegetales, provienen de estos compuestos. Dependiendo de los valores del pH, los antocianos manifiestan diferentes colores que pueden ir desde el rojo (condiciones muy ácidas) a púrpura (en condiciones de pH intermedias) y a una transición entre verde y amarillo (condiciones alcalinas). A pH ácidos la forma predominante es el catión flavilio que presenta color rojo. El color de los antocianos, también se puede ver afectado por las acilaciones o metilaciones de los grupos hidroxilo de los anillos A y B. Las antocianinas son glicósidos de los antocianos, con la molécula de azúcar unida en la posición C_3 del anillo C. La molécula de azúcar de los antocianos a veces aparece conjugada con ácidos fenólicos como el ácido ferúlico (Tsao y col., 2010).

El 90% de las antocianidinas se presentan como cianidina, delfinidina y pelargonidina en los pétalos de las flores, frutas y vegetales así como en ciertas variedades de granos (Tsao y col., 2010).

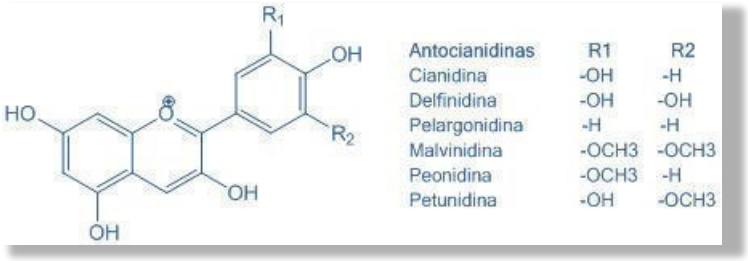

Figura 12. Estructura química de las principales antocianidinas

Strik y col. publicaron en 2007 que los cuatro principales antocianos descritos en la fresa son el 3-glucósido de cianidina, el 3-glucósido de pelargonidina, el 3-glucosido succinato de cianidina y 3-glucósido-succinato de pelargonidina que varían en función del fenotipo (Strik et al., 2007). Por otra parte, Aaby y col. en 2012 publicaron que el antociano más abundante en la fresa es el 3-glucósido de la pelargonidina (60-95%) de los antocianos totales, en segundo lugar 3-malonilglucósido de la pelargonidina (0-33,5%) (Aaby et al., 2012). Coincidiendo con esto, el contenido del 3-glucósido de pelargonidina es de 2113,59 µg L^{-1} ± 29,45, según un estudio realizado en puré de fresa producido en España (Hornedo-Ortega, Álvarez-Fernández, Cerezo, Troncoso, & García-Parrilla, 2016).

2.1.2. No Flavonoides.

2.1.2.1. Ácidos fenólicos.

Forman un grupo de polifenoles muy frecuente en la dieta, apareciendo en forma esterificada en la mayoría de los casos. En general, este término designa colectivamente a los derivados del ácido cinámico (ácido cinámico y derivados) y a los derivados del ácido benzoico. Más específicamente, están formados por dos grupos de compuestos: los derivados del ácido benzoico y los derivados del ácido cinámico (C W I Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012; Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004).

Los ácidos fenólicos más abundantes que se han descrito en la fresa son los ácidos: *p*-cumárico, cinámico *p*-hidroxibenzoico, caféico, vaníllico, protocatéquico y el 5-cafeoilquínico

(clorogénico), estando, por lo general, esterificados a azúcares. En particular, los derivados del ácido benzoico y los derivados del ácido cinámico se presentan esterificado con ácidos orgánicos (quínico, shikimico y tartárico), pudiendo hallar así a los ésteres de los ácidos cafeico, *p*-cumárico, ferúlico y del ácido gálico. Entre los ácidos fenólicos más frecuentemente descritos en la fresa se destaca el *p*-cumárico en su forma glucosilada, glucósido del ácido *p*-cumárico, que se ha descrito en la pulpa de la fruta. Además del anterior, es frecuente encontrar a los ácidos clorogénico y los p-hidroxibenzoicos (Strik, R. Howard, Hager, & Talcott, 2007).

i. Derivados del ácido benzoico.

Su estructura básica corresponde a (C6-C1) siendo este el grupo más simple, formado por un anillo de benceno como base, unido a grupo carboxílico (ácidos benzoicos). También pueden presentarse con diferentes niveles de hidroxilación. Estas moléculas pueden ser liberadas de esas uniones por hidrólisis ácida o alcalina o por la acción de enzimas (Sarkar & Shetty, 2014).

Figura 13. Estructura de los principales ácidos benzoicos C6-C1. Adaptado de (Reis Giada, 2013)

Además de los derivados del ácido benzoico que se muestran en la Figura 13, hay otro ácido que merece la pena destacar: el ácido elágico (Figura 14). Se caracteriza por tener una estructura algo más compleja, y se ha descrito en frambuesas y fresas (Kati Hanhineva, Kärenlampi, & Aharoni, 2011). Tiene la particularidad de que se puede presentar en la naturaleza libre, como glicósido o conjugado formando los taninos hidrolizables o elagitaninos.

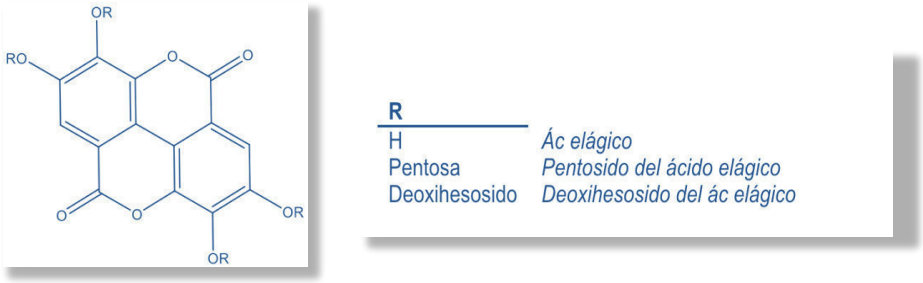

Figura 14. Estructura del ácido elágico y conjugados

ii. Derivados de ácido cinámico.

Forman el cuarto grupo de los compuestos fenólicos más abundantes en la fresa (Fig. 2). Están ampliamente distribuidos en la naturaleza en forma de conjugados en plantas y frutos, presentándose también en alimentos y bebidas (Clifford, 2000).

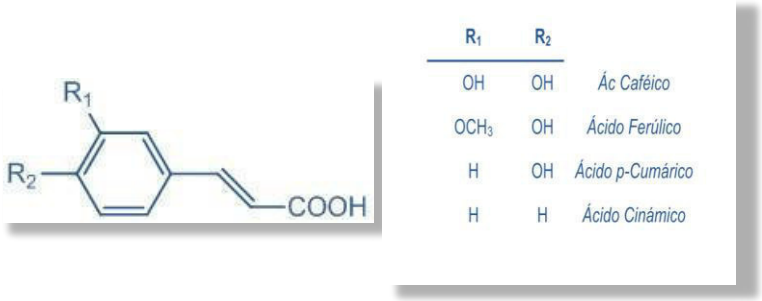

Figura 15. Estructuras de los derivados del ácido hidroxicinámico más comunes.

Los principales representantes de este grupo son el ácido cafeico, el ferúlico, el *p*-cumárico y el cinámico (Figura 15). Por lo general, aparecen en la naturaleza esterificados con el ácido quínico, el tartárico o formando glicósidos

Se dividen en dos grandes grupos: los ácidos clorogénicos y los conjugados del ácido cinámico que analizamos seguidamente.

iii. Ácidos clorogénicos.

Son una familia de ésteres formados entre ciertos ácidos *trans*-cinámicos y el ácido quínico (ácido carboxílico del 1L-1(OH),3,4/5-tetrahidroxiciclohexano), el cual tiene hidroxilaciones axiales en los carbonos 1 y 3 e hidroxilaciones ecuatoriales en los 4 y 5 (Figura 16). Cuando los productos naturales se procesan, los *trans* se convierten parcialmente en *cis* (Clifford, 2000).

El más común de los derivados del ácido hidroxicinámico es el clorogénico (ácido 5-*O*-caffeoilquínico) (5-CGA) en la naturaleza. Los ácidos clorogénicos (CGA) se subdividen por su identidad, número y posición de grupos acilo.

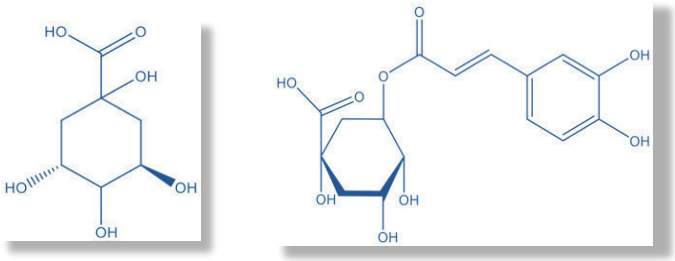

Figura 16. Estructuras del ácido quínico a la izquierda y ácido clorogénico (ácido 5-cafeoilquínico) a la derecha.

El ácido caféico es uno de los principales hidroxicinámicos en la fruta (frutos rojos) (Andrés-Lacueva et al., 2009), llegando a alcanzar el 70% del total de hidroxicinámicos. Varios estudios sostienen que el consumo diario en Alemania procedente de todos los alimentos de estos ácidos oscila entre 211 a 11 mg/día (Manach et al., 2004).

iv. Conjugados del ácido cinámico.

Los ácidos cinámicos pueden aparecer conjugados con otros ácidos diferentes del quínico, siendo estructuralmente cercanos a éste y de distribución reducida en la naturaleza.

En la fresa se ha demostrado la presencia de ésteres de (hidroxi)cinamoil glucósidos que pueden ser utilizados como precursores de diversos metabolitos secundarios, constituyentes del sabor, como son los metil y etil cinnamatos (Lunkenbein et al., 2006).

2.1.2.2. Taninos hidrolizables o elagitaninos.

Los taninos son polímeros de poifenoles y en general se clasifican en tres grupos: taninos condensados (proantocianidinas), taninos hidrolizables y taninos complejos, dependiendo del contenido en azúcar y del grado de esterificación y polimerización. Recientemente se han llegado a clasificar a los taninos hasta en cuatro grupos: taninos condensados, taninos complejos, galotaninos y elagitaninos (Aguilera-Carbo, Augur, Prado-Barragan, Favela-Torres, & Aguilar, 2008).

Los taninos hidrolizables son polímeros heterogéneos y están formados por unidades de ácido gálico o de otro ácido benzoico, unidas a un núcleo glucósido. Cuando es el ácido gálico, son conocidos como galotaninos. Son de menor tamaño que los taninos condensados y se hidrolizan con mayor facilidad por enzimas como tanasas, ácidos o álcalis. La mayoría tiene una masa molecular entre 300 y 600.

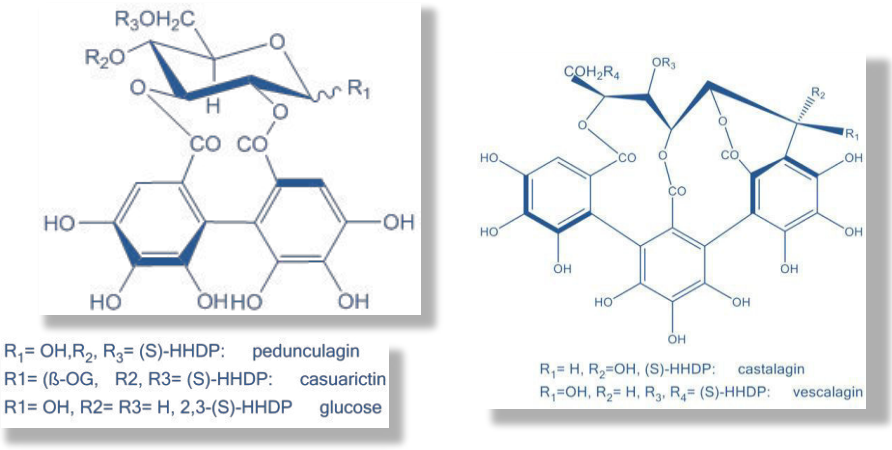

Figura 17. Principales elagitaninos monoméricos (izquierda).
Estructuras de elagitaninos C-glicósidos (derecha).

Cuando están formados por ácido elágico se denominan elagitaninos. Los simples se caracterizan por la presencia de uno o más grupos HHDP (hexahidroxidifenil) unidos a un

núcleo glucopiranósido. Se hidrolizan fácilmente liberando una molécula estable de ácido elágico como producto de la di-lactonización del HHDP.

Los elagitaninos C-glicosídos han sido detectados en numerosas familias de plantas incluyendo a las Rosáceas. Se dividen en dos grupos: los que contienen la unidad flavogaloil participando en la unión C-glucosídica como castalagin y su C-epímero vescalagin, y del tipo casuarinin, que contiene una unidad HHDP como el propio casuarinin y stachyurin.

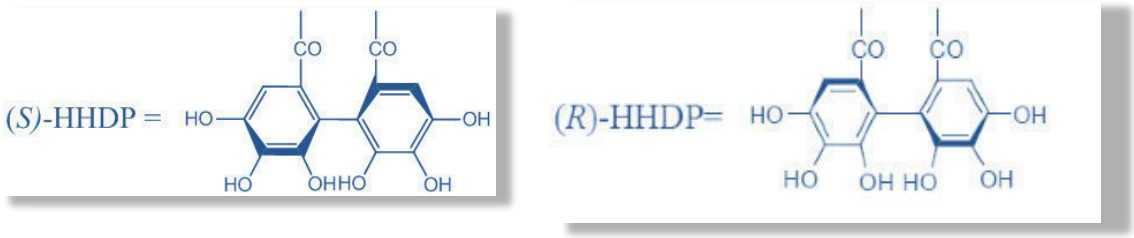

Figura 18. Representación de las dos formas quirales de la unidad HHDP.
Fuente (Yoshida et al., 2010)

En la familia Rosáceae, es común encontrar taninos oligoméricos. Este tipo de compuestos tienen diversas actividades biológicas basadas en su marcado carácter antioxidante.(Yoshida, Amakura, & Yoshimura, 2010).

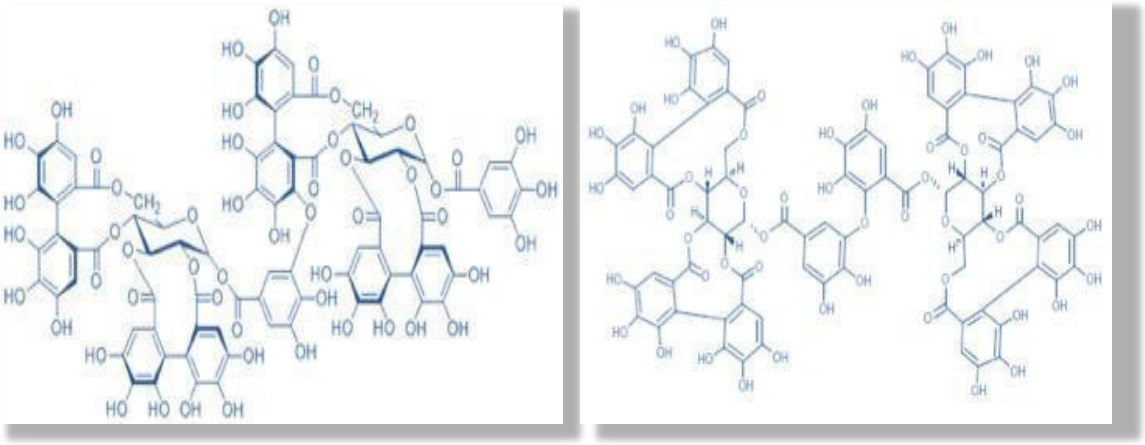

Figura 19. Estructura de sanguiin-H-6 (izda) y agrimoniin (dcha),
como ejemplo de elagitaninos oligómeros más abundantes de la fresa.

2.1.2.3. Estilbenos.

Son un grupo que se caracteriza por un esqueleto 1,2-difeniletileno (C6-C2-C6). Hay una gran variedad de compuestos formados a partir de esta sencilla estructura: monómeros y

oligómeros resultantes de las combinaciones del monómero resveratrol (dímeros, trímeros y tetrámeros).

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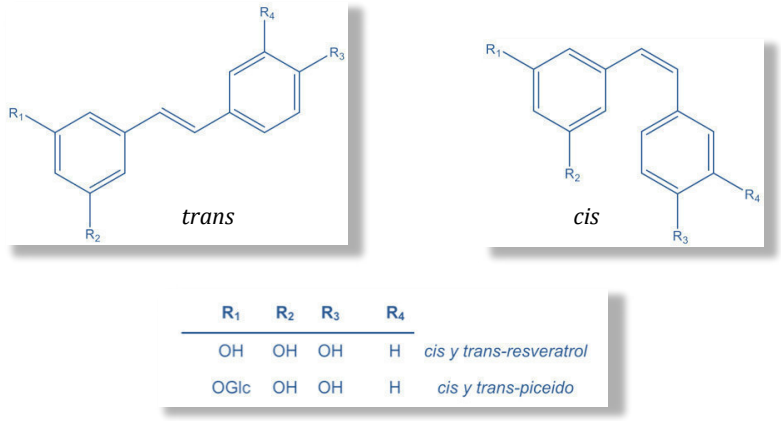
Pocas cantidades de estilbenos están presentes en la dieta de los humanos. El principal representante de este grupo es el *trans*-resveratrol y apareciendo por lo general en su forma glicosilada, también conocida como polydatin o piceido.

El resveratrol existe en la naturaleza en forma de dos isómeros *cis* y *trans* (Figura 20). El isómero más abundante en frutas y plantas es el *trans* y el *cis* es más frecuente en vinos (Wang, Chen, Wang, & Chen, 2007). Aunque en la fresa se han cuantificado ambos isómeros (Kati Hanhineva et al., 2011).

Los dos isómeros del resveratrol (Figura 20) tienen características químicas y actividades biológicas diferentes. El isómero *trans* es el más estable y la conversión a *cis* puede producirse por luz o por radiación ultravioleta (UV). El isómero *cis* se estima que proviene de la isomerización del isómero *trans* por efecto de la fermentación, como una de las formas de producción (Wang et al., 2007), ya que también está presente en la uva (Careri, Corradini, Elviri, Nicoletti, & Zagnoni, 2004). El equilibrio entre los dos isómeros se alcanza después de la exposición a la luz difusa constante y en estas condiciones más de la mitad del isómero *trans* puede cambiar a *cis* (Wang et al., 2007).



R_1	R_2	R_3	R_4	
OH	OH	OH	H	*cis y trans-resveratrol*
OGlc	OH	OH	H	*cis y trans-piceido*

Figura 20. Representación de los principales estilbenos descritos en la fresa.

El *trans*-resveratrol también ha sido descrito en la fresa con concentraciones de 830,5 ± 20,6 ng g^{-1} de peso seco (90,5 ± 11,8 ng g^{-1} de peso fresco)(Wang et al., 2007), demostrando que está presente en las semillas con una mayor concentración que en la pulpa. Por otro lado, ese mismo estudio sostuvo que los frutos aumentaban la concentración de resveratrol cuando se almacenaron en atmósferas con alto contenido de CO_2. Estas condiciones contribuirían a reducir la actividad de los radicales libres ya que disminuyen la concentración de oxígeno.

2.1.2.4 Chalconas y dihidrochalconas.

Son compuestos cuya presencia es reducida en la naturaleza, conociéndose por este motivo como flavonoides menores, a pesar de encontrarse ocasionalmente en los alimentos en concentraciones considerables (Tomás-Barberán & Clifford, 2000). Su encaje en la clasificación genera controversia, hay quien por su semejanza con la estructura C6-C3-C6 las clasifican como falvonoides (de la Rosa, Alvarez-Parrilla, & González-Aguilar, 2002; Tomás-Barberán & Clifford, 2000), y quienes atendiendo a que carecen del anillo C las clasifican como no-flavonoides (C Andrés-Lacueva et al., 2009).

Después de su formación son isomerizados por la enzima chalcona isomerasa para formar la correspondiente flavanona. Las chalconas más comunes son phloretina y su 2'-*O*-glucosido (phloridzina), chalconaringenina y arbutina (C Andrés-Lacueva et al., 2009).

La contribución de estos compuestos a la dieta mediante el consumo de fruta fresca es de escasa relevancia, aumentando su presencia en alimentos procesados. Ello es debido a que los productos elaborados a base de fruta utilizan la totalidad de la misma y sus procesos de elaboración incluyen tratamientos térmicos que inactivan las enzimas (polifenoloxidasa) que, directa o indirectamente, degradan a las dihidrochalconas (Tomás-Barberán & Clifford, 2000).

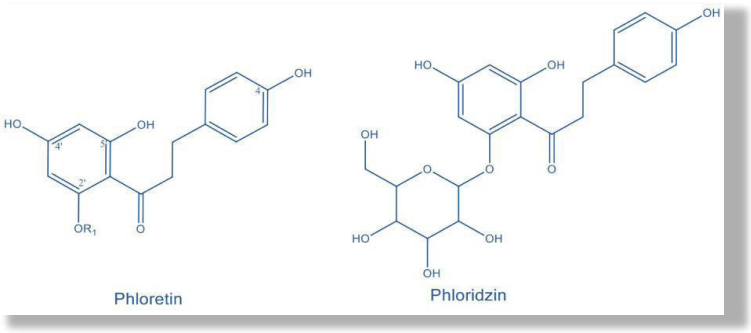


Figura 21. Estructura de la chalcona (phloretina) y su glucósido (ploridzina).

B. LA FERMENTACIÓN.

Es un proceso milenario no térmico de elaboración de alimentos a través del cual éstos sufren cambios químicos causados por la acción de las enzimas generadas por los microorganismos que intervienen en el proceso.

En los últimos cincuenta años, se ha producido una sustancial mejora de los procesos fermentativos aumentándose el contenido de compuestos beneficiosos con el objetivo de mejorar las propiedades saludables de los alimentos y para dotar al producto final de deseables características organolépticas. Esta mejora se ha logrado mediante la cuidadosa selección de los microorganismos y a través de un creciente conocimiento en profundidad del proceso de fermentación.

Los beneficios que el proceso de fermentación aporta al alimento producido se manifiestan en un enriquecimiento de su valor nutritivo, mejorando su sabor, su digestibilidad y, en definitiva, un aumento de la vida media del mismo mediante la disminución del pH y la eliminación de "antinutrientes" (van Boekel et al., 2010).

1.-Fermentación y Compuestos bioactivos.

Los bioactivos se definen como pequeñas moléculas a las que se les confiere una acción biológica y que pueden estar presentes de forma natural en los materiales vegetales o animales o ser el resultado de los cambios químicos producto del proceso fermentativo (Frias, Martinez-Villaluenga, & Peñas, 2016). Se ha comprobado la presencia de compuestos bioactivos como consecuencia del efecto de las fermentaciones, ya que algunos de ellos no estaban presentes naturalmente en los sustratos de origen.

Durante la fermentación se produce un amplio rango de metabolitos secundarios, algunos de los cuales están asociados a propiedades saludables. Se ha comprobado la modificación en el contenido de vitaminas, minerales, aminoácidos, fitoquímicos, polifenoles, ácidos grasos y polisacáridos, como consecuencia de las fermentaciones.

Los bioactivos disponibles en los alimentos fermentados pueden depender del sustrato y su variabilidad (la región geográfica de producción, madurez…), de las levaduras y bacterias utilizadas, de la disponibilidad de los sustratos específicos en el proceso de fermentación, de las condiciones ambientales tales como la estacionalidad, el método de preparación o el proceso de manufactura (Frias et al., 2016).

No todos los tipos de fermentación afectan por igual a los compuestos bioactivos. En un estudio de fermentaciones de fresa se observó que la alcohólica disminuyó en un 19% el contenido de antocianos en comparación con la fermentación acética que produjo una pérdida del 91% de antocianos (Hornedo-Ortega et al., 2017).

Los fenoles totales disminuyen con las fermentaciones acéticas, dependiendo del sustrato. En vinagres de vino tinto se ha observado una pérdida de un 13% (Cerezo et al., 2008), un 8% en vinagres de vino blanco (García-Parrilla, González, Heredia, & Troncoso, 1997) y un 13 – 60% en vinagres de fresa (Ubeda et al., 2013).

2.-La Fermentación y la actividad antioxidante.

De entre los bioactivos producidos por el efecto de la fermentación se encuentran los polifenoles, con conocidas propiedades antioxidantes que actúan como agentes reductores (eliminan radicales libres), y como quelantes de metales e inhibidores de radicales de oxígeno (Hur, Lee, Kim, Choi, & Kim, 2014).

Las enzimas producidas por los microorganismos que intervienen en el proceso de fermentación tales como glucosidasas, amilasas, celulasas, quitinasas, inulinasas, xilasas, tanasas, estearasas, invertasas o lipasas pueden hidrolizar glucósidos y romper las paredes celulares de los tejidos vegetales. La ruptura de las células libera compuestos con marcada actividad antioxidante como los polifenoles. Los cambios estructurales que la fermentación produce en los fitoquímicos contenidos en el sustrato, es otra manera en que la fermentación incrementa la actividad antioxidante del producto fermentado. Así, ácidos fenólicos y flavonoides son liberados de las estructuras celulares del sustrato vegetal durante la fermentación por la acción de enzimas hidrolíticas. Estas enzimas, al actuar sobre los fenoles glicosilados, liberan las agliconas que poseen una mayor actividad antioxidante que el glicosilado de origen (Hur et al., 2014) .

Tras la fermentación, también tiene lugar la degradación de compuestos fenólicos y es esa tasa de degradación la responsable de la disminución de la actividad antioxidante. Como ejemplo, se ha comprobado la pérdida del contenido de fenoles durante el procesamiento de aceitunas, lo que induce a una reducción en la actividad antioxidante (Othman, Roblain, Chammen, Thonart, & Hamdi, 2009). Este resultado indica que hay ciertos procesos fermentativos que ejercen efectos negativos sobre la actividad antioxidante. Por este motivo, son necesarios nuevos trabajos de investigación sobre los microorganismos y los efectos producidos por sus enzimas, a fin de aclarar los mecanismos precisos que ocurren durante la fermentación de los alimentos (Hur et al., 2014).

La Figura 22 muestra como ejemplo, dos reacciones que se producen en la fermentación para producir ácido elágico a partir de peduncalagin (elagitanino).

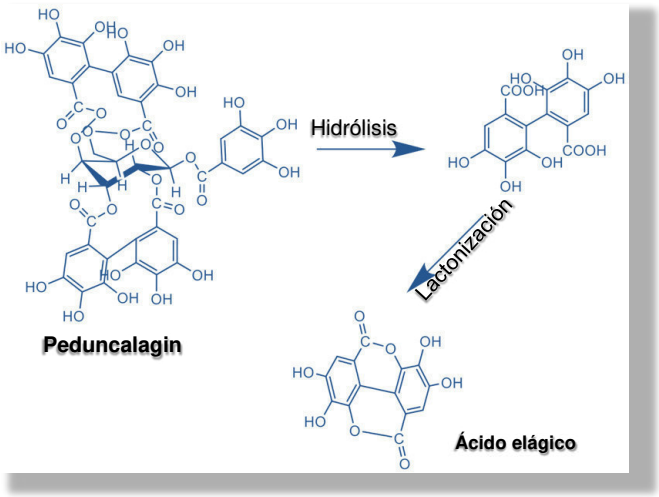


Figura 22. Esquema de la hidrólisis enzimática de un elagitanino (peduncalagin) para producir ácido elágico mediante la acción de los microorganismos. Fuente (Aguilera-Carbo et al., 2008).

3.-La Fermentación alcohólica.

Es mediada por levaduras y se caracterizan por metabolizar la glucosa y la fructosa a través del proceso de glicólisis, en etanol y dióxido de carbono. Las levaduras que más comúnmente se utilizan en este tipo de fermentaciones pertenecen al género *Saccharomyces* .A pesar de que el etanol se considera el producto final de la fermentación, se producen a su vez metabolitos que otorgan al producto final especiales características y atributos

aromáticos mediante la hidrólisis de precursores aromáticos no-volátiles. De esta forma, se liberan terpenos, fenoles, norisoprenoides y tioles, así como compuestos volátiles libres. Asimismo, los cambios fisicoquímicos de las condiciones producidas durante la fermentación, modifican progresivamente el metabolismo de las levaduras. De esta manera, compuestos responsables de los aromas del vino, pueden ser interpretados en términos de la modificación de metabolitos primarios y secundarios (Jackson, 2014).

La acción de las levaduras produce en la fermentación alcohólica una serie de compuestos que son esenciales para el olor y el sabor del alimento fermentado. Entre ellos, encontramos alcoholes diferentes al etanol, ácidos orgánicos, esteres y éteres. En la cerveza el ácido orgánico más abundante es el ácido acético, siendo cuantificado en concentraciones que van desde el 40% al 80% del total de ácidos orgánicos. Su concentración varía considerablemente en función del sustrato o de la levadura, ejerciendo una importante influencia en el pH de la bebida fermentada. También se han detectado pequeñas cantidades del ácido láctico, fórmico y succínico. (Frias et al., 2016).

4.-Metabolismo del nitrógeno.

El nitrógeno afecta a la viabilidad de las levaduras en dos aspectos fundamentales como son la producción de biomasa y la velocidad de la fermentación. Por lo tanto, el contenido de nitrógeno ejerce un efecto tanto en la regulación de la velocidad como del final de la fermentación. De hecho, la escasez de nitrógeno en el medio se ha señalado como una de las principales razones del enlentecimiento de las fermentaciones. Las fermentaciones afectadas de esta manera influyen negativamente en la calidad de los vinos ya que la presencia de azúcares residuales podrían aumentar la inestabilidad microbiológica y cambiar las propiedades organolépticas del producto final (Mas et al., 2016). El mosto contiene diferentes fuentes de nitrógeno, siendo las más importantes los aminoácidos, pero las levaduras también pueden utilizar sales de amonio Los aminoácidos aromáticos (triptófano, tirosina y fenilalanina) son las fuentes de nitrógeno que producen menor crecimiento de las levaduras (Gutiérrez, Beltran, Warringer, & Guillamón, 2013). El metabolismo de éstos aminoácidos (Figura 23) produce compuestos derivados que pueden ser importantes como factores de regulación (alcoholes superiores) o génesis de compuestos bioactivos (melatonina y serotonina) (Mas et al., 2016).

Los aminoácidos aromáticos son catabolizados mediante la ruta metabólica de Ehrlich, la cual empieza con la transaminación del grupo amino y la formación de α-ceto ácidos, tales como el indolpiruvato, fenilpiruvato y el 4-hidroxifenil piruvato desde triptófano, fenilalanina, y tirosina, respectivamente. Posteriormente, los cetoácidos son descarboxilados para formar los correspondientes aldehídos. Finalmente y ,dependiendo del estado de óxido-reducción de la célula, serán posteriormente metabolizadas a los correspondientes alcoholes aromáticos, indol 3-etanol (triptofol), feniletanol y tirosol o bien son oxidados a sus correspondientes ácidos, ácido indolacético o fenil acético y el ácido 4-hidroxifenil acético (Mas et al., 2016).

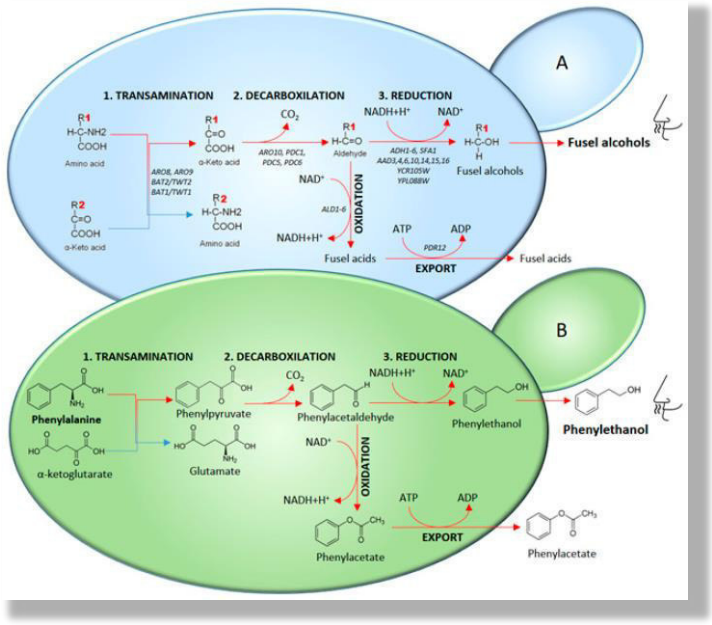


Figura 23. Ruta metabólica de Ehrlich en *Saccharomyces cerevisiae* (A) Metabolismo de los aminoácidos aromáticos (fenilalanina, tirosina y triptófano) y los genes que lo codifican (Belda et al., 2017).

5.- Bioactivos producto de la fermentación alcohólica.

i. Melatonina.

Es una molécula de bajo peso molecular que existe en organismos vivos, a la que se atribuyen muchas actividades biológicas en múltiples especies desde levaduras y bacterias a mamíferos (Rüdiger Hardeland, Pandi-Perumal, & Cardinali, 2006).

Su estructura es la de una indolamina relacionada con el metabolismo del triptófano. Se ha demostrado recientemente que es producida por levaduras del género *Saccharomyces* (Rodriguez-Naranjo, Torija, Mas, Cantos-Villar, & Garcia-Parrilla, 2012). La ruta metabólica descrita para la producción del compuesto en *Saccharomyces* es similar a la de los vertebrados, parte del aminoácido triptófano → 5-hidroxitriptófano → serotonina → N-acetilserotonina → melatonina (Rudiger Hardeland & Poeggeler, 2008). En estas levaduras se ha observado que si se suministra una cierta cantidad de 5-metoxitriptamina, es reacetilada para formar melatonina. Asimismo, cuando se suministra melatonina, las levaduras rápidamente la convierten en 5-metoxitriptamina (Rudiger Hardeland & Poeggeler, 2008). Rodriguez-Naranjo et al. (2011) confirmaron el papel crucial de éstas levaduras en la presencia de melatonina, al comprobar el aumento de la concentración en los vinos con respecto al mosto inicial. Posteriormente estos autores y otros han confirmado la síntesis de melatonina por parte de las levaduras del género Saccharomyces y no-Saccharomyces durante procesos fermentativos (Rodriguez-Naranjo et al., 2012; Vigentini et al., 2015)

Es una molécula relacionada con numerosas funciones en humanos modulando procesos fisiológicos como el ritmo circadiano, funciones reproductivas, actuando también como antioxidante. En animales, se produce en la glándula pineal, aunque también ha sido descrita en otros tejidos. Se sintetiza en concentraciones importantes en el intestino. Además de las funciones descritas, también tiene funciones antioxidantes que se relacionan con la cascada de metabolitos relativos al incremento de la longevidad. A pesar de todas las investigaciones realizadas el papel de este bioactivo en levaduras y otros microorganismos está poco claro (Mas et al., 2014).

ii. Hidroxitirosol.

Es un fenil etil alcohol 2-(3,4-dihidroxi-fenil)etanol, presente en el aceite de oliva que tiene una declaración de propiedad saludable aceptada en relación a los efectos cardioprotectivos derivados del consumo del aceite de oliva extra-virgen basados en la protección de las partículas LDL del daño oxidativo y por mantener las concentraciones del colesterol HDL en niveles normales (European Food Safety Authority (EFSA) Panel on Dietetic Products Nutrition and Allergies (NDA), 2011). En aceite se produce a partir del oleopurein (ester de hidroxitirosol y ácido elenoico) presente en el aceite de oliva (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012).

El vino parece ser otra fuente de hidroxitirosol para la dieta. En principio fue detectado en vinos italianos (Di Tommaso, Calabrese, & Rotilio, 1998) en concentraciones del orden de 4,0 mg L^{-1} en vinos tintos y de 1,9 mg L^{-1} en vinos blancos. La ruta metabólica para su producción como consecuencia de la fermentación alcohólica es a partir del aminoácido tirosina y por transaminación. descarboxilación del *p*-fenilpiruvato y reducción del *p*-hidroxifenilacetaldehido mediante la enzima alcohol deshidrogenasa (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008; Piñeiro, Cantos-Villar, Palma, & Puertas, 2011; Satoh, Tajima, Munekata, Keasling, & Lee, 2012). Tanto el tirosol como el hidroxitirosol del vino son considerados metabolitos secundarios producidos a partir de la tirosina mediante la acción de diferentes cepas de levaduras y tomando como sustrato aminoácidos (Garrido & Borges, 2013) durante la fermentación alcohólica (W. Zhu et al., 2011).

El contenido de hidroxitirosol del vino parece que está relacionado fundamentalmente con el contenido de nitrógeno de los mostos durante la fermentación alcohólica, en este sentido, es conocido que diferentes especies y cepas de levaduras tienen diferentes patrones de consumo de nitrógeno. Esto sugiere que el contenido final de hidroxitirosol y tirosol en los vinos podría estar influenciado por la ecología microbiana durante la fermentación alcohólica (Romboli, Mangani, Buscioni, Granchi, & Vincenzini, 2015).

Como bioactivo ha sido objeto de múltiples estudios, algunos de los cuales han demostrado que es un potente estimulador de la biogénesis a nivel mitocondrial en las células de la retina que contribuye a la salud ocular (L. Zhu et al., 2010), así como a la actividad anticancerígena (Roleira et al., 2015), cardioprotectora (Mnafgui et al., 2015), o relacionada con la actividad antidiabética y neuroprotectora (Fernández-Mar et al., 2012; Marhuenda et al., 2016).

C. METABOLÓMICA.

Se define como el campo de investigación que comprende la caracterización, la identificación y la cuantificación de la totalidad de pequeñas moléculas (<1500 Da) (Wishart, 2008) en un sistema biológico (Johanningsmeier, Harris, & Klevorn, 2016). Algunos autores utilizan el término metabolómica como sinónimo de metabonómica, aunque conviene resaltar sus diferencias puesto que la metabonómica se refiere específicamente a los cambios en los metabolitos de un sistema vivo relacionado con un estado patológico, un estímulo biológico o una alteración genética.

1. INTRODUCCIÓN

Desde el inicio de la aparición de las tecnologías "ómicas", una vertiginosa variedad de nuevos términos han ido aparecido en la literatura científica (foodomics, lipidomics, fluxomics, mineralomics ...). Todos centran el foco descriptivo en algunos aspectos del metabolismo o de los metabolitos que componen una matriz biológica compleja, compartiendo su poder generador de nuevos conocimientos acerca de similitudes y diferencias entre grupos definidos de muestras, abriendo nuevos campos de investigación. Por este motivo, la metabolómica y ,en general, las "ómicas", son conocidas como disciplinas generadoras de hipótesis (Johanningsmeier et al., 2016).

Algunos autores clasifican a la metabolómica en: dirigida (targeted analysis) y no dirigida (untargeted analysis), aunque existen discrepancias al respecto.

Los análisis dirigidos se centran en un grupo específico de metabolitos que, en la mayoría de los casos, requieren identificación y cuantificación. Permiten dilucidar el comportamiento de un grupo específico de compuestos en una muestra bajo condiciones específicas. Los análisis dirigidos requieren altos niveles de purificación en la mayoría de los casos, así como una extracción selectiva de los metabolitos objeto de estudio. Por contra, los análisis no dirigidos se centran en la detección del mayor número de metabolitos que sea posible para así obtener información sobre patrones de comportamiento o de identidad sin necesidad de identificar o cuantificar compuestos específicos. Por ello, y a modo de ejemplo, se ha utilizado en la identificación de patrones de comportamiento de fenómenos biológicos, tales como enfermedades de plantas y como patrones moleculares que definen enfermedades (Cevallos-cevallos, Etxeberria, Danyluk, & Rodrick, 2009).

El análisis metabolómico consiste en una secuencia de cinco etapas (Figura 24) que comprenden la preparación de la muestra, la extracción de los metabolitos, la separación de los metabolitos, la detección y el tratamiento de los datos. Sin embargo, no siempre todos estos pasos o etapas son necesarias, solo considerándose imprescindibles en los estudios metabolómicos la detección y el tratamiento de los datos. El análisis metabolómico va a depender fundamentalmente del tipo de estudio (dirigido o no-dirigido), de la instrumentación que se utilice para la separación (tipo de cromatografía) y del método de detección (espectrometría de masas frente a resonancia magnética nuclear (RMN)) (Cevallos-cevallos et al., 2009).

A continuación se van a detallar algunas de las etapas más relevantes del análisis metabolómico.

1.-Etapas del análisis metabolómico.

1.1.- Preparación de la muestra.

No existen limitaciones en cuanto al tipo de muestra que es susceptible de ser analizada en los estudios metabolómicos. Sin embargo, el tipo de muestra y los metabolitos diana determinan el método apropiado de preparación de la muestra.

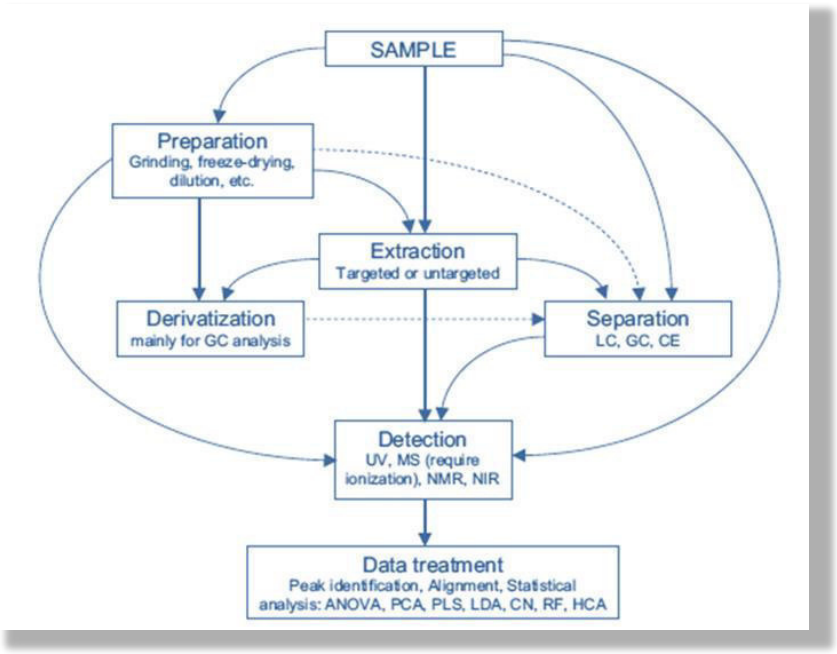


Figura 24. Representación esquemática del proceso de análisis metabolómico Fuente (Cevallos-cevallos et al., 2009).

En la preparación de la muestra, prevalece el principio general de preservar el estado original del sistema biológico tanto como sea posible, minimizando la actividad enzimática y la reactividad química que se produce durante la extracción de los metabolitos.

Los métodos de extracción más comúnmente utilizados en metabolómica son la precipitación de proteínas o las extracciones líquido-líquido. Para la extracción de los metabolitos polares se utilizan solventes orgánicos del tipo acetonitrilo, metanol o isopropanol, mientras que para la extracción de lípidos se emplean solventes con baja polaridad, o bien, una combinación de solventes. Los solventes ácidos pueden ser usados para

estabilizar cierto tipo de metabolitos, aunque pueden provocar, al mismo tiempo, la degradación de otros metabolitos causando una reducción en la sensibilidad del experimento debido al efecto de supresión del ion (X. Liu & Locasale, 2017).

Las muestras sólidas provenientes de frutas o vegetales son comúnmente molidas bajo la acción del nitrógeno líquido o liofilizadas. Una apropiada molienda aumenta la liberación de los metabolitos en el proceso de extracción.

También se suele recomendar la concentración de la muestra como método más recomendable para recabar una mayor información en el análisis (Cevallos-cevallos et al., 2009). La concentración permite mejorar los límites de detección de los equipos analíticos, realizándose a través de la evaporación de solventes mediante vacío, puesto que los métodos que emplean altas temperaturas pueden producir la degradación de los metabolitos (Pinu & Villas-Boas, 2017).

Otra opción es la utilización de la extracción en fase sólida (Figura 25). Es un método de preparación de muestra y de extracción que provoca la remoción de los metabolitos de una muestra líquida al pasar por un cartucho relleno de una sustancia adsorbente sólida, donde quedan retenidos los metabolitos diana. Además de ser un método de purificación y extracción de los metabolitos, presenta la función de concentrar la muestra. La extracción en fase sólida se clasifica en tres tipos: fase normal, fase reversa y de intercambio iónico. La fase normal se utiliza para separar metabolitos de media y baja polaridad, la fase reversa para metabolitos de alta a media polaridad y la de intercambio iónico se recomienda para metabolitos de alta polaridad (Pinu & Villas-Boas, 2017).

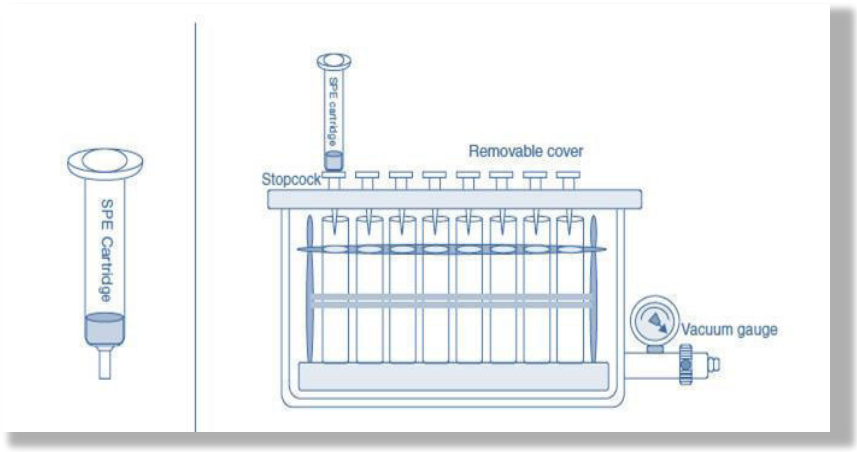


Figura 25. Esquema de la extracción en fase sólida (SPE). A la izquierda el cartucho y a la derecha el equipo de vacío para realizar las extracciones. Fuente (Villas-Boas, 2007).

1.2. Extracción de metabolitos de muestras microbianas.

Idealmente, el análisis metabolómico en un medio de fermentación debe representar el estado metabólico de una población de microorganismos en el momento exacto en el que se toma la muestra y bajo las condiciones ambientales en las que la célula se ha desarrollado. Sin embargo, muchos de los metabolitos de interés son susceptibles de degradarse por la exposición a la luz o a la temperatura, produciendo cambios en sus concentraciones que distorsionarían el estado metabólico objeto de estudio.

Para obtener unos resultados metabolómicos correctos, el metabolismo celular debe ser rápidamente detenido, después de la toma de la muestra. Esto se consigue mediante el proceso de inactivación enzimática, también conocido como "quenching".

Para identificar y cuantificar los metabolitos intracelulares se hace preciso extraerlos, llevándose a cabo esta tarea mediante el uso de solventes (orgánicos, inorgánicos no acuosos, o una mezcla de ambos) que hagan a la envoltura celular permeable, lo que permitiría la penetración de los solventes dentro del espacio intracelular, favoreciendo la recuperación de los metabolitos.

Un método ideal de extracción intracelular sería reproducible, capaz de igualar la liberación de los metabolitos de diferentes clases evitando la degradación química y bioquímica y asegurando que la extracción sea completa. La completa extracción es un hecho difícil de comprobar, ya que por lo general las concentraciones de los metabolitos son desconocidas a priori.

Las pérdidas de los metabolitos durante el proceso de extracción deben ser corregidas a través del uso de factores de recuperación específicos o mediante la aplicación de un patrón interno adecuado, como estándares isotópicamente marcados con las mismas características químicas que el compuesto a analizar (Pinu & Villas-Boas, 2017).

Un análisis metabolómico completo de células microbianas debería incluir los siguientes pasos:

i. Cultivo de los microorganismos bajo estudio, en un apropiado medio de crecimiento;

ii. Muestreo en el estado de crecimiento deseado y la inactivación de las células (quenching).
    - Separación de las células del medio de cultivo, mediante centrifugación, el sobrenadante será utilizado para el estudio metabólico del medio extracelular.
    - Las células separadas serán utilizadas para el análisis de los metabolitos intracelulares.

iii. Extracción de los metabolitos intracelulares;

iv. Análisis de los metabolitos intra- y extracelulares utilizando los instrumentos analíticos apropiados (Pinu & Villas-Boas, 2017).

1.3. Inactivación celular (quenching).

El metabolismo celular es dinámico y las concentraciones de los metabolitos son el resultado del ratio entre la formación de cada metabolito y su velocidad de conversión en otros metabolitos producto (Figura 26). La velocidad de las reacciones metabólicas depende, fundamentalmente, de las concentraciones de las enzimas y del sustrato disponible (incluyendo la disponibilidad de cofactores) y, con frecuencia, de la presencia de activadores o inhibidores.

Los metabolitos primarios están relacionados con reacciones bioquímicas que juegan un papel fundamental en la función celular, siendo intermediarios de variadas reacciones, transformándose rápidamente a consecuencia de ello en el espacio intracelular.

La mayoría de los metabolitos primarios participan en un gran número de reacciones lo que significa que se ven afectados en gran medida por las modificaciones ambientales o genéticas, siendo objeto de interés de los estudios metabolómicos. La cuantificación de dichos metabolitos requieren una toma de muestra rápida con una inactivación de las enzimas metabólicas de manera simultánea o lo más rápido posible (Villas-Boas, 2007).

Por el contrario, los metabolitos secundarios son acumulados en las células para ser secretados al medio extracelular, siendo allí transformados más lentamente. Por lo general, son producidos en el estado estacionario del crecimiento celular, pudiendo ser sensibles a altas temperaturas o a la exposición a la luz, por lo que es necesario tener especial precaución en el momento de extraerlos.

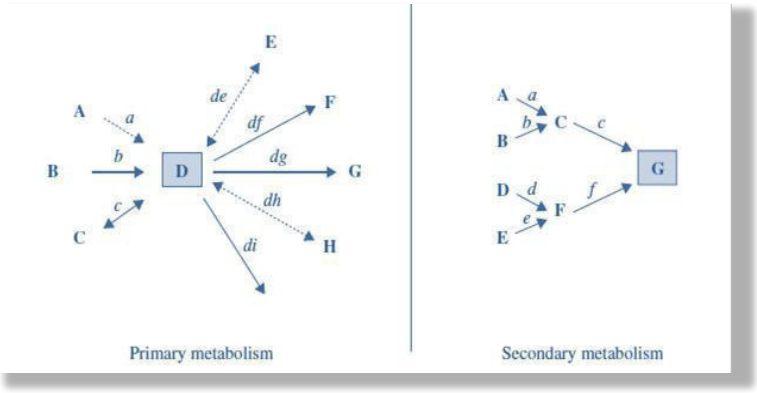


Figura 26. Esquemas del metabolismo primario y secundario. El metabolito primario D se forma a partir de los precursores A, B y C. También el metabolito D puede convertirse reversiblemente en C y es el precursor de los metabolitos E, F, G, H e I. En el metabolismo secundario, los metabolitos A y B se convierten en C y los metabolitos D y E se convierten en F. El metabolito secundario G se puede formar a partir de los precursores C y F, no es intermediario de otras reacciones, por lo que se acumula dentro de la célula o es secretado fuera. Fuente (Villas-Boas, 2007).

Los metabolitos se encuentran en el medio extracelular por haber sido secretados por las células o pueden provenir de la degradación de polímeros que están presentes en el medio como consecuencia de la lisis celular. Los cambios observados en el medio extracelular provienen de varios factores tales como la presencia de células vivas, el metabolismo celular, la lisis celular o por la secreción de enzimas. Es recomendable por ello que se separen rápidamente las células (biomasa) del contenido extracelular y se detenga la degradación mediante métodos de inactivación celular (Pinu & Villas-Boas, 2017). Así se evitan contaminaciones y el análisis metabolómico se conforma con el mayor grado de exhaustividad posible.

Los métodos de inactivación celular generalmente usan mezclas de soluciones de solventes acuosos y orgánicos en condiciones extremas de temperatura o de pH. Estas condiciones pueden producir la desestabilización de la pared celular dañando su estructura y como consecuencia de ello, producir el paso de los metabolitos hacia el medio extracelular (Figura 27).

1.4.-Métodos de disrupción celular.

La liberación de metabolitos intracelulares requiere la destrucción de la pared celular que en las levaduras se compone principalmente del complejo protein β-glucano.

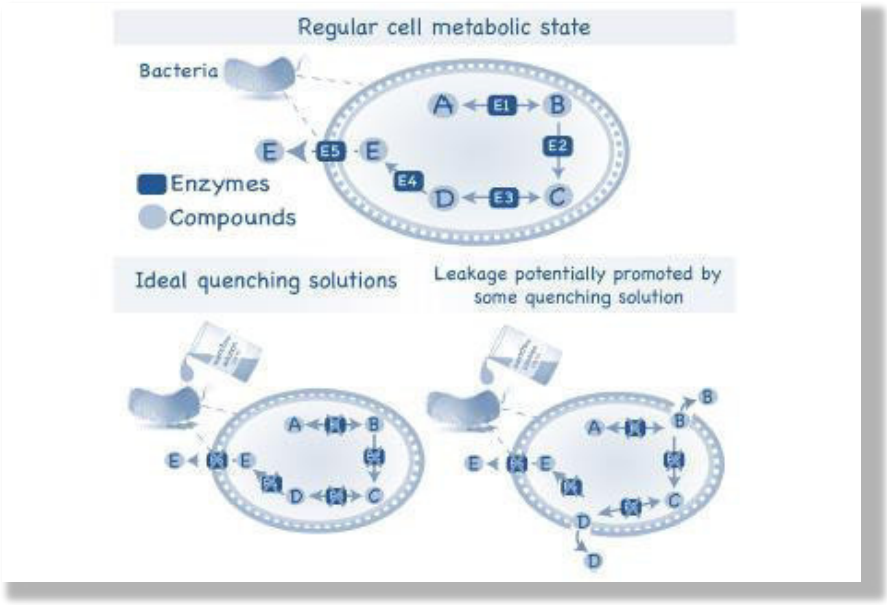


Figura 27. Estado normal de un microorganismo y la pérdida de los metabolitos ocasionado por el efecto de las soluciones de los métodos de inactivación celular. Fuente (Pinu & Villas-Boas, 2017).

Tabla 3. Detalle de algunos métodos de inactivación celular (quenching) utilizados en *Saccharomyces cerevisiae*.Fuente (Villas-Boas, 2007).

Método	Condiciones	Referencia
Ácido perclórico	0.66 M en agua 1:1 muestra: $HCLO_4$ sol. Temperatura ambiente	Larsson and Törnkvist, 1996
Metanol Frio	60% (v/v) en agua 1:4 muestra: sol. metanol -40°C	De Koning and van Dam, 1992
Metanol frío	75% (v/v) en agua /tampón 1:2 muestra: sol. metanol -40°C	Villas-Bôas et al.,2005a,b
Etanol hirviente	75% (v/v) en tampón 1:4 muestra: sol. etanol 80°C	Gonzales et al.,1997
Nitrógeno líquido	−196 °C	Mashego et al.,2003
Glicerol en frío	1:4 muestra: sol. Glicerol/ sol. Salina (3:1) -23°C 2,5 mL glicerol/sol salina 1:1 (v/v) -20°C	Villas-Bôas,2007a

Los métodos se clasifican fundamentalmente en: métodos mecánicos (emplean fuerza cortante) y métodos no mecánicos (eléctricos, físicos, químicos o enzimáticos) (Figura 28).

El empleo de los métodos mecánicos supone la destrucción no selectiva pero ofrecen la ventaja de que son fáciles de aplicar y más económicos. Por el contrario, los métodos no mecánicos son más selectivos y se suelen utilizar a escala de laboratorio debido a sus limitaciones operativas y económicas (D. Liu, Ding, Sun, Boussetta, & Vorobiev, 2016).

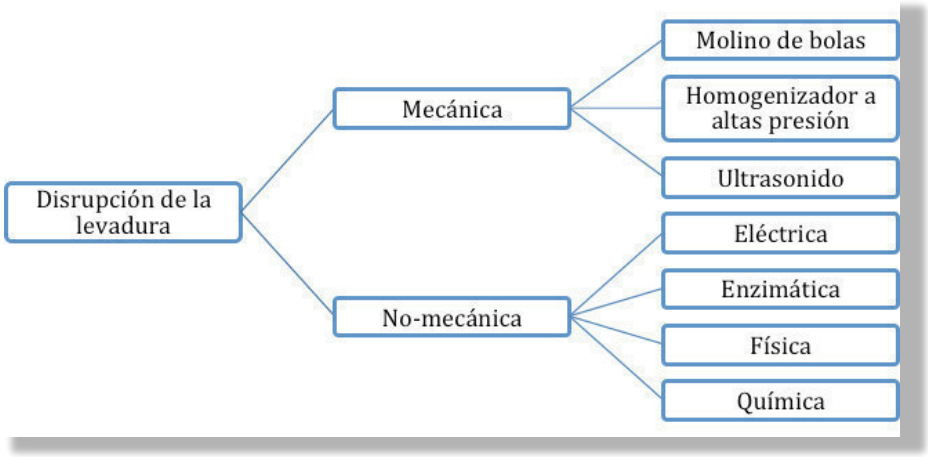


Figura 28. Clasificación de los métodos de disrupción celular utilizados con las levaduras. Fuente (D. Liu et al., 2016).

La disrupción mediante métodos mecánicos depende de las características de la pared celular, del grado de unión entre los polímeros y de la concentración de los éstos en la pared celular (Villas-Boas, 2007). A pesar de que no hay mucha información disponible relativa a la resistencia a la fragmentación de varios organismos siendo , en general, conforme al siguiente orden: Células animales > bacterias Gram-negativas > bacterias Gram-positivas > levaduras > hongos filamentosos > células vegetales (Villas-Boas, 2007). La Figura 29 muestra la composición de la envuelta celular de las levaduras.

Estos métodos no son muy usados para el análisis metabolómico, pero se ha demostrado su potencial para favorecer la fragmentación celular y para aumentar la extracción de metabolitos intracelulares de muestras biológicas, particularmente de los no polares.

Los métodos no-mecánicos, son tradicionalmente más utilizados en las extracciones intracelulares. Hacen uso de agentes químicos o físicos para hacer la pared celular más permeable y permitir la extracción de los metabolitos intracelulares del citoplasma celular.

Se clasifican en eléctricos, enzimáticos, químicos y físicos. Los métodos eléctricos, enzimáticos y físicos no son comúnmente aplicados, pero se suelen combinar con los métodos químicos para aumentar la eficiencia del proceso de extracción (especialmente los físicos). La lisis química de la pared celular constituye la mayoría de los procedimientos desarrollados

para la extracción de los metabolitos intracelulares que varían dependiendo de la estructura y la composición de la pared celular.

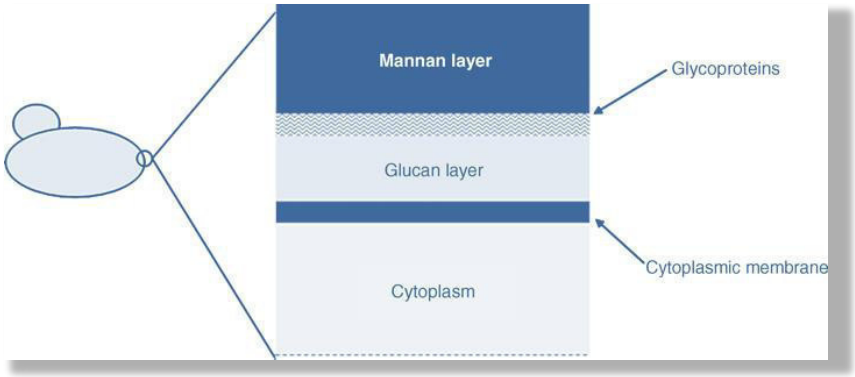


Figura 29. Esquema de la envuelta celular de las levaduras. Por lo general es más gruesa que la de una bacteria Gram-positiva y más resistente a la fragmentación mecánica que la pared bacteriana. Fuente (Villas-Boas, 2007).

Como ejemplo de una combinación de métodos físicos y químicos de disrupción celular destacamos el **Método de ciclos de congelación descongelación** (Smart, Aggio, Van Houtte, & Villas-Bôas, 2010)**.**

La pérdida de metabolitos en el proceso de extracción intracelular se debe a las características químicas de los metabolitos. Los métodos de extracción deben tender a minimizar cualquier degradación química o física de los compuestos. Este método combina la extracción usando una solución de metanol – agua con ciclos de congelación-descongelación. La congelación-descongelación aumenta la permeabilidad de la célula, debido a un proceso de expansión producido por la congelación, y posteriormente y por medio de la descongelación, se produce la disrupción de la pared celular aumentando la extracción química de los metabolitos mediante la solución acuosa de metanol en frio. Es un procedimiento simple y rápido que permite la extracción de amplio grupo de metabolitos, principalmente polares. El motivo de utilizar tan bajas temperaturas es asegurar que el metabolismo no se reactive durante el proceso de extracción (Smart et al., 2010).

A pesar de producir buenas recuperaciones de metabolitos, se ha demostrado que las extracciones con solución en 100% metanol en frío, no son suficientes para producir la disrupción de cierto tipo de células con paredes más gruesas.

1.5. Análisis.

La variabilidad de las muestras biológicas derivada de su complejidad química y del número de metabolitos a analizar, plantea un reto a la hora del análisis, no solo por los diversos problemas que se plantean a la hora de analizar un número tan elevado de metabolitos, sino también desde el punto de vista de la interpretación de los datos.

Las herramientas analíticas usualmente utilizadas en metabolómica son la espectrometría de masas y la resonancia magnética nuclear, siendo ambas empleadas de modo individualizado o asociadas a la separación cromatográfica, tanto para el análisis dirigido como para el no dirigido.

1.5.1. LC-MS.

Es una técnica analítica que asocia la espectrometría de masas con la separación por cromatografía líquida.

La espectrometría de masas conlleva la ionización de la molécula (añadiendo una carga positiva o negativa) y permitiendo el paso de esas moléculas cargadas a través de un campo magnético donde son analizadas. Las medidas, realizadas en intervalos de tiempo determinados, recogen los parámetros del ratio *m/z* de cada ión intacto y su correspondiente intensidad. Cada ión posee un tiempo de retención (RT) y un espectro de masas, los valores de cada uno de estos parámetros dependen del instrumento utilizado.

Los dos métodos para el análisis de muestras por espectrometría de masas más utilizados son MRM (Multiple Reaction Monitoring) y HRMS (High-Resolution Mass Spectrometry). Los experimentos realizados con MRM generalmente se realizan con espectrómetros de masas de triple cuadrupolo (Xu, Lu, & Rabinowitz, 2015). El primer cuadrupolo filtra los iones de una masa molecular determinada (ión parental) (frecuentemente con una resolución de 1 unidad de masa atómica). El segundo cuadrupolo fragmenta las moléculas que han sido seleccionadas y el tercer cuadrupolo selecciona los fragmentos característicos. Por tanto, antes de cada adquisición, los iones parenterales y los fragmentos de cada metabolito deben de esta definidos y optimizados previamente con sus correspondientes parámetros de energía de ionización para la fragmentación y del tiempo de retención (X. Liu & Locasale, 2017).

Tabla 4. Comparación de las tres herramientas analíticas más usadas en metabolómica. Fuente (X. Liu & Locasale, 2017)

	Pros	Contras	Aplicaciones
LC-MS	. Amplia cobertura de metabolitos . Simple preparación de la muestra . Alta sensibilidad . Flexibilidad en la separación de los compuestos y en la detección (diversas opciones en la selección de la columna fases móviles o métodos) . Amplia oferta de bases de datos y de software para el análisis de datos.	. Variación según el tipo de plata-forma . Es destructivo . Incapacidad de medir compuestos orgánicos que no forman aductos desde el ión molecular	Amplia cobertura) análisis metabolómico
GC-MS	. Amplia cobertura de metabolitos . Análisis de gases o de compuestos naturales volátiles . Alta sensibilidad . Amplia oferta de bases de datos y de software para el análisis de datos.	. Variaciones debidas al tipo de instrumento y/o condiciones . Es destructivo . No es útil para compuestos no volátiles o moléculas termolábiles . Complicaciones derivadas de la amplia oferta de derivatizaciones para un únicometabolito.	Análisis petroquímico
NMR	. Monitorización de la reacción a tiempo real en temperaturas controladas . Medidas en tiempo real *in vivo* . Detallada información estructural . No invasivo	. Baja sensibilidad . Baja cobertura de metabolitos en un único análisis . Procesamiento espectral poco automatizado . Alto coste del equipamiento y mantenimiento	. Elucidación de estructuras de nuevos compuestos . Análisis de la cinética de las reacciones químicas

Otro método muy utilizado últimamente es HRMS, que depende de la presencia de analizadores de masa de alta resolución. Uno de los más comunes analizadores de este tipo es el llamado Orbitrap® que anota las resoluciones de los iones, cuyas frecuencias suministran información de las masas moleculares (Olsen et al., 2005). Otra variedad de estos instrumentos es el TOF (tiempo de vuelo), el cual registra el tiempo que emplea un ion en atravesar un campo eléctrico (Plumb et al., 2004). Este tipo de equipos simplifican el proceso de identificación de los compuestos comparados con los equipos de baja resolución. Cuando los equipos de alta resolución se acoplan a celdas de colisión, éstas tienen la función de fragmentar los iones antes de ser enviados al analizador. Los iones parenterales proporcionan una información estructural adicional, facilitando la identificación en el procesamiento de datos (X. Liu & Locasale, 2017).

En el mercado existen numerosos paquetes informáticos para el procesamiento de los datos brutos de los análisis proporcionados por los equipos de espectrometría de masas. Por lo general, los datos comprenden el tiempo de retención, la relación *m/z* y el área del pico. Este último parámetro representa la abundancia relativa de cada metabolito en la muestra. Estas aplicaciones realizan una alineación de los picos cromatográficos seleccionados, permitiendo la identificación mediante la comparación con la fragmentación proporcionada por las bases de datos disponibles. Hay que tener en cuenta que los tiempos de retención son

altamente dependientes de la configuración del análisis de LC-MS y son difícilmente comparables con las bases de datos.

Por lo tanto, cada vez es más frecuente simultanear los análisis no dirigidos con los dirigidos o semi-dirigidos. Estos últimos funcionan como una referencia interna que incluye tanto *m/z* como el tiempo de retención medido en las mismas condiciones y equipos. Para facilitar las identificaciones, se añaden espectros MS/MS construidos con auténticos estándares de referencia y se utilizan estándares internos marcados isotópicamente.

La información obtenida de los estudios metabolómicos es tan abundante que equivale a la realización de múltiples ensayos bioquímicos por separado, lo que requiere poseer conocimientos biológicos para poder interpretar y obtener conclusiones. No siendo humanamente posible procesar de modo manual la totalidad de los datos, se hace imprescindible el uso de herramientas computacionales para el análisis posterior. Los software más recientes incluyen herramientas estadísticas tales como el análisis de componentes principales (PCA), la agrupación jerárquica (hierachical clustering) y otros test estadísticos para la visualización de datos (X. Liu & Locasale, 2017).

Se están realizando esfuerzos para avanzar en el campo de la metabolómica abarcando un mayor número de metabolitos y favoreciendo la integración de varias áreas "ómicas" para el análisis conjunto de resultados y la obtención de valiosas conclusiones (Figura 30).

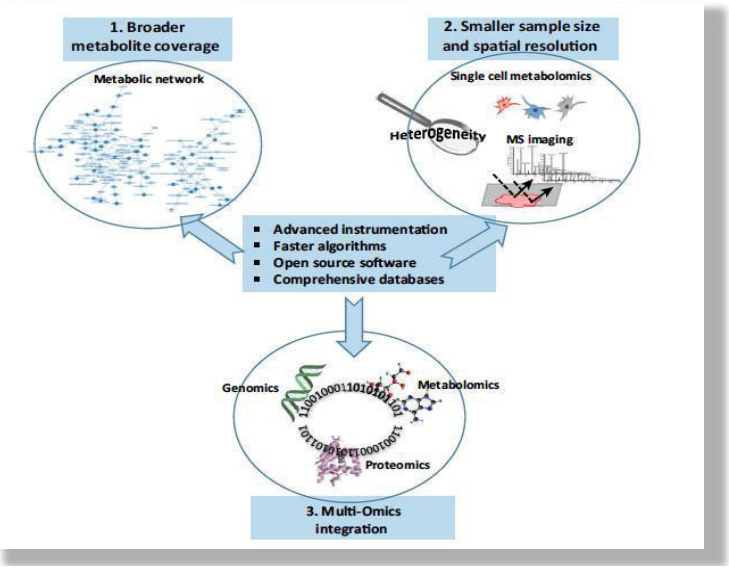


Figura 30. Tendencias en el análisis metabolómico. Las tendencias incluyen la cobertura de metabolitos para tamaños de muestras pequeños, con alta resolución espacial y la integración de datos procedentes de técnicas multiómicas. Fuente (X. Liu & Locasale, 2017).

2. JUSTIFICACIÓN Y OBJETIVOS

2

2.- JUSTIFICACIÓN Y OBJETIVOS.

La fermentación es un proceso milenario de elaboración de alimentos, que frecuentemente confiere al producto final especiales características organolépticas, y la atribución de propiedades potencialmente saludables. Este último, es un aspecto que está siendo estudiado extensivamente en la actualidad. Por otro lado la fermentación al aumentar la acidez produce una prolongación de la vida media del alimento, facilitando su almacenamiento.

España es uno de los principales productores de fresa y Andalucía es la cuarta región de producción de este fruto en el mundo (FAOSTAT, 2012). Al ser ésta una fruta que se caracteriza por su carácter perecedero, se considera recomendable el hallazgo de nuevas alternativas de aprovechamiento.

La utilización del proceso fermentativo para la producción de bebidas constituye una solución muy efectiva para la asimilación de excedentes de producción, evitando con ello la generación de pérdidas económicas.

Dada la creciente demanda de los consumidores de nuevos productos de calidad y con un plus para la salud, resulta de sumo interés, diversificar la oferta mediante la innovación en los procesos productivos con el fin ofrecer nuevos productos al mercado. Por ello, la fabricación de una nueva generación de bebidas fermentadas a base de excedentes de producción es plenamente aplicable a la fresa, mediante el uso de fermentaciones poco utilizadas hasta ahora, como son las fermentaciones glucónicas.

Estos procesos, mediados por la acción de bacterias acéticas (*Gluconobacter japonicum*) proporcionan la ventaja de mantener intacto el contenido de fructosa del sustrato mientras transforman la glucosa en ácido glucónico que actúa como eficiente regulador de la acidez, aportando particulares propiedades organolépticas (Deppenmeier, Hoffmeister, & Prust, 2002). Además, conlleva un beneficio potencial para el consumidor, por cuanto no se hace precisa la utilización de sacarosa para endulzar el alimento final. Esta particular circunstancia posibilita su consumo a personas que deban de reducir el contenido en azúcares de su dieta.

2. JUSTIFICACIÓN Y OBJETIVOS

La fresa es una fruta que, en tiempos recientes, ha sido objeto de numerosos estudios de los que se ha concluido que es una fuente de nutrientes y compuestos bioactivos de diversa índole entre los que se encuentran los polifenoles.

En la fresa encontramos polifenoles antocianos y no antocianos. La fracción antociánica está asociada al color y, ha sido objeto de numerosos estudios. Es por ello que el análisis de la composición fenólica no-antociánica de la fresa se presenta como un medio para conocer cómo los microorganismos, en concreto, levaduras y bacterias que intervienen en los procesos fermentativos, modifican la composición del sustrato mediante su metabolismo celular. Por otra parte, la caracterización química y sensorial del alimento nos puede dar una idea del interés por sus posibles beneficios para la salud derivados de su consumo y de su posible aceptación sensorial.

Además de los compuestos fenólicos, en la fresa existen otros bioactivos como la melatonina (Stürtz, Cerezo, Cantos-Villar, & Garcia-Parrilla, 2011). Bioactivo que se ha comprobado últimamente que también es producido por las levaduras, específicamente por *Saccharomyces cerevisiae* (Rodriguez-Naranjo, Torija, Mas, Cantos-Villar, & Garcia-Parrilla, 2012). Este compuesto está relacionado con el metabolismo del aminoácido aromático triptófano mediante la acción de la levadura *S. cerevisiae,* productora de fermentaciones alcohólicas y, quizás, la levadura más utilizada en la elaboración de alimentos, desde el pan a la cerveza y el vino.

Nos planteamos, por tanto, la formulación de la siguiente **hipótesis**: conociendo **el sustrato, y evaluando la acción de los microorganismos (levaduras y bacterias) que intervienen en las distintas fermentaciones (alcohólicas y glucónicas), se podría aumentar y/o modular el contenido de bioactivos de los productos resultantes.**

Para comprobar esta hipótesis se platean los siguientes **objetivos específicos**:

1. Caracterización química de una bebida elaborada a partir de fermentados de fresa.

   1.1 Caracterización química e impacto del proceso de fabricación industrial sobre el sustrato (*Fragaria* X *ananassa*).

1.2 Influencia de la fermentación glucónica mediada por *Gluconobacter japonicus* sobre el sustrato de puré de fresa.

1.3 Impacto de la fermentación alcohólica mediada por *Saccharomyces cerevisiae* sobre el sustrato de puré de fresa.

1.4 Caracterización química, estabilidad y análisis sensorial de una bebida producida a partir de fermentados del puré de fresa.

2. Síntesis de compuestos bioactivos por la acción de levaduras.

2.1. Evaluación de la producción de bioactivos, por la acción de *Saccharomyces* en el metabolismo de tirosina.

2.2. Valoración de la acción de levaduras *Saccharomyces* y no-*Saccharomyces* en la producción de metabolitos indólicos como consecuencia del metabolismo de aminoácidos aromáticos.

3. MATERIAL Y MÉTODOS

3.- MATERIAL Y MÉTODOS

Los materiales que a continuación se detallan se han empleado en la ejecución de la presente Tesis Doctoral.

1. Material y métodos correspondientes a los Capítulos I a IV.

1.1. Muestras de sustrato de fresa.

Se utilizaron muestras procedentes de los diferentes pasos que conforman el proceso industrial de elaboración del puré de fresa por la empresa Hudisa Desarrollo Industrial S.A. (Lepe, Huelva, Andalucía, España). Se emplearon las muestras procedentes de dos campañas consecutivas correspondientes a los años 2011 y 2012.

Este producto se elabora con los excedentes de producción de variedades de fresa (*Fragaria x ananassa* Duch.) que mayoritariamente se cultivan en la zona: *Camarosa, Candonga, Festival, Ventana, Splendor, Honor* y *Coral*.

El proceso comienza cuando la fruta es recibida en las instalaciones de la empresa, siendo seleccionada y limpiada. Con posterioridad, se procede a la eliminación de las hojas y tallos, y seguidamente se tritura. En este paso se realiza el tamizado con el fin de eliminar las semillas y así atender los requerimientos del mercado

Posteriormente se lleva a cabo un proceso de calentamiento a 55-65ºC durante 2 minutos con el objetivo de inactivar las enzimas presentes.

Dependiendo de la presentación del producto final, el proceso puede incluir la etapa de evaporación/concentración. Ésta consiste en la evaporación de una parte del agua intrínseca, a través de un proceso físico donde se combinan presiones de vacío con temperaturas moderadas inferiores a 75˚C. El proceso da como resultado purés con concentraciones de 11˚Brix (1,5X). Este puré, fue utilizado como sustrato para la elaboración de los fermentados glucónicos y alcohólicos por parte del grupo de investigación de la Universidad de Córdoba.

A continuación, se pasteuriza a 90ºC durante 3 minutos, y luego se reduce la temperatura a 5ºC en un período de pocos minutos.

Una parte del producto sin semillas correspondiente a la cosecha 2012 se dejó sin pasteurizar, con la finalidad de comprobar los efectos del proceso de pasteurización sobre el contenido de los fenoles no antocianos.

El producto final ha consistido en purés asépticamente envasados justo después del proceso de pasteurización, presentando una configuración con o sin semillas, según si han sido o no eliminada antes del procesamiento. Las diferentes muestras se trasladaron al laboratorio y se mantuvieron conservadas a -20ºC hasta su procesamiento. En la Figura 31 se detalla el proceso industrial.

En total se analizaron 25 muestras; 12 correspondientes a la campaña de 2011 y 13 de la de 2012. De cada cosecha se siguió el proceso a 3 sustratos diferentes compuestos por mezclas en diferentes proporciones de las variedades antes citadas, asignándosele a cada uno una letra A, B o C. Las muestras se distribuyeron de la forma que a continuación se detalla (Tabla 5):

- 6 muestras de fresa triturada de 3 sustratos diferentes (M_A, M_B y M_C). Fueron utilizadas como control del proceso de elaboración del puré de fresa, ya que no ha sufrido más manipulación que el triturado de la fruta.
- 6 muestras de inactivación enzimática (EI_A, EI_B y EI_C). Las tres muestras de la cosecha de 2011 son con semilla y las tres del 2012 son sin semilla. La inactivación enzimática es un proceso térmico que consiste en un calentamiento a una temperatura comprendida entre 55-65ºC durante 2 minutos.
- 3 muestras de pasta sin pasteurizar sin semillas (UP_A, UP_B y UP_C), solo de la campaña de 2012. Corresponden al producto de todo el proceso de fabricación hasta justo antes de la pasteurización.
- 5 muestras de puré envasado (producto final pasteurizado) con semillas (FPS_A, FPS_B y FPS_C).
- 5 muestras de puré envasado (producto final) sin semillas ($FPWS_A$, $FPWS_B$ y $FPWS_C$).

Para el análisis de los polifenoles no antocianos, todas las muestras fueron sometidas al proceso de extracción que a continuación se detalla:

- Se mezclaron 30 g de muestra con 30 mL de metanol y 1% (w/w) de ácido ascórbico, con la finalidad de evitar la oxidación.
- Esta mezcla se sometió a baño de agua con ultrasonido durante 30 min.
- Inmediatamente se centrifugó por 10 minutos a 3000 rpm.
- El sobrenadante se recolectó y el pellet fue sometido a un nuevo proceso de extracción en las mismas condiciones. Finalmente se mezclaron los sobrenadantes.
- La mezcla de los sobrenadantes obtenida se desecó utilizando un evaporador rotatorio.
- El extracto desecado se reconstituyó hasta un volumen final de 5mL con metanol/agua 1:1 (v/v) y se almacenó a -18ºC hasta el análisis.

Las extracciones se realizaron por duplicado.

El mismo proceso de extracción, exceptuando la adición de ácido ascórbico, se utilizó para la medida de la actividad antioxidante, siendo ésta realizada también por duplicado.

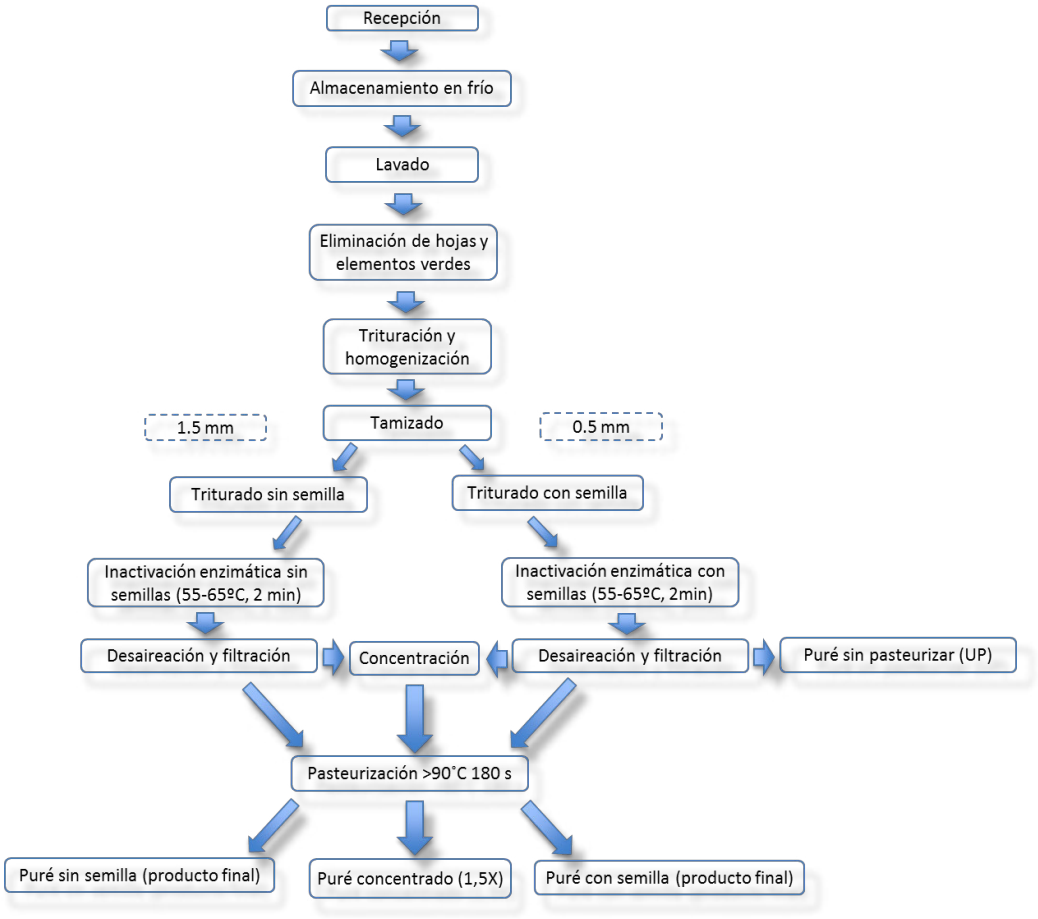


Figura 31. Diagrama del proceso de elaboración industrial del puré de fresa. HUDISA Desarrollo Industrial, S.A.

1.2. Muestras de Fermentados de Fresa.

Las fermentaciones se llevaron a cabo en el Departamento de Química Inorgánica e Ingeniería Química de la Universidad de Córdoba (Andalucía, España), a escala de laboratorio, a partir del puré de fresa descrito anteriormente. Estas actividades se han realizado dentro del marco del Proyecto del Plan Nacional "Evaluación de la Calidad y Seguridad de una Nueva Bebida Obtenida a partir de Fresa no apta para Comercialización" y, en concreto, dentro del subproyecto 3, denominado "Procesos de Fermentación para la Producción de una Nueva Bebida a partir de fresa no apta para Comercialización" (Investigador Principal: Dr. Isidoro García).

Los inóculos fueron proporcionados por el grupo de la Universidad Rovira i Virgili, (Tarragona, Cataluña, España) encargados del subproyecto 2 denominado: "Selección y Control Microbiológico para la producción de una Nueva Bebida a partir de fresa no apta para Comercialización" (Investigadora Principal: Dra. Mª Jesús Torija).

Las fermentaciones acéticas, alcohólicas y glucónicas se realizaron en cultivo sumergido. Este sistema consiste en la introducción de un cultivo de microorganismos sumergidos libremente en el líquido a fermentar que se distribuye de forma homogénea con ayuda de agitación mecánica.

En el caso de las fermentaciones acética y glucónica, el suministro de oxígeno en el fermentador se realiza de manera automática en función de las variables operacionales programadas en cada experimento. Se fija como valor constante el porcentaje de oxígeno disuelto de modo que cuando este nivel es inferior al programado, el sistema airea automáticamente restableciendo este valor.

Hay que resaltar que la fermentación acética y glucónica son procesos aerobios mientras que la alcohólica es un proceso anaerobio. Por lo tanto, el proceso de fermentación se diferencia de los anteriores en que no hay concentración de oxígeno disuelto.

En los apartados siguientes se detallan las condiciones de fermentación.

1.2.1. Fermentación Glucónica.

Para esta fermentación se utilizó la especie de bacterias acéticas *Gluconobacter japonicus* CECT 8443). Fue aislada originalmente del mosto de uva procedente de la bodega experimental Mas dels Frares (Grupo de investigación de la Universidad Rovira i Virgili) (Navarro, 2011).

Como sustrato se utilizó puré de fresa (3 L), de dos concentraciones diferentes: A (1X) y B (1,5X). Estos son purés de fresa elaborados con el proceso industrial explicado en el apartado 1. La fermentación se llevó a cabo en modo discontinuo (batch) con las condiciones que a continuación se detallan: pH 3,24; temperatura de 29ºC y 500 rpm de agitación continua.

En el fermentador se introdujeron los 3L de puré (A (1X) o B (1,5 X) y el inóculo de *G. japonicus* con la siguiente composición:

- 5% (p/v) de glucosa.
- 1% (p/v) de extracto de bacteria.
- 2% (p/v) de peptona bacteriológica.

Todo se mezcló durante un tiempo comprendido entre 20 y 30 minutos. En la Figura 32 se esquematiza el proceso.

Se realizaron cuatro ciclos de fermentaciones para cada sustrato. Cada ciclo se inicia cuando el inóculo se mezcla con el puré de fresa, siendo en este momento tomada la primera muestra que se denomina muestra inicial (I). Se considera que la fermentación termina cuando la glucosa se ha consumido en su totalidad y el pH alcanza el valor de 2,74, recogiéndose en este instante otra muestra que se denomina muestra final (F) y que coincide con el momento en que el fermentador es descargado.

Seguidamente, los fermentados se sometieron a pasteurización. El proceso de pasteurización consistió en el calentamiento del fermentado glucónico a una temperatura entre 70-80ºC por 15 minutos y, a continuación, se enfrió rápidamente. En este momento se toma otra muestra que corresponde al final del proceso de pasteurización y que se denomina (P). Seguidamente, se almacenan a -20ºC hasta su análisis.

Para los posteriores análisis, las muestras se sometieron al mismo proceso de extracción que se ha detallado para las muestras del puré de fresa.

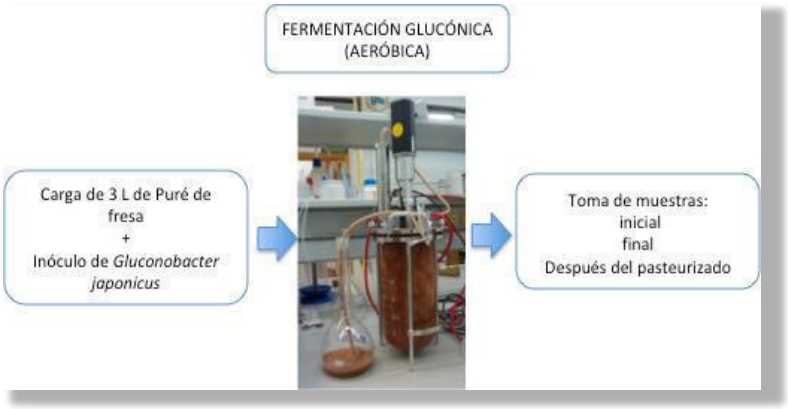


Figura 32. Esquema de las características del proceso de la fermentación glucónica. Las muestras indicadas corresponden a las evaluadas.

1.2.2. Fermentación Alcohólica.

En la elaboración de los fermentados alcohólicos se utilizó la levadura *Saccharomyces cerevisiae* (CET 13057) aisladas de la fresa (Hidalgo, Torija, Mas, & Mateo, 2013). Se realizó una fermentación sumergida en modo semicontinuo a partir de un volumen inicial de 3,6 L de puré de fresa correspondiente a dos cosechas sustrato A (1,5X) (2011) y sustrato B (1 X) (2012).

Se realizaron cuatro ciclos de fermentaciones. Las condiciones utilizadas fueron las siguientes: 29ºC de temperatura con una agitación constante de 250 rpm. Previamente a la inoculación, el medio se saturó con oxígeno solo al inicio de la fermentación. El inóculo tenía la siguiente composición:

- 10% (w/v) de glucosa.
- 0,1% (w/v) $MgSO_4$.
- 0,2% (w/v) KH_2PO_4.
- 0,3% (w/v) $(NH_4)_2SO_4$.
- 0,4% (w/v) de extracto de levadura.
- 0,36% (w/v) de peptona bacteriológica.

En el momento de la mezcla con el inóculo, se tomó la primera muestra que se denominó "inicial" (I). La fermentación se consideró finalizada cuando los azúcares fueron consumidos, alcanzando la concentración alcohólica de 3,02 g/L y 1,8 g/L para cada sustrato A y B, respectivamente. En este momento, se tomaron las muestras correspondientes al final de la fermentación, denominándose (F).

El proceso de pasteurización siguió el mismo patrón que en las fermentaciones glucónicas e, igualmente, cuando finalizó el proceso, se tomaron las muestras correspondientes, siendo estas denominadas (P).

1.2.3. Fermentación Acética.

Esta fermentación se realizó en cultivo semicontinuo (Figura 33), utilizando la bacteria acética de la especie *Acetobacter malorum* (CECT 7749) en las siguientes condiciones: 30ºC de temperatura, 70% de concentración de oxígeno disuelto y agitación continua de 500 rpm (Hidalgo et al., 2013).

Al inicio, el acetificador se cargó con 3,6 L de fermentado alcohólico de fresa del apartado anterior. Una vez que el alcohol fue consumido, se descargaron 2,6 L y se reemplazaron por un volumen igual de fermentado alcohólico de fresa.

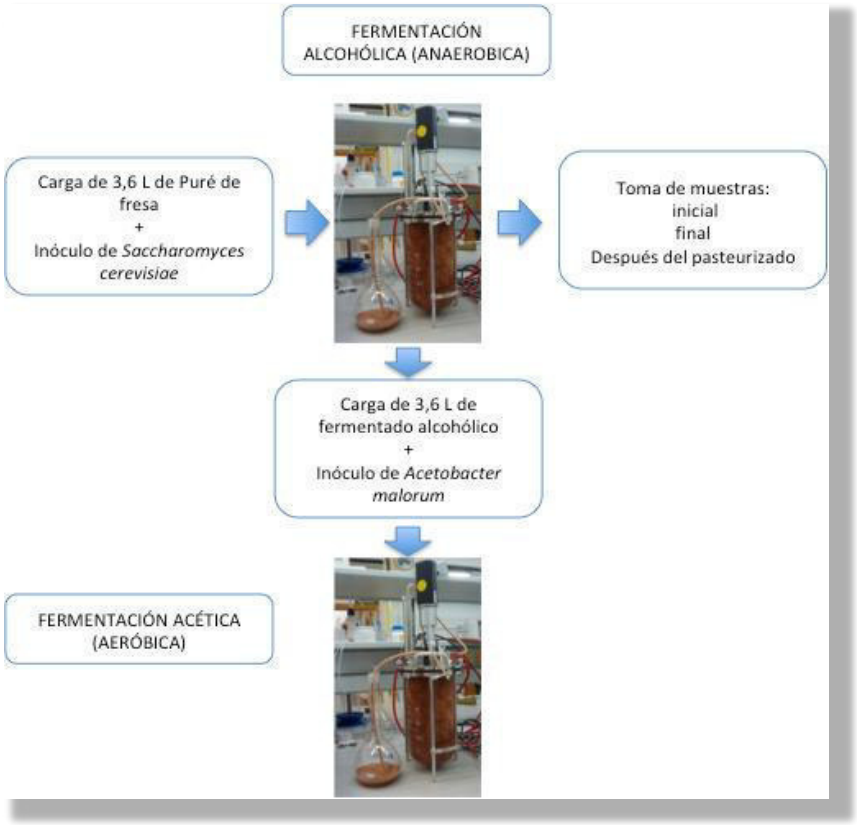


Figura 33. Esquema de las características de los procesos de fermentación alcohólica y acética. Las muestras indicadas corresponden a las analizadas.

1.2.4. Bebida de Fresa.

Se elaboró una bebida de fresa en base a fermentados glucónicos de fresa obtenidos de la fermentación del sustrato A (1 X), cuyo proceso de elaboración se detalla a continuación:

- El fermentado glucónico se centrifugó 20 minutos a 3000 rpm.
- Posteriormente, el fermentado glucónico centrifugado se diluyó con agua carbonatada en una proporción 80:20 v/v.
- El fermentado glucónico tratado se mezcló con el fermentado acético previamente centrifugado, en una proporción de 99,04: 0,96 de fermentado glucónico y fermentado acético. Como consecuencia alcanzó un contenido de ácido acético de 5,6 % p/v.
- Se agregó dimetil dicarbonato (Velcorín®) como conservante (E242) a la concentración de 230 mg L^{-1}.
- Se distribuyó en botellas ámbar previamente esterilizadas.
- Una vez distribuida en los envases, se esterilizó calentando cada envase a 90ºC durante 90 segundos, siendo inmediatamente enfriados a 20ºC o menos.

Se almacenaron en refrigeración a 4ºC o a temperatura ambiente (~ 30ºC), según el caso, y se tomó la muestra inicial justo después de la pasteurización ($R_o = F_o$). El resto de las muestras se tomaron a diferentes tiempos de 15, 30, 60 y 90 días. Nomenclatura de las muestras:

Tabla 5. Distribución de las muestras correspondientes a los Capítulos I-IV.

	Nomenclatura de las muestras		Nº de muestras
	Campaña 2011		
Capítulo I	M_A, M_B, M_C	Triturado de fresa con semilla	3 muestras
	IE_A, IE_B, IE_C	Inactivación enzimática con semillas	3 muestras
	FPS_A, FPS_B, FPS_C	Puré de fresa con semilla	3 muestras
	$FPWS_A$, $FPWS_B$, $FPWS_C$	Puré de fresa sin semilla	3 muestras
	Campaña 2012		
	M_A, M_B, M_C	Triturado de fresa con semilla	3 muestras
	IE_A, IE_B, IE_C	Inactivación enzimática	3 muestras
	UP_A, UP_B, UP_C	Puré sin pasteurizar sin semillas	3 muestras
	FPS_A, FPS_B	Puré de fresa con semilla	2 muestras
	$FPWS_A$, $FPWS_C$	Puré de fresa sin semilla	2 muestras
Capítulo II	**Sustrato A (1X)**		
	I_1, I_2, I_3, I_4	Inicial de cuatro ciclos de fermentación	4 muestras

	F_1, F_2, F_3, F_4	Final fermentación de cuatro ciclos de fermentación	4 muestras
	P_1, P_2, P_3, P_4	Pasteurizados de cuatro ciclos de fermentación	4 muestras
	Sustrato B (1,5X)		
	I_1, I_2, I_3, I_4	Inicial de cuatro ciclos de fermentación	4 muestras
	F_1, F_2, F_3, F_4	Final de fermentación de cuatro ciclos de fermentación	4 muestras
	P_1, P_2, P_3, P_4	Pasteurizados de cuatro ciclos de fermentación	4 muestras
Capítulo III	**Sustrato A (1,5X) Cosecha 2011**		
	I_1, I_2, I_3	Inicial de tres ciclos de fermentación	3 muestras
	F_1, F_2, F_3	Final de fermentación de tres ciclos de fermentación	3 muestras
	P_1, P_2, P_3	Pasteurizados de cuatro ciclos de fermentación	3 muestras
	Sustrato B (1X) Cosecha 2012		
	I_1, I_2, I_3, I_4	Inicial de cuatro ciclos de fermentación	4 muestras
	F_1, F_2, F_3, F_4	Final de fermentación de cuatro ciclos de fermentación	4 muestras
	P_1, P_2, P_3, P_4	Pasteurizados de cuatro ciclos de fermentación	4 muestras
Capítulo IV	$R_0 = F_0$	Inicial	2 muestras
	Temperatura ambiente		
	R_{15}	Almacenada durante 15 días	2 muestras
	R_{30}	Almacenada durante 30 días	2 muestras
	R_{60}	Almacenada durante 60 días	2 muestras
	R_{90}	Almacenada durante 90 días	2 muestras
	Temperatura de refrigeración (4ºC)		
	F_{15}	Almacenada durante 15 días	2 muestras
	F_{30}	Almacenada durante 30 días	2 muestras
	F_{60}	Almacenada durante 60 días	2 muestras
	F_{90}	Almacenada durante 90 días	2 muestras

1.3. Reactivos.

- Adquiridos a Sigma Aldrich (St. Louis, MO. USA): ácido gálico, ácido caféico, ácido cumárico, ácido cinámico, (-)- epicatequina, (+)-catequina, ácido clorogénico, ácido elágico, galato de (-)-epicatequina, kaempferol, glucurónido de 3-O-ß-D-kaempferol, glucósido de 3-kaempferol, trans-resveratrol, apigenina, ß-D-glucósido hidratado de

penta-O-galoil, DPPH (2,2-diphenyl-1-picrylhydrazyl), AAPH (2,2´ -diazo-bis-amidine-propane-dihydrochloride) y trolox (ácido 6-hidroxi-2,5,7,8-tetrame- tilcroman-2-carboxylico), glucósido de resveratrol, ácido quínico, brevifolina, ácido protocatéquico, isorhamnetina, (+)-taxifolina, eriodictiol, rutina, quercetina, ácido homovanillico, vainillina, ácido fórmico con calidad HPLC.

- Patrones adquiridos a Chromadex. Inc. (USA): procianidina B1.
- Patrones adquiridos a Extrasyntese (Z.I. Lyon Nord. France): luteolina, (+)-aromadendrina, floridizina, flavonomarein, glucósido de 7-*O*-apigenina.
- Acetonitrilo, ácido acético calidad HPLC y ácido fórmico con calidad HPLC adquiridos a Merck.
- Metanol con calidad de HPLC adquirido a Prolabo® (Obregón, Mexico).

1.4. Instrumentación.

- Centrífuga (Sorvall® TC Dupont).
- Evaporador rotatorio ((Büchi Rotavapor®, R- 200/205).
- Cromatógrafo líquido de alta resolución asociado a un detector de diodo (DAD) Agilent Series 1100 equipado con una bomba cuaternaria (Series 1100 G1311A), inyector automático (Series 1100 G1313A) y un desgasificador (series 1100 G1379A).
- Espectrómetro de masa Applied Biosystems QTRAP LC/MS/MS system (Foster City, USA) que consiste en un triple cuadrupolo asociado a una trampa iónica (QqQlit) y equipado con una fuente de ionización por electrospray, asociado a su vez a un cromatógrafo líquido (LC) Pelkin Elmer Series 200 HPLC system (Wellesley, USA).
- Espectrómetro de masas de alta resolución que consiste en un sistema híbrido de un cuadrupolo segmentado para la selección de iones precursores con un analizador de masas Orbitrap® de alta resolución (QExactive) Thermo Fisher Scientific (Bremen, Germany), con fuentes de ionización ESI, APCI y nanoESI asociado a un cromatógrafo líquido de alta resolución (UHPLC) Thermo Fisher Scientific (Bremen, Germany).
- Espectrómetro de masas híbrido que consta de una trampa iónica con un analizador de masas Orbitrap® (Orbitrap ELITE) con fuentes de ionización ESI, APCI y nanoESI. Este espectrómetro está además equipado con un UHPLC (Thermo Fisher Scientific (Bremen, Germany).
- Espectrofotómetro para microplacas (Synergy HT, Biotek®).
- Espectrofotómetro (Hitachi UV-2800).

2. Material y métodos correspondientes a los Capítulos V y VI.

2.1. Muestras de fermentaciones alcohólicas de uva y de mosto sintético.

Los fermentados alcohólicos con mosto de uva que se utilizaron para la realización de los experimentos del Capítulo V de la presente Tesis Doctoral se llevaron a cabo en el Instituto de Investigación y Formación Agraria y Pesquera (IFAPA), Rancho de la Merced, Junta de Andalucía, bajo la dirección de la Dra. Emma Cantos Villar. Y los fermentados en mosto sintético se llevaron a cabo en la Universidad Pablo de Olavide bajo la dirección de la Dra. Eva Valero.

2.1.1. Fermentaciones alcohólicas en mosto de uvas blancas.

Las uvas utilizadas para la elaboración del mosto se cultivaron en suelos de tipo albariza con una densidad de población de 3600 vid/Ha y pertenecían a un cultivo experimental localizado en el Rancho de la Merced. En concreto, se utilizaron 6 variedades de uvas blancas: *Corredera, Moscatel, Chardonnay, Palomino fino, Sauvignon Blanc, Vijiriega, y* una tinta, *Tempranillo*. El grado de maduración fue vigilado semanalmente y la recolección se produjo en agosto de 2015 cuando se hallaban en óptimo estado de maduración.

Una vez realizada la recolección, las uvas blancas fueron despalilladas y prensadas. Al mosto resultante se les agregaron las enzimas pectolíticas (2,5 mL h L−1, Enartis ZYM, Italia) y dióxido de azufre (Sulfosol, Sepsa-Enartis). Posteriormente, el mosto fue colocado en tanques de acero inoxidable e inoculado con levaduras de la cepa AROMA WHITE (Italy) a 18 ºC. Se consideró finalizada la fermentación cuando los azúcares alcanzaron una concentración inferior a 3 g L^{-1} (Figura 34).

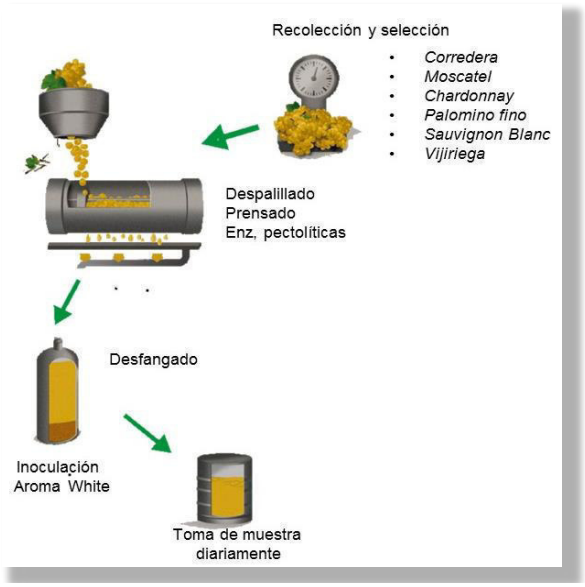


Figura 34. Esquema del proceso de las fementaciones de los mostos de uva blanca.

Las muestras se tomaron diariamente hasta que la fermentación alcohólica se consideró terminada.

Una vez tomadas las muestras, se limpiaron de posibles interferentes con el método de SPE (extracción en fase sólida) utilizando cartuchos C18 en fase reversa y aplicando vacío. El método consta de los siguientes pasos:

- Acondicionamiento con 2 mL de agua ultra pura (milli Q).
- Carga de un volumen de muestra de 500 µL.
- Lavado con 2 mL de una solución de metanol/agua 10%.
- Recogida de la muestra en 1 mL de metanol puro.

La muestra recogida, se evaporó hasta desecación utilizando un concentrador al vacío con la finalidad de recuperarla con metanol/agua 10% en un volumen tal que la muestra resultara concentrada tres veces. Las muestras así tratadas se almacenaron a -20˚C hasta el análisis.

2.1.2. Fermentaciones alcohólicas en mosto de uva *Tempranillo*.

La recolección de la uva tempranillo se realizó en condiciones de madurez óptima. El mosto se obtiene por la presión de la uva con una prensa neumática, con la adición posterior de las enzimas pectolíticas (3 mL h L^{-1}, Enartis ZYM, Italia) y de dióxido de azufre en una concentración de 40 mg L^{-1} y, posteriormente, el mosto obtenido se colocó en tanques de acero inoxidable (Figura 35).

La inoculación se realizó de cinco formas diferentes:

i. (CTQA) Control con *Saccharomyces cerevisiae* QA23.
ii. (CTRF) Control con *Saccharomyces cerevisiae* RF.
iii. (SIQA23) Inoculación secuencial, comenzando con *Torulaspora delbrueckii* y una vez que la densidad había decaído, se inoculó con *S. cerevisiae* QA23.
iv. (SIRF) Inoculación secuencial, realizándose primero con *Torulaspora delbrueckii*, y, siguiendo el mismo patrón que la anterior, con posterioridad se inoculó con *S. cerevisiae* Red Fruit.
v. (SP) Fermentación espontánea sin la inoculación de cepas comerciales.

Las muestras se tomaron diariamente hasta que se consideró finalizada la fermentación alcohólica.

Las muestras fueron tratadas antes de su análisis de la misma forma que se ha indicado en el apartado anterior.

Figura 35. Esquema de elaboración de las fermentaciones en mosto de uva *Tempranillo*.

2.1.3. Fermentación alcohólica en mosto sintético.

Se realizaron 6 fermentaciones en mosto sintético siguiendo la composición publicada por Riou y colaboradores (Tabla S2- Capítulo VI) (Riou, Nicaud, Barre, & Gaillardin, 1997), con tres cepas de levaduras, dos *S. cerevisiae* (QA23 y RED FRUIT) y la una no *Saccharomyces*, *Torulaspora delbrueckii.* Cada fermentación se realizó por duplicado en matraces Erlenmeyer. Cada uno se llenó con 750 mL de mosto sintético y, posteriormente, se inocularon con un volumen de inóculo equivalente a 10^6 células mL^{-1} (Figura 36).

Cada matraz estaba provisto de un tapón de goma con dos orificios, uno destinado a un tubo capilar por donde se libera el CO_2 producido durante la fermentación y el otro destinado a un tubo de goma, debidamente tapado, que permitiría la extracción de la muestra de forma aséptica. Los matraces se colocaron en estufa a 28 ˚C con agitación de 150 rpm.

Los matraces se pesaban diariamente para cuantificar la liberación de CO_2.

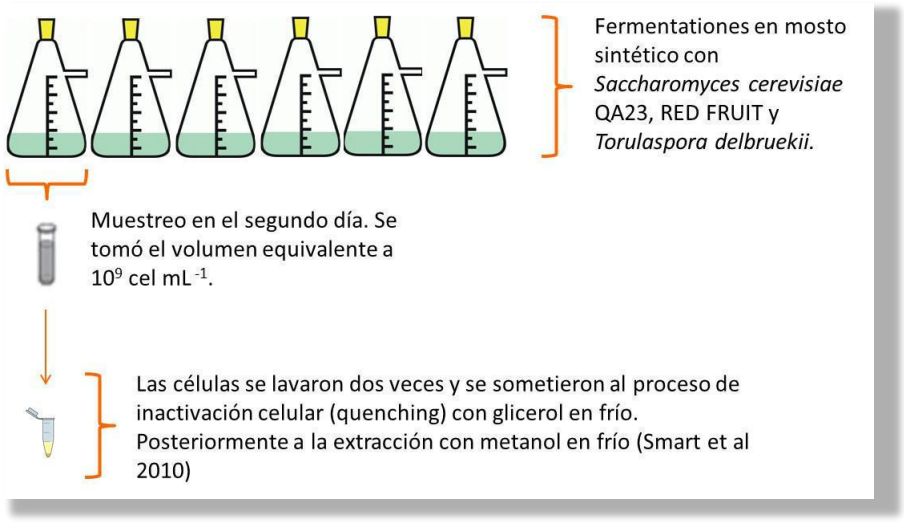


Figura 36. Esquema de las fermentaciones en mosto sintético.

3.MATERIAL Y MÉTODOS

Las muestras se tomaron al segundo día de fermentación en un volumen equivalente a 10^9 células mL^{-1}. Se lavaron dos veces con agua ultra pura y una centrifugación de 4500 rpm a 4 ˚C durante 3 minutos. Inmediatamente, el pellet de células se sometió al proceso de inactivación celular con glicerol en frío.

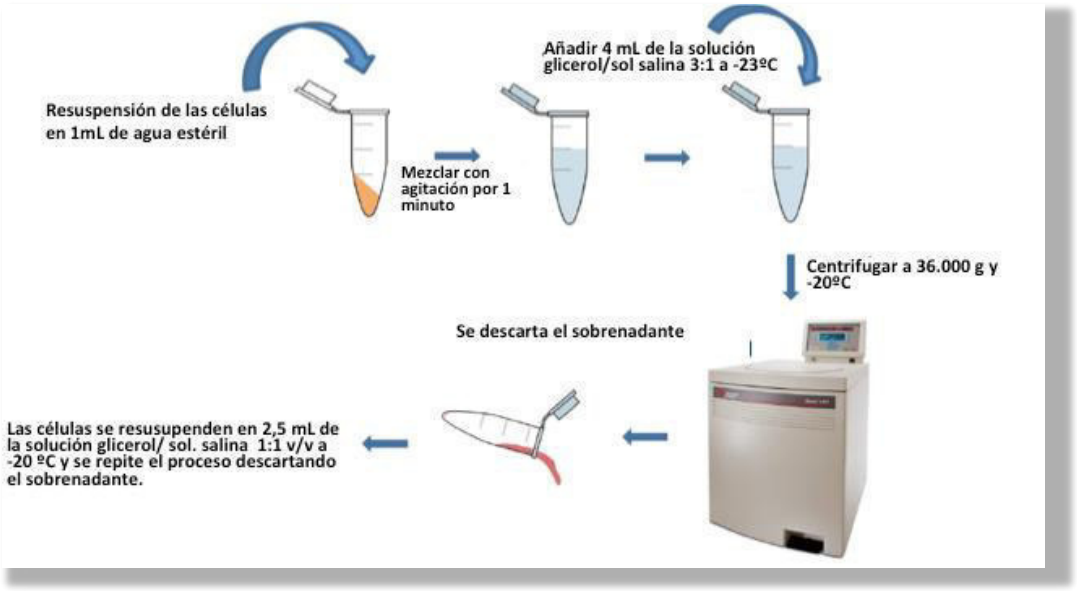


Figura 37. Esquema del proceso de inactivación actividad celular (quenching) con glicerol en frío.

Este método fue publicado por Villas-Boas (Villas-Bôas & Bruheim, 2007) (Figura 37) y consiste en agregar al pellet resuspendido en 1 mL de agua, una solución de glicerol/solución salina 3:1 v/v a una temperatura de -23 ˚C mantenido mediante un baño de etilen glicol. Después de que se haya homogeneizado, se centrifuga a 36.000 g durante 20 minutos a una temperatura de -20 ˚C. El pellet resultante se resuspendió con 2,5 mL de una solución glicerol/solución salina 1:1 v/v mantenida a -20 ˚C. Finalmente, se llevó a cabo otra centrifugación a 36.000 g durante 20 minutos a una temperatura de -20 ˚C. El pellet así tratado se reserva a -80ºC para ser sometido a la extracción intracelular

El método de extracción intracelular seleccionado consistió en una modificación realizada sobre el método publicado por Smart (Smart, Aggio, Van Houtte, & Villas-Bôas, 2010) (Figura 38). Este consiste en:

- El pellet se resuspendió con 2,5 mL de una solución de metanol /agua 50% v/v mantenida a -30˚C, en un baño de etilen glicol.

- Se realizan dos ciclos de congelación/descongelación, que consisten en congelar las células a -80 ˚C por 30 min y después se descongelan a la temperatura de 4˚C en un baño de hielo.
- Se aplica ultrasonido en un baño de hielo durante 1 minuto.
- Se centrifuga a 36.000 g durante 20 min a -20 ˚C.
- Se repite el proceso nuevamente y se recogen los dos sobrenadantes juntos.
- La muestra así extraída se somete a un proceso de limpieza mediante el sistema de extracción sólida y a la concentración posterior, tal y como se ha explicado en el aparatado 2.1.1.

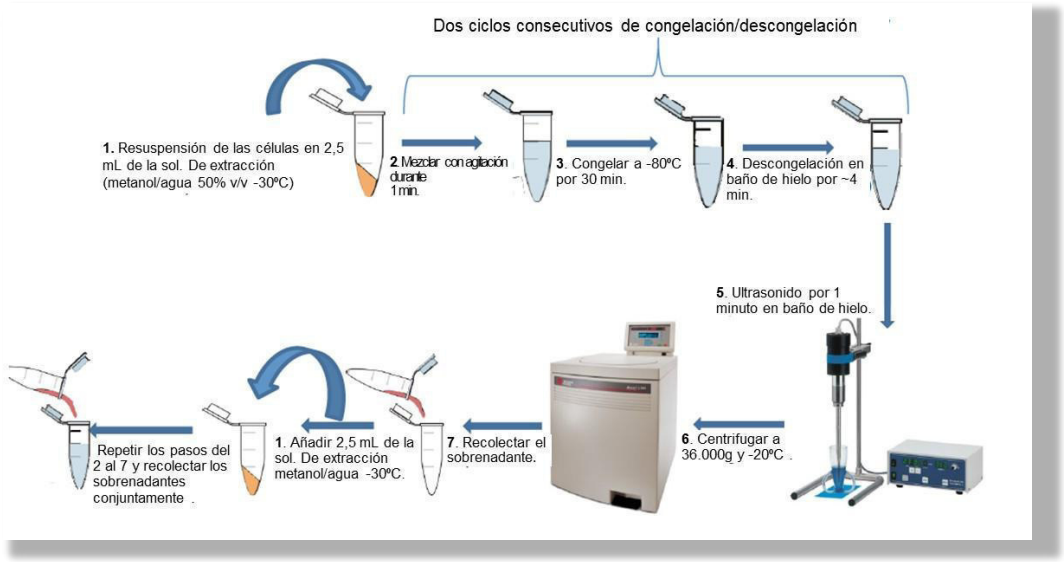


Figura 38. Esquema del proceso de la extracción intracelular de ciclos de congelación/descongelación asociados a 1 minuto de ultrasonido.

2.2. Muestras de fermentados alcohólicos en mosto sintético.

2.2.1 Muestras destinadas al análisis dirigido de metabolitos producidos en el metabolismo de los aminoácidos aromáticos.

Las fermentaciones que se realizaron para el desarrollo de los experimentos correspondientes al Capítulo VI de la presente Tesis Doctoral, se llevaron a cabo en las instalaciones de la Universidad Pablo Olavide y del CABD (Centro Andaluz de Biología del Desarrollo), bajo la supervisión de la Dra. Eva María Valero Blanco.

Para tal fin, se realizaron 6 fermentaciones en mosto sintético con la composición publicada por Riou (Riou et al., 1997) (Tabla S2- Cap VI) con dos cepas de levaduras

Saccharomyces (QA23 y RED FRUIT) y una no *Saccharomyces* (*Torulaspora delbrueckii*), con la finalidad de controlar la variabilidad biológica.

Las fermentaciones se realizaron siguiendo el mismo esquema explicado en el apartado 3.3. La toma de muestra adoptó el esquema de 2, 5 y 15 días (Figura 39).

Las muestras se tomaron en un volumen equivalente a 10^9 cel mL^{-1}; inmediatamente se centrifugaron a 4500 rpm y 4ºC por 3 minutos para separar las células del sobrenadante. El sobrenadante pasará a ser la muestra extracelular. Las células se lavaron y siguieron el mismo esquema de inactivación celular y de extracción que se ha detallado en el apartado 2.1.3 (Figuras 34 y 35).

Las muestras extracelulares se sometieron al sistema de extracción en fase sólida seguido de la evaporación con rotación al vacío, hasta la desecación. Se siguió el mismo esquema detallado en el apartado 2.1.1.

Las muestras desecadas se enviaron a la unidad de metabolómica de la Fundación Edmund Mach San Michele'all Adige (Trento-Italia), donde fueron analizadas bajo la supervisión de la Dra. Urska Vrhovsek, con la asistencia del Dr. Panagiotis Arapitsas.

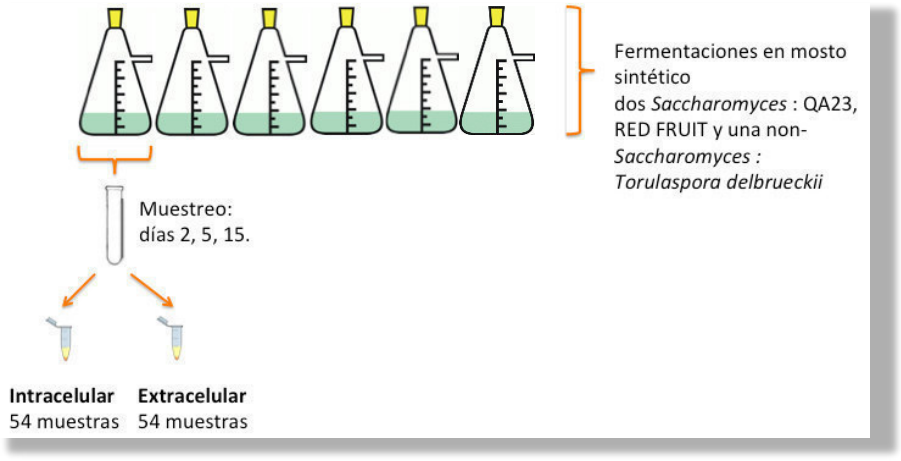


Figura 39. Esquema explicativo del diseño experimental correspondiente a la publicación 1 del Capítulo VI.

Las muestras fueron resuspendidas en una solución de nitrotirosina 9,37 ppm en metanol/agua 10% v/v que se utilizó como patrón interno. Las muestras

intracelulares se resuspendieron en un volumen tal que nos permitió concentrarlas cinco veces, mientras que las extracelulares se concentraron 3,33 veces.

2.2.2. Muestras destinadas a la optimización del método de extracción intracelular.

Se realizaron 6 fermentaciones alcohólicas en mosto sintético siguiendo el mismo diseño experimental que se ha indicado en los apartados 2.1.3 y 2.2.1. Utilizando la cepa de levadura *Saccharomyces cerevisiae* QA23 (Figura 40).

Las muestras se tomaron el día 2 de la fermentación en un volumen equivalente a 10^9 cel mL^{-1}. Se sometieron inmediatamente a centrifugación de 4500 rpm a 4ºC por 3 minutos para separar el medio extracelular (sobrenadante) de las células y, posteriormente, se lavaron dos veces con agua ultra pura con centrifugaciones a 4500 rpm a 4ºC por 3 min. Las células lavadas se sometieron a la inactivación celular mediante el método de glicerol frío descrito anteriormente en el apartado 2.1.3.

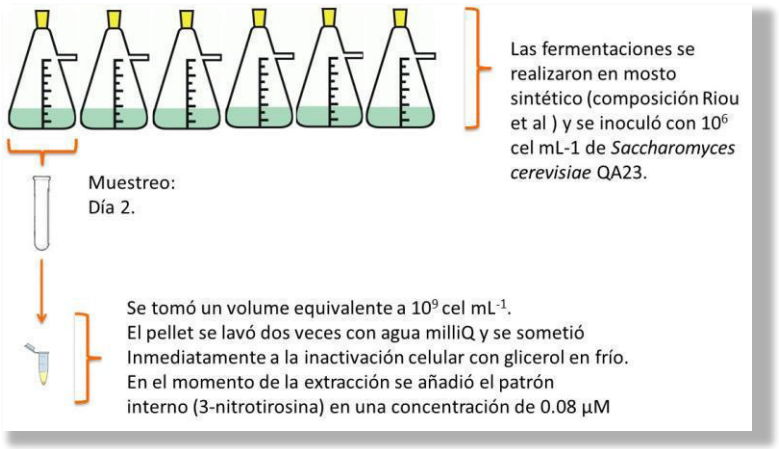


Figura 40. Esquema del diseño experimental del experimento correspondiente al Capítulo VI parte II

Las células tratadas fueron sometidas a extracción intracelular mediante la utilización de tres métodos diferentes:

- **Método de etanol en ebullición (H)** (Figura 41) (Gonzalez & Franc, 1997). Este método consiste en resuspender las células con 3 mL de una solución a 80ºC de etanol absoluto tamponado a una concentración de 70 mM con el ácido N-(2-hidroxietil) piperazina-N'-(2-etanosulfonico) (HEPES) y un volumen de solución del patrón interno (nitrotirosina) para alcanzar la concentración final de 0,08 µM. Posteriormente se encuba a 80ºC por 3 minutos para después enfriarlo en un baño de hielo durante 3 minutos. Una vez transcurrido este tiempo, la solución obtenida se evapora hasta la

sequedad con un evaporador rotativo al vacío a una temperatura de 34ºC y 2000 rpm. El residuo obtenido se resuspende en 3 mL de agua ultrapura (milliQ) y se centrifuga durante 10 minutos a 15.543 g y 4ºC. El sobrenadante es el extracto intracelular que se reserva a -80ºC hasta el análisis.

- **Método de ciclos de congelación/descongelación con 1 min de ultrasonido y centrifugación a -20ºC (LT)**.

  Este método se ha descrito en el apartado 3.3. En el momento en que las células se resuspenden con 2,5 mL de la solución de metanol en frío (~-30ºC) se añadió el volumen de la solución de nitrotirosina (patrón interno) suficiente para alcanzar la concentración final de 0,08 µM. El resto del proceso de extracción transcurrió de la forma que se ha indicado en el apartado 2.1.3.

- **Método de ciclos de congelación/descongelación con 1 min de ultrasonido y centrifugación a 4ºC (MT).**

  Este método se basa en el descrito en el apartado 3.3, con la modificación consistente en que las centrifugaciones se han realizado a 4ºC. Al igual que en el caso anterior, cuando las células se resuspenden con 2,5 mL de la solución de metanol en frío (~-30ºC) se añadió el volumen de la solución de nitrotirosina (patrón interno) en metanol suficiente para alcanzar la concentración final de 0,08 µM. El resto del proceso de extracción transcurrió de la forma que se ha indicado en el apartado 2.1.3.

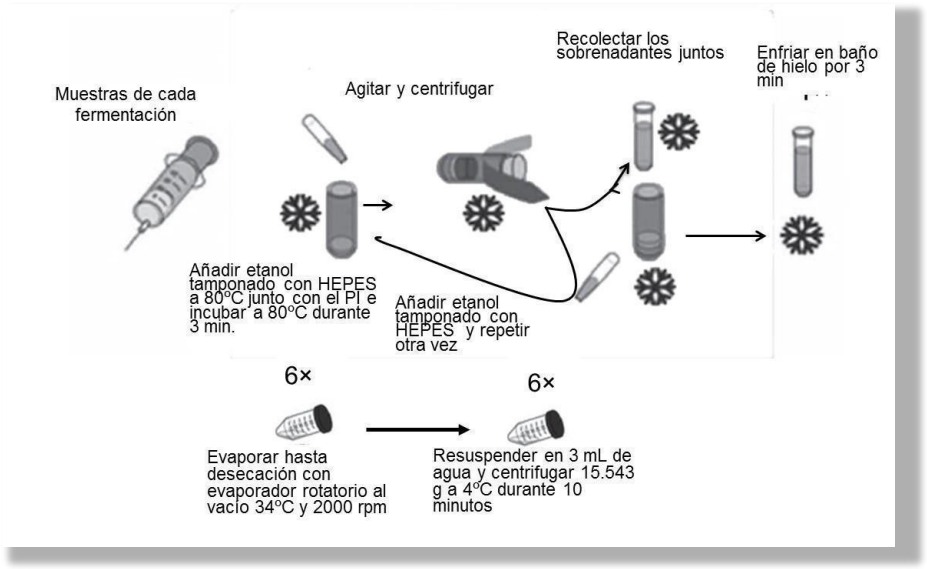


Figura 41. Esquema del proceso de extracción intracelular con etanol en ebullición. Basado en (VILLAS-BOAS, 2007)

Los extractos obtenidos se filtraron utilizando los cartuchos Phree®, con el fin de eliminar los fosfolípidos y las proteínas que provienen de la ruptura de la pared celular de las levaduras. Estos compuestos comúnmente pueden actuar como interferentes dificultando el análisis de las moléculas dianas. Los filtros Phree® filtros consisten en un relleno de circonio y sílice e incluye un primer paso de precipitación de proteínas con un disolvente orgánico. Posteriormente, los fosfolípidos son retenidos en el relleno mediante las interacciones entre el grupo fosfato de los fosfolípidos, que funcionan como una base de Lewis, y el óxido de zirconio que recubre la superficie de sílice, operando como un ácido de Lewis. Este método ofrece la ventaja de que evita las pérdidas de metabolitos que suelen producirse regularmente cuando se utilizan métodos como la extracción en fase sólida (SPE) (Reinholds, Pugajeva, Perkons, & Bartkevics, 2016).

Para la filtración se siguió el procedimiento recomendado por el fabricante que consiste en los siguientes pasos:

- Se carga de un volumen de los extractos intracelulares.
- Se añade el solvente orgánico que en este caso se trata de metanol/ácido fórmico 1% en una proporción 4:1.
- Se mezcla.
- Se procede a la filtración con vacío durante un tiempo de 5 minutos por lo menos.

La muestra filtrada se evapora hasta desecación con un evaporador rotatorio a 34ºC y 2000 rpm. El residuo obtenido se resuspende con un volumen de metanol/agua 10% acidificado con 0,1% de ácido fórmico para el análisis en modo positivo y con metanol/agua 10% acidificado con 0,1% de ácido acético para el análisis en negativo. El volumen en que se resuspende ha de ser suficiente para concentrar tres veces.

2.3. Reactivos.

Los reactivos utilizados en los experimentos de los Capítulos V y VI, así como sus procedencias están detallados en las Tabla 6., con la excepción del triptofol sulfonado que ha sido sintetizado por el Dr. Panagiotis Arapitsas (Arapitsas, Guella, & Mattivi, 2018).

2.4. Instrumentación.

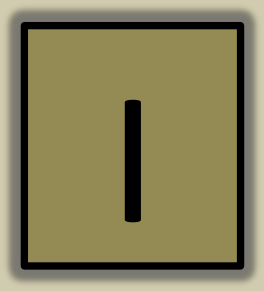
- Ultrasonido Sonoplus HD 2070, Bandelin electronic GmbH &Co. KG, Berlin, Alemania.

- Centrífuga refrigerada Sorvall LYNK 6000, ThermoFisher scientific, Waltham, MA-USA.
- Sistema de extracción de fase sólida al vacío Supelco- Visiprep.
- Cartuchos de extracción en fase sólida C18 – fase reversa- Variant-Agilent.
- Concentrador rotatorio al vacío (HyperVac-Lite, GYOZEN, Korea).
- Espectrómetro de masas de alta resolución que consiste en un sistema híbrido de un cuadrupolo segmentado para la selección de iones precursores con un analizador de masas Orbitrap® de alta resolución (QExactive) Thermo Fisher Scientific (Bremen, Germany), con fuentes de ionización ESI, APCI y nanoESI asociado a un cromatógrafo líquido de alta resolución (UHPLC) Thermo Fisher Scientific (Bremen, Germany).
- Filtros enroscables para la parte superior de frascos estériles desechables 0,2 µm Nalgene®. Thermo Fisher Scientific (Bremen, Germany).
- Espectrómetro de masas Xevo TQ-S que consiste en un triple cuadrupolo asociado a un cromatógrafo líquido de alta resolución (UHPLC) Waters Acquity (Milford, Massachusetts, USA).
- Centrífuga refrigerada modelo Allegra X-22R- Beckman Coulter.
- Contador de células automático Invitrogen Countess® Thermo Fisher Scientific (Bremen, Germany).
- Espectrofotómetro para microplacas (Synergy HT, Biotek®).
- Kits de Megazyme® para la medida de azúcares reductores, etanol y nitrógeno primario (PANOPA).

Tabla 6. Reactivos utilizados en los experimentos de los Capítulos V y VI.

Sigma-Aldrich SL Madrid, España		**Sigma-Aldrich Química SL Milán, Italia**	
Triptamina (TRYPT)	Etilen glicol	Ácido1-ciclohexano-1-carboxylico	Ácido fenil pirúvico (Ph-Py)
Ácido 3-indol acético (IAA)	Glucosa	2-Amino acetofenona (2AA)	Ácido picolínico
Ácido 3-indol butírico (IBA)	Fructosa	Ácido 2-hydroxy-fenyl acético	Ácido siquímico
Ácido 3-indolpirúvico (IPy)	Cloruro de calcio	Ácido 3-etil-indol carboxílico (E-I Ca)	Tiramina (TYRA)
Ácido 4-hidroxi-fenil acético(OH-Ph-AA)	Fosfato potásico monobásico	3-Hidroxi kinurenine (OH-KYN)	Tirosina (TYR)
5-Hidroxi-L-triptofano (OH-TRP)	Sulfato de potásio	Ácido 3-hydroxy-antranílico (OH-ANT)	Ácido fórmico (LC-MS grade)
5-Metoxitriptamina (5MOT)	Sulfato de magnesio.7H_2O	Éster etílico del ácido indol acético(IAA-EE)	Metanol (LC-MS grade)
5-Metoxi triptófano (CH_3O-TRP)	Cloruro de sodio	Ácido 3-indol láctico (ILA)	
Ácido 5-metoxi-3-indol acético (CH_3O-IAA)	Cloruro de amonio	Ácido 3-indol propiónico (IPA)	
5-Hidroxitriptofol (5-HTOL)	Cloruro de Co(II).6H_2O	3-Metoxi tiramina	**Cymit Quimica S.L., Barcelona, España.**
6-Hidroximelatonina (OH-MEL)	Sulfato de Cu(II).5H_2O	3-Metil-indol (CH_3-IND)	N-acetil-5-metoxi kynureanine hidrocloruro
DL-Kynurenine (KYN)	Ácido bórico	3-Nitrotirosina	Sulfatoxi melatonina (6-aMTs)
DL-Éster metílico del triptófano (TRP-ME)	Yoduro de potásico	Ácido 3,4-dihidroxi-3-metoxifenil propiónico	N-γ-acetil-N-2-formil-5-metoxi kynureamine
Ácido 5-hidroxi indol acético (5H-IAA)	Sulfato de manganeso	Ácido 3(2,4-dihidroxi) fenil propiónico	**Chengdu Biopurify Phytochemicals Ltd.**
Ácido kynurenico (KYNA)	Molibdato de amonio	Ácido 4-hidroxi-fenil pirúvico (OH-Ph-Py)	Hidroxitirosol (HT) standart 98%
L-Tirosina (TYR)	Sulfato de zinc H_2O	5-Metoxi triptofol	**Merck (Darmstadt, Alemania)**
L-Éster metílico de Tirosina (TYR-ME)	Cloruro de amonio	Indol 6-benziloxi-6-metoxi	Etanol grado HPLC
Melatonina (MEL)	L-prolina	Ácido abcísico	Acetonitrilo grado HPLC
N-acetil serotonina (N-SER)	L-glutamina	Ácido antranílico (ANT)	**Panreac (Barcelona, España)**
Éster etílico de N-acetil triptófano (N-TRP-EE)	L-arginina	Dopamina (DOPA)	Ácido fórmico grado HPLC
Éster etílico de N-acetil-L-tirosina (N-TYR-EE)	L-triptófano	Antranilato de etilo	**VWR International Eurolab S.L.**
Ácido fenil acético (Ph-AA)	L-alanina	Indol (IND)	Glicerol, ultrapuro 99.5%
Serotonina (SERO)	Ácido L-glutámico	Indol acetamida	
Triptófano (TRP)	L-Serina	Éster metílico del ácido indol acético (IAAME)	
Éster etílico del triptófano (TRP-EE)	Fenilalanina (PHE)	Indol carbinol (I3C)	
Triptofol (TOL)	Ácido fenil láctico (Ph-LA)	Indol carboxaldehido (ICA)	
Éster etílico de tirosina (TYR-EE)	L-Treonina	Ácido indol-2-carboxílico	
Tirosol (TYL)	L-Leucina	Indoxil sulfato	
Ácido N-(2-hidroxietil)piperazina-N'-(2-etanesulfónico)	Ácido L-aspártico	Ácido metil-indol acético (M-IAA)	
Ergosterol	L-Valina	Nicotinamida (NIC)	
Tween 80	L-Isoleucina	Ácido nicotínico	
Biotina	L-Histidina		
Pantotenato de calcio	L-Metionina		
Clorhidrato de piridoxina	L-Tirosina		
Clorhidrato de tiamina	L-Glicina		
Mioinositol	L-Lisina		
Ácido nicotínico	Ácido oleico		

CAPÍTULO PRIMERO



Effects of the strawberry (*Fragaria ananassa*) purée elaboration process on non-anthocyanin phenolic composition and antioxidant activity

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ABSTRACT

Strawberries are harvested in a short period of time frequently involving fruit surplus. This paper studies the impact of the strawberry purée elaboration process on the chemical composition of the final products. Thirty-two phenolic compounds were studied by Liquid Chromatography with Diode Array Detector (LC- DAD) and Mass Spectrometry (LC-MS). An LC-DAD method was set up and validated and the non-antho- cyanin phenolic profile was quantified at the different steps of production, for three elaboration processes and two harvests (2011 and 2012). We have tentatively identified apigenin-7-O-glucoside, luteolin-3-O- glucuronide, malonyl caffeoylquinic acid, trans-resveratrol glucoside and caffeoylglucaric isomer. (+)-Cat- echin and HHDP-galloylglucose were the most abundant phenolic compounds. The most abundant flavo-nol was kaempferol-3-glucoside. The purée maintains the fruit's non-anthocyanin phenolic composition and in vitro antioxidant activity as determined by ORAC and DPPH methods. This fact suggests that strawberry purée could be considered a valuable ingredient for producing food derivatives

Key words: Polyphenols; Validation; Liquid chromatography; Mass Spectrometry; Strawberry; Antioxidant activity.

1. INTRODUCTION

Spain (Huelva, Andalucía) is the fourth biggest strawberry (*Fragaria x ananassa*) production area in the world (FAOSTAT. 2012) . Strawberries are harvested in a very short period of time and a large amount of fruit is collected. Overproduction causes a decrease in prices and, what is more, the fruit rots and is discarded if not sold. As the strawberry is a highly perishable product, it spoils quickly, which leads to substantial economic losses.

Consequently, manufacturing derived products using strawber- ries as a raw material or ingredient drives economic profits, increases the conservation period and offers new sustainable and successful alternatives for strawberry consumption. In fact, the production of strawberry purée as an ingredient for yoghurts, mar- malades, jams, biscuit production, etc., already represents a worth- while economic solution. Strawberries are a very rich source of antioxidant compounds including vitamins C, E, b-carotene, melatonin and phenolic compounds (Oszmianski and Wojdylo, 2009; Stürtz, Cerezo, Cantos and García-Parrilla, 2011; Cerezo, Cuevas, Winterhalter, García-Parrilla and Troncoso, 2010). Among the bioactives, phenolic compounds are one of the main groups of phytochemicals present in strawberries that strongly influence quality, contributing to sensorial-organoleptic attributes and health properties (Larrosa, Tomás-Barberán, Espín, 2006; Buendia et al., 2010). The main polyphenol compounds described in strawberries are anthocyanins, flavan-3-ols, ellagitanins, glycosides of quercetin and kaempferol (Määtta, Kamal-Eldin, Kaisu and Törronen, 2004; Aaby, Mazur, Nes and Skrede, 2012).

Conversely, the phenolic profile of strawberry purée or juice, used as a raw material for further derivative products (jam, yoghurt, vinegar, syrup, etc.) has scarcely been reported in previous studies (Oszmianski et al., 2009; Hartmann, Patz, Andlauer,

70 Dietrich and Ludwig, 2008; Truchado, et al., 2012; Bodelón, Avizcuri, Fernández-
71 Zurbano, Dizy and Préstamo, 2013).
72 Treatments involved in manufacturing by-products (thermal, mechanical, etc.) are
73 known to affect polyphenolic composition (Truchado et al., 2012). Therefore, this paper
74 aims to establish the impact of industrial processing on the non-anthocyanin phenolic
75 composition and antioxidant activity of strawberry purées, by analyzing their evolution
76 throughout the production process as well as in the different final products.
77 For this purpose, an analytical Liquid Chromatography with Diode Array Detector (LC-
78 DAD) method was set up and validated, with complete identification confirmed by
79 Mass Spectrometry.
80
81 **2. MATERIALS AND METHODS**
82
83 **2.1. Chemicals**
84 The phenolic standard compounds were purchased from Fluka Sigma-Aldrich (St.
85 Louis, MO. USA) [gallic acid, caffeic acid, *p*-coumaric acid, cinnamic acid, (-)-
86 epicatechin, (+)-catechin, chlorogenic acid, ellagic acid, (-)-epicatechin gallate,
87 kaempferol, kaempferol-3-O-ß-D-glucuronide, kaempferol-3-glucoside, *trans*-
88 resveratrol, apigenin and penta-O-galloyl-ß-D-glucose hydrate] and from Chromadex®
89 Inc. (USA) [procyanidin B1]. Luteolin and apigenin-7-O-glucoside from Extrasynthese
90 (Z.I. Lyon Nord. France) DPPH (2,2-diphenyl-1-picrylhydrazyl), AAPH (2,2´-diazo-
91 bis-amidine-propane-dihydrochloride) and trolox (6-hydroxy-2,5,7,8-
92 tetramethylchroman-2-carboxylic acid) were obtained from Sigma-Aldrich (St. Louis,
93 MO. USA); acetonitrile and acetic acid (LC gradient) from Merck (Darmstadt,
94 Germany), and methanol from Prolabo® (Obregón, Mexico).

2.2. Samples

The samples used for this study were strawberry purées, provided by Hudisa Company (Lepe, Huelva, Spain). Two harvests were analyzed (2011 and 2012). Briefly, the purée elaboration process starts when the fruit is received. It is selected, cleaned and the green parts are removed. Crucial steps likely to affect the bioactive compounds of the products are mashing the flesh, enzymatic inactivation (2 min, 55 ºC – 65 ºC) and the pasteurization process (3 min, > 90 ºC). After that, the temperature is reduced to 5 ºC in a few minutes. In order to separate the flesh from the seeds, the mash is sieved, resulting in purées with or without seeds, according to the market they are destined for. Additionally, a seedless purée sample (from the 2012 harvest) was left unpasteurized.. Twenty-five samples were analyzed (12 from the 2011 harvest and 13 from 2012 harvest). They were collected at the following steps: i) mashed step (6 samples M_A, M_B, M_C); ii) enzymatic inactivation step (6 samples EI_A, EI_B, EI_C); iii) unpasteurized step (3 samples UP_A, UP_B, UP_C), only for 2012 harvest; and iv) final products consisting of purées obtained just after the pasteurization process and aseptic packaging, with seeds (5 samples FPS_A, FPS_B, FPS_C) and final products without seeds (5 samples $FPWS_A$, $FPWS_B$, $FPWS_C$). Each subscript $_{A, B, C}$ indicates samples of the same substrate. We used mashed samples as control as no thermal treatment was applied.

2.3. Sample preparation

A total of 30 g of sample was extracted in 30 mL of methanol by sonication for 30 min. Ascorbic acid 1 % (w/w) was previously added to prevent sample oxidation. It was immediately centrifuged for 10 min at 1.500 g using a Sorvall® TC Dupont Centrifuge. The supernatant was collected and the pellet re-extracted with methanol (30 mL). The

120 methanol fractions were mixed and evaporated to dryness at 37 ºC with a rotary
121 evaporator (Büchi Rotavapor®, R-200/205). The extract was reconstituted in 5 mL of
122 water/methanol 1:1 (v/v) and stored at -18 ºC until analysis. Extractions were performed
123 in duplicate. Extraction efficiency was evaluated with a solution of vainillin (46 mg/L)
124 as the internal standard. Recovery was 81.64 ± 0.40 % in accordance with the AOAC
125 requirements. The calibration data used was $y = 138.08x + 29.313$, r^2 0.999.
126 The same sample preparation process as described in this section, except for the
127 addition of ascorbic acid, was used to determine antioxidant activity.
128
129 **2.4. Chromatographic separation**
130 Chromatographic separation was performed using an LC Agilent Series 1100 system
131 equipped with a quaternary pump (Series 1100 G1311A), automatic injector (Series
132 1100 G1313A) and degasser (series 1100 G1379A). Detection was carried out using a
133 UV/Vis diode detector (Series 1100 G1379A) coupled to a Chemstation HP A.10.02
134 (HP/Agilent). The column was a Merck LiChroCART 250-4 Superspher 100 RP-18
135 1.16056.0001. The method used a binary gradient, A (glacial acetic acid/water, pH 2.65)
136 and B (20 % A + 80 % acetonitrile), programmed in the following gradients: 0 min, 100
137 % A; 5min, 98 % A + 2 % B; 10 min, 96 % A + 4 % B; 15 min, 90 % A + 10 % B; 20
138 min, 87 % A + 13 % B; 35 min, 80 % A+ 20 % B; 40 min, 70 % A + 30 % B; 45 min,
139 60 % A + 40 % B; 50 min, 100 % B; 55 min, 100 % A; 70 min, 100 % A. The flow rate
140 was 1.5 mL min^{-1}, the injection volume was 50 µL, and the temperature was set at 40
141 ºC. Each sample was analyzed twice. Identification was achieved by matching the
142 retention time and spectra of the peaks with standards. Additionally, samples were
143 spiked with standards, if they were commercially available, to achieve complete
144 identification. Quantification was performed by external calibration at 280 nm, 320 nm

and 365 nm, in accordance with the maximum absorbance of each compound, using their corresponding standards. Calibration curves were obtained by injecting standards diluted from five to eight different concentrations (R^2: 0.9949 - 0.9998). In the event of overlapping signals, either peak area or peak height was determined. A triplicate was performed at each point of the calibration curve.

The identification procedure considered that peaks showing DAD spectra similar to a phenolic compound standard but with different retention times were assigned to derivatives. Esterification with sugar causes a bathochromic shift of the maximum compared to that of the corresponding aglycone (Buendia et al., 2010). Additionally, monogalloyl glucoside, ellagic acid glycosides, ferulic acid hexoside derivative, caffeic acid hexoside, *p*-coumaroyl glucoside were quantified, assuming that they present the equal molar absorptivity to their counterpart compounds: penta-O-galloyl-β-D-glucoside, ellagic acid, caffeic acid, ferulic acid and *p*-coumaric-acid respectively. HHDP-galloylglucose was quantified assuming the same absorptivity as ellagic acid. The results were expressed as mg kg^{-1} of fresh weight (fw).

2.5. ESI-MS and MS/MS analysis

Additionally, identification was achieved by ESI-MS and MS/MS under the following conditions: MS/MS experiments performed on 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. The sample extracts were dissolved 0.1 % (v/v) with methanol:water 50 % (v/v) and analyzed in negative mode. The mass spectrometer was set to the following optimized tune parameters: curtain gas 20 psi, ion spray voltage -4500 V, source gas 20 psi.

For LC-ESI-MRM analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, ion spray voltage 4500 V, source temperature 350 ºC and source gas 60 psi. A dwell time was set at 50 ms for each transition.

Conditions of LC/MS/MS were the same as described above for LC-DAD, except flow rate was 0.2 mL min^{-1} and injection volume was 20 µL.

2.6. LC-DAD Validation Procedure

The LC-DAD method was validated to comply with the requirements of the Association of Analytical Communities (AOAC) (AOAC, 1993; González and Herrador, 2007): linearity, limits of detection and quantification, precision, and reproducibility. The limit of detection (LOD) and limit of quantification (LOQ) for each phenolic compound was calculated as the amount of compound required to produce a signal to noise ratio of 3:1 and 10:1, respectively. Selectivity is the degree to which a method can quantify the analyte accurately, in the presence of interferences, under the assay conditions for the sample matrix being studied. The recovery of the sample extraction procedure was calculated through standard addition and expressed as a percentage.

2.7. Antioxidant activity

2.7.1. ORAC test

The procedure was based on a previously reported method with slight modifications (Ou et al., 2001): 50 µL of sample or Trolox was mixed with 100 µL of fluorescein (45 nM) and 50µL of AAPH (15 mM). Florescence was recorded for 80 min (excitation wavelength was set at 485 nm and 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).

195 Fluorescein fluorescence was recorded every 5 min after addition of AAPH, until
196 fluorescence was less than 5 % of the initial reading. Final results were calculated using
197 the areas under the fluorescein decay curves, between the blank and the sample, and
198 were expressed as mmols Trolox equivalents (TE) per kg of fresh weight.
199
200 **2.7.2. DPPH method**
201 The effect of antioxidant activity on DPPH was estimated according to the procedure
202 described by Villaño, Fernández-Pachón, Moyá, Troncoso and García-Parrilla, 2007. A
203 total of 0.1 mL of sample in methanol was added to 3.9 mL of DPPH methanolic
204 solution (0.025 gL^{-1}). Absorbance at 515 nm was recorded at the start (when the sample
205 was added) and 60 min later, when the reaction reached equilibrium. We used methanol
206 as a reference. All measurements were performed in triplicate. Eight different
207 concentrations (0.9 - 0.12 mM) of Trolox were used to make the calibration curve.
208 Absorbance measurements were recorded on a Hitachi UV-2800 spectrophotometer,
209 thermostated with a Peltier system at 25 ºC.
210
211 **2.8. Statistical analysis**
212 Statistical analyses were performed by means of statistical software (Statsoft, 2001).
213 One-way analysis of variance (ANOVA) was used to test significant differences.
214
215 **3. RESULTS AND DISCUSSION**
216
217 **3.1. Method validation-calibration curve**

Table 1 displays the data for the validation method parameters, which comply with the AOAC requirements for linearity, precision, recoveries, repeatability, LOD and LOQ. The method used obtained reliable results.

3.2. Identification of phenolic compounds

A total of 32 phenolic compounds were identified through their LC elution order, UV/Vis, mass spectrometric characteristics and compared with data reported in the literature (Table 2).

3.2.1. Hydrolyzed Tannins

Peak 2 showed a $[M-H]^-$ molecular ion at *m/z* 331, and MS-MS fragments at *m/z* 271 and 169, which confirms monogalloyl glucose identification (Hanhineva et al., 2008; Sandhu and Gu, 2010). Additionally, its UV spectra were similar to those of the tannin compounds family (penta-O-galloyl-ß-D-glucoside standard). Further to this, HHDP-galloylglucoside, galloyl-bis-HHDP-glucose and bis-HHDP-glucose were identified through matching with the three deprotonated molecules $[M-H]^-$ at *m/z* 633, 934 and 783 respectively, as has previously been described in the literature (Aaby et al., 2012; Sandhu et al, 2010; Aaby, Ekeberg and Skrede, 2007a; Seeram, Lee, Scheuller and Herber, 2006). Figure 2-A shows the hypothesized structure and fragmentation of HHDP-galloylglucoside.

Another ellagitanin was identified as tris-galloyl-HHDP-hexoside (peak 7) having a $[M-H]^-$ at *m/z* 951. The fragmentation produced *m/z* 907 (loss of carboxilic group, 44 Da), *m/z* 783 (loss of gallic acid unit), *m/z* 463 (loss of tris-galloyl group, 507 Da), *m/z* 605 (deriving from *m/z* 907 though the loss HHDP unit, 302 Da) and *m/z* 301 (obtained from *m/z* 463 through the loss of a hexose unit). The product ion of *m/z* 301 was 201,

consistent with ellagic acid (Figure 2-B) (Del Bubba et al., 2012). Additionally, at 23.6 min, peak 3 showed $[M-H]^-$ at *m/z* 481 and its fragmentation produced *m/z* 301 after loss of a glucose unit (180 Da). Figure 2-C displays the fragmentation of compound identified as HHDP-glucose. This tannin has been reported before in strawberry sepals, by Hanhieva et al. (2008).

3.2.2. Flavanols and Condensed Tannins

The LC-DAD and MS analysis confirmed the presence of gallic acid, (+)-catechin, (-)-epicatechin gallate, (-)-epicatechin, procyanidin dimer and trimer (Table 2).

3.2.3. Ellagic acid and derivatives

Free ellagic acid (peak 27) was identified by its retention time, UV-visible spectrum and characteristic MS spectral data in accordance with those of the authentic standard. The MS analyses confirmed the presence of a peak at *m/z* 301, and main MS-MS fragments at *m/z* 284 and 145, consistent with ellagic acid. Additionally, peaks 9 and 23 were identified as ellagic acid derivatives, based on their similar UV-visible spectrum and their MS characteristics. Both peaks showed precursor ions at *m/z* 433 and *m/z* 447, respectively, and product ions at *m/z* 301, corresponding to ellagic acid pentoside and ellagic acid deoxyhexoside, respectively, in accordance with data reported by Aaby et al. (2012).

3.2.4. Hydroxycinnamic acids

The MS analyses confirmed the presence of *p*-coumaroylhexoside at *m/z* 325, *p*-coumaric acid at *m/z* 163, caffeic acid hexoside at *m/z* 341 and ferulic acid hexose derivative at *m/z* 449, previously reported in strawberries (Aaby et al., 2007a; Fang, Yu

and Prior, 2002; Ornelas et al., 2013). Two compounds were tentatively identified as malonyl caffeoyl quinic acid at *m/z* 439 and caffeoylglucaric isomer at *m/z* 371(Ruiz A.et al. 2013) . Due to their low concentration, it was possible to obtain only the main ion fragments of each compound: *m/z* 395 and *m/z* 209, respectively. Malonyl caffeoyl quinic acid has already been reported in blueberries, red and black currants and *Erigeron breviscapus* (Gavrilova, Kajdzanoska, Gjamovski and Stefova, 2011; Zhang, Shi, Qu and Cheng et al., 2007) and caffeoylglutaric isomer in *Berberis microphylla G. Forst* (Ruiz et al., 2013), but not in strawberries.

3.2.5. Flavonol glycosides

Kaempferol conjugates were identified as follows: kaempferol-O-coumaroyl hexoside at *m/z* 593, kaempferol-3-glucoside at *m/z* 447, and kaempferol malonylglucoside at *m/z* 489. All of them produced the same aglycone cation at *m/z* 285, in accordance with data reported before by Aaby et al., (2012, 2007a). The occurrence of kaempferol aglycon in berry fruits is infrequent. It has already been described in Finnish strawberries (Määtta et al., 2004; Ornelas et al., 2013; Häkkinen, Kärelamp, Heinonen, Mykka and Törönen, 1999), but not in varieties harvested in Spain.

Quercetin-3-glucuronide and quercetin rutinoside were also identified in our samples, showing molecular ion at *m/z* 477 (MS^2 fragment at *m/z* 301) and at *m/z* 609 (MS^2 fragments at *m/z* 301, 179 and 151), respectively, which is consistent with the literature (Aaby et al., 2007a; Seeram et al., 2006).

Apigenin-7-O-glucose and luteolin 3′-O-glucuronide were identified with authentic standards through their molecular ions $[M-H]^-$ at *m/z* 431 and *m/z* 461, respectively, and MS^2 fragments at *m/z* 269, 225 and 311 for apigenin-7-O-glucoside; *m/z* 285 and 241 for luteolin 3-O-glucuronide. Figure 2-E shows hypothetic fragment pattern. These

293 compounds have been reported before in rosemary, oregano, sage, basil and thyme
294 (Gouveia and Castilho, 2009; Hossain, Rai, Brunton, Martin-Diana and Barry-Ryan,
295 2010). Nevertheless apigenin derivatives were reported in strawberries by Ornelas et al
296 2013; and luteolin aglycone by M. Kadivec, S. M Bornsek, T. Polak, L. Demsar, J.
297 Hribar, T. Pozrl., 2013. However, as far as we know, this is the first time apigenin-7-O-
298 glucose and luteolin-3-O-glucuronide have been reported in strawberries. Figure 2-D
299 shows the hypothesized structure and fragmentation of apigenin-7-O-glucose.
300
301 *3.2.6. Stilbenes*
302 *Trans*-resveratrol glucoside, at *m/z* 389, was identified for the first time in these
303 samples, according to the authentic standard (Figure 2-F). Although small quantities of
304 *trans*-resveratrol have been found in strawberries (Ehala, Vaher and Kaljurand, 2005),
305 *trans*-resveratrol glucoside has not been identified in strawberries before.
306 To sum up, thirty-two phenolic compounds were identified in the strawberry extract.
307 Malonyl caffeoylquinic acid, caffeoylglucaric isomer, trans-resveratrol-glucoside,
308 apigenin-7-O-glucose and luteolin-3-O-glucuronide were reported for the first time in
309 strawberries.
310
311 *3.3. Non-anthocyanin phenolic composition of strawberry products*
312 Tables 3 summarize the concentrations of phenolic compounds in the strawberry purée
313 samples (2011 and 2012 harvests) during the different production steps and for the final
314 products. A total of eighteen compounds were quantified.
315 Considering our strawberry purée samples, the content of free and conjugated forms of
316 gallic acid (Tables 4-5) are similar to those already reported for the Camarosa variety
317 (132 mg kg^{-1} fw), which is the most harvested variety in Spain (Buendia et al., 2010).

318 Conversely, the content of the *Jonsok* variety is higher than in our results (333 mg kg^{-1}
319 fw) (Määtta et al., 2004).
320 The most abundant non-anthocyanic compounds quantified were (+)-catechin and
321 HHDP-galloylglucoside (182.8 – 114.3 mg kg^{-1} fw in the final product). This last one
322 has been reported before, as a major class of phenolic compounds in strawberries
323 (Aaby, Ekeberg, & et al., 2007). Indeed, we found a remarkably high flavanol content,
324 especially for (+)-catechin (123.7 – 211.8 mg kg^{-1} fw and 40.1 – 227.4 mg kg^{-1} fw in
325 the final products, 2011 and 2012 harvests, respectively), compared with reported
326 values: 4.9 - 5.8 mg kg^{-1} fw (Oszmianski & Wojdylo, 2009), 24 mg kg^{-1} fw (Määtta et
327 al., 2004), and 25 - 81 mg kg^{-1} fw (Aaby, Ekeberg, & et al., 2007).
328 Furthermore, other flavanols, such as (-)-epicatechin, (-)-epicatechin gallate and the
329 procyanidin B1, were also quantified (2.9 - 42 mg kg^{-1} fw; 9 - 45 mg kg^{-1} fw and 10 -
330 45 mg kg^{-1} fw, respectively).*p*-Coumaroyl hexoside and caffeic acid hexoside, were the
331 most abundant hydroxycinnamic acids (16.0 - 40.8 mg kg^{-1} and 48.2 - 38.5 mg $kg^{-}1$,
332 respectively, in final products).
333 The predominant flavonols in these samples were kaempferol and derivatives, in
334 contrast to data reported by other authors (Aaby, Ekeberg, & et al., 2007; Buendia et al.,
335 2010; Määtta et al., 2004; Da Silva Pinto, Lajolo, & Genovese, 2008; Del Bubba et al.,
336 2012), who found quercetin derivatives to be the main flavonol in strawberries.
337
338 *3.4. Changes in non-anthocyanin phenolic composition and antioxidant activity during*
339 *the purée elaboration process*
340 Gallic acid, monogalloylglucoside and ellagic acid decreased significantly due to
341 enzymatic inactivation ($p<0.05$) in the 2011 harvest results (Table 3). Indeed, their
342 concentrations were reduced from 10 to 68 % in respect of their content in mashed

343 samples. This is in accordance with data obtained after strawberry jam processing with
344 heat, which led to a significant decrease in total ellagic acid (83 – 25 %) (Bakkalbaşi,
345 Menteş & Artik, 2009). The mashing process causes membrane breakage, which means
346 that oxidation reactions occur more easily when heat is applied (Hartmann et al., 2008).
347 However, while gallic acid also dropped significantly in the 2012 harvest results (Table
348 3), ellagic acid remained generally unchanged, possibly underestimated due to its low
349 solubility (Aaby et al., 2012). HHDP-galloylglucoside decreased between the mashed
350 and the final product, following the same behavior in both harvests.
351 Significant changes were observed in the pasteurization process. Between the mashed
352 step and the final product (in the case of (+)-catechin the reduction was 42 - 20 % in
353 2011 and 40 - 38.6 % in 2012), most of the compounds decreased in concentration, as
354 was also reported by Hartmann et al., (2008) in strawberry juices and purées.
355 Regardless of whether the purée is processed with or without seeds, there is no
356 significant effect on the non-anthocyanic phenolic composition of the resulting purée.
357 The presence of seeds does not increase the concentration of these bioactives. This fact
358 is probably due to the seeds not being fully crushed during the process. When the seeds
359 are removed (FPWS), there is a concentration effect and higher values are produced, in
360 contrast with FPS. This is in accordance with the higher content of total
361 hydroxycinnamic compounds in FPWS when compared to FPS (21.45 - 11.97 mg kg^{-1}
362 fw in 2011 and 14.9 - 10.46 mg kg^{-1} fw in 2012). It corresponds to a reduction from
363 30.54 % to 12.6 % between the 2011 and 2012 harvests, in accordance with Aaby,
364 Wrolstad, Ekeberg and Skrede (2007).
365 Table 4 displays antioxidant activity data for the purée at the different stages.
366 Antioxidant activity decreased just slightly during the pasteurization step and no
367 significant changes were detected at any point in the process. Despite the effect of

368 processing on antioxidant activity, the final products are an excellent source of bioactive
369 substances, with antioxidant potential for further use as a raw material and ingredient in
370 derived products.
371
372 **3.5. Conclusions**
373 An analytical method to extract and determine non-anthocyanic phenolic compounds
374 from strawberries has been suitably validated. This paper reports the tentative
375 identification of malonyl caffeoylquinic acid and caffeoylglucaric isomer. Reveal the
376 identification of *trans*-resveratrol glucoside, apigenin-7-O-glucoside and luteolin-3-O-
377 glucuronide, previosly described in other sources but not in strawberries.
378 These results show that industrial processing does not break the seeds to release the
379 non-anthocyanic phenolic compounds, as may be expected, and purées with or without
380 seeds present a similar composition. The industrial process does not significantly affect
381 the non-anthocyanin phenolic profile (only *trans*-resveratrol glucoside disappears in
382 final products), and strawberry purées are a good source of phenolic compounds with
383 antioxidant potential, being of interest from a nutritional and commercial perspective as
384 ingredients or raw material for further derived food.
385
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Table 1. Validation results.

Compounds	Selectivity (R_s)	Recoveries		Intermediate precision		Repeatability		Sensitivity	
		Conc. (mg L^{-1})	%	Conc. (mg L^{-1})	CV (%)	Conc. (mg L^{-1})	CV (%)	LOD (mg kg^{-1} fw)	LOQ (mg kg^{-1} fw)
Gallic acid	7.27	110	89.41	11.55	4.19	1.44	4.68	3.03	11.6
		13.5	115.57	1.44	9.56	11.55	1.74		
Penta-Galloyl-β-D-glucoside	nd	21.25	99.37	85.60	2.02	10.7	2.15	1.39	1.69
		10.62	101.87	10.70	4.56	85.6	0.96		
(+)-Catechin	10.16	110	110.83	55.80	1. 99	6.97	9.29	11.87	37.01
		27.5	110.45	6.97	4.96	55.8	2.34		
(-)-Epicatechin	14.02	20	94.43	126.15	6.80	15.77	4.77	0.45	1.33
		10	103.65	15.79	4.91	126,15	2,83		
(-)-Epicatechin gallate	2.69	56	99.37	56.65	5.38	7.08	6.07	0.72	1.16
		8	97.11	7.08	6.41	56.65	2.96		
4-OH-benzoic acid	nd	110	90.50	110.00	6.49	13.75	7.02	nd	nd
		27.5	81.68	13.75	3.63	110	3.55		
Procyanidin B_1	1.42	80	92.9	79.80	6.70	9.975	3.15	0.17	1.51
		40	113.14	9.97	4.98	79.8	6.35		
Cinnamic acid	nd	8	99.9	12.60	3.73	2.1	7.09	0.047	0.133
		2	99.2	3.07	4.31	12.6	3.15		
Ferulic acid	nd	56	86.1	58.05	5.63	7.256	6.13	nd	nd
		14	91.4	7.26	3.72	58.05	9.25		
Chlorogenic acid	0.032	24	80,86	25.65	3.43	3.21	3.16	0.028	0.038
		6	110,39	3.21	4.75	25.65	2.67		
Caffeic acid	nd	56	101.8	56.73	3.42	7.09	4.44	0.095	0.10
		28	99.5	7.09	4.73	56.73	6.50		
***p*-Coumaric acid**	nd	30	104.5	30.33	3.98	3.79	6.13	0.008	0.053
		15	82.6	3.79	4.36	30.33	6.81		
Quercetin	nd	40	92.6	39.97	6.75	5.00	5.82	nd	nd
		20	95.5	4.99	2.41	39.97	1.17		
Kaempferol	nd	40	78.7	40.60	3.10	5.07	0.49	0.468	0.76
		20	77.3	5.07	1.90	40.6	3.46		
Ellagic acid	1.05	40	74.8	60.06	2.33	10.01	4.01	2.82	6.70
		20	81.8	10.01	5.12	60.06	6.95		

nd. "No data". Selectivity for monogalloyl glucoside, malonyl cafeoilquinic acid and kaempferol glucoside were 6.64, 3.95 and 14.24 respectively. Validations samples were determined in triplicate.

Table 2. Characterization of phenolic compounds in strawberry purée extracts using LC with diode array and electrospray ionization MS detection.

Peak	Tentative identification	Rt (min)	λmax (nm)	MW	MS (*m/z*); ID	MS^2 ions (*m/z*)	Detection	Reference
Hydroxybenzoic								
1	Gallic acid	9.5	275	170	169	**125,** 78	DAD	*
Hydrolized Tanins								
3	HHDP-glucose	23.6		482	481	**301**; 275; 249	MS	Hanhineva et al. (2008)
5	Bis-HHDP-glucose	24.5		784	783	481**; 301**	MS	Aaby et al. (2007a)
6	Monogalloyl glucose	26.6	280	332	331	**169**, 125	MS	Sandhu and Gu (2010); Hanhineva et al. (2008)
7	Tris-galloyl-HHDP-hexose	27.2		952	951	**907**; 783; 605; 463; 301; 201	MS	Bubba et al. (2008)
8	HHDP-galloylglucose	27.3		634	633	463; 481; **301**; 275	DAD-MS	Aaby et al. (2007a)
17	Galloyl-bis-HHDP-glucose	33.5		935	934	**633**; 301	MS	Aaby et al. (2007a)
Ellagic acid and derivates								
9	Ellagic acid pentoside	27.8	252;375	434	433	301	MS	Aaby et al. (2012)
23	Ellagic acid deoxyhexoside	39.4	254;370	448	447	**300**; 257	MS	Aaby et al. (2012)
27	Ellagic acid	45.0	257;358	302	301	284; 145	DAD-MS	*
Flavanols								
2	(+)-Catechin	23.0	280	290	289	245; 109	DAD-MS	*
4	(-)-Epicatechin	24.0	282	290	289	245; 109	DAD-MS	*
13	(-)-Epicatechin gallate	30.6	280	444	443	289; 169	DAD-MS	*
Condensed Tannins								
10	Procyanidin B1	28.3	280	-	577	451; 425; 407; **289**	DAD-MS	*
12	Procyanidin trimer	29.1		866	865	739; **695**; **577**; 408	MS	Aaby et al. (2007a)
Flavonols								
15	Apigenin-7-O-glucoside	32.6	-	432	431	311; **269**; 225; 270	MS	*
21	Kaempferol-3-glucoside	34.4	268;348	448	447	**285**; 257	DAD-MS	*
22	Quercetin rutinoside	38.0	-	610	609	**301**; 179; 151	MS	Seeram et al. (2006)
25	Kaempferol –O-coumaroylhexoside	41.4	-	594	593	307, **285**	DAD-MS	Bubba et al. (2008)
26	Quercetin-3-glucuronide	42.0	-	478	477	**301**; 151; 179	MS	Aaby et al. (2007a)

Table 2. Continue.

Peak	Tentative identification	Rt (min)	λmax (nm)	MW	MS (*m/z*); ID	MS^2 ions (*m/z*)	Detection	Reference
Flavonols								
28	Quercetin-O-hexoside	42.3		464	463	**300**; 271; 255; 179	MS	Gouveia et al. (2011)
29	Luteolin 3′-O-glucuronide	45.8	-	462	461	**285**; 241	MS	*
30	Kaempferol	46.7	270;375	286	285	117; 93	DAD-MS	*
31	Kaempferol malonylglucoside	47.3	-	490	489	**285**; 257	MS	Ornelas-Paz et al. (2013)
Hydroxycinnamic acid								
11	Caffeic acid hexoside	28.9		342	341	179; **161**; 135	DAD-MS	Hanhineva et al. (2008)
14	*p*-Coumaroyl hexose	32.4	311	326	325	163; **145**	DAD-MS	Aaby et al. (2007a)
16	*p*-Coumaric acid	33.3		164	163	**119**, 93	DAD-MS	*
18	Malonyl caffeoylquinic acid	33.7	323	396	439	395	DAD-MS	Gravilova et al. (2011)
19	Ferulic acid hexose derivative	34.0		450	449	**269**, 287, 193	DAD-MS	Ornelas-Paz et al. (2013)
24	Cinnamic acid	40.0	282	148	147	103, 77	DAD-MS	*
32	Caffeoylglucaric isomer	48.1	-	372	371	209	MS	Ruiz et al. (2013)
Stilbenes								
20	trans-resveratrol glucoside	34.3	313	390	389	**227**, 185	DAD-MS	*

*Identification of the compound was confirmed by the authentic standards.

Table 3. Concentrations (mg kg^{-1} of fresh weight) of non-anthocyanin phenolic compounds in strawberries purées. 2011 and 2012 harvest.

Compounds	**M_{11A}**	**M_{11B}**	**M_{11C}**	**EI_{11A}**	**EI_{11B}**	**EI_{11C}**
Gallic acid	34.04±2.0^{bd}	28.50 ±6.19^{bd}	27.2±1.3^{b}	27.1± 2.8^{ad}	16.3±2.7^{ad}	25.3±0.4^{ad}
Monogalloyl glucoside	45.26±1.17^{bde}	36.2±2.7^{de}	31.9±0.5^{bde}	25.4± 1.4^{ade}	15.7±1.7^{de}	20.1±1.4^{ade}
Ellagic acid pentoside	13.9±1.6^{de}	11.5± 0.8^{e}	10.9± 0.9^{de}	nd	nd	nd
Ellagic acid	59.2±6.8^{bde}	59.1±7.5^{be}	53.3±6.9^{be}	23.0± 3.3^{ae}	19.5±6.3^{ade}	9.6±1.9^{ae}
HHDP-galloylglucoside	150.6±12.9^{be}	113.22±10.5^{bde}	128.8±0.94^{d}	193.3± 11.0^{ade}	206.9±2.0^{ade}	160.5±8.6^{ade}
(+)-Catechin	222.6±2.2^{b}	119.2±0.4^{be}	168.1±4.9^{bde}	314.1± 10.4^{a}	239.2±4.3^{ade}	207.2±6.4^{ad}
(-)-Epicatechin	25.1±1.4	37.7± 3.0	42.0± 0.8^{bde}	25.00	nd	nd
(-)-Epicatechin gallate	38.1±0.9^{bde}	35.03±0.8^{bde}	39.7±0.5^{bde}	50.22± 0.12^{ae}	13.3±27.0	65.5±2.4^{ade}
Procyanidin B1	13.6±1.4^{de}	11.8±1.9^{bde}	10.9±0.4^{bde}	85.6± 3.7	32.4±2.3^{ae}	34.7±0.4^{ade}
Ferulic acid hexose derivative	4.6±0.4^{be}	5.1±0.14^{bde}	5.1±0.6^{be}	2.5± 0.11^{ade}	3.0±0.15^{a}	2.2±0.8^{ad}
p-Coumaroyl hexoside	38.3±0.7^{bde}	56.2±0.8^{bde}	30.2±0.3^{bde}	37.0± 2.3^{ad}	39.2±1.0^{ade}	40.9±2.9^{ade}
Caffeic acid hexoside	36.6±0.7^{be}	nd	39.1±1.4^{be}	46.6± 2.5^{a}	42.5±1.8^{e}	48.0±3.0^{ae}
p-Coumaric acid	2.6±0.07^{bde}	2.6±0.2^{bd}	1.93±0.2^{bde}	1.5± 0.2^{a}	0.8±0.02^{ade}	0.8±0.12^{a}
Cinnamic acid	0.71±0.08^{bd}	0.83± 0.07	0.64± 0.06	nd	0.48±0.00	0.33±0.10
Kaempferol	nd	nd	nd	nd	1.2±0.9^{de}	nd
Kaempferol-3-glucoside	3.26±0.16^{bde}	3.06±0.09^{bde}	3.95±0.16^{bde}	1.72± 0.16^{ae}	1.4±0.3^{ae}	3.20±0.11^{ade}
Kaempferol malonylglucoside	1.31±0.21^{d}	nd	1.67± 0.11^{be}	1.6± 0.8^{d}	nd	nd
trans-resveratrol glucoside derivative	1.23±0.02^{e}	0.7± 0.3	1.14± 0.03	nd	0.72±0.04^{d}	0.71±0.06

Table 3. (continued).

Compounds	FPS_{11A}	FPS_{11B}	FPS_{11C}	$FPWS_{11A}$	$FPWS_{11B}$	$FPWS_{11C}$
Gallic acid	21.1±1.7^{ab}	15.6±0.5^{a}	24.2±1.0^{a}	16.9±3.0^{ab}	20.3±0.5^{ab}	4.0±0.5^{ab}
Monogalloyl glucoside	34.0±1.9^{abe}	24.3±2.6^{abe}	40.30±1.05^{abe}	20.1±2.5^{abd}	29.9±0.6^{abd}	3.9±0.5^{ad}
Ellagic acid pentoside	26.29±10.01^{ae}	53.1±0.9	52.8±7.4^{ae}	37.1±2.6^{ad}	49.9±1.4^{a}	34.7±1.7^{ad}
Ellagic acid	28.1±4.3^{a}	70.5±0.6^{b}	59.5±2.4^{be}	34.0±6.6^{ab}	45.5±3.8^{ab}	31.9±2.6^{abd}
HHDP-galloylglucoside	142.8±10.9^{be}	182.8±12.3^{ab}	143.0±3.0^{a}	115.4±14.9^{abe}	133.1±1.1^{ab}	139.0±3.1^{b}
(+)-Catechin	158.03±0.02^{ad}	123.7±1.4^{abe}	160.1±1.6^{abe}	200.2±6.3^{ab}	197.72±1.06^{bd}	211.8±1.7^{ad}
(-)-Epicatechin	101.5±11.6	79.0±15.8	16.5±1.8^{ae}	15.5±0.3^{a}	14.89±0.22	13.5±0.3^{abd}
(-)-Epicatechin gallate	53.53±2.23^{a}	51.26±1.05^{ab}	54.5±0.8^{ab}	56.4±0.4^{ab}	65.1±0.4^{a}	58.5±1.0^{ab}
Procyanidin B1	23.85±0.05^{ad}	28.36±0.01^{a}	9.13±3.24^{abe}	19.98±1.02^{ad}	21.9±0.3^{ab}	22.05±1.00^{abd}
Ferulic acid hexose derivative	4.0±0.4^{bd}	2.7±0.6^{ad}	5.0±0.3^{bd}	1.6±0.2^{abd}	1.9±0.01^{abd}	1.5±0.3^{ad}
p-Coumaroyl hexoside	53.3±10.3^{abe}	46.9±0.9^{abe}	64.2±0.4^{abe}	38.4±2.4^{ad}	40.8±0.6^{abd}	34.3±1.2^{abd}
Caffeic acid hexoside	nd	nd	nd	48.2±6.8^{a}	45.8±08^{bc}	41.7±1.0^{ab}
p-Coumaric acid	0.9±0.3^{a}	1.3±0.2^{abe}	0.9±0.2^{a}	1.3±0.09^{ad}	2.1±0.03^{bd}	0.9±0.1^{a}
Cinnamic acid	0.57±0.11^{a}	0.75±0.24	0.57±0.06	0.30±0.08	0.41±0.03	0.53±0.02
Kaempferol	0.71±0.18^{d}	0.95±0.08^{be}	0.6±0.7^{be}	1.04±0.19^{d}	0.80±0.01^{bd}	0.53±0.005^{bd}
Kaempferol-3-glucoside	1.9±0.6^{a}	1.3±0.6^{ae}	2.24±0.06^{abe}	2.37±0.18^{ab}	2.37±0.05^{abd}	2.46±0.012^{abd}
Kaempferol malonylglucoside	1.34±1.15^{ab}	nd	nd	1.01±0.11	1.21±0.01	1.32±0.006^{ab}
trans-resveratrol glucoside derivative	nd	nd	0.69±0.004	0.75±0.007	0.89±0.006	nd

Table 3. (continued).

Compounds	M_{12A}	M_{12B}	M_{12C}	EI_{12A}	EI_{12B}	EI_{12C}
Gallic acid	69.4±1.8	37.7±3.3	47.0±1.5	30.7±1.3^{a}	31.6±0.6^{a}	42.8±1.5
Gallic acid derivate 1	68.0±12.7	27.1±0.7	30.4±1.1	110.4±0.9	34.1±0.7	35.3±1.6^{a}
Gallic acid derivate 2	nd	45.5±1.6	59.4±3.1	59.1±4.8	57.6±1.7^{a}	79.7±8.0
Monogalloyl glucoside	11.6±3.9	5.6± 2.4	5.9± 3.8	nd	4.6±0.5	9.9±1.7
Ellagic acid	11.2±3.0	10.5±3.4	16.4±6.9	12,2±9.5	12.3±0.5	11.7±2.1
Ellagic pentoside	12.7±1.6	7.5±2.4	10.0±0.8	9.0±0.3^{a}	8.7±0.6	11.7±1.3
Ellagic deoxyhexoside	20.6±6.4	14.6±2.0	16.4±6.9	9.8±0.7^{a}	9.8±0.7^{a}	13.1±1.5
HHDP-galloylglucoside	146.1±3.1^{cde}	127.2±18.1^{bc}	133.2±19.6^{c}	160.3±11.2^{cde}	157.2±3.6^{acd}	152.3±14.1^{ce}
(+)-Catechin	253.1±4.5	318.3±29.4	379.2±15.9	259.0±14.5	261.3±22.7	379.3±16.8
(-)-Epicatechin	4.9±0.7	2.9±1.8	12.0±2.0	10.7±0.3^{a}	6.6±2.5^{a}	10.3±1.3
(-)-Epicatechin gallate	9.3±0.9	27.2±0.1	40.3±3.7	30.2±5.6^{a}	31.3±3.8	41.5±8.5
Procyanidin B1	41.8±5.5	34.0±5.6	45.0±1.7	42.7±4.5	46.0±3.6^{a}	48.6±6.4
Ferulic acid hexose derivative	6.5±0.8^{bcde}	3.4±0.7^{cd}	5.1±0.8^{bce}	2.9±0.17^{acde}	3.1±0.11^{cd}	2.51±0.31^{ace}
p-Coumaroyl hexoside	22.4±2.2^{de}	20.3±8.6	25.5±7.6^{be}	19.9±5.6	18.0±3.5	15.1±1.6
Caffeic acid hexoside	27.2±6.9	26.5±3.7^{bd}	29.0±1.2	29.9±4.0^{c}	30.9±4.1^{d}	27.6±0.9^{e}
p-Coumaric acid	2.7±0.1^{bcde}	0.6±0.2	1.1±0.2^{ce}	0.48±0.02^{ade}	0.6±0.02^{cd}	0.7±0.4^{e}
Cinnamic acid	0.7±0.04	nd	0.8±0.04	nd	nd	0.5±0.05^{a}
Kaempferol-3-glucoside	1.2±0.7	0.5± 0.15	nd	1.2±0.03	1.1±0.3	nd
K. coumaroylglucoside	nd	nd	nd	nd	1.04±0.19	nd

Table 3. (continued).

Compounds	UP_{12A}	UP_{12B}	UP_{12C}	FPS_{12A}	FPS_{12B}	$FPWS_{12A}$	$FPWS_{12C}$
Gallic acid	nd	nd	30.4±0.7^{b}	28.2±3.4^{a}	27.5±0.5^{ab}	26.6±0.4^{ab}	27.0±0.3^{abd}
Gallic acid derivate 1	30.1±0.7^{a}	34.8±1.6	43.5±12.7	33.2±0.5^{abd}	42.2±0.7^{bd}	38.8±0.8^{bd}	39.1±2.6^{a}
Gallic acid derivate 2	37.4±4.6^{b}	31.7±2.1^{ab}	35.8±5.6^{ab}	32.8±3.4^{b}	28.5±2.1^{ab}	33.3±1.0^{b}	39.4±0.5^{ab}
Monogalloyl glucoside	4.5±1.1^{a}	4.0±0.10	2.9±0.17^{b}	nd	2.9±0.6^{b}	4.2±0.17^{a}	10.0±0.6^{d}
Ellagic acid	8.7±2.9	7.7±0.4^{b}	8.0±1.6	9,6±1.8	12.5±1.6^{c}	9.7±1.9	11.0±0.21^{bd}
Ellagic pentoside	3.7±1.3^{ab}	5.1±0.18^{b}	8.2±2.4^{b}	7.7±1.0^{abd}	10.0±3.6^{d}	8.2±1.5a	8.8±0.4^{ab}
Ellagic deoxyhexoside	4.7±0.8^{ab}	nd	nd	nd	nd	nd	nd
HHDP-galloylglucoside	63.9±9.9^{abde}	61.4±6.9^{abd}	82.7±17.9^{abe}	114.3±18.7^{abc}	118.6±6.1^{bc}	114.4±11.5^{abc}	119.0±0.58^{bc}
(+)-Catechin	258.9±21.8	41.5±5.1^{ab}	243.0±1.8^{ab}	40.1±6.8^{abd}	171.2±7.0^{abd}	147.0±8.1^{abde}	227.4±2.3
(-)-Epicatechin	2.7±0.3^{ab}	4.4±1.0^{b}	4.7±2.2^{ab}	4.5±1.9^{b}	0.7±0.2^{bd}	3.1±0.4^{ab}	5.5±0.7^{ab}
(-)-Epicatechin gallate	22.1±3.7^{a}	44.0±5.0	34.8±7.2	23.4±2.0^{a}	21.7±1.2^{b}	25.9±1.5^{a}	35.1±1.9^{a}
Procyanidin B1	30.1±0.8^{b}	34.0±5.2^{b}	27.4±7.4^{ab}	16.1±3.4^{abd}	24.9±1.6^{abd}	19.6±3.3^{bd}	25.6±1.9^{ab}
Ferulic acid hexose derivative	0.46±0.03^{abde}	0.66±0.03^{abd}	1.02 0.43^{ab}	1.28±0.22^{abce}	1.59±0.18^{abc}	0.93±0.14^{abcd}	1.16±0.10^{ace}
p-Coumaroyl hexoside	23.7±2.4^{ade}	22.3±7.2^{a}	19.1±3.3^{a}	14.2±2.8^{ac}	16.0±1.7^{a}	16.0±1.2^{ac}	15.5±1.1^{a}
Caffeic acid hexoside	18.7±1.02^{bde}	27.9±4.5^{d}	28.7±1.2	24.3±2.9^{ce}	26.1±1.5^{abc}	32.5±1.1^{cd}	29.4±0.9^{b}
p-Coumaric acid	0.4±0.06^{ade}	0.8±0.07^{bd}	0.7±0.2^{ae}	0.1±0.07^{abc}	0.11±0.04^{bc}	0.2±0.08^{abc}	0.12± 0.03^{ac}
Cinnamic acid	0.5±0.1	1.2±0.01	0.9±0.3^{b}	nd	0.5±0.01	nd	nd
Kaempferol-3-glucoside	0.9±0.02	0.9±0.03^{b}	1.1±0.6	1.7±0.4^{d}	1.9±0.08^{bd}	nd	0.6±0.05^{bd}
K. coumaroylglucoside	nd	nd	0.48±0.09	0.73±0.04	1.27±26.43^{bd}	0.41±0.03	1.24±0.92^{b}

Mean values and standard deviation.

[a] superscript letter indicate significant difference ($p< 0.05$) compared to the mashed step (M) of the same substrate,

[b] superscript letter indicate significant difference ($p<0.05$) compared to the enzymatic inactivation step (EI) of the same substrate,

[c] superscript letter indicate significant difference ($p<0.05$) compared to the unpasteurized sample (UP) of the same substrate,

[d] superscript letter indicate significant difference ($p<0.05$) compared to the purée with seeds of the same substrate (FPS), and

[e] superscript letter indicate significant difference ($p<0.05$) compared to the purée without seeds (FPWS) of the same substrate by ANOVA statistical test . HHDP-galloylglucoside was quantified as ellagic acid. Samples were determined in duplicate. nd: no data. K: kaempferol. Subscript 11 and 12 indicate the year of the harvest.

Table 4. Antioxidant activity in strawberry samples.

2011 Harvest			2012 Harvest		
Samples	**ORAC** (μmol g^{-1} fw)	**DPPH** (mmol kg^{-1} fw)	**Samples**	**ORAC** (μmol g^{-1} fw)	**DPPH** (mmol kg^{-1} fw)
$\mathbf{M_{11A}}$	12.5±.0.4	24.3±3.4[b]	$\mathbf{M_{12A}}$	13.3±1.8	16.8±2.8[b]
$\mathbf{M_{11B}}$	13.1±6.5[c]	23.45±4.01[c]	$\mathbf{M_{12B}}$	13.01±4.13	20.10±2.25[bcd]
$\mathbf{M_{11C}}$	21.3±4.6[b]	22.4±1.3[b]	$\mathbf{M_{12C}}$	16.63±2.12	19.0±3.3[bce]
$\mathbf{EI_{11A}}$	19.9±10.9[a]	32.2±1.5[ad]	$\mathbf{EI_{12A}}$	12.76±3.25	13.5±1.9[ad]
$\mathbf{EI_{11B}}$	15.5±1.8	28.47±3.23[cd]	$\mathbf{EI_{12B}}$	8.39±1.03	13.50±4.24[a]
$\mathbf{EI_{11C}}$	15.5±1.8	26.5±1.5	$\mathbf{EI_{12C}}$	16.8±1.4	22.24±3.04[ac]
$\mathbf{FPS_{11A}}$	13.92±2.62	14.63±1.01[bd]	$\mathbf{UP_{12A}}$	11.7±2.3	15.1±2.8
$\mathbf{FPS_{11B}}$	11.00±3.24[abd]	14.68±1.20[bd]	$\mathbf{UP_{12B}}$	13.20±2.06	10.5±0.6[ad]
$\mathbf{FPS_{11C}}$	8.9±1.4	23.4±1.8	$\mathbf{UP_{12C}}$	10.3±1.3	16.0±2.5[abe]
$\mathbf{FPWS_{11A}}$	15.6±2.9[b]	21.5±5.5[b]	$\mathbf{FPS_{12A}}$	17.5±2.6	16.4±1.4[b]
$\mathbf{FPWS_{11B}}$	11.5±1.5[b]	23.7±1.7[bc]	$\mathbf{FPS_{12B}}$	11.3±3.9	16.8±3.0[ac]
$\mathbf{FPWS_{11C}}$	13.7±2.4[bc]	24.5±3.7	$\mathbf{FPWS_{12A}}$	16.57±3.12	14.9±4.5
-	- - -	- - -	$\mathbf{FPWS_{12C}}$	11.51±1.09	22.6±2.5[ac]

Mean values and standard deviation.
[a] superscript letter indicate significant difference ($p< 0.05$) compared to the mashed step (M),
[b] superscript letter indicate significant difference ($p<0.05$) compared to the enzymatic inactivation step (EI),
[c] superscript letter indicate significant difference ($p<0.05$) compared to the unpasteurized step (UP),
[d] superscript letter indicate significant difference ($p<0.05$) compared to the purée with seeds (FPS),
[e] and superscript letter indicate significant difference ($p<0.05$) compared to the purée without seeds (FPWS), by ANOVA statistical test. Antioxidant samples were determined in triplicate.

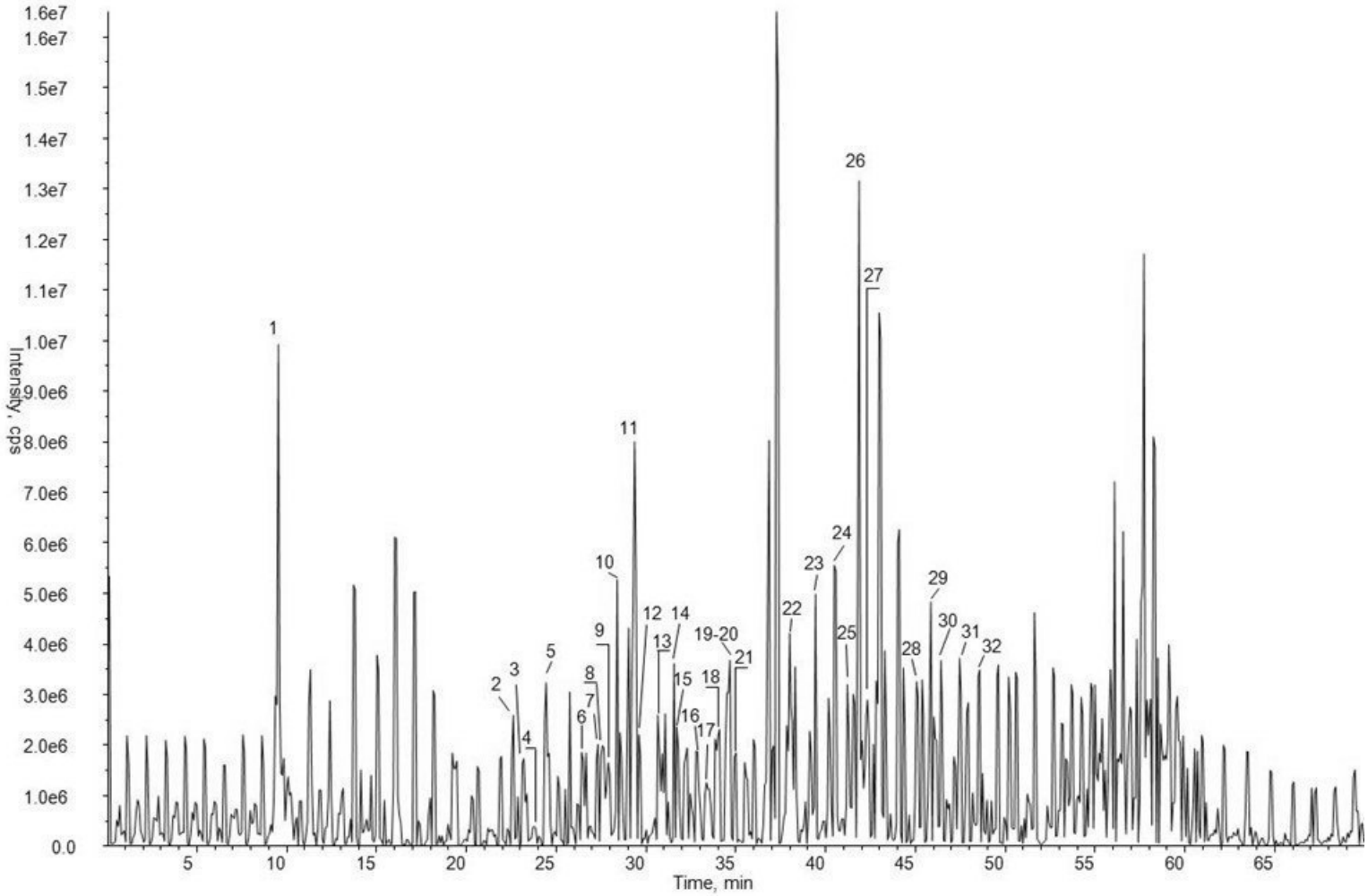

Fig. 1. LCMS chromatogram of sample. The peak numbers corresponding to displays in identification table.

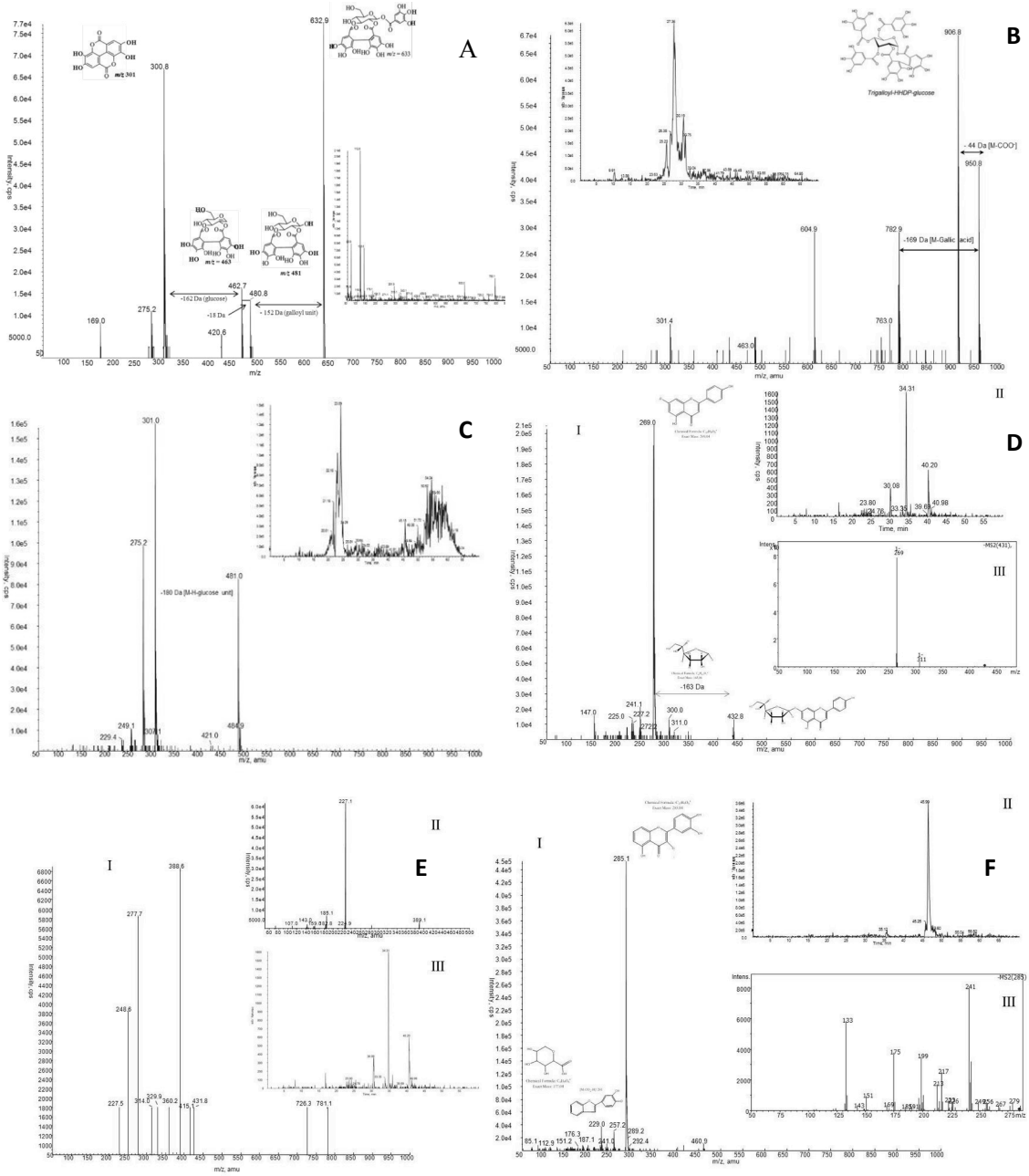

Fig. 2. LC-MSMS, structures and hypothetized fragmentations patterns (A) $[M - H]^-$ ion 633, HHDP-galloylglucose (B) $[M - H]^-$ ion 951, tris-galloyl-HHDP-hexose (C) LC- MSMS $[M - H]^-$ ion 481, HHDP glucoside (D) LC-MSMS $[M - H]^-$ ion 431, of Apigenin-7-O-glucoside in sample (I), xic chromatogram shows retention time (II) and MS2 chromatogram of apigenin-7-O-glucoside standard (III). (E) LC-MSMS $[M - H]^-$ ion 461 of Luteolin-3-O-glucuronide in sample (I), xic chromatogram shows retention time (II) and MS^3 chromatogram of luteolin aglycon standard (III) shows transition m/z 285–241. (F) LC-MS of ion precursor m/z 227 experiment of *trans*-resveratrol $[M-H]^-$ 389 (I), fragmentation pattern of standard (II) and chromatogram of MRM experiment to shows retention time in sample (III).

CAPÍTULO SEGUNDO

II

Title: Non-anthocyanin phenolic compounds and antioxidant activity of beverages obtained by gluconic fermentation of strawberry

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Non-anthocyanin phenolic compounds and antioxidant activity of beverages obtained by gluconic fermentation of strawberry.

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ABSTRACT

Strawberry is widely harvested in Spain. It is a highly perishable fruit and surplus production may be used to produce innovative foods. This paper studies the influence of gluconic fermentation by *Gluconobacter japonicus* on the non-anthocyanin content and antioxidant activity of strawberry. A total of 43 non-anthocyanin phenolic compounds were identified. To the best of our knowledge, this paper reports five compounds for the first time in strawberry and its derivatives, tentatively identified as: monogalloyl diglucose, 5-hydroxy feruloyl hexose, dihydrokaempferol hexoside, kaempferol neohesperidoside and (-)chicoric acid. It was further observed that gluconic fermentation leaves *in vitro* antioxidant activity practically unchanged.

Gluconic fermentation by *Gluconobacter japonicus* can be considered a potential tool for producing fermented beverages. It transforms glucose into gluconic acid, which allows diabetics to consume the drink, while maintaining bioactive compound concentration and antioxidant activity.

Keywords:

Gluconobacter japonicus, LC-DAD, bioactive, mass spectrometry, non-alcoholic drink

1. INTRODUCTION

Strawberry (*Fragaria x ananassa*) is widely harvested in Spain, especially in the south (Huelva). Seasonal overproduction leads to surplus fruit, which is not brought to market. In Spain the production is over 289,900 MT of fresh fruit and was the fourth largest producer in the world (FAOSTAT, 2012). As strawberry is a highly perishable product, fruits spoil quickly, leading to substantial economic losses. Therefore, the transformation of strawberry surplus into new, longer-lasting products is financially worthwhile for the industry and producers.

Fermentation is an ancient process of preserving foods. The benefits of the fermentation process are an extended shelf-life, improvements to the nutritious value of food, improved digestibility and upgraded food safety by pH reduction and elimination of antinutrients, (Balasundram, Sundram and Samman, 2006). As a result of fermentation, a range of secondary metabolites are produced. Some of these are associated with promoting health, like B vitamins and peptides released from food proteins through microbial action. It is therefore not surprising that consuming fermented food has been associated with a healthy lifestyle (Van Boekel et al., 2010).

Bioactive compounds present in food, such as polyphenols, undergo profile changes as a result of the fermentation process. For instance, there is an increase in catechin and procyanidin content during alcoholic fermentation, due to the transfer of these compounds from the solid parts of the grape into the wine (Sun et al., 2011; Ricardo da Silva, Rosec, Bourzeix, Mourgues and Moutounet 1992; Spranger, Sun, Leandro, Cavalho and Bechior, 1998). Additionally, Cerezo, Cuevas, Winterhalter, Garcia-Parrilla and Troncoso (2010a) observed a significant decrease of (+)-catechin (50%), ferulic, caftaric and caffeic acids during acetic acid fermentation.

Strawberry is a good source of bioactive compounds, such as polyphenols, which strongly influence quality and health properties (Cerezo, Cuevas, Winterhalter, Garcia-Parrilla and Troncoso, 2010b; Larrosa, Tomás-Barberán and Espín, 2006; Buendia et al., 2009). Regarding the effect of fermentation on the polyphenolic composition of strawberry substrate, it has been observed that alcoholic fermentation significantly decreases the total polyphenol index (Ubeda et al., 2013). A similar trend in the total number of monomeric anthocyanins has been observed during acetic acid fermentation (Ubeda et al., 2013). Nonetheless, few studies have been performed on the effects of fermentation on individual non-anthocyanin compounds.

Although the effect of alcoholic fermentation and acetic acid fermentation on polyphenolic compounds has been previously studied in strawberry, the effect of gluconic fermentation on the polyphenolic composition of this fruit is still unknown. Gluconic fermentation has an additional advantage from a nutritional perspective, since *Gluconobacter* strains convert glucose into mostly gluconic acid, without fermenting the fructose (Attwood, van Dijken and Pronk, 1991). Therefore, the final product will maintain the sweetness of the natural fructose content of the fruit as well as all the original health properties.

This paper aims to characterize the non-anthocyanin phenolic composition of a beverage obtained by fermentation of strawberry. Specifically, the purpose is to study the impact of gluconic fermentation process on the non-anthocyanin phenolic composition of the resulting drink.

2. MATERIALS AND METHODS

2.1. Chemicals

Standards of the non-anthocyanin compounds used were purchased from: Fluka Sigma-Aldrich (St. Louis, MO, USA) [gallic acid, caffeic acid, *p*-coumaric acid, cinnamic acid, (-)-epicatechin, (+)-catechin, chlorogenic acid, ellagic acid, (-)-epicatechin gallate, kaempferol, kaempferol-3-O-ß-D-glucuronide, kaempferol-3-glucoside, polydatin and penta-O-galloyl-ß-D-glucose hydrate]; Chromadex® Inc. (USA) [procyanidin B1], and Extrasynthese [luteolin and apigenin-7-O-glucoside]. DPPH (2,2-diphenyl-1-picrylhydrazyl), AAPH (2,2´-diazo-bis-amidine-propane-dihydrochloride) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were obtained from Sigma-Aldrich (St. Louis, MO, USA), acetonitrile and acetic acid (LC gradient) from Merck (Darmstadt, Germany), and methanol from Prolabo® (Obregón, Mexico).

2.2. Samples

Two strawberry purée substrates were fermented (A and B); these were provided by Hudisa Company (Lepe, Huelva, Spain). The purées were made with surplus production grown in the region of Huelva (Andalusia-Spain). The principals cultivated varieties were Camarosa, Candonga, Festival, Ventana, Splendor, Honor and Coral. *Gluconobacter japonicus* was used as a starter for the submerged fermentation process. Briefly, the process was optimized beforehand (Álvarez-Fernández, Hornedo-Ortega, Cerezo, Troncoso and García-Parrilla, 2014) and was as follows: 3 L of strawberry purée substrate were placed into the bioreactor and the conditions set (29 ºC, 20% O_2 and 1250 g); after 10-20 min, 125 ml of inoculum of *Gluconobacter japonicus* strain E1 were added and mixed for 20-30 min, then the initial sample was taken. The end of the fermentation process was established when the glucose had been totally consumed. Four fermentation cycles were studied for each substrate. The process stars as the strawberry purée is mixed with the inoculum (initial step) and finishes as the remaining glucose is

totally consumed (final step) when the fermentor is discharged. This process is a fermentation cycle and we studied 4 cycles. The fermented samples were supplied by the Department of Analytical Chemistry, Chemical Engineering, Faculty of Sciences, University of Cordoba.

The pasteurization process consisted of heating the sample to 70-80 ºC for 15 min and then rapidly cooling and freezing it until analysis.

We analyzed the initial and final steps of each cycle and additionally the final pasteurized sample.

Each fermentation cycle encompassed 3 analyzed samples.

Sample codes indicate: the fermentation step (I initial, F final and P pasteurized); the cycles, referred to as 1, 2, 3 and 4, and finally, the substrate, indicated by A (less-concentrated purée) or B (more-concentrated purée). A total of 24 samples were analyzed in duplicate. Table 1 displays the sample codes.

2.3. Polyphenol extraction

An aliquot of 30 g of sample was extracted in 30 mL of methanol by sonication for 30 min. Ascorbic acid 1% (w/w) was previously added to prevent compound oxidation. It was immediately centrifuged for 10 min at 1500 g using a Sorvall® TC Dupont Centrifuge. The supernatant was collected and the pellet re-extracted with methanol (30 mL). The fractions were mixed and evaporated to dryness at 37 ºC with a rotary evaporator (Büchi Rotavapor®, R-200/205). The extract was reconstituted in 5 mL of water/methanol 1:1 (v/v) and stored at -18 ºC until analysis. Extractions were performed in duplicate.

No ascorbic acid was added to the aliquot used to determine antioxidant activity, but the rest of the sample preparation process was similar to that described above.

2.4. LC/DAD, LC/MS/ESI-MS/MS and MRM analysis

Chromatographic separation was accomplished using an LC Agilent Series 1100 system equipped with a quaternary pump (Series 1100 G1311A), automatic injector (Series 1100 G1313A) and degasser (Series 1100 G1379A). Detection was with a UV/Vis diode detector (Series 1100 G1379A) coupled to a Chemstation HP A.10.02 (HP/Agilent). The column used was a Merck LiChroCART Superspher 100 [250-4 RP-18 (5µm)] 1.16056.0001. The method was a binary gradient, A (acetic acid/water, pH 2.65) and B (20% A + 80% acetonitrile), scheduled in the following gradient: 0 min, 100% A; 5min, 98% A + 2% B; 10 min, 96% A + 4% B; 15 min, 90% A + 10% B; 20 min 87% A + 13% B; 35 min, 80% A+ 20% B; 40 min, 70% A + 30% B; 45 min, 60% A + 40% B; 50 min, 100% B; 55 min 100% A; 70 min, 100% A. Each sample was analyzed twice. The injection volume was 50 µL, the flow rate was 1.5 mL min^{-1}, and the temperature was set at 40 ºC. This method has been validated before in strawberry purée analysis (Álvarez-Fernández et al. 2014). Identification was achieved by matching the retention time and spectra of the peaks with the corresponding standards. Furthermore, samples were spiked with standards, if they were commercially available, to achieve complete identification. In accordance with the maximum absorbance of each compound, quantification was performed by external calibration at 280 nm, 320 nm and 365 nm, using the corresponding standards. Calibration curves were obtained by injecting standards diluted from five to eight different concentrations (R^2: 0.9949-0.9998). A duplicate was performed at each point of the calibration curve.

LC/MS separation was performed using a Pelkin Elmer Series 200 HPLC 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 Merck LiChroCART 250-4 Superspher 100 RP-18 1.16056.0001 reversed-phase column. The flow rate was 0.4 ml min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water, and (B) water:acetonitrile 20:80 (v/v). Both components contained 0.1% formic acid (v/v). The elution profile was the same as the one used in LC/DAD analysis. The injection volume was 20 µL.

Multiple Reaction Monitoring (MRM) was applied where precursor ions and fragment ions were monitored, at Q1 and Q3, respectively, using their corresponding standards. The analysis was performed in negative ionization.

For HPLC-ESI-MRM analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, ion spray voltage 4500 V, source temperature 350 ºC and source gas 60 psi. MRM transitions were performed with the parameters shown in table 2. A dwell time was set at 50 ms for each transition.

2.5. Antioxidant activity

2.5.1. ORAC test

The method used was similar to a previously reported method, with modifications (Ou, Hampsch-Woodill and Prior, 2001): 50 µL of sample or Trolox mixed with 100 µL of fluorescein (45 nM) and 50 µL of AAPH (15 mM). Reaction was carried out for 80 min and fluorescence assessed with a fluorometer (multi-detector microplate Synergy HT, Biotek®) excitation and emission wavelengths were 485 and 528 nm respectively. The readings were taken in triplicate. Trolox was used as a calibration standard (0.5 to 9.5 µM).

The fluorescein fluorescence lectures were recorded every 5 min after addition of AAPH until they reached less than 5% of the initial reading. Results were calculated using the areas under the fluorescein decay curves between the blank and the sample and were expressed as mmols Trolox equivalents (TE) per Kg of fresh weight.

2.5.2. DPPH method

Antioxidant activity was assessed using the DPPH method, as reported by Villaño, Fernández-Pachón, Moyá, Troncoso and García-Parrilla (2007). An aliquot of 0.1 mL of sample in methanol was added to 3.9 mL of DPPH methanolic solution (0.025 gL^{-1}). Absorbance was recorded at 515 nm at the start time (when the sample was added) and 60 min later, when the reaction reached equilibrium. All measurements were taken in triplicate and methanol was used as a reference. Eight concentrations were used to make the Trolox calibration curve (0.9-0.12 mM).

All measurements were recorded on a Hitachi UV-2800 spectrophotometer thermostated with a Peltier system at 25 ºC.

2.6. Statistical analysis

Statistical analyses were performed by means of Statistica software, StatSoft, Inc. version 7 (2004). One - way analysis of variance (ANOVA) was used to test significant differences at $p<0.05$ level. Additionally, discriminant analysis was tested to build a function to discriminate between the substrate (initial) the fermented beverage (final) and the final pasteurized samples.

3. RESULTS AND DISCUSSION

3.1. Identification of compounds by LC/ESI-MS and MS/MS analysis

A total of 44 non-anthocyanin phenolic compounds were identified using LC/ESI-MS and LC-DAD methods. Table 2 displays the identified compounds and their MS/MS rupture, tentatively identified in negative ion. Figure 1 shows the corresponding mass chromatogram.

Gallic acid (peak 1), (+)-catechin (peak 6), procyanidin B1 (peak 9), caffeic acid (peak 11), (-)-epicatechin gallate (peak 20), *p*-coumaric acid (peak 22), ferulic acid (peak 24), ellagic acid (peak 25), chicoric acid (peak 29), cinnamic acid (peak 38), *trans*-resveratrol (peak 39), *p*-hydroxybenzoic acid (peak 40), *trans*-piceid (peak 42) and kaempferol (peak 44), were identified by monitoring their characteristic transitions in MRM mode and comparing their retention times with their corresponding standards. This assay was performed to confirm the identification of phenolic compounds with low concentrations or low sensitivity. The LC-DAD chromatograms were recorded at 280, 320 and 365 nm to quantitate the compounds (figure 2).

Hydroxybenzoic acids

Peak 5 had an $[M-H]^-$ ion at 299 and fragment ion at *m/z* 137, corresponding to hydroxybenzoic acid. This compound, observed in samples P, was tentatively identified as *p*-hydroxybenzoic-3-O-glucose, in accordance with previous studies in strawberry Ornelas-Paz et al. (2013).

Hydrolyzed tannins

This group includes ellagitannins and gallotannins. Peaks 4, 14, 17, 21 and 30 were identified as ellagitannins, as their MS-MS fragmentations gave *m/z* 301 corresponding to ellagic acid, in accordance with previous reports in strawberry (Aaby, Mazur, Nes and Skrede, 2012; Del Bubba et al., 2012). Peaks 18 and 37 correspond to gallotannins, as their major fragment ion is *m/z* 313, which yielded a deprotonated Gallic acid *m/z*

169 by the loss of glucose unit [M-H]$^-$ 162. Peak 17 was described as Monogalloyl glucose, which has been reported in strawberry pistil (Hanhineva et al., 2008). However, we were able to tentatively identify peak 37 as Monogalloyl diglucose with a deprotonated ion at [M-H]$^-$ 493, which has been reported in *Vitis rotundifolia* and in others plants (Sandhu & Gu, 2010; Soong & Barlow, 2005), but which has not been described before in strawberry or its derivatives products like purées, beverages or jams etc; this is an original contribution of this paper.

Hydroxycinnamic acids

Eleven compounds have been identified in this group. For the first time, we report two hydroxycinnamic derivatives in strawberry and derivatives: chicoric acid (2, 3-dicaffeoyltartaric acid) identified with authentic standards by MRM experiment (Figure 3) and 5-hydroxy feruloyl hexose. Peak 40 had an MW of 372 because an [M-H]$^-$ ion at 371 was found. The fragmentation produced MS/MS ions with *m/z* 193 and 209; fragment *m/z* 193 is characteristic of ferulic acid. The fragmentation pattern is plotted in Figure 4(A) and Figure 4 top (B) displays xic chromatogram and retention time corresponding to same fragmentation; the compound was tentatively identified as 5-hydroxyferuloyl hexose, which has been reported before in coffee beans grinder by Chandrasekara and Shahidi (2011), but not in strawberry.

Flavonols

There were fourteen compounds in this group. The main compounds were kaempferol and quercetin derivatives, thus peak 13 was tentatively identified as dihydrokaempferol hexoside, in accordance with Fischer, Carle and Kammerer (2011), reported in *Punica granatum*. This compound had an [M-H]$^-$ 449 and fragmentation pattern *m/z* 287, 269 and 259. Peak 19 exhibited a pseudo molecular ion at *m/z* 431 and MS/MS pattern with *m/z* 269; 311 and 270, and was identified as apigenin-7-O-glucoside. Peak 26 had an

$[M-H]^-$ ion at *m/z* 461, the main fragmentation product of the mono-charged pseudo-molecular ion was 285 and it presented a fragment *m/z* 241 characteristic of luteolin aglycone; this fragmentation pattern corresponds to luteolin-3-O-glucuronide (Álvarez-Fernández et al. 2014). Peak 31, which exhibited a deprotonated ion $[M-H]^-$ at 461, but with another fragmentation pattern, was identified as kaempferol 3- glucuronide (Figure 5).

A quercetin derivative was tentatively identified as Quercetin-3-(6"acetyl glucoside): it exhibited an $[M-H]^-$ at 505 and *m/z* 300 fragmentation pattern, indicating loss of 250 Da corresponding to an acetylglucose unit and with *m/z* 179 and 271 corresponding to the fragmentation pattern of quercetin. This compound has been reported before in blueberry, mulberry, kiwi fruit and *Fragaria vesca* (Mikulic-Petkovsek, Slatnar, Stampar and Veberic, 2012), but has not been determined before in *Fragaria ananassa.* Peak 33 had a pseudo molecular ion at *m/z* 491, producing an ion at *m/z* 315, with a glucuronide unit loss of 176 Da and subsequent loss of methyl from the methoxy group (15 Da). This dissociation pattern was observed by Hanhineva et al. (2008) in strawberry floral organs and tentatively identified as isorhamnetin glucuronide. Peak 35 presented kaempferol derivative with an $[M-H]^-$ at *m/z* 593, the loss of 309 Da corresponding to sugar moiety and the production of MS^2 fragmentation at *m/z* 285 corresponding to kaempferol aglicone. It was tentatively identified as kaempferol-7-O-neohesperidoside. This compound has been reported before in red and black currants, but not in strawberry (Mikulic-Petkovsek et al., 2012). These findings are a novelty of this work.

Stilbens

Peak 42 exhibited an $[M-H]^-$ ion at *m/z* 389 and MS/MS ion with *m/z* 227, this fragmentation corresponds to *trans*-piceid. It was identified by corresponding standard

and has been described before in strawberry juice (Díaz-García, Obón, Castellar, Collado and Alacid, 2013).

3.2. Changes in non-anthocyanin phenolic content and antioxidant activity though fermentation

Twenty-one identified compounds were quantified by external calibration with standards, according to the validated LC-DAD method (Álvarez-Fernández et al., 2014). Tables 3 and 4 display the phenolic compound concentrations corresponding to three steps of four fermentation cycles of two substrates (A and B), at the initial and final steps of the cycle and after the pasteurization step.

In summary, just gallic acid, HHDP galloyl-glucose, p-coumaroyl glucose, caffeic acid, kaempferol 3-hexoside and kaempferol malonyl glucoside changed significantly ($p<0.05$) during the fermentation process.

Phenolic compounds with major concentrations were (+)-catechin, *p*-coumaroyl hexoside and HHDP-galloyl-glucoside; they constituted as much as 62% of the total phenolic compounds quantified.

During the fermentation process, the content of most phenolic compounds increased. Gallic acid increased after the process in all cycles, by 36% on average. The decrease in monogalloyl glucose cannot account for this value. Enzymes, like the esterase tannase, are able to hydrolyze ester bonds to release gallic acid and sugar moieties. Bacteria, yeast or fungi in the fermentation process can produce this enzyme (Duckstein, Lorenz and Stintzing, 2012). Epicatechin gallate was considered another releaser of gallic acid. Kim, Goodner, Park, Choi and Talcott (2011) reported a decrease of epicatechin gallate and a simoultaneous increase of gallic acid after the fermentation of tea (*Camellia sinensis*). Our data do not confirm this trend.

HHDP-Galloyl-glucoside is the main hydrolyzable tannin in the samples. Its values ranged from 13.8 to 38.66 mg kg-1 fw in initial steps and it underwent a significant increase of 22.23-96.39% in the fermentation process, like most of the hydrolyzable tannins quantified.

Hydroxycinnamic acids experiment a significant increased ($p<0.05$) in general, specifically p-coumaroyl hexoside and caffeic acid. Our data are in accordance with Pérez-Gregorio, Regueiro, Alonso-González, Pastrana-Castro and Simal-Gándara (2011) who reported hydroxycinnamic acid derivatives increase after alcoholic fermentation of mulberries (*Morus nigra*). In addition, the presence of hydroxybenzoic acid may indicate the degradation of other hydroxycinnamic compounds by fermentation, as reported by Duckstein et al. (2012).

The principal flavonols in the samples were kaempferol derivatives, whose concentrations ranged from 0.4–6.20 mg kg^{-1}fw, as has been reported in strawberry purée (Álvarez-Fernández et al. 2014). Both compounds underwent a significant increase on fermentation: 1.75-2.22% on average.

3.3. Changes in non-anthocyanin phenolic content and antioxidant activity though the pasteurization process

To sum up, the pasteurization process increased contents of p-coumaroylhexoside, cinnamic acid and p-coumaric acid; and decreased caffeic acid, its hexose and procyanidin B1 contents.

The effects of the pasteurization process had significant increase in the content of p-coumaroylhexoside in cycle 2 and 4 of substrate B (15.83-14.05 % for each cycle), p-coumaric acid increase in cycle 4 of substrate B in 45.34 % and in case of cinnamic acid in cycle 4 of substrate A with an increase of 3.03%. These results are in accordance with

van Boekel et al. (2010). They reported a significant increase in total caffeoylquinic acid after the thermal process. This behavior was the result of both isomerization and hydrolysis events, leading to a substantial re-distribution of phenolic acid concentrations.

Caffeoylhexose and caffeic acid had a tendency to decrease with pasteurization process, the first one experimented significant decreased (8.03%) in cycle 4 of substrate A; caffeic acid had the same behavior in three cycles (1, 2 and 4) of substrate A (45.79-41.01 and 6.85%) and in cycle 4 of substrate B with a decrease of 1.19% .

On the other hand, procyanidin B1 underwent a significant decrease in two cycles one of each substrate; cycle 4 of substrate A had significant decrease 21.20% and cycle 4 of substrate B with decrease of 26.78% as a result of the thermal process, which is in accordance with White et al. (2011), who described how blanching caused a decrease in polymeric procyanidins. Ellagitannins showed the same trend.

3.4. Discriminant statistical analysis

A multivariate statistical analysis was applied to study the data. The data matrix was built with quantified phenolic compounds ORAC and DPPH as variables and samples as cases. Discriminant analysis was applied to both substrates in this study, and the grouping variable was the step in the process: I (initial), F (final) and P (Pasteurized). The standard method achieved better results in every analysis. All samples were correctly classified with all polyphenolic compounds as variables in the model. Figure 6 shows the scatterplot of the canonical roots obtained. As can be seen, the distance for pasteurized samples is greater than between initial and final fermentation steps. These results are reproducible for both substrates, regardless of the concentration of polyphenolic compounds in the strawberry purée.

Table 5 exhibits coefficients of variables corresponding to figure 6, indicating their relative importance in the model. The greatest values, regardless of the sign they submit, indicate their weight and importance in the model. From substrate A: (+) catechin, caffeic acid, *p*-coumaroyl hexoside, cinnamic acid, ellagic acid pentoside and (-) epicatechin, and from substrate B: castalagin, *p*-coumaroyl hexose, (+) catechin, ellagic acid, cinnamic acid and monogalloyl glucose, are the heaviest weighted in root 1 (x axis). We concluded these were the best variables to differentiate the groups in our analysis based on the criteria proposed by Kim et al., 2011.

Statistical analysis confirms that both gluconic fermentation and pasteurization change non-anthocyanin polyphenolic compounds in strawberry derived products.

3.5. Impact of gluconic fermentation and the pasteurization process on antioxidant activity

Figure 7 displays the results of antioxidant activity using two methods. The fermentation process did not change antioxidant activity regardless of the method used. Pasteurized samples present a higher value when determined by DPPH significance increased ($p < 0.05$). This result is in accordance with an increase in the contents of gallic acid and hydroxycinnamic derivatives. Hence, gluconic fermentation does helps to maintain antioxidant potential of studied beverage.

4. Conclusions

Forty-three non-anthocyanin phenolic compounds were identified and five were reported for the first time in strawberry and its derivatives; these were tentatively identified as: monogalloyl diglucose, 5-hydroxy feruroyl hexose, dihydrokaempferol

hexoside, kaempferol neohesperidoside and (-)-chicoric acid. Identification of the latter was confirmed by authentic standard.

Gluconic fermentation left *in vitro* antioxidant activity and non-anthocyanin phenolic composition practically unchanged, except for gallic acid hydroxycinnamics and kaempferol derivatives.

This paper supports gluconic fermentation of strawberry as a process that can produce beverages with a low glucose content and a high content of non-anthocyanin polyphenols, maintaining the antioxidant potential of the strawberry source. This represents an alternative use for strawberry surplus.

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Figure captions

Figure 1: LC-MS of sample P3of substrate A. The numbers indicate the compounds shown in table 2.

Figure 2: LC-DAD chromatogram: (A) at 280 nm and (B) at 320 nm. Peak numbers refer to Table 2.

Figure 3: MRM experiment to support the presence of (-)-chicoric acid, peak 29. Fragmentation pattern of standard at the top and XIC chromatogram to principal ions *m/z* 149 and 311 of samples, displaying retention times at the bottom.

Figure 4: LC-MS/MS chromatogram of an [M-H]- ion (*m/z* 385) tentatively identified as sinapic acid hexose derivative at the top and an [M-H]- ion (*m/z* 463) tentatively identified as quercetin-3-O-glucoside with hypothetic rupture at the bottom (A). LC-MS chromatogram in enhanced resolution (B).

Figure 5: LC-MS/MS of an [M-H]- ion (m/z 371) tentatively identified as 5-hydroxy feruloyl hexose at the top, an [M-H]- ion (m/z 505) tentatively identified as quercetin-3-(6"acetyl glucoside) with hypothetic rupture in the middle, and LC-MS/MS of an [M-H]- 461 identified as kaempferol glucuronide with hypothetic rupture at the bottom (A). LC-MS chromatogram in enhanced resolution (B).

Figure 6: Evolution of antioxidant activity during the process using the ORAC (A) and DPPH (B) methods. Superscript ‘c’ means significant changes between the I and P steps of the same substrate. I (initial step), F (final step) and P (pasteurized step).

µmols TEg-1fw. Micromols of Trolox equivalent per gram of fresh weight.

mmols Tkg-1fw. Millimols of Trolox per kilogram of fresh weight.

I (initial step), F (final step) and P (pasteurized step).

Figure 7: Scatterplot of canonical scores of substrate A, at the top, and substrate B, at the bottom.

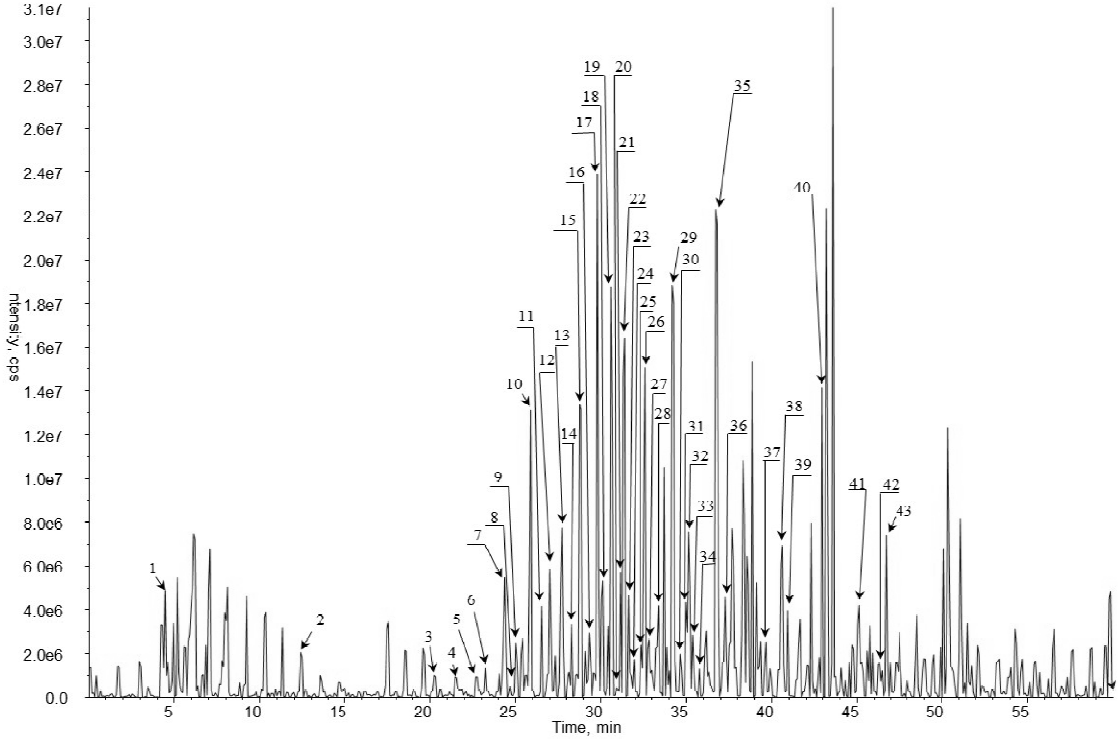

Figure 1 M. Antonia Álvarez-Fernandez

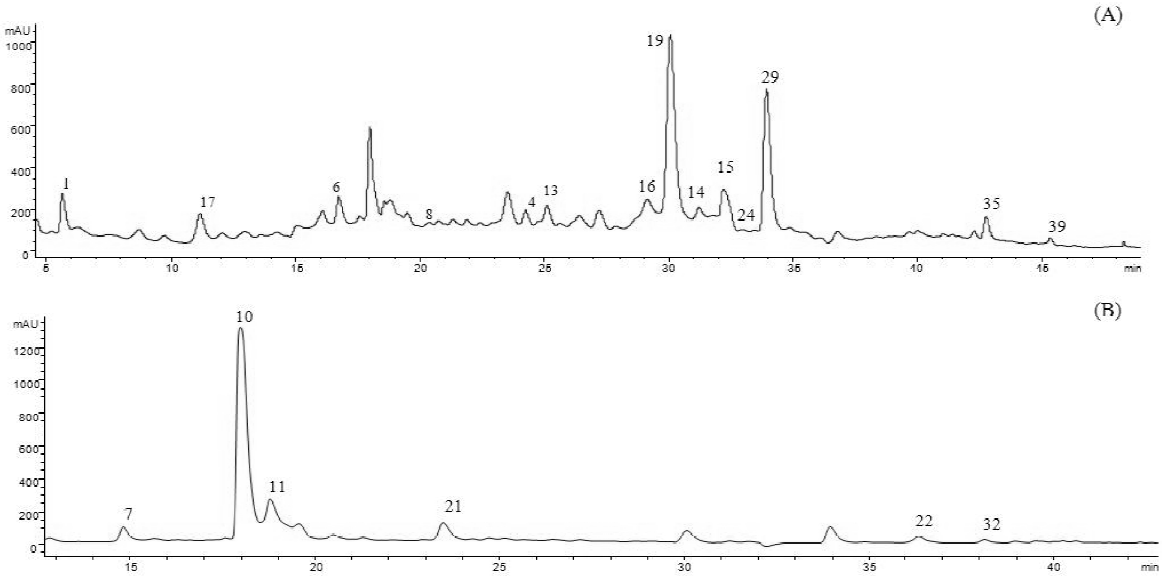

Figure 2 M. Antonia Álvarez-Fernández.

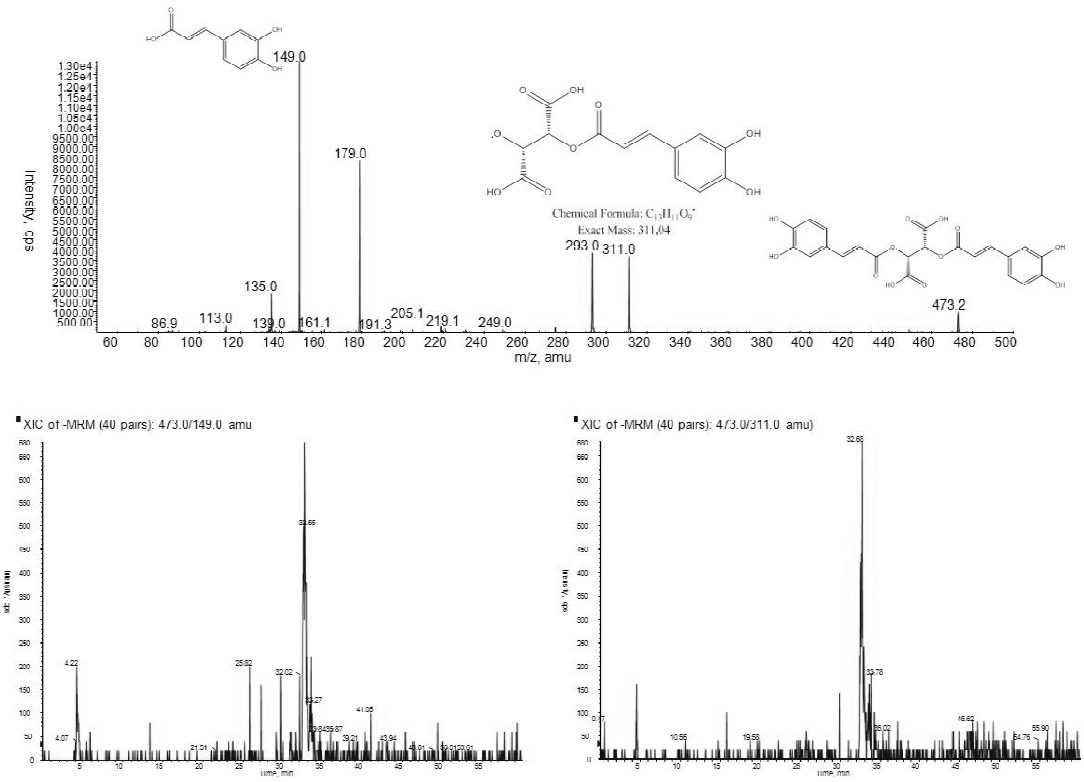

Figure 3 M. Antonia Álvarez-Fernández.

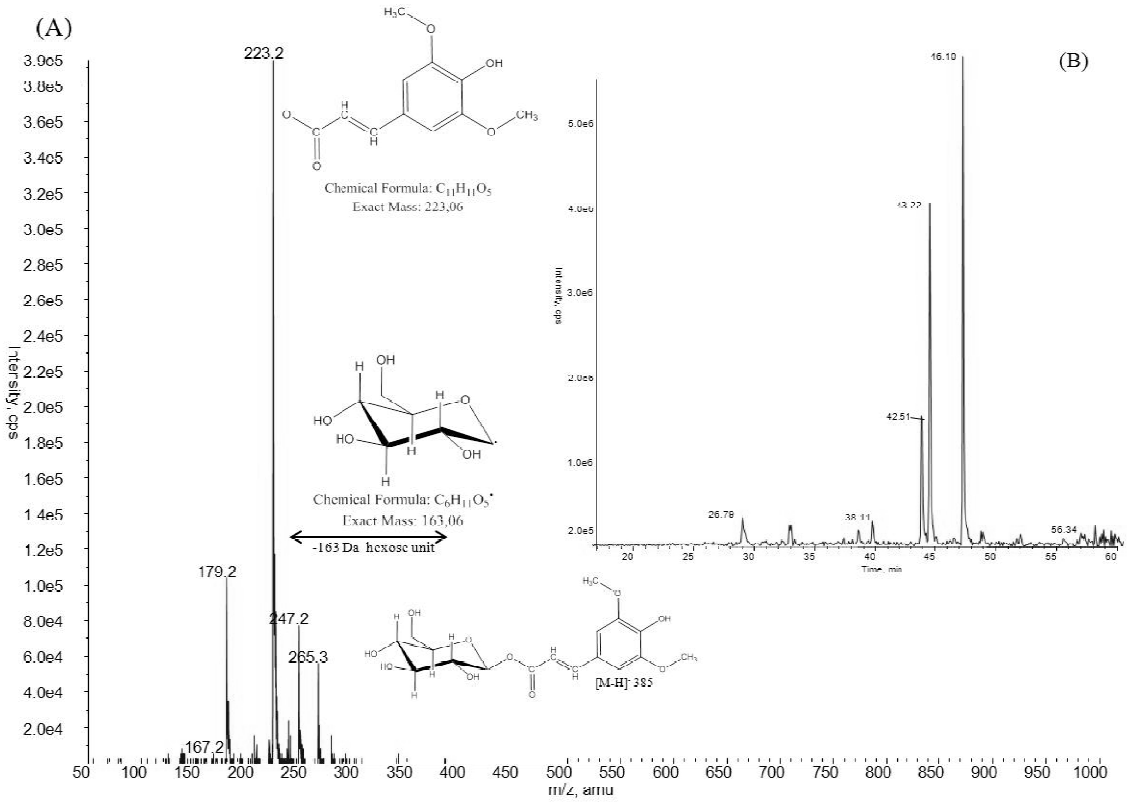

Figure 4. Top. M. Antonia Álvarez-Fernández

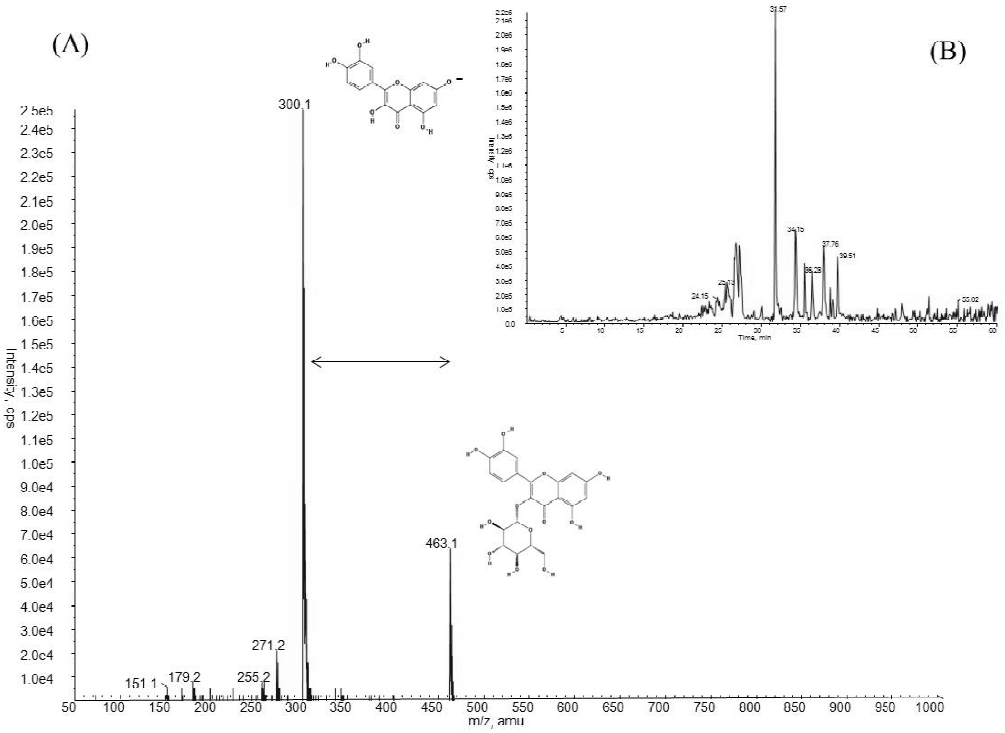

Figure 4 Bottom. M. Antonia Álvarez-Fernández.

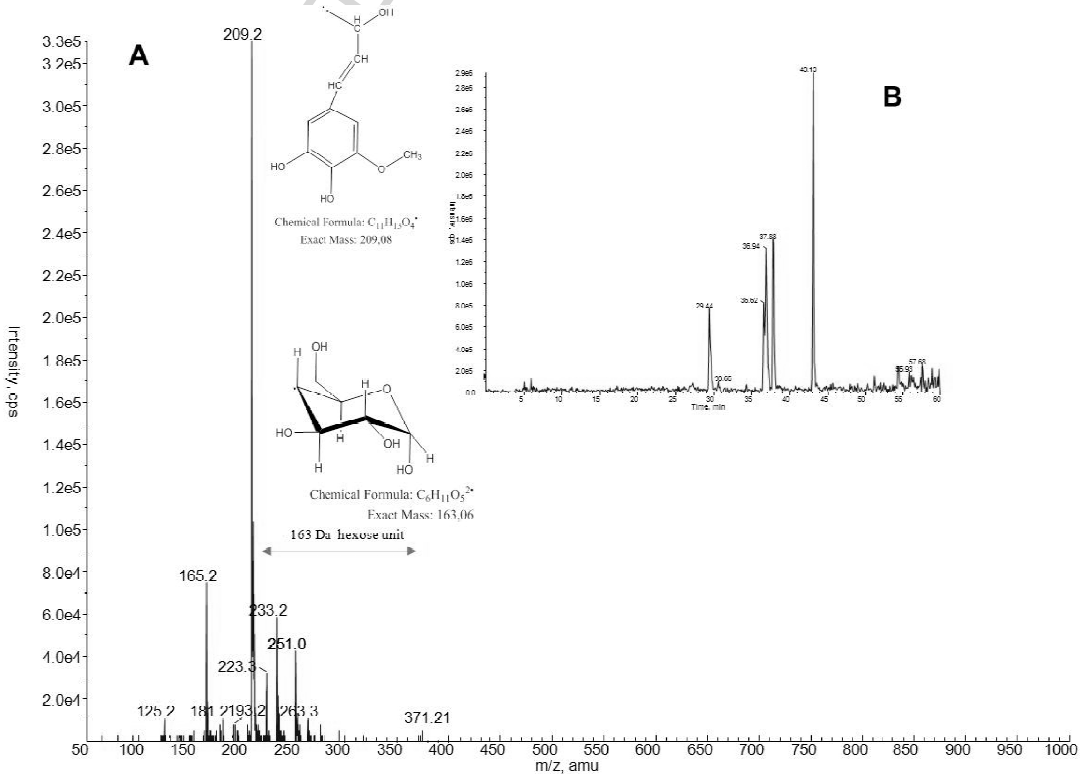

Figure 5. Top. M. Antonia Álvarez-Fernández

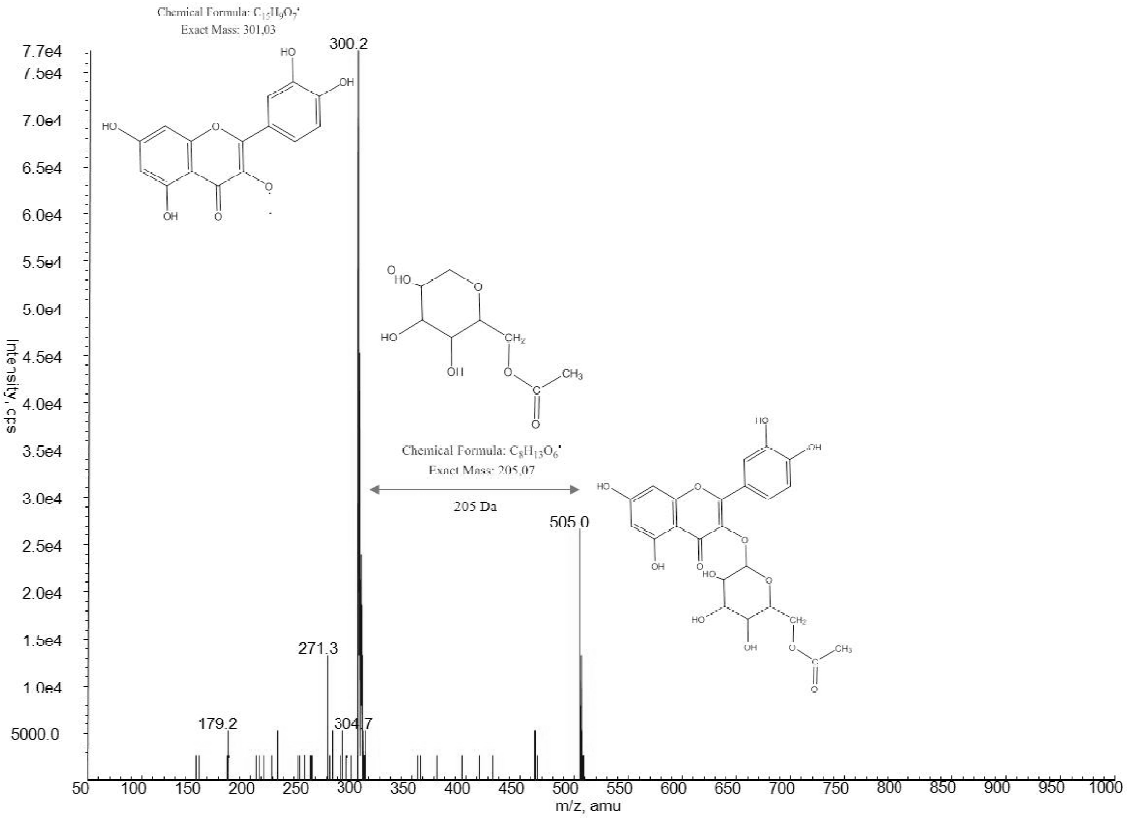

Figure 5. Middle. M. Antonia Álvarez-Fernández

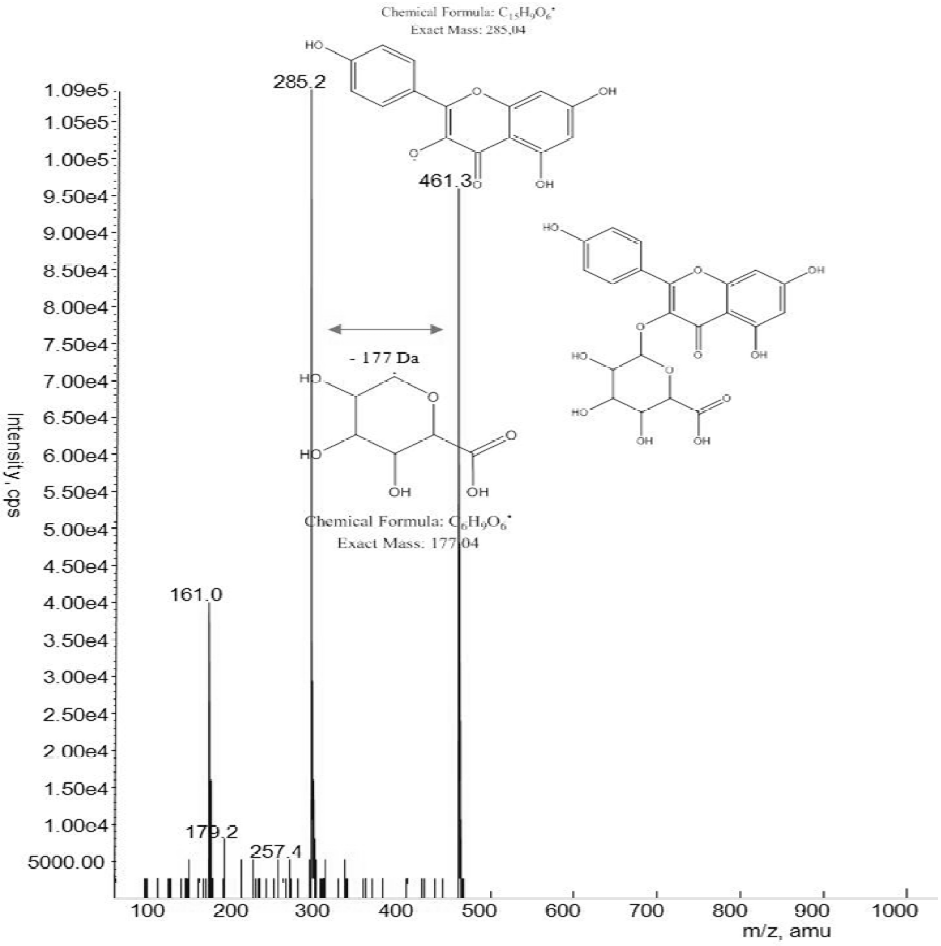

Figure 5 Bottom. M. Antonia Álvarez-Fernández.

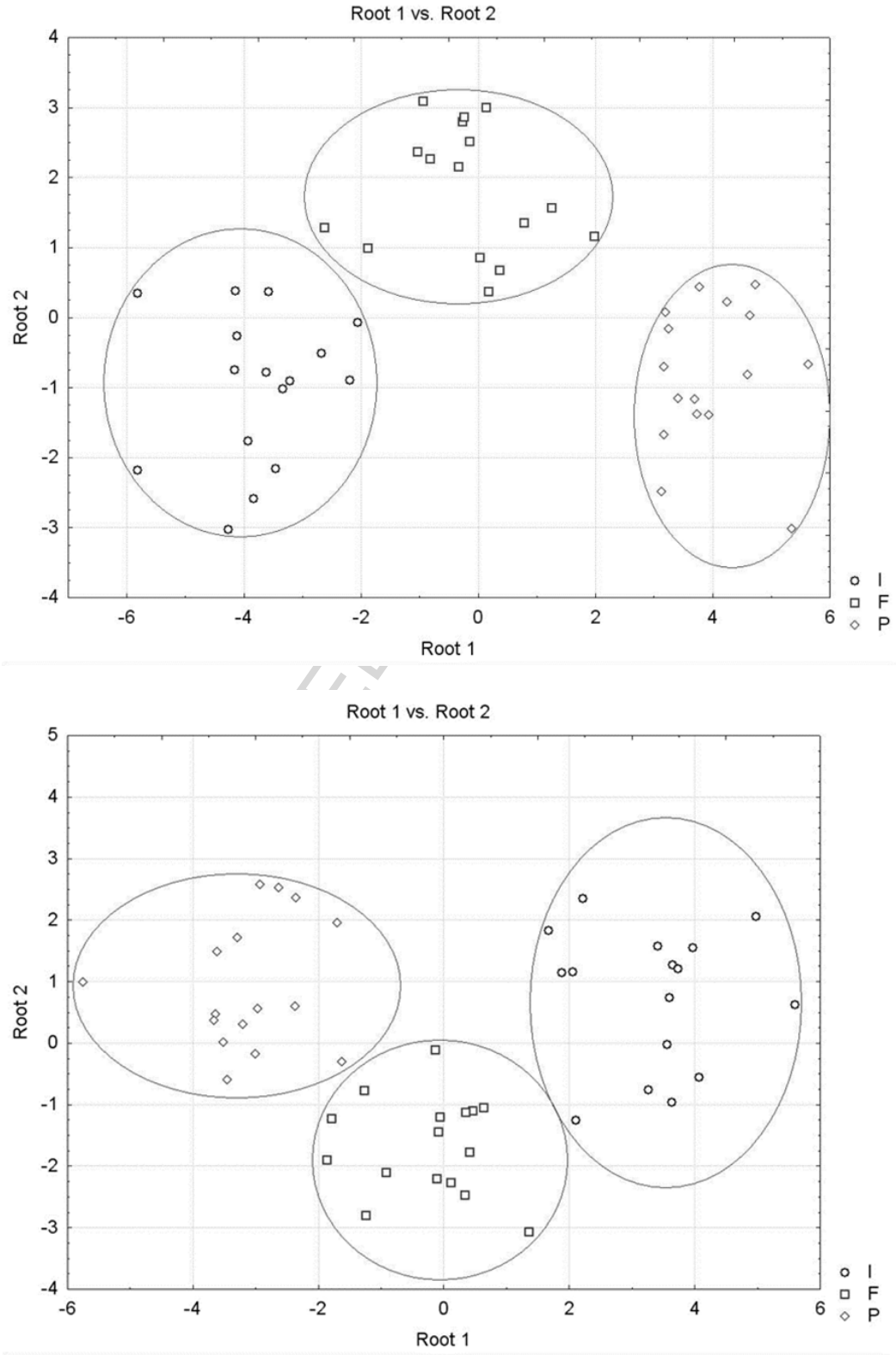

Figure 6 M. Antonia Álvarez-Fernández.

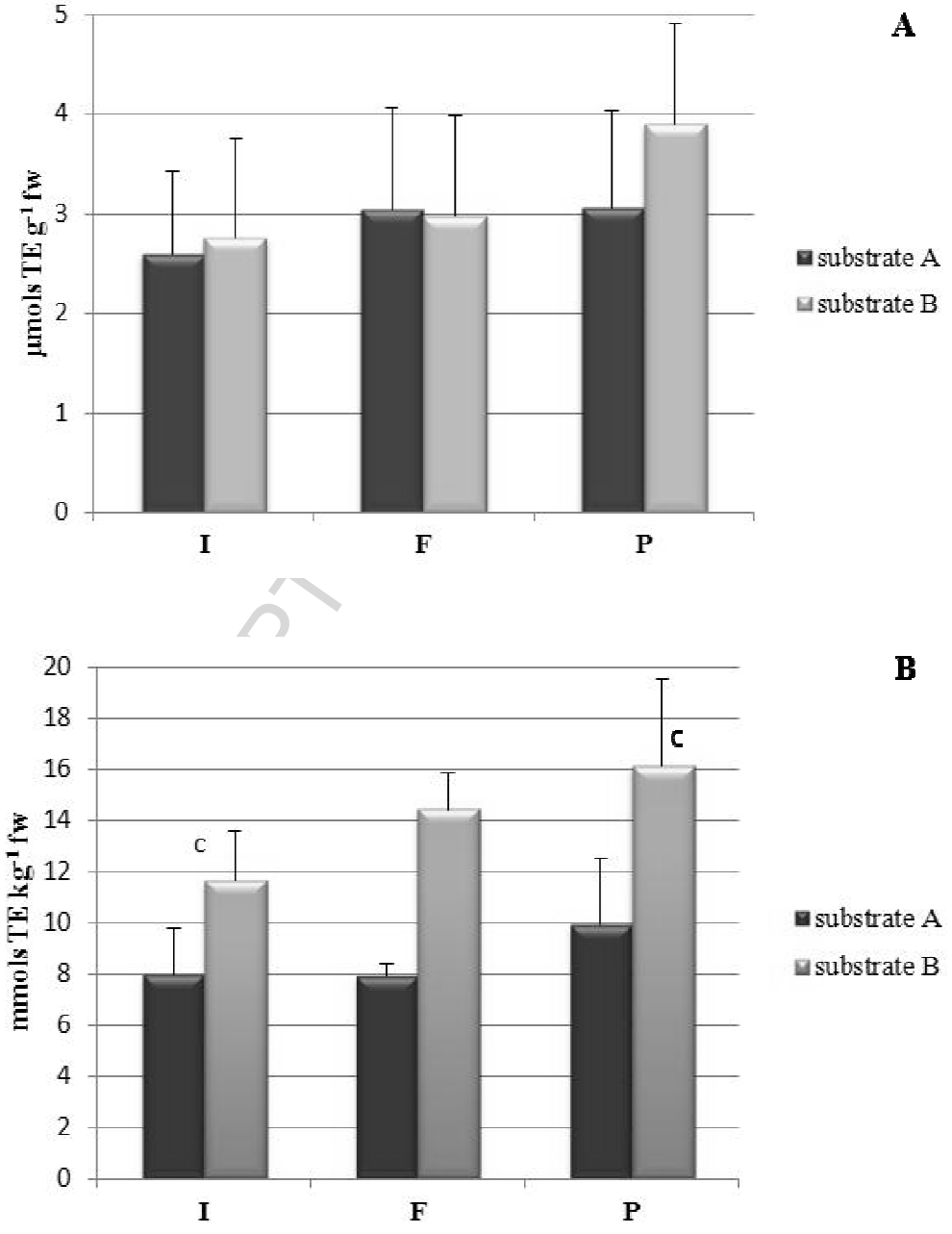

Figure 7 M. Antonia Álvarez-Fernández.

Table 1 Sample codes

Sample code	Name	Sample code	Name
	Substrate A		**Substrate B**
I1	Initial step cycle 1	**I1**	Initial step cycle 1
F1	Final step cycle 1	**F1**	Final step cycle 1
P1	Pasteurized step cycle 1	**P1**	Pasteurized final step cycle 1
I2	Initial step cycle 2	**I2**	Initial step cycle 2
F2	Final step cycle 2	**F2**	Final step cycle 2
P2	Pasteurized final step cycle 2	**P2**	Pasteurized final step cycle 2
I3	Initial step cycle 3	**I3**	Initial step cycle 3
F3	Final step cycle 3	**F3**	Final step cycle 3
P3	Pasteurized final step cycle 3	**P3**	Pasteurized final step cycle 3
I4	Initial step cycle 4	**I4**	Initial step cycle 4
F4	Final step cycle 4	**F4**	Final step cycle 4
P4	Pasteurized final step cycle 4	**P4**	Pasteurized final step cycle 4

Table 2 Identification of non-anthocyanin phenolic compounds by using their UV VIS spectral characteristics, negative ions in LC-MS and MS-MS and respective references

Peak Nº	Identification	MS Rt (min)	λmax (nm)	MW	$(M-H)^-$ (m/z)	Ms-Ms	Detection	Reference	Foods and beverages
Hydroxybenzoic acids derivatives									
1	Gallic acid	4.78	275	170	169	125; 79	DAD-MS	[a]	
5	*p*-Hydroxybenzoic-3-O-glucoside	22.11		300	299	137	MS	Ornelas-Paz et al.(2013)	*Fragaria ananassa*
39	*p*-Hydroxybenzoic acid	41.26		138	137	93; 65	MS	[a]	
Hydrolyzed Tanins									
4	Sangüin H10	21.97	232	784	783	301; 481	MS	Aaby et al. (2007)	*Fragaria ananassa*
14	Galloyl-bis-HHDP-glucose	27.53		936	935	633; 301; 783	MS	Aaby et al. (2007)	*Fragaria ananassa*
17	Castalagin	29.00		934	933	301	MS	Del Bubba et al. (2012)	*Fragaria vesca*
18	Monogalloyl glucose	29.69	280	332	331	313	MS-DAD	Hanhineva et al. (2008)	*Fragaria ananassa* flowers
21	Casuractin/Potentillin	30.23		936	935	633; 301	MS	Del Bubba et al. (2012)	*Fragaria vesca*
30	HHDP-Galloyl glucose	33.00		634	633	375; 301	MS	Aaby et al.(2007)	*Fragaria ananassa*
37	Monogalloyl diglucoside	39.83		494	493	331; 313; 161	MS	Sandhu & Gu. (2010)	*Vitis rotundifolia*
Ellagic acid and derivatives									
15	Ellagic acid pentoside	28.36	252; 375	434	433	300	MS	Del Bubba et al. (2012)	*Fragaria vesca*
16	Ellagic acid deoxyhexoside	28.88	254; 370	448	447	300; 257	MS	Aaby et al. (2012)	*Fragaria ananassa*
25	Ellagic acid	31.09	257; 358	302	301	284; 145	DAD-Ms	[a]	
Flavan 3-ols									
6	(+) Catechin	23.02	280	290	289	245; 109	DAD-MS	[a]	
8	(-) Epicatechin	24.07	282	290	289	245; 109	DAD-MS	[a]	
20	(-) Epicatechin gallate	29.82	280	444	443	289; 169	DAD-MS	[a]	
Condensed Tannins									
9	Procyanidin B1	24.4	280	578	577	288; 406	DAD-MS	[a]	
Hydroxycinnamic acids									
2	Quinic acid	10.33		192	191	111; 87;	MS	Santos et al. (2011)	*Eucalyptus globulus*
7	Cafeoylhexose	23.54		342	341	161; 179	MS	Määtta-Rihinen et al. (2004)	Finnish berries
10	*p*- Coumaroil hexose	25.66	320	326	325	187; 163; 145	DAD-MS	Aaby et al. (2012)	*Fragaria ananassa*
11	Caffeic acid	25.98	320	180	179	135; 107	MS	[a]	
12	Ferulic acid hexose derivative	26.50	320	450	449	287; 269	DAD-MS	Ornelas-Paz et al.(2013)	*Fragaria ananassa*
22	*p*- Coumaric acid	30.42	320	164	163	119; 93	DAD-MS	[a]	
24	Ferulic acid	31.00		194	193	178; 134	MS	[a]	

Table 2 Identification of non-anthocyanin phenolic compounds by using their UV VIS spectral characteristics, negative ions in LC-MS and MS-MS and respective references. Continued

Peak Nº	Identification	MS Rt (min)	λ max (nm)	MW	$(M-H)^-$ (m/z)	Ms-Ms	Detection	Reference	Foods and beverages
Hydroxycinnamic acids									
29	Chicoric acid	32.68		474	473	311; 149	MS	[a]	
38	Cinnamic acid	40.00	282	148	147	103; 77	DAD-MS	[a]	
40	5-Hydroxy feruroyl hexose	43.16		372	371	209; 193	MS	Chandrasekara et al. (2011)	Coffee bean grinder
42	Sinapic acid hexose derivative	46.10		386	385	265; 247; 223	DAD-MS	Ornelas-Paz et al.(2013)	*Fragaria ananassa*
Flavonols									
3	Quercetin rutinoside	21.69		610	609	301; 179; 151	MS	Seeram et al. (2006)	*Fragaria ananassa*
13	Dihydrokaempferol hexoside	26.52		450	449	431; 287; 269; 259; 243; 179	MS	Fischer et al. (2011)	*Punica granatum*
19	Apigenin-7-O- glucoside	29.70		432	431	270; 269; 311	MS	[a]	
23	Kaempferol hexoside	30.66		448	447	284; 255; 227	MS	Del Bubba et al. (2012)	*Fragaria vesca*
26	Luteolin-7-O- glucuronide	31.30		-	461	285; 241	MS	[a]	
27	Kaempferol coumaroyl glucoside	31.47		593	593	447; 284; 285	DAD-MS	Del Bubba et al.(2012)	*Fragaria vesca*
28	Quercetin-3-O-glucoside	31.57		464	463	300; 271; 255; 179; 151	MS	Ornelas-Paz et al.(2013)	*Fragaria ananassa*
31	Kaempferol 3-glucuronide	34.06	268; 350	462	461	285; 179; 161	DAD-MS	Seeram et al. (2006)	*Fragaria ananassa*
32	Quercetin-3-(6"acetyl glucoside)	34.57		506	505	463; 300; 271; 226; 179	MS	Mikulic-Petkovsek et al. (2012) Sun et al. (2014)	*Fragaria vesca*
33	Isorhamnetin glucuronide	34.70		491	315; 300; 271; 255; 113		MS	Hanhineva et al. (2008)	*Fragaria ananassa* flowers
34	Kaempferol-3-malonylglucoside	35.78	268; 348	534	533	285	DAD-MS	Aaby et al. (2007)	*Fragaria ananassa*
35	Kaempferol -7-O-neohesperidoside	36.75		594	593	327; 285; 257; 227; 151	MS	Mikulic-Petkovsek et al. (2012)	
36	Kaempferol acetyl hexoside	37.78		490	489	327; 285; 284; 255	MS	Del Bubba et al. (2012)	*Fragaria vesca*
43	Kaempferol	46.74	270; 375	286	285	117; 93	DAD-MS	[a]	
Stilbenes									
41	*trans*- Piceid	45.10		390	389	185; 227	MS	[a]	

[a] Identification of the compound was confirmed by the authentic standards.

Table 3 Contents of non-anthocyanin phenolic compounds in gluconic ferment of strawberry. Substrate A

Compounds	I1	F1	P1	I2	F2	P2
Gallic acid	10.10±0.045^{a}	13.23±3.55	12.48±1.12	7.3±0.22^{ab}	13.11±0.41^{ac}	11.18±0.13^{bc}
Monogalloyl glucoside	3.47±0.05^{ab}	3.23±0.64^{a}	3.72±0.13^{b}	2.54±0.12^{ab}	4.10±0.33^{a}	4.53±0.50^{b}
Ellagic acid	4.29±0.67^{b}	6.57±4.03	6.49±0.75 ^{b}	5.22±0.27 ^{b}	6.30±0.70	5.28±1.07 ^{b}
Ellagic pentoside	10.35±2.56^{a}	7.79±2.47^{a}	12.41±0.27	9.68±0.37^{a}	13.45±1.21	11.52±1.91
Ellagic deoxyhexoside	5.69±0.13^{a}	4.30±2.47^{a}	6.49±0.40	2.52±0.28	4.48±2.43	6.10±1.91
HHDP-Galloyl glucoside	38.66±8.02	29.88±11.24	36.63±4.15	20.99±3.82^{ab}	41.22±2.82^{a}	40.64±0.64^{b}
Castalagin	10.53±0.34	7.54±2.42	8.50±1.47	6.32±0.16^{ab}	9.30±0.87^{ac}	7.31±0.25^{bc}
Galloyl-bis-HHDP glucoside	5.25±0.62	4.74±1.48	6.35±1.22	4.77±0.32^{ab}	6.50±0.69^{a}	6.42±0.17^{b}
Sanguiin h10	0.77±0.02^{b}	0.89±0.13	0.83±0.03^{b}	0.76±0.16	0.85±0.04^{c}	0.77±0.02^{c}
Procyanidin B1	7.52±3.67	7.99±0.73	11.41±5.14	nd	12.75±3.75	8.83±0.55
(+)-Catechin	108.19±2.87^{a}	97.87±8.32^{a}	117.29±11.88^{b}	84.96±1.25^{b}	91.32±10.97^{c}	125.59±1.48^{bc}
(-)-Epicatechin	8.36±2.27	6.43±1.93	8.12±1.70	7.69±0.40	7.29±1.97	7.58±1.04
(-)-Epicatechin gallate	26.63±6.40	26.90±4.64	34.50±0.76	22.23±4.37^{ab}	32.86±2.39^{a}	33.86±3.47^{b}
Caffeoylhexose	1.93±0.29	2.09±0.06	2.23±0.13	1.91±0.14^{b}	2.35±0.27	2.28±0.07^{b}
p-Coumaroyl hexoside	48.45±0.73^{b}	48.27±14.66	53.86±1.94^{b}	55.37±2.69	53.82±2.06	53.19±0.44
Caffeic acid	12.61±2.95^{b}	13.78±0.31^{c}	7.47±0.33^{bc}	16.51±0.45^{ab}	8.90±0.44^{ac}	5.25±0.17^{bc}
p-Coumaric acid	1.06±0.07	0.95±0.48	1.21±0.08	1.04±0.12^{ab}	1.31±0.08^{a}	1.40±0.06^{b}
Cinnamic acid	0.83±0.04^{b}	0.99±0.29	0.98±0.03^{b}	0.44±0.02^{ab}	0.90±0.09^{a}	1.01±0.02^{b}
Kaempferol 3-hexoside	2.63±0.16	2.71±0.97	3.23±0.34	2.91±0.22^{b}	6.20±0.37	6.10±0.24^{b}
K. malonyl glucoside	0.42±0.13^{b}	0.52±0.47	1.18±0.07^{b}	1.56±0.15^{ab}	1.13±0.11^{ac}	1.83±0.07^{bc}
trans-Piceid derivative	0.63±0.001	0.61±0.03	0.63±0.01	nd	0.63±0.01	0.64±0.002

Tabla 3 Continued

Compounds	I3	F3	P3	I4	F4	P4
Gallic acid	6.3±1.62^{ab}	10.15±0.94^{a}	10.75±1.61^{b}	9.00±0.22	8.00±0.002	8.95±0.003
Monogalloyl glucoside	4.10±0.35	4.88±0.87	4.54±0.51	3.59±0.11^{ab}	6.27±0.09^{a}	4.70±0.87^{b}
Ellagic acid	4.58±0.27	5.85±0.21	5.28±0.77	5.02±0.50^{ab}	5.84±0.70^{a}	6.12±0.80^{b}
Ellagic pentoside	13.86±1.26^{a}	14.01±0.51^{a}	13.53±1.53	16.23±1.25^{b}	15.11±0.41	12.18±0.50^{b}
Ellagic deoxyhexoside	5.99±0.84	5.85±0.17^{c}	5.76±0.08^{c}	5.91±0.46^{b}	7.68±0.32^{c}	8.12±0.80^{bc}
HHDP-Galloyl glucoside	34.03±2.06^{ab}	41.60±1.39^{a}	40.97±0.92^{b}	34.03±5.68	42.27±0.56^{c}	38.82±0.97^{c}
Castalagin	9.92±2.00^{b}	8.58±0.72^{c}	6.46±0.28^{bc}	8.46±0.104^{b}	8.72±0.57^{c}	5.61±0.60^{bc}
Galloyl-bis-HHDP glucoside	5.81±0.35^{ab}	8.25±0.25^{a}	8.25±0.27^{b}	6.96±0.51^{a}	7.87±0.16^{ac}	7.01±0.20^{c}
Sanguiin h10	1.24±0.04^{a}	1.36±0.05^{ac}	1.22±0.04^{c}	1.06±0.02^{ab}	0.95±0.03^{ac}	0.75±0.03^{bc}
Procyanidin B1	15.8±4.34^{ab}	8.20±0.98^{a}	8.47±0.63^{b}	14.62±1.19^{b}	14.24±0.92^{c}	11.22±1.19^{bc}
(+)-Catechin	112.00±7.37	126.49±3.73	111.16±3.39	150.85±5.5^{ab}	139.97±5.12^{ac}	126.42±1.87^{bc}
(-)-Epicatechin	2.78±0.82^{ab}	3.91±0.03^{a}	3.91±0.38	3.28±0.09^{ab}	3.28±0.39^{ac}	3.81±0.21^{bc}
(-)-Epicatechin gallate	35.83±3.32	45.08±4.64	47.65±0.64	45.71±2.79	47.06±1.19	46.72±1.15
Caffeoylhexose	2.11±0.10^{b}	2.11±0.02	2.38±0.06^{b}	2.17±0.11	2.24±0.05^{c}	2.06±0.03^{c}
p-Coumaroyl hexoside	52.11±4.17	49.10±1.48	51.47±1.80	52.21±1.95	51.13±1.95	51.88±1.59
Caffeic acid	9.55±3.94	4.85±0.09	5.22±0.32	3.95±0.16	3.94±0.14^{c}	3.67±0.09^{bc}
p-Coumaric acid	1.06±0.18^{ab}	1.38±0.07^{a}	1.80±0.09^{b}	1.70±0.20	1.89±0.06	2.11±0.06
Cinnamic acid	0.90±0.06^{ab}	0.90±0.04^{a}	1.02±0.02^{b}	0.98±0.06	0.99±0.02^{c}	1.02±0.01^{c}
Kaempferol 3-hexoside	2.75±0.27^{ab}	3.88±0.17^{a}	3.90±0.18^{b}	3.47±0.37^{b}	3.64±0.09^{c}	4.15±0.06^{bc}
K. malonyl glucoside	1.55±0.06^{ab}	1.96±0.13^{a}	1.90±0.08^{b}	2.47±0.19^{b}	2.44±0.05^{c}	2.08±0.07^{bc}
trans-Piceid derivative	0.63±0.01	0.64±0.01	0.64±0.01	0.65±0.007^{b}	0.64±0.01^{c}	0.63±0.002^{bc}

Mean values and standard deviation.

a Superscript letter indicates a significant difference ($p< 0.05$) between the initial samples (I) and final samples (F) of the same substrate.

b Superscript letter indicates a significant difference ($p<0.05$) between the initial samples (I) and pasteurized samples (P) of the same substrate.

c Superscript letter indicates a significant difference ($p<0.05$) between the pasteurized samples (P) and final samples (F) of the same substrate, by ANOVA statistical test.

Samples were determined in duplicate. nd: no data. K: kaempferol.

Table 4 Contents of non-anthocyanin phenolic compounds in gluconic ferment of strawberry. Substrate B

Compounds	I1	F1	P1	I2	F2	P2
Gallic acid	13.91±0.93^{a}	13.37±1.38^{a}	14.37±1.31	nd	nd	nd
Monogalloyl glucoside	5.19±0.60	4.76±0.65	4.13±1.12	5.00±0.30	5.44±0.33^{c}	5.497±0.43
Ellagic acid	3.15±0.33	2.89±0.45	3.26±0.45	2.67±0.59^{ab}	3.80±0.11 ^{a}	3.93±0.30 ^{c}
Ellagic pentoside	8.42±0.49	14.37±0.62	14.52±0.84	10.70±1.16^{a}	10.73±1.65^{a}	10.35±0.93
Ellagic deoxyhexoside	5.08±0.53^{ab}	5.12±0.64^{a}	6.19±0.62^{b}	5.74±1.16	5.76±2.28	6.03±2.28
HHDP-Galloyl glucose	28.90±2.97	35.46±9.55	25.26±2.20	26.83±1.79	26.83±0.55	10.70±1.35
Castalagin	7.72±0.26^{a}	9.59±1.44^{ac}	7.25±1.09^{c}	4.00±0.31^{ab}	6.00±0.14^{a}	4.73±1.57
Galloyl-bis-HHDP glucoside	11.16±1.20^{a}	14. 38±1.42^{a}	11.93±1.65	9.48±0.61	7.96±0.62	8.96±0.69
Sanguiin	1.69±0.41^{a}	2.37±0.28^{a}	2.05±0.14	1.92±0.07^{ab}	2.74±0.25^{a}	2.38±0.76
Procyanidin B1	7.87±2.83	10.29±4.19	11.85±1.21	14.54±2.05^{ab}	8.77±2.09^{a}	4.73±0.63
(+)-Catechin	109.92±1.32	95.74±12.00	99.87±0.13	125.11±2.77^{ab}	94.40±6.21^{a}	74.04±1.57
(-)-Epicatechin	1.99±1.51	1.42±0.56	0.80±0.22	1.80±0.18^{ab}	1.33±0.01^{a}	8.96±0.69
(-)-Epicatechin gallate	20.06±0.28^{a}	31.89±2.25^{ac}	21.11±1.90^{c}	23.15±1.62	26.81±0.26	25.03±0.75^{c}
Caffeoylhexose	4.941±0.13^{ab}	5.59±0.61^{a}	5.54±0.02^{b}	5.77±0.62	6.02±0.71	4.74±0.44
p-Coumaroyl hexoside	41.73±0.66^{b}	40.73±5.38	44.71±2.10^{b}	41.75±1.23^{b}	41.43±2.66^{c}	47.99±0.71^{bc}
Caffeic acid	10.22±0.13	8.98±0.65	4.22±0.69	12.94±2.09^{ab}	13.96±3.81^{a}	17.32±3.24
p-Coumaric acid	4.68±0.62	5.13±0.63	6.04±0.28	2.98±0.08^{a}	1.46±0.80^{a}	1.60±0.17^{b}
Cinnamic acid	1.72±0.17	1.51±0.29	1.51±0.22	nd	nd	nd
Kaempferol 3- hexoside	2.65±0.82	3.21±0.95	3.21±1.00	2.12±0.08	2.24±0.10^{c}	1.22±0.066^{bc}
K. malonyl glucoside	0.96±0.47^{b}	1.40±0.053	2.08±0.94^{b}	1.99±0.95	2.51±0.09	1.23±0.09
trans-Piceid derivative	0.63±0.013	0.65±0.017	0.63±0.009	0.62±0.007	0.62±0.009	0.64±0.007^{c}

Table 4 Continued

Compounds	I3	F3	P3	I4	F4	P4
Gallic acid	9.82±0.82^{a}	11.94±0.73^{ac}	17.31±0.77^{c}	4.89±0.87^{ab}	11.69±1.24^{a}	11.90±0.54^{b}
Monogalloyl glucoside	5.24±0.18^{b}	5.24±0.007	5.43±0.13^{b}	5.44±1.42	4.87±1.67	4.09±0.01
Ellagic acid	3.38±0.29	4.63±0.03	5.74±0.84	4.59±0.29	3.19±0.34	6.80±1.38
Ellagic pentoside	7.78±0.72^{ab}	7.01±0.44^{a}	7.06±0.82^{b}	5.05±0.29	8.14±0.34^{c}	6.63±0.67^{c}
Ellagic deoxyhexoside	4.55±0.87^{ab}	7.01±0.26^{a}	9.16±1.74^{b}	3.64±0.20	3.19±0.24	3.26±0.06
HHDP-Galloyl glucose	28.41±1.73	31.41±1.92	29.13±1.36	13.80±3.93	21.73±0.12	27.09±0.12
Castalagin	4.87±0.30	5.09±0.37	4.76±0.20	9.80±1.73	8.82±0.24	9.30±0.77
Galloyl-bis-HHDP glucoside	10.51±0.37^{ab}	9.95±0.62^{ac}	9.00±0. 20^{bc}	7.42±0.37^{ab}	10.26±0.96^{a}	8.99±0.20^{b}
Sangüin	2.61±0.061	2.60±0.08	2. 71±0.08	2.38±0.46	2.61±0.08	2.71±0.04
Procyanidin B1	13.32±2.05^{ab}	8.76±2.09^{a}	7.33±0.14^{b}	9.31±1.30^{b}	6.16±0.89^{c}	4.51±0.48^{bc}
(+)-Catechin	117.13±5.073^{ab}	80.67±0.61^{a}	81.98±10.73^{b}	87.28±1.91	82.85±0.45	91.20±0.85
(-)-Epicatechin	1.80±0.18^{a}	1.33±0.011^{a}	1.30±0.90	1.61±0.055^{a}	1.74±0.055^{a}	1.33±0.027
(-)-Epicatechin gallate	26.90±0.35	26.24±1.75^{c}	27.71±0.27^{c}	25.03±1.53	27.25±0.80	25.09±0.80
Caffeoylhexose	4.06±0.19^{ab}	4.84±0.08^{a}	4.30±0.27^{b}	4.03±0.03^{b}	4.53±0.08	4.73±0.20^{b}
p-Coumaroyl hexoside	41.01±1.48	43.02±0.74	42.38±1.96	33.57±8.10^{ab}	46.24±0.78^{ac}	52.74±1.10^{bc}
Caffeic acid	21.88±1.21^{ab}	14.94±1.59^{a}	14.04±1.28^{b}	15.28±0.47^{ab}	16.67±0.56^{ac}	16.47±0.74^{bc}
p-Coumaric acid	3.18±0.47	3.35±0.60	3.98±0.60	2.11±0.90	1.72±0.37^{c}	2.50±0.30^{c}
Cinnamic acid	0.87±0.034^{b}	0.95±0.12	1.01±0.08^{b}	0.97±0.26	nd	nd
Kaempferol 3-hexoside	1.70±0.08	1.60±0.10	1.14±0.07	1.60±0.35^{b}	2.21±0.031^{c}	2.26±0.16^{bc}
K. malonyl glucoside	1.91±0.07	1.27±0.69	1.10±0.28	2.19±0.063	3.07±0.02	3.69±0.88
trans-Piceid derivative	0.57±0.001^{ab}	0.61±0.01^{a}	0.61±0.004^{b}	0.57±0.009^{b}	0.58±9.4E-04^{c}	0.59±0.002^{bc}

Mean values and standard deviation.

a Superscript letter indicate significant difference ($p< 0.05$) compared to the initial samples (I) with final (F) of the same substrate.

b Superscript letter indicate significant difference ($p<0.05$) compared to the initial samples (I) with pasteurized (P) of the same substrate.

c Superscript letter indicate significant difference ($p<0.05$) compared to the pasteurized samples (P) with final (F) of the same substrate by ANOVA statistical test .

Samples were determined in duplicate. nd: no data. K: kaempferol.

Table 5 Standardized coefficients for variables used to figure 5, substrate A on the left and B on the right.

Peak	Variable	Root 1	Root 2	Peak	Variable	Root 1	Root 2
1	Gallic acid	0,42783	0,13236	**1**	Gallic acid	-0,99578	-0,00464
18	Monogalloyl glucoside	0,05819	-0,16467	**18**	Monogalloyl glucoside	1,23600	-0,26154
9	Procyanidin B1	-0,24425	-0,32734	**9**	Procyanidin B1	0,86425	-0,35442
6	(+)-Catechin	-5,01555	1,64318	**6**	(+)-Catechin	1,57071	0,16386
8	(-)-Epicatechin	2,02656	-0,44200	**20**	(-)-Epicatechin gallate	-0,32057	1,88966
20	(-)-Epicatechin gallate	-1,02388	-1,07586	**23**	Kaempferol Hexoside	-0,94704	-0,11162
17	Castalagin	0,31782	1,40714	**25**	Ellagic acid	-1,40308	-0,20113
38	Cinnamic acid	3,05724	-0,82542	**34**	Kaempferol malonyl glucoside	0,57928	-0,12685
11	Caffeic acid	-3,52649	0,35410	**10**	*p*-Coumaroyl hexoxide	-1,71284	0,54509
22	*p*-Coumaric acid	1,62820	-0,02735	**16**	Ellagic acid deoxyhexoside	-0,71095	-0,77580
10	*p*-Coumaroyl hexoside	3,33134	-5,33952	**15**	Ellagic acid pentoside	-0,65752	0,46092
23	Kaempferol hexoside	0,91533	0,62591	**17**	Castalagin	1,90291	-2,09041
34	Kaempferol malonyl glucoside	0,02626	0,35060	**8**	(-)-Epicatechin	0,66352	0,34169
16	Ellagic acid deoxyhexoside	0,80389	-0,47424	**38**	Cinnamic acid	-1,23844	1,68992
25	Ellagic acid	-0,39361	1,68129	**30**	HHDP-Galloyl glucoside	0,70976	-0,02075
15	Ellagic acid pentoside	-2,02610	-0,57777	**14**	Galloyl-bis-HHDP glucoside	-0,05498	0,06549
7	Caffeoylhexose	-0,53288	1,70376	**4**	Sanguiin	-0,14522	0,21904
14	Galloyl-bis-HHDP glucoside	0,73210	2,43967	**42**	*trans*-Piceid	0,45134	-0,30412
4	Sanguiin	0,44502	-0,80324	**11**	Caffeic acid	0,79735	0,38949
42	*trans*-Piceid	0,37623	-1,36435	**7**	Caffeoylhexose	0,15845	0,73621
30	HHDP-Galloyl glucoside	-0,99349	0,90542	**22**	*p*-Coumaric acid	0,42381	0,34823
	DPPH	-0,05129	0,25975		DPPH	-0,41912	-0,57453
	ORAC	0,76402	0,81759		ORAC	0,43330	0,17239
	Eigenvalue	10,68040	1,78614		Eigenvalue	7,41508	1,49168
	Cum.Prop	0,85673	1,00000		Cum.Prop	0,83252	1,00000

Industrial relevance:

This paper has industrial relevance in three main aspects:

- As strawberry is a highly perishable product, fruits spoil quickly, leading to substantial economic losses. Therefore, the transformation of strawberry surplus into new, longer-lasting products is a financially worthwhile solution for the industry and producers.
- Our gluconic beverages were elaborated by traditional process using exclusively strawberry as raw material easily transferable to industrial scale.
- The main innovation aspect relies on developing an innovative product, aiming to fulfil consumers demand who are permanently seeking for different taste and flavors. At the same time its composition in sugars (non-glucose, original fructose content) makes these products very suitable for dietetic purposes.

CAPÍTULO TERCERO

Title: Composition of Nonanthocyanin Polyphenols in Alcoholic-Fermented Strawberry Products Using LC–MS (QTRAP), High-Resolution MS (UHPLC-Orbitrap-MS), LC-DAD, and Antioxidant Activity

Author: M. Antonia Álvarez-Fernández, Ana B. Cerezo, Ana M. Cañete-Rodríguez, et al

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BACK CLOSE WINDOW

Composition of non-anthocyanin polyphenols in alcoholic-fermented strawberry products, using LC-MS (QTRAP), high-resolution mass spectrometry (UHPLC-Orbitrap-MS), LC-DAD and antioxidant activity

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Composition of non-anthocyanin polyphenols in alcoholic-fermented strawberry products, using LC-MS (QTRAP), high-resolution mass spectrometry (UHPLC-Orbitrap-MS), LC-DAD and antioxidant activity.

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ABSTRACT

In this study, the non-anthocyanin profile of an alcoholic-fermented strawberry beverage was characterized. High-Performance Liquid Chromatography coupled with a Triple-Quadropole Mass Spectrometer and Ultra-High-Performance Liquid Chromatography (UHPLC) coupled with a Linear Trap Quadropole and an Orbitrap mass analyzer was used to identify non-anthocyanin phenolic compounds.

66 compounds were identified and 13 of these were identified for the first time in strawberry or its derived alcoholic fermented beverage: protocatechuic acid-4-O-ß-hexoside, brevifolin carboxylic acid, ferulic acid glucuronide, dimer caffeic acid-O-hexoside, luteolin-3´-O-xyloside, isorhamnetin3-O-glucoside, taxifolin-O-glucoside, (+)-aromadendrin rhamnoside, eriodictyol-7-O-glucoside, (+)-taxifolin, (+)-aromadendrin, eriodictyol and homovanillic acid.

The alcoholic fermentation process produced significant increases in certain compounds, such as homovanillic acid and p-hydroxybenzoic acid, while a significant decrease in Galloyl bis-HHDP-glucose was observed.

Linear Discriminant Analysis (LDA) correctly classified samples Initial, Final and Pasteurized, leading to the conclusion that alcoholic fermentation induces significant changes in composition, mainly in relation to the 19 compounds represented in the tables.

Keywords:

Alcoholic fermentation, phenolic compounds, metabolomics, *Saccharomyces cerevisiae,* mass spectometry, strawberry, yeast.

1. INTRODUCTION

Strawberry (*Fragaria x ananassa*) is widely consumed thoroughout the world, with the latest FAOSTAT report[1] showing that European production was 1,316,950 T in 2012. The USA is the leading producer globally, with Spain in fourth place. Strawberry has to be consumed shortly after it is harvested, leading to significant economic loss if it is not brought to market quickly. Therefore, it is of great industrial interest to produce innovative drinks from food surpluses that would otherwise be wasted.

The alcoholic fermentation process has been used for many centuries as a means of increasing the stability of processed foods and enhancing the shelf-life and nutritional and organoleptic qualities of food. This process entails the transformation of original substances into secondary metabolites, which can have an impact on the quality of the final product[2].

Strawberry itself is a rich source of micronutrients and phytochemicals, such as flavan-3-ols, proanthocyanins, hydroxybenzoic acids, ellagic acid, tannins, flavonols and stilbenes[3], which results from numerous biological activities. Due to its composition, consumption of this fruit is related to favorable changes in platelet function, HDL cholesterol, and blood pressure, and it has been suggested that it may play a role in preventing cardiovascular disease[4]. The antioxidant properties of strawberry have been mostly attributed to its polyphenol and metabolite content, and they are primarily responsible for its beneficial effects on health[5, 6-8].

The study of metabolite profiling (metabolomic) in fermented beverages using High-Resolution Mass Spectrometry (HRMS) instruments is a powerful tool for discovering changes during the process and predicting the nutritional quality of the final product[9]. The development of rapid technologies has assisted in the growth of metabolomics in food science[10]. Despite this, few studies have been performed on the effects of the

alcoholic fermentation of strawberry on individual non-anthocyanin phenolic compounds.

This article is primarily concerned with characterizing the content of non-anthocyanin polyphenols in alcoholic-fermented strawberry. Additionally, antioxidant activity was measured using DPPH and ORAC methods.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

The standard compounds used were purchased from Fluka Sigma-Aldrich (St. Louis, MO. USA) [gallic acid, caffeic acid, p-coumaric acid, cinnamic acid, (-)-epicatechin, (+)-catechin, chlorogenic acid, ellagic acid, (-)-epicatechin gallate, kaempferol, kaempferol-3-O-ß-D-glucuronide, kaempferol-3-glucoside, polydatin, penta-O-galloyl-ß-D-glucose hydrate, apigenin, quinic acid, brevifolin, protocatechuic acid, isorhamnetin, (+)-taxifolin, eriodictyol, rutin, quercetin, homovanillic acid and kaempferol glucuronide], from Chromadex® Inc. (USA) [procyanidin B1] and from Extrasynthese [luteolin, (+)-aromadendrin, phloridzin, flavonomarein and apigenin-7-O-glucoside]. DPPH (2,2-diphenyl-1-picrylhydrazyl), AAPH (2,2´-diazo-bis-amidine-propane-dihydrochloride) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were obtained from Sigma-Aldrich (St. Louis, MO. USA); acetonitrile, acetic acid, methanol and formic acid (LC gradient) from Merck (Darmstadt, Germany), and methanol from Prolabo® (Obregón, Mexico).

2.2. Samples

Two strawberry purée substrates (A and B) made with production surplus, grown in Huelva (Andalusia, Spain), were used as substrates of the fermentation process; they were provided by Hudisa Company (Lepe, Huelva, Spain). Substrate A corresponds to

the 2011 harvest and B to the 2012 harvest. *Saccharomyces cerevisiae* (CET 13057 isolated from native strawberry yeast)[11] was used as a starter for the submerged fermentation process. The fermentation process was as follows: 3.6 L of strawberry purée were placed into the bioreactor and the conditions set (29 ºC, 26.20 rad.s^{-1}); the medium was saturated with oxygen only at the beginning of the fermentation process, before adding the inoculum [10% (w/v) glucose, 0.1% (w/v) $MgSO_4$, 0.2% (w/v) KH_2PO_4, 0.3% (w/v) $(NH_4)_2SO_4$, 0.4% (w/v) yeast extract and 0.36% (w/v) bacteriological peptone]. At the moment when the inoculum was added, the initial samples were taken. The end of fermentation process was established when the ferment reached an alcoholic strength of 3.02-1.8 to substrate A and B respectively. The fermentation was carry out by batch and final sample was taken, before continuing with another fermentation cycle.

The pasteurization process consisted of heating the sample to 70-80 ºC for 15 min and then rapidly cooling and freezing until analysis.

Samples code show in table 1, and indicate: substrate named as A (more-concentrated purée) or B (less-concentrated purée), the cycles, referred to as 1, 2, 3, 4, and finally the fermentation step I (initial), F (final) and P (pasteurized). Each fermentation cycle encompassed 3 analyzed samples.

A total of 21 fermented samples were analyzed in duplicate. In the case of substrate A, we studied four cycles, and with substrate B, three cycles.

The fermented samples were supplied by the Department of Inorganic Chemistry and Chemical Engineering, Faculty of Science, University of Cordoba, Spain.

2.3. Sample treatment

We followed a previously reported procedure[3, 12] to extract non-anthocyanin polyphenol compounds from the samples.

2.4. LC/DAD, LC/MS/ESI-MS/MS, MRM analysis

2.4.1. LC/DAD analysis

Chromatographic separation was performed using an LC Agilent Series 1100 system with a quaternary pump (Series 1100 G1311A), automatic injector (Series 1100 G1313A) and degasser (series 1100 G1379A). Detection took place using a UV/Vis diode detector (Series 1100 G1379A) coupled to a Chemstation HP A.10.02 (HP/Agilent). The column used was a Merck LiChroCART 250-4 Superspher 100 RP-18 1.16056.0001. The method was a binary gradient and was the same as the one described and validated in [3, 12]Álvarez-Fernández et al. (2014a, b). Each sample was analyzed twice. Identification was achieved by matching the retention time and spectra of the peaks with the corresponding standards. Furthermore, samples were spiked with standards, where commercially available, to achieve complete identification. In accordance with the maximum absorbance of each compound, quantification was performed by external calibration at 280 nm, 320 nm and 365 nm, using the corresponding standards. Calibration curves were obtained by injecting standards diluted from five to eight different concentrations (R^2: 0.9949-0.9998). A duplicate was performed at each point of the calibration curve.

2.4.2. QTRAP analysis

Mass analysis was performed using a PelkinElmer Series 200 HPLC 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 (QqQLIT) mass

spectrometer equipped with an electrospray ion source. LC analyses were performed on a Merck LiChroCART 250-4 Superspher 100 RP-18 1.16056.0001 reversed-phase column. The flow rate was 400 μL min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water, and (B) water: acetonitrile 20:80 (v/v). Both phases contained 0.1% formic acid (v/v). The elution gradient was the same as the one used in the LC/DAD analysis. The injection volume was 20 μL.

A Multiple Reaction Monitoring (MRM) experiment was applied, where precursor ions and fragment ions were monitored at Q1 and Q3, respectively, using their corresponding standards.

For HPLC-ESI-MRM analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, ion spray voltage 4500 V, source temperature 350 ºC and source gas 60 psi. A dwell time was set at 50 ms for each transition.

2.4.3. High-resolution mass spectrometry (HRMS)

The experiments were performed using a Thermo Fisher Scientific (Bremen, Germany) liquid chromatography system hybrid Q-OT-qIT Mass Spectrometer (Hybrid Quadropole-Orbitrap Mass spectrometer). This bench-top UHPLC-MS/MS system combines quadropole precursor ion selection with high-resolution, accurate-mass spectrometer. Identification was performed according to mass spectra, exact mass, characteristic fragmentation and retention time. Chromatographic separation was performed on a Phenomenex Luna C18 (150 x 2.0 mm, 3μm) column. The normalized collision energy of the high-collision-induced-dissociation (CID) cell was set at 20 eV. Twenty μL of sample were injected and flow rate was 250 μL min^{-1}.

Identification in negative ionization mode using a binary gradient consisted of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid 0.0 - 2.0 min 5% B, 2.0 - 40.0 min from 5 to 90% B, 40.0 - 42.0 min from 90% to 5% B, 42.0 - 45.0 5% B.

2.5. Antioxidant activity

2.5.1. ORAC test

The method used was as previously reported, with modifications[13]: 100 μL of fluorescein (45 nM) and 50 μL of AAPH (15 mM) mixed with 50 μL of sample or Trolox. Reaction took place for a total time of 80 min. Fluorescence was assessed with a fluorometer (multi-detector microplate Synergy HT, Biotek®) with excitation, and emission wavelengths were 485 and 528 nm respectively. Trolox was used as a calibration standard (0.5 to 9.5 μM). Fluorescein fluorescence readings were recorded every 5 min after addition of AAPH until they reached less than 5% of the initial reading. Results were calculated using the areas under the fluorescein decay curves between the blank and the sample, and were expressed as μmols Trolox equivalents (TE) per g of fresh weight. The reported values are the means of at least three experiments.

2.5.2. DPPH method

Scavenging activity was also evaluated by DPPH, using a slightly modified version of the literature method[14]. 0.1 mL of sample in methanol (previously diluted 1:250) was added to 3.9 mL of DPPH methanolic solution (0.025 g L^{-1}) in a cuvette. Absorbance was recorded at 515 nm at the start time (when the sample was added) and 60 min later, when the reaction reached equilibrium. All measurements were the averages of at least three independent experiments and methanol was used as the reference. To make the Trolox calibration curve, eight concentrations were used, ranging from 0.9-0.12 mM.

All values reported were recorded on a Hitachi UV-2800 spectrophotometer, thermostated with a Peltier system at 25 ºC and expressed as mmols of Trolox equivalents (TE) per Kg of fresh weight.

2.6. Statistical analysis

Statistical analyses were performed by means of Statistica software[15]. One-way analysis of variance (ANOVA) was used to test significant differences at $p<0.05$ level. Additionally, discriminant analysis was tested, to build a function to discriminate between the substrate (initial), fermented alcohol (final) and final pasteurized samples of all cycles and the two substrates A and B.

3. Result and discussion

3.1. Identification of non-anthocyanin phenolic compounds

66 non-anthocyanin phenolic compounds and citric acid were identified in the strawberry purée and alcoholic-fermented samples. Figure 1 top displays the LC-MS (QTrap) chromatogram and Table 1 presents identification data. If standards were not available, identification was based on (I) retention time, (II) UV/Vis spectra, (III) the deprotonated ion and mass spectra, (IV) accurate mass measurements and (V) comparison with data reported in the literature[16-34]. When standards were available, identification was performed either by MRM or HRMS analysis.

As far as we know, 13 of these were identified for first time in strawberry and its derived alcoholic-fermented products: protocatechuic acid-4-*O*-ß-hexoside (Peak 16), brevifolin carboxylic acid (Peak 35), ferulic acid glucuronide (Peak 2), dimer caffeic acid-*O*-hexoside (Peak 3), luteolin-3´-*O*-xyloside (Peak 27), isorhamnetin-3-*O*-glucoside (Peak 43), taxifolin-*O*-glucoside (Peak 34), (+)-aromadendrin rhamnoside

(Peak 36), eriodictyol-7-*O*-glucoside (Peak 33), (+)-taxifolin, (+)-aromadendrin, eriodictyol and homovanillic acid.

3.1.1. Hydroxybenzoic acids and derivatives

Peak 16 was tentatively identified as protocatechuic acid-4-*O*-ß-hexoside. This compound exhibited a deprotonated $[M-H]^-$ ion at *m/z* 315 and had MS/MS fragments at *m/z* 152 $[M-2H-162]^-$ (loss of glucose moiety) and 108 $[M-H-44]^-$ (loss of CO_2); it has been previously identified in artichoke[18]. Peak 26 was tentatively identified as 1-*O*-protocatechuic-ß-xyloside. It presented a deprotonated ion at $[M-H]^-$ 285 and its fragmentation pattern was in accordance with the one reported in strawberry[33]. In our study, protocatechuic acid-4-*O*-ß-hexoside was detected in strawberry (initial samples), in the alcoholic beverages (final sample) of both substrates, and in pasteurized samples of substrate A only.

3.1.2. Hydrolized tannins

HHDP-Glucose (Peak 4) with pseudo-molecular ion *m/z* 481 produced a fragment at *m/z* 301, indicating the release of ellagic acid; this compound has been reported in strawberry before, by [25]Hanhineva et al. (2008). Peak 28 had a mono-charged pseudo-molecular ion at *m/z* 951 and MS/MS fragmentation with *m/z* 907, 783 and 301. The fragmentation pattern and MW were in agreement with those reported before for tris-galloyl-HHDP-hexose in *Fragaria vesca*[20].

Peak 35 was tentatively identified as brevifolin carboxylic acid (Figure 3). This compound exhibited a deprotonated ion $[M-H]^-$ at *m/z* 291, and its fragmentation pattern had *m/z* 247, 203, corresponding to loss of carboxylic moieties. This compound was identified in all samples of substrate A. The results indicate the presence of this compound in strawberry and its alcoholic-fermented beverages. Brevifolin carboxylic

235 acid has been reported before in pomegranate fruits[22], but as far as we know, it has not
236 yet been determined in strawberry.

237 *3.1.3. Ellagic acid and derivatives*

238 Peak 18 and 52 were tentatively identified as methyl ellagic acid derivatives. Peak 18,
239 with deprotonated $[M-H]^-$ ion 447 and fragmentation with MS/MS *m/z* 315, corresponds
240 to the loss of pentoside residue, (132 Da), and *m/z* 301 resulted from the additional loss
241 of a methyl group. This fragmentation pattern corresponds with methyl ellagic acid
242 pentoside. Methyl ellagic derivatives have been reported before in strawberry[32, 20]. The
243 second peak, number 52, was tentatively identified as dimethyl ellagic acid pentoside
244 with pseudo-molecular ion $[M-H]^-$ 461, after loss of pentose moiety gave rise to *m/z* 315
245 and two fragments, *m/z* 300 and *m/z* 145, corresponding to ellagic acid. Many
246 polyphenolic compounds occur naturally as metoxylated derivatives and exhibit a
247 fragmentation pattern where loss of the methyl fragment from the pseudo-molecular ion
248 is common, resulting in the production of the $[M-H-15]^-$ radical [20].

249 *3.1.4. Hydroxycinnamic acids*

250 Peak 2 was identified as ferulic acid glucuronide. This compound was detected only in
251 the alcoholic fermented product (final samples of two substrates). It had a deprotonated
252 $[M-H]^-$ ion 369 and exhibited a fragment with MS/MS *m/z* 193, resulting from loss of a
253 glucuronide moiety $[M-H-176]^-$. Peak 3 was tentatively identified as a dimer of caffeic
254 acid-*O*-hexoside, described before in *Helichrysum obconicum*[23]. This compound had a
255 pseudo-molecular ion $[M-H]^-$ at 683 and a fragmentation pattern with two fragments,
256 *m/z* 341 and 179, corresponding to caffeic acid hexoside and caffeic acid, respectively.
257 This was detected only in substrate B, in the initial sample (strawberry) and in alcoholic
258 beverages (final samples).

259 *3.1.5. Flavonols*

260 Peak 27 was tentatively identified as luteolin-3´-xyloside with a deprotonated ion at *m/z*
261 417. After neutral loss of the sugar moiety [M-H-132]$^-$, it results in *m/z* 285 and 241
262 fragments, which are characteristic of luteolin aglycone. Peak 43 presented a mono-
263 charged pseudo-molecular ion at *m/z* 955, and exhibited an MS/MS fragmentation
264 pattern, which matched those attributed to isorhamnetin-3-*O*-glucoside in MassBank
265 record PR040094[26] and ReSpect record PT204190[31]. It had a base peak, *m/z* 477, which
266 gave rise to a fragment at *m/z* 301, characteristic of quercetin after cleavage of the
267 molecule. This compound was present in all samples of the two substrates.

268 *3.1.6. Dihydroflavonols*

269 Peak 32 had a mono-charged ion at 449 and was tentatively identified as (+)-
270 aromadendrin hexoside, reported before in strawberry purée[12.] It presented a
271 fragmentation pattern with base peak at *m/z* 431 [M-H-18]$^-$, resulting from the
272 dehydration process. Neutral loss of a hexose moiety [M-H-162]$^-$ gave rise to a
273 fragment at *m/z* 287. The fragment at *m/z* 287 corresponded to (+)-aromadendrin
274 aglycone.

275 Peak 36 was tentatively identified as (+)-aromadendrin rhamnoside in all samples of
276 substrate A. This compound exhibited a deprotonated ion, [M-H]$^-$ 433, and presented a
277 base peak at *m/z* 287, corresponding to (+)-aromadendrin aglycone and resulting from
278 the loss of 146 Da, corresponding to dehydrated rhamnoside moiety. This compound
279 has been described before in *Eucaliptus globulus*[35].

280 Peak 34 was tentatively identified as taxifolin-*O*-glucoside in final and pasteurized
281 samples of substrate A, and initial and final samples of substrate B. It presented a
282 pseudo-molecular [M-H]$^-$ ion at 465 and MS/MS fragmentation with m/z 285, 151. The

283 MW and fragmentation pattern were in accordance with those attributed to taxifolin-*O*-
284 glucoside in grapes and red wine prepared from *Vitis vinifera*[29].

285 *3.1.7. Flavanones*

286 Peak 33 was tentatively identified as eriodictyol-7-*O*-glucoside in all samples of
287 substrate A and initial and final samples of substrate B. It exhibited a deprotonated [M-
288 H]$^-$ ion at 449, which gave rise to *m/z* 287 [M-H-162]$^-$ by neutral loss, corresponding to
289 sugar moiety (162 Da.). This ion corresponded to eriodictyol aglycone and the presence
290 of two fragments, *m/z* 151 and 135, characteristic of eriodictyol, confirmed this tentative
291 identification.

292 *3.1.8. Compounds identified by HRMS*

293 HRMS was used to confirm the identity of compounds with available standards (Figures
294 4 and 5). Eleven compounds were identified with standards, by generating the
295 molecular formula using accurate mass and matching with the isotopic pattern. The
296 results are shown in last section of Table 1. Three of these compounds, protocatechuic
297 acid, *p*-hydroxybenzoic acid and homovanillic acid (Figure 2,) were detected in I, F and
298 P samples and have been reported before as human colonic metabolites of phenolic
299 compounds[36]. Furthermore, increased concentrations of protocatechuic acid and *p*-
300 hydroxybenzoic acid have been reported before due to yeast fermentation in
301 Bokbunja[37].

302 **3.2. Quantitation of non-anthocyanin phenolic compounds in alcoholic fermented**
303 **beverages**

304 Non-anthocyanin phenolic compounds were quantified by external calibration with
305 standards, in accordance with the validated LC-DAD method reported[3]. Tables 3 and 4
306 show the concentrations of the phenolic compounds, with significant increases and

decreases after the alcoholic fermentation process ($p<0.05$). (+)-Catechin content stands out with concentrations ranging from 86.4-144.3 mg kg fw^{-1}, and along with (-)-epicatechin gallate, galloyl-bis-HHDP-glucose and *p*-coumaroyl glucose, it represents 70% of the total quantified compounds for the two substrates (supporting information). The concentration of *p*-Coumaroyl glucose and the contents of the others compound listed above are in accordance with the results of gluconic-fermented products reported[12].

The most abundant compound was galloyl bis-HHDP-glucose. This compound was described as a monomer unit of two of the most abundant ellagitannis in strawberry: agrimoniin and sanguiin H-6[38], and decreased during the alcoholic fermentation process (25-59%) in most of the cycles and for both substrates.

The concentration of Monogalloyl glucose underwent a significant increase, ranging from 20-36%, as a consequence of fermentation.

Homovanillic acid showed significant increases, 3.2-3.6% on average, for the two substrates respectively. Authors, such as Jaganath et al.[39] and Dall'Asta et al.[36], attributed the increment of this compound to be a consequence of the transformation of quercetin, rutin and other quercetin glycosides; they detected this compound as a metabolite derived from human colonic fermentation after application of a diet rich in natural polyphenols. In contrast, in this study, we detected a slight decrease or increase in rutin and quercetin glycosides.

Another compound considered to be a metabolite, produced as a consequence of human colonic fermentation, is *p*-hydroxybenzoic acid[36, 40]. It followed a similar pattern to homovanillic acid and underwent a significant increment in the majority of cycles, in both substrates, in a range of increase of 1.28% - 2.41%, in the two substrates

respectively. Additionally, Vinjanen et al.[41] reported an increase in the content of *p*-hydroxybenzoic acid in yeast fermentation of lingonberries (*Vaccinium vitis-idaea*). It is known that many compounds, such as anthocyanins and phenolics, are stored in fruits in glycosidically bound form, and fermentation-effect-produced glycosidases can release aglycones of this compounds.

3.3. LDA analysis

Quantitated phenolic compounds were used as variables for statistical analysis and grouping variables were: Initial (I), Final (F) and Pasteurized (P) steps. First, ANOVA pinpointed the variables that underwent significant change after the fermentation and/or pasteurization process, as shown in Tables 3 and 4. Secondly, 19 variables presenting significant differences were selected for discriminant analysis. Standard stepwise analysis included the whole set of 19 variables in the model, achieving 98.8% correct classification of samples. The only incorrectly classified sample is one pasteurized sample, classified as final. Figure 6 shows a scatterplot of the canonical roots obtained. As can be observed, F and P samples are closer than I (initial samples), reflecting the composition change produced by alcoholic fermentation.

3.4. Effect of alcoholic fermentation on antioxidant activity

Figure 7 displays the results of antioxidant activity, determined by two methods, ORAC and DPPH, for the two substrates. The ORAC measurements revealed a significant decrease between initial and final samples for substrate B (17.6%). The same trend was observed between initial and pasteurized samples of same substrate (15.2%). DPPH results show that only substrate A changed significantly, following a similar trend to ORAC data. A significant decrease was observed between initial and final samples (12.3%), and a decrease of and 13.9% between initial and pasteurized samples. These

355 results indicated that alcoholic fermentation caused antioxidant activity to decrease
356 slightly; in contrast with reports on gluconic fermentation, no significant changes were
357 detected[12]. Apart from non-anthocyanin polyphenolic compounds, strawberry is a
358 particularly rich source of anthocyanins[42], which are outside the scope of this paper,
359 which is focused on the identification of a large set of non-anthocyanin compounds.
360 Indeed, the extracts for obtaining the non-anthocyanin fraction may present other
361 compounds, which would account for a certain part of the antioxidant activity, which
362 could also explain this average decrease.

363 **4. Conclusions**

364 66 compounds were identified by mass spectrometry and HRMS. A total of 13
365 compounds were identified for the first time in strawberry and an alcoholic-fermented
366 product: protocatechuic acid-4-O-ß-hexoside, brevifolin carboxylic acid, ferulic acid
367 glucuronide, dimer caffeic acid-O-hexoside, luteolin-3´-O-xyloside, isorhamnetin3-O-
368 glucoside, taxifolin-O-glucoside, (+)-aromadendrin rhamnoside, eriodictyol-7-O-
369 glucoside, (+)-taxifolin, (+)-aromadendrin, eriodictyol and homovanillic acid.

370 LDA allows the correct classification of samples I, F and P as alcoholic fermentation
371 induces changes in phenolic composition, mainly related to the 19 compounds.
372 Specifically, homovanillic acid and p-hydroxybenzoic acid increased significantly in
373 almost every fermentation cycle.

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545 **Figure Captions**

546 Figure 1. MS chromatogram of a final sample. The numbers represent the compounds
547 shown in Table 1.

548 Figure 2. LC-MS/MS chromatogram of homovanillic acid. A and B represent the TIC
549 chromatogram of I and P steps of cycle 3 of B substrate, where the increase in signal is
550 shown at 28.03 min after fermentation. C represents the EPI chromatogram with the
551 fragmentation pattern of a homovanillic acid isomer.

552 Figure 3. The XIC and EPI chromatograms (A and B respectively) of brevifolin
553 carboxylic acid and its fragmentation pattern.

554 Figure 4. Mass chromatogram of eriodictyol at 25.65 min. (A) TIC chromatogram of a
555 final sample, () EPI chromatogram with fragmentation.

556 Figure 5. Mass chromatogram of apigenin. (A) TIC chromatogram and (B) EPI
557 chromatogram with fragmentation pattern.

558 Figure 6. Scatterplot of canonical scores in discriminant analysis for 2 substrates and 19
559 variables with 98.8% of classification.

560 Figure 7. Bar representation of antioxidant activity by two methods, ORAC and DPPH,
561 for the two substrates. Letters a, b and c correspond to significant differences between
562 I/F, I/P and F/P samples respectively.

563

564

Tables

Tabla 1. Samples code.

Sample code	Name	Sample code	Name
	Substrate A		**Substrate B**
I1	Initial step cycle 1	**I1**	Initial step cycle 1
F1	Final step cycle 1	**F1**	Final step cycle 1
P1	Pasteurized final step cycle 1	**P1**	Pasteurized final step cycle 1
I2	Initial step cycle 2	**I2**	Initial step cycle 2
F2	Final step cycle 2	**F2**	Final step cycle 2
P2	Pasteurized final step cycle 2	**P2**	Pasteurized final step cycle 2
I3	Initial step cycle 3	**I3**	Initial step cycle 3
F3	Final step cycle 3	**F3**	Final step cycle 3
P3	Pasteurized final step cycle 3	**P3**	Pasteurized final step cycle 3
		I4	Initial step cycle 4
		F4	Final step cycle 4
		P4	Pasteurized final step cycle 4

Table 2. Non-anthocyanin phenolic compounds in alcoholic fermentation samples in negative ionization mode by QTRAP and ORBITRAP. Expected mean, retention time (min), molecular weight, MS/MS fragments and identification. In the last section of the table, mean molecular formula, exact mass, mass accuracy (ppm), MS/MS fragments (% MS/MS base peak, and identification. Peak numbers correspond to mass chromatogram (Figure 1).

Peak Nº	Identification	MS t_R (min)	MW	$(M-H)^-$ (m/z)	Ms/Ms fragments	Ref.
Hidroxibenzoics acids and derivatives						
1	Gallic acid	4.78	170	169	125; 79	a
10	*p*-Hydroxybenzoic acid	25.07	138	137	93; 65	a
16	Protocatechuic acid 4-O-β-hexoside	28.8	316	315	152; 108	18
23	*p*-Hydroxybenzoic-3-O-glucoside	30.72	300	299	137; 93	28
26	1-O-Protocatechuyl-β-xylose	31.7	286	285	152; 108	32
Hydrolized tannins						
4	HHDP-glucose	22.67	482	481	301; 275	25
13	Monogalloyl glucose	26.00	332	331	313; 169; 151	25
20	Bis-HHDP glucose	29.84	784	783	301; 481	16
25	HHDP-Galloyl glucose	31.1	634	633	375; 301	16
28	Tris-galloyl-HHDP-hexose	33.28	952	951	907; 783; 605; 301	20
35	Brevifolin carboxylic acid	38.9	292	291	248; 247; 203	22
37	Galloyl-bis-HHDP-glucose	40.17	936	935	633;783; 301	16
39	Agrimoniin	41.2	935	934	1085; 915; 897;783; 301	32
Ellagic acid and derivatives						
17	Ellagic acid	29.71	302	301	284; 145	a
18	Methyl ellagic acid pentoside	29.79	448	447	315; 331	20
38	Ellagic acid deoxyhexoside	40.9	448	447	300; 257	17
46	Ellagic acid rhamnoside	44.9	448	447	301; 285	20
52	Dimethyl ellagic acid pentoside	47.9	462	461	300; 301; 145	20
56	Ellagic acid pentoside	54.15	434	433	300	20
Flavanols						
5	(+)-Catechin	23.02	290	289	245; 109	a
8	(-)-Epicatechin	24.07	290	289	245; 109	a
19	(-)-Epicatechin gallate	29.82	444	443	289; 169	a
Condensed tannins						
9	Procyanidin B1	24.4	578	577	288; 406	a
Hydroxycinnamic acids						
2	Ferulic acid glucuronide	10.78	370	369	193	30
3	Dimer of caffeic acid -*O*-hexoside	13.07	684	683	341; 179	24
6	Cinnamic acid	23.50	148	147	103; 77	a
7	Cafeoylhexose	23.54	342	341	161; 179	27
11	*p*- Coumaroil hexose	25.66	326	325	187; 163; 145	17
12	Caffeic acid	25.98	180	179	135; 107	a
14	Ferulic acid hexose derivative	26.50	450	449	287; 269	28
15	Galloylquinic acid	27.74	344	343	191; 169	25
22	*p*- Coumaric acid	30.42	164	163	119; 93	a
24	Ferulic acid	31.00	194	193	178; 134	a
29	(-)-Chicoric acid	33.67	474	473	311; 149	a
41	Ferulic acid hexoside	42.6	356	355	217; 193; 175; 160	25
53	5-Hydroxyferuroyl hexose	48.1	372	371	281; 251; 221; 209; 165	25
54	Sinapoyl glycoside	49.9	386	385	265; 223; 179	19

Table 2. Continued.

Peak Nº	Identification	MS t_R (min)	MW	$(M-H)^-$ (m/z)	Ms/Ms	Ref.
Flavonols						
27	Luteolin-3´-xyloside	32.49	418	417	285; 241	[32]
30	Apigenin pentose	35.01	402	401	269; 161	[21]
31	Luteolin-7-*O*- glucuronide	35.35	-	461	285; 241	[a]
40	Apigenin-7-O-glucoside	42.3	432	431	270; 269; 311	[28]
42	Quercetin-3-*O*-glucoside	43.7	464	463	300; 271; 255; 179; 151	[26]
43	Isorhamnetin 3-*O*-glucoside	43.9	956	$[2M-H]^-$ 955	301; 477	[a]
44	Quercetin	44.01	302	301	179; 151	[20]
45	Kaempferol hexoside	44.5	448	447	284; 255; 227	[32]
47	Kaempferol 3-glucuronide	46.8	462	461	285; 179; 161	[25]
48	Isorhamnetin-*O*-glucuronide	47.17	492	491	315; 300; 271; 255; 113	[a]
49	Kaempferol	47.25	286	285	117; 93	[32]
50	Quercetin-3-(6”acetyl glucoside)	47.5	506	505	463; 300; 271; 226; 179	[20]
51	Kaempferol acetyl hexoside	47.82	490	489	327; 285; 284; 255	[20]
55	Kaempferol coumaroyl glucoside	50.05	594	593	447; 284; 285	
Dihydroflavonols						
32	(+)-Aromadendrin hexoside	37.17	450	449	431; 287; 269; 259; 243; 179	[22]
34	Taxifolin- *O*-glucoside	38.5	466	465	285; 151	[29]
36	(+)-Aromadendrin rhamnoside	39.95	434	433	287	[35]
Stilbenes						
21	*trans*- Piceid	30.08	390	389	185; 227	[a]
Flavanones						
33	Eriodyctiol-7-*O*-glucoside	37.65	450	449	287; 151; 135	[26]

Table 2. Continued.

Identification	t_R (min)	Molecular formula	Exact mass [M-H]$^-$	MS/MS fragments (%)	Δmass (ppm)	Ref.
Protocatechuic acid	1.92	$C_7H_6O_4$	153.0193	109.8292(84)	1.3902	[a]
Citric acid isomer	2.77	$C_6H_8O_7$	191.0189	173.0081(2); 111.0074(100); 67.0174(1)	1.5281	32
(+)-Taxifolin	21.04	$C_{15}H_{12}O_7$	303.0510	285.0406(100); 275.0562(10); 177.0184(26); 125.0231(72)	2.8118	[a]
(+)-Aromadendrin	24.39	$C_{15}H_{12}O_6$	287.0562	259.0611(100); 243.0660(19); 125.0231(57)	4.1764	[a]
Rutin	24.75	$C_{27}H_{30}O_{16}$	609.1465	300.0275(32); 301.0355(29); 151.0024(2)	1.6171	[a]
Phloridzin	26.16	$C_{21}H_{24}O_{10}$	435.1294	273.0769(100); 167.0339(17); 125.0230(3)	0.9055	[a]
Eriodictyol	27.64	$C_{15}H_{12}O_6$	287.0562	151.0025(100); 135.0439(32); 125.0231(6)	3.6448	[a]
Homovanillic acid	28.03	$C_9H_{10}O_4$	181.0497	137.0232(66); 89.0230(64); 71.0124(82); 59.0124(100)	0.8981	[a]
Quercetin	30.16	$C_{15}H_{10}O_7$	301.0352	178.9976(22); 151.0025(32)	3.0153	[a]
Luteolin	32.83	$C_{15}H_{10}O_6$	285.0404	241.0503(1); 165.0181(3); 225.0550(1); 151.0024(1); 117.0330(1)	4.2938	[a]
Apigenin	33.24	$C_{15}H_{10}O_5$	269.0455	225.0550(1); 151.0024(1); 117.0330(1)	4.4767	[a]

[a] Identification of the compound was confirmed by the authentic standards.
Ref. reference

Table 3. Contents of non-anthocyanin phenolic compounds (mg kg fw^{-1}) in alcoholic fermentation samples of substrate A.

	Cycle 1			Cycle 2		
Compounds	**I**	**F**	**P**	**I**	**F**	**P**
(-)-Epicatechin	3.30±0.29^{ab}	5.05±0.71^{a}	4.99±0.68^{b}	4.77±0.35	4.87±0.24	4.88±0.19
Eriodictyol-7-*O*-glucoside	1.16±0.12^{ab}	2.88±0.16^{ac}	2.63±0.08^{bc}	0.90±0.20^{ab}	1.17±0.07^{ac}	1.77±0.05^{bc}
Caffeoyl hexose	1.81±0.01^{ab}	3.32±0.05^{ac}	3.53±0.03^{bc}	1.86±0.03^{ab}	2.99±0.02^{ac}	2.52±0.04^{bc}
Ferulic acid derivative	1.26±0.05^{ab}	1.57±0.03^{ac}	1.68±0.03^{bc}	1.50±0.03	1.47±0.05^{c}	1.17±0.01^{c}
Galloyl *bis*-HHDP-glucose	157.25±6.55^{ab}	95.29±1.94^{ac}	144.80±1.17^{bc}	165.44±4.52^{ab}	123.77±1.54^{ac}	127.15±0.52^{bc}
HHDP-glucose	2.85±0.44	2.37±0.20^{c}	3.13±0.18^{c}	4.64±0.67^{b}	5.52±1.65^{c}	3.29±0.25^{bc}
Homovanillic acid	9.40±0.55^{ab}	11.11±0.33^{ac}	3.76±1.04^{bc}	12.81±1.48^{ab}	20.09±1.22^{ac}	3.058±0.39^{bc}
Isorharmentin glucose	3.30±0.14^{a}	4.79±0.20^{ac}	3.48±0.21^{c}	3.80±0.13^{ab}	4.47±0.12^{a}	4.30±0.10^{b}
Kaempferol glucuronide	2.52±0.18^{ab}	3.72±0.17^{a}	3.80±0.10^{b}	2.41±0.26^{b}	2.67±0.45	3.01±0.08^{b}
Luteolin-3´-xylose	2.87±0.05^{ab}	1.92±0.18^{a}	2.02±0.04^{b}	2.17±1.08	1.23±0.10	1.26±0.04
Methyl ellagic acid	1.23±0.13^{ab}	2.22±0.08^{ac}	2.78±0.05^{bc}	2.79±0.22^{a}	3.15±0.15^{ac}	2.86±0.08^{c}
Monogalloyl glucose	5.97±2.12^{b}	7.98±0.74^{c}	9.24±0.17^{bc}	7.46±0.21^{a}	8.64±0.52^{ac}	7.58±0.22^{c}
p-Coumaroyl hexoside	73.37±0.94^{ab}	77.17±2.13^{ac}	83.07±0.32^{bc}	82.80±2.64^{ab}	84.39±0.76^{a}	70.32±1.19^{b}
Phloridzin	1.54±0.07^{ab}	3.92±0.43^{a}	3.97±0.04^{b}	2.09±0.29^{b}	2.67±0.64	3.25±0.05^{b}
p-Hydroxybenzoic acid	1.51±0.41	1.34±0.12^{c}	1.71±0.04^{c}	0.87±0.43^{a}	3.61±0.68^{ac}	1.41±0.10^{c}
Procyanidin B1	18.12±1.65^{b}	18.25±1.49^{c}	13.07±1.04^{bc}	17.85±1.47^{ab}	11.38±0.95^{ac}	13.08±0.98^{bc}
Protocatechuic acid	0.34±0.16	0.76±0.35^{c}	0.27±0.09^{c}	0.20±0.08^{b}	0.27±0.11^{b}	nd
Quercetin glucoside	1.00±0.07^{ab}	1.36±0.05^{ac}	0.50±0.09^{bc}	1.03±0.07^{ab}	1.17±0.08^{ac}	0.73±0.03^{bc}
Quercetin glucuronide	1.00±0.03^{ab}	1.21±0.09^{ac}	1.51±0.05^{bc}	1.57±0.21	1.62±0.16	1.50±0.02

Table 3. Continued

	Cycle 3		
Compounds	**I**	**F**	**P**
(-)-Epicatechin	4.26±0.10[a]	4.65±0.18[a]	4.39±0.33
Eriodictyol-7-*O*-glucoside	0.90±0.00[ab]	1.23±0.01[ac]	0.98±0.02[bc]
Caffeoyl hexose	1.86±0.00[ab]	3.19±0.04[ac]	2.98±0.13[bc]
Ferulic acid derivative	0.57±0.05[ab]	1.44±0.05[ac]	0.99±0.01[bc]
Galloyl *bis*-HHDP-glucose	145.07±10.06[b]	130.48±6.78[b]	124.00±2.91
HHDP-glucose	3.83±0.46[a]	4.94±0.19[ac]	4.02±0.21[c]
Homovanillic acid	3.06±0.39[ab]	10.30±0.20[ac]	8.79±0.75[bc]
Isorharmentin glucose	4.00±0.19[ab]	5.49±0.16[a]	3.38±0.42[b]
Kaempferol glucuronide	2.44±0.30[a]	3.69±0.06[a]	3.04±0.62
Luteolin-3′-xylose	1.15±0.08	1.22±0.05[c]	0.99±0.15[c]
Methyl ellagic acid	1.33±0.07[ab]	3.74±0.11[ac]	3.19±0.31[bc]
Monogalloyl glucose	6.05±0.07[ab]	8.23±0.29[ac]	7.19±0.36[bc]
p-Coumaroyl hexoside	77.81±5.47	79.05±0.91	72.36±5.47
Phloridzin	2.64±0.22[a]	4.22±0.03[ac]	3.37±0.61[c]
p-Hydroxybenzoic acid	6.06±0.62[b]	5.87±0.43[c]	5.33±0.50[bc]
Procyanidin B1	8.35±1.52[ab]	4.31±0.97[ac]	2.46±0.58[bc]
Protocatechuic acid	nd	6.12±0.25	6.34±0.81
Quercetin glucoside	0.75±0.03[ab]	0.55±0.05[a]	0.58±0.11[b]
Quercetin glucuronide	1.52±0.10[a]	0.99±0.02[ac]	1.83±0.41[c]

Mean values and standard deviation.

[a] Superscript letter indicates a significant difference ($p < 0.05$) between the initial samples (I) and final samples (F) of the same substrate.

[b] Superscript letter indicates a significant difference ($p<0.05$) between the initial samples (I) and pasteurized samples (P) of the same substrate.

[c] Superscript letter indicates a significant difference ($p<0.05$) between the pasteurized samples (P) and final samples (F) of the same substrate, by ANOVA statistical test. Samples were determined in duplicate.

nd: no data.

Table 4. Contents of non-anthocyanin phenolic compounds (mg kg fw^{-1}) in alcoholic fermentation samples of substrate B

	Cycle 1			Cycle 2		
Compounds	**I**	**F**	**P**	**I**	**F**	**P**
(-)-Epicatechin	3.36±0.07^{ab}	1.81±0.63^{a}	nd	2.27±0.14^{b}	2.31±0.22^{c}	nd
Eriodictyol-7-*O*-glucoside	0.43±0.03^{ab}	0.54±0.07^{ac}	0.82±0.08^{bc}	0.90±0.08^{ab}	0.32±0.03^{a}	0.26±0.04^{b}
Caffeoyl hexose	1.92±0.02^{ab}	2.84±0.04^{ac}	2.61±0.04^{bc}	nd	3.38±0.13	3.30±0.34
Ferulic acid derivative	1.72±0.09^{b}	1.57±0.07^{c}	1.42±0.03^{bc}	1.56±0.04	1.68±0.11	1.61±0.24
Galloyl *bis*-HHDP-glucose	172.93±6.31^{b}	175.30±13.31^{c}	208.15±13.65^{bc}	156.51±2.96^{b}	134.04±12.29^{c}	103.03±1.06^{bc}
HHDP-glucose	9.44±0.30^{ab}	7.22±0.88^{ac}	2.35±0.55^{bc}	3.82±0.62^{ab}	8.47±0.60^{ac}	2.73±0.44^{bc}
Homovanillic acid	5.32±0.38^{ab}	23.73±3.20^{a}	20.25±0.69^{b}	3.39±0.27^{ab}	16.83±1.23^{ac}	13.26±1.04^{bc}
Isorharmentin glucose	3.28±0.05^{a}	4.33±0.41^{a}	4.50±0.34^{b}	2.16±0.08^{ab}	4.02±0.36^{ac}	2.70±0.19^{bc}
Kaempferol glucuronide	1.64±0.13	1.64±0.19	1.65±0.13	1.73±0.09	1.87±0.16	1.88±0.10
Luteolin-3´-xylose	2.16±0.20^{ab}	2.73±0.30^{a}	3.01±0.05^{b}	2.35±0.05^{ab}	0.85±0.02^{ac}	0.54±0.10^{bc}
Methyl ellagic acid	1.19±0.06^{b}	1.16±0.02^{c}	1.36±0.04^{bc}	1.14±0.02^{b}	1.17±0.10^{c}	0.99±0.05^{bc}
Monogalloyl glucose	4.68±0.57	4.26±0.48^{c}	4.11±0.11^{c}	4.45±0.27^{a}	5.59±0.41^{a}	5.54±0.09
p-Coumaroyl hexoside	82.17±1.05^{ab}	76.81±2.62^{ac}	70.96±1.26^{bc}	79.99±1.25^{a}	84.78±2.61^{a}	81.12±7.55
Phloridzin	0.32±0.02^{ab}	1.19±0.41^{a}	0.82±0.25^{b}	1.11±0.07^{ab}	0.41±0.04^{a}	0.36±0.11^{b}
p-Hydroxybenzoic acid	1.91±0.05^{ab}	3.45±0.82^{a}	3.73±0.43^{b}	1.65±0.21^{ab}	6.64±1.02^{ac}	4.51±0.89^{bc}
Procyanidin B1	38.36±6.95^{ab}	9.30±0.44^{a}	8.99±0.37^{b}	37.26±3.89^{ab}	19.02±0.82^{a}	19.75±1.82^{b}
Protocatechuic acid	2.24±0.53	2.77±0.56	2.36±0.34	1.90±0.33^{ab}	3.69±1.36^{a}	3.70±1.13^{b}
Quercetin glucoside	1.53±0.09^{b}	1.08±0.37^{c}	1.81±0.20^{bc}	1.54±0.05^{a}	1.80±0.13^{ac}	1.50±0.14^{c}
Quercetin glucuronide	1.19±0.06^{b}	1.32±0.13	1.40±0.04^{b}	1.16±0.02^{ab}	1.66±0.07^{ac}	1.29±0.03^{bc}

Table 4. Continued.

	Cycle 3			Cycle 4		
Compounds	**I**	**F**	**P**	**I**	**F**	**P**
(-)-Epicatechin	2.17±0.15^{ab}	1.64±0.13^{a}	1.73±0.04^{b}	4.95±0.36^{ab}	3.28±0.60^{a}	3.24±0.10^{b}
Eriodictyol-7-*O*-glucoside	0.29±0.05^{ab}	0.15±0.01^{ac}	0.10±0.01^{bc}	0.91±0.06^{ab}	0.55±0.02^{ac}	0.68±0.03^{bc}
Caffeoyl hexose	1.92±0.03^{ab}	2.15±0.02^{ac}	2.21±0.03^{bc}	1.85±0.01^{ab}	2.01±0.04^{ac}	2.07±0.01^{bc}
Ferulic acid derivative	1.19±0.11^{ab}	1.48±0.05^{a}	1.45±0.02^{b}	1.05±0.02^{b}	1.04±0.02^{c}	0.72±0.06^{bc}
Galloyl *bis*-HHDP-glucose	175.91±12.83^{ab}	151.27±7.99^{ac}	118.64±3.80^{bc}	85.01±5.75^{ab}	35.35±4.25^{a}	35.77±1.84^{b}
HHDP-glucose	2.11±0.52^{ab}	1.28±0.08^{a}	1.28±0.05^{b}	2.20±0.05^{b}	2.31±0.13^{c}	1.72±0.26^{bc}
Homovanillic acid	2.24±0.31^{ab}	6.30±0.08^{a}	6.14±0.29^{b}	4.55±0.13^{ab}	9.81±1.02^{a}	10.94±0.25^{b}
Isorharmentin glucose	3.51±0.33^{a}	4.63±0.19^{ac}	3.51±0.04^{c}	3.26±0.11^{ab}	3.75±0.24^{ac}	4.42±0.05^{bc}
Kaempferol glucuronide	1.87±0.33	2.15±0.14^{c}	1.75±0.11^{c}	3.85±0.11	3.82±0.11^{c}	3.50±0.17^{c}
Luteolin-3´-xylose	1.77±0.27^{b}	1.96±0.09^{c}	1.40±0.06^{bc}	0.30±0.02^{ab}	0.18±0.01^{ac}	0.10±0.04^{bc}
Methyl ellagic acid	1.13±0.11^{ab}	1.68±0.07^{ac}	1.55±0.03^{bc}	2.02±0.24	1.65±0.11	1.86±0.14
Monogalloyl glucose	4.77±0.51	5.68±0.20	5.92±0.02	3.13±0.11^{a}	2.79±0.23^{ac}	3.33±0.17^{c}
p-Coumaroyl hexoside	77.87±1.14^{ab}	68.87±2.63^{a}	69.93±0.53^{b}	53.63±2.64^{b}	53.29±2.68^{c}	47.74±1.53^{bc}
Phloridzin	0.15±0.02^{ab}	1.76±0.13^{a}	1.62±0.02^{b}	0.35±0.25^{ab}	0.93±0.03^{a}	0.93±0.10^{b}
p-Hydroxybenzoic acid	1.23±0.24^{ab}	2.24±0.18^{a}	2.27±0.12^{b}	1.14±0.15^{ab}	1.99±0.05^{ac}	2.85±0.22^{bc}
Procyanidin B1	24.99±1.44^{ab}	17.84±1.64^{a}	15.78±0.08^{b}	11.59±4.54^{ab}	6.60±0.16^{ac}	6.16±1.52^{bc}
Protocatechuic acid	1.01±0.30	1.92±0.15	0.78±0.08	0.72±0.05^{ab}	0.48±0.03^{ac}	0.63±0.06^{bc}
Quercetin glucoside	1.56±0.14^{b}	1.68±0.11	1.38±0.04^{b}	0.72±0.40	0.52±0.06^{c}	0.42±0.01^{c}
Quercetin glucuronide	1.51±0.11^{ab}	1.70±0.10^{a}	1.30±0.03^{c}	0.81±0.03^{b}	0.81±0.03^{c}	0.73±0.04^{bc}

Mean values and standard deviation.

[a] Superscript letter indicates a significant difference ($p< 0.05$) between the initial samples (I) and final samples (F) of the same substrate.

[b] Superscript letter indicates a significant difference ($p<0.05$) between the initial samples (I) and pasteurized samples (P) of the same substrate.

[c] Superscript letter indicates a significant difference ($p<0.05$) between the pasteurized samples (P) and final samples (F) of the same substrate, by ANOVA statistical test.

Samples were determined in duplicate.

nd: no data.

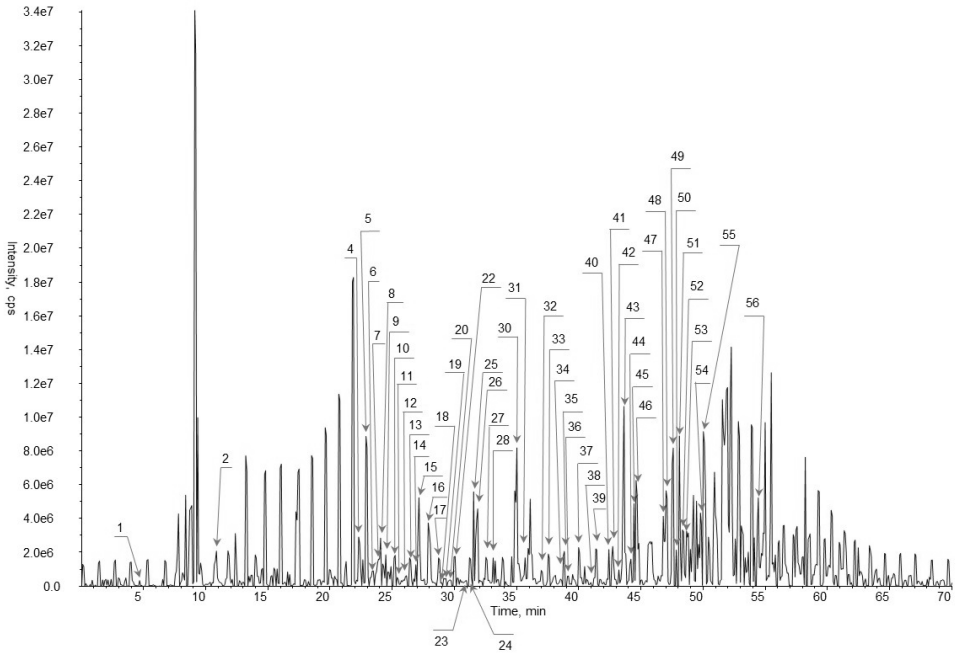


Figure 1. Álvarez-Fernández et al

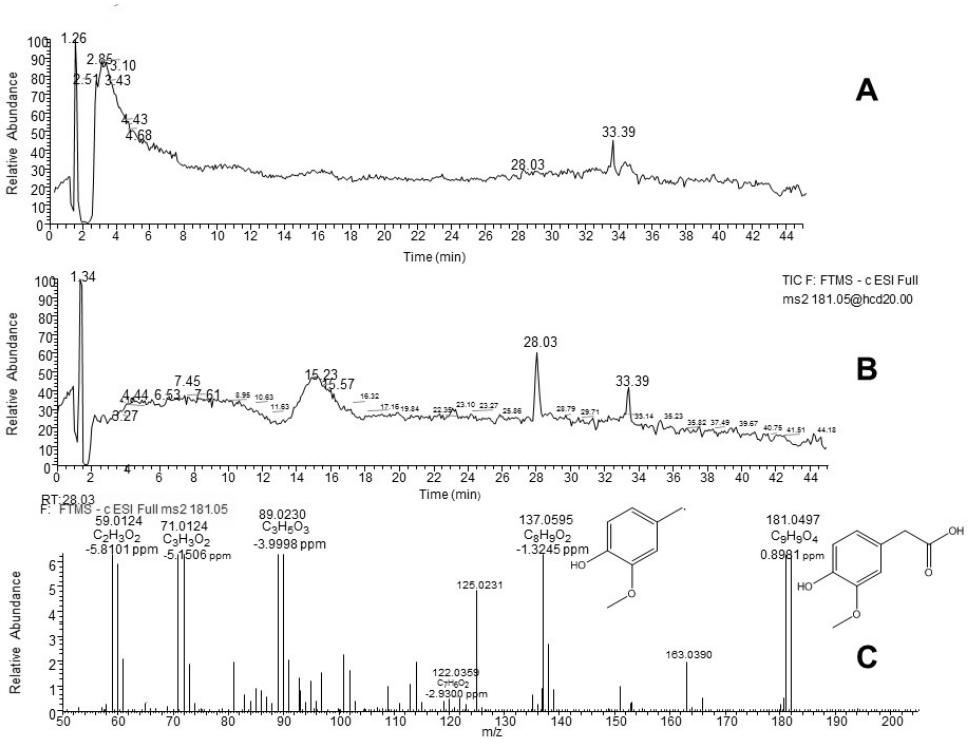


Figure 2. Álvarez-Fernández et al

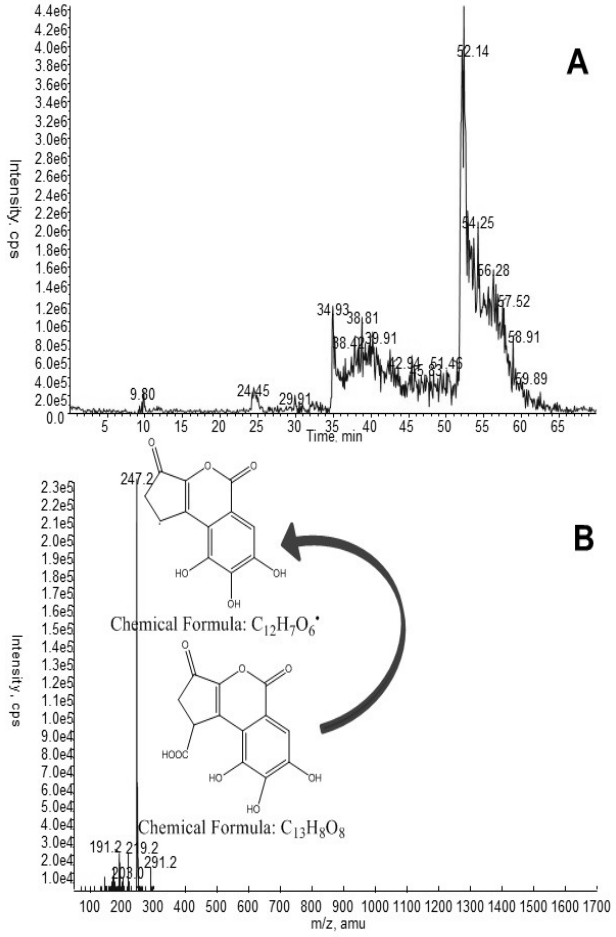


Figure 3. Álvarez-Fernández et al

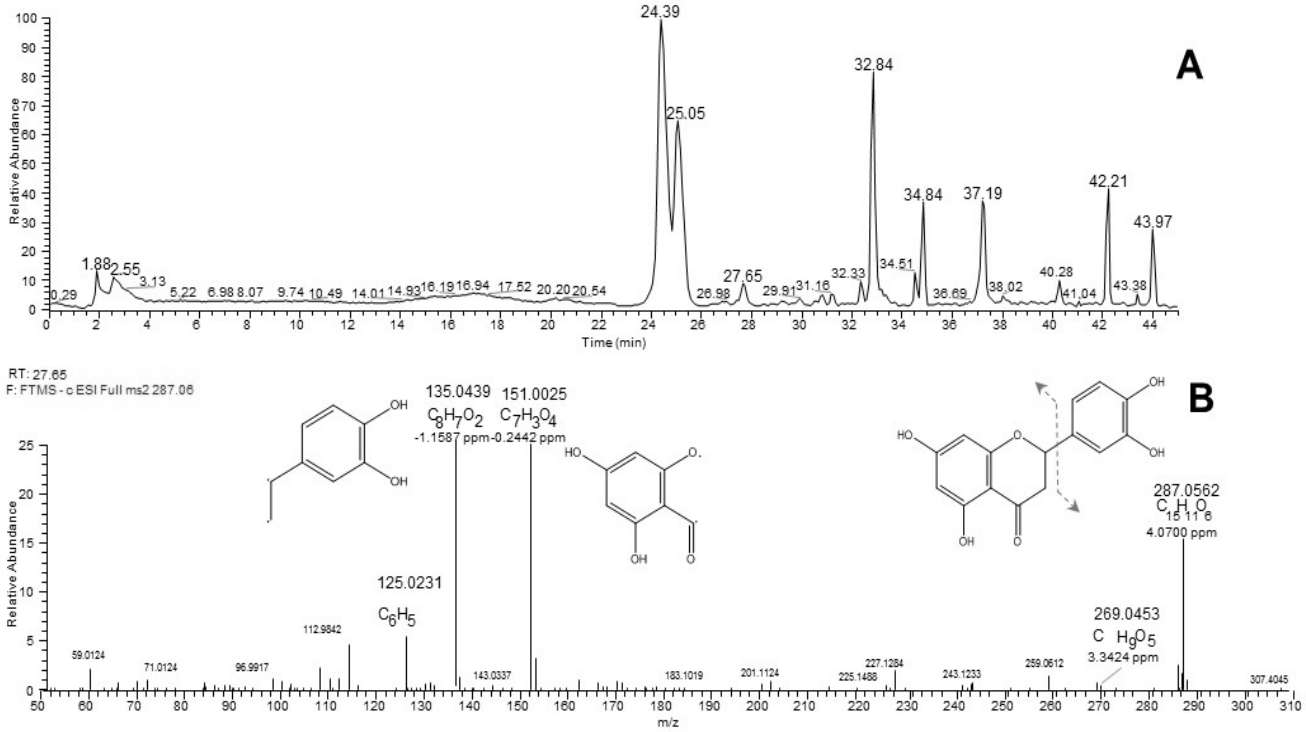


Figure 4 Álvarez-Fernández et al.

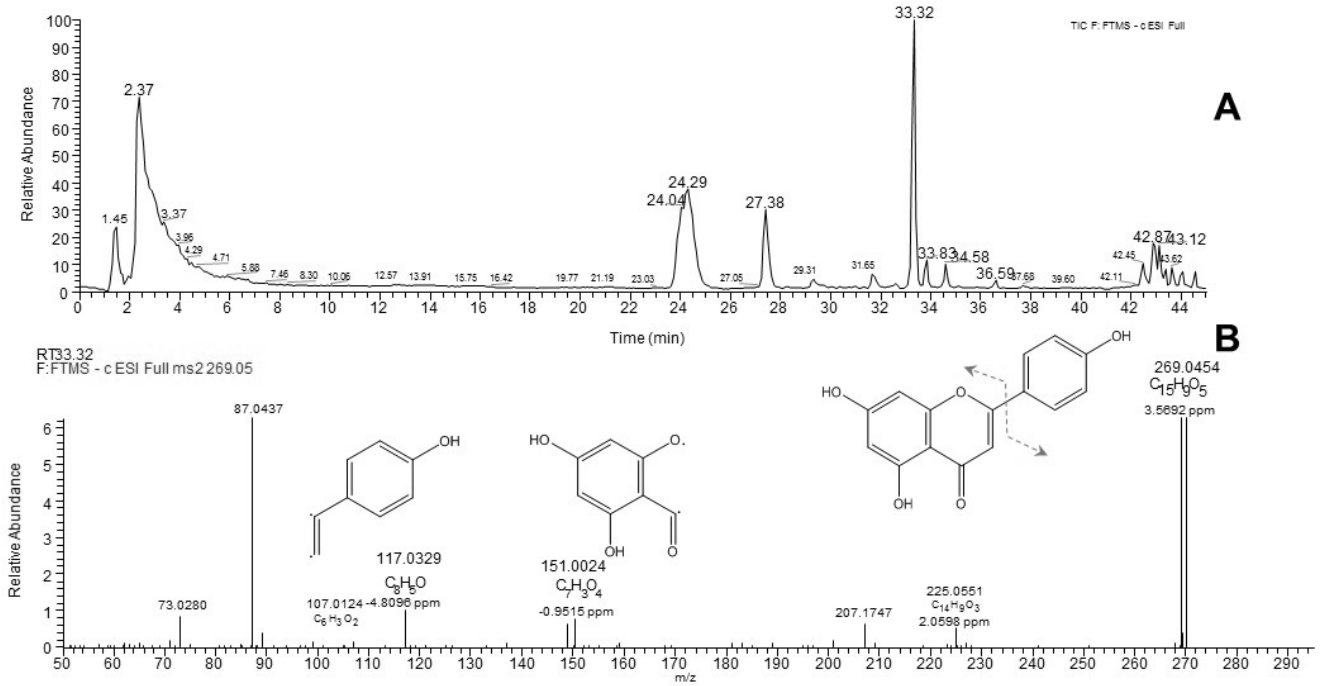


Figure 5 Álvarez-Fernández et al.

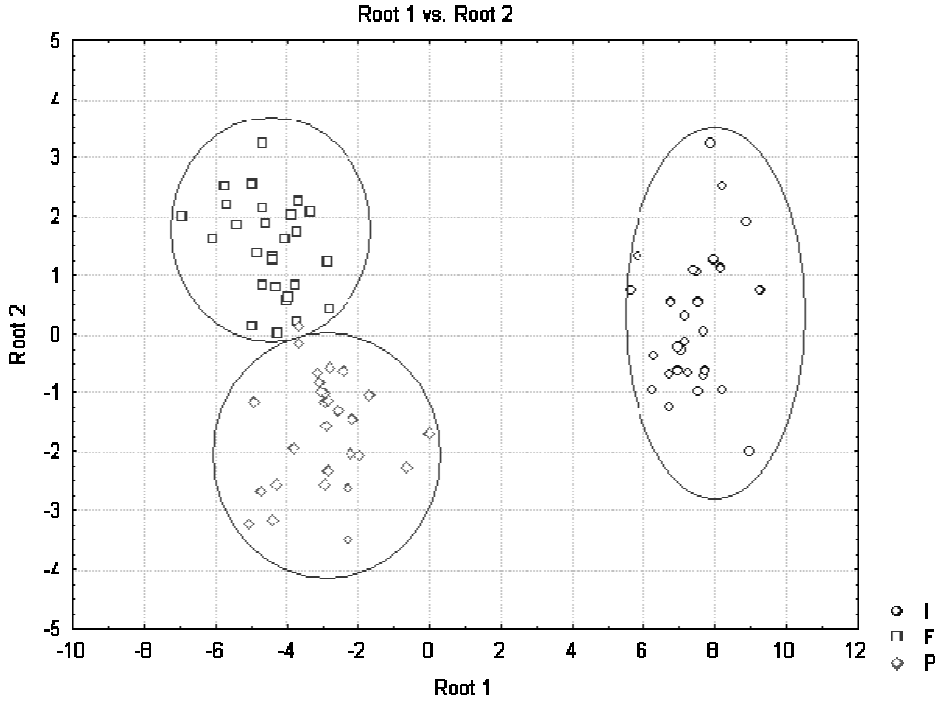


Figure 6 Álvarez-Fernández et al

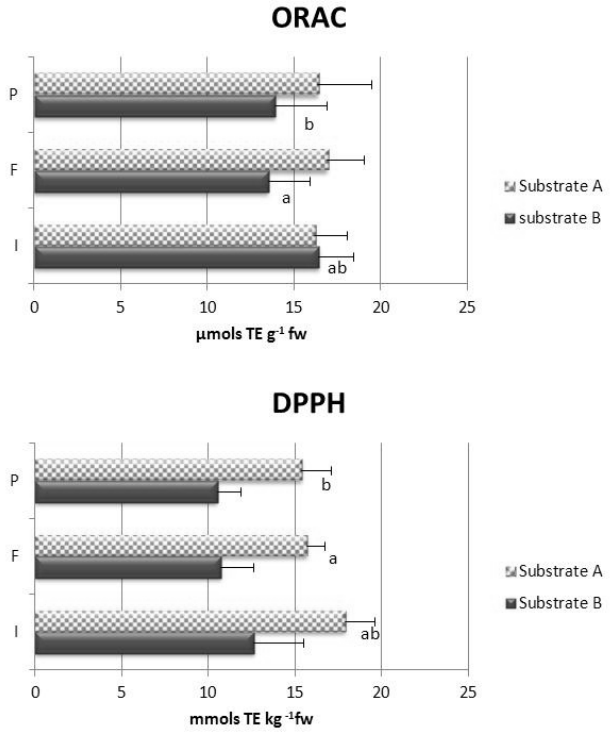
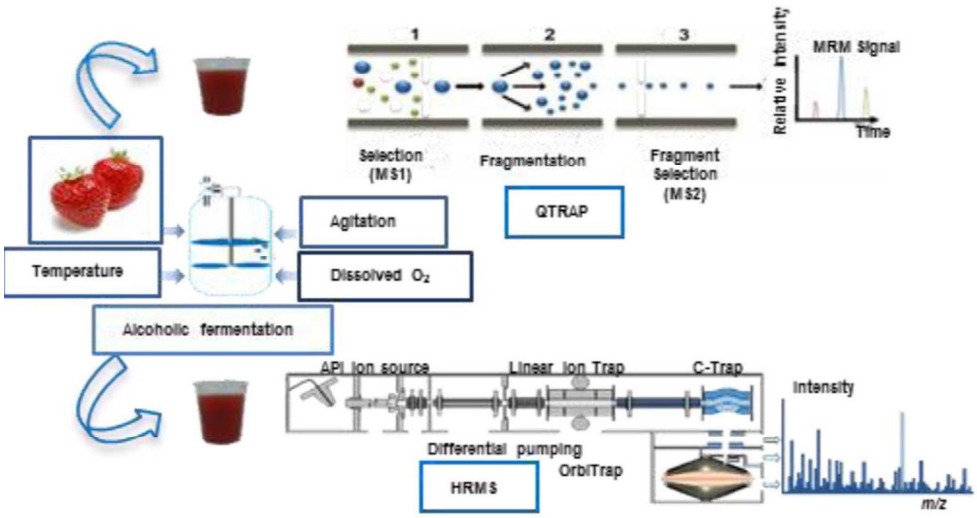


Figure 7 Álvarez-Fernández et al.



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CAPÍTULO CUARTO

IV

Determination of Nonanthocyanin Phenolic Compounds Using High-Resolution Mass Spectrometry (UHPLC-Orbitrap-MS/MS) and Impact of Storage Conditions in a Beverage made from Strawberry by Fermentation.

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ABSTRACT

Overproduction of strawberry leads to food waste, as it is very perishable. Therefore, strategies to transform it into new products are appreciated. This research focuses on characterization of the nonanthocyanin phenolic content of a beverage obtained from strawberry by gluconic and acetic fermentation and subsequently monitored for 90 days of storage, at two temperatures. Sixty-four non-anthocyanin (poly)phenols were identified by high-resolution mass spectrometry (UHPLC coupled with Linear Trap Quadropole and OrbiTrap mass analyzer) and, for the first time, four compounds were reported in beverages fermented from strawberry: aromadendrin hexoside, phloretin *2'-O*-xylosyl glucoside, dihydroferulic acid *4-O*-glucuronide and kaempferol hexosil hexoside. During the storage time the increased of protocatechuic acid content was thirteen times and condensed tannins diminish, especially procyanidin trimer. Statistical analysis showed that the composition remains unchanged until day 15 of storage at room temperature (27-30ºC) and day 30 under refrigerated conditions (4ºC).

Keywords: stability, Orbitrap mass spectrometer, gluconic fermentation, principal component analysis, sensory.

INTRODUCTION

Phenolic compounds are a group of ubiquitous compounds throughout the plant kingdom and many occur in foods. In the late 20th century, interest in food rich in phenolic compounds increased due to their antioxidant and anti-inflammatory properties, their modulation of signal transduction and their anti-microbial and anti-proliferation activities.[1] Consuming fruits with high polyphenol content has beneficial health implications.

Strawberry (*Fragaria* × *ananassa*) is a source of many phenolic compounds that have beneficial effects on health. This product is widely produced and consumed thoroughout the world and Spain one of the leading producers, with a total production of 312,500 MT[2]; this is not consumed or exported in its entirety. This situation generates overproduction and, consequently, waste, which could be avoided by creating foodstuffs that maintain unchanged the properties of the fruit used as raw material as much as possible as well as its composition in bioactives.

Fermentation is an ancient process to transform and preserve foods. It involves raw products undergoing chemical transformations as the result of the action of bacteria or yeast enzymes [3] as in case of aroma and chemical composition being a method of food processing that reduces sugar content. [4] Johnson et. al. reported that fermented berry beverages have shown increased phenolic content and higher antioxidant activity than their non-fermented counterparts. [5] In addition, in our previous work we found that after gluconic fermentation the bioactive content remained practically unchanged. [6] Therefore the study of fermented beverages deserves attention.

Reque et al reported that, under refrigeration conditions of storage, blueberries fruit and juices present good stability with respect to antioxidant capacity, but with significant anthocyanin loss, possibly due to oxidation and/or condensation reactions with other

phenolic compounds.[7] Glycosylated flavonols have also been reported to decrease during storage, while the respective aglycones increase, indicating that enzymatic hydrolysis has occurred. However, these reactions do not affect the total polyphenol content, which remains constant.[8] Conversely, studies detail the individual nonanthocyanin phenolic compounds required to gain deeper insight into chemical composition changes under effects of different temperatures during storage.

The gluconic fermentation of strawberry presents advantages from a nutritional perspective as *Gluconobacter* strains do not metabolize the fructose naturally present in fruit, so it remains in the beverage as a sweetener,[6,9] while glucose is transformed into gluconic acid.

As the gluconic fermentation is a very innovative process, few data have been reported in the literature about the effects of storage on the nonanthocyanin composition of the beverages produced by this mean, to the best of our knowledge. An accurate characterization is required to discover how time and storage conditions [refrigeration (4 ºC) and room temperature (27-30 ºC)] could affect the beverages, in order to establish the optimum conditions to maintain bioactive composition

The aims of this paper are, firstly, to establish the effects of storage for three months at refrigeration temperature (4 ºC) and room temperature (27-30 ºC) on the non-anthocyanin composition and antioxidant activity of an innovative gluconic-fermented beverage as an alternative to take advantage of overproduction of strawberry and avoid its waste and, secondly, to detail the chemical composition, to achieve complete characterization of the products.

MATERIALS AND METHODS

73 **Chemicals and Reagents.** The standard compounds used were acquired from Fluka
74 Sigma-Aldrich (St. Louis, MO. USA) [gallic acid, caffeic acid, p-coumaric acid,
75 cinnamic acid, quinic acid, (-)-epicatechin, (+)-catechin, chlorogenic acid, ellagic acid,
76 (-)-epicatechin gallate, kaempferol, kaempferol-3-glucoside, polydatin, apigenin, quinic
77 acid, brevifolin, protocatechuic acid, (+)-taxifolin, eriodictyol, rutin, quercetin,
78 homovanillic acid and naringenin], from Chromadex® Inc. (USA) [procyanidin B1] and
79 from Extrasynthese [luteolin, (+)-aromadendrin, phloridzin and flavonomarein]. 2,2-
80 Diphenyl-1-picrylhydrazyl (DPPH), 2,2´-Diazo-bis-amidine-propane-dihydrochloride
81 (AAPH), (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and
82 dimethyl dicarbonate were obtained from Sigma-Aldrich (St. Louis, MO. USA);
83 acetonitrile and formic acid (LC gradient) from Merck (Darmstadt, Germany), and
84 methanol from Prolabo (Obregón, Mexico).

85 **Samples.** A beverage was made from strawberry by gluconic fermentation, [using
86 strawberry pureé as substrate into a biorreactor, and after few minutes (10-12) was
87 added 125 ml of inoculum of G *japonicus* strain E1, the end of fermentation process
88 was established when the glucose had been totally consumed] as previously reported[6]
89 and mixed with a little proportion of acetic fermented product. The strawberry vinegar
90 was obtained by a semi-continuous fermentation by *Acetobacter malorum* (CECT
91 7749), at 30 ºC, 70% O2, 13.88 g.[10]

92 Subsequently, the product obtained was centrifuged 10 min at 1500g and diluted with
93 soda water and dimethyl dicarbonate as a preservative to stop the fermentation process.
94 The beverage thus obtained was distributed into individual bottles and pasteurized at 90
95 ºC for 90 s then placed in the refrigerator (4 ºC) or at room temperature (about 30 ºC),
96 until samples were taken (0, 15, 30, 60 and 90 days). The pasteurization process was
97 performed to mimic the industrial process to make the beverages. The initial samples

are the same for both temperature conditions (R_0/F_0). As samples were taken, they were immediately stored at -18 ºC until analysis. Table 1 details sample codes as follow: 'F' for refrigerated samples and 'R' for room temperature samples, followed by a number that indicates the storage days.

Extraction procedure. A sample of 30 g was extracted with 30 ml of methanol and 1% w/w of ascorbic acid and then 30 min of sonication. It was centrifuged for 10 min at 1500 *g*. The supernatant was collected and the pellet re-extracted with methanol according to the same procedure. The fraction was mixed, evaporated to dryness and then reconstituted in 50% (v/v) methanol.[11,12]

High-Resolution Mass Spectrometry (HRMS) Analysis. The experiments were performed using a Thermo Fisher Scientific (Bremen, Germany) liquid chromatography system hybrid Q-OT-qIT Mass Spectrometer (hybrid quadropole-Orbitrap Elite mass spectrometer). This benchtop UHPLC-MS/MS system combines quadropole precursor ion selection with a high-resolution, accurate-mass spectrometer. Identification was performed according to mass spectra, exact mass, characteristic fragmentation and retention time. Xcalibur software (version 3.0.63) was used for instrument control, data acquisition and data analysis. Internet databases of accurate mass spectrometry data, mzcloud (www.mzcloud.org), Metlin, to identify molecular characteristics (https://metlin.scripps.edu/index.php), Massbank[13] and Respect for phytochemicals,[14] were used as a reference library to identify compounds.

The UHPLC separation was performed using a binary gradient consisted of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid: 0.0–1.0 min 5% B, 1.0–12.0 min from 5 to 100% B, 12.0–13.0 min from 100% to 5% B and 13.0–15.0 min 5% B. Twenty microliters of sample were injected and flow rate was 400 μL min-1.

The eluate was analyzed using full MS and data-dependent scanning in negative mode to MS/MS analysis, and with Zorbax SB-C18 2.1x100 mm 1.8 μm column. The normalized collision energy of the high-collision-induced-dissociation (CID) cell was set at 35 eV for data-dependent scan. Helium was employed as sheath gas (53 arbitrary units), sweep gas flow 3 arbitrary units and auxiliary gas flow 14 arbitrary units. Automatic gain control was established as follows: ion trap full, SIM, and MS^n AGC target, 10,000.00; FTMS full AGC target (1000.00) and SIM, MS^n AGC target (50,000.00).

Data dependent scanning comprises a full MS scan (the range was from *m/z* 100 to 1,500 and the resolution was 30,000 FWHM), followed by a data-dependent scan (resolution of 15,000 FWHM).

To quantitate nonanthocyanin phenolic compounds, we used TraceFinder software using the corresponding aglycone when the compounds were glycosides. In case of hydrolizable tannins we used ellagic acid, and procyanidin B1 to quantitate condensed tannins. Calibration curves were obtained by injecting standards diluted from seven different concentrations (R^2 0.9995-0.9979). A triplicate was performed at each point of the calibration curve.

Sensory analysis. The sensory panel gathers 10 trained assessors, 7 females and 3 males, ranging from 22 to 45 years old selected and trained according to ISO 1993.[15] Most of them participate regularly in sensory panels of beverages and accumulates >100 h of experience in sensory analysis. Additionally, a 20 h specific training course was accomplished by every participant and consisted in ordering scales of fructose, gluconic acid, acetic acid, strawberry purées and fermented beverages from strawberry.

145 The trained panel selected the descriptors that better reflect the sensorial characteristics
146 of the beverages by open discussion with the panel leader and consensus. These
147 attributes were: strawberry taste, acidity, sweetness, strawberry aroma and overall
148 impression.

149 The panel carried out 12 triangular tests[16] to differentiate samples stored at two different
150 temperatures, room (27-30ºC) and refrigeration temperature (4ºC), and six triangular
151 tests to differentiate between times of storage.

152 Eight samples were tasted in duplicate by the sensory panel using the selected attributes
153 on a 10 cm unstructured scale ranging from 0 ("nonexistent") to 10 ("very strong"), and
154 for overall impression ranging from 0 ("dislike extremely") to 10 ("like extremely")
155 using standard wine-tasting dark cup. To evaluate the color changes, transparent glass
156 cups have been used.

157 **Antioxidant activity.** *Oxygen Radical Absorbance Capacity (ORAC method).* The
158 method used was as reported, with some modifications:[17] 100 μL of fluorescein solution
159 (45 nM) and 50 μL of AAPH (15 mM) mixed with 50 μL of sample solution or Trolox.
160 The sample solutions were made in phosphate buffer (0.022:100 v/v). Fluorescence
161 was assessed with a fluorometer (multi-detector microplate Synergy HT, Biotek) with
162 excitation, and emission wavelengths were 485 and 528 nm, respectively. Data were
163 recorded every 5 min after addition of AAPH until they reached <5% of the initial
164 value. Results were calculated using the areas under the fluorescein decay curves
165 between the blank (buffer + fluorescein + AAPH) and the sample, with Trolox as a
166 calibration standard (seven different concentration solutions ranged from 0.5 to 9.5 μM)
167 and expressed the results as micromoles of Trolox equivalents (TE) per gram of fresh
168 weight. The reported values are the means of at least three experiments.

169 *DPPH Method*. Scavenging activity was also evaluated by DPPH, using a slightly
170 modified version of the method proposed in the literature.[18] A 47.3 mg L^{-1} DPPH daily
171 prepared methanolic solution was used and stored (protected from light and
172 refrigerated). The samples were prepared in five different concentrations in methanol:
173 water 1:1 (20, 30, 40, 45 and 50 ppm); and 50 μL of the sample solution were added to
174 150 μL of DPPH solution (47.3 mg L^{-1}). A control (50 μL of methanol + 150 μL of
175 DPPH solution) and blank (200 μL of methanol) were also prepared. After shaking,
176 absorbance was determined at 515 nm every 5 min for an hour in a multidetection
177 microplate (Synergy HT, Biotek). Each measurement was taken in triplicate. The results
178 were calculated plotting percentage of inhibition 60 min against the concentration:

179 $$\% \text{ inhibition} = [(A_0 - A_E / A_0)] \times 100$$

180 where A_0 is the initial absorbance and A_E is the absorbance at 60 min.

181 IC_{50} represents the amount of sample needed to reduce the concentration of DPPH
182 radicals to half and was calculated from the curve of different dilutions. A lower value
183 of IC_{50} indicates higher antioxidant activity.[19]

184 **Statistical analysis.** Statistical analyses were performed by means of Statistica
185 software.[20] One-way analysis of variance (ANOVA) and Tukey's HSD (honest
186 significant difference) test were assessed to test significant differences at the $p < 0.05$.
187 Additionally, principal component analysis (PCA) was used for data analysis.

188 **RESULTS AND DISCUSSION**

189 **Identification of Nonanthocyanin Phenolic Compounds.** Sixty-four nonanthocyanin
190 phenolic compounds, including hydroxybenzoic acids and derivatives, hydrolyzed and
191 condensed tannins, ellagic acid and derivatives; hydroxycinnamic acids, flavonols,
192 dihydroflavanols, flavanones, flavanols, flavanones, chalcones and others like citric and

193 quinic acid were identified in the samples analyzed. Table 2 and Figure S1 summarized
194 the identified nonanthocyanin phenolic compounds.

195 Identification was based on matching the retention time, mass spectra, accurate mass
196 measurements, MS^2 analyses with standards when they were commercially available. If
197 not (as it is the case of 13 out of 64 compounds), the above mentioned parameters were
198 compared with data in the literature [21–30] and the databases cited earlier. Additionally,
199 the data of these 13 compounds are discussed as follows in this section as their novelty
200 in this product deserves more discussion. Four of these compounds, were tentatively
201 identified for the first time in a strawberry-derived product, as follows: aromadendrin
202 hexoside, phloretin 2'-O-xylosyl glucoside, dihydroferulic acid 4-O-glucuronide and
203 kaempferol hexosil hexoside. As far as we know, they have not been reported before in
204 either strawberry or its derived products.

205 *Hydroxybenzoic Acids and Derivatives.* Chromatograms in full scan MS mode and data-
206 dependent scans showed the presence of *m/z* 153.0194. It had a loss of 44u [M – H -
207 44]$^-$, characteristic of this chemical group,[29] with a product ion *m/z* 109.0295. MS^2
208 spectra exhibited a product ion *m/z* 67.0194, after a loss of 42.01, corresponding to
209 acetyl moiety. [26] This fragment pattern matches the data obtained in mzCloud for 2,4-
210 dihydroxybenzoic acid, corresponding to peak 26, as shown in Table 2.

211 *Hydrolized Tannins.* Peak 6 exhibited a $[M - H]^-$ at 343.0691, with a molecular formula
212 $C_{14}H_{16}O$ and a fragmentation pattern with a product ion $[M – H - Gall]^-$ 191.0566,
213 corresponding to the loss of galloyl moiety 152.011. This ion had a molecular formula
214 $C_7H_{11}O_6$, indicating that it is a quinic acid residue. A further two ions were present in
215 the mass spectrum: 169.0147 ($C_7H_5O_5$) and 125.0250 ($C_6H_5O_3$), which were fragments
216 characteristic of gallic monomer. This compound was tentatively identified as

galloylquinic acid, reported before in strawberry fruit and flowers,[26] but not in fermented derived products. Figure S2 shows its proposed fragmentation pattern and base peak chromatogram with t_R. Peak 27 is a dimeric ellagitannin $C_{82}H_{54}O_{52}$, tentatively identified as agrimoniin, in which the monomeric fragment corresponding to $C_{41}H_{26}O_{26}$ was detected. According to published data, this fragment must have resulted from the fracture of the C-O bond that connects the two monomers, yielding a negative ion: galloyl bis-HHDP-glucose. After loss HHDP moiety, it results in $[M - H]^-$ 633.0726 (theoretical monoisotopic mass calculated at 633.0722, with molecular formula $C_{21}H_{21}O_{18}$).[30,31]

Ellagic acid and Derivatives. Peak 42 was tentatively identified as tetramethyl ellagic acid hexose, which is reported in gluconic-fermented products for the first time, despite having been reported before in strawberry.[30] It exhibited an ion *m/z* 359.1480, with a molecular formula $C_{20}H_{23}O_6$, yielded after losing a hexose moiety (162.0528). It then lost a water moiety (18.0117) and produced an ion *m/z* 341.1363, with molecular formula $C_{20}H_{21}O_5$.

Flavanols. Peak 38 exhibited an ion *m/z* 451.1212 and a molecular formula $C_{21}H_{24}O_{11}$, and yielded an ion m/z 289.0718 $[M – H - 162]^-$ corresponding to a (+)-catechin monomer. This compound was tentatively identified as (+)-catechin-*O*-hexoside. This one has been described before in strawberry,[22] but not in gluconic-fermented products.

Hydroxycinnamic acids. Peak 40 had a pseudo-molecular ion *m/z* 337.0914 ($C_{16}H_{18}O_8$) and showed the characteristic fragmentation of a quinic acid derivative *m/z* 191.0562 ($C_7H_{11}O_6$), and monomeric ion belonging to *p*-coumaric acid m/z 163.0405 ($C_9H_7O_3$). As a result, the tentative identification was *p*-coumaroylquinic acid. This fragmentation pattern matched data obtained from mzCloud database. Peak 57 had a fragmentation

241 that indicated it was a ferulic derivative. It produced a monomeric ion, m/z 193.0503
242 ($C_{10}H_9O_4$), corresponding to ferulic acid, as a consequence of the loss of a glucuronide
243 moiety and two hydrogens $[M - 2H - Gln]^-$, producing an unsaturated bond in the
244 hydrocarbon chain. This metabolite was tentatively identified as dihydroferulic acid *4-*
245 *O*-glucuronide. As far as we know, it is the first time this compound has been described
246 in strawberry and fermented derivatives.

247 *Flavonols.* Peak 31 had an $[M - H]^-$ ion at 609.1437 with molecular formula $C_{27}H_{30}O_{16}$
248 and fragmentation pattern that matched a kaempferol derivative: an ion [M – H -–
249 2Hex]$^-$ at 285.0410 (which matches a kaempferol monomeric ion), after the loss of two
250 hexoside moieties (162.0528). This compound was putatively identified as kaempferol
251 hexosilhexoside, previously reported in mulberry. [27]

252 Peak 39 was tentatively identified as quercetin pentose glucuronide, due to the presence
253 of an ion $[M – H – Pent – Gln]^-$ 301.0428, with molecular formula $C_{15}H_9O_7$,
254 corresponding to quercetin. This compound has been described before in strawberry and
255 its flowers.[26]

256 *Condensed Tannins.* Peak 18 had a precursor ion $[M - H]^-$ at 561.1401 and presents a
257 fragmentation pathway, shown in Figure S2, that matches one reported before in
258 strawberry.[25] It was deduced to be a dimer of (epi)afzfelechin → (epi)catechin because
259 its chirality could not be discriminated by mass spectrometry. This identification was
260 confirmed by the presence of two fragments, *m/z* 271.0611 ($C_{15}H_{11}O_5$) and *m/z*
261 289.0715 ($C_{15}H_{13}O_6$), which were yielded before breakdown of the cleavage of the
262 interflavan bond. [25]

263 *Dihydroflavonols.* Peak 41, which presented a principal ion $[M - H]^-$ at *m/z* 435.0922
264 and molecular formula $C_{20}H_{20}O_{11}$, was putatively identified as (+)-taxifolin *3-O-*

arabinofuranoside, based on the production of an MS^2 fragment ion $[M - H - Pent]^-$ at 303.05, yielded by the loss of pentose monoisotopic mass 132.04, which is the major product ion, corresponding to the monomeric ion of (+)-taxifolin. Other MS^2 fragments were presented, such as $[M - H]^-$ 285.0399, 177.0192 and 125.0244, which were in accordance with the MS^2 fragmentation pathway of (+)-taxifolin (peak 37). This secondary metabolite has been reported before in strawberry.[30]

Chalcones. This is a group of compounds present in apple as a consequence of the processes related to defense against infections.[32] Peak 49 presents a pseudo-molecular ion at $[M - H]^-$ 567.1688 and an MS^2 fragment yielded after the loss of two moieties, one of pentose 132.0423 and another of galactose 162.0528 $[M - H - Galc -- Pent]^-$ at 273.0764, with molecular formula $C_{15}H_{13}O_5$. This compound was tentatively identified as phloretin *2′-O*-xylosyl-galactoside (in accordance with Metlin data). This fragment also appeared in the MS^2 spectra of peak 49, identified with standard as phloridzin (phloretin *2′*-glucoside), after loss of a glucoside moiety $[M - H - Gluc]^-$ that corresponded to a phloretin monomer.

Influence of Storage Conditions on the Nonanthocyanin Phenolic Content in Fermented Beverage. A total of 37 compounds (those above LOQ) have been quantitated. Statistical analysis was applied to reduce the variables in the model including those that contributes the most to the variance of data Table S2. Table 3 shows only those selected for the PCA, whereas the rest are displayed in Table S2. Six compounds exhibited higher concentrations in the initial samples: ellagic acid hexoside, *p*-coumaroyl hexose, ellagic acid, *p*-coumaric acid, (+)-catechin and procyanidin B_1.

Hydroxycinamics compounds analyzed as *p*-coumaric acid presented an increased during storage time that is not proportional with the decrease observed in *p*-coumaroyl

289 hexose. This behavior can be explained by disappearance of coumaroyl anthocyanins
290 during the aging process. [33]

291 At 30 ºC, (+)-catechin, procyanidin B1 and procyanidin trimer underwent a significant
292 decrease (84.5–95%) during the storage time, to the point that procyanidin trimer
293 content reached levels below the detection limit in samples R_{60} and R_{90}. At 4 ºC, it
294 decreased (42.3–36.31–78.58%), but in a lesser extent than at room temperature. The
295 reduction of the content of procyanidins (B1 and trimer) during storage is in accordance
296 with previously reported data on quince juice.[34] Another explanation of these reductions
297 could be an increase of polymeric color value, indicative of condensation reactions of
298 anthocyanins with other phenolic compounds such as procyanidins to form colored
299 polymer pigments.[34]

300 Three hydrolized tannins were quantified: an ellagitannin (bis-HHDP-glucose), a
301 gallotannin (galloylquinic acid) and brevifolin carboxylic acid; the last two increased
302 during storage time and increased more at room temperature (74–28% and 34–27%,
303 respectively). Ellagitannin started to change at 15 days, and achieved its maximum
304 decrease of 65% at room temperature (26% at 4 ºC); this decrease was associated with
305 the increase of free ellagic acid (22% at room temperature).

306 Interestingly, protocatechuic acid significantly increased after 60 days of storage (13
307 times its initial value) at 30 ºC, with its concentration reaching 11 times its initial value
308 after 90 days at 4 ºC. This increase may be due to the degradation process of
309 anthocyanin compounds (pelargonidin is a principal anthocyanin of strawberry), which
310 produced a cleavage of pelargonidin B-ring and, consequently, a molecule of
311 protocatechuic acid, as was reported in the thermal processing of food.[35] Additionally,

312 as anthocyanin are susceptible to thermal degradation, another mechanism proposed
313 describes the opening of the pyrylium ring and chalcone glycoside formation.[36]

314 Kaempferol, apigenin and their derivatives increased (24–373%) during storage,
315 increasing more at 30 ºC than 4 ºC. Conversely, the quercetin *3-O*-glucoside and rutin
316 content decreased during storage: the higher the temperature is, the greater the decrease
317 of the content of glycosides, producing an increase of the content of quercetin (Table
318 S1). The concentrations of (+)-taxifolin *7-O*-glucoside underwent a significant
319 reduction (75% at 30 ºC–51% at 4 ºC). This result is associated with the increase of
320 taxifolin (106%–41%) because of the breakdown of the glucosidic bond releasing the
321 corresponding aglycone.[8]

322 The major loss of non-anthocyanin phenolic compound content was observed at room
323 temperature. This observation was in accordance with what was reported before by
324 Oliveira et al.[37] They conclude that pasteurization treatment favors the rupture of
325 cellular structures, increasing the exposure of compounds to oxidation processes during
326 storage. This phenomenon is one of the main reasons for the loss of phenolic
327 compounds. Apart from the above mentioned changes, it can be highlighted that certain
328 (poly)phenolic compounds maintain their content practically unchanged at both tested
329 temperatures as: caffeic acid, caffeic acid hexoside and naringenin. Additionally, the
330 concentration of two compounds remained constant at refrigeration conditions
331 (phloridzin and quercetin glucuronide). All these data are shown Table S1.

332 PCA was carried out to explore the effects of storage time and temperature on the non-
333 anthocyanin phenolic compound content profile (Figure 1A and 1B). The matrix had 18
334 samples × 24 quantified non-anthocyanin phenolic compounds. The two principal
335 components (Factor 1 and Factor 2) accounted for 86.54% of the variability of the

original data. PCA showed clustering of the samples into four main groups, illustrated in Figure 1A. It was observed that samples belonging to the same storage period are very close to each other, which generate four well-separated clusters, thus highlighting the effect of time. Interestingly, R_{90} and R_{60} were very separately located from the other samples (Figure 1A). The cluster presented in the middle of the Cartesian plane, includes samples R_{30}, F_{60} and F_{90}, which indicates that storage under refrigeration has a preservative effect on non-anthocyanin phenolic composition. The last cluster consists of initial samples (represented by F_0), F_{15}, F_{30} and R_{15} with high weights on Factor 1, as determined by their high content of procyanidin B1 (17); procyanidin trimer (18) and (+)-catechin (1). In addition, the Figure 1B shows the variables separated into two groups, on the right: all compounds, which experiments a decrease; on the left, those that increased their concentrations during storage time at two temperature conditions. In all cases, the effect was greater at room temperature than refrigerated conditions; specifically the composition remains virtually unchanged until day 15 of storage at room temperature and day 30 under refrigerated conditions.

Influence of Storage Conditions in Antioxidant Activity. Figure 2 left and right panels, represent antioxidant activity by ORAC and DPPH, respectively. No significant differences were observed for ORAC results, but DPPH results were statistically different ($p < 0.05$). At room temperature, there has been observed a progressive increase until 60 days of storage and then, it decreased. At refrigeration conditions, the trend was similar.

This result was in accordance with reported before in alcoholic fermented mulberries.[18] With the fermentation process hydroxycinamic acids increased while the progressive growing in the content of protocatechuic acid indicated that it existed a degradation of anthocyanins generating another compounds with antioxidant activity.

361 A similar behavior was reported before in the analysis of antioxidant activity in
362 solutions of phenolic compounds.[38] When the storage time was prolonged, the
363 antioxidant activity decreased and so maximum value was observed (Figure 2 right,
364 maximum at 60 days and then decreased). This increase in the overall antioxidant
365 activity value could be probably explained by the formation of oligomers from free
366 polyphenols as (+)-catechin as its content decreased as showed in Table 3.

367 **Sensory Analysis.** The panel carried out 12 triangle tests to differentiate samples with
368 different storage time and 6 triangle tests to differentiate samples stored at different
369 temperatures. In general, sensory differences were perceived from 30 days of storage on
370 at room temperature, whereas the panel could not establish differences between
371 refrigerated samples whatever the storage time was.

372 On the other hand, the panel could not differentiate samples stored during 30 days at
373 different temperatures. However, differences between samples stored at 4ºC or 27-30ºC
374 for 60 could be perceived and even larger differences were perceived for samples stored
375 for 90 days.

376 The sensory profile of the beverage was built using the marks given for each attribute
377 by the panel. Figure 3 displays the spider charts for the samples stored at room
378 temperature (Figure 3A) and samples storage in refrigeration conditions (Figure 3 B).
379 The attributes (overall impression, strawberry aroma, and strawberry taste) have highest
380 marks in refrigeration conditions and the lowest marks were in samples stored at room
381 temperature. Additionally, the higher the time of storage is, the lower overall impression
382 scores are. These results should be taken into account to establish the shelf life of the
383 beverage.

In conclusion, four new compounds were identified with HRMS technique in samples of fermented strawberry beverage: aromadendrin hexoside, phloretin *2'-O*-xylosyl glucoside, dihydroferulic acid *4-O*-glucuronide and kaempferol hexosil hexoside.

Nonanthocyanin phenolic composition underwent fewer changes under refrigeration than at room temperature although 10 compounds declined when they were stored mainly at room temperature. However our results show that 14 compounds: ellagic acid, ellagic acid hexose, *p*-coumaric acid, *p*-coumaroylquinic acid, apigenin, apigenin pentose, eriodictyol glucose, kaempferol, kaempferol glucuronide, protocatechuic acid, brevifolin carboxylic acid, (+)-taxifolin, galloylquinic acid and apigenin-7-*O*-glucose increased during storage.

Strawberry fermented beverage storage period should not exceed 30 days at room temperature (27-30ºC) or 60 days at refrigeration conditions (4ºC). Gluconic fermentation is an alternative process to prevent fruit waste while elaborating a glucose free product that contains bioactive compounds.

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ASSOCIATED CONTENT

Supporting information

Table S1. concentrations of compounds whereas not part of PCA analysis. Table S2. variable contribution; based on correlations. PCA analysis. Figure S1, MS chromatograms; Figure S2 and S3 Fragmentation patterns of compounds 6 and 18. (PDF)

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Table 1. Samples Codes

storage time (days)	room temperature	refrigerated temperature
0	R_0 (initial)	F_0 (initial)
15	R_{15}	F_{15}
30	R_{30}	F_{30}
60	R_{60}	F_{60}
90	R_{90}	F_{90}

Table 2. Nonanthocyanin Phenolic Compounds Tentatively Identified in Beverages Obtained from Fermented Strawberry[a].

Peak	Tentatively identification	R_t (min)	Molecular formula	Exact-mass [M-H]$^-$	Δmass (ppm)	MS/MS fragments (relative abundance %)	Ref.
	Hydroxybenzoics Acids and Derivatives						
3	gallic acid*	1.70	$C_7H_6O_5$	169.0143	6.5199	125.0244 (100); 124.0165(0.12); 97.0295(0.11)	
8	protocatechuic acid*	3.33	$C_7H_6O_4$	153.0193	6.9744	109.0295(100); 108.0172(15)	
10	protocatechuic acid 4-*O*-*β*-hexoside	4.10	$C_{13}H_{16}O_9$	315.0727	3.3800	153.0191(100); 109.0295(9)	29
15	1-*O*-protocatechuyl-ß-xylose	4.40	$C_{12}H_{14}O_8$	285.0605	0.0279	153.0193(100); 152.0116 (31); 109.0296(6); 108.0218 (4)	30
19	*p*-hydroxybenzoic acid -*O*- glucoside	4.79	$C_{13}H_{16}O_8$	299.0778	5.6038	137.0244(100); 93.0347 (2)	39
26	2,4-dihydroxybenzoic acid	5.57	$C_7H_6O_4$	153.0194	7.6700	109.0295(100); 67.0194(2)	39
34	phloretic acid	6.30	$C_9H_{10}O_3$	165.0556	5.7668	119.0503(100); 121.0567(7); 93.0376(3)	39
51	4-hydroxybenzoic acid	7.31	$C_7H_6O_3$	137.0242	6.0310	93.0348(100)	39
	Hydrolized Tannins						
1	HHDP-glucose	1.10	$C_{20}H_{18}O_{14}$	481.0638	5.3242	300.9991(100); 275.0199(13); 249.0402(1)	26
5	tris-galloyl-HHDP-hexose	2.73	$C_{41}H_{28}O_{27}$	951.0722	1.2796	907.0834(100); 783.0674(66); 605.0583(7)	21
7	monogalloyl glucose	3.00	$C_{13}H_{16}O_{10}$	331.0677	5.2722	313.0564 (100); 169.0146 (19); 151.0050 (2); 168.0067 (71)	26
9	bis-HHDP-glucose	3.68	$C_{34}H_{24}O_{22}$	783.0680	0.5679	481.0619(21); 300.9984(100); 275.0195(22)	30
6	galloyl quinic acid	2.98	$C_{14}H_{16}O_{10}$	343.0691	2.3844	191.0566(8); 173.0459(14); 169.0147(100); 125.0250(5)	26
16	galloyl-HHDP-glucose	4.42	$C_{27}H_{22}O_{18}$	633.0721	0.2488	463.0515(9); 300.9984(100); 283.9958(1); 229.0093(1)	26
24	brevifolin carboxilic acid	5.48	$C_{13}H_8O_8$	291.0152	5.5792	247.0246 (100); 203.0351(0.2)	23
27	agrimoniin	5.58	$C_{41}H_{26}O_{26}$	934.0757	5.4095	1567.1407(81); 1265.1399(28); 1085.0741(36); 916.0588(39); 915.0543(54); 897.0430(100); 783.0692(45); 633.0726(48)	30
	Ellagic acid and Derivatives						
33	ellagic acid hexoside	6.28	$C_{20}H_{16}O_{13}$	463.0498	1.9699	300.9979(100); 299.9907(55); 283.9958(0.1)	26
42	tetramethylellagic acid hexose	6.97	$C_{26}H_{34}O_{11}$	521.2014	0.7352	359.1480 (30); 341.1363(100)	30
43	ellagic acid pentoside	7.07	$C_{19}H_{14}O_{12}$	433.0404	0.5140	300.9994(100); 299.9918(89); 283.9995(0.1)	30
48	ellagic acid deoxyhexose	7.21	$C_{20}H_{16}O_{12}$	447.0555	0.7187	300.9983(100); 283.9947(0.1); 257.0085(0.5)	26
52	ellagic acid*	7.40	$C_{14}H_6O_8$	300.9981	0.7122	300.9984(26); 283.9963(18); 257.0090(100); 229.0141(47); 185.0244(19)	

Table 2. Continued.

Peak	Tentatively identification	t_R (min)	Molecular formula	Exact mass [M-H]$^-$	Δmass (ppm)	MS/MS fragments (relative abundance %)	Ref.
	Flavanols						
17	(+)-catechin*	4.62	$C_{15}H_{14}O_6$	289.0724	6.0664	245.0820(100); 205.0508(33); 179.0352(13); 125.0248(4); 109.0297(1)	
38	(+)-catechin-*O*-hexoside	6.42	$C_{21}H_{24}O_{11}$	451.1212	3.6835	415.1114(82); 289.0718(100)	[22]
	Hydroxycinnamic acids						
13	caffeic acid hexose	4.30	$C_{15}H_{18}O_9$	341.0874	2.0342	179.0348(100); 161.0244 (60); 135.0452(8)	[26]
14	dimer of caffeic acid-*O*-hexoside	4.33	$C_{30}H_{36}O_{18}$	683.1818	2.0142	341.1058(100)	
20	*p*-coumaroyl glucose	4.99	$C_{15}H_{18}O_8$	325.0919	0.2907	265.0724(11); 235.0619(5); 205.0513(6); 187.0407(28); 163.0407(59); 145.0301(100); 119.0508(5); 117.0351(2)	[26]
22	caffeic acid*	5.28	$C_9H_8O_4$	179.0350	6.3323	135.0451 (100)	
23	ferulic acid hexose	5.30	$C_{16}H_{20}O_9$	355.1019	1.2965	217.0505(56); 193.0506(100); 175.0401(65); 160.0166(7); 134.0375(5)	[26]
35	*p*-coumaric acid*	6.33	$C_9H_8O_3$	163.0398	4.8687	119.0502 (100)	
40	*p*-coumaroylquinic acid	6.60	$C_{16}H_{18}O_8$	337.0914	1.1681	191.0562(40); 163.0405(100)	[39]
55	ferulic acid	7.60	$C_{10}H_{10}O_4$	193.0504	4.7153	149.0608 (100); 178.0265 (1); 134.0376 (1)	[39]
57	dihydroferulic acid 4-*O*-glucuronide	7.71	$C_{16}H_{20}O_{10}$	371.0985	3.4171	209.0815(18); 193.0505(100)	[40]
	Flavones						
21	luteolin-3'-xyloside	5.04	$C_{20}H_{18}O_{10}$	417.0781	5.7530	285.0612(32); 241.0715(100); 152.0116(73); 151.0402(22); 133.0296(4)	[21]
28	apigenin pentose	5.67	$C_{18}H_{26}O_{10}$	401.1449	1.6805	269.1028(100); 161.0457(22.45); 149.0458(0.6)	[29]
32	apigenin-7-O-glucoside	6.05	$C_{21}H_{20}O_{10}$	431.0970	0.5272	269.0451(100); 225.0611(68)	[14]
36	luteolin*	6.38	$C_{15}H_{10}O_6$	285.0410	5.7927	241.0507(100); 217.0507(20); 199.0402(22); 175.0403(75)	
63	apigenin*	9.26	$C_{15}H_{10}O_5$	269.0458	4.9304	225.0559(100); 151.0040(33); 149.0248(50); 117.0350(3)	
	Flavonols						
31	kaempferol hexosilhexoside	5.92	$C_{27}H_{30}O_{16}$	609.1437	2.1905	285.0410(38); 284.0334(1)	[27]
39	quercetin pentose glucuronide	6.58	$C_{26}H_{26}O_{17}$	609.1086	0.1725	301.0428(100); 178.9944(1)	[26]
44	quercetin glucuronide	7.11	$C_{21}H_{18}O_{13}$	477.0660	0.5302	301.0347(100); 178.9987(1); 151.0038(0.8)	[26]
45	isorhamentin-3-*O*-glucoside	7.16	$C_{22}H_{22}O_{12}$	477.1017	2.1743	433.1145(0.57); 301.0356(100); 178.9990(1)	[13]
46	rutin*	7.17	$C_{27}H_{30}O_{16}$	609.1439	1.7897	301.0345(100); 300.0265(0.28); 255.0289(0.31)	
47	quercetin-3-*O*-glucoside	7.20	$C_{21}H_{20}O_{12}$	463.0859	2.5155	301.0345(100); 300.0270(36); 271.0240(0.6); 255.0655(0.31); 178.9984(2.08); 151.0035(1.61)	[30]

Table 2. Continued

	Tentatively identification	t_R (min)	Molecular formula	Exact mass [M-H]-	Δmass (ppm)	MS/MS fragments (relative abundance %)	Ref.
54	kaempferol hexose*	7.59	$C_{21}H_{20}O_{11}$	447.0904	3.9017	285.0398(68); 284.0334(100); 255.0650(2); 227.0356(4)	
56	kaempferol-3-glucuronide	7.67	$C_{21}H_{18}O_{12}$	461.0714	0.0893	285.0396(100); 257.0462(0.15)	[26]
58	isorhamnetin-3-glucuronide	7.82	$C_{22}H_{20}O_{13}$	491.0816	0.7788	315.0502(100); 301.0351(1); 271.0236(0.1); 255.0305(0.1)	[26]
59	quercetin*	8.38	$C_{15}H_{10}O_7$	301.0359	5.4483	273.0399(13); 257.0451(11); 193.0140(5); 178.9984(100)	
61	kaempferol-3-coumaroylhexoside	8.66	$C_{30}H_{26}O_{13}$	593.1268	3.7043	447.0562(2); 285.0398(100); 257.0452(3); 229.0459(2)	[28]
62	kaempferol*	9.06	$C_{15}H_{10}O_6$	285.0397	1.0819	285.0398(100); 257.0453(15); 185.0608(11); 169.0660(10); 151.0037(25)	
64	galangin*	10.39	$C_{15}H_{10}O_5$	269.0456	4.1364	241.0506(27); 227.0345(100); 197.0605(39); 183.0449(77); 169.0657(10)	
	Condensed Tannins						
11	proantocyanidin trimer	4.18	$C_{45}H_{38}O_{18}$	865.1999	2.8313	739.1686(4); 695.1419(100); 587.0942(27); 575.0942(35); 543.0674(16); 287.0565(5)	[26]
12	proanthocyanidin B1*	4.28	$C_{30}H_{26}O_{12}$	577.1331	1.6199	425.0873(100); 407.0768(87); 289.0716(47)	
18	propelargonidin dimer	4.75	$C_{30}H_{26}O_{11}$	561.1401	1.7799	289.0715(100); 245.0818(7) 271.0611(14); 245.0818(7)	[25]
	Dihydroflavonols						
25	aromadendrin hexoside	5.53	$C_{21}H_{22}O_{11}$	449.1073	1.1980	287.0558(100); 259.0609(43); 125.0261	[23]
30	(+)-taxifolin-7-*O*-glucoside	5.82	$C_{21}H_{22}O_{12}$	465.1025	0.5244	285.0401(100); 177.0195(1.34)	[14]
37	(+)-taxifolin*	6.39	$C_{15}H_{12}O_7$	303.0517	5.7321	285.0403(100); 177.0195(12); 125.0247(7)	
41	(+)-taxifolin-3-*O*-arabinofuranoside	6.90	$C_{20}H_{20}O_{11}$	435.0922	0.0567	303.0503(100); 285.0399(52); 275.0559(0.5); 177.0192(4); 125.0244(1.4)	[40]
50	aromadendrin*	7.25	$C_{15}H_{12}O_6$	287.0574	5.3222	259.0613 (100); 243.0665 (18); 125.0247 (3)	
	Flavanones						
29	eriodictyol-7-*O*-glucoside	5.68	$C_{21}H_{22}O_{11}$	449.1078	0.0251	287.0557(100); 151.0038(0.3)	[13]
60	naringenin*	8.51	$C_{15}H_{12}O_5$	271.0619	6.4900	203.0364(3); 177.0196(20); 165.0197(5); 151.0039(100)	
	Chalcones						
49	phloretin-2'-*O*-xylosyl-galactoside	7.24	$C_{26}H_{32}O_{14}$	567.1688	3.5416	273.0764(100)	[40]
53	phloridzin*	7.44	$C_{21}H_{24}O_{10}$	435.1303	3.9212	273.0763(100); 167.0350(2); 125.0240(0.1)	
	Others						
2	citric acid*	1.16	$C_6H_8O_7$	191.0188	0.9689	111.0088 (100); 129.0193(3); 173.0090 (15); 67.0197(0.12)	
4	quinic acid	2.17	$C_7H_{12}O_6$	191.0560	4.9970	173.0452(51); 171.0302(19); 155.0354(6); 127.0401(53); 109.0298(16); 93.0347(32); 85.0297(100)	

[a] Δmass is deviation of the observed ion mass from the corresponding calculated monoisotopic mass. Peak is the number of the compound in the chromatogram. Ref. previous reports of compounds. *Analytes confirmed by comparing with pure standards.

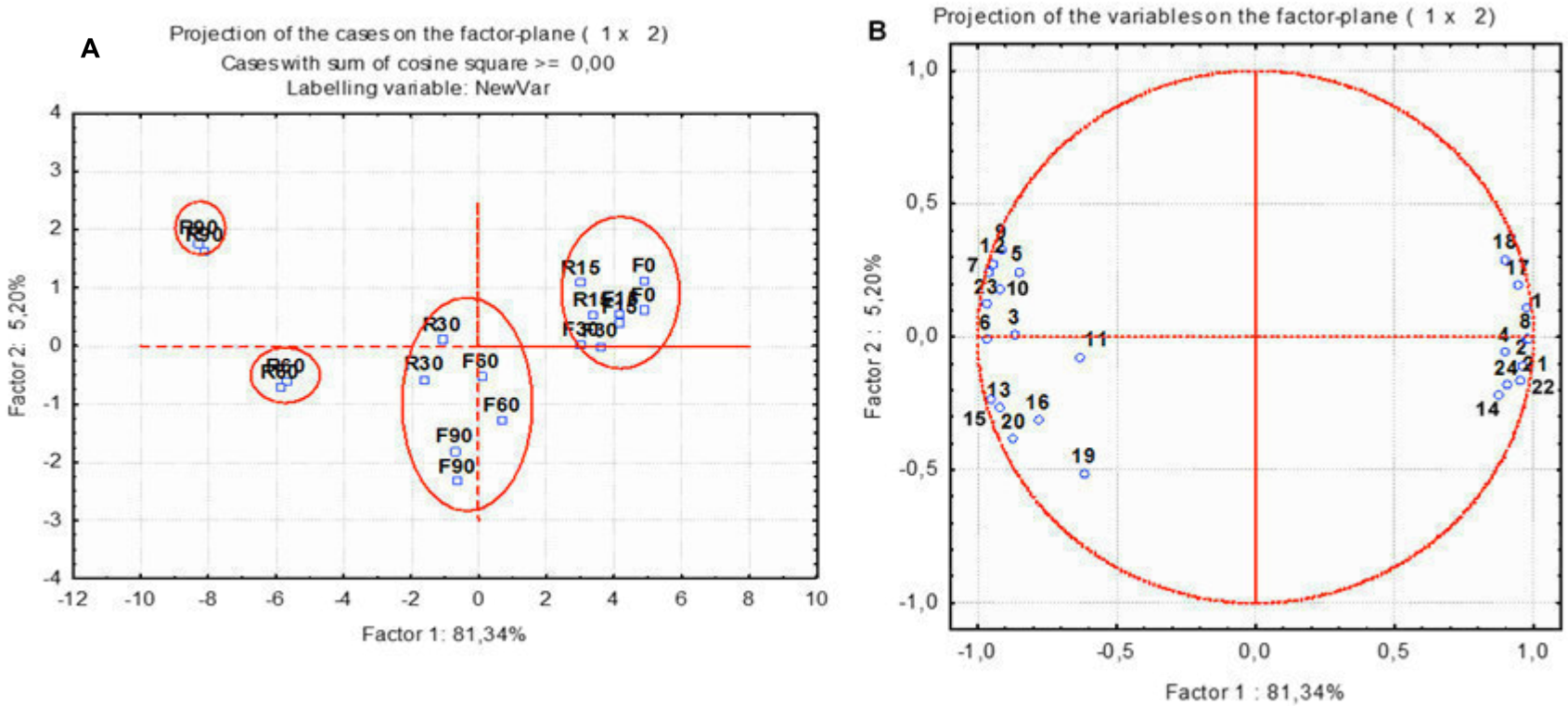
Table 3. Concentrations of Nonanthocyanin Phenolic Compounds (μg/100 g of Beverage)[a]

	R_0/F_0	R_{15}	R_{30}	R_{60}	R_{90}
(+)-catechin	92.95^{bcde}±4.02	83.89^{ae} ±0.28	61.10^{abe} ±0.09	20.47^{abce} ±0.20	14.33^{abcd} ±0.09
ellagic acid hexose	185.73^{e}±2.19	184.33^{e} ±14.22	206.65 ±5.45	212.22 ±4.41	226.05^{ab} ±0.0
ellagic acid	134.12^{cde}±6.80	132.39^{cde} ±2.06	156.49^{ab} ±0.66	151.04^{ab} ±1.10	166.69^{ab} ±3.06
bis HHDP glucose	17.22^{bcde}±0.43	13.47^{ae} ±0.11	13.35^{ae} ±1.13	11.80^{ae} ±0.55	6.04^{abcd} ±0.03
brevifolin carboxilic acid	1.72^{cde}±0.07	1.78^{cde} ±0.07	2.14^{ab} ±0.06	2.36^{ab} ±0.03	2.30^{ab} ±0.07
galloylquinic acid	1.00^{de}±0.03	1.03^{de} ±0.08	1.33^{e} ±0.17	1.65^{ab} ±0.13	1.72^{abc} ±0.03
dimer of caffeic acid-O-hexoside	7.01^{bcde}±0.42	6.76^{a} ±0.04	6.33^{ab} ±0.32	5.25^{abc} ±0.04	5.09^{abc} ±0.01
p-coumaric acid	98.53^{cde}±2.41	99.15^{cde} ±0.41	111.09^{abde} ±0.22	121.75^{abce} ±2.03	133.25^{abcd} ±0.07
p-coumaroyl hexose	141.51^{bcde}±2.44	137.32^{acde} ±0.10	135.28^{abde} ±0.98	124.39^{abce} ±0.81	120.73^{abcd} ±0.19
p-coumaroylquinic acid	49.27^{de}±1.18	48.53^{de} ±0.50	52.05^{de} ±0.18	56.65^{abce} ±0.82	62.06^{abcd} ±0.56
apigenin	0.05^{bcde}±0.00	0.06^{acde} ±0.00	0.09^{abde} ±0.00	0.11^{abc} ±0.00	0.11^{abc} ±0.00
apigenin pentose	0.72^{c}±0.02	0.73^{c} ±0.02	0.89^{ab} ±0.02	0.79 ±0.00	0.84 ±0.05
apigenin-7-*O*-glucose	1.42^{cde}±0.03	1.41^{cde} ±0.01	1.64^{abe} ±0.04	1.76^{abce} ±0.01	1.98^{abcd} ±0.02
eriodictyol glucose	20.76^{cde}±0.96	22.32^{cde} ±0.05	25.95^{abde} ±0.29	27.74^{abc} ±0.71	28.14^{abc} ±0.37
aromadendrin	52.51^{de}±0.55	51.72^{de} ±0.56	52.12^{de} ±0.47	52.12^{abc} ±0.16	48.03^{abc} ±1.08
kaempferol	6.61^{bcde}±0.17	12.46^{acde} ±0.09	18.82^{abde} ±2.60	22.72^{abce} ±0.11	24.65^{abcd} ±0.45
kaempferol glucuronide	18.76^{cde}±0.19	18.36^{cde} ±0.36	20.43^{ab} ±1.05	20.74^{ab} ±0.04	20.61^{ab} ±0.48
procyanidin B1	63.42^{cde}±3.41	62.86^{cde} ±0.30	44.83^{abde} ±0.29	4.55^{abc} ±0.19	3.11^{abc} ±0.28
procyanidin trimer	8.59^{bc}±0.99	5.79^{ac} ±0.13	2.08^{ab} ±0.01	nd	nd
protocatechuic acid	16.10^{de}±3.99	13.28^{de} ±2.27	21.90^{de} ±0.05	213.90^{abce} ±0.75	80.90^{abcd} ±1.92
quercetin 3-*O*-glucoside	2.65^{de}±0.04	2.56^{de} ±0.15	2.46^{de} ±0.03	2.20^{abce} ±0.02	1.93^{abcd} ±0.01
rutin	2.20^{bcde}±0.04	1.88^{acde} ±0.01	1.38^{abde} ±0.00	0.90^{abce} ±0.03	0.59^{abcd} ±0.04
(+)-taxifolin	0.80^{cde}±0.03	0.94^{cde} ±0.00	1.13^{abde} ±0.00	1.33^{abce} ±0.00	1.65^{abcd} ±0.11
(+)-taxifolin-7-*O*-glucoside	8.67^{bcde}±0.14	4.23^{ade} ±0.09	4.18^{ade} ±0.18	2.81^{abce} ±0.24	2.12^{abcd} ±0.08

Table 3. Continued.

	R_0/F_0	F_{15}	F_{30}	F_{60}	F_{90}
(+)-catechin	92.95^{bde}±4.02	105.95^{a} ±1.25	95.22^{bc} ±0.40	60.10^{abc} ±0.24	53.63^{abcd} ±0.04
ellagic acid hexose	185.73±2.19	195.60 ±10.05	186.99 ±1.87	193.16 ±14.1	193.44 ±9.42
ellagic acid	134.12±6.80	148.59 ±0.71	148.95 ±1.04	146.17 ±1.69	152.05 ±0.28
bis-HHDP-glucose	17.22±0.43	19.58 ±0.92	17.57 ±0.39	13.85^{abc} ±0.01	12.81^{abc} ±0.27
brevifolin carboxilic acid	1.72^{de}±0.07	1.92 ±0.10	1.86 ±0.07	2.20^{ac} ±0.00	2.18^{ac} ±0.10
galloylquinic acid	1.00±0.03	0.99 ±0.03	1.02 ±0.05	1.19 ±0.04	1.27 ±0.03
dimer of caffeic acid-*O*-hexoside	7.01^{e}±0.42	7.45^{e} ±0.17	7.23 ±0.04	6.94 ±0.14	6.65^{ab} ±0.05
p-coumaric acid	98.53±2.41	99.15^{d} ±2.16	97.37^{de} ±0.46	104.35^{bc} ±1.17	103.33^{c} ±0.27
p-coumaroyl hexose	141.51^{cde}±2.44	141.06^{cde} ±0.04	137.14^{abde} ±0.60	134.73^{abc} ±0.45	132.54^{abc} ±0.20
p-coumaroylquinic acid	49.27±1.18	48.91 ±1.33	47.70 ±0.25	50.49 ±1.72	50.03 ±1.92
apigenin	0.05^{e}±0.00	0.05^{e} ±0.00	0.05 ±0.00	0.05 ±0.00	0.06^{ab} ±0.00
apigenin pentose	0.72±0.02	0.69^{d} ±0.01	0.71 ±0.01	0.83^{b} ±0.00	0.74 ±0.07
apigenin-7-*O*-glucose	1.42±0.03	1.44 ±0.03	1.42 ±0.01	1.50 ±0.10	1.49 ±0.04
eriodictyol glucose	20.76^{bcde}±0.96	24.05^{ade} ±0.25	23.84^{ade} ±0.25	25.59^{abc} ±0.23	26.26^{abc} ±0.13
aromadendrin	52.51±0.55	53.38 ±0.64	53.24 ±0.94	52.43 ±0.06	51.92 ±0.56
kaempferol	6.61^{bcde}±0.17	9.87^{acde} ±0.06	11.95^{abde} ±0.02	16.35^{abce} ±0.10	19.97^{abcd} ±0.30
kaempferol glucuronide	18.76±0.19	19.22 ±0.36	19.81 ±0.00	20.00 ±0.26	19.97 ±0.10
procyanidin B1	63.42^{bcde}±3.41	74.90^{ade} ±0.33	72.90^{ade} ±0.18	40.65^{abce} ±0.17	23.03^{abcd} ±0.10
procyanidin trimer	8.59^{bcde}±0.99	10.53^{acde} ±0.09	8.23^{abde} ±0.08	3.04^{abce} ±0.08	1.84^{abcd} ±0.03
protocatechuic acid	16.10^{e}±3.99	14.81^{e} ±1.17	19.09^{e} ±1.21	17.55^{e} ±0.86	186.35^{abcd} ±7.99
quercetin 3-*O*-glucoside	2.65±0.04	2.60 ±0.04	2.56 ±0.04	2.47 ±0.04	2.40 ±0.01
rutin	2.20^{bcde}±0.04	1.89^{acde} ±0.07	1.79^{abde} ±0.05	1.65^{abce} ±0.00	1.44^{abcd} ±0.02
(+)-taxifolin	0.80^{cde}±0.03	0.91^{de} ±0.01	0.99^{a} ±0.01	1.09^{ab} ±0.02	1.13^{ab} ±0.02
(+)-taxifolin-7-*O*-glucoside	8.67^{bcde}±0.14	4.27^{a} ±0.03	4.12^{a} ±0.13	4.08^{a} ±0.05	4.21^{a} ±0.06

[a] Mean values and standard deviation. A letter "a" indicates a significant difference ($p < 0.05$) between the initial samples (R_0/F_0) and the other samples under the same temperature conditions. A letter "b" indicates a significant difference ($p < 0.05$) between the samples at day 15 (R_{15}/F_{15}) and the other samples under the same temperature conditions. A letter "c" indicates a significant difference ($p < 0.05$) between the samples at day 30 (R_{30}/F_{30}) and the other samples under the same temperature conditions. A letter "d" indicates a significant difference ($p < 0.05$) between the samples at day 60 (R_{60}/ F_{60}) and the other samples under the same temperature conditions, obtained through ANOVA and Tukey's HSD (honest significant difference) statistical tests. No letter indicates no significant differences. Samples were determined in duplicate. nd, no data



554 **Figure 1**. Principal component analysis: (A) is analysis of samples (R is room temperature, F is refrigerated temperature and F0 is the initial sample to two
555 conditions of temperature); (B) analysis of compounds [**1,** (+)-catechin; **2**, dimer of caffeic acid *O*-hexoside; **3**, Ellagic acid; **4** bis-HHDP-glucose; **5** ellagic
556 acid hexose; **6,** galloylquinic acid; **7,** *p*-coumaric acid; **8,** *p*-coumaroyl hexose; **9**, *p*-coumaroylquinic acid; **10**, apigenin; **11**, apigenin pentose; **12**, apigenin-7-
557 *O*-glucose; **13**, eriodictyol glucose; **14**, aromadendrin; **15**, kaempferol; **16**, kaempferol glucuronide; **17**, procyanidin B1; **18**, procyanidin trimer; **19**,
558 protocatechuic acid; **20**, brevifolin carboxylic acid; **21**, quercetin 3-*O*-glucoside; **22**, rutin; **23**, (+)-taxifolin; **24**, (+)-taxifolin-7-*O*-glucoside].

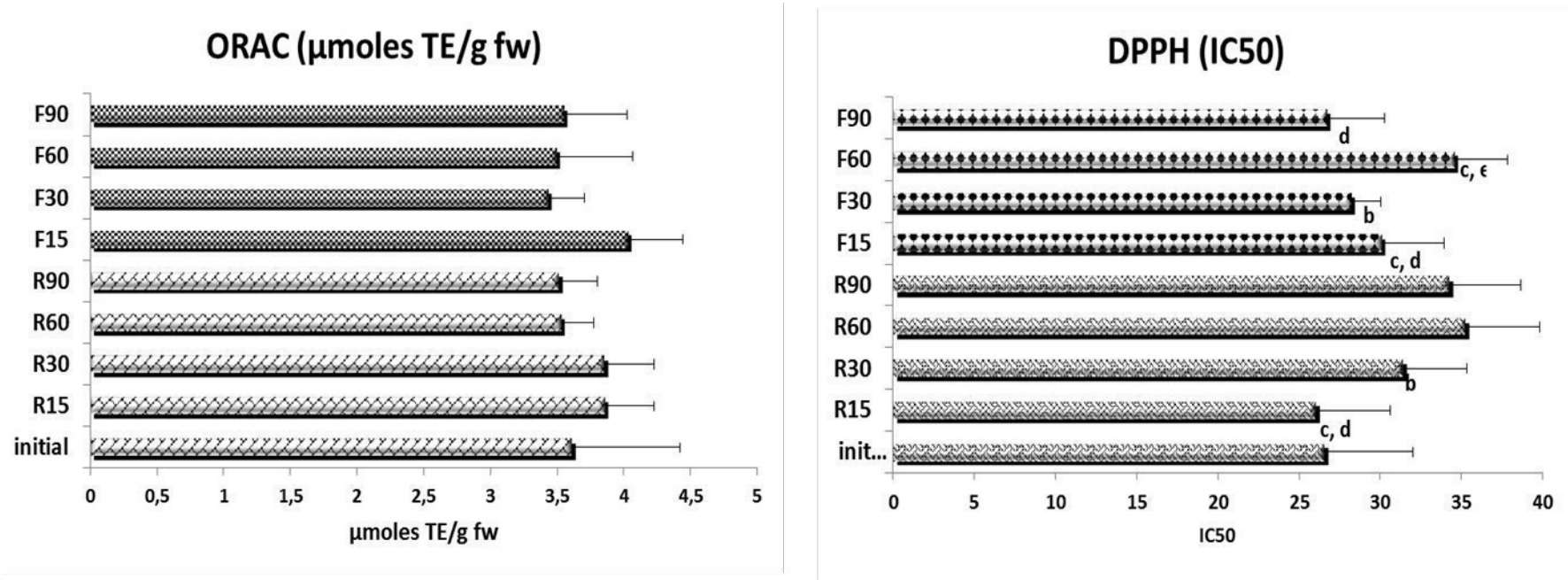


Figure 2. Left and right bar representations based on two methods: (left) ORAC; (right) DPPH; A letter "a" indicates a significant difference ($p < 0.05$) between the initial samples (R_0/F_0) and the other the samples under the same temperature conditions. A letter "b" indicates a significant difference ($p < 0.05$) between the samples stored for 15 days (R_{15}/F_{15}) and the other samples under the same temperature conditions. A letter "c" indicates a significant difference ($p < 0.05$) between the samples stored for 30 days (R_{30}/F_{30}) and the other samples under the same temperature conditions. A letter "d" indicates a significant difference ($p < 0.05$) between the samples stored for 60 days (R_{60}/ F_{60}) and the remaining samples under the same temperature conditions. A superscript letter 'e' indicates a significant difference ($p < 0.05$) between the samples stored for 90 days (R_{90}/F_{90}) and the remaining samples under the same temperature conditions. No letters indicates no significant difference.

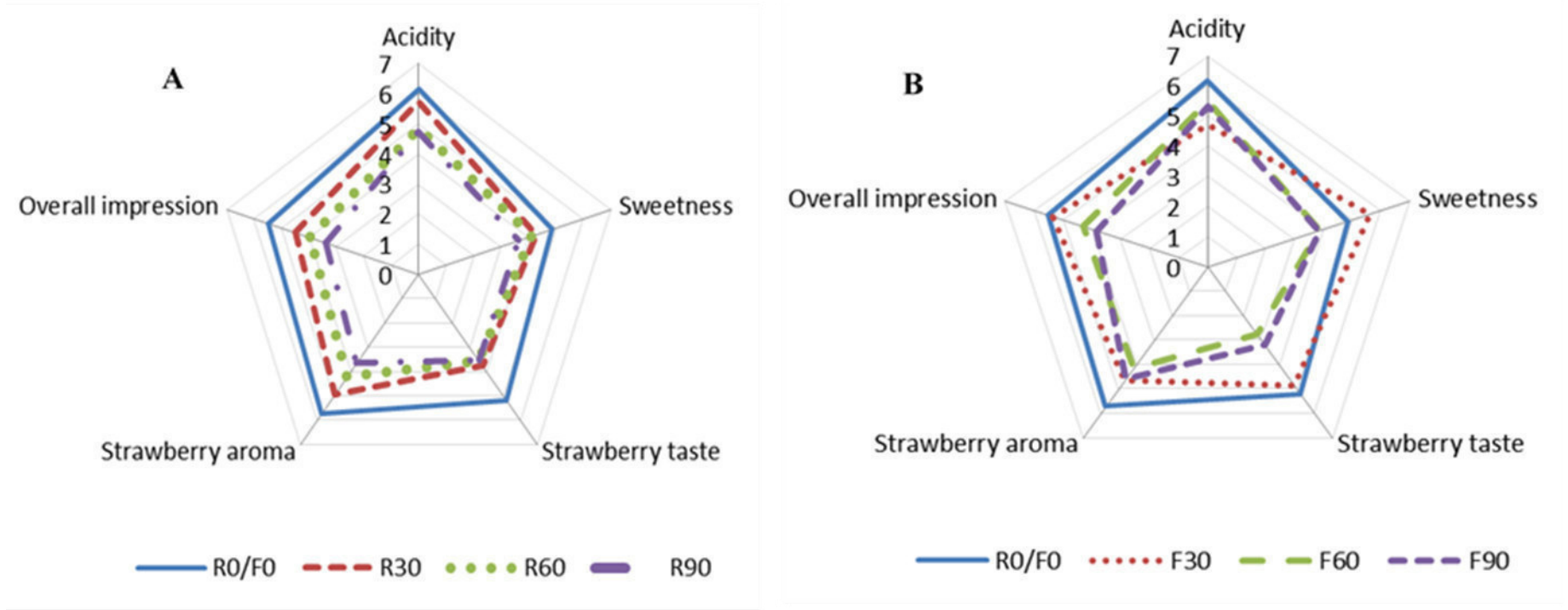


Figure 3. Sensory analysis: spider chart of samples (A) stored at room temperature (B) stored at refrigeration conditions.

TOC graphic

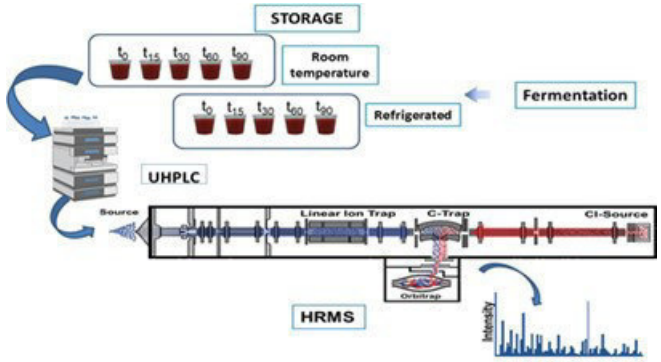

STORAGE
Room temperature
Refrigerated
Fermentation
UHPLC
Source
Linear Ion Trap
C-Trap
CI-Source
Orbitrap
HRMS
Intensity

Table S1. Concentrations of non-anthocyanin phenolic compounds (µg/100g of beverage)

	R_0/F_0	R_{15}	R_{30}	R_{60}	R_{90}
(+)-catechin-7-*O*-hexoside	$74.54^{cde} \pm 1.57$	63.46 ± 14.9	$53.26^{a} \pm 1.21$	$49.37^{a} \pm 0.23$	$48.16^{a} \pm 0.22$
propelargonidin dimer	208.70 ± 7.49	215.56 ± 2.40	221.59 ± 1.53	213.84 ± 0.40	207.70 ± 0.91
caffeic acid	$49.02^{e} \pm 4.30$	$51.66^{e} \pm 7.27$	42.07 ± 0.53	39.33 ± 4.38	$37.08^{ab} \pm 0.14$
caffeic acid hexose	$13.96^{cde} \pm 0.76$	$13.40^{de} \pm 0.35$	$12.08^{a} \pm 0.52$	$10.46^{ab} \pm 0.02$	$10.37^{ab} \pm 0.13$
galloyl-HHDP-glucose	$7.64^{ce} \pm 0.90$	7.62 ± 0.33	$9.60^{a} \pm 0.31$	9.26 ± 0.10	$9.54^{a} \pm 0.64$
tris-galloyl-HHDP-hexose	$8.05^{cde} \pm 0.43$	$8.06^{de} \pm 0.21$	$6.24^{ade} \pm 0.42$	$15.03^{abc} \pm 0.03$	$14.09^{abc} \pm 1.27$
monogalloyl glucose	$2.94^{bc} \pm 0.11$	$4.56^{acde} \pm 0.02$	$9.10^{abde} \pm 0.24$	$2.59^{bc} \pm 0.03$	$2.16^{bc} \pm 0.51$
naringenin	0.13 ± 0.00	0.14 ± 0.01	0.15 ± 0.00	0.15 ± 0.01	0.15 ± 0.00
phloridzin	$1.31^{cde} \pm 0.02$	$1.34^{de} \pm 0.00$	$1.39^{ade} \pm 0.02$	$1.25^{abce} \pm 0.01$	$1.19^{abcd} \pm 0.01$
1-*O*-protocatechuyl-β-xylose	$107.37^{de} \pm 6.19$	$102.22^{de} \pm 0.15$	$94.40^{de} \pm 5.38$	$77.58^{abc} \pm 1.85$	$77.30^{abc} \pm 0.77$
protocatechuic acid 4-*O*-β-hexoside	51.14 ± 3.47	43.89 ± 8.41	43.22 ± 0.75	36.86 ± 3.69	37.53 ± 1.90
quercetin glucuronide	$79.88^{e} \pm 2.52$	$79.35^{e} \pm 0.79$	$80.24^{e} \pm 0.94$	74.99 ± 2.31	$71.92^{abc} \pm 0.96$
quercetin	$7.86^{bcde} \pm 0.13$	$10.67^{acd} \pm 0.00$	$12.76^{abde} \pm 0.18$	$11.42^{abc} \pm 0.05$	$11.03^{ac} \pm 0.00$

Table S1. Continued.

	R_0/F_0	F_{15}	F_{30}	F_{60}	F_{90}
(+)-catechin-*O*-hexoside	74.54^{cde} ±1.57	77.05^{ac} ±0.26	75.66^{b} ± 0.36	81.65^{a} ± 3.81	83.97^{a} ± 0.30
propelargonidin dimer	208.70 ± 7.49	221.60^{e} ± 0.34	214.37^{bd} ± 1.08	210.59 ± 0.08	204.86^{b} ± 3.16
caffeic acid	49.02 ± 4.30	45.84 ± 1.96	46.51 ± 0.14	44.47 ± 0.45	47.26 ± 1.03
caffeic acid hexose	13.96 ± 0.76	14.89 ± 0.04	14.52 ± 0.59	13.80 ± 0.83	13.50 ± 0.12
galloyl-HHDP-glucose	7.64 ± 0.90	7.27 ± 0.06	7.71 ± 0.30	8.20 ± 0.01	7.29 ± 0.15
tris-galloyl-HHDP-hexose	8.05^{e} ± 0.43	7.80^{e} ± 0.27	6.81^{e} ± 0.15	7.26^{e} ± 0.14	15.87^{abcd} ± 0.16
monogalloyl glucose	2.94^{bcde} ± 0.11	3.93^{acd} ± 0.19	5.71^{abe} ± 0.37	6.21^{ae} ± 0.60	3.27^{acd} ± 0.13
naringenin	0.13 ± 0.00	0.13 ± 0.01	0.13 ± 0.00	0.14 ± 0.02	0.15 ± 0.00
phloridzin	1.31 ± 0.02	1.39 ± 0.02	1.38 ± 0.02	1.36 ± 0.00	1.37 ± 0.01
1-*O*-protocatechuyl-β-xylose	107.37 ± 6.19	112.66 ± 0.92	111.16 ± 3.59	105.73 ± 4.79	102.56 ± 1.85
protocatechuic acid 4-*O*-β-hexoside	51.14 ± 3.47	44.32 ± 9.68	53.38 ± 10.23	37.14 ± 1.53	33.93 ± 0.40
quercetin glucuronide	79.88 ± 2.52	80.37 ± 1.23	79.62 ± 1.50	79.07 ± 0.59	79.19 ± 2.79
quercetin	7.86^{bcde} ± 0.13	9.78^{acde} ± 0.13	10.67^{abd} ± 0.16	11.20^{abc} ± 0.12	11.00^{ab} ± 0.02

Mean values and standard deviation. A superscript letter 'a' indicates a significant difference ($p < 0.05$) between the initial samples (R0/F0) and the other samples under the same temperature conditions. A superscript letter 'b' indicates a significant difference ($p<0.05$) between the samples at day 15 (R15/F15) and the other samples under the same temperature conditions. A superscript letter 'c' indicates a significant difference ($p<0.05$) between the samples at day 30 (R30/F30) and the other samples under the same temperature conditions. A superscript letter 'd' indicates a significant difference ($p<0.05$) between the samples at day 60 (R60/ F60) and the other samples under the same temperature conditions, obtained through ANOVA and TUKEY'S HSD (honest significant difference) statistical tests. No superscript letter indicates no significant differences. Samples were determined in duplicate. nd: no data.

Table S2. Variable contribution, based on correlations. PCA analysis.

	Factor 1	**Factor 2**
(+)-catechin	0,048797	0,01031
dimer of caffeic acid O-hexoside	0,046931	0,008871
ellagic acid	0,038443	0,000074
bis HHDP glucose	0,041315	0,002639
ellagic acid hexose	0,0373	0,048487
galloylquinic acid	0,048282	0,000085
p-coumaric acid	0,046948	0,047426
p-coumaroyl hexose	0,048419	0,000011
p-coumaroylquinic acid	0,042466	0,087826
apigenin	0,043652	0,026696
apigenin pentose	0,0203	0,004458
apigenin-7-*O*-glucose	0,045552	0,06137
eriodictyol glucose	0,043121	0,055859
aromadendrin	0,039406	0,037925
kaempferol	0,046545	0,042664
kaempferol glucuronide	0,031157	0,077965
procyanidin B1	0,045802	0,031599
procyanidin trimer	0,041545	0,068609
protocatechuic acid	0,019564	0,211357
brevifolin carboxylic acid	0,038789	0,115917
quercetin 3-*O*-glucoside	0,046475	0,021744
rutin	0,049706	0,00003
taxifolin	0,04771	0,012071
taxifolin-7-*O*-glucoside	0,041776	0,02601

.

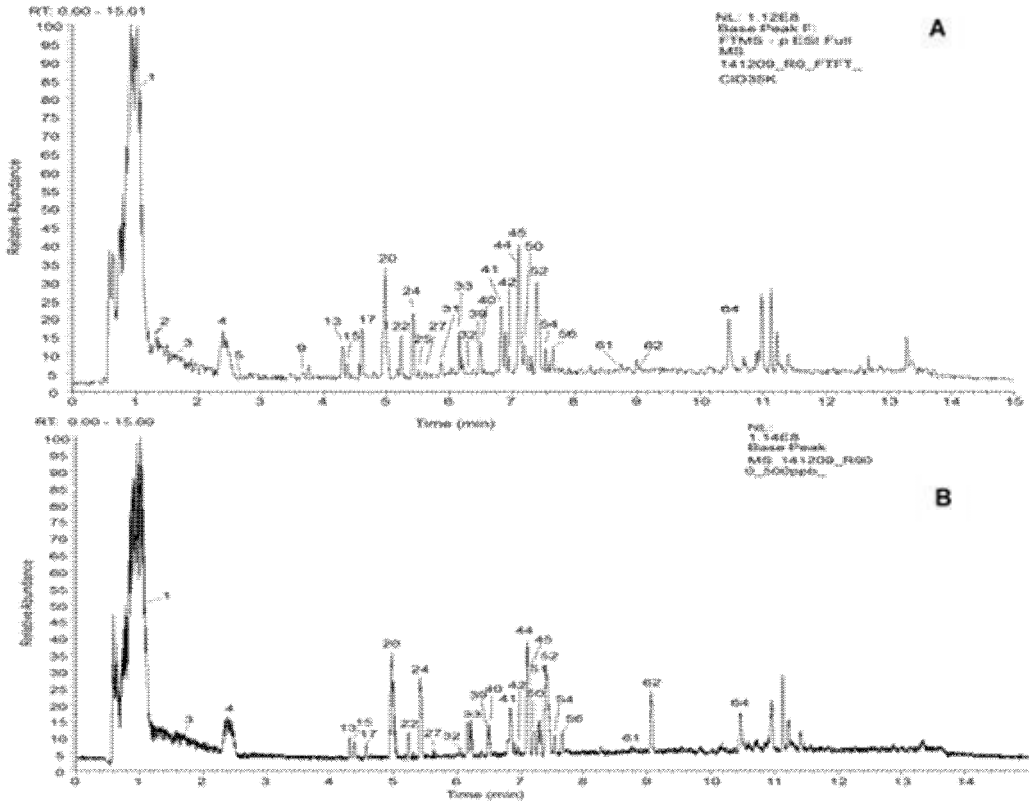


Figure S1. Total ion current chromatograms in ES (-) in full scan mode (FSM) of initial sample (A), and R90 (B). The peaks are marked with numbers that refer to the tentative identification in Table 1

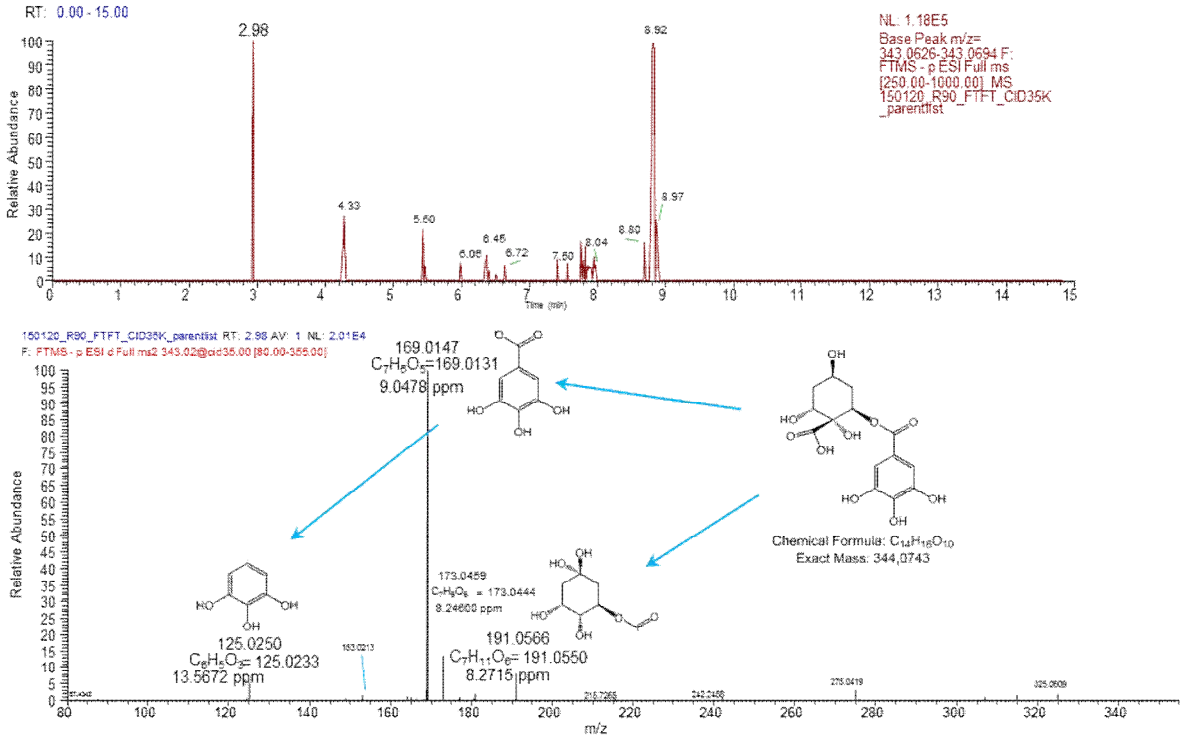


Figure S2. Full scan and MS2 spectra with characteristic fragmentation of Nº 6 compound, with tentative identification of galloyl quinic acid.

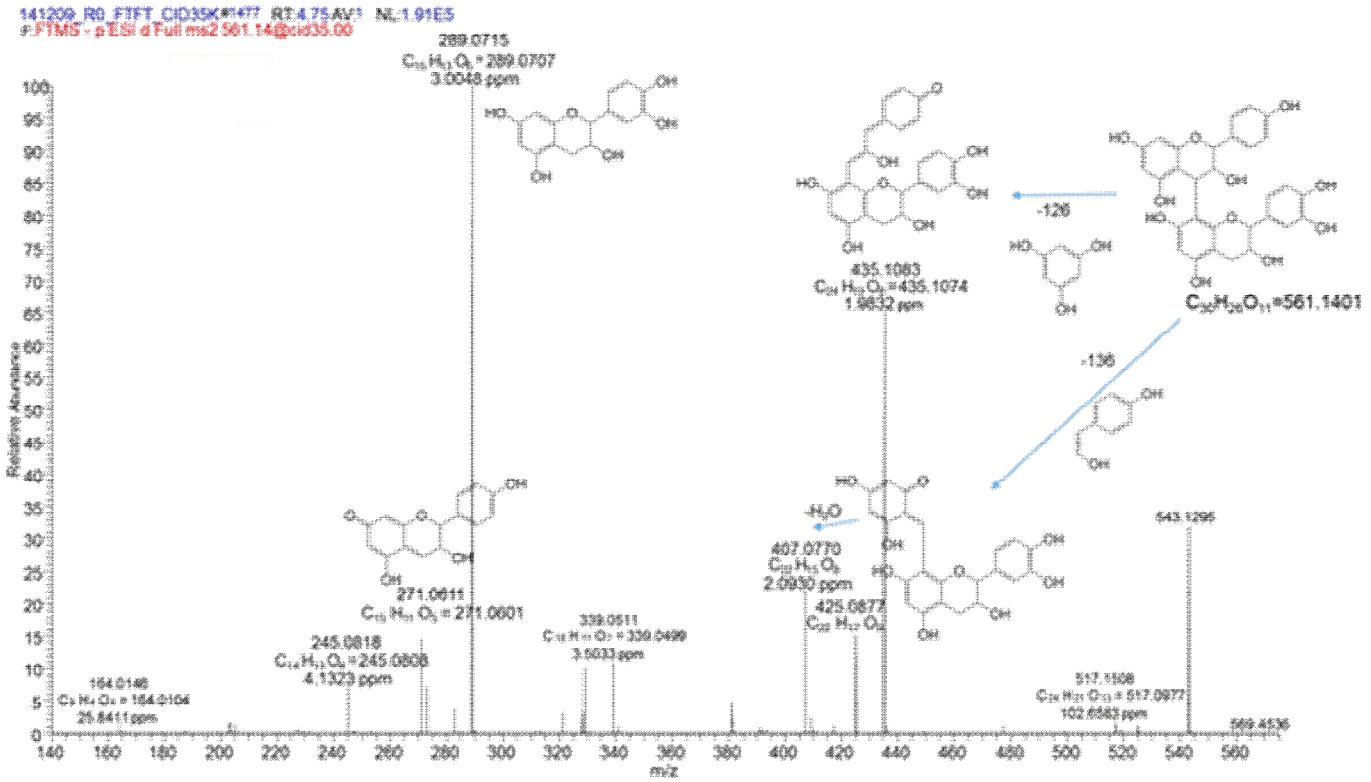


Figure S3. MS2 spectra with a proposal fragmentation pattern characteristic of propelargonidin dimer.

CAPÍTULO QUINTO

V

Title: Determination of hydroxytyrosol produced by winemaking yeasts during alcoholic fermentation using a validated UHPLC–HRMS method

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Determination of hydroxytyrosol produced by winemaking yeasts during alcoholic fermentation using a validated UHPLC-HRMS method

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Abstract

Hydroxytyrosol (HT) is a phenolic compound of recognized bioactivity that has been described in wines but little is known about its origin. This work demonstrates that yeast involved in wine making, i.e. *Saccharomyces cerevisiae* strains and the non-*Saccharomyces Torulaspora delbrueckii*, can synthesise HT, as this compound was identified in the intracellular media of three strains by means of a developed and validated UHPLC-HRMS method with LOQ and LOD of 0.108 and 0.035 ng mL^{-1} respectively. Controlled fermentations were performed with different varieties of grapes (*Corredera*, *Moscatel*, *Chardonnay*, *Palomino fino*, *Sauvignon Blanc*, *Vijiriega*, and *Tempranillo)* and synthetic must. The *Saccharomyces cerevisiae* strain QA23 was the most efficient producer of HT from tested yeasts. On the other hand, the grape variety influences HT wine concentrations. Furthermore, the maximum concentration of HT is reached between the fourth and sixth day of fermentation. This work reveals that yeasts have a great potential for the production of HT.

Keywords: *Saccharomyces*, Phenolic compound, Intracellular, Winemaking, Q-exactive, Biomass.

1. Introduction

Hydroxytyrosol (HT) 2-(3, 4-dihydroxy-phenyl) ethanol (3, 4-DHPEA), is a higher alcohol (phenyl ethyl alcohol), found in extra-virgin olive (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012) as well as in in fermented beverages such as wine (Bordiga et al., 2016). The most common synthesis pathway includes the hydroxylation of its immediate precursor, tyrosol, in the Ehrlich pathway (Figure 1). This is in turn produced from tyrosine by yeasts during alcoholic fermentation (AF), as follows: (1) transamination of tyrosine; (2) decaboxylation of *p*-hydroxyphenylpyruvate by pyruvate decarboxylase; (3) reduction of *p*-hydroxyphenylaldehyde by alcohol dehydrogenase (ADH) (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008; Pineiro, Cantos-Villar, Palma, & Puertas, 2011; Satoh, Tajima, Munekata, Keasling, & Lee, 2012). Consequently, Tyrosol and HT could be considered secondary metabolites produced from tyrosine by some yeast strains by means of a transformation of amino acids (Garrido & Borges, 2013) during alcoholic fermentation (Zhu et al., 2011). On the one hand, the content of HT appears to be related mainly to the nitrogen content in musts during alcoholic fermentation while on the other, it is well known that *Saccharomyces cerevisiae* can use tyrosine and tryptophan as a source of cellular nitrogen. Fusel alcohol such as tyrosol, HT and tryptophol respectively are the main products of its catabolism (Bordiga et al., 2016). This suggests that the final contents of HT and tyrosol in wine could be influenced by microbial activity during alcoholic fermentation (Romboli, Mangani, Buscioni, Granchi, & Vincenzini, 2015).

As a bioactive compound, HT has been object of study in many research reports. Some studies have demonstrated that HT is a potent stimulator of mitochondrial biogenesis in retinal epithelial cells that contribute to eye health (Zhu et al., 2010) as well as having anticarcinogenic (Roleira et al., 2015), cardioprotective (Mnafgui et al., 2015), antidiabetic, and neuroprotective qualities (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012; Marhuenda et al., 2016; Rigacci & Stefani, 2016). Furthermore, the EFSA (European Food Safety Authority), has admitted a claim on these healthy effects based on the protection of LDL

particles from oxidative damage and maintenance of normal blood HDL-cholesterol concentrations, as these effects were demonstrated after the consumption of extra-virgin olive oil due to its high content in HT (European Food Safety Authority [EFSA] Panel on Dietetic Products Nutrition and Allergies [NDA], 2011).

To identity, quantify, and elucidate the occurrence of HT, different analytical methods have been used in various food matrices. Thus HT has been analysed by: gas and liquid chromatography; capillary electrophoresis in wines (Piñeiro, Cantos-Villar, Palma, & Puertas, 2011); nuclear magnetic resonance in herbal medicine products (Lemonakis, Gikas, Halabalaki, & Skaltsounis, 2013). Associated techniques have also been used, such as: HPLC with GC in olive mill wastewaters (Allouche, Damak, Ellouz, & Sayadi, 2004); HPLC with fluorescence in grape leaves (Vrhovsek et al., 2012); HPLC with DAD and MS in wines (Boselli, Minardi, Giomo, & Frega, 2006). The use of HRMS associated with UHPLC has been used before in herbal medicinal products in the determination of HT (Lemonakis, Skaltsounis, Tsarbopoulos, & Gikas, 2016). This latter technique is a powerful tool to unequivocally identify and quantify compounds in different matrices, and therefore it may be useful in trying to elucidate the origin and evolution of HT in wines.

The aim of the present work is to develop and validate an UHPLC-HRMS method to assess HT and to investigate the origin of its occurrence in wines. For this purpose, the role of different strains of winemaking yeasts on the occurrence of HT in wines is studied during alcoholic fermentation (AF). Finally, the main objective is to examine the production of HT by yeasts through the evidence that the determination of this compound in the intracellular compartment provides when using a validated HRMS method coupled with UHPLC.

2. Materials and methods

2.1. Reagents and materials

97 HT standard (98%) was purchased from Chengdu Biopurify Phytochemicals Ltd. (Wenjiang
98 Zone,Chengdu, Sichuan, China), HPLC-grade methanol was acquired from Merck (Darmstadt,
99 Germany) and HPLC-grade formic acid from Panreac (Barcelona, Spain).

100 *2.2. Yeast strains*

101 The experiments on white musts were performed with the commercial wine yeast strain Enartis
102 Ferm Aroma White (Enartis). In the fermentation of *Tempranillo* must and synthetic must, three
103 different commercial strains: were used *S. cerevisiae* Lalvin YSEO QA23® (Lallemand), *S.*
104 *cerevisiae* Red Fruit RF® (Enartis) and *T. delbrueckii* TD291 Biodiva™ (Lallemand).

105 *2.3. Samples*

106 *2.3.1. Grapevine*

107 Vines were grown in a typical soil-type (*albariza)* with a plant density of 3600 vines/ha. The
108 grapes used in the fermentations belonged to an experimental cultivar located in the Rancho de
109 la Merced (IFAPA, Jerez de la Frontera, Spain), and were from seven varieties as follows:
110 *Corredera*, *Moscatel*, *Chardonnay*, *Palomino fino*, *Sauvignon Blanc*, *Vijiriega*, and
111 *Tempranillo*.

112 The degree of ripeness was followed weekly during the maturation process (data not shown).
113 Grapes were harvested at their stage of optimum maturity in August 2015.

114 *2.3.2. Alcoholic fermentation procedure*

115 *2.3.2.1. Musts of white grapes.* Grapes were harvested at optimum ripening conditions.
116 Subsequently, they were destemmed, crushed, and pressed. Then pectolitic enzymes (2.5 mL
117 hL^{-1}, Enartis ZYM, Italy) and SO_2 (Sulfosol, Sepsa-Enartis) were added into the must. After 24
118 h at 4 ºC, the must was dejuiced and placed in a 100-L steel vessel. Alcoholic fermentation (AF)
119 was carried out and monitored in vessels by yeasting (Aroma White, Italy) at 18 ºC. AF was
120 considered completed when the concentration of residual sugars was lower than 3 g L^{-1}.

121 *2.3.2.1. Musts of Tempranillo grapes. Tempranillo* grapes at optimum ripeness were harvested
122 manually in 18 kg plastic boxes; they were in good sanitary conditions and were transported to
123 the experimental winery. Musts were produced using a pneumatic press with pectolitic enzymes
124 (3 mL hL^{-1}, Enartis ZYM, Italy) and 40 mg L^{-1} of sulphur dioxide (SO2) (Sepsa- Enartis) were
125 added. Musts were placed in 15 stainless steel vats of 10-L capacity.
126 Five different methods of inoculation were used: (1) CTQA, with *Saccharomyces cerevisae*
127 QA23 yeast strain; (2) CTRF, with *Saccharomyces cerevisae* RF yeast strain; (3) SIQA23,
128 sequential inoculation first with commercial non-*Saccharomyces* strain *Torulaspora delbrueckii*
129 TD291 and later when the density had decreased by 15 points just after the start of the AF with
130 *S. cerevisiae* QA23; (4) SIRF, sequential inoculation first with commercial non-*Saccharomyces*
131 strain *Torulaspora delbrueckii* TD291 and when density had decreased by 15 points just after
132 the start of the AF with *S. cerevisiae* RF; and (5) SP, spontaneous fermentation without any
133 inoculation using commercial yeasts.

134 *2.3.3 Intracellular samples*

135 Six alcoholic fermentations were performed in synthetic must with a sugar content of 100 g L^{-1}
136 fructose and 100 g L^{-1} glucose and amino acids (purity ≥ 99 %) (Riou, Nicaud, Barre, &
137 Gaillardin, 1997) with three strains of yeast (QA23, RED FRUIT and *Torulaspora delbrueckii*).
138 The must was sterilized with bottle-top vacuum filters (Nalgene PES membrane). Each
139 Erlenmeyer flask with 750 mL of SM was inoculated with 10^6 cell mL^{-1} and capped with taps
140 equipped with a capillary to release carbon dioxide. The fermentation was monitored by
141 weighing the flasks daily before and after sampling.

142 *2.4. Sampling*

143 Samples were taken every day from inoculation until the end of AF. Samples were collected and
144 stored at -80 ºC until the analysis. The end of AF for each grape variety was different and,
145 consequently, each fermentation lasted a different number of days. The end of AF was reached
146 when the sugars were almost all consumed (lower 3 g L^{-1}). Table 1 shows the time of the

147 fermentation process and the concentrations of sugars at the end of alcoholic fermentation in
148 white grapes (Table 1a). Table 1b shows the time of fermentation and the concentration of
149 sugars (approximately 10.9 ºBe) at the end of fermentation of the 5 fermentation methods for
150 *Tempranillo* grapes.

151 *2.4.1. Intracellular metabolite extraction*

152 Samples of the intracellular compartment were collected at the second day of fermentation in a
153 volume corresponding to 10^9 cells mL^{-1}. Immediately, they were subjected to a cold glycerol-
154 saline quenching (Villas-Bôas & Bruheim, 2007), were stored at -80 ºC until the extraction
155 process was conducted. The intracellular extraction was performed following the method
156 reported by Smart et al. with minor modifications (Smart, Aggio, Van Houtte, & Villas-Bôas,
157 2010). To the cell pellets, 2.5 mL of cold methanol-water solution (50% [v/v], -30 ºC) were
158 added, mixed for 1 min and then frozen at -80 ºC. The samples were subjected to two cycles of
159 freeze-thaw (thaw in an ice bath for 4 min; then were frozen at -80 ºC for 30 min). After the last
160 cycle, they were subjected to sonication for 1 min in an ice bath using an ultrasonic liquid
161 processor (Sonicator Sonoplus HD 2070, Bandelin electronic GmbH & Co. KG, Berlin,
162 Germany). Afterwards, the samples were centrifuged at 36086*g* for 20 min at -20 ºC using a
163 refrigerated centrifuge (Sorvall LYNK 6000, ThermoFisher scientific, Waltham, MA USA).
164 Another 2.5 mL of cold methanol-water was added to the pellet and then centrifuged; the
165 supernatants were collected, pooled, and stored at -80 ºC until analysed.

166 *2.5. Sample clean up*

167 Samples were cleaned up as previously reported by Rodriguez-Naranjo, Gil-Izquierdo,
168 Troncoso, Cantos, & Garcia-Parrilla (2011) with the following modifications: C18 SPE
169 cartridges (Variant, Agilent) were conditioned with 2 mL of methanol and 2 mL of milliQ
170 water. An aliquot of 500 µL of sample was loaded followed by a washing step with 2 mL of a
171 10% v/v methanol solution. The analytes were eluted with 1 mL of methanol; afterwards
172 solvents were evaporated until dryness at 34ºC, 2000 rpm during 6 h with a vacuum

concentrator (HyperVAC-LITE, GYOZEN, Korea). Then samples were reconstituted with 167 µL of methanol/water 10% v/v and stored at -20 ºC until analysis.

2.6. UHPLC/HRMS parameters

The analysis was carried out in a UHPLC Dionex Ultimate 3000 system (Thermo Fisher Scientific (Bremen, Germany) all devices were controlled by Chromeleon Xpress Software. The column used was a ZORBAX RRHDSB-C18 (2.1×100 mm, 1.8-µm particle size) with a guard column (2.1×5 mm, 1.8-µm particle size). Column and guard column were purchased from Agilent Technologies (Waldbronn, Germany). The separation was performed using column temperature of 40 ºC, a flow of 0.5 mL min^{-1}, and injection volume of 5 µL. The chromatographic conditions consisted of two phases (A) aqueous formic solution 0.1%, and (B) solution 0.1% of formic acid in methanol and the gradient was programmed as follows: 95% A, 5% B (0 – 1 min); 0% A, 100% B (1 – 8.5 min); 95% A, 5% B (8.6 – 10 min).

A target MS^2 in negative mode with a heated ionization source HESI was selected using the transition 153 →123 HCD 100.00 in order to both identify and quantify. The main HRMS parameters were heater and capillarity temperature (400 –275 ºC respectively), spray voltage 3.0 KV; flow rates of sheath gas and auxiliary gas (65, 25 arbitrary units, respectively). Other parameters of HRMS methods were normalized collision energy (NCE) 40; S-lens RF 50% and mass resolving power (RP) 70,000 FWHM.

2.7. Statistical Analysis

Statistical analyses were performed by means of Statistica software (StatSoft, 2014). One-way analysis of variance (ANOVA) and Tukey's HSD (honest significant difference) test were assessed to test significant differences at $p < 0.05$. Additionally, principal component analysis (PCA) was used for data analysis

3. Results and discussion

3.1. Method validation

198 The validation procedure was carried out following different international guides (AOAC, 1998;
199 FDA, 2012) to establish parameters, such as: detection (LOD) and quantification (LOQ) limits,
200 precision, linearity, recovery, matrix effects, and effects of solid-phase extraction. As a blank, a
201 fermentation sample was used after cleaned using SPE, because it bears a greater similarity to
202 the matrix than to the must without fermentation.

203 The linearity, LOD, and LOQ were experimentally determined by the injection of 11 solutions
204 in the fermentation medium cleaned using SPE, as explained in Section 2.4, because it
205 reproduces matrix characteristics better than does the solvent (methanol/water 10% v/v).
206 Calibration standards of HT were prepared for each analytical batch and three replicates were
207 determined at 11 concentrations (1000, 500, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39
208 ng mL^{-1}) with 4 degrees of linearity. The detection limits were calculated based on the standard
209 deviation of the response (σ) and the slope (Ich, 2005). Calibrations curves with their slope (*S*),
210 intercept and correlation coefficient (*r*) were calculated by plotting the peak area vs. the
211 concentration of the standards using Statististica software version 12 (StatSoft, 2013).

212 $LOD = 3.3\ \sigma\ S^{-1} LOQ = 10\ \sigma\ S^{-1}$

213 The results indicated linearity by a curve with $r^2 = 0.9995$ with LOD 0.035 ng mL^{-1} and LOQ
214 0.108 ng mL^{-1}. These results improved the limits reached before by Bordiga et al. in wines using
215 HPLC-PDA-MS/MS (LOD 4 ng mL^{-1} – LOQ 11 ng mL^{-1}) (Bordiga et al., 2016) probably
216 because they determined several compounds simultaneously and our method focused on HT.

217 The intermediate precision was calculated measuring standard deviation (RSD) in a set of two
218 concentrations (0.1 - 1 ng mL^{-1}) for 5 days with 5 replicates per concentration. Repeatability
219 was assessed during a working session with 5 replicates per concentration. The data for
220 intermediate precision and repeatability are shown in Table 2.

221

The matrix effect was tested in a clean-up matrix by spiking with standard solution in 10 concentrations: 0.079, 0.158, 0.316, 0.632; 1.264, 5.056, 20.224, 80.896, 404.48, and 1011 ng mL^{-1}. The slopes resulting from the spiked matrix and calibration solutions (methanol 10% v/v) in the linear range were used to evaluate the matrix effect. The relation between the slopes was defined as (slope in solvent/slope in the spiked matrix)*100 and expressed as %ME (Trufelli, Palma, Famiglini, & Cappiello, 2011). The resulting value was 1.05%, which is considered an irrelevant value, as the calibration curves in the solvent and matrix spiked are very similar. Fig. 1 of supplementary material shows the results.

Recovery was calculated from the spiked matrix at five different concentrations ranging from 1.2 to 1011.2 ng mL^{-1}. The results were from 116 – 58% within the recommended values (40-120%) for concentrations ranging from 1 to 1000 ng mL^{-1} (Gustavo González & Ángeles Herrador, 2007). Fig. 2 of supplementary material shows the results.

SPE was used as a cleaning technique because it allowed the removal of different components that could be interfering with mass analysis. A solid-phase extraction (SPE) approach has been tested in order to avoid overestimation and sub-estimation of the quantity. Three different solutions were prepared with a concentration LOQ, LOQ + 50% and 3LOQ in methanol /water 10% v/v, which were analysed after the SPE clean-up procedure (Gasperotti, Masuero, Guella, Mattivi, & Vrhovsek, 2014). The results showed an extraction efficacy of 96.7%, 86.3%, and 143.2%, respectively. On the other hand, to evaluate the amount of analyte that is dragged in the washing step, the solutions were analysed and the HT contents were under LOQ limits, indicating the clean-up procedure was efficient.

3.2. Intracellular HT

Hydroxytyrosol is a phenolic compound that could be formed from a degradation or transformation from other polyphenolic structures present in wines (i.e. anthocyanins, (Motilva et al., 2016). Likewise it is formed from oleopurein degradation in olive oil (Charoenprasert & Mitchell, 2012). Nonetheless, different synthesis of HT has been proposed. For instance, the

metabolism in humans involves a pathway starting from dopamine, which is transformed by the monoaminoxidase to give 3,4-dihydroxyphenylacetaldehyde that can be reduced by the aldehyde reductase to HT (Pérez-Mañá et al., 2015). Additionally, the Ehrlich pathway shown in Fig. 1 relates the amino acid metabolism with HT synthesis. However, up to now, no direct evidence of this synthesis by yeast could be determined. To demonstrate that it is a metabolite formed by yeast, we analysed the intracellular media of the yeast, as this could unequivocally demonstrate its origin apart from others that might happen. Figure 2 shows three mass chromatograms of the biomass of the strains QA, RF and *T. delbrueckii* taken at day 2 of the alcoholic fermentations of synthetic must; each of these strains were analysed in duplicate. A total of six samples were analysed and HT quantified as follows: 8.6 ± 2.7 ng mL^{-1} in the intracellular media of QA at day 2 of fermentation; 106.2 ± 35.1 ng mL^{-1} in the intracellular media of RF at day 2; and 16.1 ± 2.3 ng mL^{-1} in the intracellular media of *T. delbrueckii* at day 2 of fermentation. These results demonstrate the production of HT by the strains studied, conferring yeast with a high potential as a producer of this bioactive compound.

3.3. Fermentations in Tempranillo must

Three fermentations with different inoculation processes were performed with the *Tempranillo* variety. As shown in Fig. 3A, the production of HT was influenced by the yeast strain involved in the fermentation process. The higher concentrations of HT were observed at day 5 in CTQA and at day 3 in CTRF, ranging between 400 and 235 ng mL^{-1}, respectively. Only in the case of CTQA, was the maximum HT achieved at the moment when the reducing sugars were totally consumed. When sequential fermentation was performed, lower concentrations of HT were found, as can be seen when comparing CTQA, QA and *T delbrueckii,* (41.3%; Fig. 3A left). Moreover, when SP and SIQA were compared, SP fermentation was found to show higher concentrations (24.3%) over the other fermentations. On the other hand, Fig. 3A (right) shows RF fermentations and it can be observed that concentration values in SIRF (8.51%) were lower than in CTRF. In addition, the content of HT in SP fermentation reached values 23.8% higher

than the concentration in SIRF and CTRF (16.7%). Therefore, our results imply that when *T. delbrueckii* was used in the fermentations, the HT was in a lower concentration. Romboli et al. made a similar observation in sequential fermentation with another non-*Saccharomyces* strain, *C zemplinina* (Romboli et al., 2015). These researchers reported concentrations of 18.4 mg L^{-1} for HT+ tyrosol in wines produced by *S. cerevisiae* alone, and concentrations of 5.8 mg L^{-1} of HT+tyrosol in wines produced by sequential inoculation with *C. zemplinina* and *S. cerevisiae* Sc1.

Fig. 3B shows the score plots of PCA analysis displaying on the right side the samples that were separated considering the methods of inoculation. The samples CTQA and CTRF presented the same location while the samples of sequential inoculation were separated from each other. Spontaneous fermentation was located on the bottom-left quadrant, possibly related to the influence of concentrations of days 7 and 8, which appear in the same place in the projection of cases due to their high concentrations of SP samples.

3.4. Fermentations in must of six white varieties of grapes

To study the effect of different white grapes on HT production during alcoholic fermentation, musts made from six white grape varieties were analysed after alcoholic fermentation by *S. cerevisiae* strain Aroma White. The grapes varieties were *Corredera*, *Moscatel*, *Chardonnay*, *Sauvignon Blanc*, *Palomino Fino* and *Vijiriega*, and the results are represented in a bar graph together with reducing sugars (Fig. 4A). All varieties followed a similar trend, a progressive increase until the highest concentration was reached at the fifth day (173, 159, 167, 288, 89 and 238 ng mL^{-1}, respectively) except for the variety *Chardonnay*, which presented a slight delay, and reached the highest concentration one day later (185 ng mL^{-1}). The maximum contents of HT were determined when the value of reducing sugars ranged from 53% to 35% of the initial concentration. After this point, the concentrations decreased, falling to the previous values achieved on the 3rd and 4th days (no significant differences $p < 0.05$). In fact, all these results show that the time course of hydroxytyrosol production by the AROMA WHITE strain was

affected by the grape-must composition and, consequently, by the duration of the fermentation. These results agree well with those reported before by Romboli et al. (2015), who correlated high amounts of HT with the slowness in the fermentation process. In fact, the longer the fermentation lasted, the higher the HT concentration, as with the fermentations of *Sauvignon Blanc*.

Figure 4B plots the PCA analysis, representing on the left the days of the fermentation process. The data are grouped into four clusters, two smaller for the first two days (day 1and 2) and other of last days (12, 13, 14, and 15), located on the right side of the plot. The biggest cluster is in the middle of the plot, corresponding to the interval from the 8th to the 11th days and day 3. On the left, the cluster corresponds to the interval from days 4 to 7, which are those with the highest concentrations. Figure 4B displays the projection of the varieties of grapes treated as variables (Figure 4B right). However, the Chardonnay variety is located at the bottom of the projection, probably being influenced by the location of the intermediate cluster (3, 8, 9, 10, and 11) days in which the HT content remained practically unchanged (159 to 134 ng mL^{-1}). Although further studies are required to understand how the chemical composition of the must influences the HT concentration as well as the role that this compound exerts on the yeast, it is clear from our results that the strains studied synthesise HT and therefore this compound is found in wines.

4. Conclusions

As we know, this is the first study available in which HT has been identified and quantified in an intracellular compartment of *Saccharomyces* (QA23, RF) and Non-*Saccharomyces* (*Torulaspora delbrueckii*). This could be accomplished thanks to a validated HRMS method developed specifically to diminish LOD and LOQ.

Furthermore, we ascertained that the strain is a crucial factor that influences the production of HT in wines. *Saccharomyces cerevisiae* (QA23) was a more efficient producer of HT than RF in grape musts. Sequential fermentations involving the non-*Saccharomyces* yeast, *T. delbrueckii,* adversely affect the content of HT.

Additionally, the composition of grape must affect the HT concentration. *Sauvignon Blanc* and *Vijiriega* were grape varieties from which AROMA WHITE produced higher concentrations at 5th day of alcoholic fermentation. In summary, HT was a bioactive compound produced by yeast strain that can be modulated both by the involved strain and by the composition of the must.

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556

Table 1A. Sampling and mean of reducing sugars content at the end of fermentation in different variety of grapes

Grape variety	*Palomino fino*	*Vijiriega*	*Corredera*	*Moscatel*	*Sauvignon Blanc*	*Chardonay*
Days of fermentation	7	10	11	11	13	15
Reducing Sugars (g L^{-1})	2.00	1.30	2.33	1.47	1.55	1.95

Table 1B. Sampling days and mean of the content of reducing sugars at the end of fermentation in different inoculations ways.

Fermentation	*CTQA23*	*CTRF*	*ISQA23*	*ISRF*	*Spontaneous*
Days of fermentation	13	13	14	15	13
Reducing Sugars (g L^{-1})	0.64	1.02	0.56	0.84	1.2

Table 2A. Parameters of calibration curves. LDR (linear dynamic range), LOD (limit of detection), LOQ (limit of quantitate).

				Curve		
	LDR	LOD	LOQ	(slope)	(offset)	R^2
Matrix-spiked calibration	0.079-1011.00	0.03	0.11	950135	-5070483	0.9991
Solvent calibration (methanol 10%)	0.079-1011.00	0.03	0.08	72224	-355656	0.9991

Table 2B. Accuracy and repetitivity.

	Intra-day (%RSD)	Inter-day (%RSD)	Accuracy (%RE)
Low concentration	1.6	0.5	-1
High concentration	1.4	0.5	-1

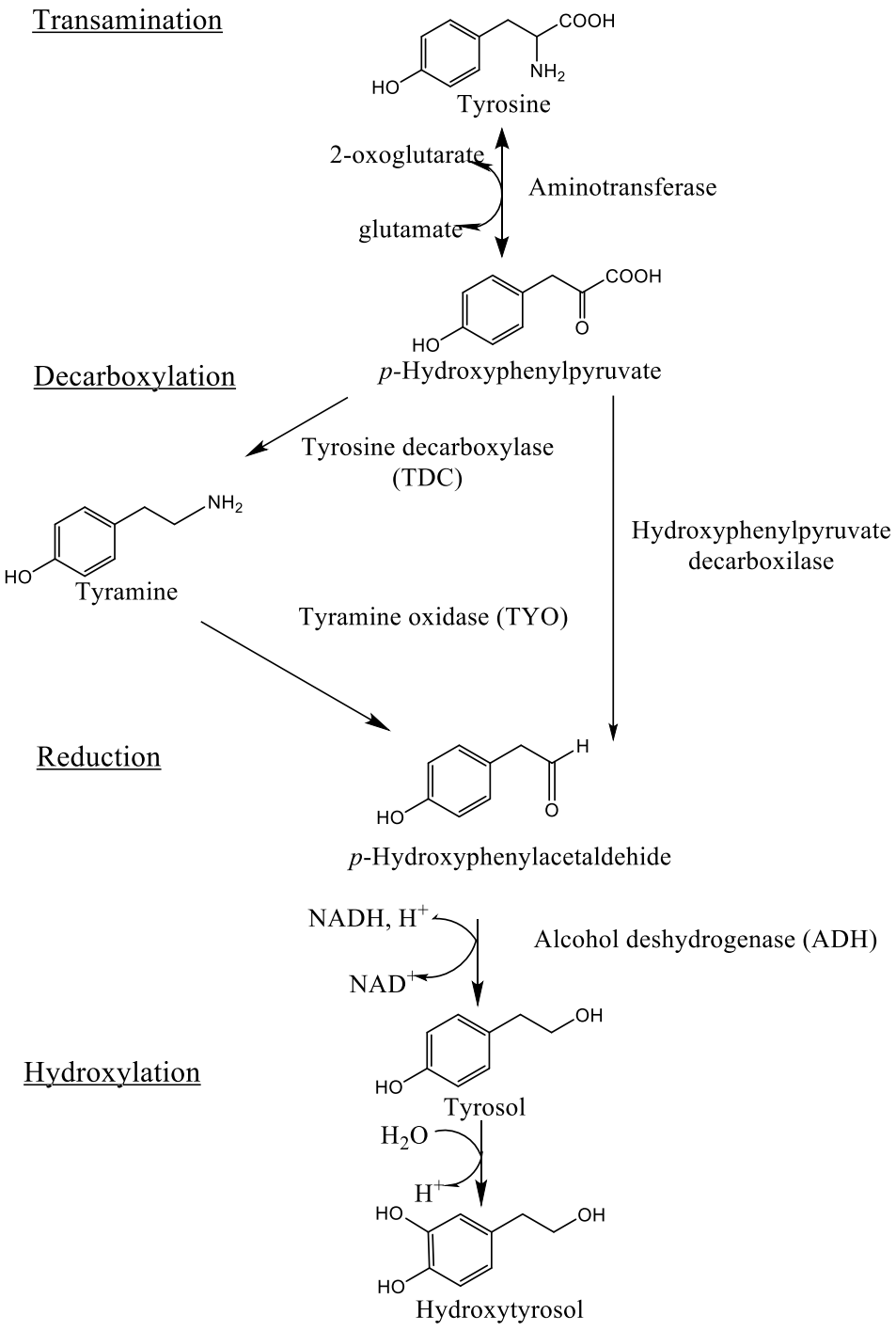

Figure 1. Ehrlich pathway for the production of hydroxytyrosol

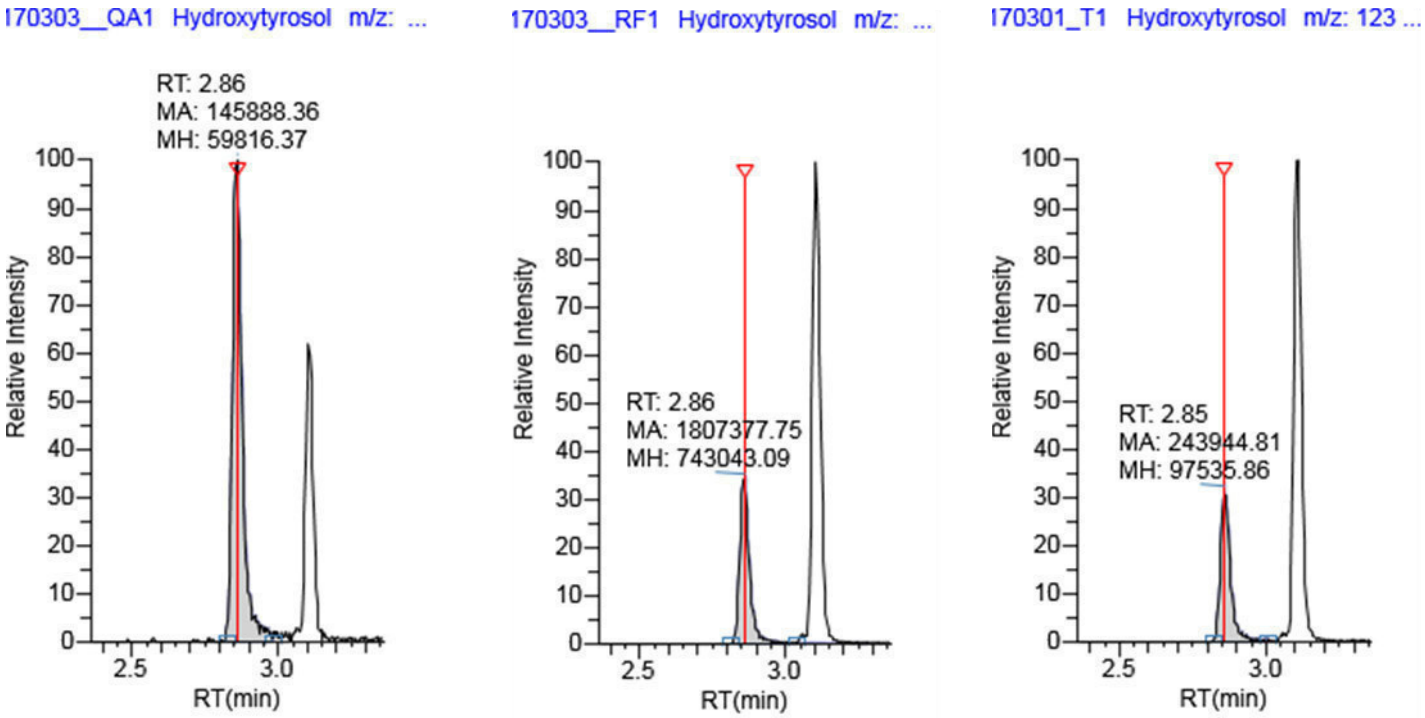

Figure 2. TIC chromatogram of HT of intracellular samples of second day of fermentation. On the left the QA sample, in the middle RF and on the right *T. delbrueckii.*

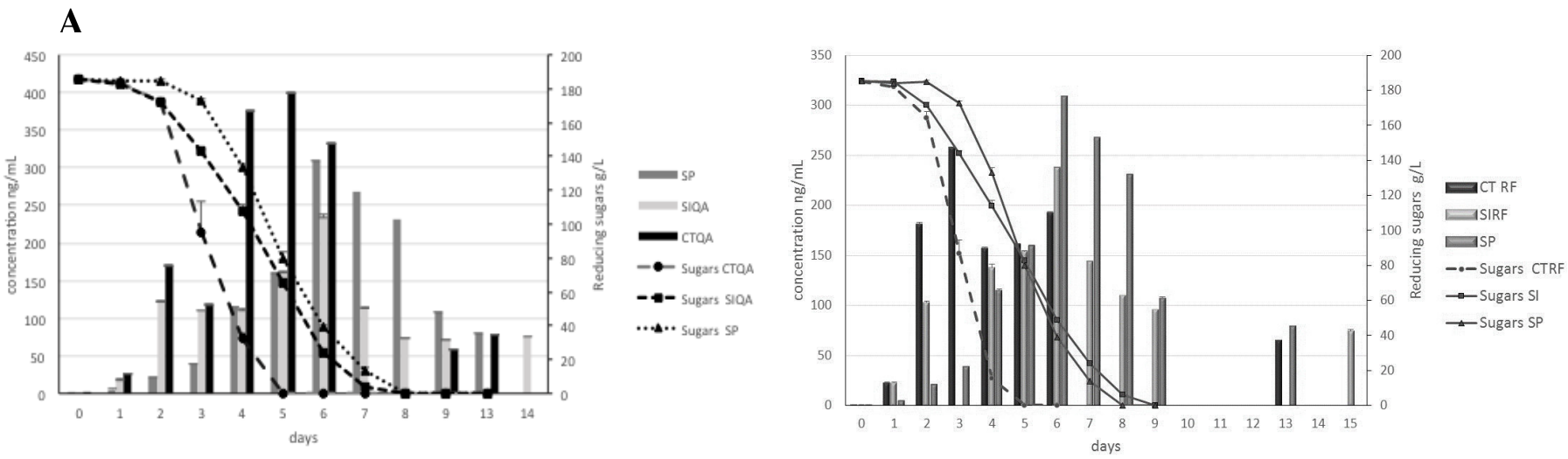

Figure 3A. Effects of two yeast strains (QA23 on the left and RF on the right) on the production of hydroxytyrosol, and time course reducing sugar in the fermentation of natural must of Tempranillo grapes. Different forms of inoculation were used: SI (sequential inoculation). SP (spontaneous fermentation). CTQA (control fermentation with *Saccharomyces cerevisiae* QA23); CTRF (control fermentation with RED FRUIT).

B

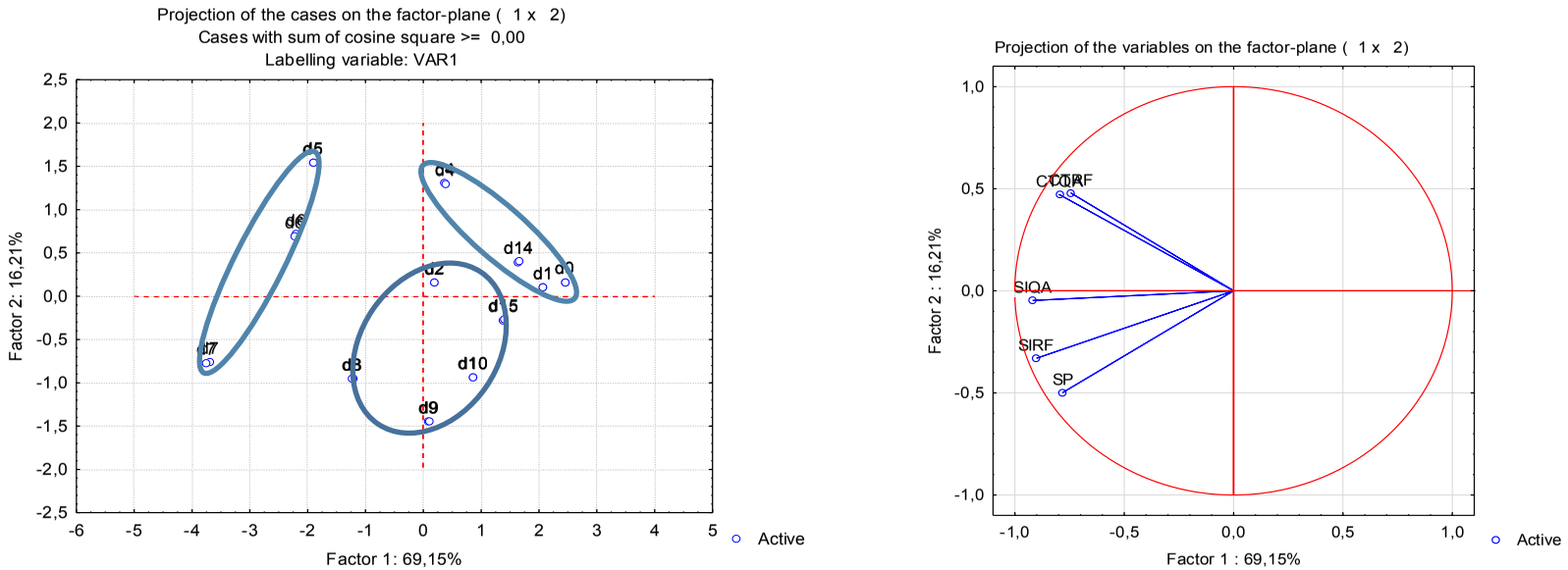

Figure 3B. Score plot of PCA analysis, on the left side, considering days of fermentation and on the right side considering different methods of inoculation

A

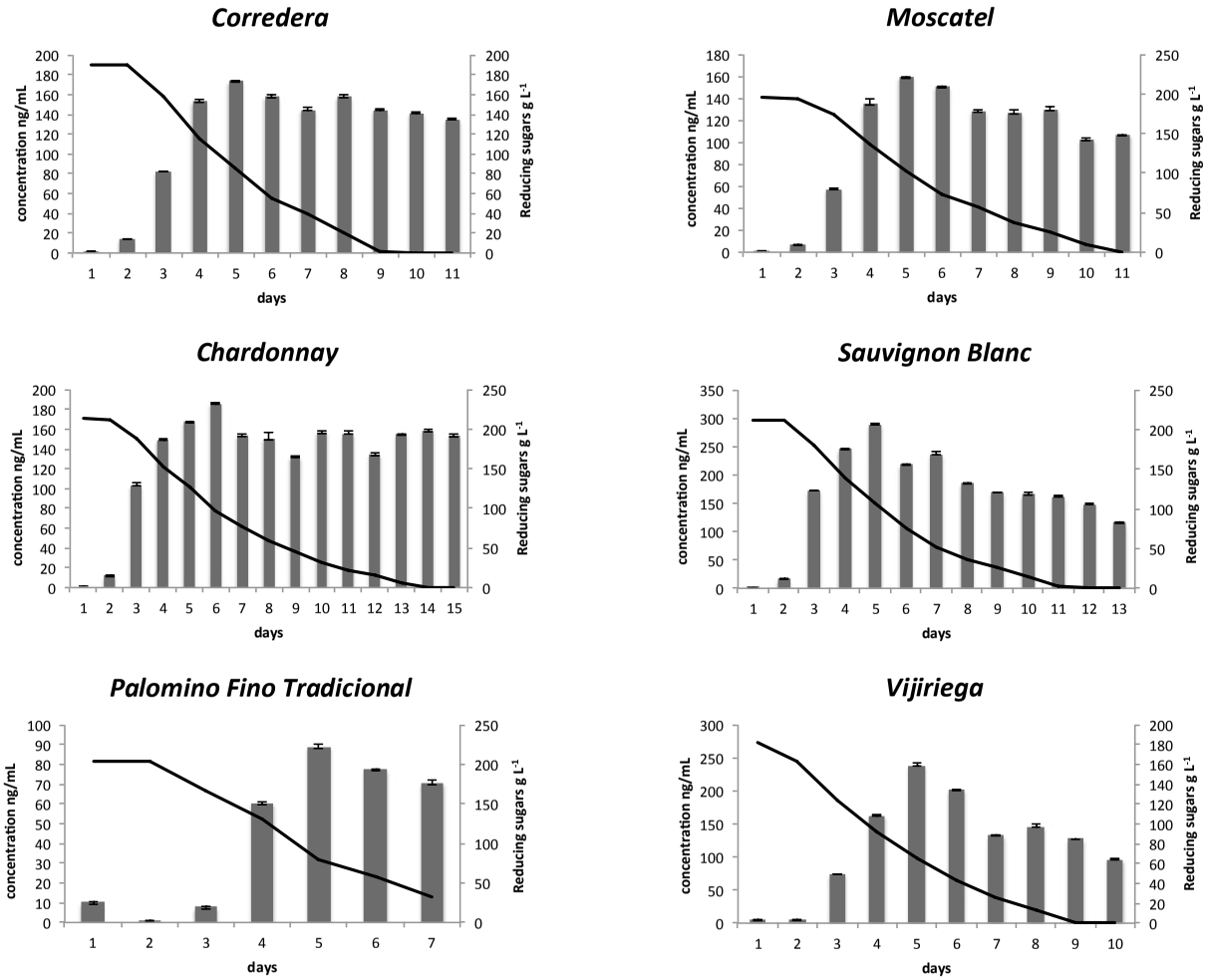

Figure 4A. Time course of HT and consumption of reducing sugars in alcoholic fermentation in natural must of six different grape varieties.

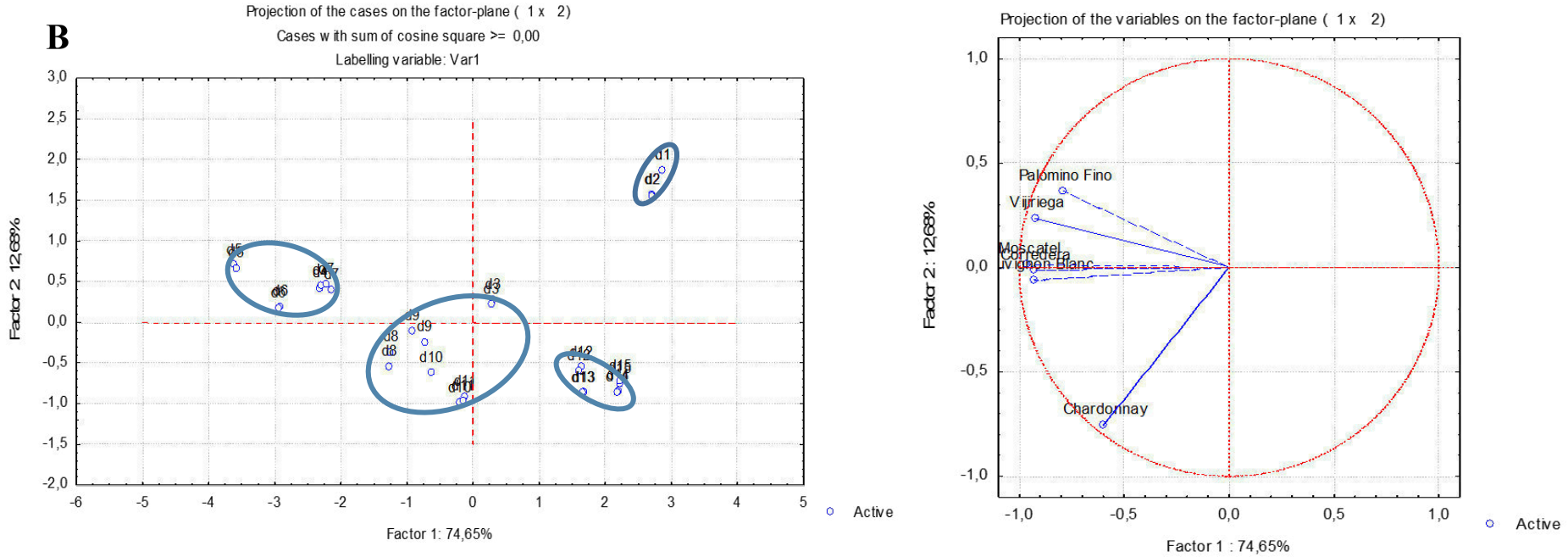

Figure 4B. Score plot of PCA analysis, on the left side considering days of fermentation and on the right considering the varieties of grapes using in the elaboration of musts.

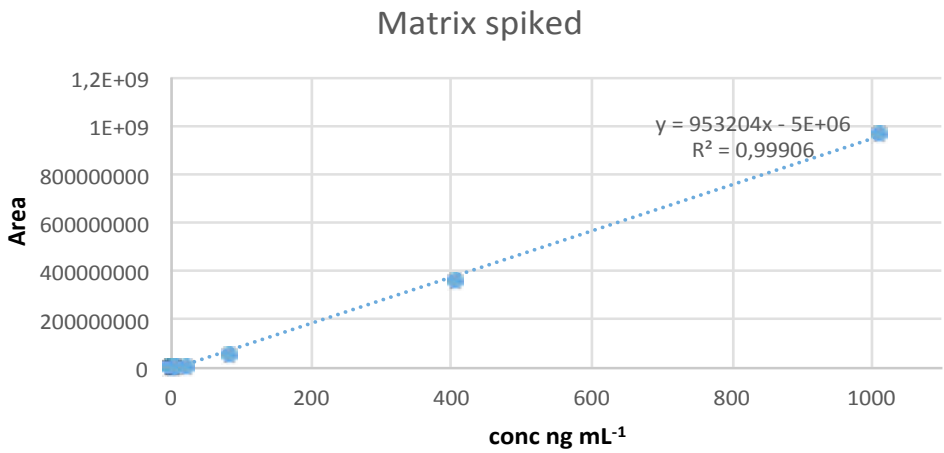

Figure 1. Representation of spiked solutions of purified matrix

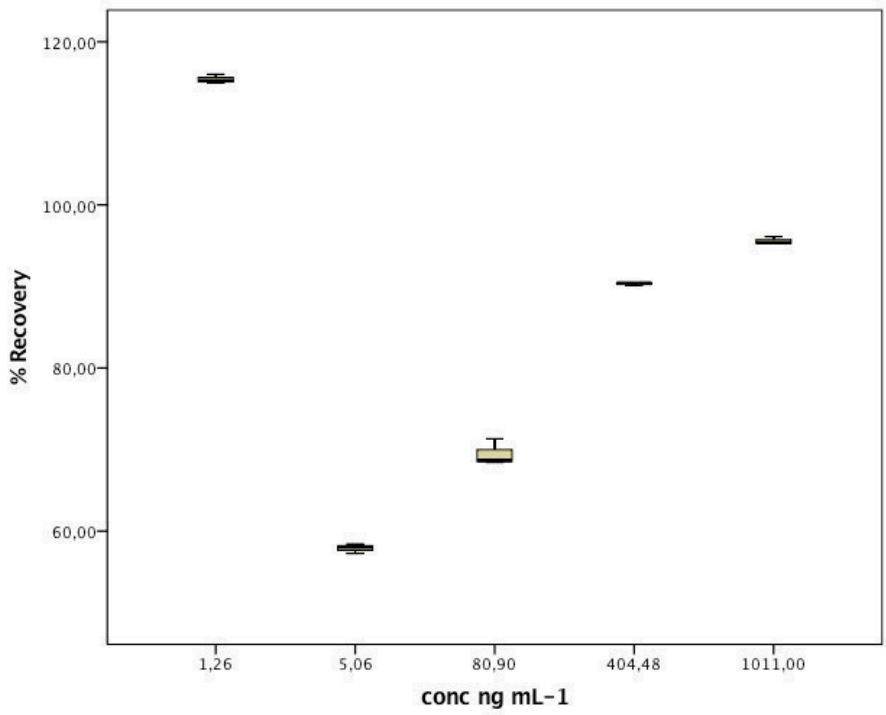

Figure 2. Distribution of the recovery (%) of hydroxytyrosol in blank matrix at five spiked concentration ranged 1.26 to 1011.00 ng mL^{-1}. The limits of AOAC are between 120 and 40% for concentrations in studied range.

CAPÍTULO SEXTO

VI

PARTE I

VI

Quantitative profiling of aromatic amino acids related compounds in intra and extracellular media produced by *Saccharomyces* and non-*Saccharomyces* winemaking strains of yeast

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Abstract

Tryptophan, phenylalanine and tyrosine play an important role as secondary source of nitrogen in yeast metabolism, able to regulate biomass production and fermentation rate. Moreover, the catabolites of these amino acids contribute to wine healthy properties and sensorial character through the yeast biotransformation of grape juice constitutes into biological active and flavour-impacting components.

An UHPLC-ESI-MS/MS method was developed for the analysis of 37 tryptophan/phenylalanine/tyrosine yeast metabolites, and was used to analyse the extra- and intracellular extracts produced by the fermentation of two *Saccharomyces* strains and one non-*Saccharomyces (Torulaspora delbrueckii)*. Between others, the results indicated the production of 2-aminoacetophenone by yeasts, intracellularly through the indole acetic acid or kynurenine pathway, and extracellularly through the tryptophol sulfonation. In addition, was pointed out the deactivation and/or detoxification of tryptophol via sulfonation, and N-acetyl tryptophan ethyl ester, N-acetyl tyrosine ethyl ester and possibly melatonin via de-acetylation.

Keywords

Nitrogen metabolism, alcoholic fermentation, sulfonation, *Torulaspora delbrueckii*, mass spectrometry, melatonin

Chemical compounds studied in this article

Tryptophan (PubChem CID: 6305); tryptophan ethyl ester (PubChem CID: 81669); 2-aminoacetophenone (PubChem CID: 11086); tryptophol (PubChem CID: 10685); N-acetyl-tyrosine ethyl ester (PubChem CID: 2004); tyrosine ethyl ester (PubChem CID: 70364); phenylalanine (PubChem CID: 2140); nicotinamide (PubChem CID: 936); indole 3-lactic acid (PubChem CID: 92904); tyrosol (PubChem CID: 10393).

1. Introduction

Over seven millennia, yeasts transform grape must into wine using a biotechnological process, where principally sugars turn to alcohol. To achieve this, yeasts use the nutrients present in the medium for growth, and in parallel produce metabolites, which led to a final hydro-alcoholic solution of special flavour and pleasant aroma (Mas et al., 2014).

The winemaking yeast *Saccharomyces cerevisiae* can grow on a diverse content of nitrogen compounds, such as purine and pyrimidine base, ammonium, urea, amino acids and small peptides. However, yeasts growth, fermentation rate and biomass yield depend on both the quantity and nature of the nitrogen source(s) available (Henschke & Jiranek, 1993). Nevertheless, the last years, non *Saccharomyces cerevisiae* yeasts received an increased interest in wine science/making, with the aim to produce better quality wines (Jolly, Augustyn, & Pretorius, 2006).

The essential amino acid tryptophan (TRP) and its related indolic metabolites (Fig. 1) (Hoenicke, Borchert, Grüning, & Simat, 2002; Kanehisa & Bork, 2003) are widely studied and they also awaken interest among researches due to their biological and pharmacological activities. Metabolites with proved biological activity (Davis & Liu, 2015; Martín-Cabrejas, Aguilera, Benítez, & Reiter, 2017) as melatonin (MEL), serotonin (SER), tryptophol (TOL), kynurenic acid (KYNA), kynurenine (KYN) and indole acetic acid (IAA) are related with TRP metabolism pathway (Fig. 1). MEL was initially classified as animal neurohormone (Martín-Cabrejas et al., 2017), but later has been found to have multiple functions and to be present in many medicinal and plant foods, wine and produced by unicellular organism as yeast (Arnao & Hernández-Ruiz, 2014; Feng, Wang, Zhao, Han, & Dai, 2014; M. Isabel Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, & Garcia-Parrilla, 2011). Via the Ehrlich pathway, the amino acids TRP, phenylalanine (PHE), and tyrosine (TYR) produce respectively the aromatic alcohols TOL, phenylethanol, and tyrosol (TYL), which as auto inducers are able to transmit information about the population density and the amount of available nitrogen synthesised from the amino acids (Chen & Fink, 2006; Martín-Cabrejas et al., 2017). In yeast, when branched-chain, aromatic, or sulfur-containing amino acids are the nitrogen source, the decarboxylation reaction contributes to low intracellular alpha-keto acid concentrations, thereby pulling the transaminating reactions

toward complete utilization of the nitrogen-donating amino acids (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008; Swiegers, Bartowsky, Henschke, & Pretorius, 2005).

The most known and studied auxin IAA has been reported as a molecule capable of stimulating the developmental transition from the vegetative yeast to the filamentous form. IAA inhibits growth at high concentrations and induces substrate adhesion at low concentrations acting as signals to modulate population growth (Engin, 2015; Martín-Cabrejas et al., 2017).

Through TRP pathway, yeast contributes to wine aroma directly by bio-transforming odourless metabolites into flavour-active as methyl mercaptan and indole (Engin, 2015) and indirectly by chemical reactions during wine aging, since the indolic metabolites are putative precursors of other aromatic substances, like 2-aminoacetophenon (2AA) (Hoenicke et al., 2002) (Fig. 1). The wine fruity aroma depends from the synthesis of higher alcohols and esters which are positively correlated with ethanol stress-tolerant yeasts, that have an enhanced expression of genes related to TRP metabolism (Engin, 2015).

In addition, indoles can react with the SO_2, added to wine for protection against oxygen and microorganisms, delivering sulfonated metabolites and effecting wine shelf life and metabolic fingerprint (Arapitsas et al., 2016).

The possibility to follow the behaviour of metabolites belonging to the Figure 1 pathways, separately and in parallel both for intracellular and extracellular media of yeast during the fermentation process is a way of gaining knowledge over the signalling and the metabolic reaction network and to improve wine - and other fermented food - nutritional value and quality. The goals of this work was i) to develop a targeted UHPLC – MS/MS method to identify and quantify as many metabolites related to aromatic amino acid metabolism as possible, and ii) apply such method to provide novel information on the aromatic amino acids metabolism. This second aim was accomplished through the analysis of the alcoholic fermentations of a synthetic medium with three winemaking yeast strains.

2. Materials and methods

102 2.1. Reagents and materials

103 All chemicals used in this study were of the highest purity grade available and purchased from
104 Sigma-Aldrich (Madrid, Spain or Milan, Italy) and Cymit Quimica S.L. Barcelona, Spain (Sup-
105 plementary Table S1), unless otherwise stated. Tryptophol sulfonated was prepared as previ-
106 ously described (Arapitsas, Guella and Mattivi, under rivision).

107 2.2. Yeast strains

108 The experiments on synthetic musts were performed with the three different commercial wine
109 yeast strain *S. cerevisiae* Lalvin YSEO QA23® (Lallemand) (QA), *S. cerevisiae* Red Fruit RF®
110 (Enartis) (RF) and *T.delbrueckii* TD291 Biodiva™ (Lallemand).

111 2.3. Alcoholic fermentation

112 Three alcoholic fermentations were performed in synthetic must (SM), prepared based on Riou
113 et al. (1997) with slight modifications (Supplementary Table S2 shows in detail the must com-
114 position). As carbon source, were added fructose and glucose (100 g L^{-1} each), the other com-
115 pounds were maintain without modification amino acids (purity ≥ 99 %) (Riou, Nicaud, Barre,
116 & Gaillardin, 1997), vitamins and anaerobic factors. The fermentations came though action of
117 three strains of yeast, the *Saccharomyces* QA23 (QA) and RED FRUIT (RF), and the *Toru-*
118 *laspora delbrueckii* (Td) without aeration, in six repetitions in Erlenmeyer flasks. To obtain
119 most reliable results possible and capture the random biological variation, six biological repli-
120 cates of each fermentation were considered (biological variability). The pH was adjusted to 3.5
121 with NaOH and after that SM was sterilized with bottle top vacuum filters (Nalgene PES mem-
122 brane). Each Erlenmeyer flask with 750 mL of SM was inoculated with 10^6 cell mL^{-1} and
123 capped with taps equipped with a capillary to release carbon dioxide. To monitor the fermenta-
124 tion, the flasks were weighed daily before and after sampling. Enzymatic kits (Megazyme Inter-
125 national, Ireland) were used to assay the reducing sugars and primary amino nitrogen (PAN)
126 content in extracellular media.

127 2.4. Extracellular and intracellular metabolite extraction

A volume of sample corresponding to 10^9 cells of each Erlenmeyer flask was taken at day 2, 5 and 15. Immediately the samples were centrifuged 4500 rpm for 3 min at 4ºC in order separate the cells from extracellular contents. The extracellular samples were collected and stored at -80ºC until the analysis.

Cells were pelleted by centrifugation 4500 rpm for 3 min at 4ºC twice with distilled water to wash them.

The washed cells were subject to a cold glycerol saline quenching procedure focussed to stop cellular metabolism and avoid the turnover of metabolites by stopping the enzymatic activity, maintaining the *in vivo* metabolite concentrations at a constant level. The procedure used was following the one reported before by Villas-Bôas et al. (Villas-Bôas & Bruheim, 2007).

The pellet was re-suspended with 1 mL of distilled water and transferred into precooled centrifuge tube containing 4 mL of cold-quenching solution [3:2 (vol/vol) glycerol:saline solution] (saline solution 0.9% wt/vol NaCl/water) maintained at -23 ºC in a refrigerated bath (ethylene glycol as cryo fluid). The solution was homogenized and then returned to the cold bath for 5 min to acclimatization. The treated samples were centrifuged at 36,086 *g* for 20 min at -20ºC (Sorvall LYNK 6000, Thermo Fisher scientific, Waltham, MA USA). The supernatant was then removed and the pellet was re-suspended in the same volume of cold glycerol and the process was repeated once again. The pellets quenched were stored at -80ºC until the extraction process was carried out. The intracellular extraction was performed following the method reported by Smart et al. with minor modifications (Smart, Aggio, Van Houtte, & Villas-Bôas, 2010). To the cell pellets, 2.5 mL of cold methanol-water solution (50% (v/v), -30ºC) were added, mixed for 1 min and then frozen at -80 ºC. The samples were subjected to two cycles of freeze-thaw (frozen at -80ºC for 30 min and then thaw in an ice bath for 4 min). After the last cycle, they were subjected to sonication for 1 min in an ice bath using an ultrasonic liquid processor (Sonicator Sonoplus HD 2070, Bandelin electronic GmbH & Co. KG, Berlin, Germany). Subsequently, the samples were centrifuged at 36,086 *g* for 20 min at -20ºC. Another 2.5 mL of cold methanol-

water was added to the pellet and then the process repeated from the cycles of freeze-thaw; the supernatants were collected, pooled and stored at -80 ºC until solid phase extraction.

2.5. Sample treatment/preparation

All extracellular samples and intracellular extracts were cleaned up as previously reported by Rodriguez-Naranjo et al. (M. Isabel Rodriguez-Naranjo et al., 2011) with the following modifications. Briefly, C18 SPE cartridges (Variant, Agilent) were conditioned with 2 mL of methanol and 2 mL of milli Q water. An aliquot of 1.5 mL of extracellular sample or 2.5 of intracellular sample was loaded followed by a washing step with 2 mL of a 10% v/v methanol solution. The analytes were eluted with 1.5 mL of methanol; afterwards solvents were evaporated until dryness at 34ºC, 2000 rpm during 6 hours with a vacuum concentrator (HyperVAC-LITE, GYOZEN, Korea). Then samples were reconstituted with 300 µL of a solution 9.37 ppm of internal standard 3-nitrotyrosine in methanol/water 10% v/v and immediately analysed.

2.6. UHPLC-MS/MS instrumental analysis

The analysis was carried out in a Waters Acquity UHPLC (Milford, Massachusetts, USA). Separation was performed in order to separate 37 metabolites and internal standard (3-nitrotyrosine). For the analysis a Waters Acquity column (Milford, Massachusetts, USA), HSS T3 (2.1×150 mm, 1.8 µm particle size) was used. The chromatographic conditions consisted in two phases (A) aqueous formic solution 0.1%, (B) solution 0.1% of formic acid in methanol and the gradient was programed as follows: 95% A, 5% B (0 min); 80% A, 20% B (1.5 min); 65% A, 35% B (2 min); 55% A, 45% B (6 min); 100% B (8 - 10 min); 95% A, 5% B (10.1- 13 min). The temperature of the column was 40ºC, the flow of 0.4 mL min $^{-1}$, the injection volume was 2 µL and 10 µL in order to allow the quantitation as a function of the concentration of the metabolites. The injection volume of 10 µL was used to quantitate all compounds except TRP and phenyl pyruvic acid (Ph-Py) where the 2µL injection volume analysis was used. The MS analysis was performed with Waters Xevo TQ (Milford, Massachusetts, USA) triple quadrupole mass spectrometer, consisted of an electrospray interface and polarity switching option. Each metabo-

lite was directly infused in MS system in negative and positive mode with 50/50 v/v of two phases A and B, in order to optimise the detection process. The two most abundant fragments were selected for each metabolite, one as quantifier (most abundant) and other as qualifier ion, using the MRM (multiple reaction monitoring) method for selective quantification. Table 1 shows MS parameters for the MRM method, retention time, and values of standard deviation (RSD) as measure of the variation of each metabolites corresponding to extracellular and intracellular QC (quality control) injections.

2.7. Statistical Analysis

Statistical analyses were performed by Statistica software (Migut, Jakubowski, & Stout, 2014) version twelve and MetaboAnalyst (Xia & Wishart, 2011). Statistical significance between groups was tested by analysis of variance ANOVA, and Tukey's HSD (honest significant difference) test. ANOVA and Tukey's were assessed to test significant differences at $p < 0.05$, the post-hoc analysis is used in conjunction with ANOVA to test which means are significantly different between analysed groups of samples (Supplementary Figs. S4 to S9). For multivariate statistical analysis, heat-maps were made by using the metaboanalyst web platform, where the data were auto-scaled and missing values were replaced by very small values (Xia & Wishart, 2011); while for the PCA plots the SIMCA-P software was used (Umetrics AB, Malmö, Sweden).

3. **Results**

3.1. UHPLC-MS/MS method

The development of the UHPLC-MS/MS method was based in previous works (Fernández-Cruz, Álvarez-Fernández, Valero, Troncoso, & García-Parrilla, 2016, 2017) which have in detail proved how adequate such system is for the analysis of tryptophan metabolites. Table 1 shows the basic instrumental parameters of the 38 analytes (37 metabolites and the IS) included in the method, selected in order to cover as possible the pathways of Figure 1. In detail, included the major metabolites of TRP catabolism: tryptophan ethyl ester (TRP-EE), tryptophan

206 methyl ester (TRP-ME), N-acetyl tryptophan ethyl ester (N-TRP-EE), TOL, 5-OH-tryptophan
207 (OH-TRP), 5-methoxy-tryptophan (CH_3O-TRP), SER, N-acetyl serotonin (N-SER), MEL, in-
208 dole pyruvic acid (IPy), indole lactic acid (ILA), IAA, 5-methoxy-indole acetic acid (CH_3O-
209 IAA), methyl indole acetic acid (M-IAA), indole acetic acid ethyl ester (IAA-EE), ethyl indole
210 carboxylic acid (E-ICa), 2AA, indole carboxaldehyde (ICA), indole proprionic acid (IPA), in-
211 dole butyric acid (IBA), KYN, KYNA, nicotinamide (NIC), anthranilic acid (ANT), and 3-OH-
212 anthranilic acid (OH-ANT). But also catabolites of the amino acid PHE: 4-OH-phenyl pyruvic
213 acid (OH-Ph-Py), 4-OH-phenyl acetic acid (OH-Ph-AA), Ph-Py, phenyl lactic acid (Ph-LA),
214 and phenyl acetic acid (Ph-AA). And finally the catabolites of the amino acid TYR: tyrosine
215 ethyl ester (TYR-EE), N-acetyl-tyrosine ethyl ester (N-TYR-EE) and TYL. As expected the
216 method qualitative characteristic were comparable with the previous methods (Fernández-Cruz
217 et al., 2016, 2017). Based in our former experience in high-throughput targeted analysis
218 (Ehrhardt, Arapitsas, Stefanini, Flick, & Mattivi, 2014), to control the robustness of the LC-MS
219 system and its signal stability a) 3-nitrotyrosine was added as internal standard, b) the order of
220 sample injection was randomized and c) a QC sample was injected every 10 real sample injec-
221 tions (instrumental variability). The QC samples were prepared as pooled mix of all extracellu-
222 lar (QC_{ex}) and intracellular (QC_{in}) samples separately. Finally, three technical replicates, of each
223 sample were injected. In order to verify instrumental accuracy and stability, 33 injections of the
224 QC were carried out as part of the analysis method, through the measure of concentrations of
225 almost all metabolites of interest (Ehrhardt et al., 2014). Table 1 displays the values of standard
226 deviation (RSD) as measure of the variation corresponding to intracellular and extracellular QC
227 injections. The RSD for the IS was 9% for extracellular and 4% for intracellular.

228 3.2. Intracellular versus extracellular metabolic profile

229 From the 37 metabolites of Table 1, 20 were detected and quantified in the intracellular sam-
230 ples, and 26 in the extracellular samples. The heat-maps of Figure 2 provided a visually intuitive
231 overview of the data set with hierarchical clustering and gave a simple view of the trends of the
232 studied compounds concentrations across the different sampling times. The heat-map (Figure 2)

includes all the samples and gives information about the differences between the extracellular and intracellular metabolites. The amounts of TOL, NIC, TYR-EE, TYL and PHE were significant higher in the intracellular samples, while most of the others were higher in the extracellular samples (Figs. 2, and Supplementary Figs. S11-S15).

3.3. Intracellular metabolic profile

Figure 3A provides a more detailed picture in concern the intracellular behaviour of the various metabolite in correspond the sampling time and the yeast strain. The highest concentrations of TRP and PHE occurred at the second day in intracellular samples of RF fermentations, could be result of the amino acid transporter permeases activity, which facility the inclusion of two amino acids to the intracellular space (Crépin, Nidelet, Sanchez, Dequin, & Camarasa, 2012). During the early phases of fermentation, TRP and PHE are not the source of nitrogen used in the first instance (Quirós et al., 2013). Intracellularly, TRP and PHE together with KYN were the only metabolites, which concentrations were higher at the first sampling point and then decreased, while the concentration of most of the others metabolites quantified increased. KYN exhibit concentrations enclosed between 1.31-1.13 and 0.91 μg $L^{-1}10^9$ cells at 2nd day for QA, RF and Td respectively and concentrations of 1.36- 1.01 and 0,79 μg $L^{-1}10^9$ cells at 15th day, as shows the heat-map hierarchical clustering of figure 3 left, the increased was higher in RF and Td than QA. These results are in accordance with Shin, Mariko, Keiji, Sano, and Umezawa (1991) who reported an increase of TRP and KYN in intracellular pool. On the other hand, TOL and TYL had major concentrations at 5th and/or 15th days. TOL in QA and Td samples reached concentrations between 99– 311 μg $L^{-1}10^9$ cells, and 106 – 2869 μg $L^{-1}10^9$ cells respectively. The highest value of concentrations of TYL ranged 68 – 193 μg $L^{-1}10^9$ cells in RF fermentations, the only case with significant differences (Supplementary Figs. S9-S14). Higher alcohols are used as environmental cues in regulation of morphogenesis by encouraging a transition from the unicellular to a multicellular filamentous morphotype in *Saccharomyces cerevisiae* (Dufour & Rao, 2011).

3.4. Extracellular metabolic profile

According to the heat-map hierarchical clustering of Figure 3B, a group of metabolites enclosed IPy, ICA, CH_3O-IAA, IAA and IAA-EE. Interestingly, the same metabolites are clearly connected also in the metabolic pathways described in Figure 1 (pink arrows). All these compounds appeared in higher concentrations at 2^{nd} day of extracellular samples of Td, but they followed the same trend in extracellular samples at 2^{nd} day in RF.

Then the hierarchical cluster analysis (Figure 3B) pointed out a second group of metabolites, which included two subgroups: a) OH-Ph-Py, Ph-Py and N-TRP-EE; and b) ANT, TOL, N-TYR-EE and TYL. Chemically this group enclosed the N-acetyl ethyl esters of the amino acids tryptophan and tyrosine (N-TRP-EE and N-TYR-EE); the two phenyl-pyruvic acid metabolites (OH-Ph-Py and Ph-Py); two aromatic alcohols (TYL and TOL); and anthranilic acid (ANT). All they exhibited a similar behaviour in extracellular samples of *Saccharomyces cerevisiae* strains, since at the 2^{nd} day had higher concentrations, which progressively went diminishing. This reduction of the concentration was faster in case of OH-Ph-Py, Ph-Py and N-TRP-EE. The amino benzoic acid ANT has a roll as environmental cue modulating the growth and inducing morphological transitions in *Saccharomyces* and another fungi (Prusty, Grisafi, & Fink, 2004). In addition, this compound was also correlated with OH-Ph-Py and Ph-Py through the phenylalanine pathway (Figure 1, right box); in our analysis was quantified in all extracellular samples, but exhibited a statistically significant difference only for the two *Saccharomyces* strains, QA23 and RF with concentrations ranged in 108– 167– 101μg L^{-1} and 61– 57– 46 μg L^{-1} respectively (Supplementary Figs. S11-S15). Worth to notice that the N-acetyl derivatives of tryptophan and tyrosine, N-TRP-EE and N-TYR-EE, had the opposite trend in respect their analogues, TRP-EE and TYR-EE, most probably due to a deacetylation enzymatic process similar to those reported by Kradolfer et al, which act in the production of TOL from TRP (Kradolfer, Niederberger, & Htitter, 1982) (Figure 4).

These two last metabolites, TRP-EE and TYR-EE, clustered together with the sulfonated tryptophol (TOL-SO_3H) according to the hierarchical cluster analysis of the Figure 3B, showing a maximum concentration for the last point (15 days) of our experiment. The sulfonation of TOL

was lately discovered as a reaction occurring in bottled white wines in the presence of O_2 where SO_2 was added. Here we report for the first time that this reaction can occur also in the context of an alcoholic fermentation where the presence of SO_2 is due to the sulfur metabolism of yeast. The sources of this metabolism were the sulfur amino acids and the sulfated salts of the synthetic must composition. As far as the presence of O_2, the reducing sugars were already consumed after the 5th day (Supplementary Fig. S1), so in absent of CO_2 formation limited amounts of O_2 could enter into the fermentation flask through the capillary between the 5th and the 15th day, favouring the sulfonation of TOL. The concentration of TOL-SO_3H ranged between 0.54 - 2.24 μg L^{-1} 10^9 with a trend to increase in Td, intracellular samples, the only case where the evolution presented statistical significant differences (Supplementary Fig S11). In extracellular samples, TOL-SO_3H ranged 75 - 590 μg L^{-1} in Td, again with a statistical significant difference (Supplementary Fig. S14).

A group of metabolites clustered close to the previous one in extracellular metabolome, thus with similar trend, enclosed 2AA, E-ICa, PHE and NIC. 2AA in extracellular samples increased significantly through time, with concentrations range between 0.3 – 1.2 μg L^{-1} in QA, 54 – 258 μg L^{-1} in RF and 36. – 704 μg L^{-1} in Td samples (Fig. 2, Supplementary Figs. S11-S15). The heat-maps (Fig. 2B and C) show higher concentration at 15th day both for intracellular and extracellular samples. 2AA can be chemically produced by oxidative degradation of IAA and KYN, as suggested by Hoenicke et al. (Figure 1). However, 2AA can be also produce enzymatically by ANT (Hoenicke et al., 2002).

Finally, the last cluster of the first section of the hierarchical analysis of extracellular samples (Figure 3 right) contained TRP-ME, TRP, KYN, KYNA, ILA, IBA and MEL, that was almost similar to the last one of the heat map of all samples (Figure 2). In intracellular samples, MEL had concentrations lower than LOQ in most samples, which made difficult to follow the evolution though the sampling time. In extracellular samples MEL was quantified in all sampling days with concentrations of 0.04 – 0.08 – 0.22 μg L^{-1} in QA, 0.15 – 0.10 – 0.16 μg L^{-1} in RF and in case of *T delbrueckii*, could be quantified at 2nd and 15th days with concentrations of 0.14 –

314 0.08 μg L^{-1} respectively. At 15th day of QA extracellular samples the concentration of MEL was
315 higher, the content of reducing sugars and nitrogen concentrations were low (Supplementary
316 Figs. S1-S2). These results are consistent with those reported by Rodriguez-Naranjo, Torija,
317 Mas, Cantos-Villar, & Garcia-Parrilla (2012), who concluded *S. cerevisiae var bayanus* QA
318 strain had the ability of produce MEL when the reducing sugars have been totally consumed.
319 Other compounds included in this cluster were KYN and KYNA related to the TRP metabolism
320 via kynurenine pathway, which produces nicotin amide dinucleotide (NAD) an important cofac-
321 tor for oxidoreductases enzymes. NAD is produced though synthesis of quinolinic acid, a pre-
322 cursor which is associated with several neurological diseases and is thought to be the major link
323 between KYN pathway and inflammatory response in humans (Davis & Liu, 2015). KYN
324 reached concentrations in the range between 0.33 – 1.58 μg L^{-1} in QA and 0.39 – 0.53 μg L^{-1} in
325 RF, the two cases with significant differences in extracellular samples only for 5th and 15th days.

326 4. **Discussion**

327 The possibility to follow the intra- and extra-cellular behaviour of metabolic pathways in paral-
328 lel during fermentation is a great advantage. The sets of information obtained in return are com-
329 plementary to each other and help to extract a more complete view of the yeast metabolism.
330 Even though the culture media influences the intracellular metabolism, cell has the ability to
331 adjust rapidly minimal changes through very fast turn over mechanisms. Generally, the concen-
332 tration of the metabolites in the cell is lower than their concentration to the extracellular envi-
333 ronment.

334 The PCA graph of Figure 5A shows the loading bi plot including both samples and compounds,
335 and summarises the behaviour of the metabolic profile between intra- and extra-cellular metabo-
336 lism and the different time points. In fact, the component 1 separated the intracellular samples
337 located in the left from the extracellular samples laid on the right. On the other hand, the com-
338 ponent 2 separated the extracellular samples by days, where the 2nd day samples were located on
339 the upper right side of the PCA and the 5th and 15th days samples were located on the bottom of
340 the right side of the plot. As for the heat-map of Figure 2, also here, the intracellular samples

341 metabolic profile was characterized by TYL and TOL, IAA-EE, TYR-EE, PHE and NIC. The
342 grouping of the intracellular samples in one dense cluster, in respect the extracellular samples,
343 was one more proof of the intracellular metabolism plasticity and of the ability of the yeast to
344 maintain a tightly regulated composition in a drastically changing environment. As for the heat-
345 map of Figure 2, also here, between the metabolites characterising intracellular samples were
346 two higher alcohols for the Ehrlich pathway (TOL and TYL) and PHE, NIC.

347 The PCA bi-plot of Figure 5B provided a complementary visualization of the data in respect to
348 Figure 3B, summarising the extracellular behaviour of samples and metabolites. This PCA plot
349 is not a zoom of the Figure 5A PCA plot, since only the data of the extra-cellular samples were
350 used to produce it. The first component separated the sampling points (2, 5 and 15 days), while
351 the second component separated *Torulaspora delbrueckii* from the two *Saccharomyces cere-*
352 *visiae* yeasts. The combination of the various data offered the possibility to make several con-
353 siderations as far as extracellular metabolism. The amino acids TRP and PHE together with the
354 "kind of amino acid" KYN (Shin, Mariko; Keiji, Sano; and Umezawa, 1991), were consumed in
355 order to produce several metabolites belonging to the catabolism of Figure 1. Via Ehrlich path-
356 way, TRP produced intracellular the quorum-sensing TOL, which was released in the extracel-
357 lular environment. However, TOL wasn't accumulated but reacted with SO_2 and delivered
358 TOL-SO_3H. Next to this TRP mechanism, we could also speculate that IAA was first produced
359 in the cell, then released in the media, then chemically sulfonated, and finally breakdown to
360 2AA. Even though, the presence of 2AA in intracellular samples indicates a possible direct pro-
361 duction of yeast metabolism. Indeed, 2AA can also be enzymatically synthesised from ANT,
362 through the KYN pathway of TRP (Fig. 1) (Hoenicke et al., 2002). 2AA is directly associated
363 with the wine fault called "untypical ageing off-flavour"(Hoenicke et al., 2002), and as to our
364 knowledge this is the first time that it was detected as an intracellular metabolite. Further re-
365 search in 2AA intracellular biosynthesis should be of great importance for the wine production,
366 especially for the yeast choice. By taking in consideration that the known chemical production
367 of 2AA pass through the sulfonation of IAA, and that IAA and TOL have the same indolic

skeleton, we could postulate that TOL-SO_3H chemical breakdown could also deliver 2AA or other similar metabolites. TOL-SO_3H and 2AA clustered together with other final products in both the Figure 3 right heat-map and Figure 5B PCA plot. While mainly TOL and IAA and secondly KYN characterised more the first days fermentation samples.

Amino acids ethyl ester is a very important group of basic metabolites present in wine. Their concentration increases mainly in the second half of the fermentation, and even more when yeast fermentation ceased, and in periods of wine yeast contact (Fernández-Cruz et al., 2016, 2017; Herraiz & Ough, 1993). Figure 4 shows that while during fermentation the concentration of N-TYR-EE and N-TRP-EE decreased, the concentration of TYR-EE and TRP-EE increased. This could be an indication that the ethyl esters of TRP and TYR occurred not by the esterification of the free amino acids, but through the de-acetylation of the N-acetyl ethyl esters of the amino acid. In addition, the process of de-acetylation of N-TYR-EE may occur inside the cell, so higher concentration of TYR-EE was measured in intracellular samples. Such a biosynthetic path seems valid only for the two *Saccharomyces* strains where also the extracellular TRP levels were much lower (Figs. 3 and 4). N-TYR-EE participate to the regulation of the TRP synthesis in yeasts, as tryptophan synthase inhibitor (Betz, Hinze, & Holzer, 1974; Saheki & Holzer, 1974), and both PCA (Fig. 5B) and hierarchical cluster analysis (Fig. 3B) grouped N-TYR-EE and N-TRP-EE together with the quorum sensing metabolites TYL and TOL. All four metabolites presented their highest concentration the period of *Saccharomyces* exponential growth. On the other hand, the de-acetylated TYR-EE and TRP-EE clustered together with other final products (e.i. TOL-SO_3H and 2AA), where their higher concentration was measured at the last sampling point (Fig. 3). Generally, metabolites sulfonation or de-acetylation are mechanisms of deactivation and/or detoxification. This could be an indication that N-TYR-EE and N-TRP-EE play a strategic role in yeast mechanism, machinery and regulation; and so influence the fermented food quality. However, further experiments are necessary to validate such hypothesis.

393 The clustering of NIC and PHE with other final products of the yeast metabolism possible at-
394 tested a de-novo synthesis (Figs 1, 3B and 5B)(Braus, 1991). The confirmation of such hypothe-
395 sis could also be enhanced by the intracellular behaviour of NIC and PHE (Figs 2 and 5A).

396 Finally, MEL is located near the centre, between 2nd and 5th day samples (Fig 5B). There are
397 some hypotheses, which could explain those findings: i) MEL play a role as growth signal, the
398 production of this indole amine may be correlated with yeast-growth phase (Rodriguez-Naranjo
399 et al., 2012). ii) The possibility the MEL binds to specific protein, which plays, as carrier to
400 released MEL and establish equilibrium between consumed free MEL (Tan, Reiter, &
401 Manchester, 2002). iii) MEL is an intermediate metabolite, which rapidly is transformed,
402 thought de-acetylation pathway; like N-TRP-EE and N-TYR-EE. Some of the de-acetylation
403 pathway metabolites were included in the method (5-methoxy tryptophan and 5-methoxyindole
404 acetic acid) but their concentrations were below quantifications threshold. This one is the most
405 likely due to this pathway was described in fish, mammalian and pineal glands of both *Anolis*
406 *carolinensis* and *Sceloporus jarrooi* (Grace, Besharse, Biology, City, & Biology, 1994), and in
407 yeast (Sprenger, Hardeland, Fuhrberg, & Han, 1999).

408 ## 5. Conclusions

409 This study was focused in elucidating a comprehensive status of yeast metabolism by combin-
410 ing intra- and extra-cellular metabolomic dataset of tryptophan/phenylalanine/tyrosine metabo-
411 lism, through the development of a fast and sensitive LC-MS analytical method, able to measure
412 38 analytes in 13 minutes. In total the concentrations of 26 metabolites were monitored (26 ex-
413 tra- and 20 intra-cellular) during the alcoholic fermentation produced by three winemaking
414 strains of yeast, two *Saccharomyces* and one non-*Saccharomyces* specifically *Torulaspora del-*
415 *brueckii*.

416 The extracellular extract appeared richer both in term of concentration and number of metabo-
417 lites; however the alcohols TOL and TYL, the amino acid PHE, and NIC shown higher concen-
418 tration in intracellular. Generally, during the fermentation, the amino acids PHE and TRP were

419 decreasing in the intracellular compartment, while they were increasing in the extracellular me-
420 dium.

421 As far as our knowledge is concerning, this is the first time that 2AA and TOL-SO_3H have been
422 detected and quantified in intracellular samples which indicates that they could be produced by
423 yeast.

424 Some findings indicated the deactivation/detoxification of metabolites like a) the quorum sens-
425 ing high alcohol TOL by sulfonation to TOL-SO_3H and b) the regulators N-TYR-EE and N-
426 TRP-EE by de-acetylation to TYR-EE and TRP-EE. Moreover, we could speculate the forma-
427 tion of MEL in the beginning of the fermentation, followed by its de-acetylation; and the pro-
428 duction of 2AA through TOL sulfonation. Other findings pointed out a de-novo synthesis of the
429 amino acid PHE. In addition, the activation of the KYN pathway was measured during the yeast
430 fermentation, especially for the non-*Saccharomyces Torulaspora delbrueckii.*

431 This new knowledge helps for a better understanding and monitor in concern how yeasts, which
432 often participating in several foodstuffs production, may modify, enrich and benefit their nutri-
433 tion value and sensorial character.

434

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443

444 **Conflict of interest**

445 Declaration of interest: none

446

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558

559

Figure Captions

Figure 1. Scheme of proposal pathway of tryptophan (TRP), tyrosine (TYR), phenylalanine (PHE) and related compounds. Purple arrows indicate Ehrlich pathway of tryptophan. Pink arrows indicate the compounds enclosed in a cluster of heat-map of extracellular samples. The box on the left enclosed the kynurenine pathway. The box on the right, enclosed the reactions of compounds related to TYR and PHE metabolism.

Figure 2. Clustered heatmap of the measured metabolites including both extracellular and intracellular samples of the three yeast strains (the *Saccharomyces* strains QA23 and RF, and the To*rulaspora delbrueckii* Td) at three time points of the fermentation process (2, 5 and 15 days). TOL: tryptophol; NIC: nicodinamide; TYR-EE: tyrosine ethyl ester; TYL: tyrosol; IAA-EE: indole acetic acid methyl ester; PHE: phenylalanine; OH-Ph-Py: OH-phenyl-pyruvic acid; N-TRP-EE: N-acetyl-tryptophan ethyl ester; Ph-Py: phenyl-pyruvic acid; KYNA: kynurenic acid; N-TYR-EE: N-acetyl-tyrosine ethyl ester; ANT: anthranilic acid; CH3O-IAA: methoxy-indole acetic acid; IPY: indole pyruvic acid; IAA: indole acetic acid; ICA: indole carboxyladehyde; 2AA: 2-aminoacetophenone; E-ICA ethyl indole carboxyladehyde; ILA: indole lactic acid; TRP-EE: tryptophan ethyl ester; TOL-SO3H: sulfonated tryptophol; TRP-ME: tryptophan methyl ester; TRP: tryptophan; KYN: kynurenine; IBA: indole butyric acid; MEL: melatonine.

Figure 3. Clustered heatmaps of the measured metabolites separately for the intracellular (A) and extracellular (B) samples of the three yeast strains (the *Saccharomyces* strains QA23 and RF, and the To*rulaspora delbrueckii* Td) at three time points of the fermentation process (2, 5 and 15 days). TOL: tryptophol; NIC: nicodinamide; TYR-EE: tyrosine ethyl ester; TYL: tyrosol; IAA-EE: indole acetic acid methyl ester; PHE: phenylalanine; OH-Ph-Py: OH-phenyl-pyruvic acid; N-TRP-EE: N-acetyl-tryptophan ethyl ester; Ph-Py: phenyl-pyruvic acid; KYNA: kynurenic acid; N-TYR-EE: N-acetyl-tyrosine ethyl ester; ANT: anthranilic acid; CH3O-IAA: methoxy-indole acetic acid; IPY: indole pyruvic acid; IAA: indole acetic acid; ICA: indole carboxyladehyde; 2AA: 2-aminoacetophenone; E-ICA ethyl indole carboxyladehyde; ILA: indole lactic acid; TRP-EE: tryptophan ethyl ester; TOL-SO3H: sulfonated tryptophol; TRP-ME: tryp-

587 tophan methyl ester; TRP: tryptophan; KYN: kynurenine; IBA: indole butyric acid; MEL: mela-
588 tonine.

589 **Figure 4.** Time course of de-acetylation process: (A) N-acetyl tyrosine ethyl ester (N-TYR-EE)
590 and (B) N-acetyl tryptophan ethyl ester (N-TRP-EE). * Means statistically significant differ-
591 ences $p<0.05$. QA means *Saccharomyces cerevisiae* Lalvin YSEO QA23® (Lallemand), RF
592 means *Saccharomyces cerevisiae* Red Fruit RF® (Enartis) and *Torulaspora delbrueckii*

593 **Figure 5.** PCA plots based on the measured metabolites of the three yeast strains (the *Sac-*
594 *charomyces* strains QA23 and RF, and the To*rulaspora delbrueckii* Td) at three time points of
595 the fermentation process (2, 5 and 15 days); A) including both extracellular and intracellular
596 samples, and B) only for the extracellular samples. TOL: tryptophol; NIC: nicodinamide; TYR-
597 EE: tyrosine ethyl ester; TYL: tyrosol; IAA-EE: indole acetic acid methyl ester; PHE: phenyla-
598 lanine; OH-Ph-Py: OH-phenyl-pyruvic acid; N-TRP-EE: N-acetyl-tryptophan ethyl ester; Ph-
599 Py: phenyl-pyruvic acid; KYNA: kynurenic acid; N-TYR-EE: N-acetyl-tyrosine ethyl ester;
600 ANT: anthranilic acid; CH3O-IAA: methoxy-indole acetic acid; IPY: indole pyruvic acid; IAA:
601 indole acetic acid; ICA: indole carboxyladehyde; 2AA: 2-aminoacetophenone; E-ICA ethyl
602 indole carboxyladehyde; ILA: indole lactic acid; TRP-EE: tryptophan ethyl ester; TOL-SO3H:
603 sulfonated tryptophol; TRP-ME: tryptophan methyl ester; TRP: tryptophan; KYN: kynurenine;
604 IBA: indole butyric acid; MEL: melatonine.

Table 1. UHPLC–ESI-MS/MS conditions for quantification and identification of metabolites and internal standards (IS)

#	Metabolite (abbreviation)	RT (min)	ESI mode	Cone voltage (V)	Quantifier MRM (collision energy)	Qualifier MRM (collision energy)	LOQ (µg L^{-1})	QCex µg L^{-1} (%RSD)	QCin µg L^{-1} (%RSD)
1	nicotinamide (NIC)	1.50	+	28	123→ 79(14)	123→95(14)	4.2	14.81 (9)	5.73 (10)
2	5-OH-tryptophan (OH-TRP)	2.16	+	14	221→162(18)	221→133(24)	3.9	n.q.	n.q.
3	5-CH_3O-tryptophan (CH_3O-TRP)	2.16	+	14	235→176(18)	235→148(24)	10.50	n.q.	n.q.
4	kynurenine (KYN)	2.37	+	14	209→146(16)	209→136(12)	16.40	n.q.	n.q.
5	phenylalanine (PHE)	2.65	+	16	166→120(12)	1661→03(24)	44.90	130 (12)	70 (9)
6	3-nitrotyrosine (IS)	2.76	+	16	227→181(14)	227→117(24)	3.90	7300 (9)	5740 (4)
7	4-OH-phenyl pyruvic ac (OH-Ph-Py)	2.79	+	12		181→106(20)	1185.90	44100 (25)	n.q.
			-	8	179→106(14)				
8	3-OH-anthranilic acid (OH-ANT)	3.01	+	12	154→79(24)	154→108(22)	3.20	n.q.	n.q.
9	tryptophan (TRP)	3.07	+	12	205→146(18)	205→117(26)	11.20	1170 (20)	190 (3)
10	tyrosine ethyl ester (TYR-EE)	3.08	+	18	210→136(14)	210→90(26)	13.00	n.q.	n.q.
11	N-acetyl serotonin (N-SER)	3.27	+	16	219→160(16)	219→132(26)	9.10	n.q.	n.q.
12	tyrosol (TYL)	3.37	+	24	139→102(18)	139→93(12)	36.00	n.q.	n.q.
13	tryptophol sulphonate (TOL-SO_3H)	3.41	+	8	240→160(22)	240→130(28)	4.2	100 (20)	n.q.
14	kynurenic ac (KYNA)	3.46	+	22	190→143(14)	190→116(32)	10.30	54.58 (31)	n.q.
15	4-OH-phenyl acetic ac (OH-Ph-AA)	3.54	+	12	153→107(8)	153→76(24)	1262.33	n.q.	n.q.
16	tryptophan methyl ester (TRP-ME)	3.70	+	12	219→144(30)	219→160(18)	24.06	n.q.	n.q.
17	phenyl pyruvic ac (Ph-Py)	3.97	+	10		164→90(20)	43.20	n.q.	n.q.
			-	14	163→90(10)				
18	indole pyruvic ac (IPy)	4.21	+	16	204→130(22)	204→158(12)	0.77	n.q.	n.q.
19	anthranilic ac (ANT)	4.23	+	12	138→92(22)	138→64(26)	9.40	n.q.	n.q.

Table 1. Continued

#	Metabolite (abbreviation)	RT (min)	ESI mode	Cone voltage (V)	Quantifier MRM (collision energy)	Qualifier MRM(collision energy)	LOQ (µg L^{-1})	QCex µg L^{-1} (%RSD)	QCin µg L^{-1} (%RSD)
20	tryptophan ethyl ester (TRP-EE)	4.62	+	14	233→174(14)	233→159(20)	27.36	n.q.	n.q.
21	phenyl lactic acid (Ph-LA)	4.80	-	14	165→103(16)	165→119(16)	208.50	850 (12)	n.q.
22	3-indole lactic acid (ILA)	4.80	+	18	206→117(20)	206→160(10)	0.45	58.36 (4)	20.64 (28)
23	N-acetyl-L-tyrosine ethyl ester (N-TYR-EE)	4.99	+	14	253→136(22)	253→178(12)	1.70	1.75 (9)	n.d.
24	indole carboxaldehyde (ICA)	5.13	+	22	146→118(14)	146→90(24)	0.09	236.78 (2)	12.35 (8)
25	melatonin (MEL)	5.20	+	16	233→174(14)	233→159(28)	0.65	n.q.	n.q.
26	5-CH_3O-indole acetic acid (CH_3O-IAA)	5.30	+	18	206→160(18)	206→145(28)	0.16	3.60 (9)	12.21 (12)
27	tryptophol (TOL)	5.56	+	14	162→144(20)	162→127(22)	71.00	5840 (3)	80 (4)
28	3-indole acetic acid (IAA)	5.58	+	18	176→102(28)	176→130(12)	0.52	9.31 (5)	74.30 (4)
29	phenyl acetic acid (Ph-AA)	5.62	+	12	137→90(12)	137→64(30)	166.90	n.q.	n.q.
30	2-aminoacetophenone (2AA)	5.68	+	20	136→117(20)	136→42(16)	0.15	1.20 (16)	n.d.
31	indole (IND)	6.90	+	30	118→118(16)	118→90(18)	0.19	n.q.	n.q.
32	3-indole propionic acid (IPA)	7.12	+	12	190→130(14)	190→54(20)	0.19	n.q.	n.q.
33	N-acetyl tryptophan ethyl ester (N-TRP-EE)	7.61	+	18	275→159(22)	275→201(12)	0.50	0.21 (25)	n.d.
34	3-indole butyric acid (IBA)	7.78	+	18	204→130(22)	204→144(22)	0.08	2.58 (26)	n.d.
35	methyl-indole acetic acid (M-IAA)	7.80	+	14	190→130(10)	190→103(32)	2.80	3.59 (9)	1.22 (13)
36	3-CH_3-indole (CH_3-IND)	8.06	+	34	132→103(20)	132→76(22)	6.60	n.q.	n.q.
37	3-indole acetic acid ethyl ester (IAA-EE)	8.11	+	14	204→130(14)	204→102(36)	0.06	3.00 (12)	3.91 (3)
38	3-ethyl-indole carboxylic acid (E-ICa)	8.15	+	16	190→118(18)	190→162(12)	0.18	16.28 (10)	1.48 (37)

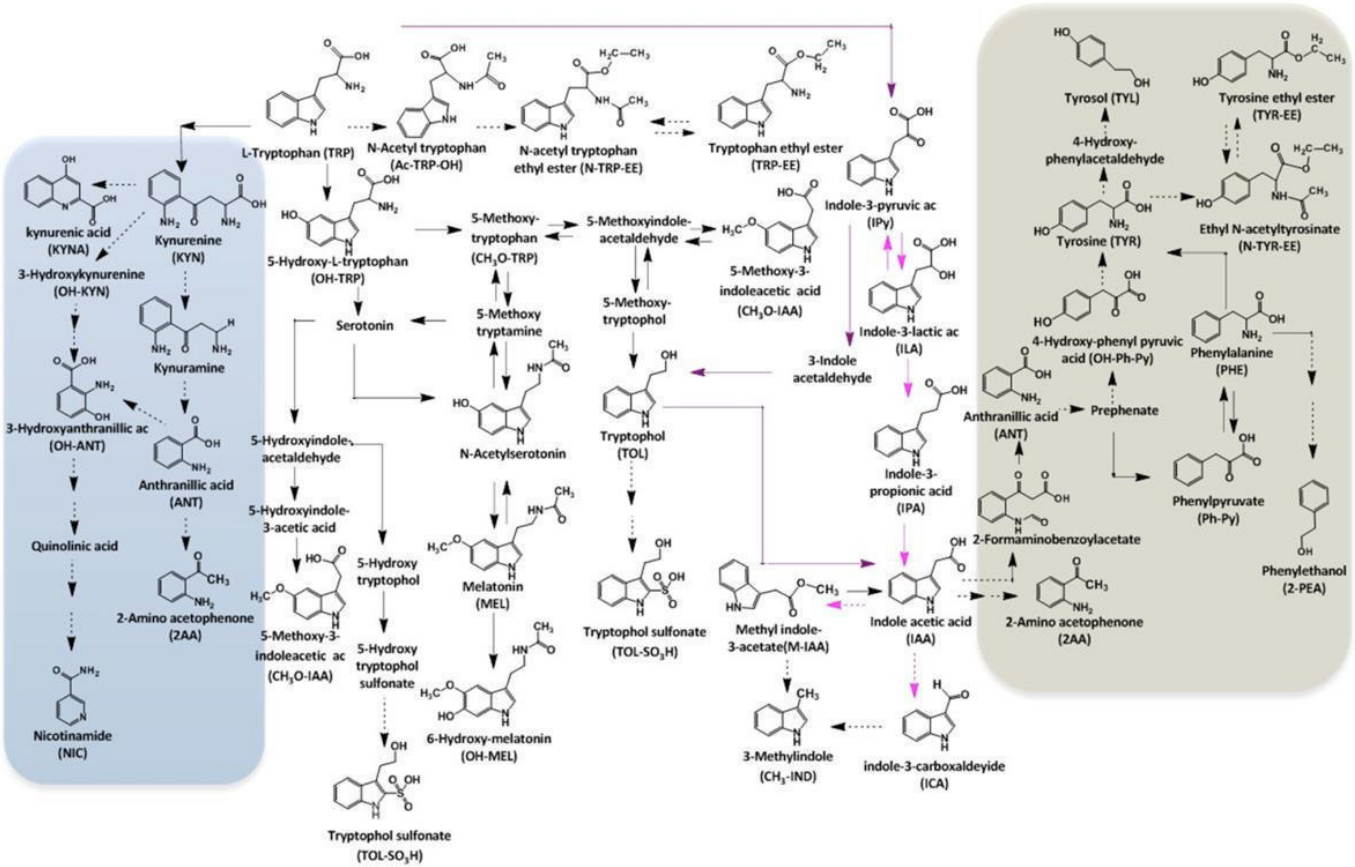

Figure 1. Scheme of proposal pathway of tryptophan (TRP), tryrosine (TYR), phenylalanine (PHE) and related compounds. Purple arrows indicate Ehrlich pathway of tryptophan. Pink arrows indicate the compounds enclosed in a cluster of heat-map of extracellular samples. The box on the left enclosed the kynurenine pathway. The box on the right, enclosed the reactions of compounds related to TYR and PHE metabolism

Figure(s)

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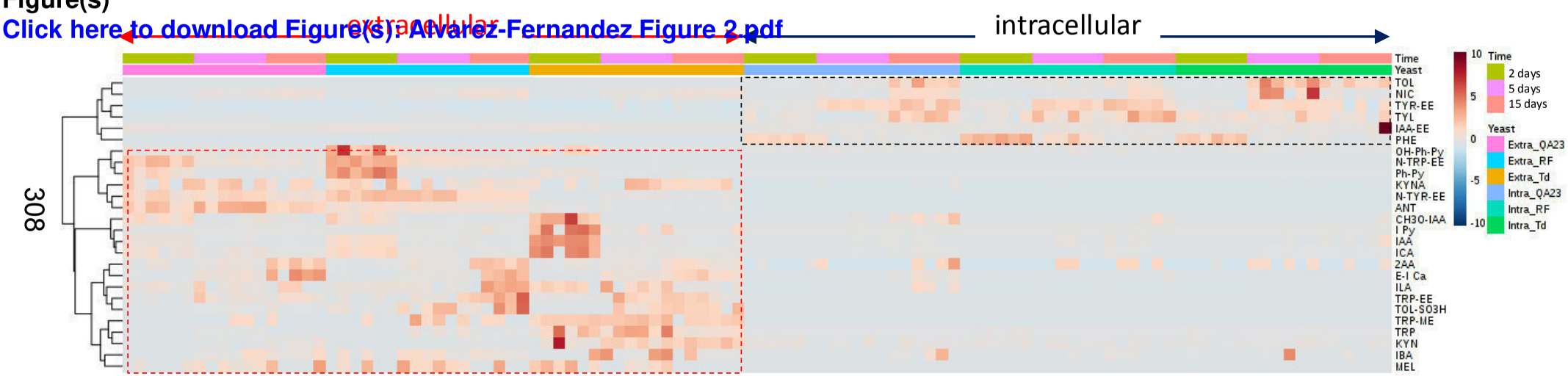

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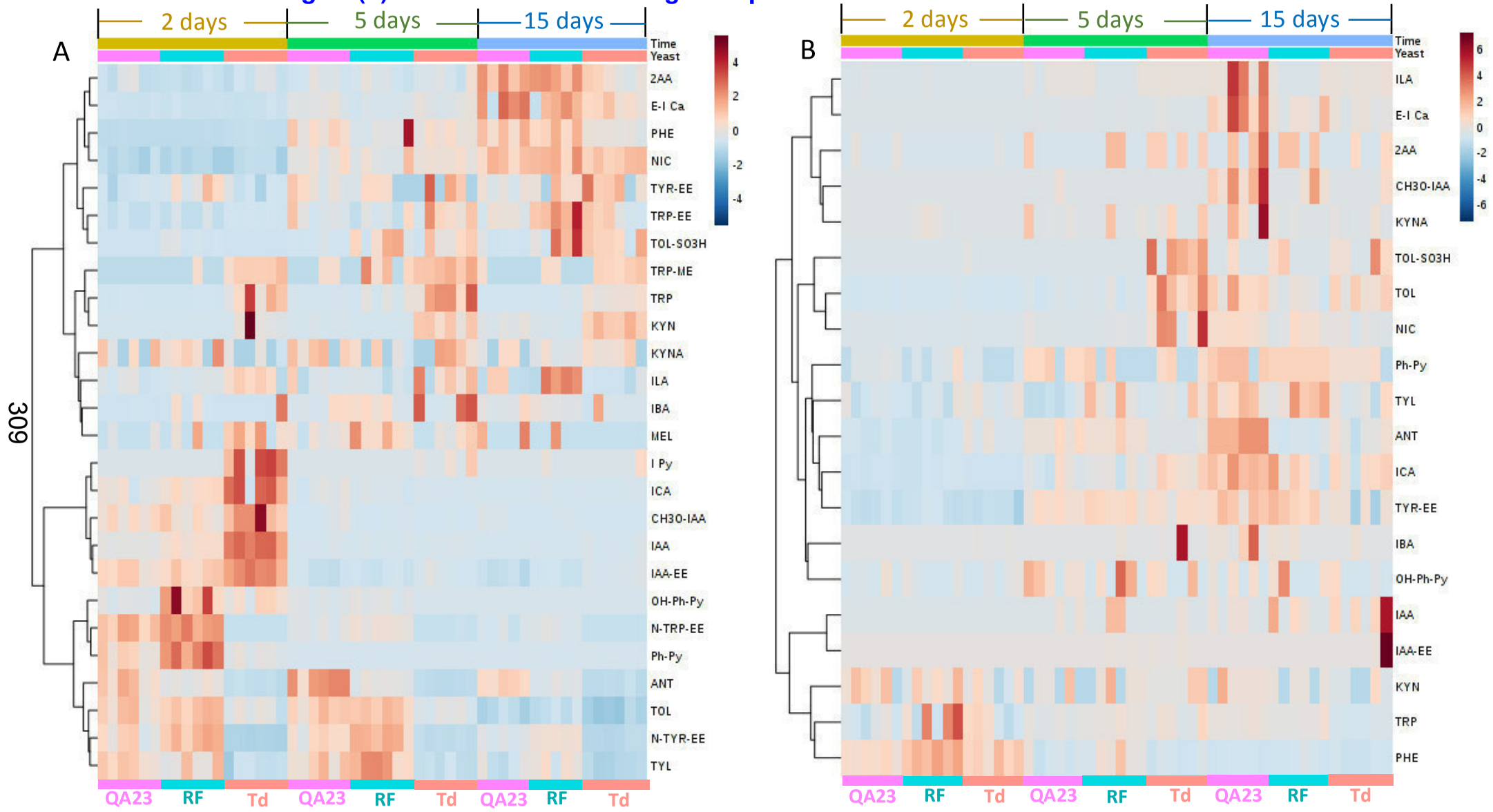

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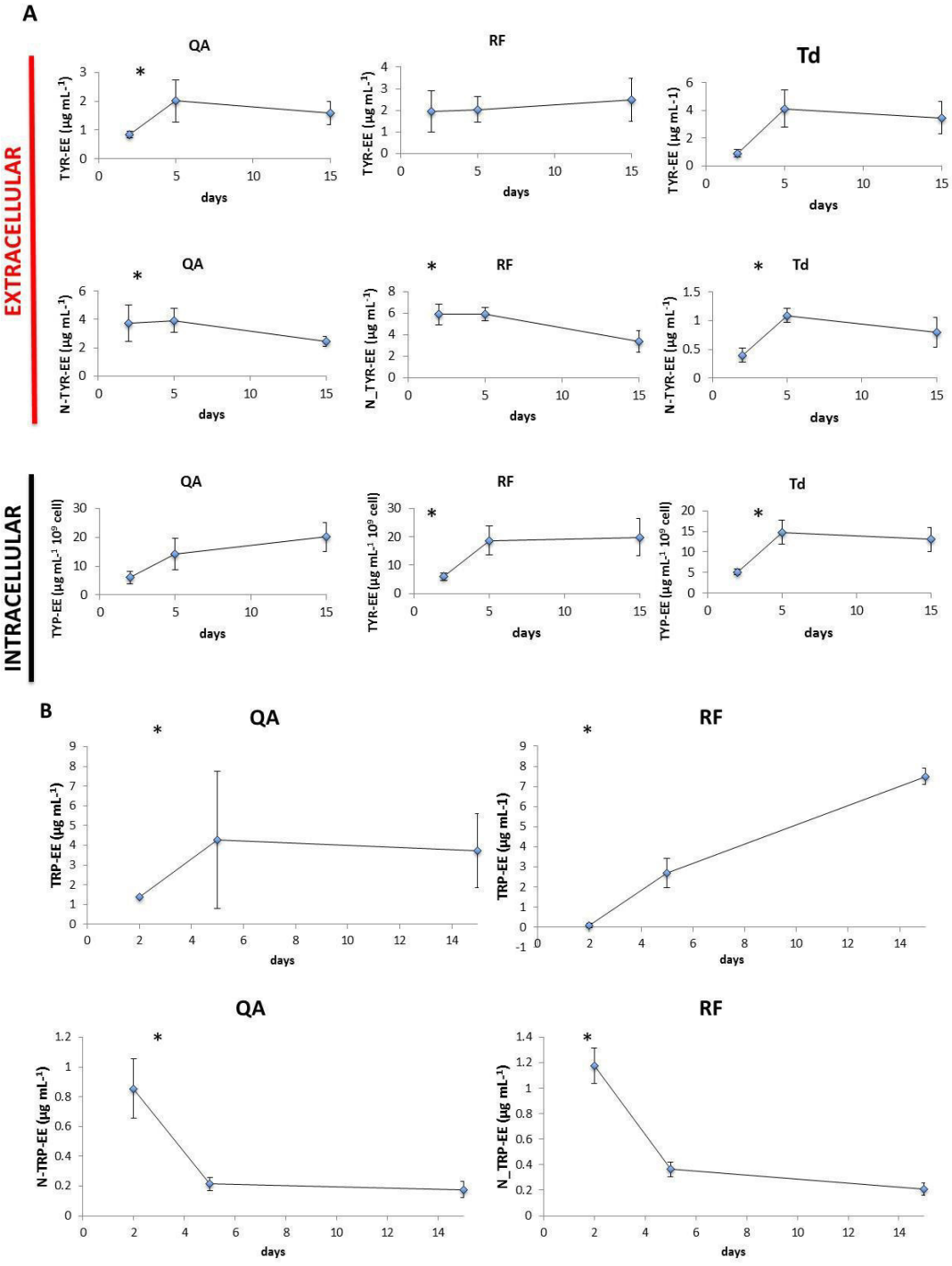

Figure(s)

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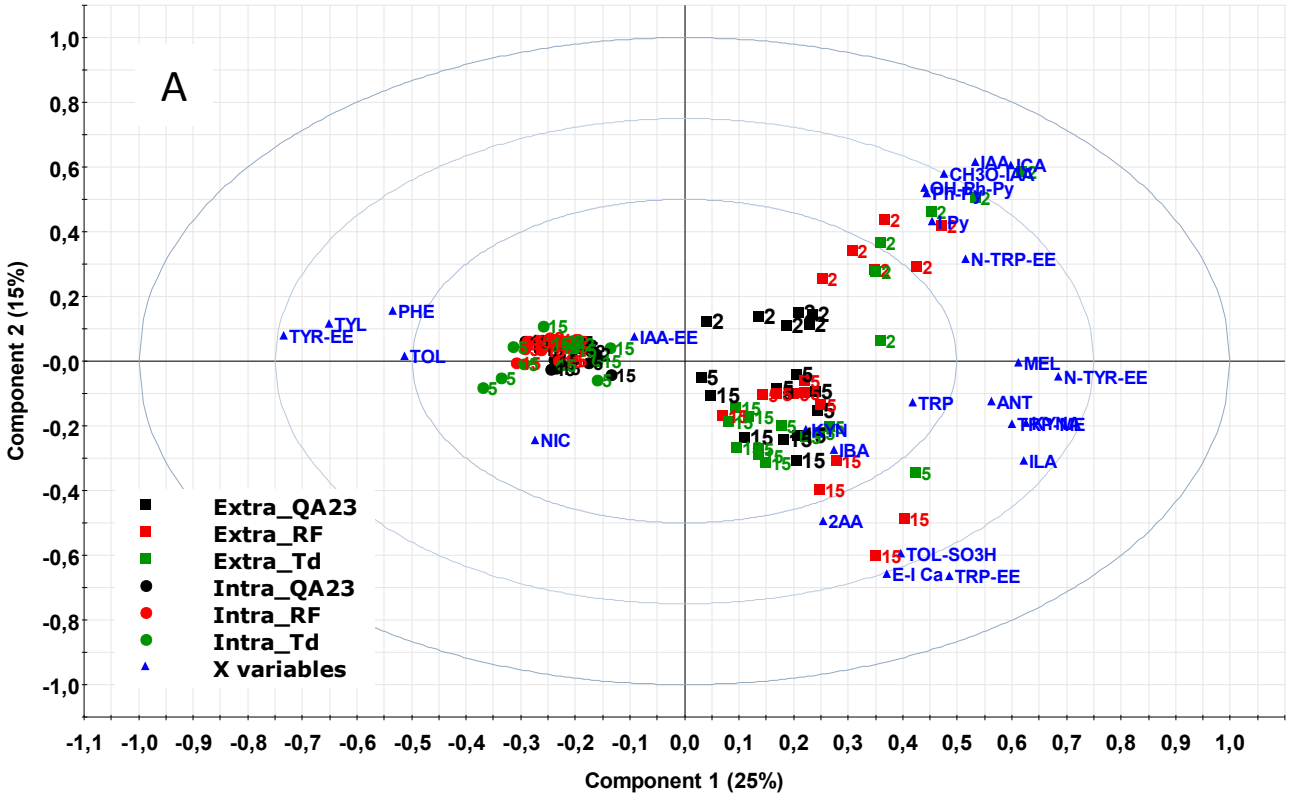


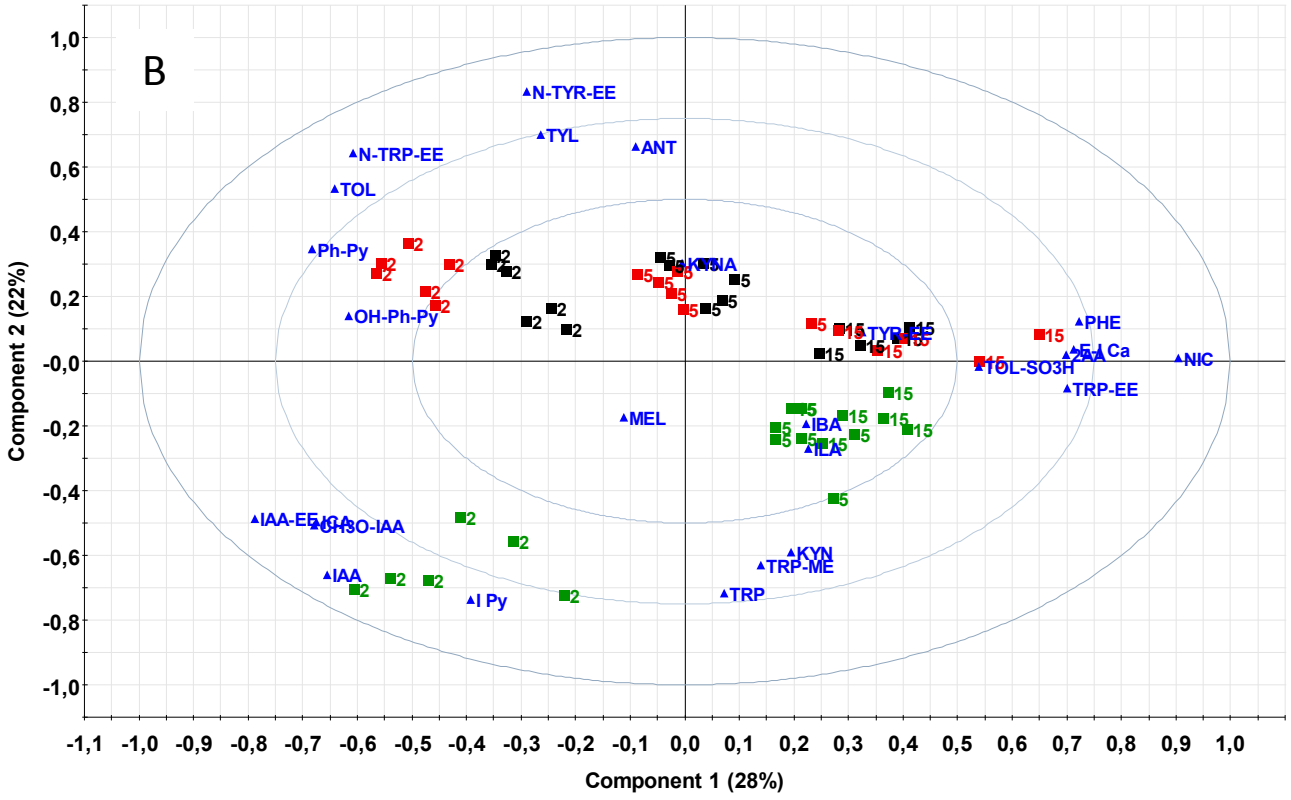


Table S1. Standards

Sigma-Aldrich SL Madrid, Spain	**Sigma-Aldrich Quimica SL Milan, Italy**	
tryptamine (TRYPT)	1-cyclohexene-1-carboxylic acid	phenyl pyruvic acid (Ph-Py)
3-Indole acetic acid (IAA)	2-amino acetophenone (2AA)	picolinic acid
3-indole butyric acid (IBA)	2-hydroxy-phenyl acetic acid	shikimic acid
3-indole pyruvic acid (IPy)	3-ethyl-indole carboxylic acid (E-I Ca)	tryptophan methyl ester (TRP ME)
4-hydroxy-phenyl acetic acid (OH-Ph-AA)	3-hydroxy kynurenine (OH-KYN)	tyramine (TYRA)
5-hydroxy-L-tryptophan (OH-TRP)	3-hydroxy-anthranilic acid (OH-ANT)	tyrosine (TYR)
5-Methoxytryptamine (5MOT)	3-indole acetic acid ethyl ester (IAA-EE)	tyrosine methyl ester (TYRME)
5-Metoxy tryptophan (CH_3O-TRP)	3-indole lactic acid (ILA)	formic acid (LC-MS grade)
5-metoxy-3-indole acetic acid (CH_3O-IAA)	3-indole propionic acid (IPA)	methanol (LC-MS grade)
5-Metoxytryptophol (5-HTOL	3-methoxy tyramine	
6-Hydroxymelatonin (OH-MEL)	3-methyl-indole (CH_3-IND)	
DL-kynurenine (KYN)	3-nitrotyrosine(IS)	
DL-tryptophan methyl ester (TRP-ME)	3,4-dihydroxy-3-methoxyphenyl propionic acid	
hydroxy indole -3-acetic acid (5H-IAA)	3(2,4-dihydroxy) phenyl propionic acid	
kynurenic acid (KYNA)	4-hydroxy-phenyl pyruvic acid (OH-Ph-Py)	
L-Tyrosine (TYR)	5-methoxy tryptophol	
L-tyrosine methyl ester (TYR-ME)	6-benzyloxy-6-methoxy indole	
melatonin (MEL)	abscisic acid	
N-acetyl serotonin (N-SER)	anthranilic acid (ANT)	
N-acetyl tryptophan ethyl ester (N-TRP-EE)	dopamine (DOPA)	
N-acetyl-L-tyrosine ethyl ester (N-TYR-EE)	ethyl anthranilate	
phenyl acetic acid (Ph-AA)	indole (IND)	
serotonine (SERO)	indole acetamide	
tryptophan (TRP)	indole acetic acid methyl ester (IAAME)	
tryptophan ethyl ester (TRP-EE)	indole carbinol (I3C)	
tryptophol (TOL)	indole carboxaldehyde (ICA)	
tyrosine ethyl ester (TYR-EE)	indole-2-carboxylic acid	
tyrosol (TYL)	indoxyl sulphate	
	methyl-indole acetic acid (M-IAA)	
Cymit Quimica S.L., Barcelona, Spain.	nicotinamide (NIC)	
N-acetyl-5-methoxy kynureanine hydrochloride (AMK)	nicotinic acid	
sulfatoxy melatonin (6-aMTs)	phenyl alanine (PHE)	
N-γ-acetyl-N-2-formyl-5-methoxy kynureamine (AFMK)	phenyl lactic acid (Ph-LA)	

Table S2. Composition of synthetic must.

	Compound	g L^{-1}
	Glucose	100
	Fructose	100
Mineral Salts	$CaCl_2$	0.155
	$KH_2 \cdot PO_4$	0.75
	K_2SO_4	0.5
	$MgSO_4 \cdot 7H_2O$	0.25
	NaCl	0.2
	NH_4Cl	0.46
Trace Elements	$COCl_2 \cdot 6H_2O$	0.4
	$CuSO_4 \cdot 5H_2O$	1
	H_3BO_3	1
	KI	1
	$MnSO_4 \cdot H_2O$	4
	$(NH_4)6Mo_7O_{24}$	1
	$ZnSO_4 \cdot H_2O$	4
Nitrogen sources		% wt/wt
	ammoniacal nitrogen 18.6% wt/wt	18.6
	NH_4Cl	20.5
	L-proline	16.9
	L-glutamine	1.25
	L-arginine	6
	L-tryptophan	4.9
	L-alanine	4
	L-glutamic acid	2.6
	L-serine	2.6
	L-threonine	1.6
	L-leucine	1.5
	L-aspartic acid	1.5
	L-valine	1.3
	L-phenylalanine	1.1
	L-isoleucine	1.1
	L-histidine	1.1
	L-methionine	0.6
	L-tyrosine	0.6
	L-glycine	0.6
	L-lysine	0.6
Aerobics Factors		g 100 mL^{-1}
	Oleic acid	0.5
	Ergosterol	1.5
	Tween 80	0.5
Vitamins		mg mL^{-1}
	Biotin	0.003
	Calcium pantothenate	1.5
	Chlorohydrate pyridoxine	0.25
	Chlorohydrate thiamine	0.25
	Myoinositol	20
	Nicotinic acid	2

Table S3. Calibration curve information

#	Metabolite	Degree of linearity	LOD (µg L^{-1})	LOQ (µg L^{-1})	*a* (slope)	*b*	R^2
1	nicotinamide (NIC)	5	1.39	4.2	26513.9	-42.33	0.9968
2	5-OH-tryptophan (OH-TRP)	5	1.29	3.90	52940.5	304.35	0.9937
3	5-CH_3O-tryptophan (CH_3O-TRP)	4	3.47	10.5	122841	483.08	0.9836
4	kynurenine (KYN)	5	5.41	16.40	42790.8	526.02	0.9973
5	phenylalanine (PHE)	4	14.82	44.9	249486	17879.20	0.9286
6	3-nitrotyrosine (IS)	5	1.29	3.90	177335	187.22	0.9481
7	4-OH-phenyl pyruvic acid (OH-Ph-Py)	2	391.35	1185.9	3300.82	2873.89	0.9436
8	3-OH-anthranilic acid (OH-ANT)	5	1.06	3.2	72356	466.56	0.8761
9	tryptophan (TRP)	4	3.70	11.2	98314.8	4081.96	0.9877
10	tyrosine ethyl ester (TYR-EE)	3.5	39.4	13.00	369979	2051.90	0.8211
11	N-acetyl serotonin (N-SER)	6	3.00	9.1	44447.7	978.03	0.9233
12	tyrosol (TYL)	4	11.88	36	2436.93	1894.53	0.9762
13	tryptophol sulphonate (TOL-SO_3H)	4	1.39	4.2	4381.18	121.92	0.9531
14	kynurenic acid (KYNA)	3	3.40	10.30	23423.8	2061.11	0.9259
15	4-OH-phenyl acetic acid (OH-Ph-AA)	4	416.57	1262.33	3300.82	2873.89	0.9714
16	tryptophan methyl ester (TRP-ME)	4	72.9	24.06	252041	243204	0.9464
17	phenyl pyruvic acid (Ph-Py)	4	14.26	43.2	302509	-111.49	0.9730
18	indole pyruvic acid (IPy)	3	0.77	0.24	10091	9560.45	0.9977
19	anthranilic acid (ANT)	4	3.10	9.4	110637	729.00	0.9900
20	tryptophan ethyl ester (TRP-EE)	4	82.9	27.36	174531	574258	0.9666
21	phenyl lactic acid (Ph-LA)	3	68.81	208.5	1784.52	155.74	0.9986
22	3-indole lactic acid (ILA)	4	0.14	0.45	105262	46607514	0.9969
23	N-acetyl-L-tyrosine ethyl ester (N-TYR-EE)	5	0.56	1.7	384108	155.93	0.9970
24	indole carboxaldehyde (ICA)	5	0.03	0.09	666814	-11821468	0.9996
25	melatonin (MEL)	5	0.21	0.65	189985	-122.89	0.9993
26	5-CH_3O-indole acetic acid (CH_3O-IAA)	5	0.05	0.16	801.95	37458.61	0.9991

Table S1. Continued

#	Metabolite	Degree of linearity	LOD (µg L^{-1})	LOQ (µg L^{-1})	*a* (slope)	*b*	R^2
27	tryptophol (TOL)	5	23.43	71.00	39496.2	2187.56	0.9758
28	3-indole acetic acid (IAA)	5	0.17	0.52	31.7	18572.02	0.9932
29	phenyl acetic acid (Ph-AA)	3	55.08	166.9	4120.67	1687.99	0.9133
30	2-aminoacetophenone (2AA)	4	0.04	0.15	734168	6005504	0.9995
31	indole (IND)	3	0.06	0.19	10736	-5728107	0.9989
32	3-indole propionic acid (IPA)	5	0.06	0.19	282.95	38674.00	0.9988
33	N-acetyl tryptophan ethyl ester (N-TRP-EE)	5	1.5	0.50	146982	3.48	0.9994
34	3-indole butyric acid (IBA)	4	0.02	0.08	133.08	-1775.78	0.9997
35	methyl-indole acetic acid (M-IAA)	5	0.9	2.8	901707	784.32	0.9973
36	3-CH_3-indole (CH_3-IND)	4	2.18	6.60	89361.3	589.29	0.9942
37	3-indole acetic acid ethyl ester (IAA-EE)	5	0.02	0.06	907.12	495.01	0.9998
38	3-ethyl-indole carboxylic acid (E-ICa)	5	0.06	0.18	495659	-21751351	0.9988

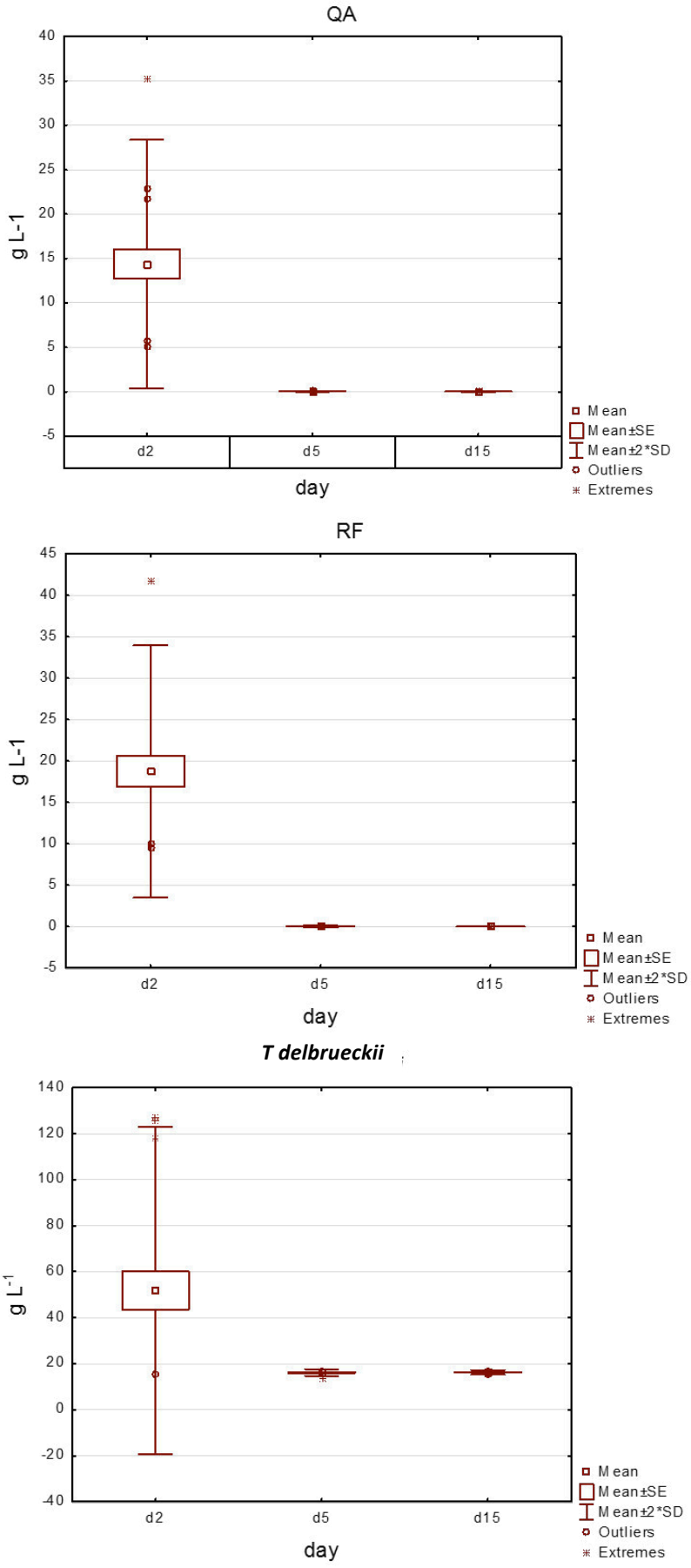


Figure S1. Graphic representation of variability of reducing sugar consumption through sampling time of the six folds of the three strain of yeast studied

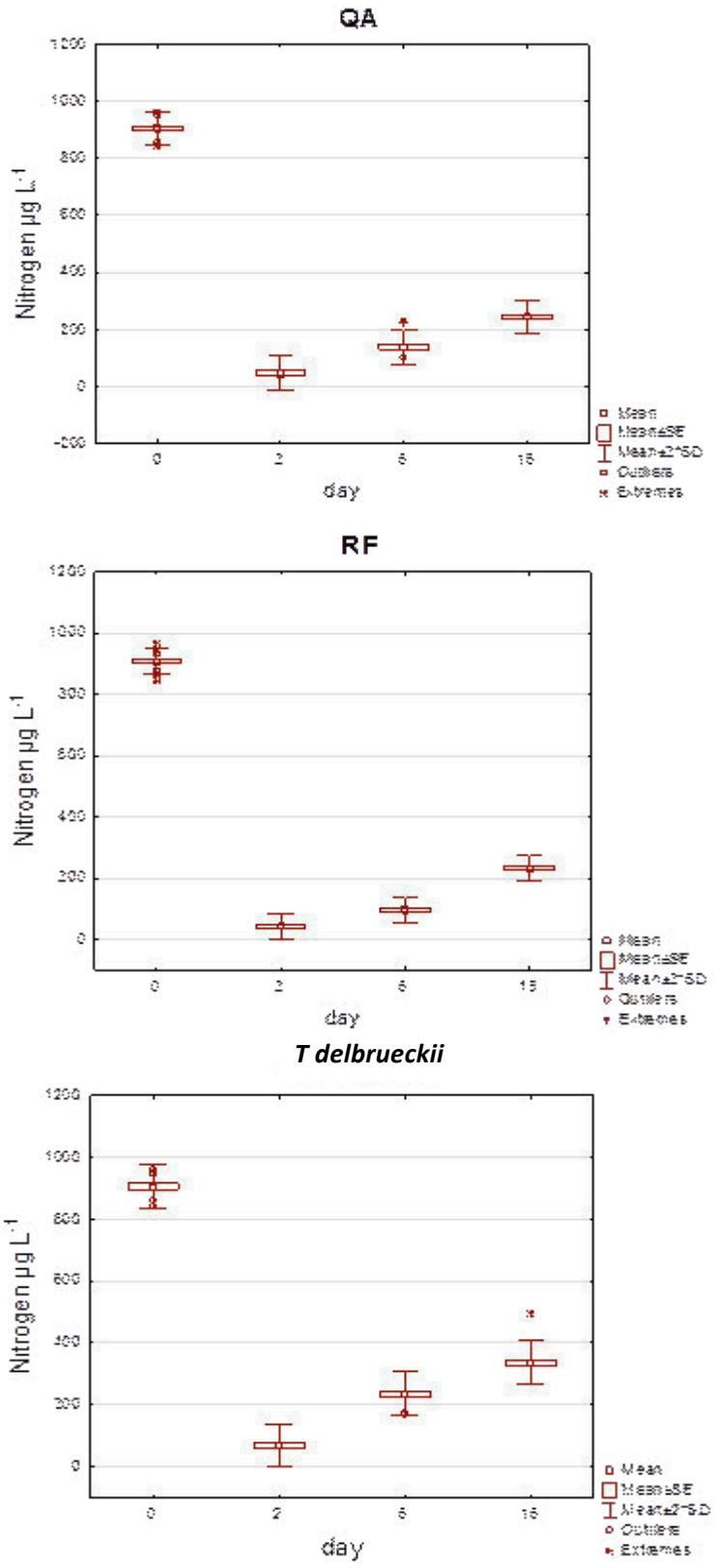
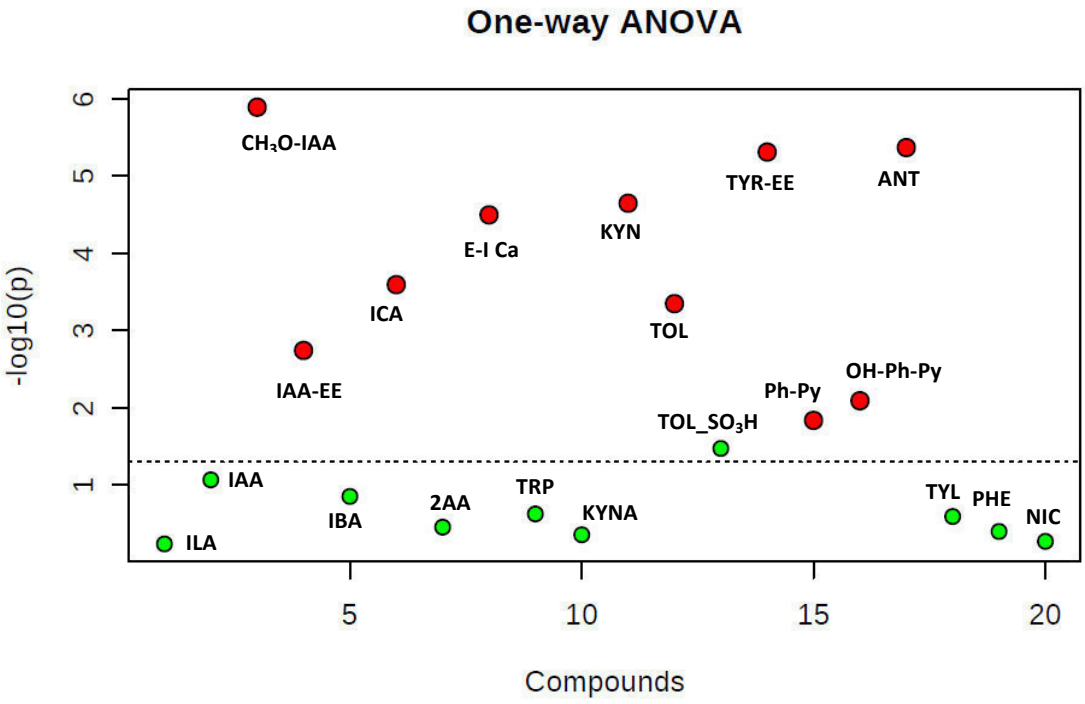
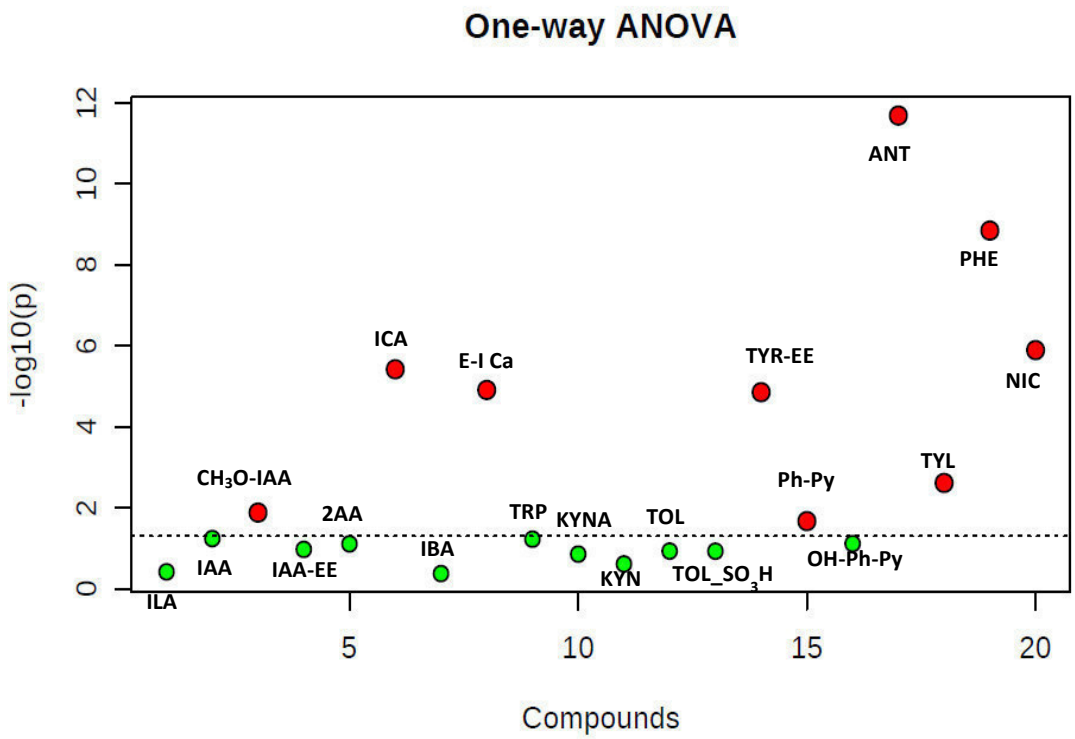


Figure S2. Graphic representation of variability of nitrogen concentration (µg L^{-1}) of extracellular samples through the sampling days.



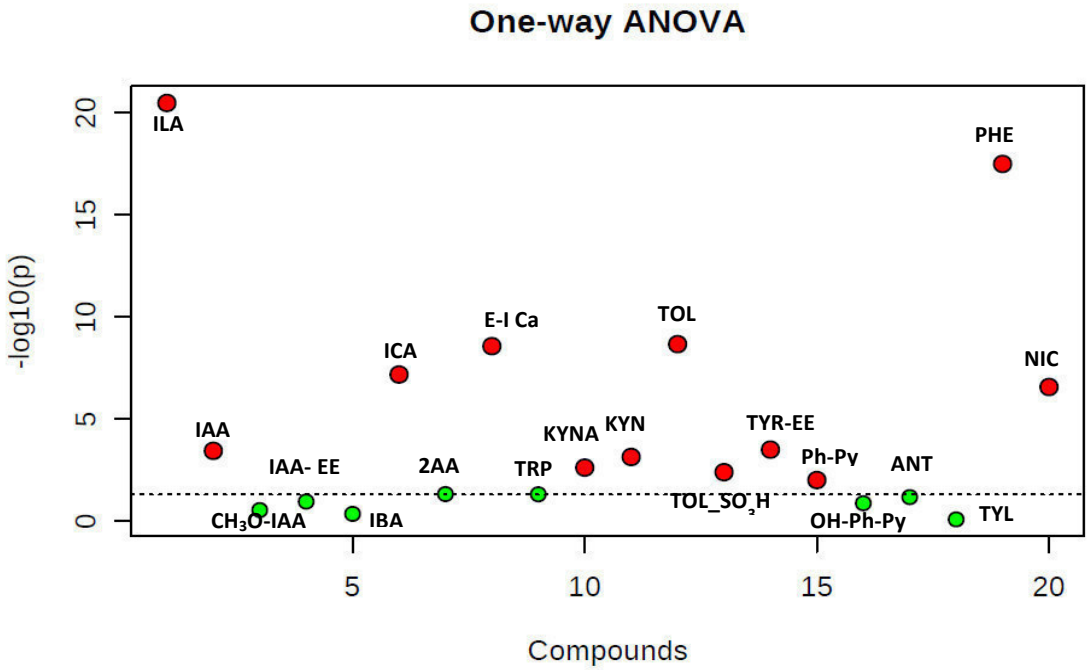
Name	f.value	p.value	-log10(p)	FDR	Tukey's HSD
CH_3O-IAA	25.975	1.0403e-07	6.9828	2.0806e-06	3-1; 3-2
E-I Ca	17.104	6.0035e-06	5.2216	6.0035e-05	3-1; 3-2
ANT	10.443	0.00026508	3.5766	0.0015412	2-1; 3-1
KYN	10.206	0.00030824	3.5111	0.0015412	2-1; 3-1
TYR-EE	9.3655	0.00053117	3.2748	0.0018477	2-1; 3-1
TOL	9.3008	0.0005543	3.2563	0.0018477	2-1; 3-2
ICA	6.9755	0.0027521	2.5603	0.0078631	3-1; 3-2
IAA-EE	6.4943	0.003906	2.4083	0.009765	3-1; 3-2
OH-Ph-Py	4.3867	0.019728	1.7049	0.04384	3-1; 3-2

Figure S3. ANOVA analysis of QA intracellular samples. The points highlighted in red are the significant compounds selected based on the default p value threshold (0.05), which is marked by a dashed line. And table with statistical data of compounds with significant differences.



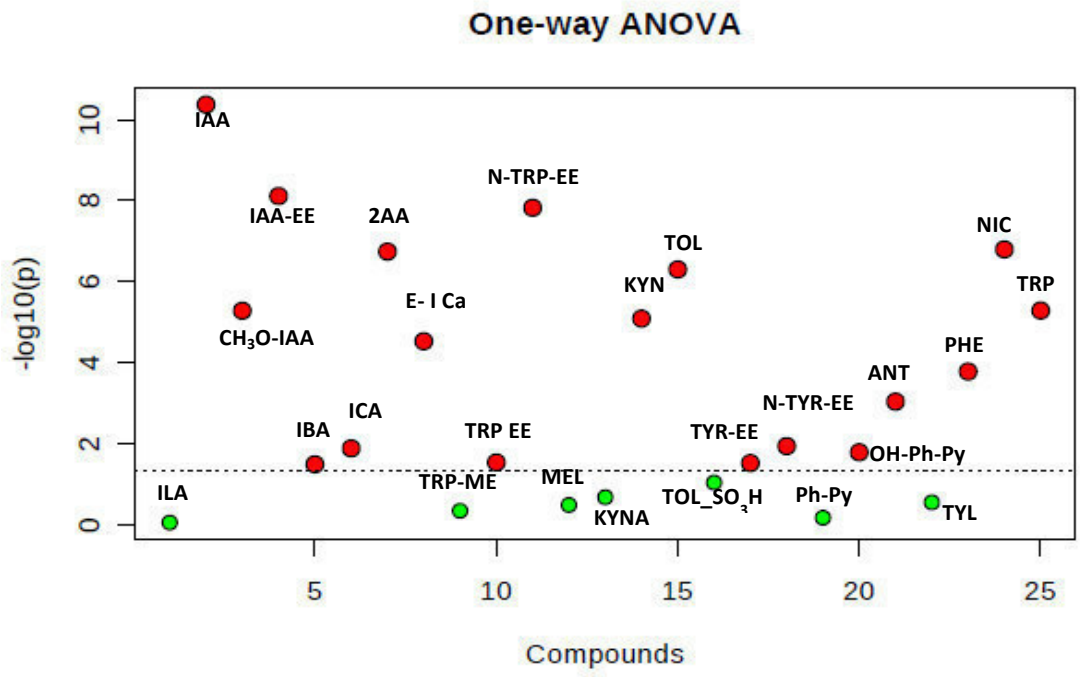
Name	f.value	p.value	-log10(p)	FDR	Tukey's HSD
ANT	63.826	2.1093e-12	11.676	4.2186e-11	2-1; 3-2
PHE	38.478	1.4551e-09	8.8371	1.4551e-08	2-1; 3-1; 3-2
NIC	20.474	1.2949e-06	5.8878	8.6326e-06	3-1; 3-2
ICA	18.18	3.853e-06	5.4142	1.9265e-05	2-1; 3-1; 3-2
E-I Ca	15.867	1.2449e-05	4.9049	4.7112e-05	3-1; 3-2
TYR- EE	15.626	1.4134e-05	4.8497	4.7112e-05	2-1; 3-1
TYL	7.1726	0.0024514	2.6106	0.0070041	2-1; 3-1
CH_3O-IAA	4.9081	0.013215	1.8789	0.033037	3-1; 3-2
Ph-Py	4.2982	0.021419	1.6692	0.047599	3-1

Figure S4. ANOVA analysis of RF intracellular samples and data of compounds with significant differences ($p<0.05$).



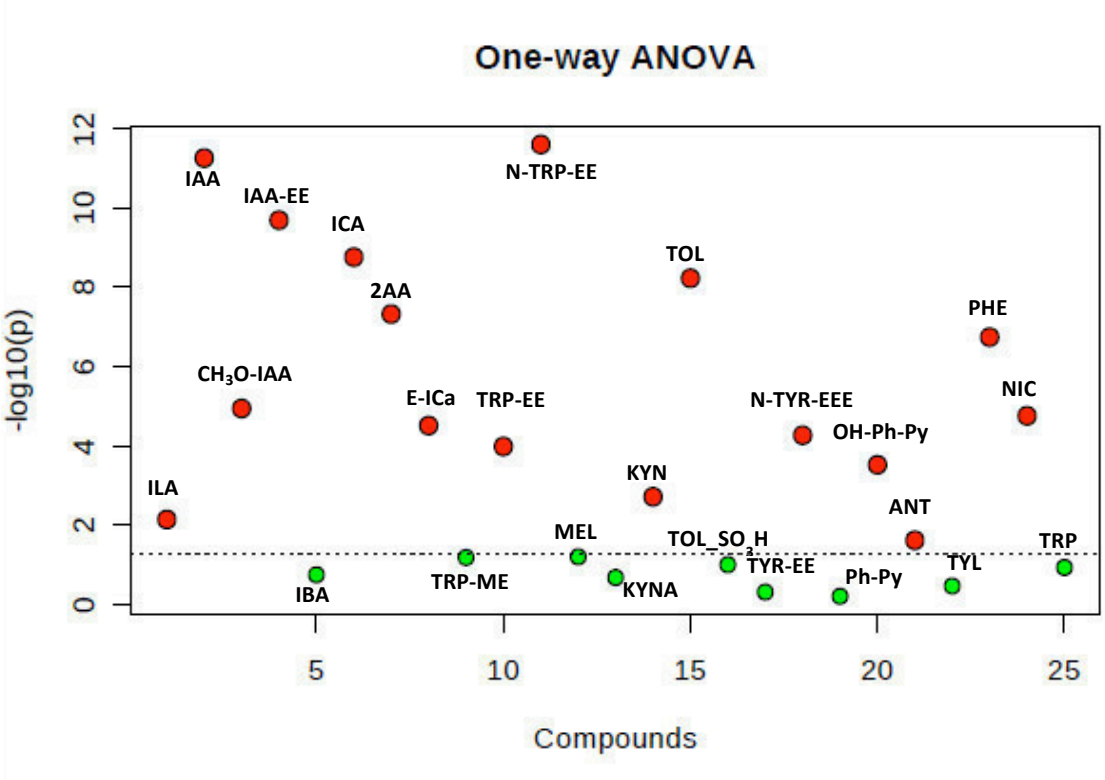
Name	f.value	p.value	-log10(p)	FDR	Tukey's HSD
ILA	217.7	3.4425e-21	20.463	6.8849e-20	2-1; 3-1
PHE	144.41	3.3249e-18	17.478	3.3249e-17	2-1; 3-1
TOL	35.831	2.2098e-09	8.6557	1.3832e-08	2-1; 3-1; 3-2
E-I Ca	35.175	2.7664e-09	8.5581	1.3832e-08	3-1; 3-2
ICA	26.658	6.7632e-08	7.1698	2.7053e-07	2-1; 3-1
NIC	23.347	2.7657e-07	6.5582	9.2191e-07	2-1; 3-2
TYR-EE	10.078	0.00032073	3.4939	0.00091637	2-1; 3-1
IAA	9.8625	0.0003689	3.4331	0.00092224	3-1; 3-2
KYN	8.8237	0.00073568	3.1333	0.0016348	3-2
KYNA	7.1091	0.00244	2.6126	0.0048801	2-1; 3-2
TOL SO_3H	6.4112	0.004068	2.3906	0.0073963	2-1; 3-2
Ph-Py	5.241	0.0099072	2.004	0.016512	2-1; 3-1

Figure S5. ANOVA analysis of *Torulaspora delbruekii* intracellular samples and data of compounds with significant differences ($p<0.05$).



Name	f.value	p.value	-log10(p)	FDR	Tukey's HSD
IAA	132.53	4.2757e-11	10.369	1.0689e-09	2-1; 3-1; 3-2
IAA-EE	67.975	7.765e-09	8.1099	9.7062e-08	2-1; 3-1
N-TRP-EE	62.186	1.5161e-08	7.8193	1.2634e-07	2-1; 3-1
NIC	45.007	1.6165e-07	6.7914	9.1852e-07	2-1; 3-1; 3-2
2AA	44.208	1.837e-07	6.7359	9.1852e-07	3-1; 3-2
TOL	38.286	5.0593e-07	6.2959	2.108e-06	3-1; 3-2
TRP	27.028	5.2501e-06	5.2798	1.6564e-05	2-1; 3-2
CH_3O-IAA	26.989	5.3004e-06	5.2757	1.6564e-05	2-1; 3-1
KYN	25.162	8.3059e-06	5.0806	2.3072e-05	3-1; 3-2
E-I Ca	20.419	3.0195e-05	4.5201	7.5488e-05	3-1; 3-2
PHE	15.118	0.00016883	3.7725	0.00038371	2-1; 3-1
ANT	10.809	0.0009357	3.0289	0.0019494	2-1; 3-2
N-TYR-EE	5.834	0.011775	1.9291	0.022643	3-2
ICA	5.6206	0.013375	1.8737	0.023884	3-1
OH-Ph-Py	5.2553	0.016713	1.7769	0.027855	3-1; 3-2
TRP-EE	4.356	0.029693	1.5273	0.045521	2-1
TYR-EE	4.2932	0.030955	1.5093	0.045521	2-1
IBA	4.1915	0.033128	1.4798	0.046011	3-1

Figure S6. ANOVA analysis of QA extracellular samples and data of compounds with significant differences ($p<0.05$)



Name	f.value	p.value	-log10(p)	FDR	Tukey's HSD
N-TRP-EE	217.41	2.5173e-12	11.599	6.2932e-11	2-1; 3-1; 3-2
IAA	196.34	5.519e-12	11.258	6.8987e-11	2-1; 3-1
IAA-EE	122.17	2.0358e-10	9.6913	1.6965e-09	2-1; 3-1
ICA	91.553	1.7389e-09	8.7597	1.0868e-08	2-1; 3-1
TOL	77.391	5.9348e-09	8.2266	2.9674e-08	3-1; 3-2
2AA	57.833	4.7549e-08	7.3229	1.9812e-07	3-1; 3-2
PHE	47.715	1.807e-07	6.7431	6.4534e-07	2-1; 3-1; 3-2
CH_3O-IAA	25.189	1.1396e-05	4.9433	3.5612e-05	2-1; 3-1
NIC	23.476	1.7414e-05	4.7591	4.8373e-05	2-1; 3-1; 3-2
E-I Ca	21.31	3.0801e-05	4.5114	7.7003e-05	3-1; 3-2
N-TYR-EE	19.331	5.3888e-05	4.2685	0.00012247	3-1; 3-2
TRP-EE	17.195	0,00010333	3.9858	0.00021526	3-1; 3-2
OH-Ph-Py	14,061	0.00029904	3.5243	0.00057508	2-1; 3-1
KYN	9.4862	0.0019194	2.7168	0.0034274	3-1; 3-2
ILA	6.8293	0.0071739	2.1442	0.011957	3-1; 3-2
ANT	4.75	0.024024	1.6194	0.037538	3-1

Figure S7. ANOVA analysis of RF extracellular samples and data of compounds with significant differences ($p<0.05$)

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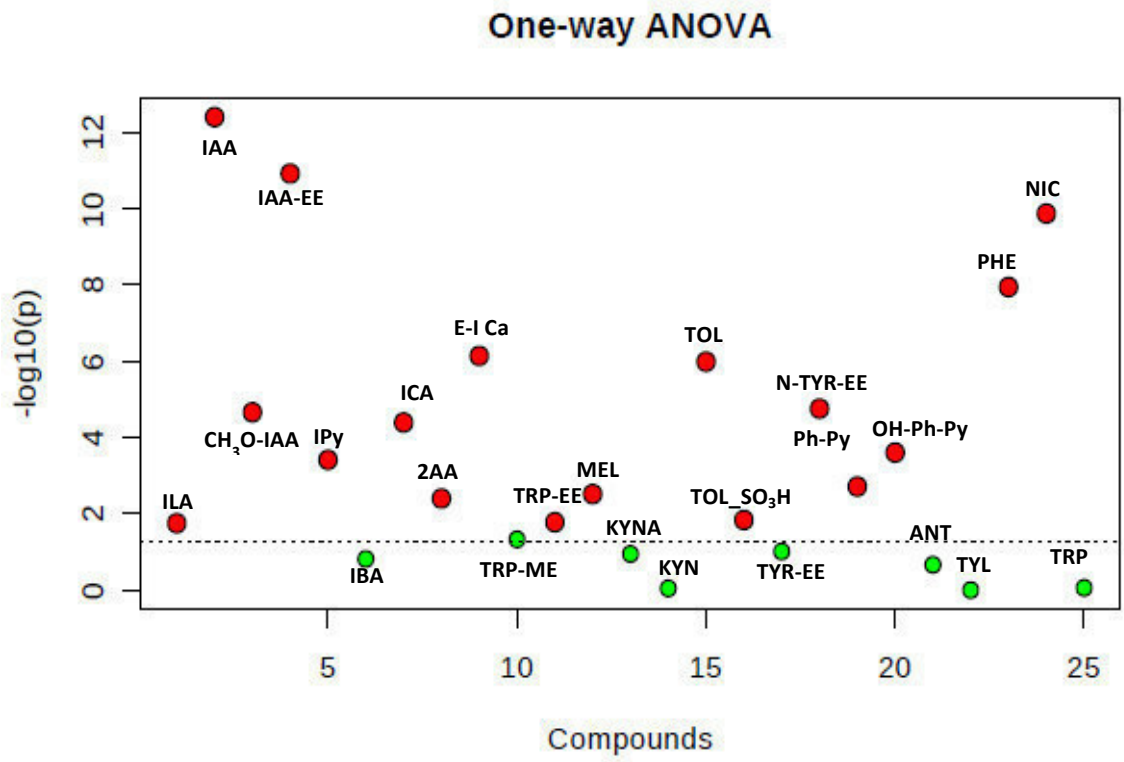

Name	f.value	p.value	-log10(p)	FDR	Tukey's HSD
IAA	276.19	3.9428e-13	12.404	9.8571e-12	2-1; 3-1
IAA-EE	177.6	1.1913e-11	10.924	1.4892e-10	2-1; 3-1
NIC	129.25	1.332e-10	9.8755	1.11e-09	2-1; 3-1; 3-2
PHE	70.818	1.1264e-08	7.9483	7.0401e-08	2-1; 3-1
E-I Ca	38.892	7.1768e-07	6.1441	3.5884e-06	2-1; 3-1; 3-2
TOL	36.875	1.0202e-06	5.9913	4.2507e-06	3-1; 3-2
N-TYR-EE	23.486	1.7371e-05	4.7602	6.2039e-05	2-1; 3-1; 3-2
CH_3O-IAA	22.628	2.1665e-05	4.6642	6.7702e-05	2-1; 3-1
ICA	20.347	4.0243e-05	4.3953	0.00011179	2-1; 3-1
OH-Ph-Py	14,604	0.00024618	3.6087	0.00061546	2-1; 3-1
IPy	13.397	0.00038181	3.4182	0.00086774	2-1; 3-1
Ph-Py	9.4916	0.0019146	2.7179	0.0039888	2-1; 3-1
MEL	8.5267	0.0030146	2.5208	0.0057974	3-1
2AA	7.9826	0.0039404	2.4045	0.0070365	3-1; 3-2
TOL SO_3H	5.5924	0.0144	1.8416	0.024	2-1; 3-1
TRP-EE	5.3645	0.016486	1.7829	0.025759	2-1
ILA	5.2593	0.017561	1.7554	0.025825	3-2

Figure S8. ANOVA analysis of *Torulaspora delbruekii* extracellular samples, and table with data of compounds with significant differences ($p<0.05$).

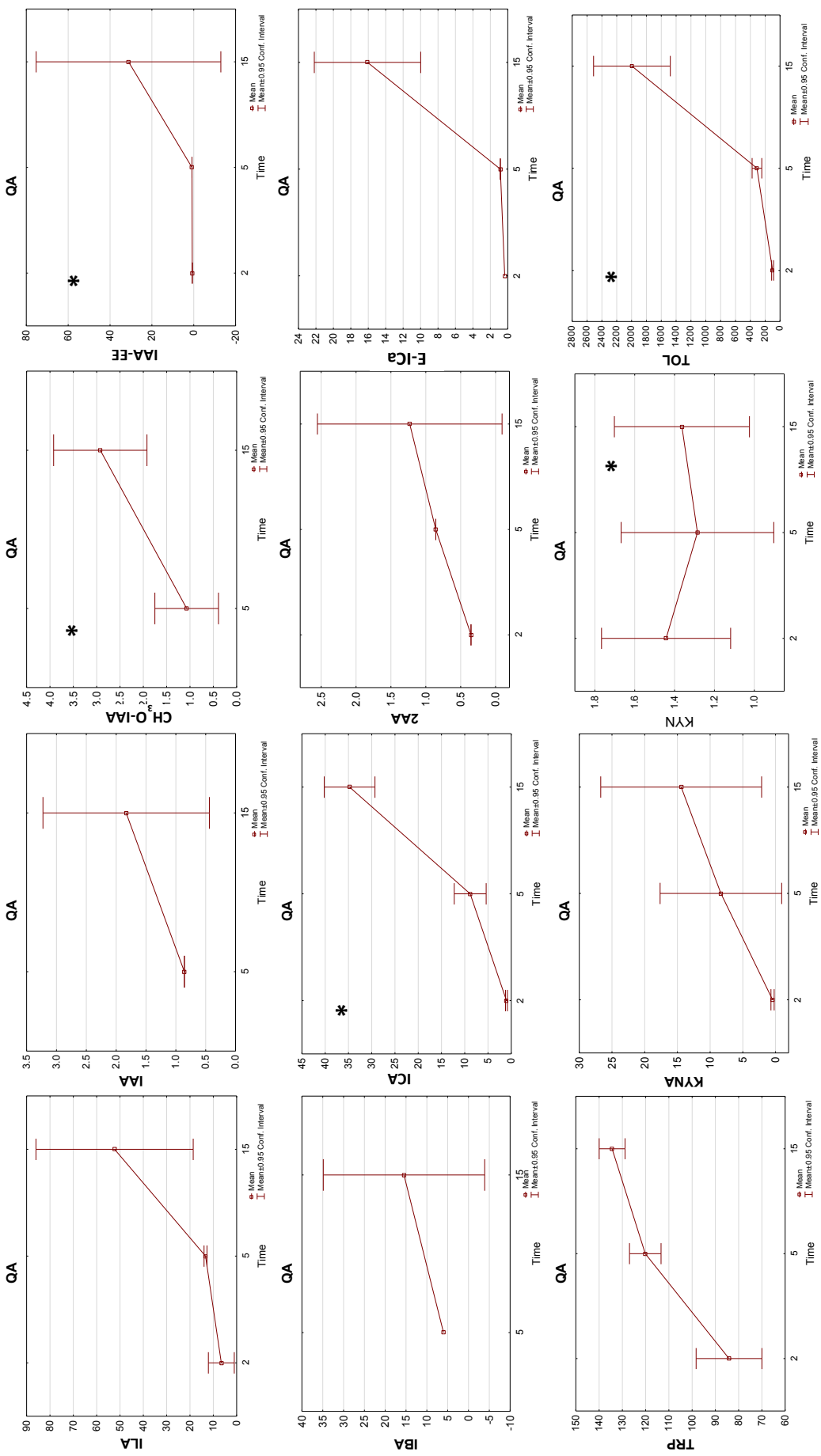

Figure S9. Time curse of compounds in QA intracellular samples. * means significant differences ($p<0.05$).

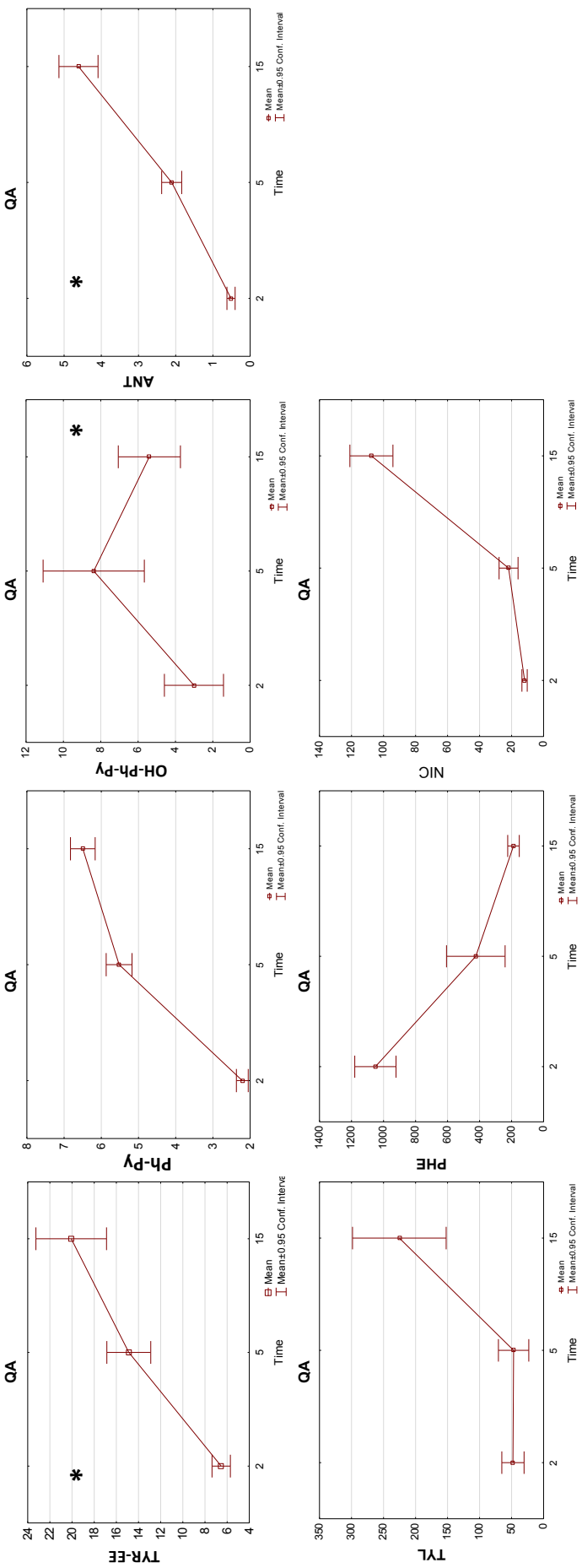

Figure S9. Continued.

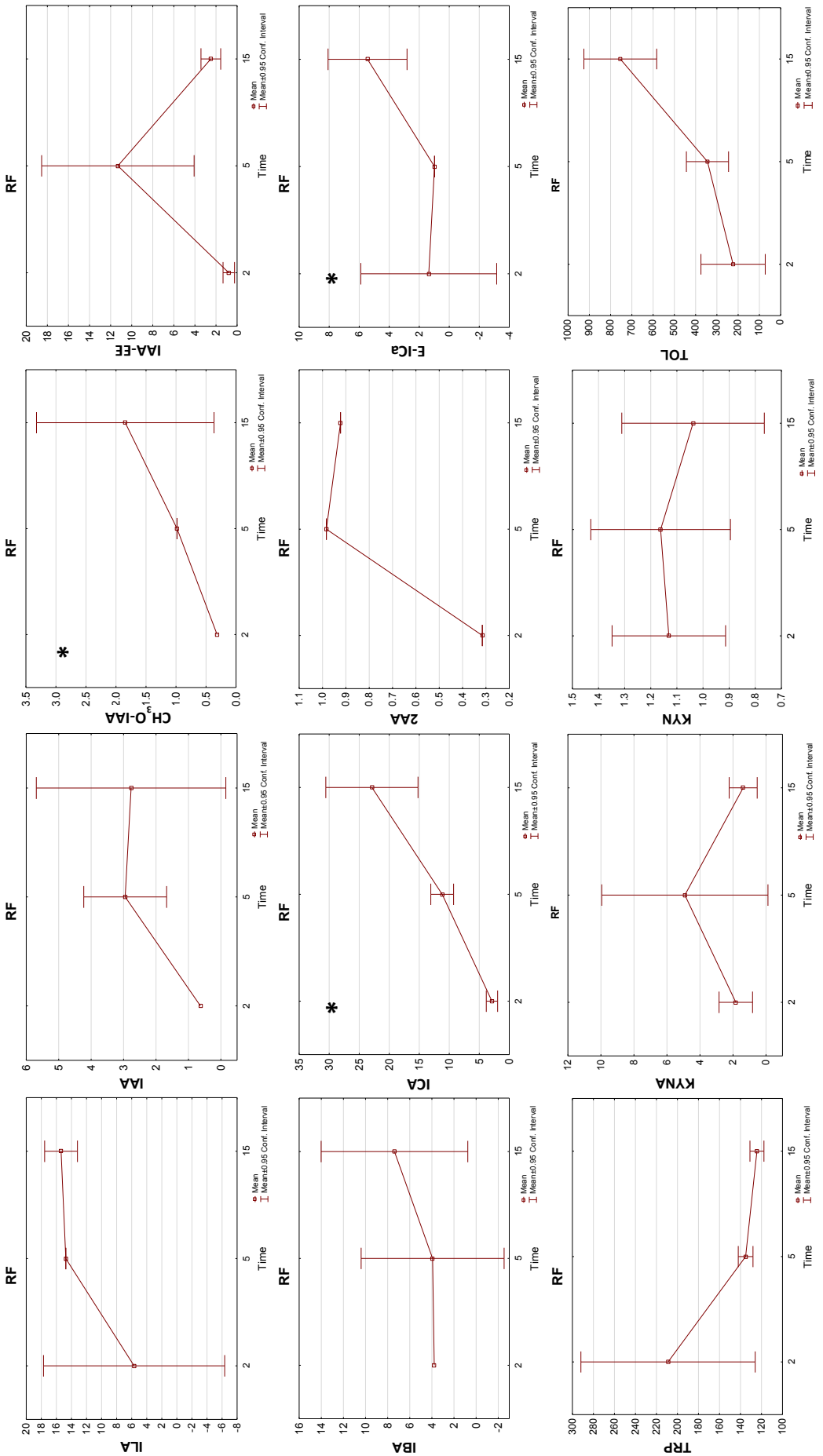

Figure S10. Time curse of compounds in RF intracellular samples. * Indicates significant differences ($p<0.05$).

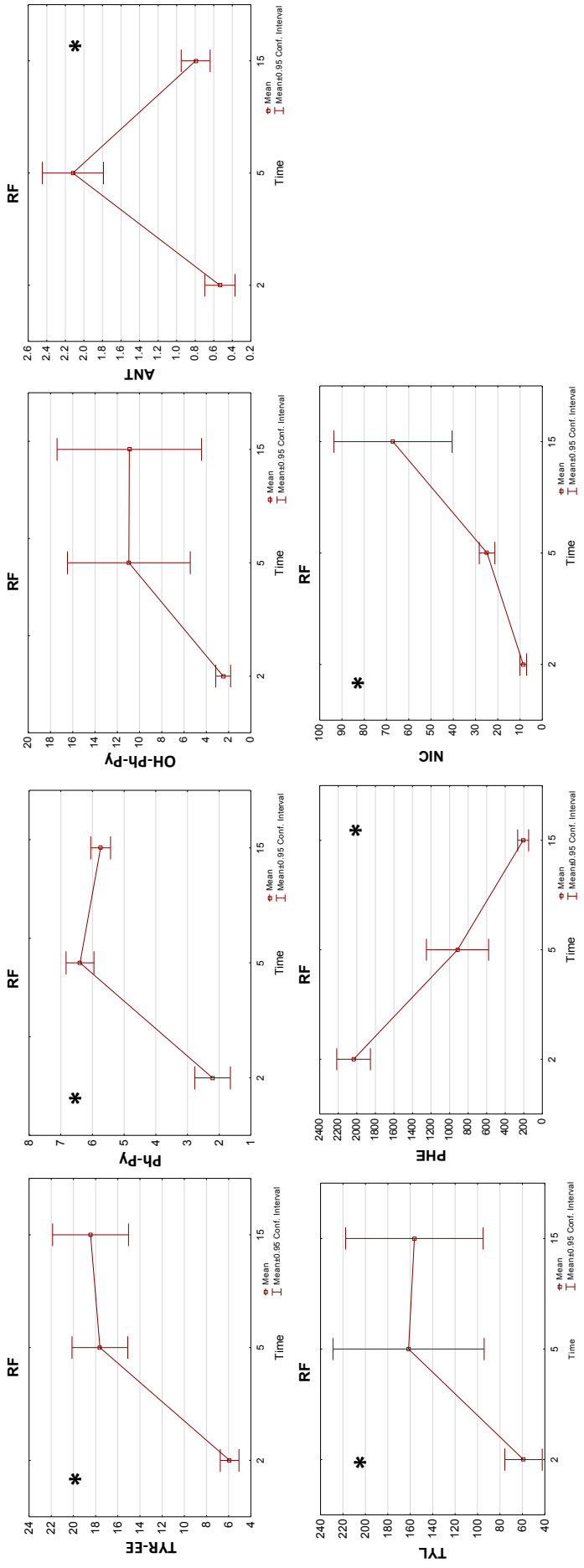

Figure S10. Continued.

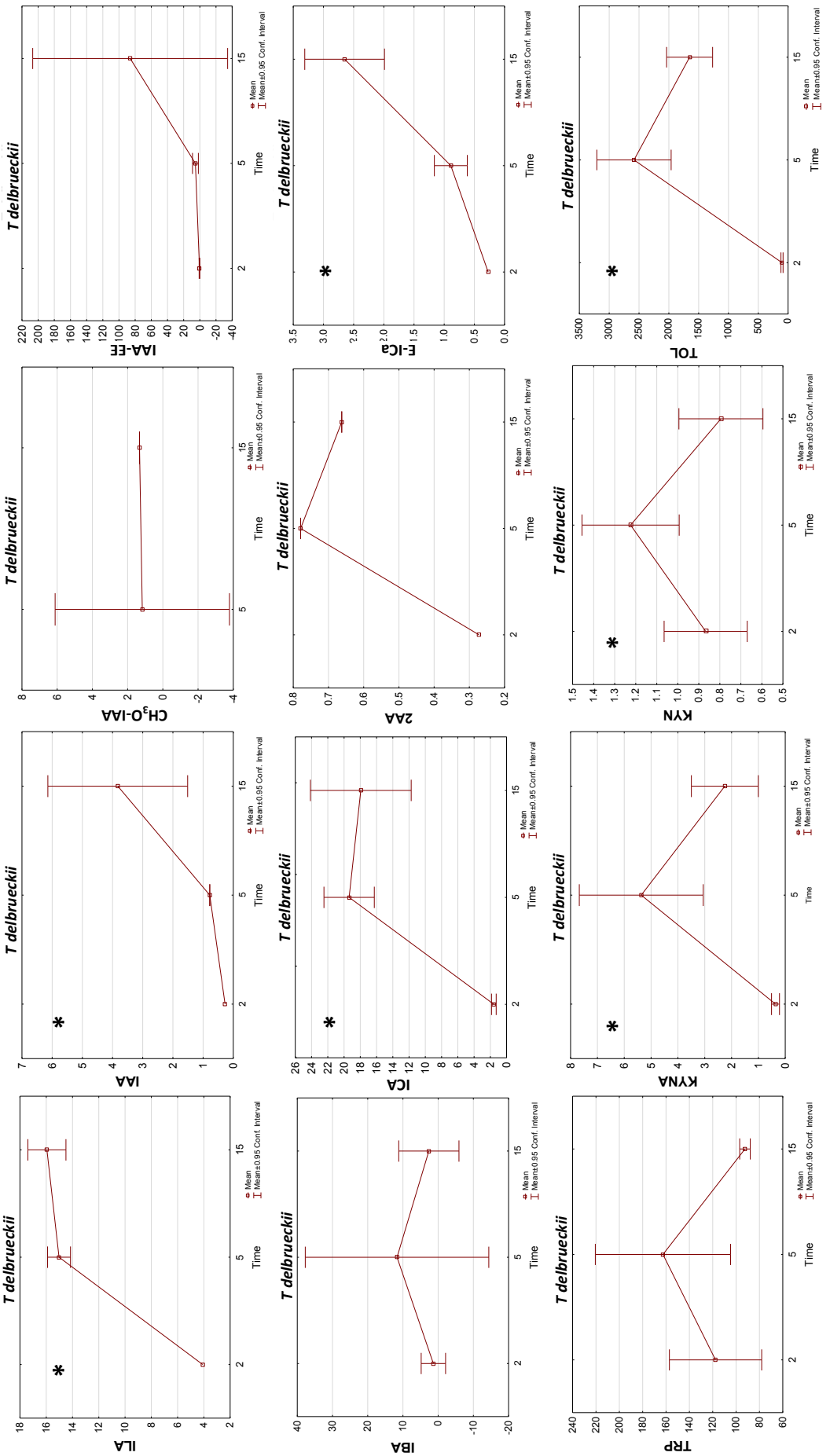

Figure S11. Time curse of compounds in *T delbrueckii* intracellular samples. * Indicates significant differences ($p<0.05$).

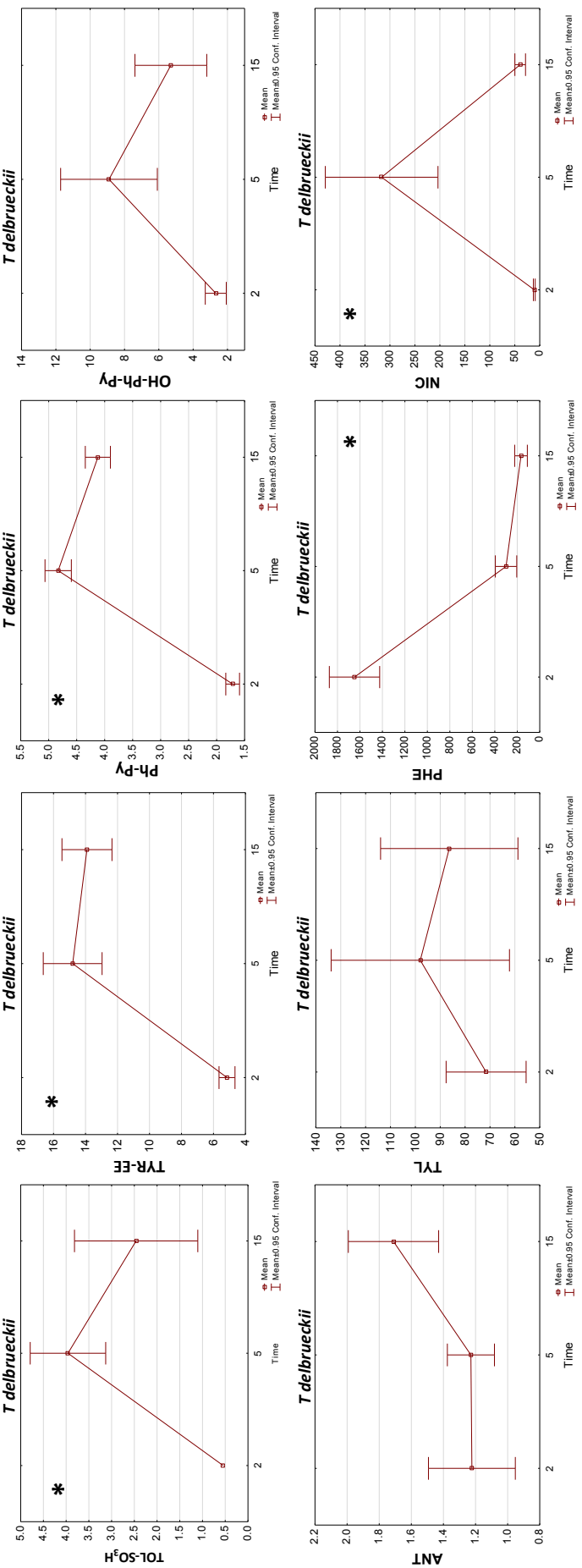

Figure S11. Continued

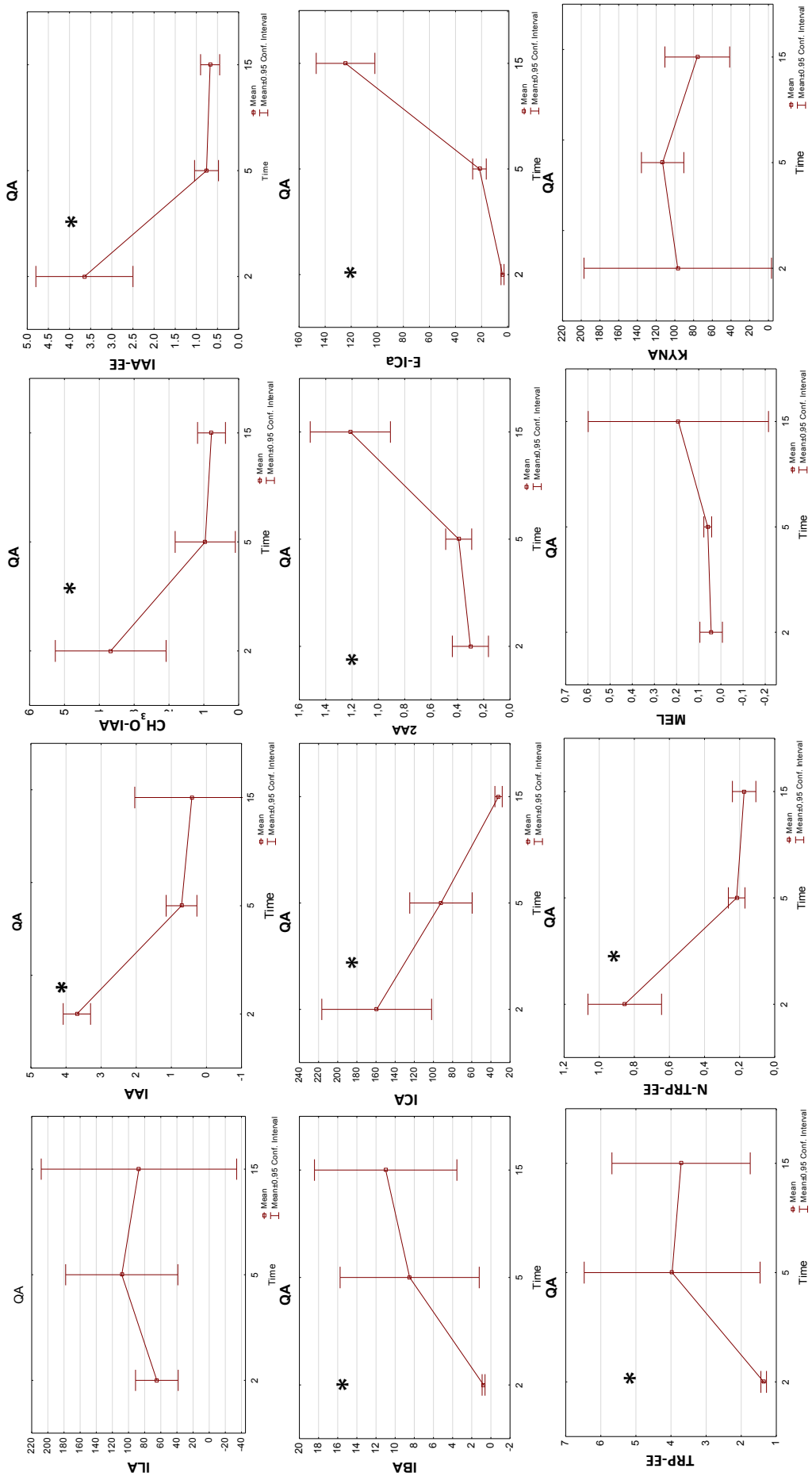

Figure S12. Time curse of compounds in QA extracellular samples. * Indicates significant differences ($p<0.05$).

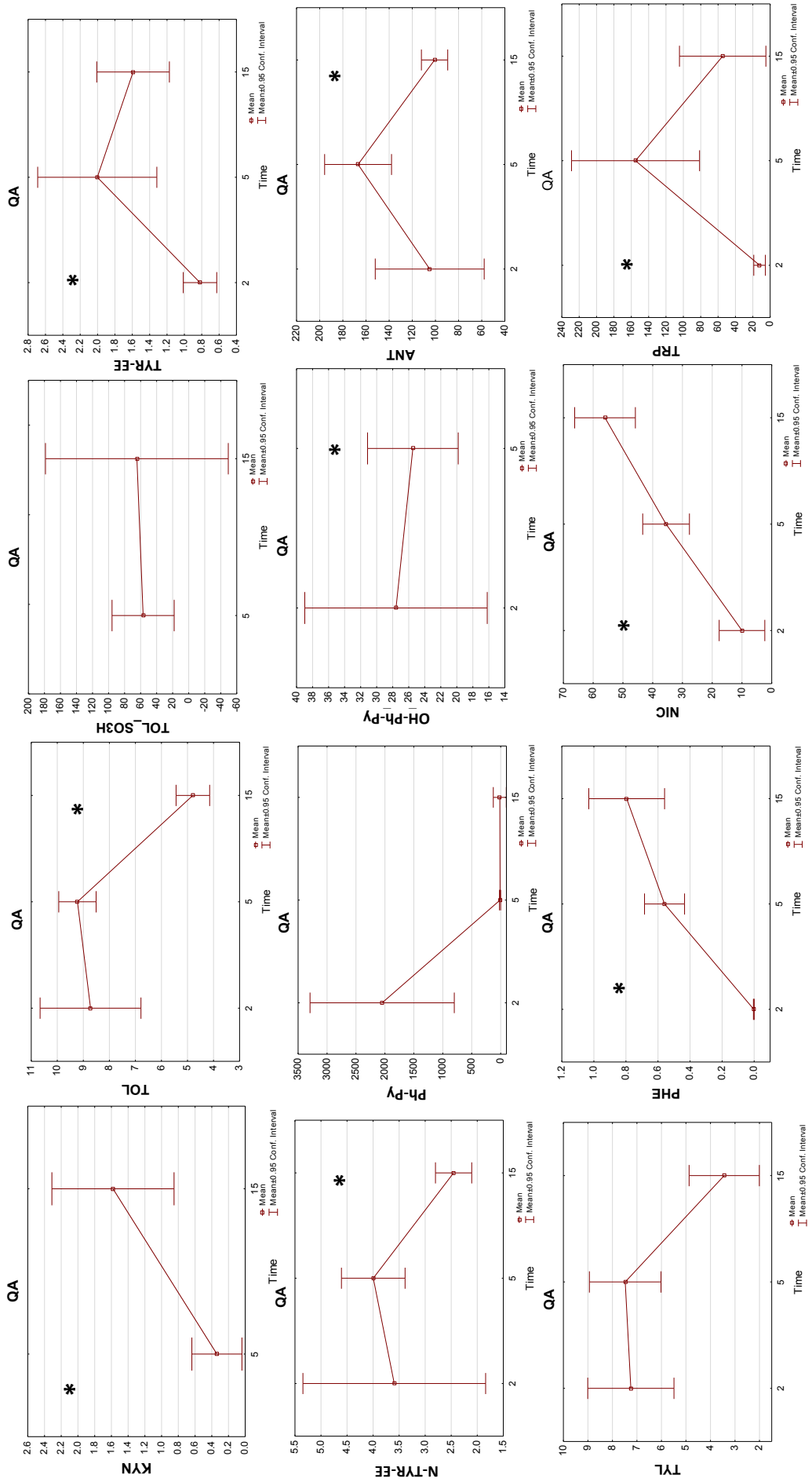

Figure S12. Continued.

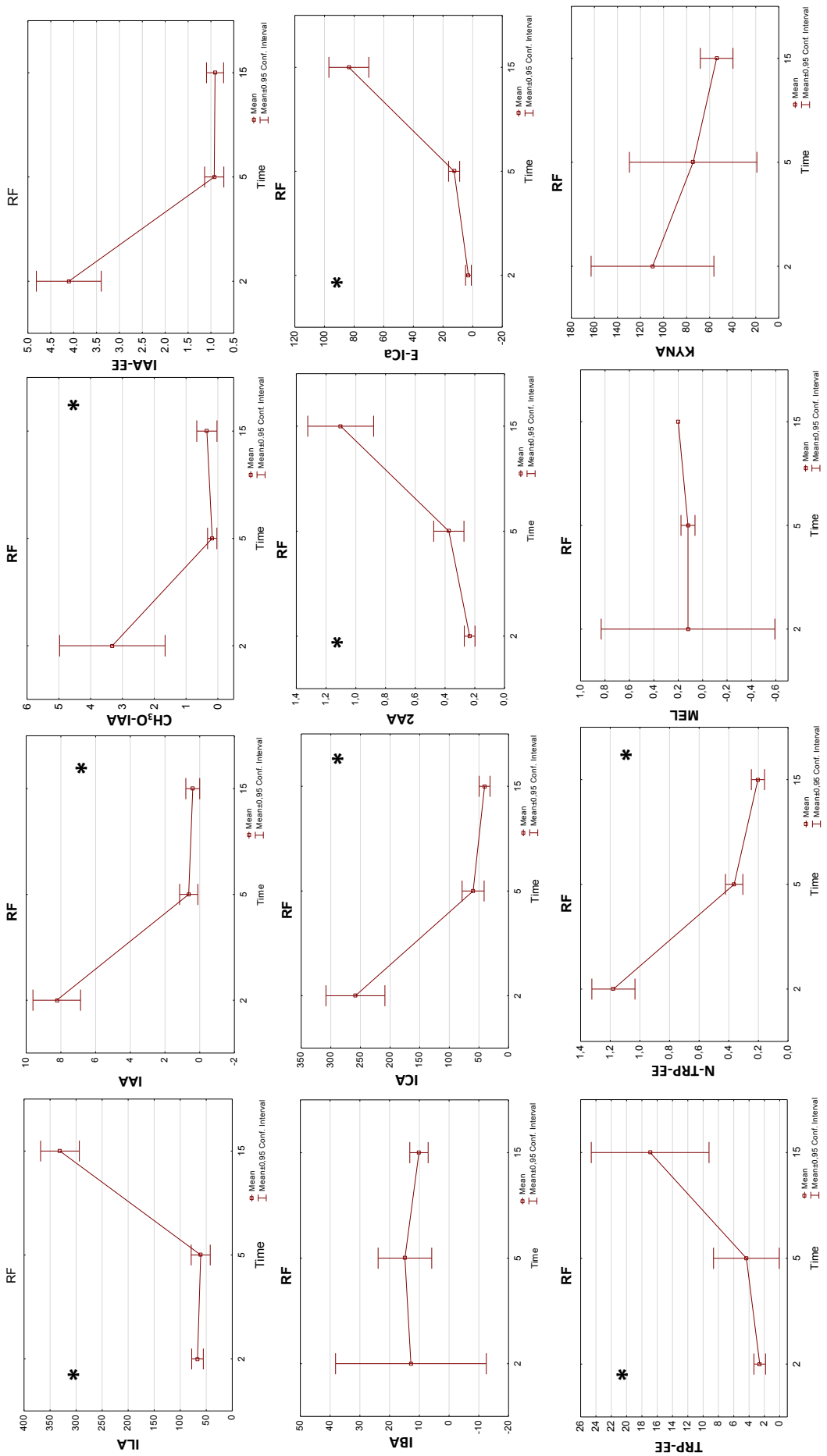

Figure S13. Time curse of compounds in RF extracellular samples. * Indicates significant differences ($p<0.05$).

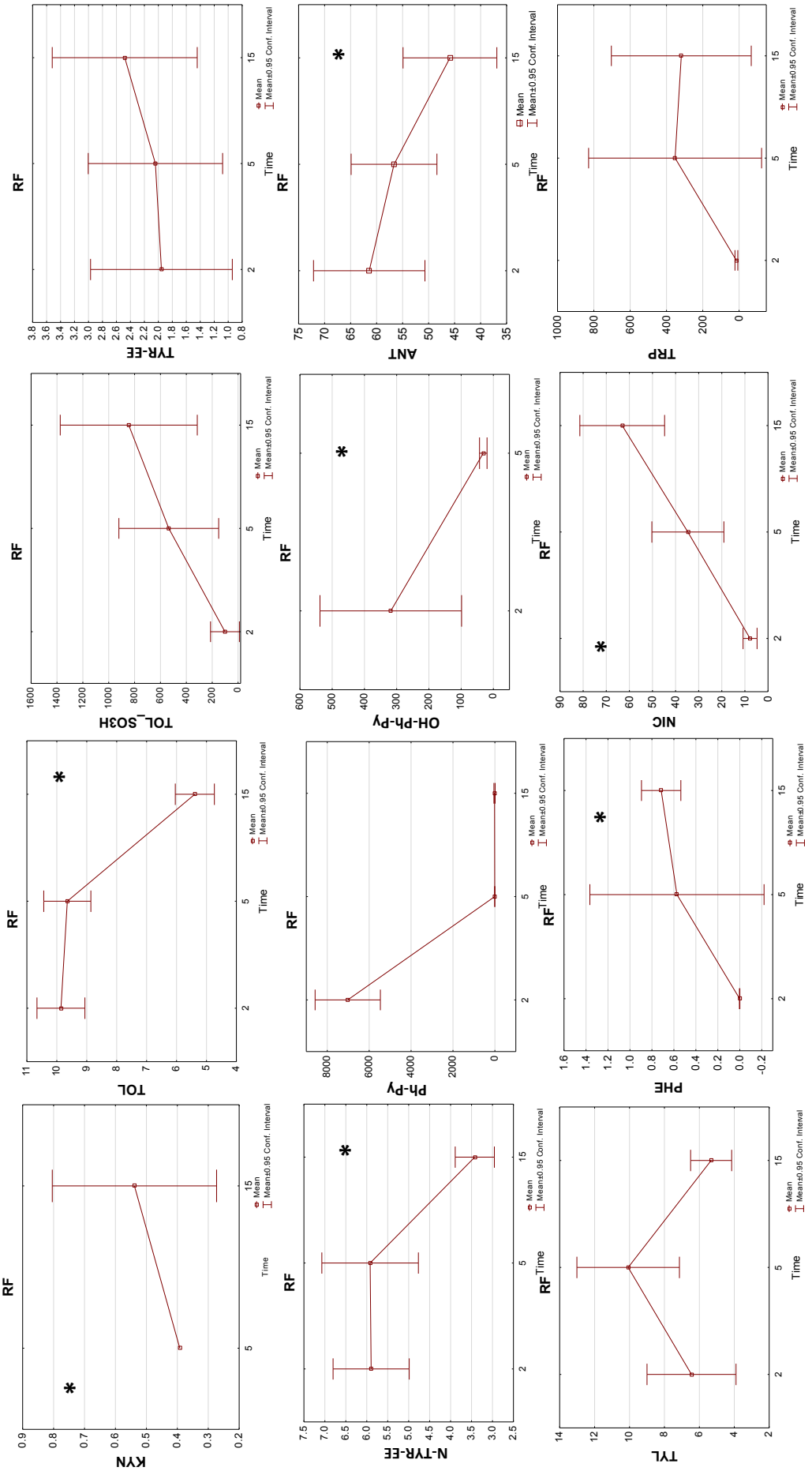

Figure S13. Continued

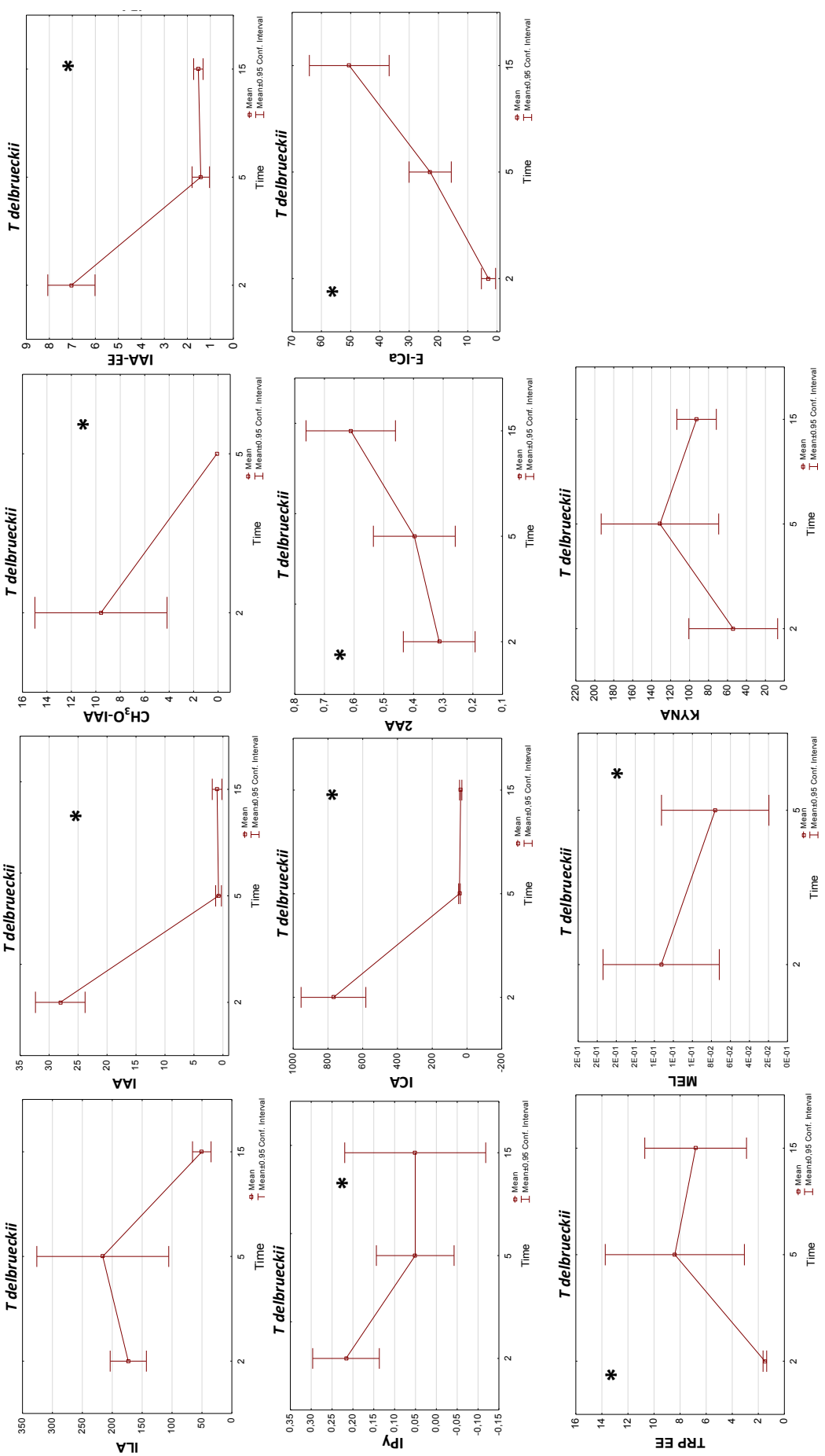

Figure S14. Time curse of compounds in *T delbrueckii* extracellular samples. * Indicates significant differences ($p<0.05$).

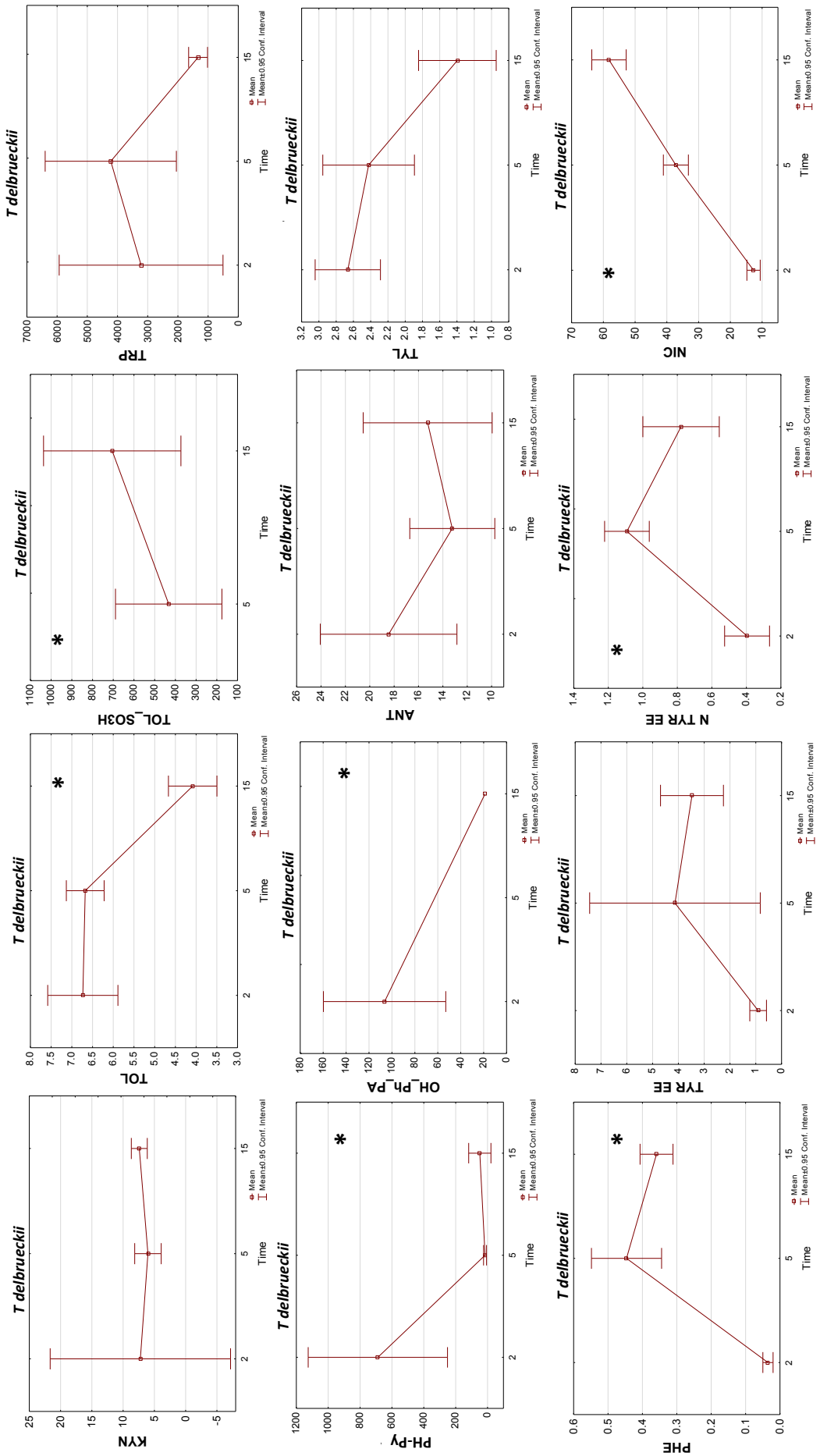

Figure S14. Continued.

PARTE II

VI

Optimisation of an extraction method of melatonin- and tryptophan-related metabolites from yeast's intracellular compartment for analysis by LC-HRMS.

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Abstract

Together with other molecules derived from aromatic amino acid that may have an impact on health or sensory properties. The synthesis of melatonin by yeasts has attracted scientific interest. However, the analysis of melatonin and related compounds represents an analytical challenge due to the very low (ng g^{-1} – pg mL^{-1}) concentrations expected. Thanks to UHPLC/HRMS with regard to separation and identification this challenge has, in part, been successfully solved. It still, however, requires a prior sample treatment procedure in order to avoid interferences.

This present work focuses on the optimisation of a procedure for extracting melatonin from the intracellular compartment of yeast, measuring the effect of temperature on the integrity of Melatonin-, Tryptophan- and Tyrosine-related metabolites. This is followed by the development and validation of two UHPLC/HRMS methods capable of measuring 13 metabolites in both positive and negative modes. The validation provided optimum values of LOD for the 15 metabolites (7.4 10^{-6} to 0.1 µg L^{-1}), LOQ (2 10-^{5} to 0.02 µg L^{-1}) precision (11 to 0.5% RSD) and repeatability (12 to 0.5% RSD). The results proved that low-temperature methods were more effective, providing better precision for 16 metabolites. The high-temperature extraction method may yield enhanced compounds concentration since they could come from cell wall macromolecules degradation. The removal of the interfering molecules enabled matrix effects to be kept at low levels (between -20% and 20%). The proposed methodology could be applied to explore metabolic pathways of melatonin and related compounds in yeast

Keywords: *Saccharomyces cerevisiae*, tryptophan, serotonin, phospholipids, matrix effect, intracellular extraction

1, Introduction

Tryptophan (TRP), is the precursor of such compounds as Melatonin (MLT), Serotonin (5-HT), Kynurenic acid (KYNA), Kynurenine (KYN), all of which have important biological activities and health implications [1,2]. MLT was originally known as indolamine pineal hormone in mammals, while simultaneously being ubiquitous in many organisms of the animal and plant kingdoms [3]. Recently, it has been demonstrated that MLT could be synthesised by yeast during alcoholic fermentation [4]. MLT is a tiny molecule with a molecular weight of 232 Daltons, and a solubility of 0.1 mg/mL in water and 8 mg/mL in ethanol. Solutions of melatonin are light-sensitive and readily oxidisable [5]. Its chemical structure contains an indolic ring and two residues, *O*-methyl and *N*-acetyl that confer an amphiphilic character, enabling it to pass through cell walls and to be transported by biological fluids [6]. As an antioxidant, MLT is an effective protector of lipid membrane structure, proteins, and of DNA against free radical oxidation [7]. The major pathway for mammals to synthesise MLT from TRP occurs in the following order of reactions: *5*-hydroxylation to produce 5-hydroxytryptophan; decarboxylation to produce 5-HT (5-hydroxytryptamine); *N*-acetylation to produce *N*-acetylserotonine; and *O*-methylation to produce MLT [3]. This pathway is shared by yeast [3]. Recently, however, some modifications have been proposed, especially with regard to the two last steps [8,9]. The authors argue that serotonin *O*-methylation is possible in order to produce 5-methoxytryptamine, followed by N-acetylation to produce MLT. In addition to MLT, there are other TRP-related molecules of interest, such as KYN and KYNA which are involved in the kynurenine pathway [10]. This pathway accounts for the catabolism of ~99% of the ingested TRP, which is used for protein synthesis by mammals, but it could also be involved in nicotinamide adenine dinucleotide synthesis. This catabolic route of TRP is also shared by yeast [11,12]. Both KYN and KYNA are connected to indoleamines and TRP by pyrrole ring cleavage reactions, enzymatically catalysed by indole-amine 2, 3-dioxigenase (IDO). These reactions were first demonstrated for tryptamine and 5-HT and later also for MLT [10].

Compounds such as Tryptophol (TOL), Tryptophan ethyl ester (TRP-EE), N-acetyl tryptophan ethyl ester and Indole acetic acid (IAA) are related to TRP yeast metabolism and have been detected in wines in concentrations of 2.2 ng mL^{-1} [13]. TOL, a higher alcohol produced by the Ehrlich pathway which is the most relevant metabolism pathway of TRP in yeast, has a signalling role as its counterpart produced from Tyrosine (TYR), Tyrosol (TYL). Both TOL and TYL are compounds capable of transmitting information concerning population density and the amount of available nitrogen [14]. Another Ehrlich-pathway-related compound is hydroxytyrosol (HT), a product of TYL hydroxylation with important reported health effects [15]. It has recently been demonstrated to be produced by yeast [16].

In order to understand the significance of the abovementioned compounds derived from aromatic amino acids and to unravel the synthetic pathways, it is essential to employ validated methods for their accurate, reliable analysis. Despite the recent advances in their analysis, there still remain some challenges to be overcome, as described below.

MLT has been reported to be an amphiphilic molecule present in low concentrations in many of the food samples in which it has been studied. In fruits and plants, for instance, it was quantified at concentrations of ng g^{-1}; in beverages in amounts of µg g^{-1} and pg mL^{-1} [17] and in biological samples at concentrations of ng per 8-hour periods in human urine [18]. As a result, techniques such as ELISA and HRMS (High Resolution Mass Spectrometry) have been used to analyse such low concentrations [13,19]. A sample treatment involving a concentration step is, however, generally required.

In order to enhance analytical performance, several extraction methods have been reported in the literature, such as extractions with 10% sodium carbonate in bananas [20] or extractions with methanol and C_{18} cartridges used in many fruit substrates [21] and in wine [13,22]. Moreover, immune affinity purification was used in biological samples. This system consists of sorbent-bound specific antibodies for MLT extraction with optimal results, but only for this compound [23]. The success of the intracellular extraction method depends directly on the microorganism's cell envelope structure and on the chemical nature of the target metabolites.

Many intracellular extraction methods have been proposed. The most popular, boiling ethanol, has been in use for many years. This method is useful for extracting water-soluble intracellular metabolites such as ɤ-aminobutyric acid, aspartic acid, glycine and lactic acid. However, it has a poor recovery yield [24] for metabolites such as phosphorylated metabolites, nucleotides and tricarboxylic acids. The most widely-used intracellular extraction methods were compared for detecting various yeast metabolites such as amino acids, sugars, sugar phosphates, sugar alcohols and peptides [25]. Following a comparative test of four different methods (boiling ethanol, freeze–thaw cycles, pure methanol and pure methanol coupled to sonication) with metabolomic analysis, Duportet concluded that one of principal factors influencing intracellular extraction was the differential composition of metabolites and that it was not possible to eliminate the influence of the biological matrix [26]. Therefore, it would be worth trying a method combining freezing/thawing cycles with ultrasound, since the mechanical methods could have a great potential for enhancing intracellular metabolites extraction, particularly non-polar compounds [27].

The purpose of this work is to optimise the procedure for the intracellular extraction of MLT/ TRP- and TYR-related metabolites from yeast. The selected methods were the classic boiling ethanol method and two methods involving freeze-thaw cycles associated with ultrasound at two different temperatures of centrifugation. The determinations were performed using two validated UHPLC-MS methods capable of quantifying 13 compounds in both positive and negative modes.

The results of this work could be useful to researchers in the field of microbial metabolomics, as they assess these metabolites more accurately in order to explore and improve knowledge of the metabolomics pathway and their role.

2. Materials and methods

2.1. Reagents and materials

125 The chemicals used in this work were of the highest grade of purity and purchased from
126 Sigma- Aldrich (Madrid, Spain), Chengdu Biopurify Phytochemicals Ltd. (Wenjiang
127 Zone, Chengdu-Sichuan, China), Merck (Darmstadt, Germany) and VWR International
128 Eurolab S.L. (Barcelona – Spain), unless otherwise stated. (Supplementary Material
129 Table S1)

130 **2.2. Alcoholic fermentation procedure**

131 The alcoholic fermentations were performed in synthetic must (SM), prepared as described by
132 Riou et al. (1997), with slight modifications (Supplementary Table S2 shows the must
133 composition in detail). Fructose and glucose were added as carbon source (100 g L^{-1} each), the
134 other compounds were unmodified: amino acids (purity ≥ 99 %), vitamins and anaerobic factors
135 [28]. The SM was sterilised with bottle top vacuum filters (Nalgene PES membrane) after
136 adjusting the pH to 3.5 with NaOH. Each Erlenmeyer flask was filled with 750 mL of SM, The
137 inoculation was performed with 10^6cell mL^{-1} and the Erlenmeyer flasks were then capped with
138 tops equipped with a capillary for releasing carbon dioxide. The fermentations were due to the
139 action of *Saccharomyces* QA23 strains of yeast in six replicates in order to consider biological
140 variability. The flasks were weighed before and after the samples were taken in order to monitor
141 the fermentation.

142 **2.3. Intracellular extraction methods.**

143 **2.3.1. Cold glycerol quenching procedure**

144 On day two of fermentation, a sample volume containing 10^9 cells (previously counted with an
145 automated Invitrogen Countess cell counter) was taken from each Erlenmeyer flask. The
146 samples were immediately centrifuged at 4500 rpm for 3 min at 4º C to separate the cells from
147 extracellular media. Cells were pelleted twice by centrifugation 4500 rpm for 3 min at 4ºC with
148 distilled water to wash them in order to prevent contaminations from the extracellular
149 metabolites.

150 The washed cells were subjected to a cold glycerol saline quenching procedure in order to stop
151 enzymatic activity, maintaining the *in vivo* metabolite concentrations at a constant level. The
152 procedure used was that reported by Villas-Bôas et al. [29].

153 The pellet was re-suspended with 1 mL of distilled water and transferred into a pre-cooled
154 centrifuge tube containing 4 mL of cold-quenching solution [3:2 (vol/vol) glycerol:saline
155 solution] maintained at -23 ºC in a refrigerated bath using ethylene glycol as cryo fluid. The
156 solution was homogenised and returned to the cold bath for 5 min. The treated samples were
157 centrifuged at 36,086 *g* for 20 min at -20ºC (Sorvall LYNK 6000, Thermo Fisher scientific,
158 Waltham, MA USA). The supernatant was removed and the pellet was then re-treated with 2.5
159 mL of cold washing solution [1:1 (vol/vol) glycerol/saline solution] maintained at -20ºC. They
160 were then centrifuged under the same conditions. The supernatant was discarded and the pellet
161 was stored at -80 ºC until extraction.

162 **2.3.2. Boiling ethanol extraction (H)**

163 The boiling ethanol extraction procedure was performed following the method reported by
164 Gonzalez et al [30]. Three mL of N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
165 (HEPES) ethanol boiling solution (absolute ethanol buffered with 70 mM HEPES) and a
166 volume of methanol internal standard (3-nitrotyrosine) solution to obtain a final concentration
167 of 0.08 μM were added to the cell pellet, and incubated at 80ºC for 3 min. After cooling in an
168 ice bath for 3 min, the solution was evaporated until dry at 34ºC, and 2000 rpm with a vacuum
169 concentrator (Hyper VAC-LITE, GYOZEN, Korea). The residue was re-suspended to a final
170 volume of 3 mL with ultrapure Mille Q water and was then centrifuged for 10 min at 15,543 *g*
171 and 4ºC. The supernatant was collected and stored at -80ºC, until clean-up.

172 **2.3.4. Freezing temperature extraction (LT)**

173 Intracellular extraction was performed following the method reported by Smart et al. (2010)
174 with some modifications [31]. 2.5 mL of cold methanol-water solution (50% (v/v), -30ºC) was
175 added to the cell pellet, together with a volume of IS methanolic solution to obtain a final

concentration of 0.08 μM. Afterwards, the sample was mixed for 1 min with vortex and then frozen at -80 ºC. The samples were subjected to two freeze-thaw cycles (frozen at -80ºC for 30 min and then thawed in an ice bath for 4 min). After the last cycle they were subjected to sonication for 1 min in an ice bath using an ultrasonic liquid processor (Sonicator Sonoplus HD 2070, Bandelin electronic GmbH & Co. KG, Berlin, Germany). The samples were then centrifuged at 36,086 *g* for 20 min at -20ºC. Another aliquot of 2.5 mL of cold methanol-water was added to the pellet and the same process was repeated from the freeze-thaw cycles; the supernatants were collected, pooled and stored at -80 ºC until clean-up. The aim of this kind of extraction process is to render the cell walls more permeable to organic solvents, and thus enable intracellular metabolites releasing while preserving their chemical integrity [31].

2.3.5. Refrigeration temperature extraction (MT)

This method follows the same procedure as the above, but changing the centrifugation temperature. After two cycles of freezing-thaw and one min of ultrasound, the samples were centrifuged at 36,086 *g* for 20 min at 4ºC. Subsequently, the procedure was performed in the same way, and finally the extracts were stored at -80ºC until clean-up.

2.3.6. Clean-up and concentration procedure

After every treatment described above, the samples were cleaned up using 1 mL zirconia-coated Phree™ cartridge in order to avoid the presence of phospholipids and proteins due to the fragmentation of cell walls [32,33]. The procedure was performed in four steps following the manufacturer's protocol. When the filtration process had finished, the samples were speed vac evaporated to dryness at 34˚C and 2000 rpm to be later re-suspended with a mobile phase in order to obtain extracts that have been thrice concentrated.

2.4. UHPLC/HRMS analysis

The analysis was performed in a UHPLC Dionex Ultimate 3000 system (Thermo Fisher Scientific-Bremen, Germany) consisting of a binary pump, cooling autosampler, online vacuum degasser, and column oven, coupled to a hybrid quadrupole-Orbitrap Q Exactive mass

spectrometer (Thermo Fisher Scientific-Bremen, Germany). The column used for the analysis was Zorbax RRHDSB-C18 (2.1 × 5mm, 1.8 μm particle size) with a guard column (2.1 ×, 1.8 μm particle size), both purchased from Agilent Technologies (Waldbronm, Germany). The separation was undertaken at a column temperature of 40ºC, a flow 0.5 mL min^{-1}, and an injection volume of 5 μL. The chromatographic conditions for positive analysis consisted of two phases (A) aqueous formic acid solution 0.1 %, and (B) methanolic formic acid solution 0.1%, the gradient was programed as follows: 95% A (0 -1min), 0% A (1- 12 min), 95% A (12.1 – 15 min). Electrospray positive ionisation mode was applied with the parameters in the source set as follows: capillary voltage at 0.1 V; sheath gas flow rate 60.13; aux gas flow rate 24.85 and sweep gas flow rate 0.07, all in arbitrary units. To quantify in positive, a full scan was used, with the most important transition as the confirming ion. To quantify compounds as 5-HT, the most important product ion was used, due to the fact that that kind of compound suffers ionisation at source and the pseudomolecular ion is impossible to detect. For analysis a window of 30 sec and lens of 50 was used. Table 1 shows MS parameters, retention time and standard deviation values (% RSD) as a measure of the intra- and inter-day variation of each metabolite measured, based on quality control samples.

In the negative analysis phase (A) was aqueous acetic solution 0.2 % and (B) acetonitrile, while the chromatographic gradient was set as follows: 95% (A) (0 – 1 min), 0% (A) (1 – 8.5 min), 95% (A) (8.6 – 10 min). The electrospray negative ionisation mode was performed with the parameters in the source set as follows: capillary voltage at 0.1 V; sheath gas flow rate 60.15; aux gas flow rate 25.03 and sweep gas flow rate 0.04 all in arbitrary unit. The two most important transitions were selected for each metabolite, a quantifier (the most abundant) and another as a qualifier ion in order to perform data-dependent scanning as the quantifying method. All samples were analysed in duplicate and in randomised sequences. Table 1 shows the MS parameters.

2.5. Statistical analysis

228 Statistical analyses were performed using MetaboAnalyst [34] and Statistica software [35].
229 Statistical significance ($p < 0.05$) between groups was tested by ANOVA analysis of variance
230 and Tukey's HSD (honest significant difference) test (Figure S1). The heat-map was produced
231 as a multivariate statistical analysis where the data were auto-scaled and missing values were
232 replaced by very low values.

233 **3. Results and Discussion**

234 **3.1. Validation method and Matrix effects**

235 Figure 1 displays possible pathways involving aromatic amino acid derivatives described in
236 yeast (KEGG: Encyclopaedia of Genes and Genomes [36,37]). One of the pathways of TRP
237 metabolism is involved in the synthesis of 5-HT and is related to the production of metabolites
238 such as: 5HTP, 5HIAA, MLT and 5MIAA. 5MIAA is the principal degradation product of MLT
239 metabolism and 5-HT [9]. The most relevant TRP metabolism route in humans is the
240 kynurenine pathway [38], which has also been described in yeast. In yeast only 2% of TRP
241 follows this metabolism route [39]; this pathway transforms TRP into two compounds: KYN
242 and KYNA which are included in the targeted method.

243 Furthermore, TOL and TYL, the principal products of the Ehrlich pathway from TRP and TYR,
244 respectively, have also been included in this study. In yeast, the Ehrlich pathway is the most
245 important metabolism route producing higher alcohols such as TOL and TYL from aromatic
246 amino acids (TRP and TYR), together with IAA. Finally HT was selected since, as recent
247 research has demonstrated [16], it is a TYL derivative and is produced by *Saccharomyces*
248 *cerevisiae.*

249 It is important to emphasise that validated analytical methods able to determine very low
250 concentrations would help to elucidate those metabolic pathways involved in the production of
251 bioactive compounds derived from aromatic amino acids. With the development and validation
252 of these methods, we try to address this issue. Therefore, the validation procedure was
253 performed following the AOAC guidelines [40]. The parameters measured were as follows:

254 detection and quantification limits (LOD, LOQ), precision, linearity, recovery, matrix effect and
255 the efficiency of the extraction procedure through the internal standard (3-nitrotyrosine). Table
256 1 shows the LOD and LOQ calculated for 15 compounds and the internal standard based on the
257 standard deviation of the response (σ) and the slope of the calibration curve. The linearity data
258 are shown in Table S1 (supplementary material). The precision was measured using QC (quality
259 control) prepared as a pool of all samples. In order to measure instrumentational stability and
260 accuracy, QC samples were injected every four injections in duplicate, in positive and negative
261 mode. The measure of precision was expressed as intraday and interday (% RSD) data (Table 1)
262 [41]. In addition, all values were highly satisfactory and ranged from 0.48 to 12.49 and matched
263 the requirements of AOAC with regard to their concentration levels [42].

264 Due to the fact that the extraction process entailed the breaking of cell walls, it is not surprising
265 that the resulting extract contains phospholipids (PLs) and proteins, the main compounds of the
266 cell walls. As they are known to exert significant interference in the analysis by reverse phase
267 chromatographic methods coupled with mass spectrometry, it is advisable to remove them.
268 Therefore, removing PLs and proteins prior to analysis could be the most effective way of
269 reducing the matrix effects from the endogenous cellular extracts [33]. By these means, a
270 sample clean-up with Phree^{TM} filtration was introduced [43]. By coating silica with zirconia,
271 this filtration has the advantage of high sorbent selectivity. First, the organic solvent precipitates
272 the proteins and then the PLs are retained through interactions between the phosphate moiety of
273 phospholipids (Lewis base) and zirconium oxide (Lewis acid) coated on the silica surface.
274 Purifying phospholipids with proteins precipitation as a clean-up method, instead of SPE (solid
275 phase extraction), avoids a loss of metabolites traditionally associated with the use of
276 conventional SPE [43].

277 The matrix effect was examined by comparing the MS/MS response (peak areas) of an analyte
278 at two concentrations spiked post-extraction into a sample extract (PES), to the MS/MS
279 response of the same analyte at the same two concentrations in the neat mobile phase solution
280 (MFS). Following the sample preparation procedure, the samples (PES and MFS) were

concentrated three times and re-suspended with methanol 0.1% formic acid (FA) for positive analysis. For the negative analysis, they were re-suspended with acetonitrile with 5% acetic acid. A previous clean-up sample was used as a blank. The matrix effect was calculated using the following equation:

$$\% \text{ Matrix Effect (\%ME)} = ((\text{PES} / \text{MFS}) - 1) \times 100$$

where MFS is the Matrix Free Sample response and PES is the Post-Extracted spiked Sample response.

This value is also known as absolute ME [19]. A suppression or enhancement is considered acceptable if the matrix effect range is from -20% to 20%. Percentage values of ME higher than 20% or lower than -20% indicate a strong matrix effect [44]. Table 2 shows the recovery and matrix effect values for every analyte in the method.

3.2. Comparison of Extraction Efficiency.

As was described in materials and methods, all the extraction procedures consist of several steps before the sample was injected, which may involve substantial metabolite losses. In order to assess this technical variability, a known concentration of IS (3-nitrotyrosine) was added at the beginning of the sample preparation process. 3-nitrotyrosine was selected as IS due to its structural similarities, its stability characteristics in the UHPLC-MS method, its low cost and the fact that before it is added, it is not present in the samples. Indeed, the recovery values RE (%) obtained after the intracellular extraction process with the three methods were 46% (±2.08) in boiling ethanol extraction, 53% (±1.64) in MT and 45% (±1.02) in LT extraction. Figure 1 shows the variability plot of 3-nitrotyrosine comparing the three methods. The data were analysed by ANOVA/Tukey's HSD test and no significant differences were detected between the three methods. Moreover, taking into account that the role of the IS mimics the compounds analysed as closely as possible, and the fact that the procedure could affect all the analytes in the same proportion, correction with IS enables this technical variation to be corrected. As a result, the analyte concentrations in samples have to be normalised due to the abundance of IS [31].

The extraction methods' efficiency was defined as a method's ability to release analytes from the cells [45]. In certain situations as, for example, is the case of when the substrate is converted into intermediates or macromolecules which are subsequently broken down as a consequence of the extraction procedure to produce analytes, the efficiency could be wrongly assessed. This misleading situation can be counteracted by the efficiency measurement, calculated against the median of each analyte's concentrations. The efficiency factor was defined as:

$$\text{efficiency vs median} = x/\text{median}$$

These results are comparative efficiency values that depend on the extraction method used [45]. Figure 2 shows the extraction plots for the normalised efficiencies of 15 compounds included in the work. The efficiency profiles for all extraction methods are very similar, with values near 1 for most metabolites. The H extraction profile shows a clear discrimination between two amino acids [L-TRP ((>2 standard deviation) and TYR (>1 standard deviation)] compared with all remaining metabolites. It is possible that the temperature of the boiling ethanol may break down the proteins, releasing amino acids and increasing the measured concentrations, thus yielding incorrect efficiency values. On the other hand, the two phenolic compounds TYL (>0.5 standard deviation) and HT (> 1 standard deviation) presented higher efficiency than the other compounds and other methods, but lower efficiency than the two aromatic amino acids.

As can be observed, the two low-temperature extraction methods, LT and MT presented a similar profile. The profile of LT extractions is noticeably the flatter (Figure 2) indicating excellent reproducibility. With the advantage of preserving the integrity of the metabolites, this avoids false positive results and provides an exact vision of the intracellular content.

Figure 3 shows a heat map that provides an overview of the data set with hierarchical clustering. The overall results of the quantification of each metabolite are represented for every extraction and the six replications are expressed in μg L^{-1}. The first cluster enclosed TRP, TYR, HT and TYL, all having higher concentrations in H extraction. The concentrations values measured in each method (H, MT and LT) were as follows: 387 – 45 - 51 μg L^{-1} for TRP; 966 – 170 – 198

μg L^{-1} for TYR; 24 – 3 – 9 μg L^{-1} for HT and finally 144 – 72 – 70 μg L^{-1} for TYL. As can be observed, the differences between the H and LT methods are outstanding. Indeed, in the case of TRP the factor is as high as eight. It seems plausible to suggest that the high levels come from the decomposition of macromolecules such as proteins, as stated earlier with regard to the high extraction efficiency. This observation matched similar conclusions reported earlier by Canelas et al. [45]. These authors suggest that the hydrolysis of as little as 0.2% of the cell protein would be sufficient to explain the highest percentages of amino acids in extractions using hot water. It is conceivable that the high TYL and HT concentrations might have a similar explanation, reflecting that they would be the product of macromolecules degradation. However, the exact biomass components generated by thermal hydrolysis remain to be elucidated.

A second cluster contained KYN, KYNA and TOL. These presented higher concentrations (163, 3 and 164 μg L^{-1}) in H extraction for the three compounds, respectively, but with a remarkable variability (101, 1 and 75 deviation respectively). With the two low-temperature methods, KYN presented the same concentration and low deviation (0.59 for MT and 0.55 for LT). For KYNA 114 μg L^{-1} ± 37 with the MT extraction and 127 μg L^{-1} ± 36 with LT, and for TOL 140 μg L^{-1} ± 35 with the MT and 140 μg L^{-1} ± 17 with LT extraction.

The third cluster, comprising TEE, MLT and NTEE, had very similar results in the three extractions. The concentrations were 3μg L^{-1} on average for TEE, the same for the three methods, for MLT (27, 28 and 30 μg L^{-1}) and in case of NTEE 9 μg L^{-1} with H extraction and 10 μg L^{-1} with two low-temperature extraction methods. The values were closer to each other, but the variability was better with low-temperature than with H extraction especially in MLT (8, 5 and 4 for each method respectively).

Finally, the last two clusters contained 5HTP, 5-HT, 5HIAA, 5MIAA and IAA, all of which had the highest concentrations in MT extractions (101, 3, 22, 358 and 14 μg L^{-1}, respectively) with better reproducibility (47, 1, 2, 40 and 3 deviation, respectively), whereas with H the concentrations obtained were 83, 3, 14, 243 and 8 μg L^{-1} with large deviations 55, 2, 1.5, 95 and 4, respectively, with the sole exception of 5-HT which had the same concentration in all cases,

but better variability in MT extraction. The compounds included in these two clusters have indolic structures in common and exhibited lower concentrations in H than in MT extraction. This might be due to the high temperatures that may produce degradation, as reported earlier for IAA [46]. The low deviations with the two low-temperature methods indicate that, in contrast with Canelas et al. [38], enzymatic activity stopped, probably due to the fact that cold glycerol quenching was more efficient than the pure methanol quenching at -40ºC method that they used [47].

It is interesting to highlight that the concentrations of MLT obtained with low -temperature methods ranging from 28 to 30 μg L^{-1} are significantly higher than other previously-reported values in extracellular media – even with an identical yeast strain [13,48]. Moreover, the detection and quantification of MLT and 5-HT in the intracellular media is an advance in our knowledge of the metabolism of *S. cerevisiae.* Indeed, the changes introduced in the sample preparation and in the analytical method could help the research community, since there are few references related to producing these two compounds by yeast [49].

4. Conclusions

This study demonstrates how the temperature of the extraction methods could affect the extractive efficiency of 15 melatonin/tryptophan- and tyrosine-related metabolites through the development of two validated and sensitive UHPLC/MS analytical methods.

The proposed sample preparation scheme is based on removing phospholipids and proteins and enabling obtain good values for matrix effects to be obtained, indicating that the possible impact of ion enhancement or ion suppression were small and unable to produce distortive behaviour on the quantification process.

The levels of melatonin measured with low-temperature extractions were higher than others previously reported in extracellular media, showing that low-temperature intracellular extraction methods are more suitable for studying melatonin and its related compounds.

385 It is obvious that extraction efficiency depends on the metabolites' characteristics, yet for the
386 metabolites analysed in this work, extractions at low temperatures had a better efficiency and
387 more satisfactory repeatability values than extraction methods using boiling ethanol. The low
388 deviations indicate that, contrary to the results obtained by other authors, the enzymatic activity
389 was stopped. Higher temperatures raise the risk of overestimating some metabolites – possibly
390 due to macromolecules hydrolysis. Due to the small differences between both low-temperature
391 extractions methods, MT extraction can be used without incurring a greater risk of losses in
392 comparison with LT and, furthermore, the extraction process of these bioactive compounds has
393 the advantage of using more affordable equipment.

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Table 1 UHPLC–HRMS conditions for quantifying and identifying compounds and internal standards (IS).

Compound (acronyms)	RT (min)	ESI mode	Quantifier	Confirming ion	LOQ (µg L^{-1})	LOD (µg L^{-1})	Intra day (%RSD)	Inter day (%RSD)
tyrosine (TYR)	0.84	+	182.0811	182.0811→165.0546	0.05	0.02	0.92	0.62
serotonine (5-HT)	0.87	+	160.0756	177.1022→160.0756	0.02	0.08	5.08	0.84
5-hydroxy tryptophan (5HTP)	1.26	+	221.0920	221.0920→204.0655	0.03	0.01	5.03	9.60
kynurenine (KYN)	1.50	+	209.0921	209.0920→192.0655	0.04	0.01	1.60	2.04
hydroxytyrosol (HT)	2.03	−	153.0629→123.0440	153.0629→109.2840	0.10	0.03	0.86	2.25
3-nitrotyrosine (IS)	2.42	+	227.0662	227.0662→181.0604	0.02	0.01	0.89	0.86
tyrosol (TYL)	2.85	−	137.0680→119.0502	137.0680→108.0217	0.39	0.13	1.55	1.58
tryptophan (TRP)	3.00	+	205.0971	205.0971→188.0706	0.12	0.04	1.58	1.34
5-hydroxy indole acetic acid (5HIAA)	3.83	+	192.0655	192.0655→146.0600	0.40	0.13	1.15	1.50
tryptophan ethyl ester (TEE)	4.84	+	233.1284	233.1284→216.1019	0.16	0.05	2.56	0.97
kynurenic acid (KYNA)	4.91	+	190.0498	190.0498→162.0549	2.20E-05	7.40E-06	12.49	11.56
5-methoxy indole acetic acid (5MIAA)	5.70	+	206.0811	206.0811→160.0754	0.07	0.02	0.48	0.94
3-indole acetic acid (IAA)	5.76	+	176.0706	176.0706→130.0651	0.08	0.02	3.87	9.40
tryptophol (TOL)	5.76	+	162.0913	162.0913→144.0807	0.10	0.03	0.55	0.92
melatonin (MLT)	5.79	+	233.1284	233.1284→174.0913	0.11	0.04	1.13	0.50
N-acetyl tryptophan ethyl ester (N-TEE)	6.91	+	275.1390	275.1390→201.1024	0.08	0.03	1.88	1.90

Table 2 Table 2 Matrix effects parameters. ME (%) entity means that ME (%)>100 = there is a signal enhancement and if ME (%) < 100 = a signal suppression [18].

Metabolite (acronims)	conc ($\mu g\ L^{-1}$)	Recovery (%)	Matrix effect
5-methoxy indole acetic acid (5MIAA)	247	89.22	-10.77
5-hydroxy indole acetic acid (5HIAA)	247	126.95	24.88
	4	104.62	6.99
5-hydroxy tryptophan (5HTP)	247	100.77	1.11
	4	132.81	15.56
3-indole acetic acid (IAA)	247	92.42	11.87
	1	120.64	15.46
kynurenic acid (KYNA)	247	110.06	-1.75
	4	89.69	-10.3
kynurenine (KYN)	247	100.08	0.67
	4	92.78	-7.21
tryptophan (TRP)	247	99.41	-0.59
	4	136.38	36.38
tryptophan ethyl ester (TEE)	247	93.91	-6.08
	4	92.90	-7.1
tyrosine (TYR)	247	166.88	-7.23
	1	61.25	-22.14
melatonin (MLT)	247	89.32	-10.68
	4	117.86	17.86
N-acetyl tryptophan ethyl ester (N-TEE)	247	82.69	-15.08
	1	82.65	-19.07
3-nitrotyrosine (IS)	247	95.40	-4.59
serotonine (5-HT)	247	97.34	-0.75
	1	64.26	-21.03
tryptophol (TOL)	247	101.21	1.54
hydroxytryptophol (HT)	60	129.84	-3.61
	0.5	106.93	9.49
tyrosol (TYL)	200	105.62	16.03
	0.5	120.32	-0.22

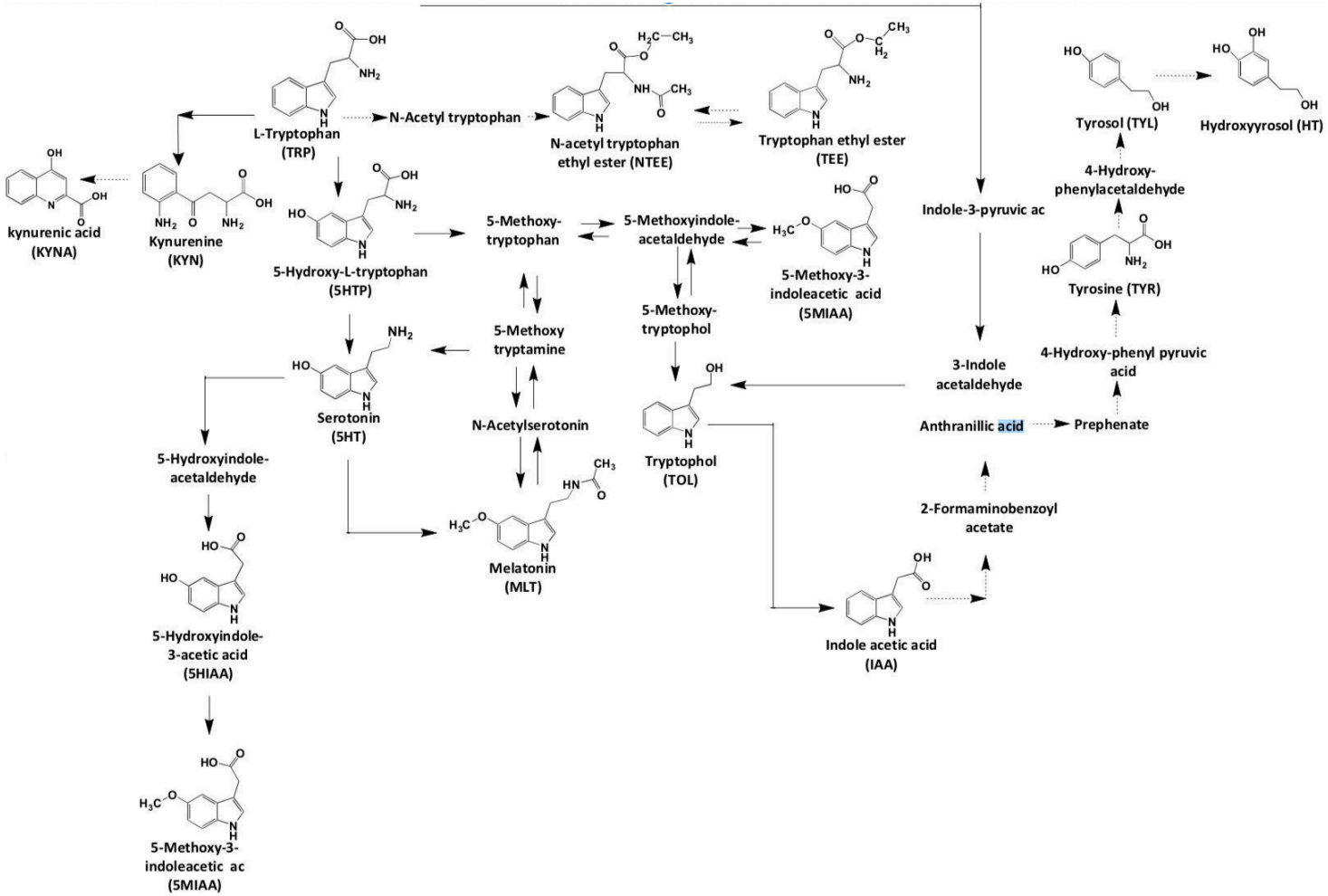


Figure 1. Compounds in the analytical method and their relationship to MEL/L-TRP and TYR metabolism. Compounds included in the quantitate method appear with structural formula.

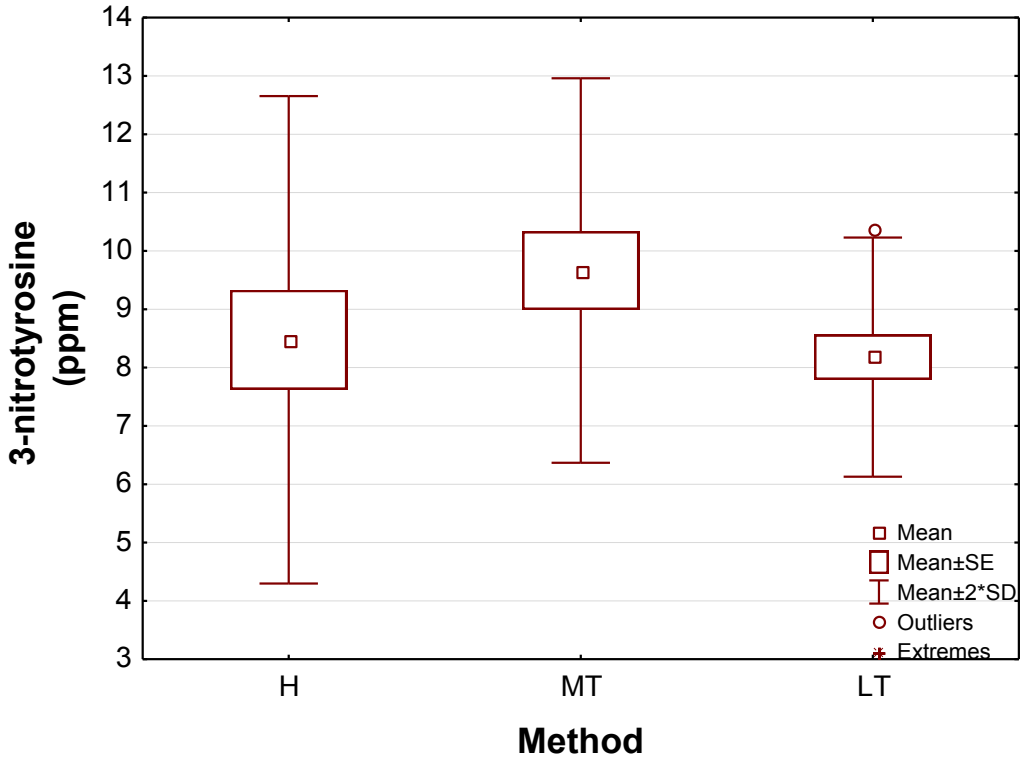


Figure 2. Box plot of variability of 3-nitrotyrosine (IS) in the three extraction methods studied: Boiling ethanol (H), Freezing-thaw method at -20˚C (LT) and Freezing-thaw method at 4˚C (MT).

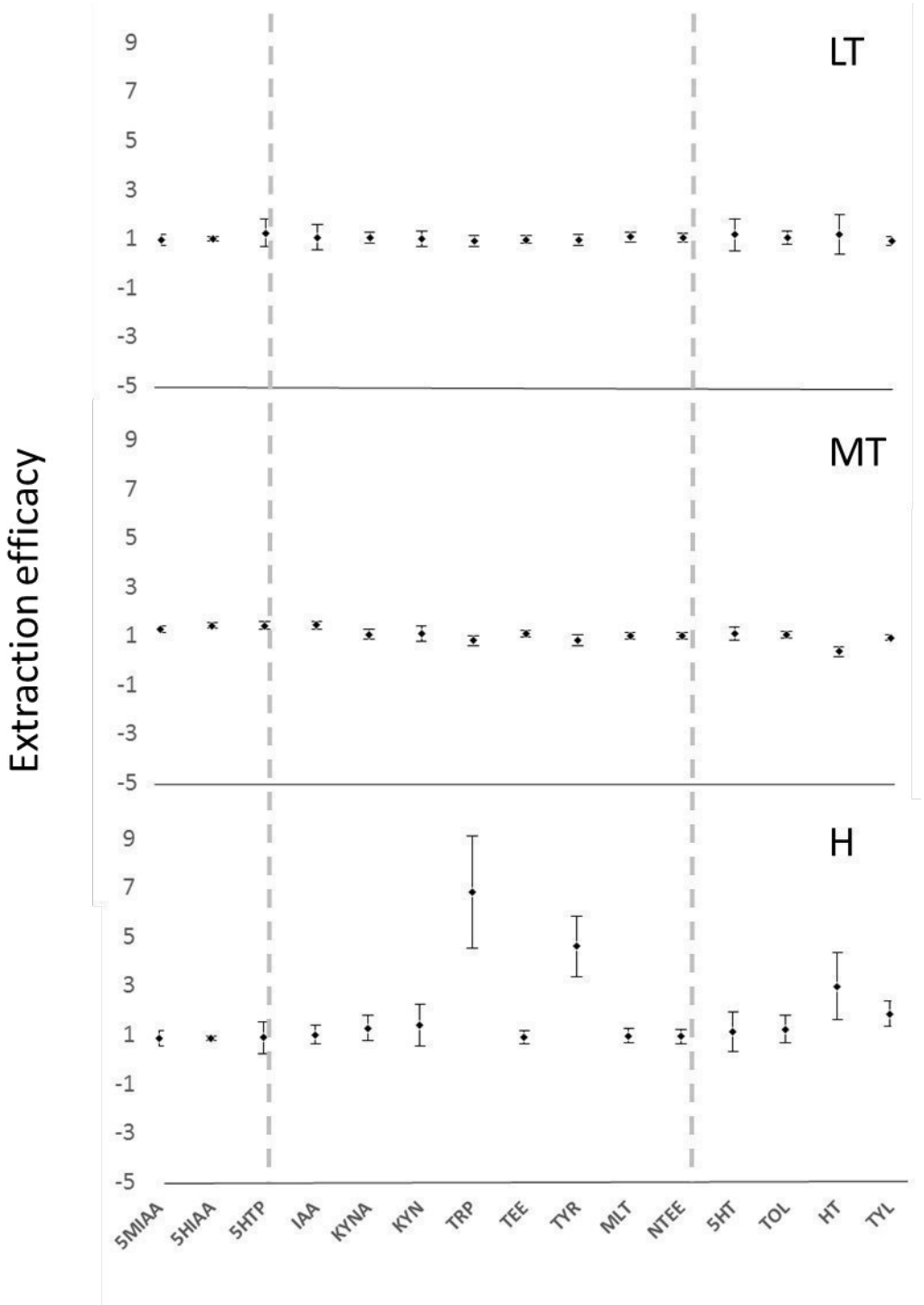


Figure 3. Comparative extraction efficiencies for 15 metabolites and for each extraction method. Data are means and standard deviations of normalised data by median of six replicates (see Results and Discussion for definitions of efficiency). Dashed grey lines are for guidance.

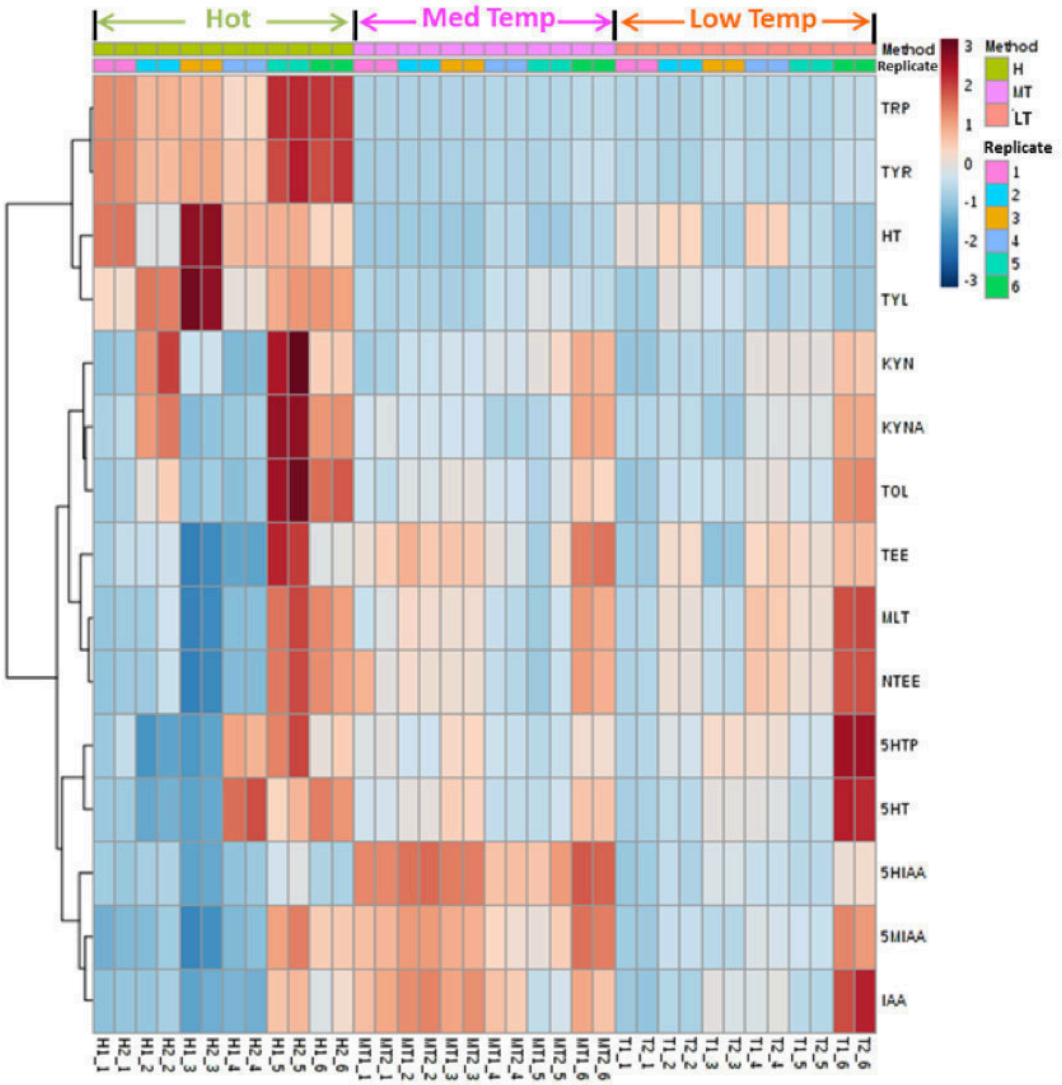


Figure 4. Heat map, representing concentrations expressed in µg L-1 related to the method; boiling ethanol (Hot), freezing thaw at 4˚C (Medium Temperature), freezing thaw at -20˚C (Low temperature).

Table S1. Standards and reagents.

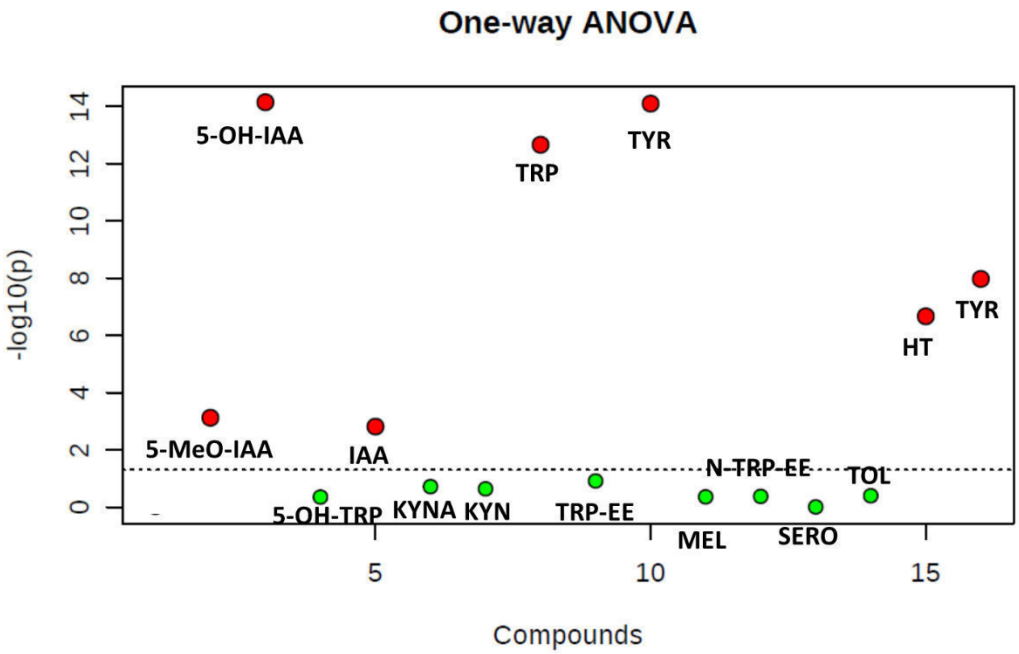
Sigma-Aldrich SL Madrid, Spain		**Chengdu Biopurify Phytochemicals Ltd. (Wenjiang Zone, Chengdu-Sichuan, China)**
5-methoxy indole acetic acid (5MIAA)	L-proline	Hydroxytyrosol (HT) standard 98%
5-hydroxy indole acetic acid (5HIAA)	L-glutamine	**Merck (Darmstadt, Germany)**
5-hydroxy tryptophan (5HTP)	L-arginine	Ethanol HPLC grade
3-indole acetic acid (IAA)	L-tryptophan	Acetonitrile HPLC grade
Kynurenic acid (KYNA)	L-alanine	Panreac (Barcelona, Spain)
Kynurenine (KYN)	L-glutamic acid	Formic acid
Tryptophan (TRP)	L-serine	**VWR International Eurolab S.L.**
Tryptophan ethyl ester (TEE)	L-threonine	**(Barcelona-Spain)**
Tyrosine (TYR)	L-leucine	Glycerol, ultrapure 99.5%
Melatonin (MLT)	L-aspartic acid	
N-acetyl tryptophan ethyl ester (N-TEE)	L-valine	
3-nitrotyrosine (IS)	L-phenylalanine	
Serotonin (5-HT)	L-isoleucine	
Tryotophol (TOL)	L-histidine	
Tyrosol (TYL)	L-methionine	
N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES)	L-tyrosine	
Ethylene glycol	L-glycine	
Glucose	L-lysine	
Fructose	Oleic acid	
Calcium chloride	Ergosterol	
Monobasic potassium phosphate	Tween 80	
Potassium sulfate	Biotin	
Magnesium sulfate heptahydrate	Calcium pantothenate	
Sodium chloride	Chlorohydrate	
Ammonium chloride	Chlorohydrate thiamine	
Cobalt(II) chloride hexahydrate	Myoinositol	
Copper(II) sulfate pentahydrate	Nicotinic acid	
Boric acid		
Potassium iodide		
Manganese sulfate		
Ammonium molybdate		
Zinc Sulfate Monohydrate		
Ammonium chloride		

Table S2. Synthetic must composition

	Compound	g L^{-1}
	Glucose	100
	Fructose	100
Mineral Salts	$CaCl_2$	0.155
	$KH_2 \cdot PO_4$	0.75
	K_2SO_4	0.5
	$MgSO_4 \cdot 7H_2O$	0.25
	NaCl	0.2
	NH_4Cl	0.46
Trace Elements	$COCl_2 \cdot 6H_2O$	0.4
	$CuSO_4 \cdot 5H_2O$	1
	H_3BO_3	1
	KI	1
	$MnSO_4 \cdot H_2O$	4
	$(NH_4)6Mo_7O_{24}$	1
	$ZnSO_4 \cdot H_2O$	4
Nitrogen sources		% wt/wt
	ammoniacal nitrogen 18.6% wt/wt	18.6
	NH_4Cl	20.5
	L-proline	16.9
	L-glutamine	1.25
	L-arginine	6
	L-tryptophan	4.9
	L-alanine	4
	L-glutamic acid	2.6
	L-serine	2.6
	L-threonine	1.6
	L-leucine	1.5
	L-aspartic acid	1.5
	L-valine	1.3
	L-phenylalanine	1.1
	L-isoleucine	1.1
	L-histidine	1.1
	L-methionine	0.6
	L-tyrosine	0.6
	L-glycine	0.6
	L-lysine	0.6
Aerobics Factors		g 100 mL^{-1}
	Oleic acid	0.5
	Ergosterol	1.5
	Tween 80	0.5
Vitamins		mg mL^{-1}
	Biotin	0.003
	Calcium pantothenate	1.5
	Chlorohydrate pyridoxine	0.25
	Chlorohydrate thiamine	0.25
	Myoinositol	20
	Nicotinic acid	2

Table S3 Calibration curve information.

Metabolite (acronims)	Degree of linearity	*a* (slope)	*b*	R^2
5-methoxy indole acetic acid (5-MeO-IAA)	6	4.58E+05	1.53E+05	0.9985
5-hydroxy indole acetic acid (5-OH-IAA)	5	2.64E+05	-3.24E+06	0.9456
5-hydroxy tryptophan (5-OH-TRP)	6	3.50E+05	2.25E+05	0.9992
3-indole acetic acid (IAA)	5	4.57E+05	1.45E+06	0.9898
kynurenic acid (KYNA)	5	2.31E+07	-8.23E+05	0.9983
kynurenine (KYN)	6	1.86E+05	-1.14E+05	0.9991
tryptophan (TRP)	6	7.07E+05	-1.51E+05	0.9951
tryptophan ethyl ester (TRP-EE)	6	2.84E+06	1.93E+06	0.9927
tyrosine (TYR)	6	5.86E+05	3.73E+05	0.9990
melatonin (MEL)	6	3.36E+06	3.68E+06	0.9952
N-acetyl tryptophan ethyl ester (N-TRP-EE)	6	9.72E+04	3.58E+06	0.9888
3-nitrotyrosine (IS)	6	4.57E+05	-2.48E+05	0.9996
serotonine (SER)	6	3.89E+06	1.48E+04	0.9978
tryptophol (TOL)	6	4.22E+05	1.50E+06	0.9958
hydroxytryptophol (HT)	5	2.46E+05	-7.86E+04	0.9998
tyrosol (TYL)	4	2.15E+02	-7.59E+03	0.9992



Name	f. Value	p.value	-log10	FDR	Post-hoc test
TYR	108.1	1.05E-15	14.979	1.68E-14	LT-H; MT-H
L_TRP	86.109	3.05E-14	13.516	2.44E-13	LT-H; MT-H
5OH_IAA	40.198	8.57E-10	9.0671	4.57E-09	LT-H; MT-H;
TYL	28.741	4.12E-08	7.3848	1.65E-07	LT-H; MT-H
HT	26.705	9.07E-08	7.0426	2.90E-07	LT-H; MT-H;
5MeO_IAA	6.5555	0.0038186	2.4181	0.010183	LT-H; MT-H
NTRPEE	5.8167	0.0065918	2.181	0.013209	MT-H; MT-LT
MEL	5.8141	0.0066045	2.1802	0.013209	MT-H; MT-LT
IAA	4.7602	0.014839	1.8286	0.02638	LT-H

Figure S1. Anova and Tukey's test for all samples. The points in red are the significant compounds selected base on the default p value threshold (0.05), which is marked by dashed line. The table contains the statistical data of compounds with significant differences.

4. DISCUSIÓN/DISCUSSION

4

4.- DISCUSIÓN GENERAL

La presente Tesis Doctoral se estructura en dos partes. La primera se fundamenta en la evaluación de diferentes productos fermentados elaborados a partir de un puré de fresa. Ello nos ha permitido conocer sus características con el fin de elaborar una bebida con alto potencial bioactivo que sirva de aprovechamiento de los excedentes de producción de una fruta de marcado carácter perecedero.

La segunda parte se dedica a comprobar la producción de compuestos bioactivos por las levaduras utilizadas en la fermentación alcohólica.

Siendo España uno de los principales productores mundiales de fresa, resulta de sumo interés desarrollar productos que permitan optimizar el aprovechamiento de esta fruta. Entre ellos destaca el puré de fresa, que sirve de base para la elaboración de numerosas variedades de productos alimenticios (helados, batidos, yogures, confituras, rellenos, galletas, pasteles...).

I. Por ello cabe plantearse **en qué medida los procesos industriales de elaboración de este puré de fresa afectan a su composición y si preservan las cualidades de la fruta de la que se obtiene**. La respuesta a este interrogante es fundamental, tanto para valorar su composición en nutrientes y compuestos bioactivos, como para determinar sus potencialidades en la elaboración de productos innovadores exigidos por un mercado cada vez más exigente.

Entre los compuestos bioactivos de la fresa destacan los polifenoles. Los polifenoles antocianos han sido objeto de múltiples estudios ya que son los responsables del color rojo, presentándose en este fruto en cantidades apreciables. Como quiera que éstos han sido objeto de estudio en otra tesis defendida recientemente (Hornedo et al., 2016), esta Tesis Doctoral se centra en el análisis de los polifenoles no antocianos.

A tal fin, se analizó la composición de los polifenoles no antocianos en cada paso de la fabricación del puré de fresa (véase apartado 1 de Material y Métodos). Las etapas de elaboración estudiadas comprenden: triturado de la fruta, inactivación enzimática (55-65ºC, 2

min) y el producto final pasteurizado (véase Tabla 5 de distribución de muestras, apartado 1 de Material y Métodos y Anexo 1, publicación 1).

A continuación, se analizaron los fermentados glucónicos (Anexo 1, publicación 2) y alcohólicos (Anexo 1, publicación 3) realizados por el Grupo de Investigación del Departamento de Química Inorgánica e de Ingeniería Química de la Universidad de Córdoba liderado por el Dr. Isidoro García. Para realizar las fermentaciones se emplearon los inóculos de bacterias y levaduras (*Gluconobacter japonicus y Saccharomyces cerevisiae*, respectivamente) aislados previamente por el grupo de investigación de la Universidad Rovira i Virgili dirigido por la Dra. Mª Jesús Torrija y por el Dr. Albert Mas, con quienes colaboramos en el desarrollo del proyecto AGL2010-22152-01 (véase apartado 2 de Material y Métodos).

Seguidamente, se analizaron las muestras de la bebida cuya composición consistió en una proporción 80:20 v/v del fermentado glucónico previamente centrifugado y agua carbonatada mezclada con una pequeña proporción de vinagre de fresa (0,05% de ácido acético) que, al disminuir el pH, actúa como conservante. Con el propósito de garantizar la conservación del producto, se incorporó a la fórmula de la bebida el Velcorin® (dimetil dicarbonato) en una concentración de 230 mg L^{-1}. Posteriormente se pasteurizó para imitar el proceso de elaboración industrial de una bebida comercializada (véase apartado 1.2.4 de Material y métodos, Tabla 5 de muestras y Anexo 1, publicación 4)

Para la identificación de los compuestos polifenólicos no antocianos en los artículos que constituyen los Capítulos I- IV, se utilizaron diferentes técnicas e instrumentación cada vez más sofisticadas a medida que la Universidad ha ido adquiriendo nuevos equipos.

En los Capítulos I y II se utilizó la cromatografía de líquidos de alta resolución acoplada a un detector de diodos, HPLC-DAD con la finalidad de identificar y cuantificar, y la espectrometría de masas QTRAP [triple cuadrupolo híbrido con una trampa iónica (QqQ_{LIT})] para confirmar la identificación. Se hizo uso del método MRM (multiple reaction monitoring) de espectometría de masas, que consiste en la selección de un ión deseado (precursor) y varios fragmentos MS/MS (producto), teniendo en cuenta que la fragmentación (fragmentos y sus abundancias) es una característica propia de cada metabolito.

La combinación de las dos técnicas nos permitió identificar 32 compuestos no antocianos en las muestras de fresa y puré correspondientes al Capítulo I.

Mediante esta tarea identificativa pudimos obtener los siguientes resultados:

- 13 compuestos fueron identificados frente a patrones con una pureza comprendida entre el 95 – 99%.
- 19 fueron identificados contrastando sus fragmentaciones con la literatura científica.

Respecto a los fermentados glucónicos, la identificación nos proporcionó un total de 43 compuestos polifenólicos no antocianos distribuidos de la siguiente manera:

- 16 compuestos se identificaron comparando con patrones de alta pureza (frente a patrones con purezas de 95-99%).
- 27 fueron identificados mediante la comparación con la fragmentación publicada en las bases de datos y en la literatura.

Posteriormente, para los trabajos de investigación que conforman los Capítulos III y IV, comenzamos a utilizar el espectrómetro de masas QExactive perteneciente a una nueva generación de equipos (HRMS) de espectrometría de masas de alta resolución que nos proporciona valores de masa exacta con un nivel de precisión de hasta cuatro decimales en comparación con el valor teórico, con resoluciones de hasta 140.000 a *m/z* 200 y que permite analizar un rango de masas comprendido entre *m/z* 50 – 6.000.

Esta nueva tecnología nos permitió obtener la identificación de 66 compuestos en muestras de fermentados alcohólicos pertenecientes al Capítulo III distribuidos como sigue:

- 26 compuestos fueron identificados frente a patrones de purezas 95-99%.
- 40 compuestos se identificaron utilizando la literatura y las bases de datos Massbank (Horai et al., 2010) y ReSpect for phytochemicals (Sawada et al., 2012).

En el análisis de los compuestos polifenólicos no antocianos de las muestras de bebida se lograron identificar 64 compuestos distribuidos de la siguiente forma:

- 19 compuestos se identificaron frente a patrones de purezas 95/99%.
- 45 compuestos fueron identificados utilizando la literatura y las bases de datos disponibles en internet mzcloud (www.mzcloud.org) y Metlin (https://metlin.scripps.edu/index.php). Además, se consultaron las bases de datos Massbank (Horai et al., 2010) y ReSpect for phytochemicals (Sawada et al., 2012).

Es de destacar que por primera vez (a la fecha de publicación de los artículos) se han identificado en la fresa o productos derivados de ella, los compuestos que se muestran a continuación en la Tabla.

Compuestos inéditos en la fresa o productos derivados en la fecha de publicación de los artículos.

	Compuestos	Compuestos	Compuestos
Capítulo I	glucósido de trans-resveratrol 7-*O*-glucósido de apigenina 3-*O*-glucuronido de luteonina isómero del cafeoil glucárico ácido malonil cafeoilquínico	ácido 4-O-ß-hexósido del protocatéquico ácido carboxílico de brevifolina glucurónido del ácido ferúlico dímero del *O*-hexósido del ácido caféico	hexósido de aromadendrina glalactósido de 2'-*O*-xilosil floretina 4-*O*-glucurónido del ácido dihidroferúlico hexosil-hexósido de kaempferol
Capítulo II	diglucósido del monogaloil 5-hidroxi feruroil hexosa hexósido de dihidrokaempferol kaempferol neohesperidosido ácido chicórico	**Capítulo III** 3´-*O*-xilósido de la luteína glucósido de 3-*O*-isorhamnetina glucósido de *O*-taxifolina rhamnósido de (+)-aromadendrina glucósido de 7-*O*-eriodictiol eriodictiol ácido homovanílico	**Capítulo IV**

Mediante la cuantificación de los compuestos fenólicos no antocianos se pretendía comprobar si los diferentes pasos del proceso industrial de fabricación del puré de fresa implican una pérdida del potencial bioactivo de la fresa. Tras la cuantificación de compuestos fenólicos antocianos en 25 muestras de distintas etapas del proceso de producción industrial del puré de fresa proporcionado por HUDISA, S.A correspondientes a las campañas de 2011 y 2012 (Figura 31 y Tabla 5), se observó que, como consecuencia del proceso de inactivación enzimática, los compuestos ácido gálico, glucósido de monogaloil y el ácido elágico disminuyeron en sus concentraciones en comparación con el triturado de fresa (muestras control). Una vez realizado el proceso de pasteurización, se apreció una disminución en el contenido de (+)-catequina entre un 20-42 %. Estos decrecimientos podrían deberse a la liberación de los compuestos de las estructuras celulares como consecuencia del triturado de la fruta pues, éstos, al quedar expuestos, son más susceptibles a las reacciones de oxidación facilitadas por la acción del calor (Hartmann, Patz, Andlauer, Dietrich, & Ludwig, 2008). Además, se constató que la presencia de semillas no aumentó el contenido de compuestos fenólicos, como cabría esperar. Incluso el hecho de eliminarlas produjo un efecto de concentración en la mayoría de los compuestos. Esto, junto con los eventos de hidrólisis e isomerizaciones propias de los procesos térmicos (van Boekel et al., 2010), produjeron que los hidroxicinámicos en su conjunto, sufrieran un sustancial incremento, lo que supone una diferencia de un 30,5% en la cosecha de 2011 y de 12,6% en la de 2012. Este comportamiento fue observado anteriormente por Aaby en la fresa (Aaby, Ekeberg, & Skrede, 2007).

La medida de la actividad antioxidante *in vitro* utilizando dos métodos DPPH (2,2-difenil-1-picrilhidracilo) y ORAC (capacidad de absorción de radicales oxígeno) referidas en la literatura (Fernández-Pachón, Villaño, García-Parrilla, & Troncoso, 2004; Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007), nos permitió observar solo un pequeño descenso con el proceso de pasteurización cuyos valores no tuvieron significación estadística.

Lo anteriormente expuesto nos permite concluir que el proceso industrial de elaboración del puré de fresa mantiene en su producto final las propiedades antioxidantes de la fruta de origen.

La fermentación es un proceso milenario empleado para la elaboración de los alimentos con la finalidad de aportarles especiales características organolépticas facilitando, además, la conservación de los mismos.

II. Por ello se planteó **evaluar de qué forma la fermentación del puré de fresa afecta a la composición de polifenoles no antocianos, a fin de evaluar si supone una alternativa para la elaboración de nuevos alimentos preservando su potencial bioactivo.**

Tomando como sustrato de partida el puré de fresa, se realizaron dos tipos de fermentaciones: fermentación alcohólica y fermentación glucónicas. Éstas últimas, producidas por bacterias acéticas pertenecientes al género *Gluconobacter*, que transforman la glucosa en ácido glucónico, respetando el contenido original de fructosa. Específicamente, se eligió *Gluconobacter japonicus* (Grupo de investigación de la Universidad Rovira i Virgili) para elaborar el inóculo de las fermentaciones.

Los resultados de este estudio pusieron de manifiesto que la fermentación glucónica produjo un incremento en la mayoría de los compuestos polifenoles no antocianos estudiados (el mayor aumento lo presentó el ácido gálico con un 36%). Esta observación podría explicarse por la acción enzimática. Enzimas como las esterasas tanasas, propias de bacterias, actúan hidrolizando las uniones ésteres liberando las unidades de ácido gálico y las fracciones de azúcares. La presencia de metabolitos como el hidroxibenzoico (identificado no cuantificado), indican la degradación de compuestos hidroxicinámicos como consecuencia del proceso fermentativo (Duckstein, Lorenz, & Stintzing, 2012).

Por otra parte, se observó que la fermentación alcohólica produjo aumentos significativos en un grupo de compuestos formado por el monogaloil glucósido, el ácido homovanillico y el ácido *p*-hidroxibenzoico. El aumento del monogalioil glucósido (20-36%) probablemente esté relacionado con la degradación del contenido de otros galotaninos. En cuanto al aumento del ácido homovanillico (3,2-3,6%) se debe a la transformación de quercetina, rutina y otros glicósidos de la quercetina, tal y como afirman los estudios de Jaganath et al. y de Dall'Asta et al. (Dall'Asta et al., 2012; Jaganath, Mullen, Lean, Edwards, & Crozier, 2009), aunque, en nuestro caso, los cambios observados en el contenido del glucósido de quercetina no confirman esta afirmación, pues probablemente existan otros glicósidos que, sin haber sido detectados, su transformación contribuya al aumento del ácido homovanilico. Asimismo, el ácido *p*-hidroxibenzoico sufrió un aumento (1,2-2,4%), éste se ha descrito como un metabolito secundario de la biodegradación de otros polifenoles hidroxicinámicos (Duckstein, Lorenz, & Stintzing, 2012).

La actividad antioxidante *in vitro* medida mediante dos técnicas DPPH y ORAC, nos permitió constatar que las fermentaciones glucónicas, aun cuando afectan significativamente a las concentraciones de un mayor número de compuestos, mantienen la capacidad antioxidante prácticamente sin cambios.

Por otro lado, la fermentación alcohólica supuso un descenso [2,3 mmols TE kg^{-1} pf sustrato A y 2.9 µmols TE g^{-1} pf en el sustrato B (diferencias estadísticamente significativas)] de la capacidad antioxidante, que posiblemente sea debida a pérdidas importantes en el contenido de polifenoles antocianos (Hornedo et al., 2016).

La pasteurización de los fermentados se realizó con el objeto de paralizar la actividad microbiana (calentamiento 70-80ºC por 15 min., seguido de un rápido enfriamiento). Este proceso en los fermentados alcohólicos produjo una disminución en el contenido de compuestos como la phloridizina, derivados del kaempferol, del caféico y de la procianidina B1. Esta disminución del contenido de procianidina B1 (27-51%) concuerda con otros estudios (White, Howard, & Prior, 2011), que describieron cómo el calor utilizado en el proceso de blanqueamiento (proceso térmico aplicado por poco tiempo sobre los vegetales) produjo una disminución en el contenido de las procianidinas poliméricas.

En los fermentados glucónicos la pasteurización produjo un aumento en el contenido de compuestos hidroxicinámicos como el *p*-coumaroil hexosa (14-16%), el cinámico (3%) y el *p*-

coumárico (45%). El aumento de éstos tres compuestos podría explicarse mediante la producción de isomerizaciones y procesos hidrolíticos generados por el efecto del calor que producen una redistribución sustancial de las concentraciones de los compuestos fenólicos (Van Boekel et al., 2010).

La pasteurización del puré de fresa, al igual que la de los fermentados glucónicos, no produjo cambios significativos de la actividad antioxidante. La explicación a este fenómeno puede provenir de la liberación de las agliconas como producto de la hidrólisis térmica de los compuestos glicosilados. Las agliconas, por lo general, tienen mayor actividad antioxidante que el glicosilado de origen (Hur, Lee, Kim, Choi, & Kim, 2014; Van Boekel et al., 2010).

El hecho de que la fermentación glucónica mantuviera la actividad antioxidante sin cambios con respecto al puré de fresa y sea un proceso que conserva el contenido de fructosa de la fruta de origen, nos permitió decantarnos por la utilización del fermentado glucónico de fresa como base fundamental para la elaboración de una bebida.

El diseño de la bebida se basó en las pruebas del panel de cata de nuestro Grupo de Investigación (Derivados de la uva, Área de Nutrición y Bromatología, Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal) (AGR-167) y las pruebas de preferencia y aceptabilidad fueron realizadas por los alumnos de la Facultad de Farmacia de la Universidad de Sevilla.

III. Una vez elaborada la bebida, nos formulamos tres cuestiones. Una referida a **qué tiempo de vida útil tendría una bebida elaborada de esta manera. La segunda, sobre cómo pueden afectar las condiciones de almacenamiento al contenido polifenólico no antociano y a la actividad antioxidante de la misma y, por último, en qué medida las condiciones de almacenamiento afectan sensorialmente a la bebida.**

Para responder a todas estas cuestiones, se evaluó el almacenamiento de la bebida en dos condiciones de temperatura: refrigeración (4ºC) y temperatura ambiente (27 – 30ºC) en muestras tomadas al inicio (justo después de la pasteurización) a 15, 30, 60 y 90 días de almacenamiento.

Realizada la cuantificación de los polifenoles no antocianos en la bebida, se observó que, comparándola con el puré de fresa, el fermentado glucónico y la muestra inicial de la

bebida, compuestos como la (+)-catequina y taninos condensados como la procianidina B1 y el trímero de la procianidina sufrieron importantes disminuciones en sus concentraciones como consecuencia del tiempo y de las condiciones de almacenamiento, con la práctica desaparición de (+)-catequina y procianidina B1 que presentaron pérdidas a temperatura ambiente de 84,5 y 95% respectivamente. Se apreciaron aumentos en las concentraciones de compuestos como el ácido elágico, *p*-cumárico y el ácido protocatéquico (13 veces la concentración inicial). Los compuestos más estables durante el período de almacenamiento a las dos temperaturas se detallan en la tabla S1 (Capítulo IV). De estos compuestos cabe destacar que dos taninos hidrolizables presentaron comportamientos dispares: el tris-galoil-HHDP-hexosa, que experimentó un aumento de concentración a partir de los 60 días de almacenamiento a temperatura ambiente y a partir de los 90 en refrigeración, y el monogaloil glucosa que, por el contrario, experimentó un decrecimiento significativo en el mismo tiempo de almacenamiento. Probablemente, la disminución de este último compuesto se haya producido a expensas del aumento del primero. Además hay que tener en cuenta que a estas pérdidas también contribuyen las manipulaciones realizadas en el proceso de elaboración de la bebida como la centrifugación del fermentado glucónico y la dilución con agua carbonatada.

Asimismo se observó que la mayor pérdida de polifenoles no antocianos se manifestó a temperatura ambiente. Esta disminución puede deberse al efecto de la pasteurización que, favoreciendo la ruptura de las estructuras celulares, aumentó la exposición de los compuestos a los procesos oxidativos durante el almacenamiento, más favorecidos a temperatura ambiente que en refrigeración (Oliveira, Almeida, & Pintado, 2013).

Los resultados de la valoración sensorial de la bebida basados en los atributos (impresión general, aroma a fresa y sabor a fresa) otorgaron una mejor puntuación a las muestras de bebida conservadas a temperatura de refrigeración que en las almacenadas a temperatura ambiente, siendo la muestra almacenada a temperatura ambiente durante 90 días la que tuvo peor valoración general. En base a estos resultados, el período de tiempo óptimo de almacenamiento de la bebida no debería exceder de los 30 días a temperatura ambiente (27 – 30ºC) o 60 días en condiciones de refrigeración (4ºC).

IV. La segunda parte de la tesis doctoral trata de responder a la siguiente cuestión: **en qué medida la presencia de determinados compuestos bioactivos en productos fermentados pueden ser consecuencia de la síntesis directa de las levaduras a partir de nutrientes precursores (aminoácidos aromáticos)**.

En concreto, se ha estudiado la producción de hidroxitirosol (HT) por acción de las levaduras vínicas. Este compuesto bioactivo ha sido muy estudiado ya que está presente en el aceite de oliva, otorgándole propiedades saludables (Andreadou et al., 2011; European Food Safety Authority (EFSA) Panel on Dietetic Products Nutrition and Allergies (NDA), 2011). Es un polifenol que anteriormente se ha descrito en vinos (Bordiga et al., 2016; Di Tommaso, Calabrese, & Rotilio, 1998), y que se produce mediante la ruta de Ehrlich, usando a la tirosina como sustrato por la acción de levaduras del género *Saccharomyces* (Zhu et al., 2010).

Los resultados obtenidos de las extracciones intracelulares nos demostraron que levaduras del género *Saccharomyces*, específicamente las cepas QA23 y Red Fruit así como la levadura no *Saccharomyces Torulaspora delbrueckii,* son capaces de producir HT en las cantidades de 8,6; 106,2 y 16,1 ng mL^{-1}, respectivamente, en el día 2 de la fermentación alcohólica (22,7; 26,1 y 33,72 g L^{-1} de azúcares reductores respectivamente).

Se estudiaron cuatro diferentes formas de inoculación en mosto de uva tinta *Tempranillo*, dos con cultivos puros QA23 y Red Fruit y dos secuenciales con *Torulaspora delbrueckii* primero seguido de una *Saccharomyces* (QA23 o Red Fruit) además de una fermentación espontánea sin inoculación (véase Material y Métodos apartado 2.1.2). Los resultados constataron que, dependiendo de la levadura utilizada, el contenido de HT varía. Así, las mayores concentraciones se obtuvieron bajo la acción de QA23. En las inoculaciones secuenciales (cuando la *T. delbrueckii* es incluida), se apreció una disminución del contenido de HT en comparación con las fermentaciones que cursaron bajo la única intervención de una de las cepas de levaduras del género *Saccharomyces* estudiadas (QA23 y Red Fruit). A la misma conclusión llegó Romboli en las inoculaciones secuenciales realizadas con *C. Zemplinina* en vino (Romboli, Mangani, Buscioni, Granchi, & Vincenzini, 2015).

Asimismo, se realizó un estudio para contemplar el efecto de otras variables como la variedad de uva de la especie *Vitis vinífera (Corredera, Moscatel, Chardonnay, Palomino fino, Sauvignon Blanc y Vijiriega)*, utilizada para la elaboración del mosto utilizando en la fermentación la cepa de levaduras AROMA WHITE (véase el apartado 2.1.2 de Material y Métodos). De los resultados obtenidos del estudio de mostos de 6 variedades de uvas blancas, se concluyó que la variedad de uva y la duración de la fermentación son otros factores que afectan la producción de HT. La variedad de uva que tuvo una mayor concentración de HT fue

la *Sauvignon Blanc*. También fue la variedad que produjo fermentaciones más largas (Romboli et al., 2015).

Una vez analizada la producción de HT por parte de las levaduras quisimos hacer extensivo el seguimiento de la producción de bioactivos sintetizados en el metabolismo de los aminoácidos aromáticos (triptófano y fenilalanina) incluyendo asimismo al precursor inmediato del HT, el tirosol, proveniente del metabolismo de la tirosina.

Este planteamiento nos ha permitido ahondar en el conocimiento de la producción de bioactivos como la melatonina que se ha descrito recientemente como producto del metabolismo del triptófano bajo la acción de las levaduras *Saccharomyces* (Rodriguez-Naranjo, Torija, Mas, Cantos-Villar, & Garcia-Parrilla, 2012; Sprenger, Hardeland, Fuhrberg, & Han, 1999). Sin embargo, hay muchos aspectos fundamentales sobre la síntesis de la melatonina y otros bioactivos indólicos por la acción de las levaduras que son completamente desconocidos, como el motivo fisiológico o metabólico que lleva a las levaduras a producir estos compuestos, así como también las rutas o mecanismos moleculares que intervienen en la síntesis de los mismos.

V. Realizado el planteamiento anterior, nos formulamos la siguiente interrogante: **en qué momento se producen y qué destino catabólico tienen los compuestos implicados en las rutas metabólicas de los aminoácidos aromáticos.** (Objeto de los proyectos coordinados AGL2013-47300-C3-2-R, AGL 2016-77505-C3-2R.)

Para tal fin se desarrolló un método de análisis de espectrometría de masas capaz de detectar y cuantificar 38 metabolitos (37 metabolitos y el estándar interno) en 13 minutos. Se analizaron muestras intra y extracelulares obtenidas de fermentaciones alcohólicas en mosto sintético producidas por tres cepas de levaduras, dos *Saccharomyces* (QA23 y Red Fruit) y una no *Saccharomyces* (*Torulaspora delbrueckii*) (véase apartado 2.2.1 de Material y Métodos).

Los resultados de este trabajo nos permitieron identificar y cuantificar por primera vez dos compuestos en el medio intracelular de las levaduras: la 2-aminoacetofenona y el triptofol sulfonado. La 2-aminoacetofenona es una molécula que forma parte de un grupo de sustancias conocidas como UTA "untypical aging off flavour" capaces de imprimir un característico y desagradable olor a los alimentos fermentados. Ambos compuestos se han descrito anteriormente como productos de reacciones químicas directamente relacionadas con la presencia de sulfitos (SO_2) agregados a los vinos con finalidad de protegerlos de la acción del oxígeno y de los microorganismos (Arapitsas et al., 2016; Hoenicke et al., 1999). El hecho de haber

cuantificado estas moléculas en el medio intracelular, nos permite sostener que, además de la reacción de síntesis antes descrita, estos compuestos se sintetizan al final de la fermentación alcohólica en presencia de pequeñas cantidades de oxígeno donde el aporte de azufre solo puede provenir del metabolismo de los aminoácidos azufrados y/o de las sales sulfatadas que forman parte de la composición del mosto sintético. Este comportamiento entendemos que es debido al metabolismo directo de las levaduras.

Asimismo, se pudo comprobar que los ésteres etílicos del triptófano y de la tirosina se producen por la reacción de de-acetilación de los derivados N-acetilados correspondientes ya que, como muestra la Figura 4-Capítulo VI-parte I, a medida que disminuyen los compuestos N-acetilados, aumenta la concentración de los esteres etílicos.

La realización de este trabajo nos permitió comprobar que hubo compuestos a los que fue imposible hacer un seguimiento a través del tiempo, pues las bajas concentraciones no superaron los límites de cuantificación. Posiblemente, la eliminación de interferentes disminuya los límites facilitando la cuantificación. Este hecho nos llevó a plantearnos en qué medida la presencia de artefactos y elementos interferentes de las muestras pueden afectar a la debida fragmentación, imposibilitando la detección y la cuantificación.

Teniendo en cuenta que las extracciones intracelulares dependen fundamentalmente de las características estructurales del microorganismo así como de las propiedades químicas de las moléculas que se quieren analizar, es, por tanto, interesante evaluar las extracciones enfocadas en los microorganismos así como en las moléculas específicas.

VI. Surge, por tanto, la necesidad de **determinar los niveles de eficiencia de los métodos extractivos utilizados y en qué medida afectan a las moléculas objeto de investigación.**

Así nos propusimos la optimización del método de extracción intracelular, mediante la evaluación de la eficiencia de tres métodos basados en los anteriormente publicados y comúnmente utilizados (Gonzalez & Franc, 1997; Smart, Aggio, Van Houtte, & Villas-Bôas, 2010), con la introducción, además, de la eliminación de fosfolípidos y proteínas en el proceso de preparación de la muestra. Estos compuestos son conocidos interferentes en los análisis de muestras biológicas, cuando son analizados con espectrometría de masas (Carmical & Brown, 2016) (véase apartado 2.2.2 de Materiales y Métodos).

Los resultados pusieron de manifiesto que la eliminación de fosfolípidos y proteínas permitió mantener el efecto matriz en valores muy cercanos al intervalo comprendido entre 20 y -20% lo que indica que, en estos niveles, las distorsiones en la detección y en la cuantificación son inapreciables (Álvarez E, Madrid Y, 2016).

Por otra parte, los métodos a bajas temperaturas fueron más efectivos en la extracción de compuestos relacionados con el metabolismo del triptófano, la tirosina y la melatonina, mientras que el método a alta temperatura podría producir falsos positivos al sobreestimar algunos metabolitos como los aminoácidos, ya que éstos pueden provenir de la degradación de macromoléculas como las proteínas por el efecto del calor (Canelas et al., 2009). Así, el triptófano y la tirosina produjeron, respectivamente, concentraciones ocho y cinco veces superiores a las obtenidas con las extracciones a bajas temperaturas.

Finalmente, las pequeñas diferencias obtenidas en las concentraciones de los metabolitos extraídos con los dos métodos de extracción a bajas temperaturas nos permiten utilizar el método con centrifugaciones a 4ºC, sin riesgo de grandes pérdidas de metabolitos y con la ventaja de poder utilizar equipos de centrifugación más asequibles.

5. CONCLUSIONES/CONCLUSIONS

5

5.-CONCLUSIONES.

1. A través del uso de la espectrometría de masas se han identificado tentativamente por primera vez a la fecha de las publicaciones 25 fenoles no antoncianos en el puré de fresa y sus derivados fermentados, distribuidos como sigue: 12 flavonoides, 7 ácidos hidroxicinámicos, 2 taninos, 1 chalcona, 1 estilbeno, 1 ácido hidroxibenzoico y 1 hidroxifenilacético.

2. La caracterización de la composición de los polifenoles no antocianos del puré de fresa demostró que se caracteriza por ser especialmente abundante en (+)-catequina y HHDP galoil glucósido y además, el proceso industrial de su elaboración es respetuoso con la composición de los polifenoles no antocianos, preservando la actividad antioxidante de la fruta de origen sin cambios significativos. Por este motivo, el puré de fresa es una fuente de compuestos fenólicos con potencial antioxidante apto para ser utilizado como ingrediente de alimentos derivados.

3. La fermentación alcohólica del puré de fresa causó cambios estadísticamente significativos en las concentraciones de 19 compuestos, siendo los más notables los incrementos en las concentraciones del ácido homovaníllico y el *p*-hidroxibenzóico y el decrecimiento del glucósido de galoil bis-HHDP. Por contra, la fermentación glucónica provocó escasas modificaciones, manteniendo el contenido de fructosa y la actividad antioxidante del puré. La fermentación glucónica es, por tanto, una buena alternativa para el aprovechamiento de los excedentes de producción de la fresa.

4. El estudio de la estabilidad de los compuestos polifenólicos no antocianos y la valoración sensorial de la bebida determinaron que para preservar tanto la composición de fenoles como la capacidad antioxidante, el almacenamiento a temperatura ambiente no debe exceder de 30 días ni de 60 días en refrigeración.

5. La identificación y cuantificación del hidroxitirosol en el medio intracelular de las levaduras *Saccharomyces cerevisiae* (QA23 y RED FRUIT) y en la no *Saccharomyces* (*Torulaspora delbrueckii*), permite afirmar que éste es un bioactivo producido por ellas durante la fermentación del mosto. Se comprobó que la levadura de la cepa QA23 fue la más eficiente

productora de hidroxirosol. Del mismo modo, el vino de la variedad de uva *Sauvignon Blanc* fue el que presentó mayores concentraciones de hidroxitirosol.

6. El estudio del metabolismo de los aminoácidos aromáticos en mostos sintéticos fermentados por acción de las levaduras *S. cerevisae* (QA23 y RED FRUIT) y no *Saccharomyces* (*Torulaspora delbrueckii*), mediante un método de LC-MS (QTRAP), permitió por primera vez hasta la fecha, detectar y cuantificar en el medio intracelular los compuestos 2-aminoacetofenona y triptofol sulfonado, lo que permite afirmar que estos dos compuestos son producidos directamente por las levaduras estudiadas.

7. Como consecuencia del experimento anteriormente descrito y mediante la interrelación de los resultados de la cuantificación efectuada en el medio intra y extracelular podemos deducir que durante la fermentación se produce:

- La sulfonación del Triptofol con la consiguiente formación de triptofol sulfonado.
- La deacetilación de los derivados N-acetilados etil ésteres del triptófano y de la tirosina para producir sus respectivos ésteres etílicos.
- La síntesis de novo de fenilalanina.

8. Para optimizar la detección y cuantificación de metabolitos de interés como melatonina y serotonina, se eliminaron moléculas interferentes como fosfolípidos de membrana y proteínas. Una vez eliminados, se comprobó que el efecto matriz observado se situaba entre los valores aceptados de -20 y 20%. Ello nos indica que los efectos distorsionantes de supresión del ión (ion supression) o de mejora de la señal del ion (ion enhanced) son inapreciables mejorándose la detección y la cuantificación de los compuestos.

9. Los métodos de extracción intracelular de metabolitos a bajas temperaturas (-20ºC, 4ºC) son más eficientes y preservan la integridad de los metabolitos, mientras que el método clásico utilizando etanol en ebullición (80ºC) produce la sobreestimación del contenido de aminoácidos, debido a la degradación de las proteínas por el efecto del calor. Así, el triptófano y la tirosina produjeron, respectivamente, concentraciones ocho y cinco veces superiores a las obtenidas con las extracciones a bajas temperaturas.

5.-CONCLUSIONS.

1. Through the use of mass spectrometry, the following compounds have been tentatively identified for the first time at the date of publication: 25 non-anthocyanin phenolic compounds in strawberry purée and derivatives fermented products, distributed as follows: 12 flavonoids, 7 hydroxycinnamics acids, 2 hydrolyzed tannins, 1 chalcone, 1 stilbene, 1 hydroxybenzoic acid and 1 hydroxyphenilacetic acid.

2. The characterization of the non-anthocyanin phenolic composition of the strawberry purée demonstrated that it is especially rich in (+)-catechin and HHDPgalloylglucoside, additionally, the industrial elaboration process preserves the non-anthocyanin phenolic composition and maintains the antioxidant activity of the fruit used in its original state. For this reason, the strawberry purée is a source of phenolic compounds with an antioxidant potential suitable to be used as an ingredient for derived food.

3. The alcoholic fermentation produced statistically significant changes in the concentrations of 19 non-anthocyanin phenolic compounds, most notably the increase of concentrations of homovanilic acid and *p*-hydroxybenzoic and decreased of bis-HHDP-galloylglucoside. In contrast, the gluconic fermentation hardly provoked any modifications, preserving the fructose contend and the purée antioxidant activity. The gluconic fermentation is thus a valuable approach for the use of the strawberry surplus production.

4. The study of the stability of the non-anthocyanin phenolic compounds and of sensory evaluation in the fermented beverage revealed that in order to preserve both the phenolic composition and the antioxidant capacity, the storage at room temperature should not exceed 30 days or 60 days at refrigeration temperature.

5. The identification and quantification of hydroxytyrosol in the intracellular media of *Saccharomyces cerevisiae* strains of yeast (QA23 and RED FRUIT) and of non-*Saccharomyces* (*Torulaspora delbrueckii*) demonstrates that this compound is produced by yeast during the fermentation of the must. QA23 strain of yeast was the most efficient producer of

hydroxytyrosol. Likewise, must from *Sauvignon Blanc* grape variety presented the highest concentrations of hydroxytyrosol.

6. We followed the metabolism of the aromatic amino acids by the yeast strains of *S. cerevisiae* (QA23 and RED FRUIT) and non-*Saccharomyces* (*Torulaspora delbrueckii*), applying a LC-MS (QTRAP) method. Two compounds, 2-aminoacetophenone and tryptophol sulfonated, were detected and quantified for the first time in the intracellular medium, hence demonstrating that these two compounds are produced directly by the studied yeasts.

7. As a consequence of the experiment described above and through the connection with the results of the intra- and extracellular media quantification, we can deduce that during the fermentation process the following occurs:

- The sulfonation of tryptophol to produce tryptophol sulfonated.
- The de-acetylation of the N-acetylated ethyl esters of both tryptophol and tyrosine yielding the correspondent ethyl esters.
- Phenylalanine undergoes a synthesis de novo.

8. In order to optimize the detection and the quantification of the studied metabolites such as melatonin and serotonin, we removed the interfering molecules (of cell walls and proteins). Once removed, we found out that the matrix effect was closely the accepted values of -20% to 20%. This indicates that the possible impact of ion enhancement or ion suppression were imperceptible, improving the detection and the quantification of the compounds.

9. The intracellular extraction methods at low temperatures (-20ºC, 4ºC) are more efficient and preserve the integrity of the studied metabolites, whilst the traditional boiling ethanol method (80ºC) produced the overestimation of amino acids due to the breakdown of proteins favoured by heat. By these means, tryptophan and tyrosine concentration increased by eight and five times, respectively compared to those obtained with low temperature extractions.

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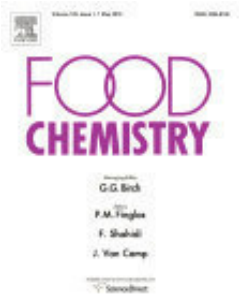
ANEXO I

I



Title: Effects of the strawberry (Fragaria ananassa) purée elaboration process on non-anthocyanin phenolic composition and antioxidant activity

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ABSTRACT

Strawberries are harvested in a short period of time frequently involving fruit surplus. This paper studies the impact of the strawberry purée elaboration process on the chemical composition of the final products. Thirty-two phenolic compounds were studied by Liquid Chromatography with Diode Array Detector (LC-DAD) and Mass Spectrometry (LC-MS). An LC-DAD method was set up and validated and the non-anthocyanin phenolic profile was quantified at the different steps of production, for three elaboration processes and two harvests (2011 and 2012). We have tentatively identified apigenin-7-O-glucoside, luteolin-3-O-glucuronide, malonyl caffeoylquinic acid, *trans*-resveratrol glucoside and caffeoylglucaric isomer. (+)-Catechin and HHDP-galloylglucose were the most abundant phenolic compounds. The most abundant flavonol was kaempferol-3-glucoside.

The purée maintains the fruit's non-anthocyanin phenolic composition and *in vitro* antioxidant activity as determined by ORAC and DPPH methods. This fact suggests that strawberry purée could be considered a valuable ingredient for producing food derivatives.

1. Introduction

Spain (Huelva, Andalucía) is the fourth biggest strawberry (*Fragaria x ananassa*) production area in the world (FAOSTAT, 2012). Strawberries are harvested in a very short period of time and a large amount of fruit is collected. Overproduction causes a decrease in prices and, what is more, the fruit rots and is discarded if not sold. As the strawberry is a highly perishable product, it spoils quickly, which leads to substantial economic losses.

Consequently, manufacturing derived products using strawberries as a raw material or ingredient drives economic profits, increases the conservation period and offers new sustainable and successful alternatives for strawberry consumption. In fact, the production of strawberry purée as an ingredient for yoghurts, marmalades, jams, biscuit production, etc., already represents a worthwhile economic solution.

Strawberries are a very rich source of antioxidant compounds including vitamins C, E, β-carotene, melatonin and phenolic compounds (Oszmianski & Wojdylo, 2009; Stürtz, Cerezo, Cantos-Villar, & Garcia-Parrilla, 2011; Cerezo, Cuevas, Winterhalter, García-Parrilla, & Troncoso, 2010). Among the bioactives, phenolic compounds are one of the main groups of phytochemicals present in strawberries that strongly influence quality, contributing to sensorial-organoleptic attributes and health properties (Larrosa, Tomás-Barberán, & Espín, 2006; Buendia et al., 2010). The main polyphenol compounds described in strawberries are anthocyanins, flavan-3-ols, ellagitanins, glycosides of quercetin and kaempferol (Aaby, Mazur, Nes, & Skrede, 2012; Määttа, Kamal-Eldin, Kaisu, & Törronen, 2004).

Conversely, the phenolic profile of strawberry purée or juice, used as a raw material for further derivative products (jam, yoghurt, vinegar, syrup, etc.) has scarcely been reported in previous studies (Oszmianski & Wojdylo, 2009; Hartmann, Patz, Andlauer, Dietrich, & Ludwig, 2008; Truchado et al., 2012; Bodelón, Avizcuri, Fernández-Zurbano, Dizy, & Préstamo, 2013).

Treatments involved in manufacturing by-products (thermal, mechanical, etc.) are known to affect polyphenolic composition (Truchado et al., 2012). Therefore, this paper aims to establish the impact of industrial processing on the non-anthocyanin phenolic composition and antioxidant activity of strawberry purées, by analyzing their evolution throughout the production process as well as in the different final products.

For this purpose, an analytical Liquid Chromatography with Diode Array Detector (LC-DAD) method was set up and validated, with complete identification confirmed by Mass Spectrometry.

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2. Materials and methods

2.1. Chemicals

The phenolic standard compounds were purchased from Fluka Sigma–Aldrich (St. Louis, MO. USA) [gallic acid, caffeic acid, *p*-coumaric acid, cinnamic acid, (-)-epicatechin, (+)-catechin, chlorogenic acid, ellagic acid, (-)-epicatechin gallate, kaempferol, kaempferol-3-O-ß-D-glucuronide, kaempferol-3-glucoside, *trans*-resveratrol, apigenin and penta-O-galloyl-ß-D-glucose hydrate] and from Chromadex® Inc. (USA) [procyanidin B1]. Luteolin and apigenin-7-O-glucoside from Extrasynthese (Z.I. Lyon Nord. France) DPPH (2,2-diphenyl-1-picrylhydrazyl), AAPH (2,2́-diazo-bis-amidine-propane-dihydrochloride) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were obtained from Sigma–Aldrich (St. Louis, MO. USA), acetonitrile and acetic acid (LC gradient) from Merck (Darmstadt, Germany), and methanol from Prolabo® (Obregón, Mexico).

2.2. Samples

The samples used for this study were strawberry purées, provided by Hudisa Company (Lepe, Huelva, Spain). Two harvests were analyzed (2011 and 2012). Briefly, the purée elaboration process starts when the fruit is received. It is selected, cleaned and the green parts are removed. Crucial steps likely to affect the bioactive compounds of the products are mashing the flesh, enzymatic inactivation (2 min, 55 °C–65 °C) and the pasteurization process (3 min, >90 °C). After that, the temperature is reduced to 5 °C in a few minutes. In order to separate the flesh from the seeds, the mash is sieved, resulting in purées with or without seeds, according to the market they are destined for. Additionally, a seedless purée sample (from the 2012 harvest) was left unpasteurized. Twenty-five samples were analyzed (12 from the 2011 harvest and 13 from 2012 harvest). They were collected at the following steps: (i) mashed step (6 samples M_A, M_B, M_C), (ii) enzymatic inactivation step (6 samples EI_A, EI_B, EI_C), (iii) unpasteurized step (3 samples UP_A, UP_B, UP_C), only for 2012 harvest; and (iv) final products consisting of purées obtained just after the pasteurization process and aseptic packaging, with seeds (5 samples FPS_A, FPS_B, FPS_C) and final products without seeds (5 samples $FPWS_A$, $FPWS_B$, $FPWS_C$). Each subscript $_{A, B, C}$ indicates samples of the same substrate. We used mashed samples as control as no thermal treatment was applied.

2.3. Sample preparation

A total of 30 g of sample was extracted in 30 mL of methanol by sonication for 30 min. Ascorbic acid 1% (w/w) was previously added to prevent sample oxidation. It was immediately centrifuged for 10 min at 1.500 g using a Sorvall® TC Dupont Centrifuge. The supernatant was collected and the pellet re-extracted with methanol (30 mL). The methanol fractions were mixed and evaporated to dryness at 37 °C with a rotary evaporator (Büchi Rotavapor®, R-200/205). The extract was reconstituted in 5 mL of water/methanol 1:1 (v/v) and stored at −18 °C until analysis. Extractions were performed in duplicate. Extraction efficiency was evaluated with a solution of vainillin (46 mg/L) as the internal standard. Recovery was 81.64 ± 0.40% in accordance with the AOAC requirements. The calibration data used was $y = 138.08x + 29.313$, r^2 0.999.

The same sample preparation process as described in this section, except for the addition of ascorbic acid, was used to determine antioxidant activity.

2.4. Chromatographic separation

Chromatographic separation was performed using an LC 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 a UV/Vis diode detector (Series 1100 G1379A) 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. The method used a binary gradient, A (glacial acetic acid/water, pH 2.65) and B (20% A + 80% acetonitrile), programmed in the following gradients: 0 min, 100% A; 5 min, 98% A + 2% B; 10 min, 96% A + 4% B; 15 min, 90% A + 10% B; 20 min, 87% A + 13% B; 35 min, 80% A + 20% B; 40 min, 70% A + 30% B; 45 min, 60% A + 40% B; 50 min, 100% B; 55 min, 100% A; 70 min, 100% A. The flow rate was 1.5 mL min^{-1}, the injection volume was 50 μL, and the temperature was set at 40 °C. Each sample was analyzed twice. Identification was achieved by matching the retention time and spectra of the peaks with standards. Additionally, samples were spiked with standards, if they were commercially available, to achieve complete identification. Quantification was performed by external calibration at 280 nm, 320 nm and 365 nm, in accordance with the maximum absorbance of each compound, using their corresponding standards. Calibration curves were obtained by injecting standards diluted from five to eight different concentrations (R^2: 0.9949–0.9998). In the event of overlapping signals, either peak area or peak height was determined. A triplicate was performed at each point of the calibration curve.

The identification procedure considered that peaks showing DAD spectra similar to a phenolic compound standard but with different retention times were assigned to derivatives. Esterification with sugar causes a bathochromic shift of the maximum compared to that of the corresponding aglycone (Buendia et al., 2010). Additionally, monogalloyl glucoside, ellagic acid glycosides, ferulic acid hexoside derivative, caffeic acid hexoside, *p*-coumaroyl glucoside were quantified, assuming that they present the equal molar absorptivity to their counterpart compounds: penta-O-galloyl-β-D-glucoside, ellagic acid, caffeic acid, ferulic acid and *p*-coumaric-acid respectively. HHDP-galloylglucose was quantified assuming the same absorptivity as ellagic acid. The results were expressed as mg kg^{-1} of fresh weight (fw).

2.5. ESI–MS and MS/MS analysis

Additionally, identification was achieved by ESI–MS and MS/MS under the following conditions: MS/MS experiments performed on 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. The sample extracts were dissolved 0.1% (v/v) with methanol:water 50% (v/v) and analyzed in negative mode. The mass spectrometer was set to the following optimized tune parameters: curtain gas 20 psi, ion spray voltage −4500 V, source gas 20 psi.

For LC–ESI–MRM analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, ion spray voltage 4500 V, source temperature 350 °C and source gas 60 psi. A dwell time was set at 50 ms for each transition.

Conditions of LC/MS/MS were the same as described above for LC-DAD, except flow rate was 0.2 mL min^{-1} and injection volume was 20 μL.

2.6. LC-DAD validation procedure

The LC-DAD method was validated to comply with the requirements of the Association of Analytical Communities (AOAC) (AOAC, 1993; González & Herrador, 2007): linearity, limits of detection

and quantification, precision, and reproducibility. The limit of detection (LOD) and limit of quantification (LOQ) for each phenolic compound was calculated as the amount of compound required to produce a signal to noise ratio of 3:1 and 10:1, respectively. Selectivity is the degree to which a method can quantify the analyte accurately, in the presence of interferences, under the assay conditions for the sample matrix being studied. The recovery of the sample extraction procedure was calculated through standard addition and expressed as a percentage.

2.7. Antioxidant activity

2.7.1. ORAC test

The procedure was based on a previously reported method with slight modifications (Ou, Hampsch-Woodill, & Prior, 2001): 50 μL of sample or Trolox was mixed with 100 μL of fluorescein (45 nM) and 50 μL of AAPH (15 mM). Florescence was recorded for 80 min (excitation wavelength was set at 485 nm and 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).

Fluorescein fluorescence was recorded every 5 min after addition of AAPH, until fluorescence was less than 5% of the initial reading. Final results were calculated using the areas under the fluorescein decay curves, between the blank and the sample, and were expressed as mmols Trolox equivalents (TE) per kg of fresh weight.

2.7.2. DPPH method

The effect of antioxidant activity on DPPH was estimated according to the procedure described by Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007. A total of 0.1 mL of sample in methanol was added to 3.9 mL of DPPH methanolic solution (0.025 gL^{-1}). Absorbance at 515 nm was recorded at the start (when the sample was added) and 60 min later, when the reaction reached equilibrium. We used methanol as a reference. All measurements were performed in triplicate. Eight different concentrations (0.9–0.12 mM) of Trolox were used to make the calibration curve.

Absorbance measurements were recorded on a Hitachi UV-2800 spectrophotometer, thermostated with a Peltier system at 25 °C.

2.8. Statistical analysis

Statistical analyses were performed by means of statistical software (Statsoft, 2001). One-way analysis of variance (ANOVA) was used to test significant differences.

3. Results and discussion

3.1. Method validation-calibration curve

Table 1 displays the data for the validation method parameters, which comply with the AOAC requirements for linearity, precision, recoveries, repeatability, LOD and LOQ. The method used obtained reliable results.

3.2. Identification of phenolic compounds

A total of 32 phenolic compounds were identified through their LC elution order, UV/Vis, mass spectrometric characteristics (Fig. 1) and compared with data reported in the literature (Table 2).

Table 1
Validation results.

Compounds	Selectivity (R_s)	Recoveries		Intermediate precision		Repeatability		Sensitivity	
		Conc. ($mg\ L^{-1}$)	%	Conc. ($mg\ L^{-1}$)	CV (%)	Conc. ($mg\ L^{-1}$)	CV (%)	LOD ($mg\ kg^{-1}$ fw)	LOQ ($mg\ kg^{-1}$ fw)
Gallic acid	7.27	110	89.41	11.55	4.19	1.44	4.68	3.03	11.6
		13.5	115.57	1.44	9.56	11.55	1.74		
Penta-Galloyl-β-D-glucoside	nd	21.25	99.37	85.60	2.02	10.7	2.15	1.39	1.69
		10.62	101.87	10.70	4.56	85.6	0.96		
(+)-Catechin	10.16	110	110.83	55.80	1.99	6.97	9.29	11.87	37.01
		27.5	110.45	6.97	4.96	55.8	2.34		
(-)-Epicatechin	14.02	20	94.43	126.15	6.80	15.77	4.77	0.45	1.33
		10	103.65	15.79	4.91	126.15	2.83		
(-)-Epicatechin gallate	2.69	56	99.37	56.65	5.38	7.08	6.07	0.72	1.16
		8	97.11	7.08	6.41	56.65	2.96		
4-OH-benzoic acid	nd	110	90.50	110.00	6.49	13.75	7.02	nd	nd
		27.5	81.68	13.75	3.63	110	3.55		
Procyanidin B_1	1.42	80	92.9	79.80	6.70	9.975	3.15	0.17	1.51
		40	113.14	9.97	4.98	79.8	6.35		
Cinnamic acid	nd	8	99.9	12.60	3.73	2.1	7.09	0.047	0.133
		2	99.2	3.07	4.31	12.6	3.15		
Ferulic acid	nd	56	86.1	58.05	5.63	7.256	6.13	nd	nd
		14	91.4	7.26	3.72	58.05	9.25		
Chlorogenic acid	0.032	24	80.86	25.65	3.43	3.21	3.16	0.028	0.038
		6	110.39	3.21	4.75	25.65	2.67		
Caffeic acid	nd	56	101.8	56.73	3.42	7.09	4.44	0.095	0.10
		28	99.5	7.09	4.73	56.73	6.50		
p-Coumaric acid	nd	30	104.5	30.33	3.98	3.79	6.13	0.008	0.053
		15	82.6	3.79	4.36	30.33	6.81		
Quercetin	nd	40	92.6	39.97	6.75	5.00	5.82	nd	nd
		20	95.5	4.99	2.41	39.97	1.17		
Kaempferol	nd	40	78.7	40.60	3.10	5.07	0.49	0.468	0.76
		20	77.3	5.07	1.90	40.6	3.46		
Ellagic acid	1.05	40	74.8	60.06	2.33	10.01	4.01	2.82	6.70
		20	81.8	10.01	5.12	60.06	6.95		

nd. "No data". Selectivity for monogalloyl glucoside, malonyl cafeoilquinic acid and kaempferol glucoside were 6.64, 3.95 and 14.24 respectively. Validations samples were determined in triplicate.

Table 2
Characterization of phenolic compounds in strawberry purée extracts using LC with diode array and electrospray ionization MS detection.

Peak	Tentative identification	Rt (min)	max (nm)	MW	MS (*m/z*), ID	MS^2 ions (*m/z*)	Detection	Reference
Hydroxybenzoic								
1	Gallic acid	9.5	275	170	169	125; 78	DAD	*
Hydrolized Tanins								
3	HHDP-glucose	23.6		482	481	**301**; 275; 249	MS	Hanhineva et al. (2008)
5	Bis-HHDP-glucose	24.5		784	783	481; 301	MS	Aaby, Ekeberg, and et al. (2007)
6	Monogalloyl glucose	26.6	280	332	331	**169**; 125	MS	Sandhu and Gu (2010), Hanhineva et al. (2008)
7	Tris-galloyl-HHDP-hexose	27.2		952	951	**907**; 783; 605; 463; 301; 201	MS	Del Bubba et al. (2012)
8	HHDP-galloylglucose	27.3		634	633	463; 481; **301**; 275	DAD-MS	Aaby, Ekeberg, and et al. (2007)
17	Galloyl-bis-HHDP-glucose	33.5		935	934	**633**; 301	MS	Aaby, Ekeberg, and et al. (2007)
Ellagic acid and derivates								
9	Ellagic acid pentoside	27.8	252;375	434	433	301	MS	Aaby et al. (2012)
23	Ellagic acid deoxyhexoside	39.4	254;370	448	447	**300**; 257	MS	Aaby et al. (2012)
27	Ellagic acid	45.0	257;358	302	301	284; 145	DAD-MS	*
Flavanols								
2	(+)-Catechin	23.0	280	290	289	245; 109	DAD-MS	*
4	(-)-Epicatechin	24.0	282	290	289	245; 109	DAD-MS	*
13	(-)-Epicatechin gallate	30.6	280	444	443	289; 169	DAD-MS	*
Condensed tannins								
10	Procyanidin B1	28.3	280	–	577	451; 425; 407; **289**	DAD-MS	*
12	Procyanidin trimer	29.1		866	865	739; **695**; **577**; 408	MS	Aaby, Ekeberg, and et al. (2007)
Flavonols								
15	Apigenin-7-O-glucoside	32.6	–	432	431	311; **269**; 225; 270	MS	*
21	Kaempferol-3-glucoside	34.4	268;348	448	447	**285**; 257	DAD-MS	*
22	Quercetin rutinoside	38.0	–	610	609	**301**; 179; 151	MS	Seeram et al. (2006)
25	Kaempferol –O-coumaroyl hexoside	41.4	–	594	593	307; **285**	DAD-MS	Del Bubba et al. (2012)
26	Quercetin-3-glucuronide	42.0	–	478	477	**301**; 151; 179	MS	Aaby, Ekeberg, and et al. (2007)
28	Quercetin-O-hexoside	42.3		464	463	**300**; 271; 255; 179	MS	Gouveia et al. (2009)
29	Luteolin 3́-O-glucuronide	45.8	–	462	461	**285**; 241	MS	*
30	Kaempferol	46.7	270;375	286	285	117; 93	DAD-MS	*
31	Kaempferol malonylglucoside	47.3	–	490	489	**285**; 257	MS	Ornelas-Paz et al. (2013)
Hydroxycinnamic acid								
11	Caffeic acid hexoside	28.9		342	341	179; **161**; 135	DAD-MS	Hanhineva et al. (2008)
14	*p*-Coumaroyl hexose	32.4	311	326	325	163; **145**	DAD-MS	Aaby, Ekeberg, and et al. (2007)
16	*p*-Coumaric acid	33.3		164	163	**119;** 93	DAD-MS	*
18	Malonyl caffeoylquinic acid	33.7	323	396	439	395	DAD-MS	Gavrilova et al. (2011)
19	Ferulic acid hexose derivative	34.0		450	449	**269;** 287; 193	DAD-MS	Ornelas-Paz et al. (2013)
24	Cinnamic acid	40.0	282	148	147	103; 77	DAD-MS	*
32	Caffeoylglucaric isomer	48.1	–	372	371	209	MS	Ruiz et al. (2013)
Stilbens								
20	*trans*-Resveratrol glucoside	34.3	313	390	389	**227**; 185	DAD-MS	*

* Identification of the compound was confirmed by the authentic standards. The most abundant ions are shown in bold.

3.2.1. Hydrolyzed Tannins

Peak 2 showed a $[M\text{-}H]^-$ molecular ion at *m/z* 331, and MS-MS fragments at *m/z* 271 and 169, which confirms monogalloyl glucose identification (Hanhineva et al., 2008; Sandhu & Gu, 2010). Additionally, its UV spectra were similar to those of the tannin compounds family (penta-O-galloyl-ß-D-glucoside standard). Further to this, HHDP-galloylglucoside, galloyl-bis-HHDP-glucose and bis-HHDP-glucose were identified through matching with the three deprotonated molecules $[M\text{-}H]^-$ at *m/z* 633, 934 and 783 respectively, as has previously been described in the literature (Aaby et al., 2012; Sandhu & Gu, 2010; Aaby, Ekeberg, & Skrede, 2007; Seeram, Lee, Scheuller, & Herber, 2006). Fig. 2-A shows the hypothesized structure and fragmentation of HHDP-galloylglucoside.

Another ellagitanin was identified as tris-galloyl-HHDP-hexoside (peak 7) having a $[M\text{-}H]^-$ at *m/z* 951. The fragmentation produced *m/z* 907 (loss of carboxilic group, 44 Da), *m/z* 783 (loss of gallic acid unit), *m/z* 463 (loss of tris-galloyl group, 507 Da), *m/z* 605 (deriving from *m/z* 907 though the loss HHDP unit, 302 Da) and *m/z* 301 (obtained from *m/z* 463 through the loss of a hexose unit). The product ion of *m/z* 301 was 201, consistent with ellagic acid (Fig. 2-B) (Del Bubba et al., 2012). Additionally, at 23.6 min, peak 3 showed $[M\text{-}H]^-$ at *m/z* 481 and its fragmentation produced *m/z* 301 after loss of a glucose unit (180 Da). Fig. 2-C displays the fragmentation of compound identified as HHDP-glucose. This tannin has been reported before in strawberry sepals, by Hanhineva et al. (2008).

3.2.2. Flavanols and condensed tannins

The LC-DAD and MS analysis confirmed the presence of gallic acid, (+)-catechin, (-)-epicatechin gallate, (-)-epicatechin, procyanidin dimer and trimer (Table 2).

3.2.3. Ellagic acid and derivatives

Free ellagic acid (peak 27) was identified by its retention time, UV–visible spectrum and characteristic MS spectral data in accordance with those of the authentic standard. The MS analyses confirmed the presence of a peak at *m/z* 301, and main MS-MS fragments at *m/z* 284 and 145, consistent with ellagic acid. Additionally, peaks 9 and 23 were identified as ellagic acid derivatives, based on their similar UV–visible spectrum and their MS

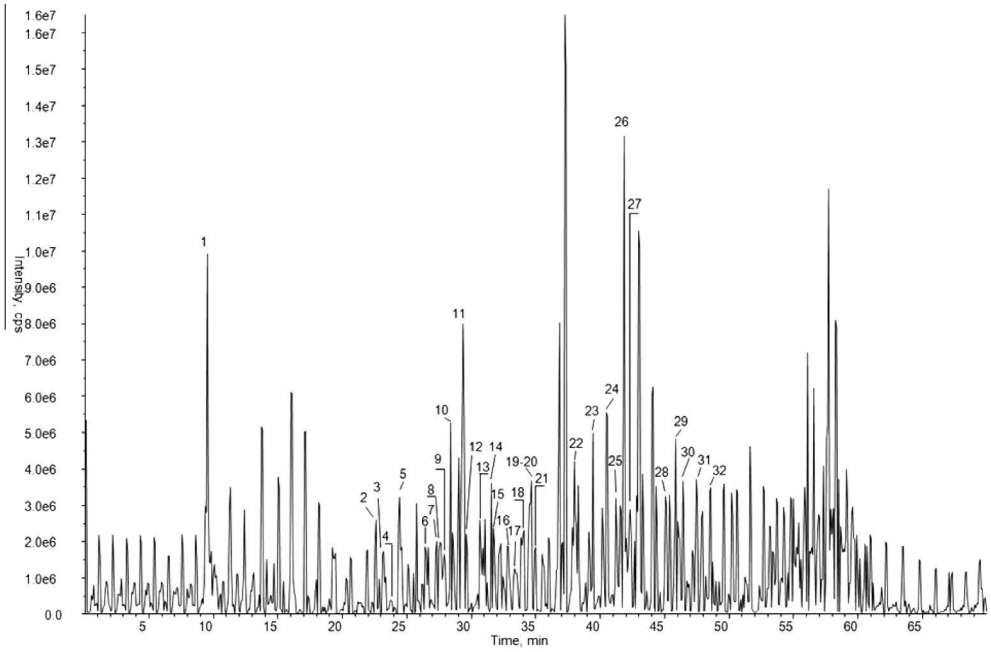

Fig. 1. LCMS chromatogram of sample. The peak numbers corresponding to displays in identification table.

characteristics. Both peaks showed precursor ions at *m/z* 433 and *m/z* 447, respectively, and product ions at *m/z* 301, corresponding to ellagic acid pentoside and ellagic acid deoxyhexoside, respectively, in accordance with data reported by Aaby et al. (2012).

3.2.4. Hydroxycinnamic acids

The MS analyses confirmed the presence of *p*-coumaroyl hexoside at *m/z* 325, *p*-coumaric acid at *m/z* 163, caffeic acid hexoside at *m/z* 341 and ferulic acid hexose derivative at *m/z* 449, previously reported in strawberries (Aaby, Ekeberg, & et al., 2007; Fang, Yu, & Prior, 2002; Ornelas-Paz et al., 2013). Two compounds were tentatively identified as malonyl caffeoylquinic acid at *m/z* 439 and caffeoylglucaric isomer at *m/z* 371 (Ruiz et al., 2013). Due to their low concentration, it was possible to obtain only the main ion fragments of each compound: *m/z* 395 and *m/z* 209, respectively. Malonyl caffeoylquinic acid has already been reported in blueberries, red and black currants and *Erigeron breviscapus* (Gavrilova, Kajdzanoska, Gjamovski, & Stefova, 2011; Zhang, Shi, Qu, Cheng, 2007) and caffeoylglucaric isomer in *Berberis microphylla G. Forst* (Ruiz et al., 2013), but not in strawberries.

3.2.5. Flavonol glycosides

Kaempferol conjugates were identified as follows: kaempferol-O-coumaroyl hexoside at *m/z* 593, kaempferol-3-glucoside at *m/z* 447, and kaempferol malonylglucoside at *m/z* 489. All of them produced the same aglycone cation at *m/z* 285, in accordance with data reported before by Aaby et al. (2012), Aaby et al. (2007). The occurrence of kaempferol aglycon in berry fruits is infrequent. It has already been described in Finnish strawberries (Määtta et al., 2004; Ornelas-Paz et al., 2013; Häkkinen, Kärelamp, Heinonen, Mykka, & Törönen, 1999), but not in varieties harvested in Spain.

Quercetin-3-glucuronide and quercetin rutinoside were also identified in our samples, showing molecular ion at *m/z* 477 (MS^2 fragment at *m/z* 301) and at *m/z* 609 (MS^2 fragments at *m/z* 301, 179 and 151), respectively, which is consistent with the literature (Aaby, Ekeberg, & et al., 2007; Seeram et al., 2006).

Apigenin-7-O-glucose and luteolin 3′-O-glucuronide were identified with authentic standards through their molecular ions $[M-H]^-$ at *m/z* 431 and *m/z* 461, respectively, and MS^2 fragments at *m/z* 269, 225 and 311 for apigenin-7-O-glucoside; *m/z* 285 and 241 for luteolin 3-O-glucuronide. Fig. 2-E shows hypothetic fragment pattern. These compounds have been reported before in rosemary, oregano, sage, basil and thyme (Gouveia & Castilho, 2009; Hossain, Rai, Brunton, Martin-Diana, & Barry-Ryan, 2010). Nevertheless apigenin derivatives were reported in strawberries by Ornelas-Paz et al., 2013; and luteolin aglycone by Kadivec et al., 2013. However, as far as we know, this is the first time apigenin-7-O-glucose and luteolin-3-O-glucuronide have been reported in strawberries. Fig. 2-D shows the hypothesized structure and fragmentation of apigenin-7-O-glucose.

3.2.6. Stilbens

Trans-resveratrol glucoside, at *m/z* 389, was identified for the first time in these samples, according to the authentic standard (Fig. 2-F). Although small quantities of *trans*-resveratrol have been found in strawberries (Ehala, Vaher, & Kaljurand, 2005), *trans*-resveratrol glucoside has not been identified in strawberries before.

To sum up, thirty-two phenolic compounds were identified in the strawberry extract. Malonyl caffeoylquinic acid, caffeoylglucaric isomer, *trans*-resveratrol-glucoside, apigenin-7-O-glucose and luteolin-3-O-glucuronide were reported for the first time in strawberries.

3.3. Non-anthocyanin phenolic composition of strawberry products

Tables 3 summarize the concentrations of phenolic compounds in the strawberry purée samples (2011 and 2012 harvests) during the different production steps and for the final products. A total of eighteen compounds were quantified.

Considering our strawberry purée samples, the content of free and conjugated forms of gallic acid (Table 4) and 5 are similar to those already reported for the Camarosa variety (132 mg kg^{-1} fw), which is the most harvested variety in Spain (Buendia et al., 2010). Conversely, the content of the *Jonsok* variety is higher than in our results (333 mg kg^{-1} fw) (Määtta et al., 2004).

The most abundant non-anthocyanic compounds quantified were (+)-catechin and HHDP-galloylglucoside (182.8–114.3 mg kg^{-1} fw in the final product). This last one has been reported before, as a

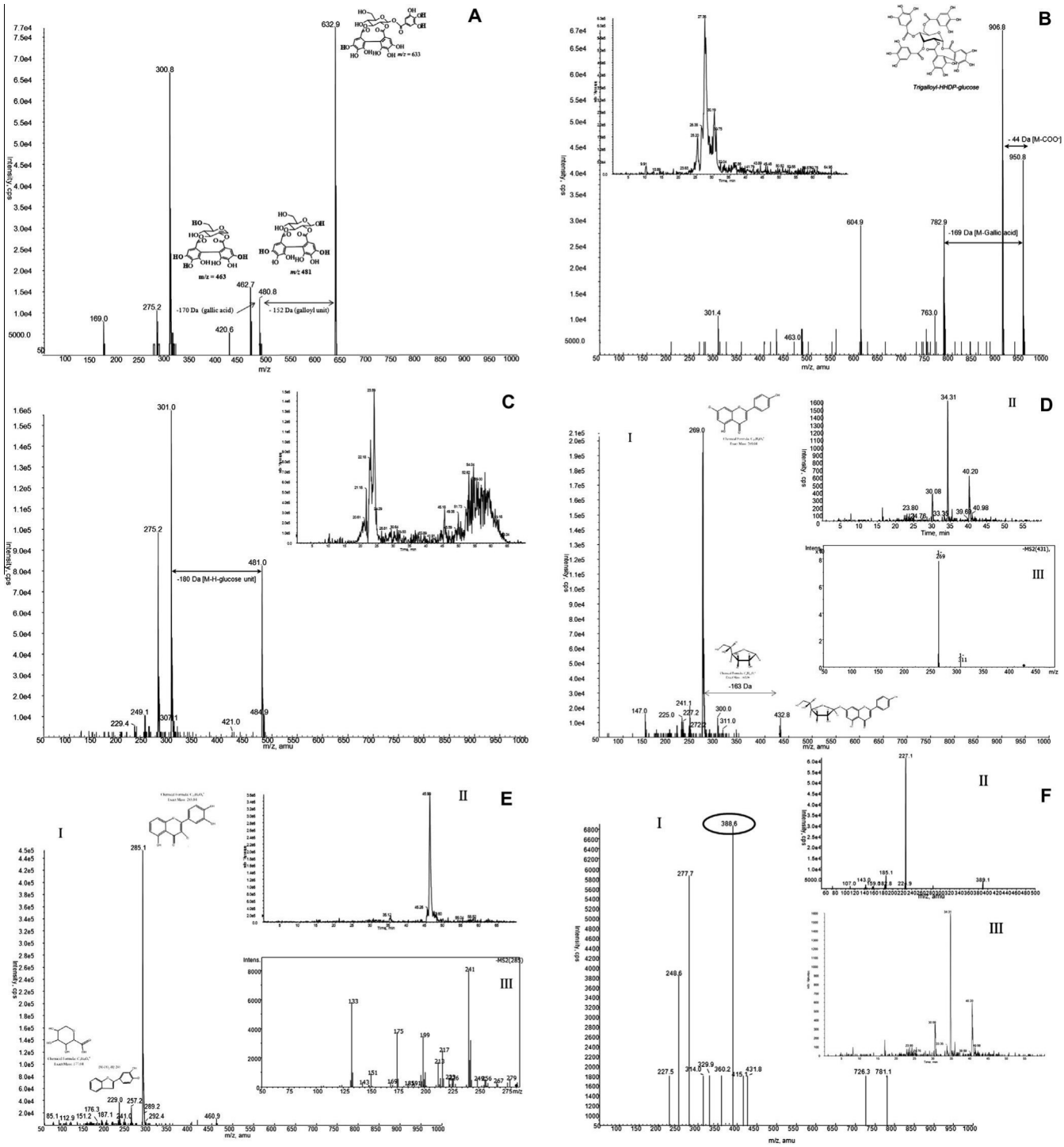

Fig. 2. LC-MSMS, structures and hypothetized fragmentations patterns (A) $[M - H]^-$ ion 633, HHDP-galloylglucose (B) $[M - H]^-$ ion 951, tris-galloyl-HHDP-hexose (C) LC-MSMS $[M - H]^-$ ion 481, HHDP glucoside (D) LC-MSMS $[M - H]^-$ ion 431, of Apigenin-7-O-glucoside in sample (I), xic chromatogram shows retention time (II) and MS^2 chromatogram of apigenin-7-O-glucoside standard (III). (E) LC-MSMS $[M - H]^-$ ion 461 of Luteolin-3-O-glucuronide in sample (I), xic chromatogram shows retention time (II) and MS^3 chromatogram of luteolin aglycon standard (III) shows transition *m/z* 285–241. (F) LC-MS of ion precursor *m/z* 227 experiment of *trans*-resveratrol $[M-H]^-$ 389 (I), fragmentation pattern of standard (II) and chromatogram of MRM experiment to shows retention time in sample (III).

major class of phenolic compounds in strawberries (Aaby, Ekeberg, & et al., 2007). Indeed, we found a remarkably high flavanol content, especially for (+)-catechin (123.7–211.8 mg kg^{-1} fw and 40.1–227.4 mg kg^{-1} fw in the final products, 2011 and 2012 harvests, respectively), compared with reported values: 4.9–5.8 mg kg^{-1} fw (Oszmianski & Wojdylo, 2009), 24 mg kg^{-1} fw (Määttä et al., 2004), and 25–81 mg kg^{-1} fw (Aaby, Ekeberg, & et al., 2007).

Furthermore, other flavanols, such as (-)-epicatechin, (-)-epicatechin gallate and the procyanidin B1, were also quantified (2.9–42 mg kg^{-1} fw; 9–45 mg kg^{-1} fw and 10–45 mg kg^{-1} fw, respectively). *p*-Coumaroyl hexoside and caffeic acid hexoside, were the most abundant hydroxycinnamic acids (16.0–40.8 mg kg^{-1} and 48.2–38.5 mg kg^{-1}, respectively, in final products).

Table 3
Concentrations (mg kg^{-1} of fresh weight) of non-anthocyanin phenolic compounds in strawberries purées. 2011 and 2012 harvest.

Compounds	M_{11A}	M_{11B}	M_{11C}	EI_{11A}	EI_{11B}	EI_{11C}	
Gallic acid	34.04±2.0^{bd}	28.50±6.19^{bd}	27.2±1.3^{b}	27.1±2.8^{ad}	16.3±2.7^{ad}	25.3±0.4^{ad}	
Monogalloyl glucoside	45.26 ± 1.17^{bde}	36.2 ± 2.7^{de}	31.9 ± 0.5^{bde}	25.4 ± 1.4^{ade}	15.7 ± 1.7^{de}	20.1 ± 1.4^{ade}	
Ellagic acid pentoside	13.9 ± 1.6^{de}	11.5 ± 0.8^{e}	10.9 ± 0.9^{de}	nd	nd	nd	
Ellagic acid	59.2 ± 6.8^{bde}	59.1 ± 7.5^{be}	53.3 ± 6.9^{be}	23.0±3.3^{ae}	19.5 ± 6.3^{ade}	9.6 ± 1.9^{ae}	
HHDP-galloylglucoside	150.6 ± 12.9^{be}	113.22 ± 10.5^{bde}	128.8 ± 0.94^{d}	193.3 ± 11.0^{ade}	206.9 ± 2.0^{ade}	160.5 ± 8.6^{ade}	
(+)-Catechin	222.6±2.2^{b}	119.2 ± 0.4^{be}	168.1 ± 4.9^{bde}	314.1 ± 10.4^{a}	239.2 ± 4.3^{ade}	207.2 ± 6.4^{ad}	
(-)-Epicatechin	25.1 ± 1.4	37.7 ± 3.0	42.0 ± 0.8^{bde}	25.00	nd	nd	
(-)-Epicatechin gallate	38.1 ± 0.9^{bde}	35.03 ± 0.8^{bde}	39.7 ± 0.5^{bde}	50.22 ± 0.12^{ae}	13.3 ± 27.0	65.5 ± 2.4^{ade}	
Procyanidin B1	13.6 ± 1.4^{de}	11.8 ± 1.9^{bde}	10.9 ± 0.4^{bde}	85.6 ± 3.7	32.4 ± 2.3^{ae}	34.7 ± 0.4^{ade}	
Ferulic acid hexose derivative	4.6 ± 0.4^{be}	5.1 ± 0.14^{bde}	5.1 ± 0.6^{be}	2.5 ± 0.11^{ade}	3.0 ± 0.15^{a}	2.2 ± 0.8^{ad}	
p-Coumaroyl hexoside	38.3 ± 0.7^{bde}	56.2 ± 0.8^{bde}	30.2 ± 0.3^{bde}	37.0 ± 2.3^{ad}	39.2 ± 1.0^{ade}	40.9 ± 2.9^{ade}	
Caffeic acid hexoside	36.6 ± 0.7^{be}	nd	39.1 ± 1.4^{be}	46.6 ± 2.5^{a}	42.5 ± 1.8^{e}	48.0 ± 3.0^{ae}	
p-Coumaric acid	2.6 ± 0.07^{bde}	2.6 ± 0.2^{bd}	1.93 ± 0.2^{bde}	1.5 ± 0.2^{a}	0.8 ± 0.02^{ade}	0.8 ± 0.12^{a}	
Cinnamic acid	0.71 ± 0.08^{bd}	0.83 ± 0.07	0.64 ± 0.06	nd	0.48 ± 0.00	0.33 ± 0.10	
Kaempferol	nd	nd	nd	nd	1.2 ± 0.9^{de}	nd	
Kaempferol-3-glucoside	3.26 ± 0.16^{bde}	3.06 ± 0.09^{bde}	3.95 ± 0.16^{bde}	1.72 ± 0.16^{ae}	1.4 ± 0.3^{ae}	3.20 ± 0.11^{ade}	
Kaempferol malonylglucoside	1.31 ± 0.21^{d}	nd	1.67 ± 0.11^{be}	1.6 ± 0.8^{d}	nd	nd	
trans-Resveratrol glucoside derivative	1.23 ± 0.02^{e}	0.7 ± 0.3	1.14 ± 0.03	nd	0.72 ± 0.04^{d}	0.71 ± 0.06	
Compounds	FPS_{11A}	FPS_{11B}	FPS_{11C}	$FPWS_{11A}$	$FPWS_{11B}$	$FPWS_{11C}$	
Gallic acid	21.1 ± 1.7^{ab}	15.6 ± 0.5^{a}	24.2 ± 1.0^{a}	16.9 ± 3.0^{ab}	20.3 ± 0.5^{ab}	4.0 ± 0.5^{ab}	
Monogalloyl glucoside	34.0 ± 1.9^{abe}	24.3 ± 2.6^{abe}	40.30 ± 1.05^{abe}	20.1 ± 2.5^{abd}	29.9 ± 0.6^{abd}	3.9 ± 0.5^{ad}	
Ellagic acid pentoside	26.29 ± 10.01^{ae}	53.1 ± 0.9	52.8 ± 7.4^{ae}	37.1 ± 2.6^{ad}	49.9 ± 1.4^{a}	34.7 ± 1.7^{ad}	
Ellagic acid	28.1 ± 4.3^{a}	70.5 ± 0.6^{b}	59.5 ± 2.4^{be}	34.0 ± 6.6^{ab}	45.5 ± 3.8^{ab}	31.9 ± 2.6^{abd}	
HHDP-galloylglucoside	142.8 ± 10.9^{be}	182.8 ± 12.3^{ab}	143.0 ± 3.0^{a}	115.4 ± 14.9^{abe}	133.1 ± 1.1^{ab}	139.0 ± 3.1^{b}	
(+)-Catechin	158.03 ± 0.02^{ad}	123.7 ± 1.4^{abe}	160.1 ± 1.6^{abe}	200.2 ± 6.3^{ab}	197.72v1.06^{bd}	211.8 ± 1.7^{ad}	
(-)-Epicatechin	101.5 ± 11.6	79.0 ± 15.8	16.5v1.8^{ae}	15.5 ± 0.3^{a}	14.89 ± 0.22	13.5 ± 0.3^{abd}	
(-)-Epicatechin gallate	53.53 ± 2.23^{a}	51.26 ± 1.05^{ab}	54.5 ± 0.8^{ab}	56.4 ± 0.4^{ab}	65.1 ± 0.4^{a}	58.5 ± 1.0^{ab}	
Procyanidin B1	23.85v0.05^{ad}	28.36 ± 0.01^{a}	9.13 ± 3.24^{abe}	19.98 ± 1.02^{ad}	21.9 ± 0.3^{ab}	22.05 ± 1.00^{abd}	
Ferulic acid hexose derivative	4.0 ± 0.4^{bd}	2.7 ± 0.6^{ad}	5.0 ± 0.3^{bd}	1.6 ± 0.2^{abd}	1.9 ± 0.01^{abd}	1.5 ± 0.3^{ad}	
p-Coumaroyl hexoside	53.3v10.3^{abe}	46.9 ± 0.9^{abe}	64.2 ± 0.4^{abe}	38.4 ± 2.4^{ad}	40.8 ± 0.6^{abd}	34.3 ± 1.2^{abd}	
Caffeic acid hexoside	nd	nd	nd	48.2 ± 6.8^{a}	45.8 ± 08^{bc}	41.7 ± 1.0^{ab}	
p-Coumaric acid	0.9 ± 0.3^{a}	1.3 ± 0.2^{abe}	0.9 ± 0.2^{a}	1.3 ± 0.09^{ad}	2.1 ± 0.03^{bd}	0.9 ± 0.1^{a}	
Cinnamic acid	0.57 ± 0.11^{a}	0.75 ± 0.24	0.57 ± 0.06	0.30 ± 0.08	0.41 ± 0.03	0.53 ± 0.02	
Kaempferol	0.71 ± 0.18^{d}	0.95 ± 0.08^{be}	0.6 ± 0.7^{be}	1.04 ± 0.19^{d}	0.80 ± 0.01^{bd}	0.53 ± 0.005^{bd}	
Kaempferol-3-glucoside	1.9 ± 0.6^{a}	1.3 ± 0.6^{ae}	2.24 ± 0.06^{abe}	2.37 ± 0.18^{ab}	2.37 ± 0.05^{abd}	2.46 ± 0.012^{abd}	
Kaempferol malonylglucoside	1.34 ± 1.15^{ab}	nd	nd	1.01 ± 0.11	1.21 ± 0.01	1.32 ± 0.006^{ab}	
trans-Resveratrol glucoside derivative	nd	nd	0.69 ± 0.004	0.75 ± 0.007	0.89 ± 0.006	nd	
Compounds	M_{12A}	M_{12B}	M_{12C}	EI_{12A}	EI_{12B}	EI_{12C}	
Gallic acid	69.4 ± 1.8	37.7 ± 3.3	47.0 ± 1.5	30.7 ± 1.3^{a}	31.6 ± 0.6^{a}	42.8 ± 1.5	
Gallic acid derivate 1	68.0 ± 12.7	27.1 ± 0.7	30.4 ± 1.1	110.4v0.9	34.1 ± 0.7	35.3 ± 1.6^{a}	
Gallic acid derivate 2	nd	45.5 ± 1.6	59.4 ± 3.1	59.1 ± 4.8	57.6 ± 1.7^{a}	79.7 ± 8.0	
Monogalloyl glucoside	11.6 ± 3.9	5.6 ± 2.4	5.9 ± 3.8	nd	4.6 ± 0.5	9.9 ± 1.7	
Ellagic acid	11.2 ± 3.0	10.5 ± 3.4	16.4 ± 6.9	12,2 ± 9.5	12.3 ± 0.5	11.7 ± 2.1	
Ellagic pentoside	12.7 ± 1.6	7.5 ± 2.4	10.0 ± 0.8	9.0 ± 0.3^{a}	8.7 ± 0.6	11.7 ± 1.3	
Ellagic deoxyhexoside	20.6 ± 6.4	14.6 ± 2.0	16.4 ± 6.9	9.8 ± 0.7^{a}	9.8 ± 0.7^{a}	13.1 ± 1.5	
HHDP-galloylglucoside	146.1 ± 3.1^{cde}	127.2 ± 18.1^{bc}	133.2 ± 19.6^{c}	160.3 ± 11.2^{cde}	157.2 ± 3.6^{acd}	152.3 ± 14.1^{ce}	
(+)-Catechin	253.1 ± 4.5	318.3 ± 29.4	379.2 ± 15.9	259.0 ± 14.5	261.3 ± 22.7	379.3 ± 16.8	
(-)-Epicatechin	4.9 ± 0.7	2.9 ± 1.8	12.0 ± 2.0	10.7 ± 0.3^{a}	6.6 ± 2.5^{a}	10.3 ± 1.3	
(-)-Epicatechin gallate	9.3 ± 0.9	27.2 ± 0.1	40.3 ± 3.7	30.2 ± 5.6^{a}	31.3 ± 3.8	41.5 ± 8.5	
Procyanidin B1	41.8 ± 5.5	34.0 ± 5.6	45.0 ± 1.7	42.7 ± 4.5	46.0 ± 3.6^{a}	48.6v6.4	
Ferulic acid hexose derivative	6.5 ± 0.8^{bcde}	3.4 ± 0.7^{cd}	5.1 ± 0.8^{bce}	2.9 ± 0.17^{acde}	3.1 ± 0.11^{cd}	2.51 ± 0.31^{ace}	
p-Coumaroyl hexoside	22.4 ± 2.2^{de}	20.3 ± 8.6	25.5 ± 7.6^{be}	19.9 ± 5.6	18.0 ± 3.5	15.1 ± 1.6	
Caffeic acid hexoside	27.2 ± 6.9	26.5 ± 3.7^{bd}	29.0 ± 1.2	29.9 ± 4.0^{c}	30.9 ± 4.1^{d}	27.6 ± 0.9^{e}	
p-Coumaric acid	2.7 ± 0.1^{bcde}	0.6 ± 0.2	1.1 ± 0.2^{ce}	0.48 ± 0.02^{ade}	0.6 ± 0.02^{cd}	0.7 ± 0.4^{e}	
Cinnamic acid	0.7 ± 0.04	nd	0.8 ± 0.04	nd	nd	0.5 ± 0.05^{a}	
Kaempferol-3-glucoside	1.2 ± 0.7	0.5 ± 0.15	nd	1.2 ± 0.03	1.1 ± 0.3	nd	
K. coumaroylglucoside	nd	nd	nd	nd	1.04 ± 0.19	nd	
Compounds	UP_{12A}	UP_{12B}	UP_{12C}	FPS_{12A}	FPS_{12B}	$FPWS_{12A}$	$FPWS_{12C}$
Gallic acid	nd	nd	30.4 ± 0.7^{b}	28.2 ± 3.4^{a}	27.5 ± 0.5^{ab}	26.6 ± 0.4^{ab}	27.0 ± 0.3^{abd}
Gallic acid derivate 1	30.1 ± 0.7^{a}	34.8 ± 1.6	43.5 ± 12.7	33.2 ± 0.5^{abd}	42.2 ± 0.7^{bd}	38.8 ± 0.8^{bd}	39.1 ± 2.6^{a}
Gallic acid derivate 2	37.4 ± 4.6^{b}	31.7 ± 2.1^{ab}	35.8 ± 5.6^{ab}	32.8 ± 3.4^{b}	28.5 ± 2.1^{ab}	33.3 ± 1.0^{b}	39.4 ± 0.5^{ab}
Monogalloyl glucoside	4.5 ± 1.1^{a}	4.0 ± 0.10	2.9 ± 0.17^{b}	nd	2.9 ± 0.6^{b}	4.2 ± 0.17^{a}	10.0 ± 0.6^{d}
Ellagic acid	8.7 ± 2.9	7.7 ± 0.4^{b}	8.0 ± 1.6	9,6 ± 1.8	12.5 ± 1.6^{c}	9.7 ± 1.9	11.0 ± 0.21^{bd}
Ellagic pentoside	3.7 ± 1.3^{ab}	5.1 ± 0.18^{b}	8.2 ± 2.4^{b}	7.7 ± 1.0^{abd}	10.0 ± 3.6^{d}	8.2 ± 1.5a	8.8 ± 0.4^{ab}
Ellagic deoxyhexoside	4.7 ± 0.8^{ab}	nd	nd	nd	nd	nd	nd
HHDP-galloylglucoside	63.9 ± 9.9^{abde}	61.4 ± 6.9^{abd}	82.7 ± 17.9^{abe}	114.3 ± 18.7^{abc}	118.6 ± 6.1^{bc}	114.4 ± 11.5^{abc}	119.0 ± 0.58^{bc}
(+)-Catechin	258.9 ± 21.8	41.5 ± 5.1^{ab}	243.0 ± 1.8^{ab}	40.1 ± 6.8^{abd}	171.2 ± 7.0^{abd}	147.0 ± 8.1^{abde}	227.4 ± 2.3
(-)-Epicatechin	2.7 ± 0.3^{ab}	4.4 ± 1.0^{b}	4.7 ± 2.2^{ab}	4.5 ± 1.9^{b}	0.7 ± 0.2^{bd}	3.1 ± 0.4^{ab}	5.5 ± 0.7^{ab}
(-)-Epicatechin gallate	22.1 ± 3.7^{a}	44.0 ± 5.0	34.8 ± 7.2	23.4 ± 2.0^{a}	21.7 ± 1.2^{b}	25.9 ± 1.5^{a}	35.1 ± 1.9^{a}
Procyanidin B1	30.1 ± 0.8^{b}	34.0 ± 5.2^{b}	27.4 ± 7.4^{ab}	16.1 ± 3.4^{abd}	24.9 ± 1.6^{abd}	19.6 ± 3.3^{bd}	25.6 ± 1.9^{ab}
Ferulic acid hexose derivative	0.46 ± 0.03^{abde}	0.66 ± 0.03^{abd}	1.02 ± 0.43^{ab}	1.28 ± 0.22^{abce}	1.59 ± 0.18^{abc}	0.93 ± 0.14^{abcd}	1.16 ± 0.10^{ace}

Table 3 (*continued*)

Compounds	M_{11A}	M_{11B}	M_{11C}	EI_{11A}	EI_{11B}	EI_{11C}	
p-Coumaroyl hexoside	23.7 ± 2.4[ade]	22.3 ± 7.2[a]	19.1 ± 3.3[a]	14.2 ± 2.8[ac]	16.0 ± 1.7[a]	16.0 ± 1.2[ac]	15.5 ± 1.1[a]
Caffeic acid hexoside	18.7 ± 1.02[bde]	27.9 ± 4.5[d]	28.7 ± 1.2	24.3 ± 2.9[ce]	26.1 ± 1.5[abc]	32.5 ± 1.1[cd]	29.4 ± 0.9[b]
p-Coumaric acid	0.4 ± 0.06[ade]	0.8 ± 0.07[bd]	0.7 ± 0.2[ae]	0.1 ± 0.07[abc]	0.11 ± 0.04[bc]	0.2 ± 0.08[abc]	0.12 ± 0.03[ac]
Cinnamic acid	0.5 ± 0.1	1.2 ± 0.01	0.9 ± 0.3[b]	nd	0.5 ± 0.01	nd	nd
Kaempferol-3-glucoside	0.9 ± 0.02	0.9 ± 0.03[b]	1.1 ± 0.6	1.7 ± 0.4[d]	1.9 ± 0.08[bd]	nd	0.6 ± 0.05[bd]
K. coumaroylglucoside	nd	nd	0.48 ± 0.09	0.73 ± 0.04	1.27 ± 26.43[bd]	0.41 ± 0.03	1.24 ± 0.92[b]

Mean values and standard deviation.
[a] Superscript letter indicate significant difference ($p < 0.05$) compared to the mashed step (M) of the same substrate,
[b] Superscript letter indicate significant difference ($p < 0.05$) compared to the enzymatic inactivation step (EI) of the same substrate,
[c] Superscript letter indicate significant difference ($p < 0.05$) compared to the unpasteurized sample (UP) of the same substrate,
[d] Superscript letter indicate significant difference ($p < 0.05$) compared to the purée with seeds of the same substrate (FPS),
[e] Superscript letter indicate significant difference ($p < 0.05$) compared to the purée without seeds (FPWS) of the same substrate by ANOVA statistical test. HHDP-galloylglucoside was quantified as ellagic acid. Samples were determined in duplicate. nd: no data. K: kaempferol. Subscript 11 and 12 indicate the year of the harvest.

Table 4
Antioxidant activity in strawberry samples.

2011 Harvest			2012 Harvest		
Samples	ORAC (μmol g^{-1} fw)	DPPH (mmol kg^{-1} fw)	Samples	ORAC (μmol g^{-1} fw)	DPPH (mmol kg^{-1} fw)
M_{11A}	12.5 ± .0.4	24.3 ± 3.4[b]	M_{12A}	13.3 ± 1.8	16.8 ± 2.8[b]
M_{11B}	13.1 ± 6.5[c]	23.45 ± 4.01[c]	M_{12B}	13.01 ± 4.13	20.10 ± 2.25[bcd]
M_{11C}	21.3 ± 4.6[b]	22.4 ± 1.3[b]	M_{12C}	16.63 ± 2.12	19.0 ± 3.3[bce]
EI_{11A}	19.9 ± 10.9[a]	32.2 ± 1.5[ad]	EI_{12A}	12.76 ± 3.25	13.5 ± 1.9[ad]
EI_{11B}	15.5 ± 1.8	28.47 ± 3.23[cd]	EI_{12B}	8.39 ± 1.03	13.50 ± 4.24[a]
EI_{11C}	15.5 ± 1.8	26.5 ± 1.5	EI_{12C}	16.8 ± 1.4	22.24 ± 3.04[ac]
FPS_{11A}	13.92 ± 2.62	14.63 ± 1.01[bd]	UP_{12A}	11.7 ± 2.3	15.1 ± 2.8
FPS_{11B}	11.00 ± 3.24[abd]	14.68 ± 1.20[bd]	UP_{12B}	13.20 ± 2.06	10.5 ± 0.6[ad]
FPS_{11C}	8.9 ± 1.4	23.4 ± 1.8	UP_{12C}	10.3 ± 1.3	16.0 ± 2.5[abe]
$FPWS_{11A}$	15.6 ± 2.9[b]	21.5 ± 5.5[b]	FPS_{12A}	17.5 ± 2.6	16.4 ± 1.4[b]
$FPWS_{11B}$	11.5 ± 1.5[b]	23.7 ± 1.7[bc]	FPS_{12B}	11.3 ± 3.9	16.8 ± 3.0[ac]
$FPWS_{11C}$	13.7 ± 2.4[bc]	24.5 ± 3.7	$FPWS_{12A}$	16.57 ± 3.12	14.9 ± 4.5
–	–	–	$FPWS_{12C}$	11.51 ± 1.09	22.6 ± 2.5[ac]

Mean values and standard deviation.
[A] Superscript letter indicate significant difference ($p < 0.05$) compared to the mashed step (M),
[B] Superscript letter indicate significant difference ($p < 0.05$) compared to the enzymatic inactivation step (EI),
[C] Superscript letter indicate significant difference ($p < 0.05$) compared to the unpasteurized step (UP),
[D] Superscript letter indicate significant difference ($p < 0.05$) compared to the purée with seeds (FPS),
[E] Superscript letter indicate significant difference ($p < 0.05$) compared to the purée without seeds (FPWS), by ANOVA statistical test. Antioxidant samples were determined in triplicate.

The predominant flavonols in these samples were kaempferol and derivatives, in contrast to data reported by other authors (Aaby, Ekeberg, & et al., 2007; Buendia et al., 2010; Määtta et al., 2004; Da Silva Pinto, Lajolo, & Genovese, 2008; Del Bubba et al., 2012), who found quercetin derivatives to be the main flavonol in strawberries.

3.4. Changes in non-anthocyanin phenolic composition and antioxidant activity during the purée elaboration process

Gallic acid, monogalloylglucoside and ellagic acid decreased significantly due to enzymatic inactivation ($p < 0.05$) in the 2011 harvest results (Table 3). Indeed, their concentrations were reduced from 10% to 68% in respect of their content in mashed samples. This is in accordance with data obtained after strawberry jam processing with heat, which led to a significant decrease in total ellagic acid (83–25%) (Bakkalbaşi, Menteş, & Artik, 2009). The mashing process causes membrane breakage, which means that oxidation reactions occur more easily when heat is applied (Hartmann et al., 2008). However, while gallic acid also dropped significantly in the 2012 harvest results (Table 3), ellagic acid remained generally unchanged, possibly underestimated due to its low solubility (Aaby et al., 2012). HHDP-galloylglucoside decreased between the mashed and the final product, following the same behavior in both harvests.

Significant changes were observed in the pasteurization process. Between the mashed step and the final product (in the case of (+)-catechin the reduction was 42–20% in 2011 and 40–38.6% in 2012), most of the compounds decreased in concentration, as was also reported by Hartmann et al. (2008) in strawberry juices and purées.

Regardless of whether the purée is processed with or without seeds, there is no significant effect on the non-anthocyanic phenolic composition of the resulting purée. The presence of seeds does not increase the concentration of these bioactives. This fact is probably due to the seeds not being fully crushed during the process. When the seeds are removed (FPWS), there is a concentration effect and higher values are produced, in contrast with FPS. This is in accordance with the higher content of total hydroxycinnamic compounds in FPWS when compared to FPS (21.45–11.97 mg kg^{-1} fw in 2011 and 14.9–10.46 mg kg^{-1} fw in 2012). It corresponds to a reduction from 30.54% to 12.6% between the 2011 and 2012 harvests, in accordance with Aaby, Wrolstad, Ekeberg, and Skrede (2007).

Table 4 displays antioxidant activity data for the purée at the different stages. Antioxidant activity decreased just slightly during the pasteurization step and no significant changes were detected at any point in the process. Despite the effect of processing on antioxidant activity, the final products are an excellent source of bioactive substances, with antioxidant potential for further use as a raw material and ingredient in derived products.

4. Conclusions

An analytical method to extract and determine non-anthocyanic phenolic compounds from strawberries has been suitably validated. This paper reports the tentative identification of malonyl caffeoylquinic acid and caffeoylglucaric isomer. Reveal the identification of *trans*-resveratrol glucoside, apigenin-7-O-glucoside and luteolin-3-O-glucuronide, previously described in other sources but not in strawberries.

These results show that industrial processing does not break the seeds to release the non-anthocyanic phenolic compounds, as may be expected, and purées with or without seeds present a similar composition. The industrial process does not significantly affect the non-anthocyanin phenolic profile (only *trans*-resveratrol glucoside disappears in final products), and strawberry purées are a good source of phenolic compounds with antioxidant potential, being of interest from a nutritional and commercial perspective as ingredients or raw material for further derived food.

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Non-anthocyanin phenolic compounds and antioxidant activity of beverages obtained by gluconic fermentation of strawberry

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ABSTRACT

Strawberry is widely harvested in Spain. It is a highly perishable fruit and surplus production may be used to produce innovative foods. This paper studies the influence of gluconic fermentation by *Gluconobacter japonicus* on the non-anthocyanin content and antioxidant activity of strawberry. A total of 43 non-anthocyanin phenolic compounds were identified. To the best of our knowledge, this paper reports five compounds for the first time in strawberry and its derivatives, tentatively identified as: monogalloyl diglucose, 5-hydroxy feruloyl hexose, dihydrokaempferol hexoside, kaempferol neohesperidoside and (−)chicoric acid. It was further observed that gluconic fermentation leaves in vitro antioxidant activity practically unchanged.
Gluconic fermentation by *Gluconobacter japonicus* can be considered a potential tool for producing fermented beverages. It transforms glucose into gluconic acid, which allows diabetics to consume the drink, while maintaining bioactive compound concentration and antioxidant activity.
Industrial relevance: As strawberry is a highly perishable product, fruits spoil quickly, leading to substantial economic losses. Therefore, the transformation of strawberry surplus into new, longer-lasting products is a financially worthwhile solution for the industry and producers. Our gluconic beverages were elaborated by traditional process using exclusively strawberry as raw material easily transferable to industrial scale. The main innovation aspect relies on developing an innovative product, aiming to fulfill consumers demand who are permanently seeking for different taste and flavors. At the same time its composition in sugars (non-glucose, original fructose content) makes these products very suitable for dietetic purposes.

1. Introduction

Strawberry (*Fragaria* × *ananassa*) is widely harvested in Spain, especially in the south (Huelva). Seasonal overproduction leads to surplus fruit, which is not brought to market. In Spain the production is over 289,900 MT of fresh fruit and was the fourth largest producer in the world (FAOSTAT, 2012). As strawberry is a highly perishable product, fruits spoil quickly, leading to substantial economic losses. Therefore, the transformation of strawberry surplus into new, longer-lasting products is financially worthwhile for the industry and producers.

Fermentation is an ancient process of preserving foods. The benefits of the fermentation process are an extended shelf-life, improvements to the nutritious value of food, improved digestibility and upgraded food safety by pH reduction and elimination of antinutrients (Balasundram, Sundram, & Samman, 2006). As a result of fermentation, a range of secondary metabolites are produced. Some of these are associated with promoting health, like B vitamins and peptides released from food proteins through microbial action. It is therefore not surprising that consuming fermented food has been associated with a healthy lifestyle (Van Boekel et al., 2010).

Bioactive compounds present in food, such as polyphenols, undergo profile changes as a result of the fermentation process. For instance, there is an increase in catechin and procyanidin content during alcoholic fermentation, due to the transfer of these compounds from the solid parts of the grape into the wine (Ricardo-da-Silva, Rosec, Bourzeix, Mourgues, & Moutounet, 1992; Spranger, Sun, Leandro, Cavalho, & Bechior, 1998; Sun et al., 2011). Additionally, Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, and Troncoso (2010a) observed a significant decrease of (+)-catechin (50%), ferulic, caftaric and caffeic acids during acetic acid fermentation.

Strawberry is a good source of bioactive compounds, such as polyphenols, which strongly influence quality and health properties (Buendia et al., 2009; Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, & Troncoso, 2010b; Larrosa, Tomás-Barberán, & Espín, 2006). Regarding the effect of fermentation on the polyphenolic composition of strawberry substrate, it has been observed that alcoholic fermentation significantly decreases the total polyphenol index (Ubeda et al., 2013). A

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similar trend in the total number of monomeric anthocyanins has been observed during acetic acid fermentation (Ubeda et al., 2013). Nonetheless, few studies have been performed on the effects of fermentation on individual non-anthocyanin compounds.

Although the effect of alcoholic fermentation and acetic acid fermentation on polyphenolic compounds has been previously studied in strawberry, the effect of gluconic fermentation on the polyphenolic composition of this fruit is still unknown. Gluconic fermentation has an additional advantage from a nutritional perspective, since *Gluconobacter* strains convert glucose into mostly gluconic acid, without fermenting the fructose (Attwood, van Dijken, & Pronk, 1991). Therefore, the final product will maintain the sweetness of the natural fructose content of the fruit as well as all the original health properties.

This paper aims to characterize the non-anthocyanin phenolic composition of a beverage obtained by fermentation of strawberry. Specifically, the purpose is to study the impact of gluconic fermentation process on the non-anthocyanin phenolic composition of the resulting drink.

2. Materials and methods

2.1. Chemicals

Standards of the non-anthocyanin compounds used were purchased from: Fluka Sigma-Aldrich (St. Louis, MO, USA) [gallic acid, caffeic acid, *p*-coumaric acid, cinnamic acid, (−)-epicatechin, (+)-catechin, chlorogenic acid, ellagic acid, (−)-epicatechin gallate, kaempferol, kaempferol-3-O-β-D-glucuronide, kaempferol-3-glucoside, polydatin and penta-O-galloyl-β-D-glucose hydrate]; Chromadex® Inc. (USA) [procyanidin B1], and Extrasynthese [luteolin and apigenin-7-O-glucoside]. DPPH (2,2-diphenyl-1-picrylhydrazyl), AAPH (2,2′-diazo-bis-amidine-propane-dihydrochloride) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were obtained from Sigma-Aldrich (St. Louis, MO, USA), acetonitrile and acetic acid (LC gradient) from Merck (Darmstadt, Germany), and methanol from Prolabo® (Obregón, Mexico).

2.2. Samples

Two strawberry purée substrates were fermented (A and B); these were provided by Hudisa Company (Lepe, Huelva, Spain). The purées were made with surplus production grown in the region of Huelva (Andalusia — Spain). The principals cultivated varieties were Camarosa, Candonga, Festival, Ventana, Splendor, Honor and Coral. *Gluconobacter japonicus* was used as a starter for the submerged fermentation process. Briefly, the process was optimized beforehand (Álvarez-Fernández, Hornedo-Ortega, Cerezo, Troncoso, & García-Parrilla, 2014) and was as follows: 3 L of strawberry purée substrate was placed into the bioreactor and the conditions set (29 °C, 20% O_2 and 1250 g); after 10–20 min, 125 mL of inoculum of *G. japonicus* strain E1 was added and mixed for 20–30 min, then the initial sample was taken. The end of the fermentation process was established when the glucose had been totally consumed. Four fermentation cycles were studied for each substrate. The process stars as the strawberry purée is mixed with the inoculum (initial step) and finishes as the remaining glucose is totally consumed (final step) when the fermentor is discharged. This process is a fermentation cycle and we studied 4 cycles. The fermented samples were supplied by the Department of Analytical Chemistry, Chemical Engineering, Faculty of Sciences, University of Cordoba.

The pasteurization process consisted of heating the sample to 70–80 °C for 15 min and then rapidly cooling and freezing it until analysis.

We analyzed the initial and final steps of each cycle and additionally the final pasteurized sample.

Each fermentation cycle encompassed 3 analyzed samples.

Sample codes indicate: the fermentation step (I initial, F final and P pasteurized); the cycles, referred to as 1, 2, 3 and 4, and finally, the substrate, indicated by A (less-concentrated purée) or B (more-concentrated purée). A total of 24 samples were analyzed in duplicate. Table 1 displays the sample codes.

2.3. Polyphenol extraction

An aliquot of 30 g of sample was extracted in 30 mL of methanol by sonication for 30 min. Ascorbic acid 1% (w/w) was previously added to prevent compound oxidation. It was immediately centrifuged for 10 min at 1500 *g* using a Sorvall® TC Dupont Centrifuge. The supernatant was collected and the pellet re-extracted with methanol (30 mL). The fractions were mixed and evaporated to dryness at 37 °C with a rotary evaporator (Büchi Rotavapor®, R-200/205). The extract was reconstituted in 5 mL of water/methanol 1:1 (v/v) and stored at −18 °C until analysis. Extractions were performed in duplicate.

No ascorbic acid was added to the aliquot used to determine antioxidant activity, but the rest of the sample preparation process was similar to that described above.

2.4. LC/DAD, LC/MS/ESI-MS/MS and MRM analysis

Chromatographic separation was accomplished using an LC Agilent Series 1100 system equipped with a quaternary pump (Series 1100 G1311A), automatic injector (Series 1100 G1313A) and degasser (Series 1100 G1379A). Detection was with a UV/vis diode detector (Series 1100 G1379A) coupled to a Chemstation HP A.10.02 (HP/Agilent). The column used was a Merck LiChroCART Superspher 100 [250-4 RP-18 (5 μm)] 1.16056.0001. The method was a binary gradient, A (acetic acid/water, pH 2.65) and B (20% A + 80% acetonitrile), scheduled 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; 20 min 87% A + 13% B; 35 min, 80% A + 20% B; 40 min, 70% A + 30% B; 45 min, 60% A + 40% B; 50 min, 100% B; 55 min 100% A; 70 min, and 100% A. Each sample was analyzed twice. The injection volume was 50 μL, the flow rate was 1.5 mL min^{-1}, and the temperature was set at 40 °C. This method has been validated before in strawberry purée analysis (Álvarez-Fernández et al., 2014). Identification was achieved by matching the retention time and spectra of the peaks with the corresponding standards. Furthermore, samples were spiked with standards, if they were commercially available, to achieve complete identification. In accordance with the maximum absorbance of each compound, quantification was performed by external calibration at 280 nm, 320 nm and 365 nm, using the corresponding standards. Calibration curves were obtained by injecting standards diluted from five to eight different concentrations (R^2: 0.9949–0.9998). A duplicate was performed at each point of the calibration curve.

Table 1
Sample codes.

Sample code	Name	Sample code	Name
	Substrate A		Substrate B
I1	Initial step cycle 1	**I1**	Initial step cycle 1
F1	Final step cycle 1	**F1**	Final step cycle 1
P1	Pasteurized step cycle 1	**P1**	Pasteurized final step cycle 1
I2	Initial step cycle 2	**I2**	Initial step cycle 2
F2	Final step cycle 2	**F2**	Final step cycle 2
P2	Pasteurized final step cycle 2	**P2**	Pasteurized final step cycle 2
I3	Initial step cycle 3	**I3**	Initial step cycle 3
F3	Final step cycle 3	**F3**	Final step cycle 3
P3	Pasteurized final step cycle 3	**P3**	Pasteurized final step cycle 3
I4	Initial step cycle 4	**I4**	Initial step cycle 4
F4	Final step cycle 4	**F4**	Final step cycle 4
P4	Pasteurized final step cycle 4	**P4**	Pasteurized final step cycle 4

Table 2
Identification of non-anthocyanin phenolic compounds by using their UV–vis spectral characteristics, negative ions in LC–MS and MS–MS and respective references.

Peak N°	Identification	MS Rt (min)	λmax (nm)	MW	$(M-H)^-$ (m/z)	Ms–Ms	Detection	Reference	Foods and beverages
Hydroxybenzoic acid derivatives									
1	Gallic acid	4.78	275	170	169	125; 79	DAD–MS	[a]	
5	*p*-Hydroxybenzoic-3-O-glucoside	22.11		300	299	137	MS	Ornelas-Paz et al. (2013)	*Fragaria ananassa*
39	*p*-Hydroxybenzoic acid	41.26		138	137	93; 65	MS	[a]	
Hydrolyzed tannins									
4	Sangüin H10	21.97	232	784	783	301; 481	MS	Aaby, Ekeberg, and Skrede (2007)	*Fragaria ananassa*
14	Galloyl-bis-HHDP-glucose	27.53		936	935	633; 301; 783	MS	Aaby et al. (2007)	*Fragaria ananassa*
17	Castalagin	29.00		934	933	301	MS	Del Bubba et al. (2012)	*Fragaria vesca*
18	Monogalloyl glucose	29.69	280	332	331	313	MS–DAD	Hanhineva et al. (2008)	*Fragaria ananassa* flowers
21	Casuricitin/potentillin	30.23		936	935	633; 301	MS	Del Bubba et al. (2012)	*Fragaria vesca*
30	HHDP-galloyl glucose	33.00		634	633	375; 301	MS	Aaby et al. (2007)	*Fragaria ananassa*
37	Monogalloyl diglucoside	39.83		494	493	331; 313; 161	MS	Sandhu and Gu (2010)	*Vitis rotundifolia*
Ellagic acid and derivatives									
15	Ellagic acid pentoside	28.36	252; 375	434	433	300	MS	Del Bubba et al. (2012)	*Fragaria vesca*
16	Ellagic acid deoxyhexoside	28.88	254; 370	448	447	300; 257	MS	Aaby et al. (2012)	*Fragaria ananassa*
25	Ellagic acid	31.09	257; 358	302	301	284; 145	DAD–Ms	[a]	
Flavan 3-ols									
6	(+) Catechin	23.02	280	290	289	245; 109	DAD–MS	[a]	
8	(−) Epicatechin	24.07	282	290	289	245; 109	DAD–MS	[a]	
20	(−) Epicatechin gallate	29.82	280	444	443	289; 169	DAD–MS	[a]	
Condensed tannins									
9	Procyanidin B1	24.4	280	578	577	288; 406	DAD–MS	[a]	
Hydroxycinnamic acids									
2	Quinic acid	10.33		192	191	111; 87;	MS	Santos, Freire, Domingues, Silvestre, and Neto (2011)	*Eucalyptus globulus*
7	Cafeoylhexose	23.54		342	341	161; 179	MS	Määttä-Riihinen, Kamal-Eldin, and Törrönen (2004)	Finnish berries
10	*p*-Coumaroil hexose	25.66	320	326	325	187; 163; 145	DAD–MS	Aaby et al. (2012)	*Fragaria ananassa*
11	Caffeic acid	25.98	320	180	179	135; 107	MS	[a]	
12	Ferulic acid hexose derivative	26.50	320	450	449	287; 269	DAD–MS	Ornelas-Paz et al. (2013)	*Fragaria ananassa*
22	*p*-Coumaric acid	30.42	320	164	163	119; 93	DAD–MS	[a]	
24	Ferulic acid	31.00		194	193	178; 134	MS	[a]	
29	Chicoric acid	32.68		474	473	311; 149	MS	[a]	
38	Cinnamic acid	40.00	282	148	147	103; 77	DAD–MS	[a]	
40	5-Hydroxy feruroyl hexose	43.16		372	371	209; 193	MS	Chandrasekara and Shahidi (2011)	Coffee bean grinder
42	Sinapic acid hexose derivative	46.10		386	385	265; 247; 223	DAD–MS	Ornelas-Paz et al. (2013)	*Fragaria ananassa*
Flavonols									
3	Quercetin rutinoside	21.69		610	609	301; 179; 151	MS	Seeram, Lee, Scheuller, and Heber (2006)	*Fragaria ananassa*
13	Dihydrokaempferol hexoside	26.52		450	449	431; 287; 269; 259; 243; 179	MS	Fischer et al. (2011)	*Punica granatum*
19	Apigenin-7-O- glucoside	29.70		432	431	270; 269; 311	MS	[a]	
23	Kaempferol hexoside	30.66		448	447	284; 255; 227	MS	Del Bubba et al. (2012)	*Fragaria vesca*
26	Luteolin-7-O- glucuronide	31.30		-	461	285; 241	MS	[a]	
27	Kaempferol coumaroyl glucoside	31.47		593	593	447; 284; 285	DAD–MS	Del Bubba et al. (2012)	*Fragaria vesca*
28	Quercetin-3-O-glucoside	31.57		464	463	300; 271; 255; 179; 151	MS	Ornelas-Paz et al. (2013)	*Fragaria ananassa*
31	Kaempferol 3-glucuronide	34.06	268; 350	462	461	285; 179; 161	DAD–MS	Seeram et al. (2006)	*Fragaria ananassa*
32	Quercetin-3-(6″acetyl glucoside)	34.57		506	505	463; 300; 271; 226; 179	MS	Mikulic-Petkovsek et al. (2012); Sun, Liu, Yang, Slovin, and Chen (2014)	*Fragaria vesca*
33	Isorhamnetin glucuronide	34.70			491	315; 300; 271; 255; 113	MS	Hanhineva et al. (2008)	*Fragaria ananassa* flowers
34	Kaempferol-3-malonylglucoside	35.78	268; 348	534	533	285	DAD–MS	Aaby et al. (2007)	*Fragaria ananassa*
35	Kaempferol-7-O-neohesperidoside	36.75		594	593	327; 285; 257; 227; 151	MS	Mikulic-Petkovsek et al. (2012)	
36	Kaempferol acetyl hexoside	37.78		490	489	327; 285; 284; 255	MS	Del Bubba et al. (2012)	*Fragaria vesca*
43	Kaempferol	46.74	270; 375	286	285	117; 93	DAD–MS	[a]	
Stilbenes									
41	*trans*-Piceid	45.10		390	389	185; 227	MS	[a]	

[a] Identification of the compound was confirmed by the authentic standards.

LC/MS separation was performed using a Pelkin Elmer Series 200 HPLC 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 Merck LiChroCART 250–4 Superspher 100 RP-18 1.16056.0001 reversed-

phase column. The flow rate was 0.4 mL min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water, and (B) water:acetonitrile 20:80 (v/v). Both components contained 0.1% formic acid (v/v). The elution profile was the same as the one used in LC/DAD analysis. The injection volume was 20 μL.

Multiple Reaction Monitoring (MRM) was applied where precursor ions and fragment ions were monitored, at Q1 and Q3, respectively, using their corresponding standards. The analysis was performed in negative ionization.

For HPLC–ESI-MRM analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, ion spray voltage 4500 V, source temperature 350 °C and source gas 60 psi. MRM transitions were performed with the parameters shown in Table 2. A dwell time was set at 50 ms for each transition.

2.5. Antioxidant activity

2.5.1. ORAC test

The method used was similar to a previously reported method, with modifications (Ou, Hampsch-Woodill, & Prior, 2001): 50 μL of sample or Trolox mixed with 100 μL of fluorescein (45 nM) and 50 μL of AAPH (15 mM). Reaction was carried out for 80 min and fluorescence was assessed with a fluorometer (multi-detector microplate Synergy HT, Biotek®) excitation and emission wavelengths were 485 and 528 nm respectively. The readings were taken in triplicate. Trolox was used as a calibration standard (0.5 to 9.5 μM).

The fluorescence lectures were recorded every 5 min after addition of AAPH until they reached less than 5% of the initial reading. Results were calculated using the areas under the fluorescein decay curves between the blank and the sample and were expressed as mmols Trolox equivalents (TE) per kg of fresh weight.

2.5.2. DPPH method

Antioxidant activity was assessed using the DPPH method, as reported by Villaño, Fernández-Pachón, Moyá, Troncoso, and García-Parrilla (2007). An aliquot of 0.1 mL of sample in methanol was added to 3.9 mL of DPPH methanolic solution (0.025 gL^{-1}). Absorbance was recorded at 515 nm at the start time (when the sample was added) and 60 min later, when the reaction reached equilibrium. All measurements were taken in triplicate and methanol was used as a reference. Eight concentrations were used to make the Trolox calibration curve (0.9–0.12 mM).

All measurements were recorded on a Hitachi UV-2800 spectrophotometer thermostated with a Peltier system at 25 °C.

2.6. Statistical analysis

Statistical analyses were performed by means of Statistica software, StatSoft Inc. (2004). One-way analysis of variance (ANOVA) was used to test significant differences at $p < 0.05$ level. Additionally, discriminant analysis was tested to build a function to discriminate between the substrate (initial) of the fermented beverage (final) and the final pasteurized samples.

3. Results and discussion

3.1. Identification of compounds by LC/ESI-MS and MS/MS analysis

A total of 44 non-anthocyanin phenolic compounds were identified using LC/ESI-MS and LC–DAD methods. Table 2 displays the identified compounds and their MS/MS rupture, tentatively identified in negative ion. Fig. 1 shows the corresponding mass chromatogram.

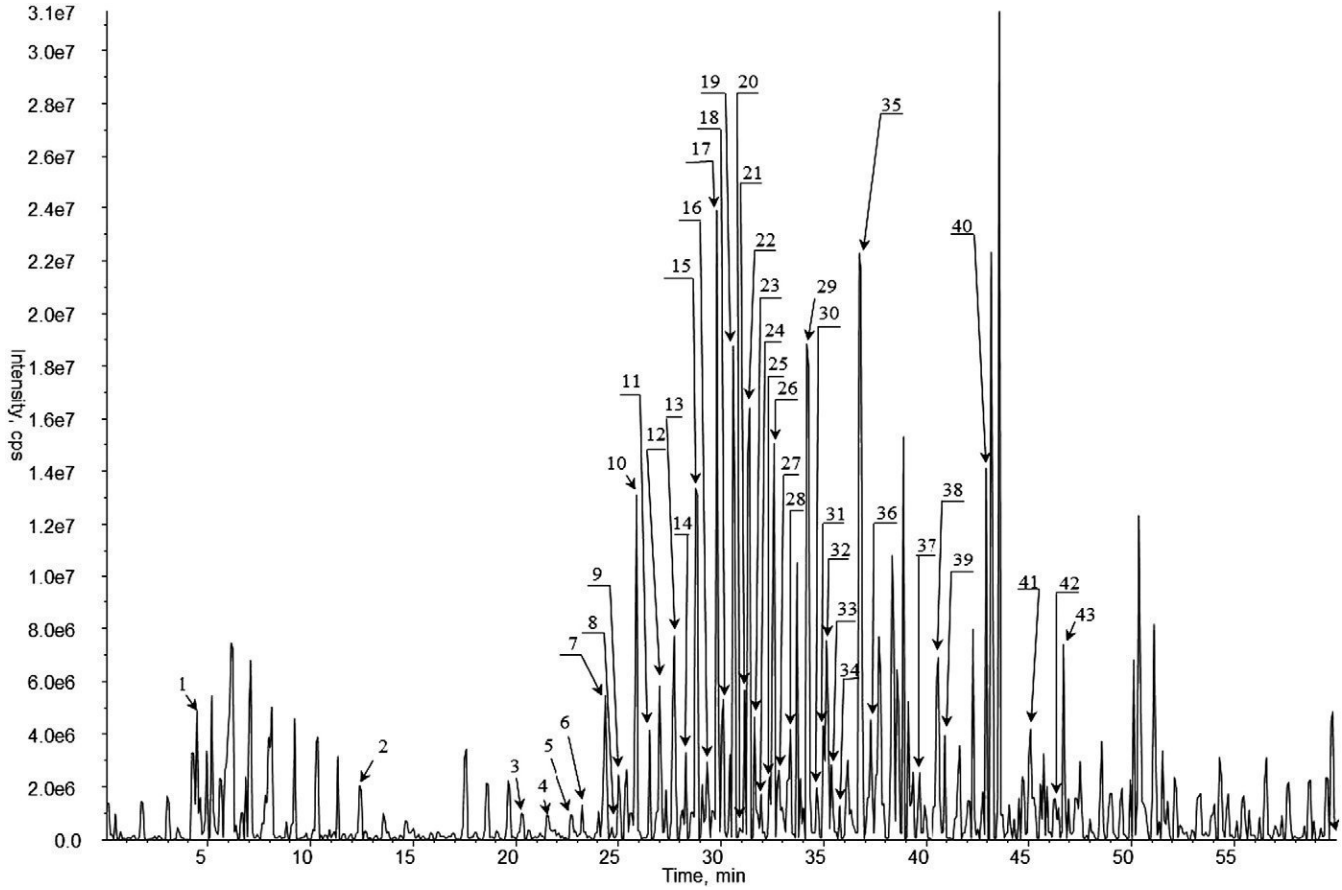


Fig. 1. LC–MS of sample P3 of substrate A. The numbers indicate the compounds shown in Table 2.

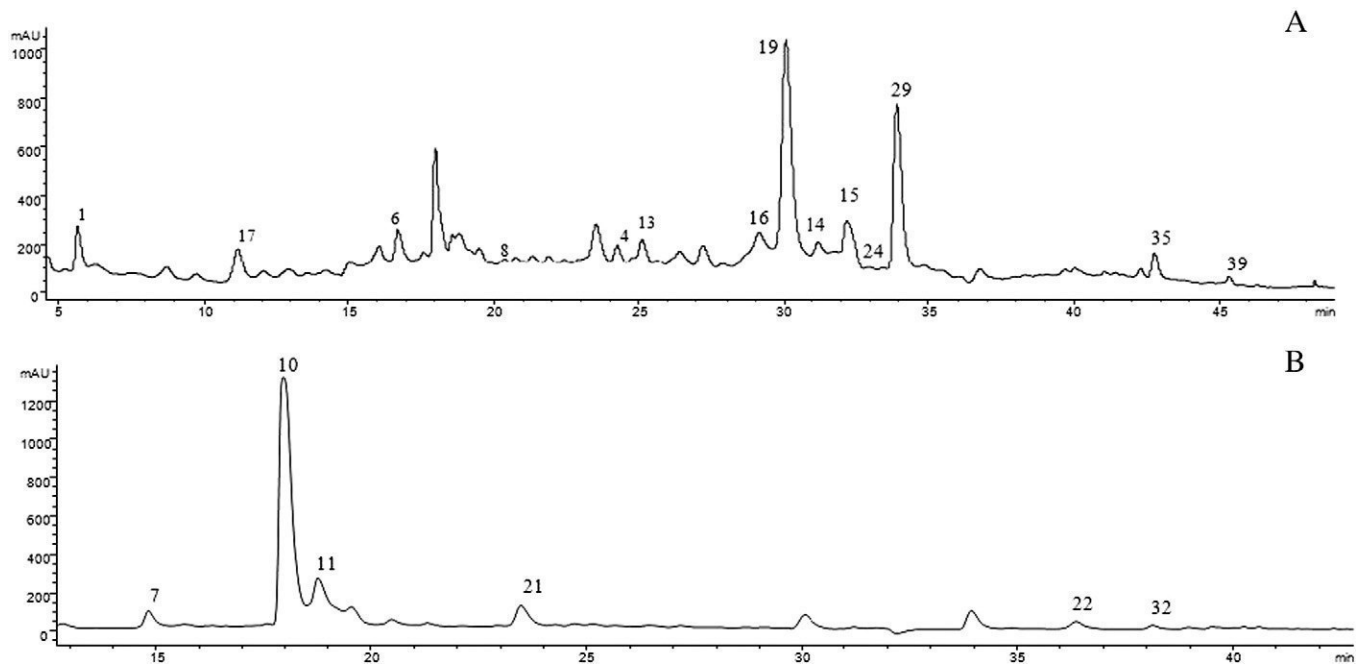


Fig. 2. LC–DAD chromatogram: (A) at 280 nm and (B) at 320 nm. Peak numbers refer to Table 2.

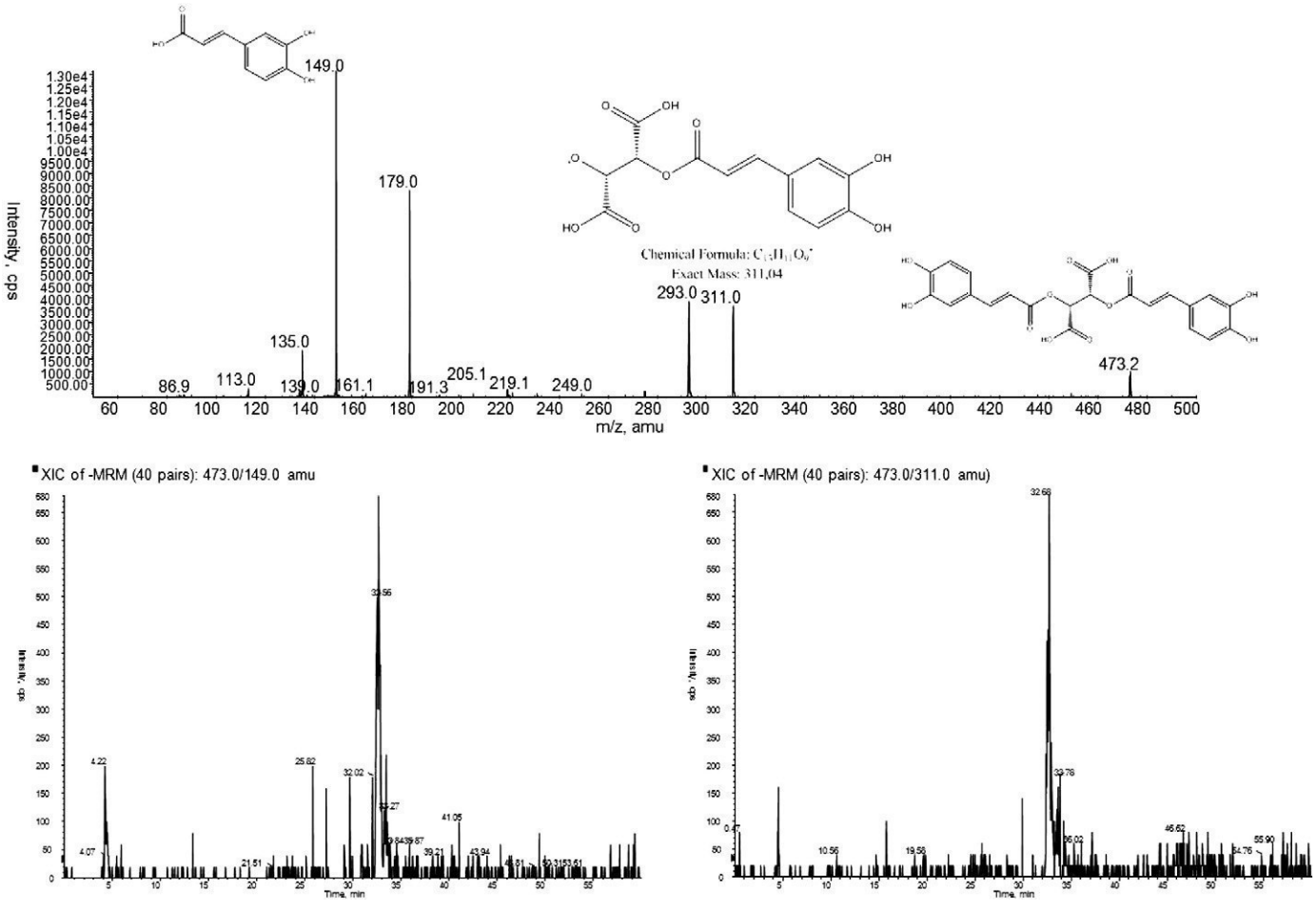
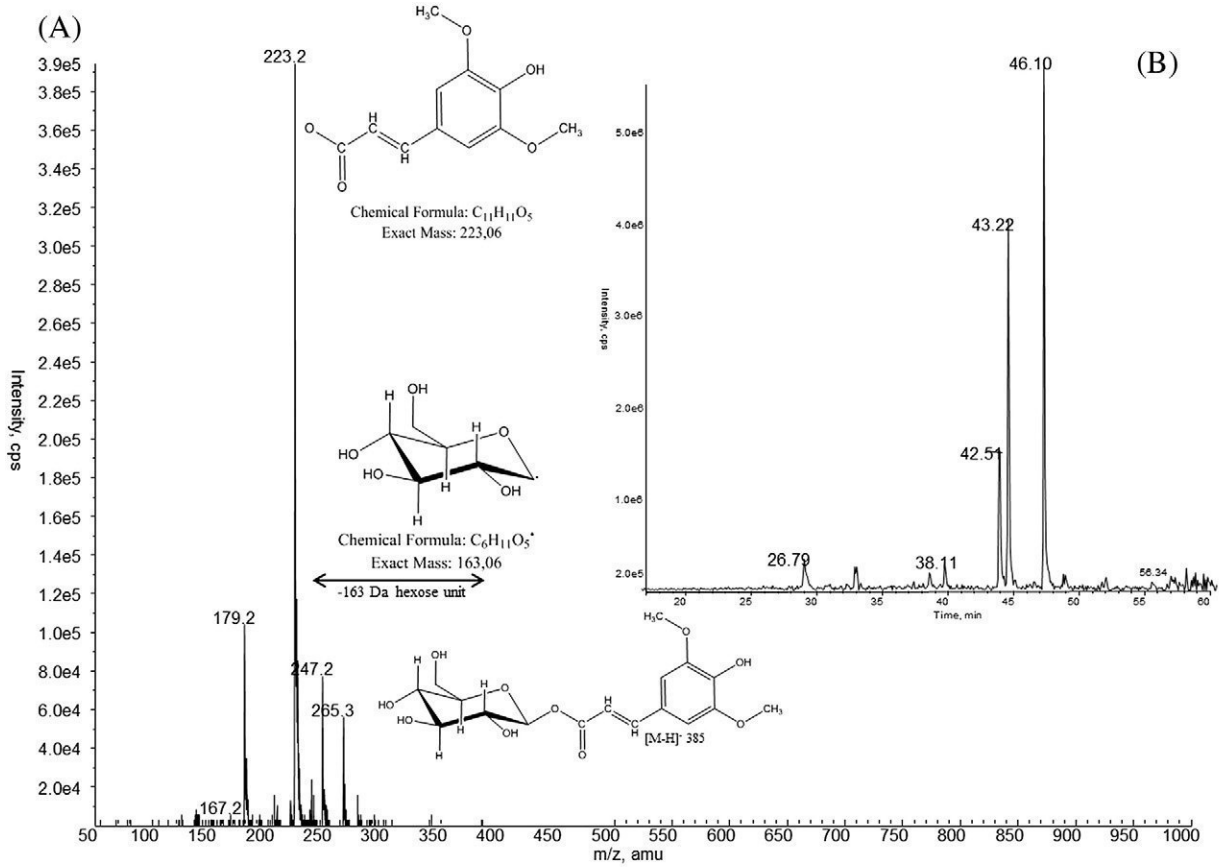
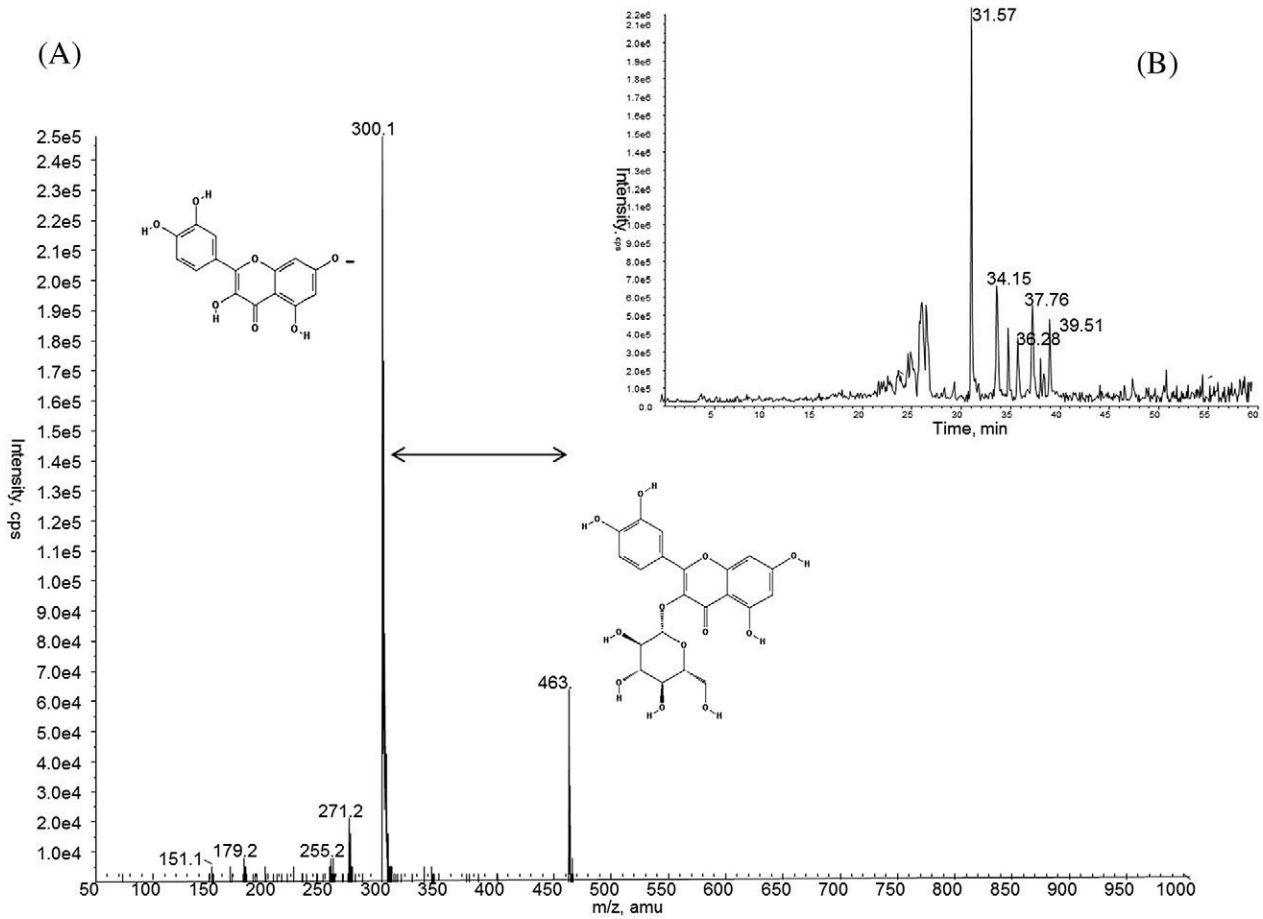


Fig. 3. MRM experiment to support the presence of (−)-chicoric acid, peak 29. Fragmentation pattern of standard at the top and XIC chromatogram to principal ions *m/z* 149 and 311 of samples, displaying retention times at the bottom.


(A)
(B)
223.2
179.2
247.2
265.3
167.2
Chemical Formula: C11H11O5
Exact Mass: 223,06
Chemical Formula: C6H11O5•
Exact Mass: 163,06
-163 Da hexose unit
[M-H]- 385
46.10
43.22
42.51
26.79
38.11
56.34
Time, min
Intensity, cps
m/z, amu


(A)
(B)
300.1
463
271.2
255.2
179.2
151.1
31.57
34.15
37.76
39.51
36.28
Time, min
Intensity, cps
m/z, amu

Gallic acid (peak 1), (+)-catechin (peak 6), procyanidin B1 (peak 9), caffeic acid (peak 11), (−)-epicatechin gallate (peak 20), *p*-coumaric acid (peak 22), ferulic acid (peak 24), ellagic acid (peak 25), chicoric acid (peak 29), cinnamic acid (peak 38), *trans*-resveratrol (peak 39), *p*-hydroxybenzoic acid (peak 40), *trans*-piceid (peak 42) and kaempferol (peak 44), were identified by monitoring their characteristic transitions in MRM mode and comparing their retention times with their corresponding standards. This assay was performed to confirm the identification of phenolic compounds with low concentrations or low sensitivity. The LC–DAD chromatograms were recorded at 280, 320 and 365 nm to quantitate the compounds (Fig. 2).

3.1.1. Hydroxybenzoic acids

Peak 5 had an $[M-H]^-$ ion at 299 and fragment ion at *m/z* 137, corresponding to hydroxybenzoic acid. This compound, observed in sample P, was tentatively identified as *p*-hydroxybenzoic-3-O-glucose, in accordance with previous studies in strawberry Ornelas-Paz et al. (2013).

3.1.2. Hydrolyzed tannins

This group includes ellagitannins and gallotannins. Peaks 4, 14, 17, 21 and 30 were identified as ellagitannins, as their MS–MS fragmentations gave *m/z* 301 corresponding to ellagic acid, in accordance with previous reports in strawberry (Aaby, Mazur, Nes, & Skrede, 2012; Del Bubba et al., 2012). Peaks 18 and 37 correspond to gallotannins, as their major fragment ion is *m/z* 313, which yielded a deprotonated Gallic acid *m/z* 169 by the loss of glucose unit $[M-H]^-$ 162. Peak 17 was described as monogalloyl glucose, which has been reported in strawberry pistil (Hanhineva et al., 2008). However, we were able to tentatively identify peak 37 as monogalloyl diglucose with a deprotonated ion at $[M-H]^-$ 493, which has been reported in *Vitis rotundifolia* and in others plants (Sandhu & Gu, 2010; Soong & Barlow, 2005), but which has not been described before in strawberry or its derivative products like purées, beverages or jams etc.; this is an original contribution of this paper.

3.1.3. Hydroxycinnamic acids

Eleven compounds have been identified in this group. For the first time, we report two hydroxycinnamic derivatives in strawberry and derivatives: chicoric acid (2, 3-dicaffeoyltartaric acid) identified with authentic standards by MRM experiment (Fig. 3) and 5-hydroxy feruloyl hexose. Peak 40 had an MW of 372 because an $[M-H]^-$ ion at 371 was found. The fragmentation produced MS/MS ions with *m/z* 193 and 209; fragment *m/z* 193 is characteristic of ferulic acid. The fragmentation pattern is plotted in Fig. 4 top (A) and (B) displays xic chromatogram and retention time corresponding to same fragmentation; the compound was tentatively identified as 5-hydroxyferuloyl hexose, which has been reported before in coffee beans grinder by Chandrasekara and Shahidi (2011), but not in strawberry.

3.1.4. Flavonols

There were fourteen compounds in this group. The main compounds were kaempferol and quercetin derivatives, thus peak 13 was tentatively identified as dihydrokaempferol hexoside, in accordance with Fischer, Carle, and Kammerer (2011), as reported in *Punica granatum*. This compound had an $[M-H]^-$ 449 and fragmentation pattern *m/z* 287, 269 and 259. Peak 19 exhibited a pseudo molecular ion at *m/z* 431 and MS/MS pattern with *m/z* 269; 311 and 270, and was identified as apigenin-7-O-glucoside. Peak 26 had an $[M-H]^-$ ion at *m/z* 461, the main fragmentation product of the mono-charged pseudo-molecular ion was 285 and it presented a fragment *m/z* 241 characteristic of luteolin aglycone; this fragmentation pattern corresponds to luteolin-3-O-glucuronide (Álvarez-Fernández et al., 2014). Peak 31, which exhibited a deprotonated ion $[M-H]^-$ at 461, but with another fragmentation pattern, was identified as kaempferol 3-glucuronide (Fig. 5).

A quercetin derivative was tentatively identified as quercetin-3-(6″ acetyl glucoside): it exhibited an $[M-H]^-$ at 505 and *m/z* 300 fragmentation pattern, indicating loss of 250 Da corresponding to an acetylglucose unit and with *m/z* 179 and 271 corresponding to the fragmentation pattern of quercetin. This compound has been reported before in blueberry, mulberry, kiwi fruit and *Fragaria vesca* (Mikulic-Petkovsek, Slatnar, Stampar, & Veberic, 2012), but has not been determined before in *Fragaria ananassa*. Peak 33 had a pseudo molecular ion at *m/z* 491, producing an ion at *m/z* 315, with a glucuronide unit loss of 176 Da and subsequent loss of methyl from the methoxy group (15 Da). This dissociation pattern was observed by Hanhineva et al. (2008) in strawberry floral organs and tentatively identified as isorhamnetin glucuronide. Peak 35 presented kaempferol derivative with an $[M-H]^-$ at *m/z* 593, the loss of 309 Da corresponding to sugar moiety and the production of MS^2 fragmentation at *m/z* 285 corresponding to kaempferol aglicone. It was tentatively identified as kaempferol-7-O-neohesperidoside. This compound has been reported before in red and black currants, but not in strawberry (Mikulic-Petkovsek et al., 2012). These findings are a novelty of this work.

3.1.5. Stilbenes

Peak 42 exhibited an $[M-H]^-$ ion at *m/z* 389 and MS/MS ion with *m/z* 227, this fragmentation corresponds to *trans*-piceid. It was identified by corresponding standard and has been described before in strawberry juice (Díaz-García, Obón, Castellar, Collado, & Alacid, 2013).

3.2. Changes in non-anthocyanin phenolic content and antioxidant activity though fermentation

Twenty-one identified compounds were quantified by external calibration with standards, according to the validated LC–DAD method (Álvarez-Fernández et al., 2014). Tables 3 and 4 display the phenolic compound concentrations corresponding to three steps of four fermentation cycles of two substrates (A and B), at the initial and final steps of the cycle and after the pasteurization step.

In summary, just gallic acid, HHDP galloyl-glucose, *p*-coumaroyl glucose, caffeic acid, kaempferol 3-hexoside and kaempferol malonyl glucoside changed significantly ($p < 0.05$) during the fermentation process.

Phenolic compounds with major concentrations were (+)-catechin, *p*-coumaroyl hexoside and HHDP-galloyl-glucoside; they constituted as much as 62% of the total phenolic compounds quantified.

During the fermentation process, the content of most phenolic compounds increased. Gallic acid increased after the process in all cycles, by 36% on average. The decrease in monogalloyl glucose cannot account for this value. Enzymes, like the esterase tannase, are able to hydrolyze ester bonds to release gallic acid and sugar moieties. Bacteria, yeast or fungi in the fermentation process can produce this enzyme (Duckstein, Lorenz, & Stintzing, 2012). Epicatechin gallate was considered another releaser of gallic acid. Kim, Goodner, Park, Choi, and Talcott (2011) reported a decrease of epicatechin gallate and a simoultaneous increase of gallic acid after the fermentation of tea (*Camellia sinensis*). Our data do not confirm this trend.

HHDP-galloyl-glucoside is the main hydrolyzable tannin in the samples. Its values ranged from 13.8 to 38.66 mg kg^{-1} fw in initial steps and it underwent a significant increase of 22.23–96.39% in the fermentation process, like most of the hydrolyzable tannins quantified.

Fig. 4. LC–MS/MS chromatogram of an $[M-H]^-$ ion (*m/z* 385) tentatively identified as sinapic acid hexose derivative at the top and an $[M-H]^-$ ion (*m/z* 463) tentatively identified as quercetin-3-O-glucoside with hypothetic rupture at the bottom (A). LC–MS chromatogram in enhanced resolution (B).

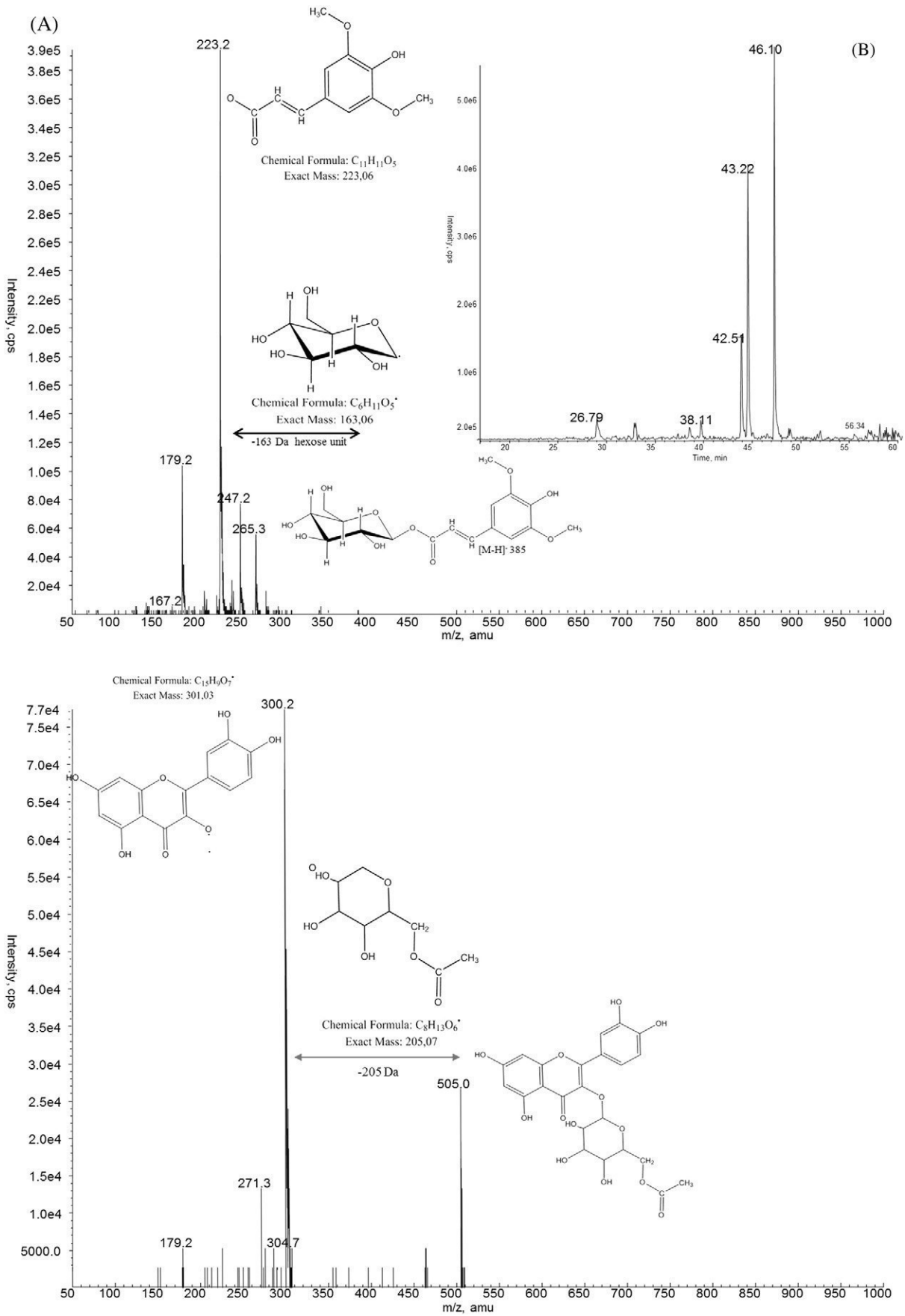


Fig. 5. LC–MS/MS of an [M−H]⁻ ion (*m/z* 371) tentatively identified as 5-hydroxy feruloyl hexose at the top, an [M−H]⁻ ion (*m/z* 505) tentatively identified as quercetin-3-(6″acetyl glucoside) with hypothetic rupture in the middle, and LC−MS/MS of an [M−H]⁻ 461 identified as kaempferol glucuronide with hypothetic rupture at the bottom (A). LC–MS chromatogram in enhanced resolution (B).

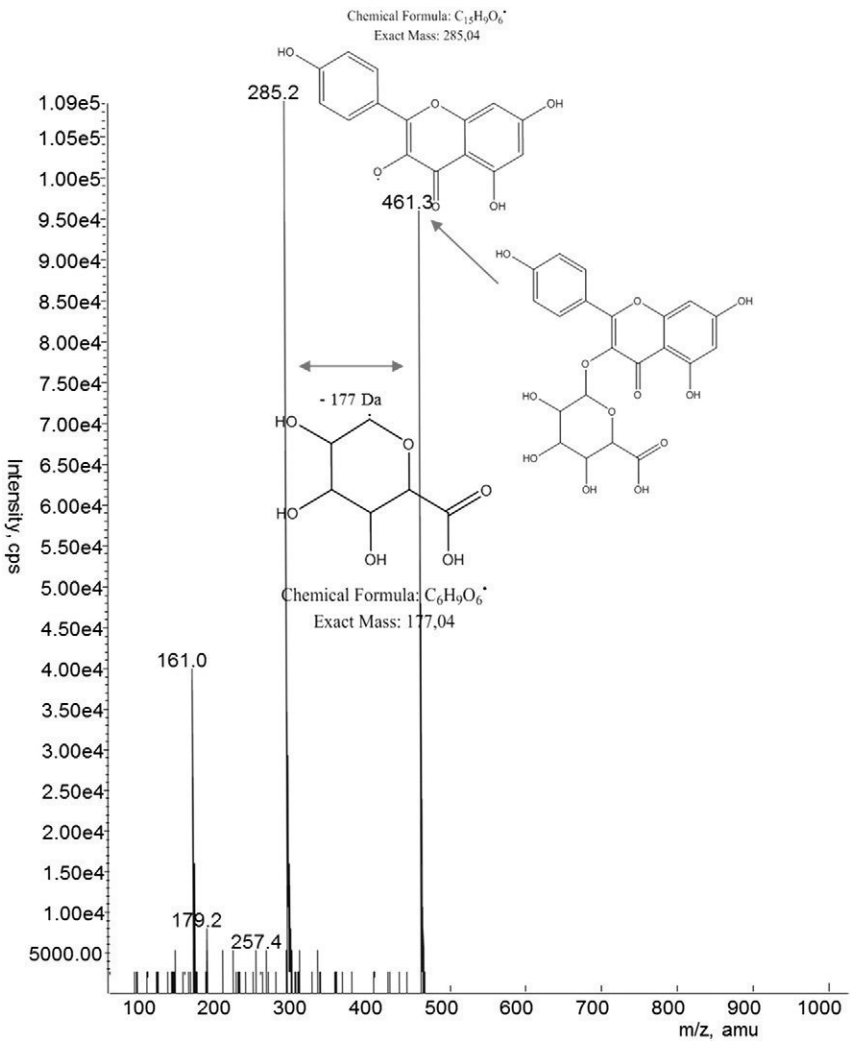


Fig. 5 (*continued*).

Hydroxycinnamic acids experiment a significant increase ($p < 0.05$) in general, specifically *p*-coumaroyl hexoside and caffeic acid. Our data are in accordance with Pérez-Gregorio, Regueiro, Alonso-González, Pastrana-Castro, and Simal-Gándara (2011) who reported hydroxycinnamic acid derivatives increase after alcoholic fermentation of mulberries (*Morus nigra*). In addition, the presence of hydroxybenzoic acid may indicate the degradation of other hydroxycinnamic compounds by fermentation, as reported by Duckstein et al. (2012).

The principal flavonols in the samples were kaempferol derivatives, whose concentrations ranged from 0.4–6.20 mg kg^{-1} fw, as has been reported in strawberry purée (Álvarez-Fernández et al., 2014). Both compounds underwent a significant increase on fermentation: 1.75–2.22% on average.

3.3. Changes in non-anthocyanin phenolic content and antioxidant activity through the pasteurization process

To sum up, the pasteurization process increased contents of *p*-coumaroylhexoside, cinnamic acid and *p*-coumaric acid; and decreased caffeic acid, its hexose and procyanidin B1 contents.

The effects of the pasteurization process had a significant increase in the content of *p*-coumaroylhexoside in cycles 2 and 4 of substrate B (15.83–14.05% for each cycle), *p*-coumaric acid increase in cycle 4 of substrate B in 45.34% and in case of cinnamic acid in cycle 4 of substrate A with an increase of 3.03%. These results are in accordance with Van Boekel et al. (2010). They reported a significant increase in total caffeoylquinic acid after the thermal process. This behavior was the result of both isomerization and hydrolysis events, leading to a substantial re-distribution of phenolic acid concentrations.

Caffeoylhexose and caffeic acid had a tendency to decrease with pasteurization process, the first one experimented a significant decrease (8.03%) in cycle 4 of substrate A; caffeic acid had the same behavior in three cycles (1, 2 and 4) of substrate A (45.79–41.01 and 6.85%) and in cycle 4 of substrate B with a decrease of 1.19%.

On the other hand, procyanidin B1 underwent a significant decrease in two cycles one of each substrate; cycle 4 of substrate A had a significant decrease of 21.20% and cycle 4 of substrate B with a decrease of 26.78% as a result of the thermal process, which is in accordance with White, Howard, and Prior (2011), who described how blanching caused a decrease in polymeric procyanidins. Ellagitannins showed the same trend.

3.4. Discriminant statistical analysis

A multivariate statistical analysis was applied to study the data. The data matrix was built with quantified phenolic compounds ORAC and DPPH as variables and samples as cases. Discriminant analysis was

applied to both substrates in this study, and the grouping variable was the step in the process: I (initial), F (final) and P (pasteurized). The standard method achieved better results in every analysis. All samples were correctly classified with all polyphenolic compounds as variables in the model. Fig. 6 shows the scatterplot of the canonical roots obtained. As can be seen, the distance for pasteurized samples is greater than between initial and final fermentation steps. These results are reproducible for both substrates, regardless of the concentration of polyphenolic compounds in the strawberry purée.

Table 5 exhibits coefficients of variables corresponding to Fig. 6, indicating their relative importance in the model. The greatest values, regardless of the sign they submit, indicate their weight and importance in the model. From substrate A: (+) catechin, caffeic acid, *p*-coumaroyl hexoside, cinnamic acid, ellagic acid pentoside and (−) epicatechin, and from substrate B: castalagin, *p*-coumaroyl hexose, (+) catechin, ellagic acid, cinnamic acid and monogalloyl glucose, are the heaviest weighted in root 1 (x axis). We concluded that these were the best variables to differentiate the groups in our analysis based on the criteria proposed by Kim et al. (2011).

Statistical analysis confirms that both gluconic fermentation and pasteurization change non-anthocyanin polyphenolic compounds in strawberry derived products.

3.5. Impact of gluconic fermentation and the pasteurization process on antioxidant activity

Fig. 7 displays the results of antioxidant activity using two methods. The fermentation process did not change antioxidant activity regardless of the method used. Pasteurized samples present a higher value when determined by DPPH significance increased ($p < 0.05$). This result is in accordance with an increase in the contents of gallic acid and hydroxycinnamic derivatives. Hence, gluconic fermentation does help to maintain antioxidant potential of studied beverage.

4. Conclusions

Forty-three non-anthocyanin phenolic compounds were identified and five were reported for the first time in strawberry and its

Table 3
Contents of non-anthocyanin phenolic compounds in gluconic ferment of strawberry. Substrate A.

Compounds	I1	F1	P1	I2	F2	P2
Gallic acid	10.10 ± 0.045^{a}	13.23 ± 3.55	12.48 ± 1.12	7.3 ± 0.22^{ab}	13.11 ± 0.41^{ac}	11.18 ± 0.13^{bc}
Monogalloyl glucoside	3.47 ± 0.05^{ab}	3.23 ± 0.64^{a}	3.72 ± 0.13^{b}	2.54 ± 0.12^{ab}	4.10 ± 0.33^{a}	4.53 ± 0.50^{b}
Ellagic acid	4.29 ± 0.67^{b}	6.57 ± 4.03	6.49 ± 0.75 ^{b}	5.22 ± 0.27^{b}	6.30 ± 0.70	5.28 ± 1.07 ^{b}
Ellagic pentoside	10.35 ± 2.56^{a}	7.79 ± 2.47^{a}	12.41 ± 0.27	9.68 ± 0.37^{a}	13.45 ± 1.21	11.52 ± 1.91
Ellagic deoxyhexoside	5.69 ± 0.13^{a}	4.30 ± 2.47^{a}	6.49 ± 0.40	2.52 ± 0.28	4.48 ± 2.43	6.10 ± 1.91
HHDP-galloyl glucoside	38.66 ± 8.02	29.88 ± 11.24	36.63 ± 4.15	20.99 ± 3.82^{ab}	41.22 ± 2.82^{a}	40.64 ± 0.64^{b}
Castalagin	10.53 ± 0.34	7.54 ± 2.42	8.50 ± 1.47	6.32 ± 0.16^{ab}	9.30 ± 0.87^{ac}	7.31 ± 0.25^{bc}
Galloyl-bis-HHDP glucoside	5.25 ± 0.62	4.74 ± 1.48	6.35 ± 1.22	4.77 ± 0.32^{ab}	6.50 ± 0.69^{a}	6.42 ± 0.17^{b}
Sanguiin h10	0.77 ± 0.02^{b}	0.89 ± 0.13	0.83 ± 0.03^{b}	0.76 ± 0.16	0.85 ± 0.04^{c}	0.77 ± 0.02^{c}
Procyanidin B1	7.52 ± 3.67	7.99 ± 0.73	11.41 ± 5.14	nd	12.75 ± 3.75	8.83 ± 0.55
(+)-Catechin	108.19 ± 2.87^{a}	97.87 ± 8.32^{a}	117.29 ± 11.88^{b}	84.96 ± 1.25^{b}	91.32 ± 10.97^{c}	125.59 ± 1.48^{bc}
(−)-Epicatechin	8.36 ± 2.27	6.43 ± 1.93	8.12 ± 1.70	7.69 ± 0.40	7.29 ± 1.97	7.58 ± 1.04
(−)-Epicatechin gallate	26.63 ± 6.40	26.90 ± 4.64	34.50 ± 0.76	22.23 ± 4.37^{ab}	32.86 ± 2.39^{a}	33.86 ± 3.47^{b}
Caffeoylhexose	1.93 ± 0.29	2.09 ± 0.06	2.23 ± 0.13	1.91 ± 0.14^{b}	2.35 ± 0.27	2.28 ± 0.07^{b}
p-Coumaroyl hexoside	48.45 ± 0.73^{b}	48.27 ± 14.66	53.86 ± 1.94^{b}	55.37 ± 2.69	53.82 ± 2.06	53.19 ± 0.44
Caffeic acid	12.61 ± 2.95^{b}	13.78 ± 0.31^{c}	7.47 ± 0.33^{bc}	16.51 ± 0.45^{ab}	8.90 ± 0.44^{ac}	5.25 ± 0.17^{bc}
p-Coumaric acid	1.06 ± 0.07	0.95 ± 0.48	1.21 ± 0.08	1.04 ± 0.12^{ab}	1.31 ± 0.08^{a}	1.40 ± 0.06^{b}
Cinnamic acid	0.83 ± 0.04^{b}	0.99 ± 0.29	0.98 ± 0.03^{b}	0.44 ± 0.02^{ab}	0.90 ± 0.09^{a}	1.01 ± 0.02^{b}
Kaempferol 3-hexoside	2.63 ± 0.16	2.71 ± 0.97	3.23 ± 0.34	2.91 ± 0.22^{b}	6.20 ± 0.37	6.10 ± 0.24^{b}
K. malonyl glucoside	0.42 ± 0.13^{b}	0.52 ± 0.47	1.18 ± 0.07^{b}	1.56 ± 0.15^{ab}	1.13 ± 0.11^{ac}	1.83 ± 0.07^{bc}
trans-Piceid derivative	0.63 ± 0.001	0.61 ± 0.03	0.63 ± 0.01	nd	0.63 ± 0.01	0.64 ± 0.002
Compounds	I3	F3	P3	I4	F4	P4
Gallic acid	6.3 ± 1.62^{ab}	10.15 ± 0.94^{a}	10.75 ± 1.61^{b}	9.00 ± 0.22	8.00 ± 0.002	8.95 ± 0.003
Monogalloyl glucoside	4.10 ± 0.35	4.88 ± 0.87	4.54 ± 0.51	3.59 ± 0.11^{ab}	6.27 ± 0.09^{a}	4.70 ± 0.87^{b}
Ellagic acid	4.58 ± 0.27	5.85 ± 0.21	5.28 ± 0.77	5.02 ± 0.50 ^{ab}	5.84 ± 0.70 ^{a}	6.12 ± 0.80 ^{b}
Ellagic pentoside	13.86 ± 1.26 ^{a}	14.01 ± 0.51 ^{a}	13.53 ± 1.53	16.23 ± 1.25 ^{b}	15.11 ± 0.41	12.18 ± 0.50 ^{b}
Ellagic deoxyhexoside	5.99 ± 0.84	5.85 ± 0.17 ^{c}	5.76 ± 0.08 ^{c}	5.91 ± 0.46 ^{b}	7.68 ± 0.32 ^{c}	8.12 ± 0.80^{bc}
HHDP-galloyl glucoside	34.03 ± 2.06^{ab}	41.60 ± 1.39^{a}	40.97 ± 0.92^{b}	34.03 ± 5.68	42.27 ± 0.56^{c}	38.82 ± 0.97^{c}
Castalagin	9.92 ± 2.00^{b}	8.58 ± 0.72^{c}	6.46 ± 0.28^{bc}	8.46 ± 0.104^{b}	8.72 ± 0.57^{c}	5.61 ± 0.60^{bc}
Galloyl-bis-HHDP glucoside	5.81 ± 0.35^{ab}	8.25 ± 0.25^{a}	8.25 ± 0.27^{b}	6.96 ± 0.51^{a}	7.87 ± 0.16^{ac}	7.01 ± 0.20^{c}
Sanguiin h10	1.24 ± 0.04^{a}	1.36 ± 0.05^{ac}	1.22 ± 0.04^{c}	1.06 ± 0.02^{ab}	0.95 ± 0.03^{ac}	0.75 ± 0.03^{bc}
Procyanidin B1	15.8 ± 4.34^{ab}	8.20 ± 0.98^{a}	8.47 ± 0.63^{b}	14.62 ± 1.19^{b}	14.24 ± 0.92^{c}	11.22 ± 1.19^{bc}
(+)-Catechin	112.00 ± 7.37	126.49 ± 3.73	111.16 ± 3.39	150.85 ± 5.5^{ab}	139.97 ± 5.12^{ac}	126.42 ± 1.87^{bc}
(−)-Epicatechin	2.78 ± 0.82^{ab}	3.91 ± 0.03^{a}	3.91 ± 0.38	3.28 ± 0.09^{ab}	3.28 ± 0.39^{ac}	3.81 ± 0.21^{bc}
(−)-Epicatechin gallate	35.83 ± 3.32	45.08 ± 4.64	47.65 ± 0.64	45.71 ± 2.79	47.06 ± 1.19	46.72 ± 1.15
Caffeoylhexose	2.11 ± 0.10^{b}	2.11 ± 0.02	2.38 ± 0.06^{b}	2.17 ± 0.11	2.24 ± 0.05^{c}	2.06 ± 0.03^{c}
p-Coumaroyl hexoside	52.11 ± 4.17	49.10 ± 1.48	51.47 ± 1.80	52.21 ± 1.95	51.13 ± 1.95	51.88 ± 1.59
Caffeic acid	9.55 ± 3.94	4.85 ± 0.09	5.22 ± 0.32	3.95 ± 0.16	3.94 ± 0.14^{c}	3.67 ± 0.09^{bc}
p-Coumaric acid	1.06 ± 0.18^{ab}	1.38 ± 0.07^{a}	1.80 ± 0.09^{b}	1.70 ± 0.20	1.89 ± 0.06	2.11 ± 0.06
Cinnamic acid	0.90 ± 0.06^{ab}	0.90 ± 0.04^{a}	1.02 ± 0.02^{b}	0.98 ± 0.06	0.99 ± 0.02^{c}	1.02 ± 0.01^{c}
Kaempferol 3-hexoside	2.75 ± 0.27^{ab}	3.88 ± 0.17^{a}	3.90 ± 0.18^{b}	3.47 ± 0.37^{b}	3.64 ± 0.09^{c}	4.15 ± 0.06^{bc}
K. malonyl glucoside	1.55 ± 0.06^{ab}	1.96 ± 0.13^{a}	1.90 ± 0.08^{b}	2.47 ± 0.19^{b}	2.44 ± 0.05^{c}	2.08 ± 0.07^{bc}
trans-Piceid derivative	0.63 ± 0.01	0.64 ± 0.01	0.64 ± 0.01	0.65 ± 0.007^{b}	0.64 ± 0.01^{c}	0.63 ± 0.002^{bc}

Mean values and standard deviation.
a superscript letter indicates a significant difference ($p < 0.05$) between the initial samples (I) and final samples (F) of the same substrate.
b superscript letter indicates a significant difference ($p < 0.05$) between the initial samples (I) and pasteurized samples (P) of the same substrate.
c superscript letter indicates a significant difference ($p < 0.05$) between the pasteurized samples (P) and final samples (F) of the same substrate, by ANOVA statistical test.
Samples were determined in duplicate. nd: no data. K: kaempferol.

Table 4
Contents of non-anthocyanin phenolic compounds in gluconic ferment of strawberry. Substrate B.

Compounds	I1	F1	P1	I2	F2	P2
Gallic acid	13.91 ± 0.93^{a}	13.37 ± 1.38^{a}	14.37 ± 1.31	nd	nd	nd
Monogalloyl glucoside	5.19 ± 0.60	4.76 ± 0.65	4.13 ± 1.12	5.00 ± 0.30	5.44 ± 0.33^{c}	5.497 ± 0.43
Ellagic acid	3.15 ± 0.33	2.89 ± 0.45	3.26 ± 0.45	2.67 ± 0.59^{ab}	3.80 ± 0.11 ^{a}	3.93 ± 0.30 ^{c}
Ellagic pentoside	8.42 ± 0.49	14.37 ± 0.62	14.52 ± 0.84	10.70 ± 1.16^{a}	10.73 ± 1.65^{a}	10.35 ± 0.93
Ellagic deoxyhexoside	5.08 ± 0.53^{ab}	5.12 ± 0.64^{a}	6.19 ± 0.62^{b}	5.74 ± 1.16	5.76 ± 2.28	6.03 ± 2.28
HHDP-galloyl glucose	28.90 ± 2.97	35.46 ± 9.55	25.26 ± 2.20	26.83 ± 1.79	26.83 ± 0.55	10.70 ± 1.35
Castalagin	7.72 ± 0.26^{a}	9.59 ± 1.44^{ac}	7.25 ± 1.09^{c}	4.00 ± 0.31^{ab}	6.00 ± 0.14^{a}	4.73 ± 1.57
Galloyl-bis-HHDP glucoside	11.16 ± 1.20^{a}	14. 38 ± 1.42^{a}	11.93 ± 1.65	9.48 ± 0.61	7.96 ± 0.62	8.96 ± 0.69
Sanguiin	1.69 ± 0.41^{a}	2.37 ± 0.28^{a}	2.05 ± 0.14	1.92 ± 0.07^{ab}	2.74 ± 0.25^{a}	2.38 ± 0.76
Procyanidin B1	7.87 ± 2.83	10.29 ± 4.19	11.85 ± 1.21	14.54 ± 2.05^{ab}	8.77 ± 2.09^{a}	4.73 ± 0.63
(+)-Catechin	109.92 ± 1.32	95.74 ± 12.00	99.87 ± 0.13	125.11 ± 2.77^{ab}	94.40 ± 6.21^{a}	74.04 ± 1.57
(−)-Epicatechin	1.99 ± 1.51	1.42 ± 0.56	0.80 ± 0.22	1.80 ± 0.18^{ab}	1.33 ± 0.01^{a}	8.96 ± 0.69
(−)-Epicatechin gallate	20.06 ± 0.28^{a}	31.89 ± 2.25^{ac}	21.11 ± 1.90^{c}	23.15 ± 1.62	26.81 ± 0.26	25.03 ± 0.75^{c}
Caffeoylhexose	4.941 ± 0.13^{ab}	5.59 ± 0.61^{a}	5.54 ± 0.02^{b}	5.77 ± 0.62	6.02 ± 0.71	4.74 ± 0.44
p-Coumaroyl hexoside	41.73 ± 0.66^{b}	40.73 ± 5.38	44.71 ± 2.10^{b}	41.75 ± 1.23^{b}	41.43 ± 2.66^{c}	47.99 ± 0.71^{bc}
Caffeic acid	10.22 ± 0.13	8.98 ± 0.65	4.22 ± 0.69	12.94 ± 2.09^{ab}	13.96 ± 3.81^{a}	17.32 ± 3.24
p-Coumaric acid	4.68 ± 0.62	5.13 ± 0.63	6.04 ± 0.28	2.98 ± 0.08^{a}	1.46 ± 0.80^{a}	1.60 ± 0.17^{b}
Cinnamic acid	1.72 ± 0.17	1.51 ± 0.29	1.51 ± 0.22	nd	nd	nd
Kaempferol 3- hexoside	2.65 ± 0.82	3.21 ± 0.95	3.21 ± 1.00	2.12 ± 0.08	2.24 ± 0.10^{c}	1.22 ± 0.066^{bc}
K. malonyl glucoside	0.96 ± 0.47^{b}	1.40 ± 0.053	2.08 ± 0.94^{b}	1.99 ± 0.95	2.51 ± 0.09	1.23 ± 0.09
trans-Piceid derivative	0.63 ± 0.013	0.65 ± 0.017	0.63 ± 0.009	0.62 ± 0.007	0.62 ± 0.009	0.64 ± 0.007^{c}
Compounds	I3	F3	P3	I4	F4	P4
Gallic acid	9.82 ± 0.82^{a}	11.94 ± 0.73^{ac}	17.31 ± 0.77^{c}	4.89 ± 0.87^{ab}	11.69 ± 1.24^{a}	11.90 ± 0.54^{b}
Monogalloyl glucoside	5.24 ± 0.18^{b}	5.24 ± 0.007	5.43 ± 0.13^{b}	5.44 ± 1.42	4.87 ± 1.67	4.09 ± 0.01
Ellagic acid	3.38 ± 0.29	4.63 ± 0.03	5.74 ± 0.84	4.59 ± 0.29	3.19 ± 0.34	6.80 ± 1.38
Ellagic pentoside	7.78 ± 0.72^{ab}	7.01 ± 0.44^{a}	7.06 ± 0.82^{b}	5.05 ± 0.29	8.14 ± 0.34^{c}	6.63 ± 0.67^{c}
Ellagic deoxyhexoside	4.55 ± 0.87^{ab}	7.01 ± 0.26^{a}	9.16 ± 1.74^{b}	3.64 ± 0.20	3.19 ± 0.24	3.26 ± 0.06
HHDP-galloyl glucose	28.41 ± 1.73	31.41 ± 1.92	29.13 ± 1.36	13.80 ± 3.93	21.73 ± 0.12	27.09 ± 0.12
Castalagin	4.87 ± 0.30	5.09 ± 0.37	4.76 ± 0.20	9.80 ± 1.73	8.82 ± 0.24	9.30 ± 0.77
Galloyl-bis-HHDP glucoside	10.51 ± 0.37^{ab}	9.95 ± 0.62^{ac}	9.00 ± 0. 20^{bc}	7.42 ± 0.37^{ab}	10.26 ± 0.96^{a}	8.99 ± 0.20^{b}
Sangüin	2.61 ± 0.061	2.60 ± 0.08	2. 71 ± 0.08	2.38 ± 0.46	2.61 ± 0.08	2.71 ± 0.04
Procyanidin B1	13.32 ± 2.05^{ab}	8.76 ± 2.09^{a}	7.33 ± 0.14^{b}	9.31 ± 1.30^{b}	6.16 ± 0.89^{c}	4.51 ± 0.48^{bc}
(+)-Catechin	117.13 ± 5.073^{ab}	80.67 ± 0.61^{a}	81.98 ± 10.73^{b}	87.28 ± 1.91	82.85 ± 0.45	91.20 ± 0.85
(−)-Epicatechin	1.80 ± 0.18^{a}	1.33 ± 0.011^{a}	1.30 ± 0.90	1.61 ± 0.055^{a}	1.74 ± 0.055^{a}	1.33 ± 0.027
(−)-Epicatechin gallate	26.90 ± 0.35	26.24 ± 1.75^{c}	27.71 ± 0.27^{c}	25.03 ± 1.53	27.25 ± 0.80	25.09 ± 0.80
Caffeoylhexose	4.06 ± 0.19^{ab}	4.84 ± 0.08^{a}	4.30 ± 0.27^{b}	4.03 ± 0.03^{b}	4.53 ± 0.08	4.73 ± 0.20^{b}
p-Coumaroyl hexoside	41.01 ± 1.48	43.02 ± 0.74	42.38 ± 1.96	33.57 ± 8.10^{ab}	46.24 ± 0.78^{ac}	52.74 ± 1.10^{bc}
Caffeic acid	21.88 ± 1.21^{ab}	14.94 ± 1.59^{a}	14.04 ± 1.28^{b}	15.28 ± 0.47^{ab}	16.67 ± 0.56^{ac}	16.47 ± 0.74^{bc}
p-Coumaric acid	3.18 ± 0.47	3.35 ± 0.60	3.98 ± 0.60	2.11 ± 0.90	1.72 ± 0.37^{c}	2.50 ± 0.30^{c}
Cinnamic acid	0.87 ± 0.034^{b}	0.95 ± 0.12	1.01 ± 0.08^{b}	0.97 ± 0.26	nd	nd
Kaempferol 3-hexoside	1.70 ± 0.08	1.60 ± 0.10	1.14 ± 0.07	1.60 ± 0.35^{b}	2.21 ± 0.031^{c}	2.26 ± 0.16^{bc}
K. malonyl glucoside	1.91 ± 0.07	1.27 ± 0.69	1.10 ± 0.28	2.19 ± 0.063	3.07 ± 0.02	3.69 ± 0.88
trans-Piceid derivative	0.57 ± 0.001^{ab}	0.61 ± 0.01^{a}	0.61 ± 0.004^{b}	0.57 ± 0.009^{b}	0.58 ± 9.4E−04^{c}	0.59 ± 0.002^{bc}

Mean values and standard deviation.
a superscript letter indicate significant difference ($p < 0.05$) compared to the initial samples (I) with final (F) of the same substrate.
b superscript letter indicate significant difference ($p < 0.05$) compared to the initial samples (I) with pasteurized (P) of the same substrate.
c superscript letter indicate significant difference ($p < 0.05$) compared to the pasteurized samples (P) with final (F) of the same substrate by ANOVA statistical test.
Samples were determined in duplicate. nd: no data. K: kaempferol.

derivatives; these were tentatively identified as: monogalloyl diglucose, 5-hydroxy feruroyl hexose, dihydrokaempferol hexoside, kaempferol neohesperidoside and (−)-chicoric acid. Identification of the latter was confirmed by authentic standard.

Gluconic fermentation left *in vitro* antioxidant activity and non-anthocyanin phenolic composition was practically unchanged, except for gallic acid hydroxycinnamics and kaempferol derivatives.

This paper supports gluconic fermentation of strawberry as a process that can produce beverages with a low glucose content and a high content of non-anthocyanin polyphenols, maintaining the antioxidant potential of the strawberry source. This represents an alternative use for strawberry surplus.

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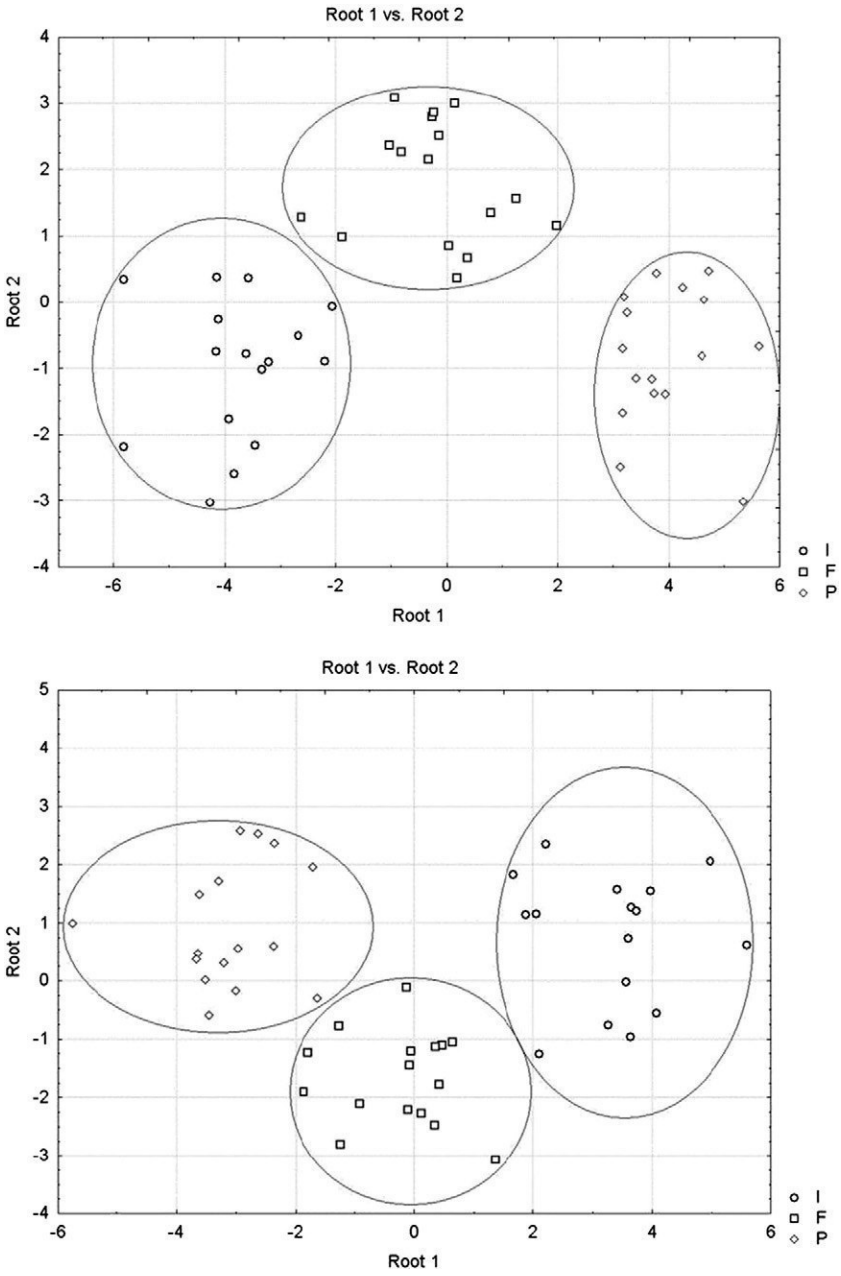

Fig. 6. Scatterplot of canonical scores of substrate A, at the top, and substrate B, at the bottom.

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Table 5
Standardized coefficients for variables used to Fig. 5, substrate A on the left and B on the right.

Peak	Variable	Root 1	Root 2	Peak	Variable	Root 1	Root 2
1	Gallic acid	0.42783	0.13236	**1**	Gallic acid	−0.99578	−0.00464
18	Monogalloyl glucoside	0.05819	−0.16467	**18**	Monogalloyl glucoside	1.23600	−0.26154
9	Procyanidin B1	−0.24425	−0.32734	**9**	Procyanidin B1	0.86425	−0.35442
6	(+)-Catechin	−5.01555	1.64318	**6**	(+)-Catechin	1.57071	0.16386
8	(−)-Epicatechin	2.02656	−0.44200	**20**	(−)-Epicatechin gallate	−0.32057	1.88966
20	(−)-Epicatechin gallate	−1.02388	−1.07586	**23**	Kaempferol Hexoside	−0.94704	−0.11162
17	Castalagin	0.31782	1.40714	**25**	Ellagic acid	−1.40308	−0.20113
38	Cinnamic acid	3.05724	−0.82542	**34**	Kaempferol malonyl glucoside	0.57928	−0.12685
11	Caffeic acid	−3.52649	0.35410	**10**	*p*-Coumaroyl hexoxide	−1.71284	0.54509
22	*p*-Coumaric acid	1.62820	−0.02735	**16**	Ellagic acid deoxyhexoside	−0.71095	−0.77580
10	*p*-Coumaroyl hexoside	3.33134	−5.33952	**15**	Ellagic acid pentoside	−0.65752	0.46092
23	Kaempferol hexoside	0.91533	0.62591	**17**	Castalagin	1.90291	−2.09041
34	Kaempferol malonyl glucoside	0.02626	0.35060	**8**	(−)-Epicatechin	0.66352	0.34169
16	Ellagic acid deoxyhexoside	0.80389	−0.47424	**38**	Cinnamic acid	−1.23844	1.68992
25	Ellagic acid	−0.39361	1.68129	**30**	HHDP-galloyl glucoside	0.70976	−0.02075
15	Ellagic acid pentoside	−2.02610	−0.57777	**14**	Galloyl-bis-HHDP glucoside	−0.05498	0.06549
7	Caffeoylhexose	−0.53288	1.70376	**4**	Sanguiin	−0.14522	0.21904
14	Galloyl-bis-HHDP glucoside	0.73210	2.43967	**42**	*trans*-Piceid	0.45134	−0.30412
4	Sanguiin	0.44502	−0.80324	**11**	Caffeic acid	0.79735	0.38949
42	*trans*-Piceid	0.37623	−1.36435	**7**	Caffeoylhexose	0.15845	0.73621
30	HHDP-galloyl glucoside	−0.99349	0.90542	**22**	*p*-Coumaric acid	0.42381	0.34823
	DPPH	−0.05129	0.25975		DPPH	−0.41912	−0.57453
	ORAC	0.76402	0.81759		ORAC	0.43330	0.17239
	Eigenvalue	10.68040	1.78614		Eigenvalue	7.41508	1.49168
	Cum.Prop	0.85673	1.00000		Cum.Prop	0.83252	1.00000

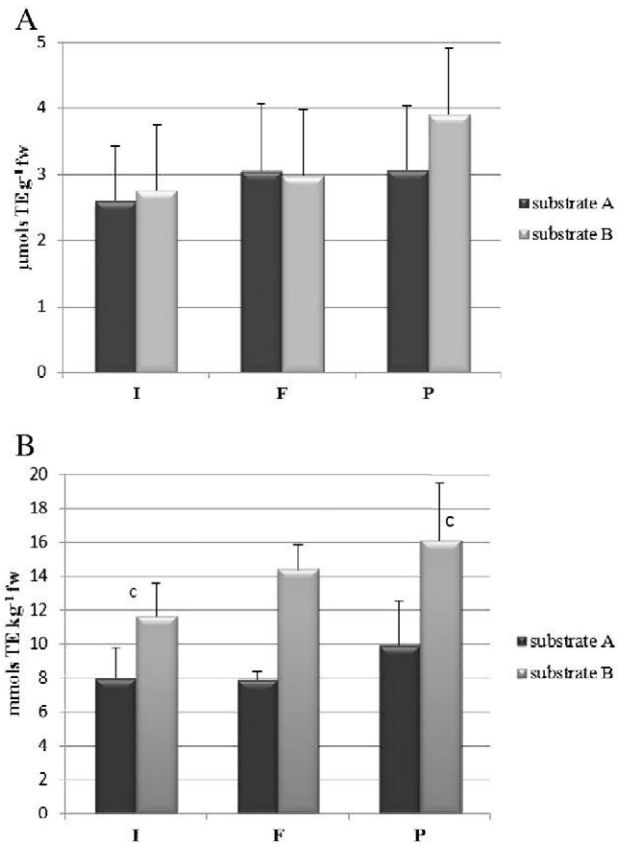

Fig. 7. Evolution of antioxidant activity during the process using the ORAC (A) and DPPH (B) methods. Superscript 'c' means significant changes between the I and P steps of the same substrate. I (initial step), F (final step) and P (pasteurized step). µmols TEg-1fw. Micromols of Trolox equivalent per gram of fresh weight. mmols Tkg-1fw. Millimols of Trolox per kilogram of fresh weight. I (initial step), F (final step) and P (pasteurized step).

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Composition of Nonanthocyanin Polyphenols in Alcoholic-Fermented Strawberry Products Using LC–MS (QTRAP), High-Resolution MS (UHPLC-Orbitrap-MS), LC-DAD, and Antioxidant Activity

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Supporting Information

ABSTRACT: In this study, the nonanthocyanin (poly)phenolic profile of an alcoholic-fermented strawberry beverage was characterized. High-performance liquid chromatography coupled with a triple-quadropole mass spectrometer and ultra-high-performance liquid chromatography coupled with a linear trap quadropole and an Orbitrap mass analyzer was used to identify nonanthocyanin phenolic compounds. Sixty-six compounds were identified, and 13 of these were identified for the first time in strawberry or its derived alcoholic fermented beverage: protocatechuic acid-4-*O*-*β*-hexoside, brevifolin carboxylic acid, ferulic acid glucuronide, dimer caffeic acid-*O*-hexoside, luteolin-3′-*O*-xyloside, isorhamnetin 3-*O*-glucoside, taxifolin-*O*-glucoside, (+)-aromadendrin rhamnoside, eriodictyol-7-*O*-glucoside, (+)-taxifolin, (+)-aromadendrin, eriodictyol, and homovanillic acid. The alcoholic fermentation process produced significant increases in certain compounds, such as homovanillic acid and p-hydroxybenzoic acid, while a significant decrease in galloyl bis-HHDP-glucose was observed. Linear discriminant analysis correctly classified samples initial, final, and pasteurized, which led to the conclusion that alcoholic fermentation induces significant changes in composition, mainly in relation to the 19 compounds represented in the tables of this work.

KEYWORDS: *alcoholic fermentation, phenolic compounds, metabolomics, Saccharomyces cerevisiae, mass spectometry, strawberry, yeast*

1. INTRODUCTION

Strawberry (*Fragaria x ananassa*) is widely consumed thoroughout the world, with the latest FAOSTAT report[1] showing that European production was 1,316,950 T in 2012. The USA is the leading producer globally, with Spain in fourth place. Strawberry must be consumed shortly after it is harvested, which leads to significant economic loss if it is not brought to market quickly. Therefore, it is of great industrial interest to produce innovative drinks from food surpluses that would otherwise be wasted.

The alcoholic fermentation process has been used for many centuries as a means of increasing the stability of processed foods and enhancing the shelf life and nutritional and organoleptic qualities of food. This process entails the transformation of original substances into secondary metabolites, which can have an impact on the quality of the final product.[2]

Strawberry itself is a rich source of micronutrients and phytochemicals, such as flavan-3-ols, proanthocyanins, hydroxybenzoic acids, ellagic acid, tannins, flavonols, and stilbenes,[3] which result from numerous biological activities. Because of its composition, consumption of this fruit is related to favorable changes in platelet function, HDL cholesterol, and blood pressure, and it has been suggested that it may play a role in preventing cardiovascular disease.[4] The antioxidant properties of strawberry have been mostly attributed to its polyphenol and metabolite content, and they are primarily responsible for its beneficial effects on health.[5−8]

The study of metabolite profiling (metabolomic) in fermented beverages using high-resolution mass spectrometry (HRMS) instruments is a powerful tool for discovering changes during the process and predicting the nutritional quality of the final product.[9] The development of rapid technologies has assisted in the growth of metabolomics in food science.[10] Despite this, few studies have been performed on the effects of the alcoholic fermentation of strawberry on individual non-anthocyanin phenolic compounds.

This article is primarily concerned with characterizing the content of nonanthocyanin polyphenols in alcoholic-fermented strawberry. Additionally, antioxidant activity was measured using DPPH (2,2-diphenyl-1-picrylhydrazyl) and Oxygen Radical Absorbance Capacity (ORAC) methods.

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Table 1. Sample Code

substrate A		substrate B	
sample code	name	sample code	name
I1	initial step cycle 1	I1	initial step cycle 1
F1	final step cycle 1	F1	final step cycle 1
P1	pasteurized final step cycle 1	P1	pasteurized final step cycle 1
I2	initial step cycle 2	I2	initial step cycle 2
F2	final step cycle 2	F2	final step cycle 2
P2	pasteurized final step cycle 2	P2	pasteurized final step cycle 2
I3	initial step cycle 3	I3	initial step cycle 3
F3	final step cycle 3	F3	final step cycle 3
P3	pasteurized final step cycle 3	P3	pasteurized final step cycle 3
		I4	initial step cycle 4
		F4	final step cycle 4
		P4	pasteurized final step cycle 4

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. The standard compounds used were purchased from Fluka Sigma-Aldrich (St. Louis, MO, USA) [gallic acid, caffeic acid, p-coumaric acid, cinnamic acid, (−)-epicatechin, (+)-catechin, chlorogenic acid, ellagic acid, (−)-epicatechin gallate, kaempferol, kaempferol-3-*O*-*β*-D-glucuronide, kaempferol-3-glucoside, polydatin, penta-*O*-galloyl-*β*-D-glucose hydrate, apigenin, quinic acid, brevifolin, protocatechuic acid, isorhamnetin, (+)-taxifolin, eriodictyol, rutin, quercetin, homovanillic acid, and kaempferol glucuronide], Chromadex Inc. (USA) [procyanidin B1], and Extrasynthese [luteolin, (+)-aromadendrin, phloridzin, flavonomarein, and apigenin-7-*O*-glucoside]. DPPH, AAPH (2,2′-diazo-bis-amidine-propane-dihydrochloride), and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were obtained from Sigma-Aldrich (St. Louis, MO, USA); acetonitrile, acetic acid, methanol, and formic acid (LC gradient) from Merck (Darmstadt, Germany), and methanol from Prolabo (Obregón, Mexico).

2.2. Samples. Two strawberry purée substrates (A and B) made with production surplus, grown in Huelva (Andalusia, Spain), were used as substrates of the fermentation process were analyzed and previously reported.[3] The principal cultivated varieties were Camarosa, Candonga, Festival, Ventana, Splendor, Honor, and Coral. They were provided by Hudisa Company (Lepe, Huelva, Spain). Substrate A corresponds to the 2011 harvest and B to the 2012 harvest. *Saccharomyces cerevisiae* (CET 13057 isolated from native strawberry yeast)[11] was used as a starter for the submerged fermentation process. The fermentation process was as follows: 3.6 L of strawberry purée was placed into the bioreactor and the conditions set (29 °C, 26.20 rad s^{-1}); the medium was saturated with oxygen only at the beginning of the fermentation process, before the inoculum was added [10% (w/v) glucose, 0.1% (w/v) $MgSO_4$, 0.2% (w/v) KH_2PO_4, 0.3% (w/v) $(NH_4)_2SO_4$, 0.4% (w/v) yeast extract, and 0.36% (w/v) bacteriological peptone]. At the moment when the inoculum was added, the initial samples were taken. The end of the fermentation process was established when the ferment reached alcoholic strengths of 3.02 and 1.8 for substrates A and B, respectively. The fermentation was carried out by batch, and a final sample was taken before the process continued with another fermentation cycle.

The pasteurization process consisted of heating the sample to 70−80 °C for 15 min and then rapidly cooling and freezing until analysis. Sample codes are shown in Table 1 and indicate: substrate named as A (more-concentrated purée) or B (less-concentrated purée); the cycles, referred to as 1, 2, 3, and 4; and finally the fermentation step I (initial), F (final), and P (pasteurized). Each cycle encompassed three analyzed samples.

Samples were analyzed in duplicate. In the case of substrate A, we studied four cycles, and with substrate B, three cycles. The fermented samples were supplied by the Department of Inorganic Chemistry and Chemical Engineering, Faculty of Science, University of Cordoba, Spain.

2.3. Sample Treatment. We followed a previously reported procedure[3,12] to extract nonanthocyanin polyphenol compounds from the samples.

2.4. Liquid Chromatography with Diode Array Detection (LC-DAD), Liquid Chromatography−Mass Spectrometry/Elec-

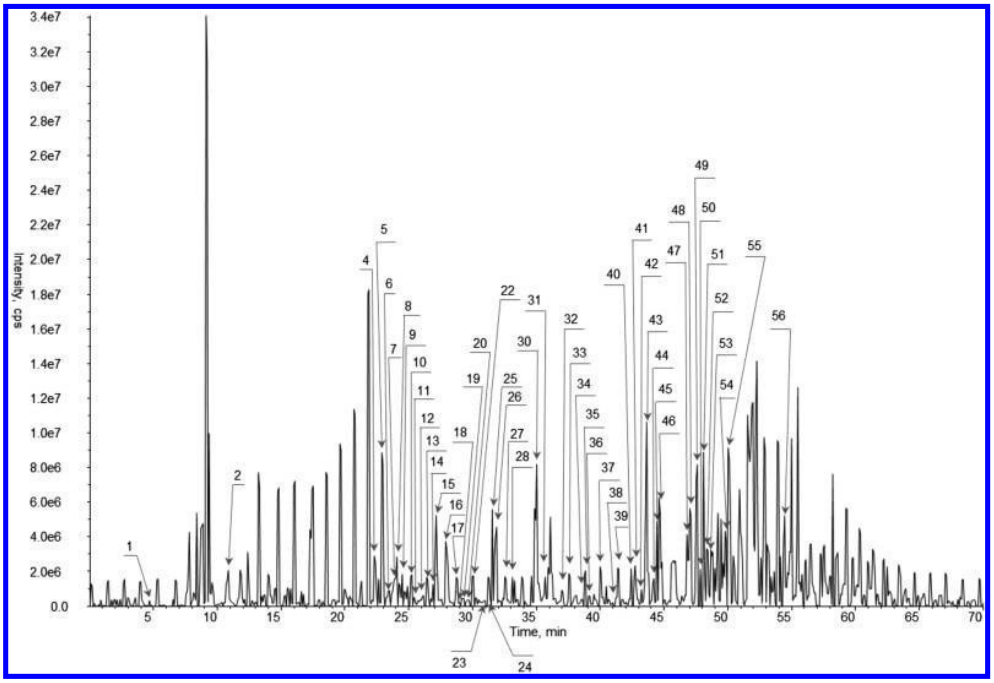

Figure 1. MS chromatogram of a final sample. The numbers represent the compounds shown in Table 2.

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Table 2. Nonanthocyanin Phenolic Compounds in Alcoholic Fermentation Samples in negative ionization mode by QTRAP. Expected Mean, Retention Time (min), Molecular Weight, MS/MS Fragments, Identification and Reference. Peak Numbers Correspond to Mass Chromatogram (Figure 1)

peak N°	identification	MS t_R (min)	MW	$(M-H)^-$ (m/z)	MS/MS fragments	ref[a]
Hydroxybenzoics Acids and Derivatives						
1	gallic acid	4.78	170	169	125; 79	a
10	*p*-hydroxybenzoic acid	25.07	138	137	93; 65	a
16	protocatechuic acid 4-*O*-*β*-hexoside	28.8	316	315	152; 108	18
23	*p*-hydroxybenzoic-3-*O*-glucoside	30.72	300	299	137; 93	28
26	1-*O*-protocatechuyl-*β*-xylose	31.7	286	285	152; 108	32
Hydrolyzed Tannins						
4	HHDP-glucose	22.67	482	481	301; 275	25
13	monogalloyl glucose	26.00	332	331	313; 169; 151	25
20	bis-HHDP glucose	29.84	784	783	301; 481	16
25	HHDP-galloyl glucose	31.1	634	633	375; 301	16
28	Tris-galloyl-HHDP-hexose	33.28	952	951	907; 783; 605; 301	20
35	brevifolin carboxylic acid	38.9	292	291	248; 247; 203	22
37	galloyl-bis-HHDP-glucose	40.17	936	935	633;783; 301	16
39	agrimoniin	41.2	935	934	1085; 915; 897;783; 301	32
Ellagic Acid and Derivatives						
17	ellagic acid	29.71	302	301	284; 145	a
18	methyl ellagic acid pentoside	29.79	448	447	315; 331	20
38	ellagic acid deoxyhexoside	40.9	448	447	300; 257	17
46	ellagic acid rhamnoside	44.9	448	447	301; 285	20
52	dimethyl ellagic acid pentoside	47.9	462	461	300; 301; 145	20
56	ellagic acid pentoside	54.15	434	433	300	20
Flavanols						
5	(+)-catechin	23.02	290	289	245; 109	a
8	(−)-epicatechin	24.07	290	289	245; 109	a
19	(−)-epicatechin gallate	29.82	444	443	289; 169	a
Condensed Tannins						
9	procyanidin B1	24.4	578	577	288; 406	a
Hydroxycinnamic Acids						
2	ferulic acid glucuronide	10.78	370	369	193	30
3	dimer of caffeic acid-*O*-hexoside	13.07	684	683	341; 179	24
6	cinnamic acid	23.50	148	147	103; 77	a
7	cafeoylhexose	23.54	342	341	161; 179	27
11	*p*-coumaroyl hexose	25.66	326	325	187; 163; 145	17
12	caffeic acid	25.98	180	179	135; 107	a
14	ferulic acid hexose derivative	26.50	450	449	287; 269	28
15	galloylquinic acid	27.74	344	343	191; 169	25
22	*p*-coumaric acid	30.42	164	163	119; 93	a
24	ferulic acid	31.00	194	193	178; 134	a
29	(−)-chicoric acid	33.67	474	473	311; 149	a
41	ferulic acid hexoside	42.6	356	355	217; 193; 175; 160	25
53	5-hydroxyferuroyl hexose	48.1	372	371	281; 251; 221; 209; 165	25
54	sinapoyl glycoside	49.9	386	385	265; 223; 179	19
Flavonols						
27	luteolin-3′-xyloside	32.49	418	417	285; 241	
30	apigenin pentose	35.01	402	401	269; 161	32
31	luteolin-7-*O*-glucuronide	35.35		461	285; 241	21
40	apigenin-7-*O*-glucoside	42.3	432	431	270; 269; 311	a
42	quercetin-3-*O*-glucoside	43.7	464	463	300; 271; 255; 179; 151	28
43	isorhamnetin 3-*O*-glucoside	43.9	956	$[2M-H]^-$ 955	301; 477	26
44	quercetin	44.01	302	301	179; 151	a
45	kaempferol hexoside	44.5	448	447	284; 255; 227	20
47	kaempferol 3-glucuronide	46.8	462	461	285; 179; 161	32
48	isorhamnetin-*O*-glucuronide	47.17	492	491	315; 300; 271; 255; 113	25
49	kaempferol	47.25	286	285	117; 93	a
50	quercetin-3-(6′′acetyl glucoside)	47.5	506	505	463; 300; 271; 226; 179	32
51	kaempferol acetyl hexoside	47.82	490	489	327; 285; 284; 255	20
55	kaempferol coumaroyl glucoside	50.05	594	593	447; 284; 285	20

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Table 2. continued

peak N°	identification	MS t_R (min)	MW	$(M-H)^-$ (m/z)	MS/MS fragments	ref[a]
Dihydroflavonols						
32	(+)-aromadendrin hexoside	37.17	450	449	431; 287; 269; 259; 243; 179	22
34	taxifolin-*O*-glucoside	38.5	466	465	285; 151	29
36	(+)-aromadendrin rhamnoside	39.95	434	433	287	35
Stilbenes						
21	*trans*-piceid	30.08	390	389	185; 227	a
Flavanones						
33	eriodyctiol-7-*O*-glucoside	37.65	450	449	287; 151; 135	26

[a]The letter a indicates that identification of the compound was confirmed by the authentic standard.

Table 3. Nonanthocyanin Phenolic Compounds in Alcoholic Fermentation Samples in negative ionization mode by ORBITRAP. Expected Mean Molecular Formula, Exact Mass, Mass Accuracy (ppm), MS/MS Fragments (%), Identification, and Reference[a]

identification	t_R (min)	molecular formula	exact mass $(M-H)^-$	MS/MS fragments	Δmass (ppm)	ref[a]
protocatechuic acid	1.92	$C_7H_6O_4$	153.0193	109.8292(84)	1.3902	a
citric acid isomer	2.77	$C_6H_8O_7$	191.0189	173.0081(2); 111.0074(100); 67.0174(1)	1.5281	32
dimer of caffeic acid-*O*-hexoside	13.07	$C_{30}H_{36}O_{18}$	683.1818	341.0872(100)	0.0406	24
(+)-taxifolin	21.04	$C_{15}H_{12}O_7$	303.0510	285.0406(100); 275.0562(10); 177.0184(26); 125.0231(72)	2.8118	a
(+)-aromadendrin	24.39	$C_{15}H_{12}O_6$	287.0562	259.0611(100); 243.0660(19); 125.0231(57)	4.1764	a
rutin	24.75	$C_{27}H_{30}O_{16}$	609.1465	300.0275(32); 301.0355(29); 151.0024(2)	1.6171	a
phloridzin	26.16	$C_{21}H_{24}O_{10}$	435.1294	273.0769(100); 167.0339(17); 125.0230(3)	0.9055	a
eriodictyol	27.64	$C_{15}H_{12}O_6$	287.0562	151.0025(100); 135.0439(32); 125.0231(6)	3.6448	a
homovanillic acid	28.03	$C_9H_{10}O_4$	181.0497	137.0232(66); 89.0230(64); 71.0124(82); 59.0124(100)	0.8981	a
protocatechuic acid 4-*O*-*β*-hexoside	28.8	$C_{13}H_{15}O_9$	315.0715	153.0191(100); 109.0295(9)	1.3131	18
quercetin	30.16	$C_{15}H_{10}O_7$	301.0352	178.9976(22); 151.0025(32)	3.0153	a
luteolin-3′-xyloside	32.49	$C_{20}H_{18}O_{10}$	417.0810	285.0612(100); 241.0715(32); 133.0296(4); 151.0402(22)	1.4360	
luteolin	32.83	$C_{15}H_{10}O_6$	285.0404	241.0503(1); 165.0181(3); 225.0550(1); 151.0024(1); 117.0330(1)	4.2938	a
apigenin	33.24	$C_{15}H_{10}O_5$	269.0455	225.0550(1); 151.0024(1); 117.0330(1)	4.4767	a
eriodyctiol-7-*O*-glucoside	37.65	$C_{21}H_{22}O_{11}$	449.1075	287.0558(100); 269.0452(39); 243.0662(1.50)	0.7903	26
(+)-taxifolin-*O*-glucoside	38.50	$C_{21}H_{22}O_{12}$	465.1025	285.0401(100); 303.0507(11); 151.0038(32.20)	0.5244	29
brevifolin carboxylic acid	38.90	$C_{13}H_8O_8$	291.0139	247.0246(100); 203.0357	1.0700	22
ferulic acid hexoside	42.62	$C_{16}H_{20}O_9$	355.1027	193.0507(100); 175.0403(69); 160.0168(8)	1.0239	25
isorhamnetin 3-*O*-glucoside	43.90	$C_{22}H_{22}O_{12}$	447.0558	301.0344(100); 315.0344(8); 151.0037(0.4)	0.0322	26

[a]The letter a indicates that identification of the compound was confirmed by the authentic standards.

trospray Ionization Tandem Mass Spectrometry (LC−MS/ESI−MS/MS), Multiple Reaction Monitoring (MRM) Analysis. *2.4.1. LC-DAD Analysis.* Chromatographic separation was performed using an LC Agilent Series 1100 system with a quaternary pump (Series 1100 G1311A), automatic injector (Series 1100 G1313A), and degasser (series 1100 G1379A). Detection took place using a UV−vis diode detector (Series 1100 G1379A) coupled to a Chemstation HP A.10.02 (HP/Agilent). The column used was a Merck LiChroCART 250−4 Superspher 100 RP-18 1.16056.0001. The method was a binary gradient and was the same as the one described and validated by Álvarez-Fernández et al.[3,12] Each sample was analyzed twice. Identification was achieved by matching the retention time and spectra of the peaks with the corresponding standards. Furthermore, samples were spiked with standards, where commercially available, to achieve complete identification. In accordance with the maximum absorbance of each compound, quantification was performed by external calibration at 280 nm, 320 nm, and 365 nm using the corresponding standards. Calibration curves were obtained by injecting standards diluted from five to eight different concentrations (R^2: 0.9949−0.9998). A duplicate was performed at each point of the calibration curve.

2.4.2. QTRAP Analysis. Mass analysis was performed using a PelkinElmer Series 200 HPLC 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 (QqQLIT) mass spectrometer equipped with an electrospray ion source. LC analyses were performed on a Merck LiChroCART 250−4 Superspher 100 RP-18 1.16056.0001 reversed-phase column. The flow rate was 400 μL min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water and (B) water/acetonitrile 20:80 (v/v). Both phases contained 0.1% formic acid (v/v). The elution gradient was the same as the one used in the LC-DAD analysis. The injection volume was 20 μL.

A MRM experiment was applied, where precursor ions and fragment ions were monitored at Q1 and Q3, respectively, using their corresponding standards.

For HPLC−ESI−MRM analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas 35 psi, ion spray voltage 4500 V, source temperature 350 °C, and source gas 60 psi. A dwell time was set at 50 ms for each transition.

2.4.3. High-Resolution Mass Spectrometry (HRMS). The experiments were performed using a Thermo Fisher Scientific (Bremen, Germany) LC system hybrid quadropole-orbitrap mass spectrometer (Q-OT-qIT). This benchtop UHPLC−MS/MS system combines quadropole precursor ion selection with high-resolution, accurate-mass spectrometer. Identification was performed according to mass spectra, exact mass, characteristic fragmentation, and retention time. Chromatographic separation was performed on a Phenomenex Luna

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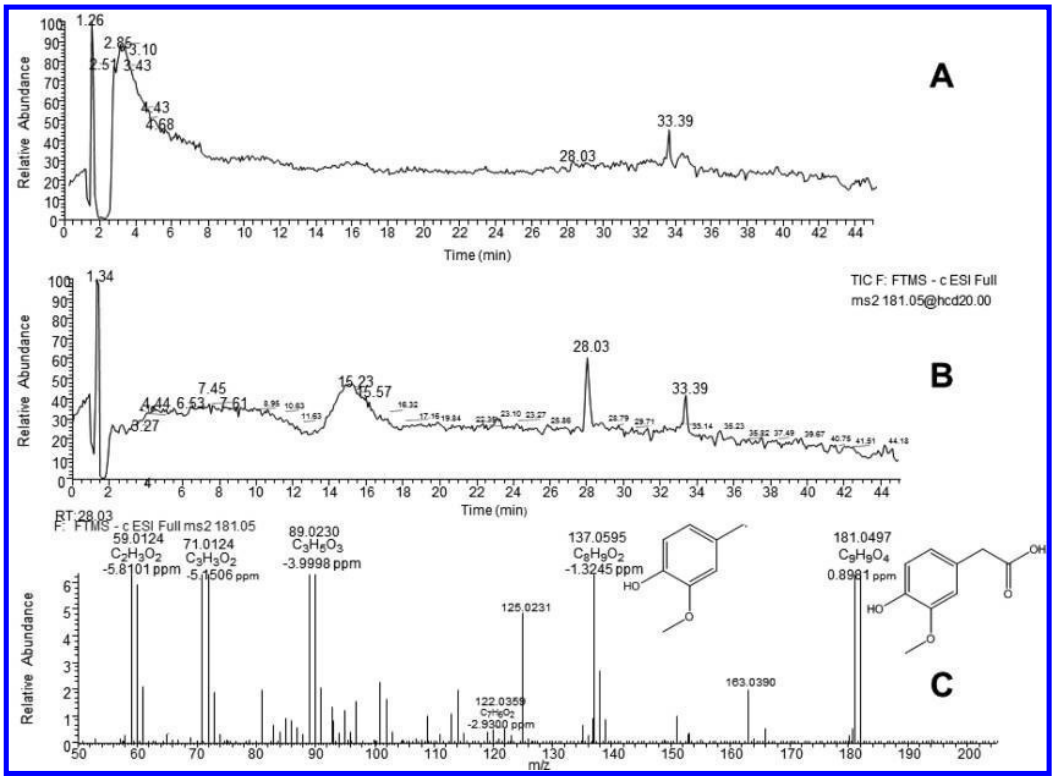

Figure 2. LC−MS/MS chromatogram of homovanillic acid. Panels A and B represent the TIC chromatogram of I and P steps of cycle three of substrate B, where the increase in signal is shown at 28.03 min after fermentation. Panel C represents the EPI chromatogram with the fragmentation pattern of a homovanillic acid isomer.

C18 (150 mm × 2.0 mm, 3 μm) column. The normalized collision energy of the high-collision-induced-dissociation (CID) cell was set at 20 eV. Twenty microliters of sample was injected, and the flow rate was 250 μL min^{-1}.

Identification in negative ionization mode using a binary gradient consisted of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid 0.0−2.0 min 5% B, 2.0−40.0 min from 5−90% B, 40.0−42.0 min from 90−5% B, 42.0−45.0 5% B.

2.5. Antioxidant Activity. *2.5.1. ORAC Test.* The method used was as previously reported with the following modifications:[13] 100 μL of fluorescein (45 nM) and 50 μL of AAPH (15 mM) mixed with 50 μL of sample or Trolox. The reaction took place for a total time of 80 min. Fluorescence was assessed with a fluorometer (multidetector microplate Synergy HT, Biotek) with excitation, and emission wavelengths were 485 and 528 nm, respectively. Trolox was used as a calibration standard (0.5−9.5 μM). Fluorescein fluorescence readings were recorded every 5 min after addition of AAPH until they reached less than 5% of the initial reading. Results were calculated using the areas under the fluorescein decay curves between the blank and the sample and were expressed as micromols Trolox equivalents (TE) per gram of fresh weight. The reported values are the means of at least three experiments.

2.5.2. DPPH Method. Scavenging activity was also evaluated by DPPH using a slightly modified version of the literature method.[14] A 0.1 mL of sample in methanol (previously diluted 1:250) was added to 3.9 mL of DPPH methanolic solution (0.025 g L^{-1}) in a cuvette. Absorbance was recorded at 515 nm at the start time (when the sample was added) and 60 min later, when the reaction reached equilibrium. All measurements were the averages of at least three independent experiments, and methanol was used as the reference. To make the Trolox calibration curve, eight concentrations were used, which ranged from 0.9−0.12 mM. All values reported were recorded on a Hitachi UV-2800 spectrophotometer, thermostated with a Peltier system at 25 °C, and expressed as mmol of TE per kg of fresh weight.

2.6. Statistical Analysis. Statistical analyses were performed by means of Statistica software.[15] One-way analysis of variance (ANOVA) was used to test significant differences at the $p < 0.05$ level. Additionally, linear discriminant analysis (LDA) was tested to build a function to discriminate between the substrate (initial), fermented alcohol (final), and final pasteurized samples of all cycles and the two substrates A and B.

3. RESULTS AND DISCUSSION

3.1. Identification of Nonanthocyanin Phenolic Compounds. Sixty-six nonanthocyanin phenolic compounds and citric acid were identified in the strawberry purée and alcoholic-fermented samples. Figure 1 displays the LC−MS (QTrap) chromatogram, and Table 2 and 3 presents identification data. If standards were not available, identification was based on (I) retention time, (II) UV−vis spectra, (III) the deprotonated ion and mass spectra, (IV) accurate mass measurements, and (V) comparison with data reported in the literature.[16−34] When standards were available, identification was performed either by MRM or HRMS analysis.

As far as we know, 13 of these were identified for first time in strawberry and its derived alcoholic-fermented products: protocatechuic acid-4-*O*-β-hexoside (Peak 16), brevifolin carboxylic acid (Peak 35), ferulic acid glucuronide (Peak 2), dimer caffeic acid-*O*-hexoside (Peak 3), luteolin-3′-*O*-xyloside (Peak 27), isorhamnetin-3-*O*-glucoside (Peak 43), taxifolin-*O*-glucoside (Peak 34), (+)-aromadendrin rhamnoside (Peak 36), eriodictyol-7-*O*-glucoside (Peak 33), (+)-taxifolin, (+)-aromadendrin, eriodictyol, and homovanillic acid.

3.1.1. Hydroxybenzoic Acids and Derivatives. Peak 16 was tentatively identified as protocatechuic acid-4-*O*-β-hexoside. This compound exhibited a deprotonated [M-H]$^-$ ion at *m/z*

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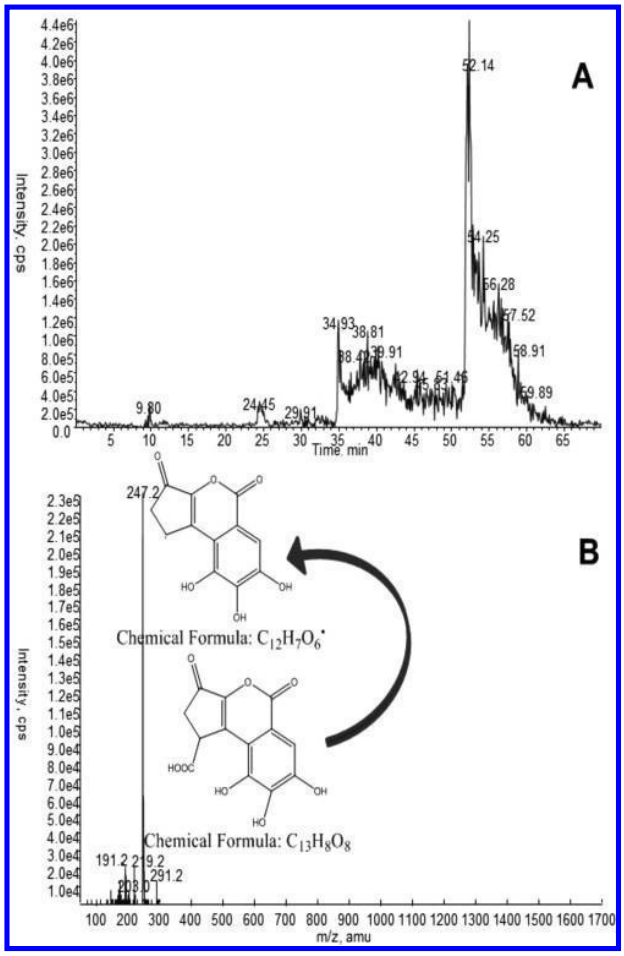

Figure 3. XIC and EPI chromatograms (A and B respectively) of brevifolin carboxylic acid and its fragmentation pattern.

315 and had MS/MS fragments at m/z 152 $[M\text{-}2H\text{-}162]^-$ (loss of glucose moiety) and 108 $[M\text{-}H\text{-}44]^-$ (loss of CO_2); it has been previously identified in artichoke.[18] Peak 26 was tentatively identified as 1-*O*-protocatechuic-*β*-xyloside. It presented a deprotonated ion at $[M\text{-}H]^-$ 285, and its fragmentation pattern was in accordance with the one reported in strawberry.[33] In our study, protocatechuic acid-4-*O*-*β*-hexoside was detected in strawberry (initial samples), in the alcoholic beverages (final sample) of both substrates, and in pasteurized samples of substrate A only.

3.1.2. Hydrolized Tannins. HHDP-Glucose (Peak 4) with pseudomolecular ion m/z 481 produced a fragment at m/z 301, which indicates the release of ellagic acid; this compound has been reported in strawberry before by Hanhineva et al.[25] Peak 28 had a pseudomolecular ion at m/z 951 and MS/MS fragmentation with m/z 907, 783, and 301. The fragmentation pattern and MW were in agreement with those reported before for Tris-galloyl-HHDP-hexose in *Fragaria vesca*.[20]

Peak 35 was tentatively identified as brevifolin carboxylic acid (Figure 3). This compound exhibited a deprotonated ion $[M\text{-}H]^-$ at m/z 291, and its fragmentation pattern had m/z 247, 203, which correspond to loss of carboxylic moieties. This compound was identified in all samples of substrate A. The results indicate the presence of this compound in strawberry and its alcoholic-fermented beverages. Brevifolin carboxylic acid has been reported before in pomegranate fruits,[22] but as far as we know, it has not yet been determined in strawberry.

3.1.3. Ellagic Acid and Derivatives. Peaks 18 and 52 were tentatively identified as methyl ellagic acid derivatives. Peak 18, with deprotonated $[M\text{-}H]^-$ ion 447 and fragmentation with MS/MS m/z 315, corresponds to the loss of pentoside residue, (132 Da), and m/z 301 resulted from the additional loss of a methyl group. This fragmentation pattern corresponds with methyl ellagic acid pentoside. Methyl ellagic derivatives have been reported before in strawberry.[32,20] The second peak, number 52, was tentatively identified as dimethyl ellagic acid pentoside with pseudomolecular ion $[M\text{-}H]^-$ 461, after loss of pentose moiety gave rise to m/z 315 and two fragments, m/z 300 and m/z 145, corresponding to ellagic acid. Many polyphenolic compounds occur naturally as metoxylated derivatives and exhibit a fragmentation pattern where loss of the methyl fragment from the pseudomolecular ion is common, which results in the production of the $[M\text{-}H\text{-}15]^-$ radical.[20]

3.1.4. Hydroxycinnamic Acids. Peak 2 was identified as ferulic acid glucuronide. This compound was detected only in the alcoholic fermented product (final samples of two substrates). It had a deprotonated $[M\text{-}H]^-$ ion 369 and exhibited a fragment with MS/MS m/z 193, which resulted from loss of a glucuronide moiety $[M\text{-}H\text{-}176]^-$. Peak 3 was tentatively identified as a dimer of caffeic acid-*O*-hexoside, described before in *Helichrysum obconicum*.[23] This compound had a pseudomolecular ion $[M\text{-}H]^-$ at 683 and a fragmentation pattern with two fragments, m/z 341 and 179, corresponding to caffeic acid hexoside and caffeic acid, respectively. This was detected only in substrate B, in the initial sample (strawberry) and alcoholic beverages (final samples).

3.1.5. Flavonols. Peak 27 was tentatively identified as luteolin-3′-xyloside with a deprotonated ion at m/z 417. After neutral loss of the sugar moiety $[M\text{-}H\text{-}132]^-$, it results in m/z 285 and 241 fragments, which are characteristic of luteolin aglycone. Peak 43 presented a pseudomolecular ion at m/z 955 and exhibited an MS/MS fragmentation pattern, which matched those attributed to isorhamnetin-3-*O*-glucoside in MassBank record PR040094[26] and ReSpect record PT204190.[31] It had a base peak, m/z 477, which gave rise to a fragment at m/z 301, characteristic of quercetin after cleavage of the molecule. This compound was present in all samples of the two substrates.

3.1.6. Dihydroflavonols. Peak 32 had an ion at 449 and was tentatively identified as (+)-aromadendrin hexoside, reported before in strawberry purée.[12] It presented a fragmentation pattern with base peak at m/z 431 $[M\text{-}H\text{-}18]^-$, which resulted from the dehydration process. Neutral loss of a hexose moiety $[M\text{-}H\text{-}162]^-$ gave rise to a fragment at m/z 287. The fragment at m/z 287 corresponded to (+)-aromadendrin aglycone.

Peak 36 was tentatively identified as (+)-aromadendrin rhamnoside in all samples of substrate A. This compound exhibited a deprotonated ion, $[M\text{-}H]^-$ 433, and presented a base peak at m/z 287, corresponding to (+)-aromadendrin aglycone and resulting from the loss of 146 Da, corresponding to dehydrated rhamnoside moiety. This compound has been described before in *Eucaliptus globulus*.[35]

Peak 34 was tentatively identified as taxifolin-*O*-glucoside in final and pasteurized samples of substrate A and initial and final samples of substrate B. It presented a pseudomolecular $[M\text{-}H]^-$ ion at 465 and MS/MS fragmentation with m/z 285, 151. The MW and fragmentation pattern were in accordance with those

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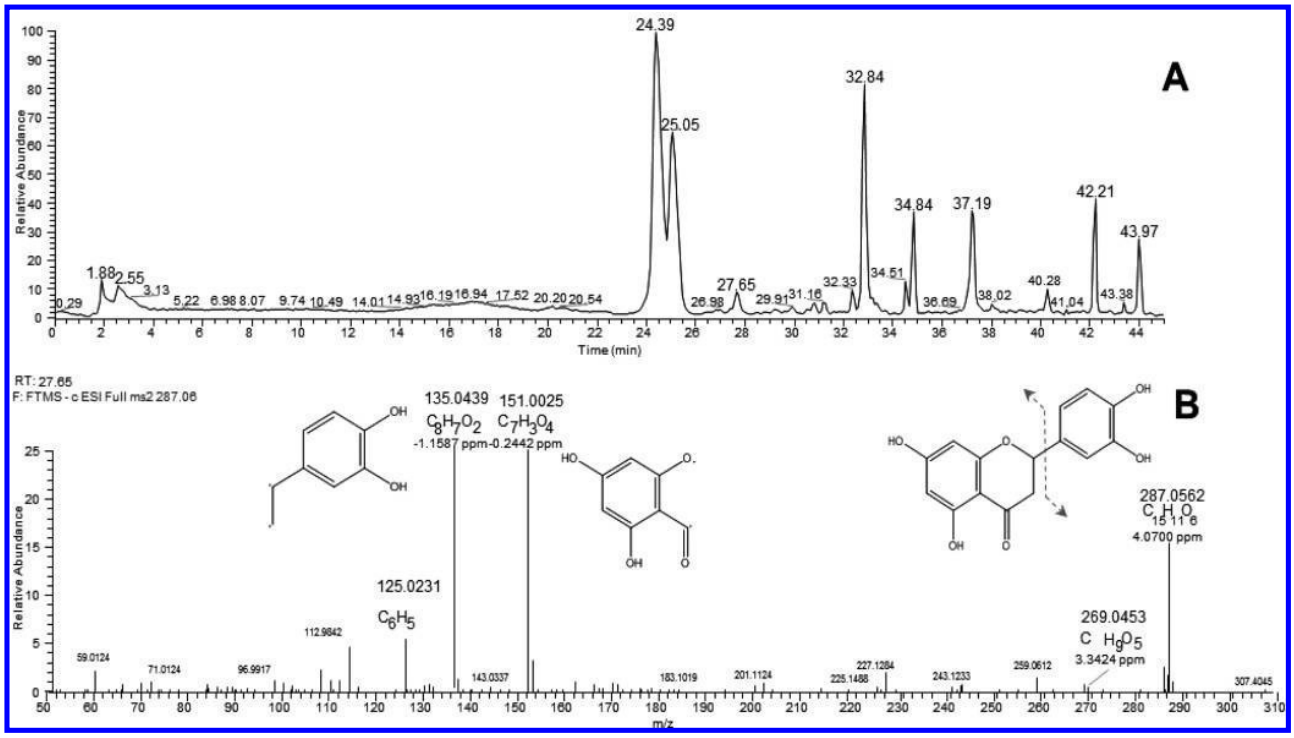

Figure 4. Mass chromatogram of eriodictyol at 25.65 min. (A) TIC chromatogram of a final sample, (B) EPI chromatogram with fragmentation.

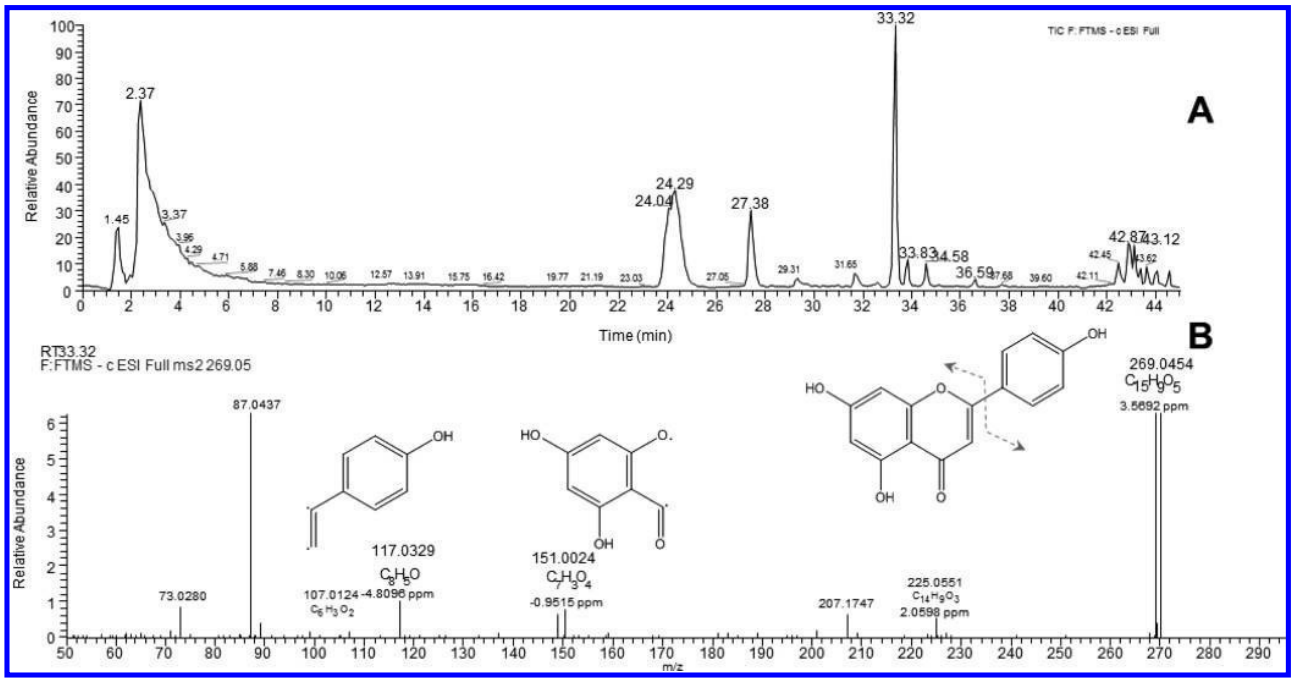

Figure 5. Mass chromatogram of apigenin. (A) TIC chromatogram and (B) EPI chromatogram with fragmentation pattern.

attributed to taxifolin-*O*-glucoside in grapes and red wine prepared from *Vitis vinifera*.[29]

3.1.7. Flavanones. Peak 33 was tentatively identified as eriodictyol-7-*O*-glucoside in all samples of substrate A and initial and final samples of substrate B. It exhibited a deprotonated $[M\text{-}H]^-$ ion at 449, which gave rise to m/z 287 $[M\text{-}H\text{-}162]^-$ by neutral loss, corresponding to sugar moiety (162 Da.). This ion corresponded to eriodictyol aglycone, and the presence of two fragments, m/z 151 and 135, characteristic of eriodictyol, confirmed this tentative identification.

3.1.8. Compounds Identified by HRMS. HRMS was used to confirm the identity of compounds with available standards (Figures 4 and 5). Eleven compounds were identified with standards by generating the molecular formula using accurate mass and matching with the isotopic pattern. The results are shown in Table 3. Three of these compounds, protocatechuic

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Table 4. Contents of Nonanthocyanin Phenolic Compounds (mg kg fw^{-1}) in Alcoholic Fermentation Samples of Substrate A[a]

	Cycle 1			Cycle 2			Cycle 3		
Compounds	I	F	P	I	F	P	I	F	P
(-)-Epicatechin	3.30±0.29^{ab}	5.05±0.71^{a}	4.99±0.68^{b}	4.77±0.35	4.87±0.24	4.88±0.19	4.26±0.10^{a}	4.65±0.18^{a}	4.39±0.33
Eriodictyol-7-*O*-glucoside	1.16±0.12^{ab}	2.88±0.16^{ac}	2.63±0.08^{bc}	0.90±0.20^{ab}	1.17±0.07^{ac}	1.77±0.05^{bc}	0.90±0.00^{ab}	1.23±0.01^{ac}	0.98±0.02^{bc}
Caffeoyl hexose	1.81±0.01^{ab}	3.32±0.05^{ac}	3.53±0.03^{bc}	1.86±0.03^{ab}	2.99±0.02^{ac}	2.52±0.04^{bc}	1.86±0.00^{ab}	3.19±0.04^{ac}	2.98±0.13^{bc}
Ferulic acid derivative	1.26±0.05^{ab}	1.57±0.03^{ac}	1.68±0.03^{bc}	1.50±0.03	1.47±0.05^{c}	1.17±0.01^{c}	0.57±0.05^{ab}	1.44±0.05^{ac}	0.99±0.01^{bc}
Galloyl *bis*-HHDP-glucose	157.25±6.55^{ab}	95.29±1.94^{ac}	144.80±1.17^{bc}	165.44±4.52^{ab}	123.77±1.54^{ac}	127.15±0.52^{bc}	145.07±10.06^{b}	130.48±6.78^{b}	124.00±2.91
HHDP-glucose	2.85±0.44	2.37±0.20^{c}	3.13±0.18^{c}	4.64±0.67^{b}	5.52±1.65^{c}	3.29±0.25^{bc}	3.83±0.46^{a}	4.94±0.19^{ac}	4.02±0.21^{c}
Homovanillic acid	9.40±0.55^{ab}	11.11±0.33^{ac}	3.76±1.04^{bc}	12.81±1.48^{ab}	20.09±1.22^{ac}	3.058±0.39^{bc}	3.06±0.39^{ab}	10.30±0.20^{ac}	8.79±0.75^{bc}
Isorharmentin glucose	3.30±0.14^{a}	4.79±0.20^{ac}	3.48±0.21^{c}	3.80±0.13^{ab}	4.47±0.12^{a}	4.30±0.10^{b}	4.00±0.19^{ab}	5.49±0.16^{a}	3.38±0.42^{b}
Kaempferol glucuronide	2.52±0.18^{ab}	3.72±0.17^{a}	3.80±0.10^{b}	2.41±0.26^{b}	2.67±0.45	3.01±0.08^{b}	2.44±0.30^{a}	3.69±0.06^{a}	3.04±0.62
Luteolin-3′-xylose	2.87±0.05^{ab}	1.92±0.18^{a}	2.02±0.04^{b}	2.17±1.08	1.23±0.10	1.26±0.04	1.15±0.08	1.22±0.05^{c}	0.99±0.15^{c}
Methyl ellagic acid	1.23±0.13^{ab}	2.22±0.08^{ac}	2.78±0.05^{bc}	2.79±0.22^{a}	3.15±0.15^{ac}	2.86±0.08^{c}	1.33±0.07^{ab}	3.74±0.11^{ac}	3.19±0.31^{bc}
Monogalloyl glucose	5.97±2.12^{b}	7.98±0.74^{c}	9.24±0.17^{bc}	7.46±0.21^{a}	8.64±0.52^{ac}	7.58±0.22^{c}	6.05±0.07^{ab}	8.23±0.29^{ac}	7.19±0.36^{bc}
p-Coumaroyl hexoside	73.37±0.94^{ab}	77.17±2.13^{ac}	83.07±0.32^{bc}	82.80±2.64^{ab}	84.39±0.76^{a}	70.32±1.19^{b}	77.81±5.47	79.05±0.91	72.36±5.47
Phloridzin	1.54±0.07^{ab}	3.92±0.43^{a}	3.97±0.04^{b}	2.09±0.29^{b}	2.67±0.64	3.25±0.05^{b}	2.64±0.22^{a}	4.22±0.03^{ac}	3.37±0.61^{c}
p-Hydroxybenzoic acid	1.51±0.41	1.34±0.12^{c}	1.71±0.04^{c}	0.87±0.43^{a}	3.61±0.68^{ac}	1.41±0.10^{c}	6.06±0.62^{b}	5.87±0.43^{c}	5.33±0.50^{bc}
Procyanidin B1	18.12±1.65^{b}	18.25±1.49^{c}	13.07±1.04^{bc}	17.85±1.47^{ab}	11.38±0.95^{ac}	13.08±0.98^{bc}	8.35±1.52^{ab}	4.31±0.97^{ac}	2.46±0.58^{bc}
Protocatechuic acid	0.34±0.16	0.76±0.35^{c}	0.27±0.09^{c}	0.20±0.08^{b}	0.27±0.11^{b}	nd	nd	6.12±0.25	6.34±0.81
Quercetin glucoside	1.00±0.07^{ab}	1.36±0.05^{ac}	0.50±0.09^{bc}	1.03±0.07^{ab}	1.17±0.08^{ac}	0.73±0.03^{bc}	0.75±0.03^{ab}	0.55±0.05^{a}	0.58±0.11^{b}
Quercetin glucuronide	1.00±0.03^{ab}	1.21±0.09^{ac}	1.51±0.05^{bc}	1.57±0.21	1.62±0.16	1.50±0.02	1.52±0.10^{a}	0.99±0.02^{ac}	1.83±0.41^{c}

[a]Mean values and standard deviation. Superscript a indicates a significant difference ($p < 0.05$) between the initial samples (I) and final samples (F) of the same substrate. Superscript b indicates a significant difference ($p < 0.05$) between the initial samples (I) and pasteurized samples (P) of the same substrate. Superscript c indicates a significant difference ($p < 0.05$) between the pasteurized samples (P) and final samples (F) of the same substrate, by ANOVA statistical test. Samples were determined in duplicate. nd, no data.

acid, *p*-hydroxybenzoic acid, and homovanillic acid (Figure 2), were detected in I, F, and P samples and have been reported before as human colonic metabolites of phenolic compounds.[36] Furthermore, increased concentrations of protocatechuic acid and *p*-hydroxybenzoic acid have been reported before due to yeast fermentation in Bokbunja.[37]

3.2. Quantitation of Nonanthocyanin Phenolic Compounds in Alcoholic Fermented Beverages. Nonanthocyanin phenolic compounds were quantified by external calibration with standards, in accordance with the validated LC-DAD method reported.[3] Tables 4 and 5 show the concentrations of the phenolic compounds, with significant increases and decreases after the alcoholic fermentation process ($p < 0.05$). (+)-Catechin content stands out with concentrations ranging from 86.4−144.3 mg kg fw^{-1}, and along with (−)-epicatechin gallate, galloyl-bis-HHDP-glucose, and *p*-coumaroyl glucose, it represents 70% of the total quantified compounds for the two substrates (Supporting Information). The concentration of *p*-coumaroyl glucose and the contents of the others compound listed above are in accordance with the results of gluconic-fermented products reported.[12]

In accordance with the report on sweet wine by Figueredo-González et al.,[38] the condensed tannins detected in ours samples was mainly dimer, specifically procyanidin B1 and appear in concentrations ranged between 14.8 and 28.0 mg kg fw^{-1} on average in initial samples for substrates A and B, respectively.

The most abundant compound was galloyl bis-HHDP-glucose. This compound was described as a monomer unit of two of the most abundant ellagitannis in strawberry, agrimoniin and sanguiin H-6,[39] and decreased during the alcoholic fermentation process (25−59%) in most of the cycles and for both substrates.

The concentration of monogalloyl glucose underwent a significant increase, which ranged from 20−36%, as a consequence of fermentation.

Homovanillic acid showed significant increases, 3.2−3.6% on average, for the two substrates, respectively. Authors such as Jaganath et al.[40] and Dall'Asta et al.[36] attributed the increment of this compound to be a consequence of the transformation of quercetin, rutin, and other quercetin glycosides; they detected this compound as a metabolite derived from human colonic fermentation after application of a diet rich in natural polyphenols. In contrast, in this study, we detected a slight decrease or increase in rutin and quercetin glycosides.

Another compound considered to be a metabolite, produced as a consequence of human colonic fermentation, is *p*-hydroxybenzoic acid.[36,41] It followed a similar pattern to homovanillic acid and underwent a significant increment in the majority of cycles, in both substrates, in a range of increase of 1.28−2.41%. Additionally, Vinjanen et al.[42] reported an increase in the content of *p*-hydroxybenzoic acid in yeast fermentation of lingonberries (*Vaccinium vitis-idaea*). It is known that many compounds, such as anthocyanins and phenolics, are stored in fruits in glycosidically bound form, and fermentation-effect-produced glycosidases can release aglycones of these compounds.

The effects of the pasteurization process were slight and scarce, and when they happened tended to decrease concentrations, for example, in the cases of phloridzin and kaempferol derivatives. Their concentrations experienced reductions in all cycles in amounts ranging from 2−13% for the two substrates.

3.3. LDA Analysis. Quantitated phenolic compounds were used as variables for statistical analysis, and grouping variables were initial (I), final (F), and pasteurized (P) steps. First, ANOVA pinpointed the variables that underwent significant change after the fermentation or pasteurization process, as shown in Tables 4 and 5. Second, 19 variables presenting significant differences were selected for discriminant analysis. Standard stepwise analysis included the whole set of 19 variables in the model, which achieved 98.8% correct

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Table 5. Contents of Nonanthocyanin Phenolic Compounds (mg kg fw^{-1}) in Alcoholic Fermentation Samples of Substrate B[a]

	Cycle 1			Cycle 2		
Compounds	I	F	P	I	F	P
(-)-Epicatechin	3.36±0.07^{ab}	1.81±0.63^{a}	nd	2.27±0.14^{b}	2.31±0.22^{c}	nd
Eriodictyol-7-*O*-glucoside	0.43±0.03^{ab}	0.54±0.07^{ac}	0.82±0.08^{bc}	0.90±0.08^{ab}	0.32±0.03^{a}	0.26±0.04^{b}
Caffeoyl hexose	1.92±0.02^{ab}	2.84±0.04^{ac}	2.61±0.04^{bc}	nd	3.38±0.13	3.30±0.34
Ferulic acid derivative	1.72±0.09^{b}	1.57±0.07^{c}	1.42±0.03^{bc}	1.56±0.04	1.68±0.11	1.61±0.24
Galloyl *bis*-HHDP-glucose	172.93±6.31^{b}	175.30±13.31^{c}	208.15±13.65^{bc}	156.51±2.96^{b}	134.04±12.29^{c}	103.03±1.06^{bc}
HHDP-glucose	9.44±0.30^{ab}	7.22±0.88^{ac}	2.35±0.55^{bc}	3.82±0.62^{ab}	8.47±0.60^{ac}	2.73±0.44^{bc}
Homovanillic acid	5.32±0.38^{ab}	23.73±3.20^{a}	20.25±0.69^{b}	3.39±0.27^{ab}	16.83±1.23^{ac}	13.26±1.04^{bc}
Isorharmentin glucose	3.28±0.05^{a}	4.33±0.41^{a}	4.50±0.34^{b}	2.16±0.08^{ab}	4.02±0.36^{ac}	2.70±0.19^{bc}
Kaempferol glucuronide	1.64±0.13	1.64±0.19	1.65±0.13	1.73±0.09	1.87±0.16	1.88±0.10
Luteolin-3′-xylose	2.16±0.20^{ab}	2.73±0.30^{a}	3.01±0.05^{b}	2.35±0.05^{ab}	0.85±0.02^{ac}	0.54±0.10^{bc}
Methyl ellagic acid	1.19±0.06^{b}	1.16±0.02^{c}	1.36±0.04^{bc}	1.14±0.02^{b}	1.17±0.10^{c}	0.99±0.05^{bc}
Monogalloyl glucose	4.68±0.57	4.26±0.48^{c}	4.11±0.11^{c}	4.45±0.27^{a}	5.59±0.41^{a}	5.54±0.09
p-Coumaroyl hexoside	82.17±1.05^{ab}	76.81±2.62^{ac}	70.96±1.26^{bc}	79.99±1.25^{a}	84.78±2.61^{a}	81.12±7.55
Phloridzin	0.32±0.02^{ab}	1.19±0.41^{a}	0.82±0.25^{b}	1.11±0.07^{ab}	0.41±0.04^{a}	0.36±0.11^{b}
p-Hydroxybenzoic acid	1.91±0.05^{ab}	3.45±0.82^{a}	3.73±0.43^{b}	1.65±0.21^{ab}	6.64±1.02^{ac}	4.51±0.89^{bc}
Procyanidin B1	38.36±6.95^{ab}	9.30±0.44^{a}	8.99±0.37^{b}	37.26±3.89^{ab}	19.02±0.82^{a}	19.75±1.82^{b}
Protocatechuic acid	2.24±0.53	2.77±0.56	2.36±0.34	1.90±0.33^{ab}	3.69±1.36^{a}	3.70±1.13^{b}
Quercetin glucoside	1.53±0.09^{b}	1.08±0.37^{c}	1.81±0.20^{bc}	1.54±0.05^{a}	1.80±0.13^{ac}	1.50±0.14^{c}
Quercetin glucuronide	1.19±0.06^{b}	1.32±0.13	1.40±0.04^{b}	1.16±0.02^{ab}	1.66±0.07^{ac}	1.29±0.03^{bc}

	Cycle 3			Cycle 4		
Compounds	I	F	P	I	F	P
(-)-Epicatechin	2.17±0.15^{ab}	1.64±0.13^{a}	1.73±0.04^{b}	4.95±0.36^{ab}	3.28±0.60^{a}	3.24±0.10^{b}
Eriodictyol-7-*O*-glucoside	0.29+0.05^{ab}	0.15+0.01^{ac}	0.10+0.01^{bc}	0.91+0.06^{ab}	0.55+0.02^{ac}	0.68+0.03^{bc}
Caffeoyl hexose	1.92±0.03^{ab}	2.15±0.02^{ac}	2.21±0.03^{bc}	1.85±0.01^{ab}	2.01±0.04^{ac}	2.07±0.01^{bc}
Ferulic acid derivative	1.19±0.11^{ab}	1.48±0.05^{a}	1.45±0.02^{b}	1.05±0.02^{b}	1.04±0.02^{c}	0.72±0.06^{bc}
Galloyl *bis*-HHDP-glucose	175.91±12.83^{ab}	151.27±7.99^{ac}	118.64±3.80^{bc}	85.01±5.75^{ab}	35.35±4.25^{a}	35.77±1.84^{b}
HHDP-glucose	2.11±0.52^{ab}	1.28±0.08^{a}	1.28±0.05^{b}	2.20±0.05^{b}	2.31±0.13^{c}	1.72±0.26^{bc}
Homovanillic acid	2.24±0.31^{ab}	6.30±0.08^{a}	6.14±0.29^{b}	4.55±0.13^{ab}	9.81±1.02^{a}	10.94±0.25^{b}
Isorharmentin glucose	3.51±0.33^{a}	4.63±0.19^{ac}	3.51±0.04^{c}	3.26±0.11^{ab}	3.75±0.24^{ac}	4.42±0.05^{bc}
Kaempferol glucuronide	1.87±0.33	2.15±0.14^{c}	1.75±0.11^{c}	3.85±0.11	3.82±0.11^{c}	3.50±0.17^{c}
Luteolin-3′-xylose	1.77±0.27^{b}	1.96±0.09^{c}	1.40±0.06^{bc}	0.30±0.02^{ab}	0.18±0.01^{ac}	0.10±0.04^{bc}
Methyl ellagic acid	1.13±0.11^{ab}	1.68±0.07^{ac}	1.55±0.03^{bc}	2.02±0.24	1.65±0.11	1.86±0.14
Monogalloyl glucose	4.77±0.51	5.68±0.20	5.92±0.02	3.13±0.11^{a}	2.79±0.23^{ac}	3.33±0.17^{c}
p-Coumaroyl hexoside	77.87±1.14^{ab}	68.87±2.63^{a}	69.93±0.53^{b}	53.63±2.64^{b}	53.29±2.68^{c}	47.74±1.53^{bc}
Phloridzin	0.15±0.02^{ab}	1.76±0.13^{a}	1.62±0.02^{b}	0.35±0.25^{ab}	0.93±0.03^{a}	0.93±0.10^{b}
p-Hydroxybenzoic acid	1.23±0.24^{ab}	2.24±0.18^{a}	2.27±0.12^{b}	1.14±0.15^{ab}	1.99±0.05^{ac}	2.85±0.22^{bc}
Procyanidin B1	24.99±1.44^{ab}	17.84±1.64^{a}	15.78±0.08^{b}	11.59±4.54^{ab}	6.60±0.16^{ac}	6.16±1.52^{bc}
Protocatechuic acid	1.01±0.30	1.92±0.15	0.78±0.08	0.72±0.05^{ab}	0.48±0.03^{ac}	0.63±0.06^{bc}
Quercetin glucoside	1.56±0.14^{b}	1.68±0.11	1.38±0.04^{b}	0.72±0.40	0.52±0.06^{c}	0.42±0.01^{c}
Quercetin glucuronide	1.51±0.11^{ab}	1.70±0.10^{a}	1.30±0.03^{c}	0.81±0.03^{b}	0.81±0.03^{c}	0.73±0.04^{bc}

[a]Mean values and standard deviation. Superscript a indicates a significant difference ($p < 0.05$) between the initial samples (I) and final samples (F) of the same substrate. Superscript b indicates a significant difference ($p < 0.05$) between the initial samples (I) and pasteurized samples (P) of the same substrate. Superscript c indicates a significant difference ($p < 0.05$) between the pasteurized samples (P) and final samples (F) of the same substrate, by ANOVA statistical test. Samples were determined in duplicate. nd, no data.

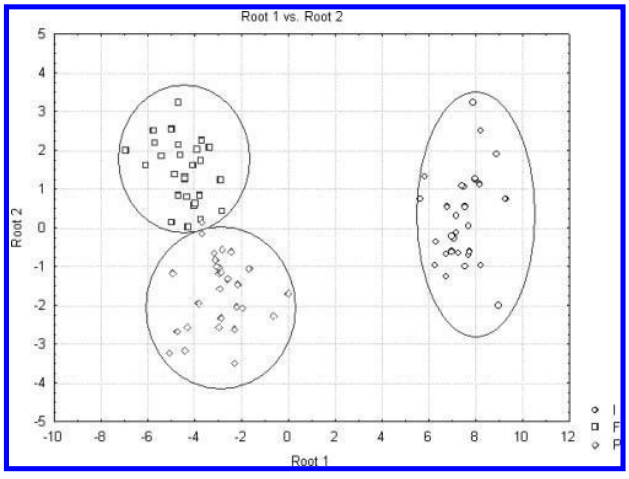

Figure 6. Scatterplot of canonical scores in discriminant analysis for two substrates and 19 variables with 98.8% of classification.

classification of samples. The only incorrectly classified sample was one pasteurized sample, classified as final. Figure 6 shows a scatterplot of the canonical roots obtained. As can be observed, F and P samples are closer than I (initial samples), which reflects the composition change produced by alcoholic fermentation.

3.4. Effect of Alcoholic Fermentation on Antioxidant Activity. Figure 7 displays the results of antioxidant activity determined by two methods, ORAC and DPPH, for the two substrates. The ORAC measurements revealed a significant decrease between initial and final samples for substrate B (17.6%). The same trend was observed between initial and pasteurized samples of the same substrate (15.2%). DPPH results show that only substrate A changed significantly and followed a similar trend to the ORAC data. A significant decrease was observed between initial and final samples (12.3%), and a decrease of and 13.9% between initial and pasteurized samples was observed. These results indicated that alcoholic fermentation caused antioxidant activity to decrease slightly; in contrast with reports on gluconic fermentation[12] and

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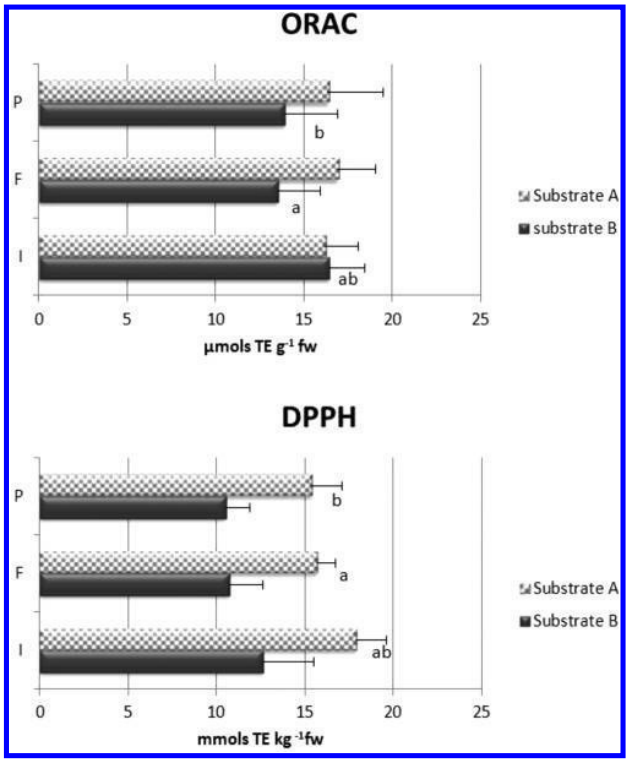

Figure 7. Bar representation of antioxidant activity by two methods, ORAC and DPPH, for the two substrates. Letters a, b, and c correspond to significant differences between I/F, I/P, and F/P samples, respectively.

alcoholic fermentation in mulberries,[43] no significant changes were detected. Apart from nonanthocyanin polyphenolic compounds, strawberry is a particularly rich source of anthocyanins,[44] which are outside the scope of this paper, which is focused on the identification of a large set of nonanthocyanin compounds. Indeed, the extracts for obtaining the nonanthocyanin fraction may present other compounds, which would account for a certain part of the antioxidant activity, which could also explain this average decrease.

3.5. Conclusions. Sixty-six compounds were identified by MS and HRMS. A total of 13 compounds was identified for the first time in strawberry and an alcoholic-fermented product: protocatechuic acid-4-*O*-*β*-hexoside, brevifolin carboxylic acid, ferulic acid glucuronide, dimer caffeic acid-*O*-hexoside, luteolin-3′-*O*-xyloside, isorhamnetin3-*O*-glucoside, taxifolin-*O*-glucoside, (+)-aromadendrin rhamnoside, eriodictyol-7-*O*-glucoside, (+)-taxifolin, (+)-aromadendrin, eriodictyol, and homovanillic acid.

LDA allows the correct classification of samples I, F, and P as alcoholic fermentation induces changes in phenolic composition, mainly related to the 19 compounds. Specifically, homovanillic acid and *p*-hydroxybenzoic acid increased significantly in almost every fermentation cycle.

■ ASSOCIATED CONTENT

Supporting Information

Contents of nonanthocyanin phenolic compounds that are not reported in this work for substrates A and B. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Determination of Nonanthocyanin Phenolic Compounds Using High-Resolution Mass Spectrometry (UHPLC-Orbitrap-MS/MS) and Impact of Storage Conditions in a Beverage Made from Strawberry by Fermentation

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Supporting Information

ABSTRACT: Overproduction of strawberry leads to food waste, as it is very perishable. Therefore, strategies to transform it into new products are appreciated. This research focuses on characterization of the nonanthocyanin phenolic content of a beverage obtained from strawberry by gluconic and acetic fermentation and subsequently monitored for 90 days of storage, at two temperatures. Sixty-four nonanthocyanin (poly)phenols were identified by high-resolution mass spectrometry (UHPLC coupled with linear trap quadrupole and Orbitrap mass analyzer) and, for the first time, four compounds were reported in beverages fermented from strawberry: aromadendrin hexoside, phloretin 2′-*O*-xylosyl glucoside, dihydroferulic acid 4-*O*-glucuronide, and kaempferol hexosyl hexoside. During the storage time the increase in protocatechuic acid content was 13 times and condensed tannins diminished, especially procyanidin trimer. Statistical analysis showed that the composition remains unchanged until day 15 of storage at room temperature (27−30 °C) and until day 30 under refrigerated conditions (4 °C).

KEYWORDS: *stability, Orbitrap mass spectrometer, gluconic fermentation, principal component analysis, sensory*

INTRODUCTION

Phenolic compounds are a group of ubiquitous compounds throughout the plant kingdom and many occur in foods. In the late 20th century, interest in food rich in phenolic compounds increased due to their antioxidant and anti-inflammatory properties, their modulation of signal transduction, and their antimicrobial and antiproliferation activities.[1] Consuming fruits with high polyphenol content has beneficial health implications.

Strawberry (*Fragaria* × *ananassa*) is a source of many phenolic compounds that have beneficial effects on health. This product is widely produced and consumed thoroughout the world, and Spain is one of the leading producers, with a total production of 312,500 MT;[2] this is not consumed or exported in its entirety. This situation generates overproduction and, consequently, waste, which could be avoided by creating foodstuffs that maintain unchanged the properties of the fruit used as raw material as much as possible as well as its composition in bioactives.

Fermentation is an ancient process to transform and preserve foods. It involves raw products undergoing chemical transformations as the result of the action of bacteria or yeast enzymes[3] as in the case of aroma and chemical composition, being a method of food processing that reduces sugar content.[4] Johnson et al. reported that fermented berry beverages have shown increased phenolic content and higher antioxidant activity than their nonfermented counterparts.[5] In addition, in our previous work we found that after gluconic fermentation, the bioactive content remained practically unchanged.[6] Therefore, the study of fermented beverages deserves attention.

Reque et al. reported that, under refrigeration conditions of storage, blueberries fruits and juices present good stability with respect to antioxidant capacity, but with significant anthocyanin loss, possibly due to oxidation and/or condensation reactions with other phenolic compounds.[7] Glycosylated flavonols have also been reported to decrease during storage, whereas the respective aglycones increase, indicating that enzymatic hydrolysis has occurred. However, these reactions do not affect the total polyphenol content, which remains constant.[8] Conversely, studies detail that the individual nonanthocyanin phenolic compounds are required to gain deeper insight into chemical composition changes under effects of different temperatures during storage.

The gluconic fermentation of strawberry presents advantages from a nutritional perspective as *Gluconobacter* strains do not metabolize the fructose naturally present in fruit, so it remains in the beverage as a sweetener,[6,9] whereas glucose is transformed into gluconic acid.

As gluconic fermentation is a very innovative process, few data have been reported in the literature about the effects of storage on the nonanthocyanin composition of the beverages produced by this means, to the best of our knowledge. An accurate characterization is required to discover how time and storage conditions [refrigeration (4 °C) and room temperature

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(27−30 °C)] could affect the beverages to establish the optimum conditions to maintain bioactive composition.

The aims of this paper are, first, to establish the effects of storage for 3 months at refrigeration temperature (4 °C) and room temperature (27−30 °C) on the nonanthocyanin composition and antioxidant activity of an innovative gluconic-fermented beverage as an alternative to take advantage of overproduction of strawberry and avoid its waste and, second, to detail the chemical composition to achieve complete characterization of the products.

MATERIALS AND METHODS

Chemicals and Reagents. The standard compounds used were acquired from Fluka Sigma-Aldrich (St. Louis, MO, USA) [gallic acid, caffeic acid, *p*-coumaric acid, cinnamic acid, quinic acid, (−)-epicatechin, (+)-catechin, chlorogenic acid, ellagic acid, (−)-epicatechin gallate, kaempferol, kaempferol-3-glucoside, polydatin, apigenin, quinic acid, brevifolin, protocatechuic acid, (+)-taxifolin, eriodictyol, rutin, quercetin, homovanillic acid, and naringenin;, from Chromadex Inc. (USA) (procyanidin B1). From Extrasynthese [luteolin, (+)-aromadendrin, phloridzin, and flavonomarein]. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-diazo-bis(amidine-propane-dihydrochloride) (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and dimethyl dicarbonate were obtained from Sigma-Aldrich (St. Louis, MO, USA), acetonitrile and formic acid (LC gradient) were from Merck (Darmstadt, Germany), and methanol was from Prolabo (Obregón, Mexico).

Samples. A beverage was made from strawberry by gluconic fermentation [using strawberry pureé as substrate into a biorreactor, and after few minutes (10−12) was added 125 mL of inoculum of G *japonicus* strain E1; the end of the fermentation process was established when the glucose had been totally consumed] as previously reported[6] and mixed with a small proportion of acetic fermented product. The strawberry vinegar was obtained by a semicontinuous fermentation by *Acetobacter malorum* (CECT 7749), at 30 °C, 70% O_2, 13.88 g.[10]

Subsequently, the product obtained was centrifuged 10 min at 1500*g* and diluted with soda water and dimethyl dicarbonate as a preservative to stop the fermentation process. The beverage thus obtained was distributed into individual bottles, pasteurized at 90 °C for 90 s, and then placed in the refrigerator (4 °C) or at room temperature (about 30 °C), until samples were taken (0, 15, 30, 60, and 90 days). The pasteurization process was performed to mimic the industrial process to make the beverages. The initial samples are the same for both temperature conditions (R_0/F_0). As samples were taken, they were immediately stored at −18 °C until analysis. Table 1 details sample codes as follows: F indicates refrigerated samples and R, room temperature samples, followed by a subscript number that indicates the storage days.

Table 1. Sample Codes

storage time (days)	room temperature	refrigerated temperature
0	R_0 (initial)	F_0 (initial)
15	R_{15}	F_{15}
30	R_{30}	F_{30}
60	R_{60}	F_{60}
90	R_{90}	F_{90}

Extraction Procedure. A sample of 30 g was extracted with 30 mL of methanol and 1% w/w of ascorbic acid and then 30 min of sonication. It was centrifuged for 10 min at 1500*g*. The supernatant was collected and the pellet re-extracted with methanol according to the same procedure. The fraction was mixed, evaporated to dryness, and then reconstituted in 50% (v/v) methanol.[11,12]

High-Resolution Mass Spectrometry (HRMS) Analysis. The experiments were performed using a Thermo Fisher Scientific (Bremen, Germany) liquid chromatography system hybrid Q-OT-qIT mass spectrometer (hybrid quadrupole-Orbitrap Elite mass spectrometer). This benchtop UHPLC-MS/MS system combines quadrupole precursor ion selection with a high-resolution, accurate-mass spectrometer. Identification was performed according to mass spectra, exact mass, characteristic fragmentation, and retention time. Xcalibur software (version 3.0.63) was used for instrument control, data acquisition, and data analysis. Internet databases of accurate mass spectrometry data, mzcloud (www.mzcloud.org), Metlin, to identify molecular characteristics (https://metlin.scripps.edu/index.php) and Massbank[13] and ReSpect for phytochemicals,[14] were used as a reference library to identify compounds.

The UHPLC separation was performed using a binary gradient consisting of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid: 0.0−1.0 min, 5% B; 1.0−12.0 min, from 5 to 100% B; 12.0−13.0 min, from 100%to 5% B; and 13.0−15.0 min, 5% B. Twenty microliters of sample was injected, and flow rate was 400 μL min^{-1}. The eluate was analyzed using full MS and data-dependent scanning in negative mode to MS/MS analysis and with a Zorbax SB-C18 2.1 × 100 mm, 1.8, μm column. The normalized collision energy of the high-collision-induced-dissociation (CID) cell was set at 35 eV for data-dependent scan. Helium was employed as sheath gas (53 arbitrary units), sweep gas flow was 3 arbitrary units, and auxiliary gas flow was 14 arbitrary units. Automatic gain control was established as follows: ion trap full, SIM, and MS^n AGC target, 10,000.00; FTMS full AGC target (1000.00) and SIM, MS^n AGC target (50,000.00).

Data-dependent scanning comprises a full MS scan (the range was from m/z 100 to 1500 and the resolution was 30,000 FWHM), followed by a data-dependent scan (resolution of 15,000 FWHM).

To quantitate nonanthocyanin phenolic compounds, we used TraceFinder software using the corresponding aglycone when the compounds were glycosides. In the case of hydrolyzable tannins we used ellagic acid and procyanidin B1 to quantitate condensed tannins. Calibration curves were obtained by injecting standards diluted from seven different concentrations (R^2 = 0.9995−0.9979). A triplicate was performed at each point of the calibration curve.

Sensory Analysis. The sensory panel gathers 10 trained assessors, 7 females and 3 males, ranging from 22 to 45 years old, selected and trained according to ISO 1993.[15] Most of them participate regularly in sensory panels of beverages and have accumulated >100 h of experience in sensory analysis. Additionally, a 20 h specific training course was accomplished by every participant and consisted in ordering scales of fructose, gluconic acid, acetic acid, strawberry purées, and fermented beverages from strawberry.

The trained panel selected the descriptors that better reflect the sensorial characteristics of the beverages by open discussion with the panel leader and consensus. These attributes were strawberry taste, acidity, sweetness, strawberry aroma, and overall impression.

The panel carried out 12 triangular tests[16] to differentiate samples stored at two different temperatures, room (27−30 °C) and refrigeration temperature (4 °C), and six triangular tests to differentiate between times of storage.

Eight samples were tasted in duplicate by the sensory panel using the selected attributes on a 10 cm unstructured scale ranging from 0 ("nonexistent") to 10 ("very strong") and for overall impression ranging from 0 ("dislike extremely") to 10 ("like extremely") using standard wine-tasting dark cups. To evaluate color changes, transparent glass cups have been used.

Antioxidant Activity. *Oxygen Radical Absorbance Capacity (ORAC Method).* The method used was as reported, with some modifications:[17] 100 μL of fluorescein solution (45 nM) and 50 μL of AAPH (15 mM) mixed with 50 μL of sample solution or Trolox. The sample solutions were made in phosphate buffer (0.022:100 v/v). Fluorescence was assessed with a fluorometer (multidetector microplate Synergy HT, Biotek) with excitation and emission wavelengths of 485 and 528 nm, respectively. Data were recorded every 5 min after the addition of AAPH until they reached <5% of the initial value. Results were calculated using the areas under the fluorescein decay curves between the blank (buffer + fluorescein + AAPH) and the sample, with Trolox as a calibration standard (seven different

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Table 2. Nonanthocyanin Phenolic Compounds Tentatively Identified in Beverages Obtained from Fermented Strawberry[a]

peak	tentative identification	t_R (min)	mol formula	exact mass $[M-H]^-$	Δmass (ppm)	MS/MS fragments (rel abundance %)	ref
			Hydroxybenzoic Acids and Derivatives				
3	gallic acid[b]	1.70	$C_7H_6O_5$	169.0143	6.5199	125.0244 (100); 124.0165 (0.12); 97.0295 (0.11)	
8	protocatechuic acid[b]	3.33	$C_7H_6O_4$	153.0193	6.9744	109.0295 (100); 108.0172 (15)	
10	protocatechuic acid 4-*O*-*β*-hexoside	4.10	$C_{13}H_{16}O_9$	315.0727	3.3800	153.0191 (100); 109.0295 (9)	29
15	1-*O*-protocatechuyl-*β*-xylose	4.40	$C_{12}H_{14}O_8$	285.0605	0.0279	153.0193 (100); 152.0116 (31); 109.0296 (6); 108.0218 (4)	30
19	*p*-hydroxybenzoic acid-*O*-glucoside	4.79	$C_{13}H_{16}O_8$	299.0778	5.6038	137.0244(100); 93.0347 (2)	39
26	2,4-dihydroxybenzoic acid	5.57	$C_7H_6O_4$	153.0194	7.6700	109.0295 (100); 67.0194 (2)	39
34	phloretic acid	6.30	$C_9H_{10}O_3$	165.0556	5.7668	119.0503 (100); 121.0567 (7); 93.0376 (3)	39
51	4-hydroxybenzoic acid	7.31	$C_7H_6O_3$	137.0242	6.0310	93.0348 (100)	39
			Hydrolyzed Tannins				
1	HHDP-glucose	1.10	$C_{20}H_{18}O_{14}$	481.0638	5.3242	300.9991 (100); 275.0199 (13); 249.0402 (1)	26
5	tris-galloyl-HHDP-hexose	2.73	$C_{41}H_{28}O_{27}$	951.0722	1.2796	907.0834 (100); 783.0674 (66); 605.0583 (7)	21
7	monogalloyl glucose	3.00	$C_{13}H_{16}O_{10}$	331.0677	5.2722	313.0564 (100); 169.0146 (19); 151.0050 (2); 168.0067 (71)	26
9	bis-HDHP-glucose	3.68	$C_{34}H_{24}O_{22}$	783.0680	0.5679	481.0619 (21); 300.9984 (100); 275.0195 (22)	30
6	galloylquinic acid	2.98	$C_{14}H_{16}O_{10}$	343.0691	2.3844	191.0566 (8); 173.0459 (14); 169.0147 (100); 125.0250 (5)	26
16	galloyl-HHDP-glucose	4.42	$C_{27}H_{22}O_{18}$	633.0721	0.2488	463.0515 (9); 300.9984 (100); 283.9958 (1); 229.0093 (1)	26
24	brevifolin carboxylic acid	5.48	$C_{13}H_8O_8$	291.0152	5.5792	247.0246 (100); 203.0351 (0.2)	23
27	agrimoniin	5.58	$C_{41}H_{26}O_{26}$	934.0757	5.4095	1567.1407 (81); 1265.1399 (28); 1085.0741 (36); 916.0588 (39); 915.0543 (54); 897.0430 (100); 783.0692(45); 633.0726(48)	30
			Ellagic Acid and Derivatives				
33	ellagic acid hexoside	6.28	$C_{20}H_{16}O_{13}$	463.0498	1.9699	300.9979 (100); 299.9907 (55); 283.9958 (0.1)	26
42	tetramethylellagic acid hexose	6.97	$C_{26}H_{34}O_{11}$	521.2014	0.7352	359.1480 (30); 341.1363 (100)	30
43	ellagic acid pentoside	7.07	$C_{19}H_{14}O_{12}$	433.0404	0.5140	300.9994 (100); 299.9918 (89); 283.9995 (0.1)	30
48	ellagic acid deoxyhexose	7.21	$C_{20}H_{16}O_{12}$	447.0555	0.7187	300.9983 (100); 283.9947 (0.1); 257.0085 (0.5)	26
52	ellagic acid[b]	7.40	$C_{14}H_6O_8$	300.9981	0.7122	300.9984 (26); 283.9963 (18); 257.0090 (100); 229.0141 (47); 185.0244 (19)	
			Flavanols				
17	(+)-catechin[b]	4.62	$C_{15}H_{14}O_6$	289.0724	6.0664	245.0820 (100); 205.0508 (33); 179.0352 (13); 125.0248 (4); 109.0297 (1)	
38	(+)-catechin-*O*-hexoside	6.42	$C_{21}H_{24}O_{11}$	451.1212	3.6835	415.1114 (82); 289.0718 (100)	22
			Hydroxycinnamic Acids				
13	caffeic acid hexose	4.30	$C_{15}H_{18}O_9$	341.0874	2.0342	179.0348 (100); 161.0244 (60); 135.0452 (8)	26
14	dimer of caffeic acid-*O*-hexoside	4.33	$C_{30}H_{36}O_{18}$	683.1818	2.0142	341.1058 (100)	
20	*p*-coumaroyl glucose	4.99	$C_{15}H_{18}O_8$	325.0919	0.2907	265.0724 (11); 235.0619 (5); 205.0513 (6); 187.0407 (28); 163.0407 (59); 145.0301(100); 119.0508 (5); 117.0351 (2)	26
22	caffeic acid[b]	5.28	$C_9H_8O_4$	179.0350	6.3323	135.0451 (100)	
23	ferulic acid hexose	5.30	$C_{16}H_{20}O_9$	355.1019	1.2965	217.0505 (56); 193.0506 (100); 175.0401(65); 160.0166 (7); 134.0375 (5)	26
35	*p*-coumaric acid[b]	6.33	$C_9H_8O_3$	163.0398	4.8687	119.0502 (100)	
40	*p*-coumaroylquinic acid	6.60	$C_{16}H_{18}O_8$	337.0914	1.1681	191.0562 (40); 163.0405(100)	39
55	ferulic acid	7.60	$C_{10}H_{10}O_4$	193.0504	4.7153	149.0608 (100); 178.0265 (1); 134.0376 (1)	39
57	dihydroferulic acid 4-*O*-glucuronide	7.71	$C_{16}H_{20}O_{10}$	371.0985	3.4171	209.0815 (18); 193.0505 (100)	40
			Flavones				
21	luteolin-3′-xyloside	5.04	$C_{20}H_{18}O_{10}$	417.0781	5.7530	285.0612 (32); 241.0715 (100); 152.0116 (73); 151.0402 (22); 133.0296 (4)	21
28	apigenin pentose	5.67	$C_{18}H_{26}O_{10}$	401.1449	1.6805	269.1028 (100); 161.0457 (22.45); 149.0458 (0.6)	29
32	apigenin-7-*O*-glucoside	6.05	$C_{21}H_{20}O_{10}$	431.0970	0.5272	269.0451 (100); 225.0611 (68)	14
36	luteolin[b]	6.38	$C_{15}H_{10}O_6$	285.0410	5.7927	241.0507 (100); 217.0507 (20); 199.0402 (22); 175.0403 (75)	
63	apigenin[b]	9.26	$C_{15}H_{10}O_5$	269.0458	4.9304	225.0559 (100); 151.0040 (33); 149.0248 (50); 117.0350 (3)	

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Table 2. continued

peak	tentative identification	t_R (min)	mol formula	exact mass $[M-H]^-$	Δmass (ppm)	MS/MS fragments (rel abundance %)	ref
			Flavonols				
31	kaempferol hexosylhexoside	5.92	$C_{27}H_{30}O_{16}$	609.1437	2.1905	285.0410 (38); 284.0334 (1)	27
39	quercetin pentose glucuronide	6.58	$C_{26}H_{26}O_{17}$	609.1086	0.1725	301.0428 (100); 178.9944 (1)	26
44	quercetin glucuronide	7.11	$C_{21}H_{18}O_{13}$	477.0660	0.5302	301.0347 (100); 178.9987 (1); 151.0038 (0.8)	26
45	isorhamnentin-3-*O*-glucoside	7.16	$C_{22}H_{22}O_{12}$	477.1017	2.1743	433.1145 (0.57); 301.0356 (100); 178.9990 (1)	13
46	rutin[b]	7.17	$C_{27}H_{30}O_{16}$	609.1439	1.7897	301.0345 (100); 300.0265 (0.28); 255.0289 (0.31)	
47	quercetin-3-*O*-glucoside	7.20	$C_{21}H_{20}O_{12}$	463.0859	2.5155	301.0345 (100); 300.0270 (36); 271.0240 (0.6); 255.0655 (0.31); 178.9984 (2.08); 151.0035 (1.61)	30
54	kaempferol hexose[b]	7.59	$C_{21}H_{20}O_{11}$	447.0904	3.9017	285.0398 (68); 284.0334 (100); 255.0650 (2); 227.0356 (4)	
56	kaempferol-3-glucuronide	7.67	$C_{21}H_{18}O_{12}$	461.0714	0.0893	285.0396 (100); 257.0462 (0.15)	26
58	isorhamnetin-3-glucuronide	7.82	$C_{22}H_{20}O_{13}$	491.0816	0.7788	315.0502 (100); 301.0351 (1); 271.0236 (0.1); 255.0305 (0.1)	26
59	quercetin[b]	8.38	$C_{15}H_{10}O_7$	301.0359	5.4483	273.0399 (13); 257.0451 (11); 193.0140 (5); 178.9984 (100)	
61	kaempferol-3-coumaroylhexoside	8.66	$C_{30}H_{26}O_{13}$	593.1268	3.7043	447.0562 (2); 285.0398 (100); 257.0452 (3); 229.0459 (2)	28
62	kaempferol[b]	9.06	$C_{15}H_{10}O_6$	285.0397	1.0819	285.0398 (100); 257.0453 (15); 185.0608 (11); 169.0660 (10); 151.0037 (25)	
64	galangin[b]	10.39	$C_{15}H_{10}O_5$	269.0456	4.1364	241.0506 (27); 227.0345 (100); 197.0605 (39); 183.0449 (77); 169.0657 (10)	
			Condensed Tannins				
11	proanthocyanidin trimer	4.18	$C_{45}H_{38}O_{18}$	865.1999	2.8313	739.1686 (4); 695.1419 (100); 587.0942 (27); 575.0942 (35); 543.0674 (16); 287.0565 (5)	26
12	proanthocyanidin B1[b]	4.28	$C_{30}H_{26}O_{12}$	577.1331	1.6199	425.0873 (100); 407.0768 (87); 289.0716 (47)	
18	propelargonidin dimer	4.75	$C_{30}H_{26}O_{11}$	561.1401	1.7799	289.0715 (100); 245.0818 (7) 271.0611 (14); 245.0818 (7)	25
			Dihydroflavonols				
25	aromadendrin hexoside	5.53	$C_{21}H_{22}O_{11}$	449.1073	1.1980	287.0558 (100); 259.0609 (43); 125.0261	23
30	(+)-taxifolin-7-*O*-glucoside	5.82	$C_{21}H_{22}O_{12}$	465.1025	0.5244	285.0401 (100); 177.0195 (1.34)	14
37	(+)-taxifolin[b]	6.39	$C_{15}H_{12}O_7$	303.0517	5.7321	285.0403 (100); 177.0195 (12); 125.0247 (7)	
41	(+)-taxifolin-3-*O*-arabinofuranoside	6.90	$C_{20}H_{20}O_{11}$	435.0922	0.0567	303.0503 (100); 285.0399 (52); 275.0559 (0.5); 177.0192 (4); 125.0244 (1.4)	40
50	aromadendrin[b]	7.25	$C_{15}H_{12}O_6$	287.0574	5.3222	259.0613 (100); 243.0665 (18); 125.0247 (3)	
			Flavanones				
29	eriodictyol-7-*O*-glucoside	5.68	$C_{21}H_{22}O_{11}$	449.1078	0.0251	287.0557 (100); 151.0038 (0.3)	13
60	naringenin[b]	8.51	$C_{15}H_{12}O_5$	271.0619	6.4900	203.0364 (3); 177.0196 (20); 165.0197 (5); 151.0039 (100)	
			Chalcones				
49	phloretin-2′-*O*-xylosyl-galactoside	7.24	$C_{26}H_{32}O_{14}$	567.1688	3.5416	273.0764 (100)	40
53	phloridzin[b]	7.44	$C_{21}H_{24}O_{10}$	435.1303	3.9212	273.0763 (100); 167.0350 (2); 125.0240 (0.1)	
			Others				
2	citric acid[b]	1.16	$C_6H_8O_7$	191.0188	0.9689	111.0088 (100); 129.0193 (3); 173.0090 (15); 67.0197 (0.12)	
4	quinic acid	2.17	$C_7H_{12}O_6$	191.0560	4.9970	173.0452 (51); 171.0302 (19); 155.0354 (6); 127.0401 (53); 109.0298 (16); 93.0347 (32); 85.0297 (100)	37

[a]Δmass is deviation of the observed ion mass from the corresponding calculated monoisotopic mass. Peak is the number of the compound in the chromatogram. Ref. previous reports of compounds. [b]Analytes confirmed by comparison with pure standards.

concentration solutions ranged from 0.5 to 9.5 μM) and expressed the results as micromoles of Trolox equivalents (TE) per gram of fresh weight. The reported values are the means of at least three experiments.

DPPH Method. Scavenging activity was also evaluated by DPPH, using a slightly modified version of the method proposed in the literature.[18] A 47.3 mg L^{-1} DPPH daily prepared methanolic solution was used and stored (protected from light and refrigerated). The samples were prepared in five different concentrations in methanol: water 1:1 (20, 30, 40, 45, and 50 ppm); and 50 μL of the sample solution was added to 150 μL of DPPH solution (47.3 mg L^{-1}). A control (50 μL of methanol + 150 μL of DPPH solution) and blank (200 μL of methanol) were also prepared. After shaking, absorbance was determined at 515 nm every 5 min for an hour in a multidetection microplate (Synergy HT, Biotek). Each measurement was taken in triplicate. The results were calculated by plotting percentage of inhibition at 60 min against the concentration

$$\%\ \text{inhibition} = [(A_0 - A_E/A_0)] \times 100$$

where A_0 is the initial absorbance and A_E is the absorbance at 60 min.

IC_{50} represents the amount of sample needed to reduce the concentration of DPPH radicals to half and was calculated from the

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curve of different dilutions. A lower value of IC_{50} indicates higher antioxidant activity.[19]

Statistical Analysis. Statistical analyses were performed by means of Statistica software.[20] One-way analysis of variance (ANOVA) and Tukey's HSD (honest significant difference) test were assessed to test significant differences at $p < 0.05$. Additionally, principal component analysis (PCA) was used for data analysis.

RESULTS AND DISCUSSION

Identification of Nonanthocyanin Phenolic Compounds. Sixty-four nonanthocyanin phenolic compounds, including hydroxybenzoic acids and derivatives, hydrolyzed and condensed tannins, ellagic acid and derivatives, hydroxycinnamic acids, flavonols, dihydroflavanols, flavanones, flavanols, flavanones, chalcones, and others such as citric and quinic acid, were identified in the samples analyzed. Table 2 and Figure S1 summarize the identified nonanthocyanin phenolic compounds.

Identification was based on matching the retention time, mass spectra, accurate mass measurements, and MS^2 analyses with standards when they were commercially available. If not (as was the case for 13 of 64 compounds), the above-mentioned parameters were compared with data in the literature[21−30] and the databases cited earlier. Additionally, the data of these 13 compounds are discussed as follows in this section as their novelty in this product deserves more discussion. Four of these compounds were tentatively identified for the first time in a strawberry-derived product, as follows: aromadendrin hexoside, phloretin 2′-*O*-xylosyl glucoside, dihydroferulic acid 4-*O*-glucuronide, and kaempferol hexosyl-hexoside. As far as we know, they have not been reported before in either strawberry or its derived products.

Hydroxybenzoic Acids and Derivatives. Chromatograms in full scan MS mode and data-dependent scans showed the presence of m/z 153.0194. It had a loss of 44 u $[M - H - 44]^-$, characteristic of this chemical group,[29] with a product ion m/z 109.0295. MS^2 spectra exhibited a product ion m/z 67.0194, after a loss of 42.01, corresponding to an acetyl moiety.[26] This fragment pattern matches the data obtained in mzCloud for 2,4-dihydroxybenzoic acid, corresponding to peak 26, as shown in Table 2.

Hydrolyzed Tannins. Peak 6 exhibited a $[M - H]^-$ at 343.0691, with a molecular formula $C_{14}H_{16}O$ and a fragmentation pattern with a product ion $[M - H - Gall]^-$ 191.0566, corresponding to the loss of a galloyl moiety, 152.011. This ion had a molecular formula $C_7H_{11}O_6$, indicating that it is a quinic acid residue. A further two ions were present in the mass spectrum: 169.0147 ($C_7H_5O_5$) and 125.0250 ($C_6H_5O_3$), which were fragments characteristic of gallic monomer. This compound was tentatively identified as galloylquinic acid, reported before in strawberry fruit and flowers,[26] but not in fermented derived products. Figure S2 shows its proposed fragmentation pattern and base peak chromatogram with t_R. Peak 27 is a dimeric ellagitannin $C_{82}H_{54}O_{52}$, tentatively identified as agrimoniin, in which the monomeric fragment corresponding to $C_{41}H_{26}O_{26}$ was detected. According to published data, this fragment must have resulted from the fracture of the C−O bond that connects the two monomers, yielding a negative ion: galloyl bis-HHDP-glucose. After the loss of the HHDP moiety, it results in $[M - H]^-$ 633.0726 (theoretical monoisotopic mass calculated at 633.0722, with molecular formula $C_{21}H_{21}O_{18}$).[30,31]

Ellagic Acid and Derivatives. Peak 42 was tentatively identified as tetramethyl ellagic acid hexose, which is reported in gluconic-fermented products for the first time, despite having been reported before in strawberry.[30] It exhibited an ion m/z 359.1480, with a molecular formula $C_{20}H_{23}O_6$, yielded after losing a hexose moiety (162.0528). It then lost a water moiety (18.0117) and produced an ion m/z 341.1363, with molecular formula $C_{20}H_{21}O_5$.

Flavanols. Peak 38 exhibited an ion m/z 451.1212 and a molecular formula $C_{21}H_{24}O_{11}$ and yielded an ion m/z 289.0718 $[M - H - 162]^-$ corresponding to a (+)-catechin monomer. This compound was tentatively identified as (+)-catechin-*O*-hexoside. This one has been described before in strawberry,[22] but not in gluconic-fermented products.

Hydroxycinnamic Acids. Peak 40 had a pseudomolecular ion m/z 337.0914 ($C_{16}H_{18}O_8$) and showed the characteristic fragmentation of a quinic acid derivative m/z 191.0562 ($C_7H_{11}O_6$) and a monomeric ion belonging to *p*-coumaric acid m/z 163.0405 ($C_9H_7O_3$). As a result, the tentative identification was *p*-coumaroylquinic acid. This fragmentation pattern matched data obtained from the mzCloud database. Peak 57 had a fragmentation that indicated it was a ferulic derivative. It produced a monomeric ion, m/z 193.0503 ($C_{10}H_9O_4$), corresponding to ferulic acid, as a consequence of the loss of a glucuronide moiety and two hydrogens $[M - 2H - Gln]^-$, producing an unsaturated bond in the hydrocarbon chain. This metabolite was tentatively identified as dihydroferulic acid 4-*O*-glucuronide. As far as we know, this is the first time this compound has been described in strawberry and fermented derivatives.

Flavonols. Peak 31 had an $[M - H]^-$ ion at 609.1437 with molecular formula $C_{27}H_{30}O_{16}$ and fragmentation pattern that matched a kaempferol derivative: an ion $[M - H - 2Hex]^-$ at 285.0410 (which matches a kaempferol monomeric ion), after the loss of two hexoside moieties (162.0528). This compound was putatively identified as kaempferol hexosylhexoside, previously reported in mulberry.[27]

Peak 39 was tentatively identified as quercetin pentose glucuronide, due to the presence of an ion $[M - H - Pent - Gln]^-$ 301.0428, with molecular formula $C_{15}H_9O_7$, corresponding to quercetin. This compound has been described before in strawberry and its flowers.[26]

Condensed Tannins. Peak 18 had a precursor ion $[M - H]^-$ at 561.1401 and presents a fragmentation pathway, shown in Figure S2, that matches one reported before in strawberry.[25] It was deduced to be a dimer of (epi)afzfelechin → (epi)catechin because its chirality could not be discriminated by mass spectrometry. This identification was confirmed by the presence of two fragments, m/z 271.0611 ($C_{15}H_{11}O_5$) and m/z 289.0715 ($C_{15}H_{13}O_6$), which were yielded before breakdown of the cleavage of the interflavan bond.[25]

Dihydroflavonols. Peak 41, which presented a principal ion $[M - H]^-$ at m/z 435.0922 and molecular formula $C_{20}H_{20}O_{11}$, was putatively identified as (+)-taxifolin 3-*O*-arabinofuranoside, based on the production of an MS^2 fragment ion $[M - H - Pent]^-$ at 303.05, yielded by the loss of pentose monoisotopic mass 132.04, which is the major product ion, corresponding to the monomeric ion of (+)-taxifolin. Other MS^2 fragments were presented, such as $[M - H]^-$ 285.0399, 177.0192, and 125.0244, which were in accordance with the MS^2 fragmentation pathway of (+)-taxifolin (peak 37). This secondary metabolite has been reported before in strawberry.[30]

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Table 3. Concentrations of Nonanthocyanin Phenolic Compounds (μg/100 g of Beverage)[a]

	R_0/F_0	R_{15}	R_{30}	R_{60}	R_{90}
(+)-catechin	92.95bcde ± 4.02	83.89ae ± 0.28	61.10abe ± 0.09	20.47abce ± 0.20	14.33abcd ± 0.09
ellagic acid hexose	185.73e ± 2.19	184.33e ± 14.22	206.65 ± 5.45	212.22 ± 4.41	226.05ab ± 0.0 9
ellagic acid	134.12cde ± 6.80	132.39cde ± 2.06	156.49ab ± 0.66	151.04ab ± 1.10	166.69ab ± 3.06
bis-HHDP glucose	17.22bcde ± 0.43	13.47ae ± 0.11	13.35ae ± 1.13	11.80ae ± 0.55	6.04abcd ± 0.03
brevifolin carboxylic acid	1.72cde ± 0.07	1.78cde ± 0.07	2.14ab ± 0.06	2.36ab ± 0.03	2.30ab ± 0.07
galloylquinic acid	1.00de ± 0.03	1.03de ± 0.08	1.33e ± 0.17	1.65ab ± 0.13	1.72abc ± 0.03
dimer of caffeic acid-*O*-hexoside	7.01bcde ± 0.42	6.76a ± 0.04	6.33ab ± 0.32	5.25abc ± 0.04	5.09abc ± 0.01
p-coumaric acid	98.53cde ± 2.41	99.15cde ± 0.41	111.09abde ± 0.22	121.75abce ± 2.03	133.25abcd ± 0.07
p-coumaroyl hexose	141.51bcde ± 2.44	137.32acde ± 0.10	135.28abde ± 0.98	124.39abce ± 0.81	120.73abcd ± 0.19
p-coumaroylquinic acid	49.27de ± 1.18	48.53de ± 0.50	52.05de ± 0.18	56.65abce ± 0.82	62.06abcd ± 0.56
apigenin	0.05bcde ± 0.00	0.06acde ± 0.00	0.09abde ± 0.00	0.11abc ± 0.00	0.11abc ± 0.00
apigenin pentose	0.72c ± 0.02	0.73c ± 0.02	0.89ab ± 0.02	0.79 ± 0.00	0.84 ± 0.05
apigenin-7-*O*-glucose	1.42cde ± 0.03	1.41cde ± 0.01	1.64abe ± 0.04	1.76abce ± 0.01	1.98abcd ± 0.02
eriodictyol glucose	20.76cde ± 0.96	22.32cde ± 0.05	25.95abde ± 0.29	27.74abc ± 0.71	28.14abc ± 0.37
aromadendrin	52.51de ± 0.55	51.72de ± 0.56	52.12de ± 0.47	52.12abc ± 0.16	48.03abc ± 1.08
kaempferol	6.61bcde ± 0.17	12.46acde ± 0.09	18.82abde ± 2.60	22.72abce ± 0.11	24.65abcd ± 0.45
kaempferol glucuronide	18.76cde ± 0.19	18.36cde ± 0.36	20.43ab ± 1.05	20.74ab ± 0.04	20.61ab ± 0.48
procyanidin B1	63.42cde ± 3.41	62.86cde ± 0.30	44.83abde ± 0.29	4.55abc ± 0.19	3.11abc ± 0.28
procyanidin trimer	8.59bc ± 0.99	5.79ac ± 0.13	2.08ab ± 0.01	nd	nd
protocatechuic acid	16.10de ± 3.99	13.28de ± 2.27	21.90de ± 0.05	213.90abce ± 0.75	80.90abcd ± 1.92
quercetin 3-*O*-glucoside	2.65de ± 0.04	2.56de ± 0.15	2.46de ± 0.03	2.20abce ± 0.02	1.93abcd ± 0.01
rutin	2.20bcde ± 0.04	1.88acde ± 0.01	1.38abde ± 0.00	0.90abce ± 0.03	0.59abcd ± 0.04
(+)-taxifolin	0.80cde ± 0.03	0.94cde ± 0.00	1.13abde ± 0.00	1.33abce ± 0.00	1.65abcd ± 0.11
(+)-taxifolin-7-*O*-glucoside	8.67bcde ± 0.14	4.23ade ± 0.09	4.18ade ± 0.18	2.81abce ± 0.24	2.12abcd ± 0.08
	R_0/F_0	F_{15}	F_{30}	F_{60}	F_{90}
(+)-catechin	92.95bde ± 4.02	105.95a ± 1.25	95.22bc ± 0.40	60.10abc ± 0.24	53.63abcd ± 0.04
ellagic acid hexose	185.73 ± 2.19	195.60 ± 10.05	186.99 ± 1.87	193.16 ± 14.17	193.44 ± 9.42
ellagic acid	134.12 ± 6.80	148.59 ± 0.71	148.95 ± 1.04	146.17 ± 1.69	152.05 ± 0.28
bis-HHDP-glucose	17.22 ± 0.43	19.58 ± 0.92	17.57 ± 0.39	13.85abc ± 0.01	12.81abc ± 0.27
brevifolin carboxylic acid	1.72de ± 0.07	1.92 ± 0.10	1.86 ± 0.07	2.20ac ± 0.00	2.18ac ± 0.10
galloylquinic acid	1.00 ± 0.03	0.99 ± 0.03	1.02 ± 0.05	1.19 ± 0.04	1.27 ± 0.03
dimer of caffeic acid-*O*-hexoside	7.01e ± 0.42	7.45e ± 0.17	7.23 ± 0.04	6.94 ± 0.14	6.65ab ± 0.05
p-coumaric acid	98.53 ± 2.41	99.15d ± 2.16	97.37de ± 0.46	104.35bc ± 1.17	103.33c ± 0.27
p-coumaroyl hexose	141.51cde ± 2.44	141.06cde ± 0.04	137.14abde ± 0.60	134.73abc ± 0.45	132.54abc ± 0.20
p-coumaroylquinic acid	49.27 ± 1.18	48.91 ± 1.33	47.70 ± 0.25	50.49 ± 1.72	50.03 ± 1.92
apigenin	0.05e ± 0.00	0.05e ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.06ab ± 0.00
apigenin pentose	0.72 ± 0.02	0.69d ± 0.01	0.71 ± 0.01	0.83b ± 0.00	0.74 ± 0.07
apigenin-7-*O*-glucose	1.42 ± 0.03	1.44 ± 0.03	1.42 ± 0.01	1.50 ± 0.10	1.49 ± 0.04
eriodictyol glucose	20.76bcde ± 0.96	24.05ade ± 0.25	23.84ade ± 0.25	25.59abc ± 0.23	26.26abc ± 0.13
aromadendrin	52.51 ± 0.55	53.38 ± 0.64	53.24 ± 0.94	52.43 ± 0.06	51.92 ± 0.56
kaempferol	6.61bcde ± 0.17	9.87acde ± 0.06	11.95abde ± 0.02	16.35abce ± 0.10	19.97abcd ± 0.30
kaempferol glucuronide	18.76 ± 0.19	19.22 ± 0.36	19.81 ± 0.00	20.00 ± 0.26	19.97 ± 0.10
procyanidin B1	63.42bcde ± 3.41	74.90ade ± 0.33	72.90ade ± 0.18	40.65abce ± 0.17	23.03abcd ± 0.10
procyanidin trimer	8.59bcde ± 0.99	10.53acde ± 0.09	8.23abde ± 0.08	3.04abce ± 0.08	1.84abcd ± 0.03
protocatechuic acid	16.10e ± 3.99	14.81e ± 1.17	19.09e ± 1.21	17.55e ± 0.86	186.35abcd ± 7.99
quercetin 3-*O*-glucoside	2.65 ± 0.04	2.60 ± 0.04	2.56 ± 0.04	2.47 ± 0.04	2.40 ± 0.01
rutin	2.20bcde ± 0.04	1.89acde ± 0.07	1.79abde ± 0.05	1.65abce ± 0.00	1.44abcd ± 0.02
(+)-taxifolin	0.80cde ± 0.03	0.91de ± 0.01	0.99a ± 0.01	1.09ab ± 0.02	1.13ab ± 0.02
(+)-taxifolin-7-*O*-glucoside	8.67bcde ± 0.14	4.27a ± 0.03	4.12a ± 0.13	4.08a ± 0.05	4.21a ± 0.06

[a]Mean values and standard deviation. A letter "a" indicates a significant difference ($p < 0.05$) between the initial samples (R_0/F_0) and the other samples under the same temperature conditions. A letter "b" indicates a significant difference ($p < 0.05$) between the samples at day 15 (R_{15}/F_{15}) and the other samples under the same temperature conditions. A letter "c" indicates a significant difference ($p < 0.05$) between the samples at day 30 (R_{30}/F_{30}) and the other samples under the same temperature conditions. A letter"d" indicates a significant difference ($p < 0.05$) between the samples at day 60 (R_{60}/F_{60}) and the other samples under the same temperature conditions, obtained through ANOVA and Tukey's HSD (honest significant difference) statistical tests. No letter indicates no significant differences. Samples were determined in duplicate. nd, no data.

Chalcones. This is a group of compounds present in apple as a consequence of the processes related to defense against infections.[32] Peak 49 presents a pseudomolecular ion at $[M - H]^-$ 567.1688 and an MS^2 fragment yielded after the loss of two moieties, one of pentose 132.0423 and another of galactose 162.0528 $[M - H - Galc - Pent]^-$ at 273.0764, with molecular formula $C_{15}H_{13}O_5$. This compound was tentatively identified as phloretin 2′-*O*-xylosyl-galactoside (in accordance

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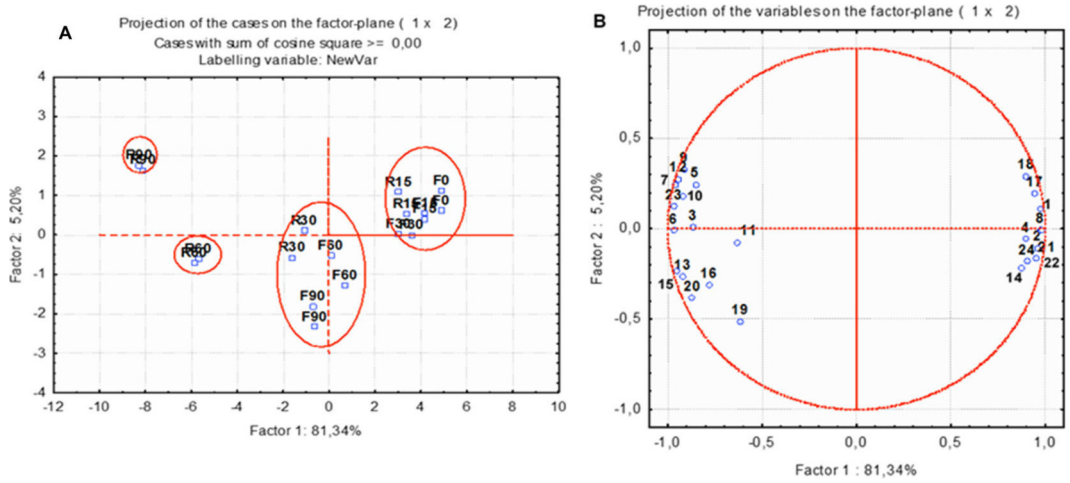

Figure 1. Principal component analysis: (A) analysis of samples (R is room temperature, F is refrigerated temperature, and F_0 is the initial sample to two conditions of temperature); (B) analysis of compounds [**1**, (+)-catechin; **2**, dimer of caffeic acid *O*-hexoside; **3**, ellagic acid; **4**, bis-HHDP-glucose; **5**, ellagic acid hexose; **6**, galloylquinic acid; **7**, *p*-coumaric acid; **8**, *p*-coumaroyl hexose; **9**, *p*-coumaroylquinic acid; **10**, apigenin; **11**, apigenin pentose; **12**, apigenin-7-*O*-glucose; **13**, eriodictyol glucose; **14**, aromadendrin; **15**, kaempferol; **16**, kaempferol glucuronide; **17**, procyanidin B1; **18**, procyanidin trimer; **19**, protocatechuic acid; **20**, brevifolin carboxylic acid; **21**, quercetin 3-*O*-glucoside; **22**, rutin; **23**, (+)-taxifolin; **24**, (+)-taxifolin-7-*O*-glucoside].

with Metlin data). This fragment also appeared in the MS^2 spectra of peak 49, identified with standard as phloridzin (phloretin 2′-glucoside), after the loss of a glucoside moiety $[M - H - Gluc]^-$ that corresponded to a phloretin monomer.

Influence of Storage Conditions on the Nonanthocyanin Phenolic Content in Fermented Beverage. A total of 37 compounds (those above LOQ) have been quantitated. Statistical analysis was applied to reduce the variables in the model including those that contribute most to the variance of data (Table S2). Table 3 shows only those selected for the PCA, whereas the rest are displayed in Table S2. Six compounds exhibited higher concentrations in the initial samples: ellagic acid hexoside, *p*-coumaroyl hexose, ellagic acid, *p*-coumaric acid, (+)-catechin, and procyanidin B1.

Hydroxycinamics compounds analyzed as *p*-coumaric acid presented an increase during storage time that is not proportional with the decrease observed in *p*-coumaroyl hexose. This behavior can be explained by the disappearance of coumaroyl anthocyanins during the aging process.[33]

At 30 °C, (+)-catechin, procyanidin B1, and procyanidin trimer underwent a significant decrease (84.5−95%) during the storage time, to the point that procyanidin trimer content reached levels below the detection limit in samples R_{60} and R_{90}. At 4 °C, it decreased (42.3−36.31−78.58%), but to a lesser extent than at room temperature. The reduction of the content of procyanidins (B1 and trimer) during storage is in accordance with previously reported data on quince juice.[34] Another explanation of these reductions could be an increase of polymeric color value, indicative of condensation reactions of anthocyanins with other phenolic compounds such as procyanidins to form colored polymer pigments.[34]

Three hydrolyzed tannins were quantified: an ellagitannin (bis-HHDP-glucose), a gallotannin (galloylquinic acid), and brevifolin carboxylic acid; the last two increased during storage time and increased more at room temperature (74−28 and 34−27%, respectively). Ellagitannin started to change at 15 days and achieved its maximum decrease of 65% at room temperature (26% at 4 °C); this decrease was associated with the increase of free ellagic acid (22% at room temperature).

Interestingly, protocatechuic acid significantly increased after 60 days of storage (13 times its initial value) at 30 °C, with its concentration reaching 11 times its initial value after 90 days at 4 °C. This increase may be due to the degradation process of anthocyanin compounds (pelargonidin is a principal anthocyanin of strawberry), which produced a cleavage of pelargonidin B-ring and, consequently, a molecule of protocatechuic acid, as was reported in the thermal processing of food.[35] Additionally, as anthocyanins are susceptible to thermal degradation, another proposed mechanism describes the opening of the pyrylium ring and chalcone glycoside formation.[36]

Kaempferol, apigenin, and their derivatives increased (24−373%) during storage, increasing more at 30 °C than at 4 °C. Conversely, the quercetin 3-*O*-glucoside and rutin contents decreased during storage: the higher the temperature is, the greater the decrease of the content of glycosides, producing an increase of the content of quercetin (Table S1). The concentrations of (+)-taxifolin 7-*O*-glucoside underwent a significant reduction (75% at 30 °C, 51% at 4 °C). This result is associated with the increase of taxifolin (106−41%) because of the breakdown of the glucosidic bond releasing the corresponding aglycone.[8]

The major loss of nonanthocyanin phenolic compound content was observed at room temperature. This observation was in accordance with what was reported before by Oliveira et al.[37] They concluded that pasteurization treatment favors the rupture of cellular structures, increasing the exposure of compounds to oxidation processes during storage. This phenomenon is one of the main reasons for the loss of phenolic compounds. Apart from the above-mentioned changes, it can be highlighted that certain (poly)phenolic compounds maintain their content practically unchanged at both tested temperatures as caffeic acid, caffeic acid hexosided and naringenin. Additionally, the concentration of two compounds remained constant at refrigeration conditions

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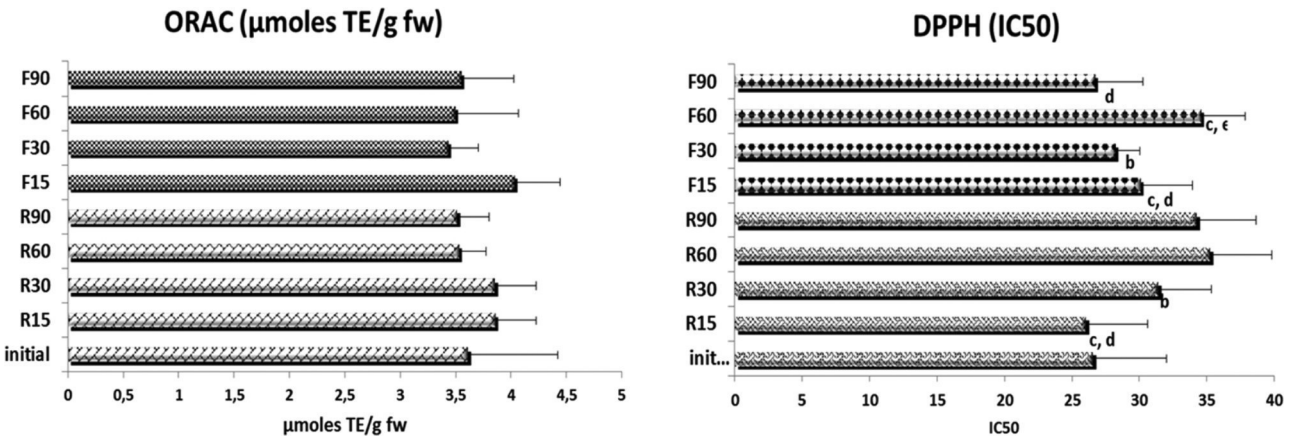

Figure 2. Bar representations based on two methods: (left) ORAC; (right) DPPH. A letter "a" indicates a significant difference ($p < 0.05$) between the initial samples (R_0/F_0) and the other the samples under the same temperature conditions. A letter "b" indicates a significant difference ($p < 0.05$) between the samples stored for 15 days (R_{15}/F_{15}) and the other samples under the same temperature conditions. A letter "c" indicates a significant difference ($p < 0.05$) between the samples stored for 30 days (R_{30}/F_{30}) and the other samples under the same temperature conditions. A letter "d" indicates a significant difference ($p < 0.05$) between the samples stored for 60 days (R_{60}/ F_{60}) and the remaining samples under the same temperature conditions. A letter "e" indicates a significant difference ($p < 0.05$) between the samples stored for 90 days (R_{90}/F_{90}) and the remaining samples under the same temperature conditions. No letter indicates no significant difference.

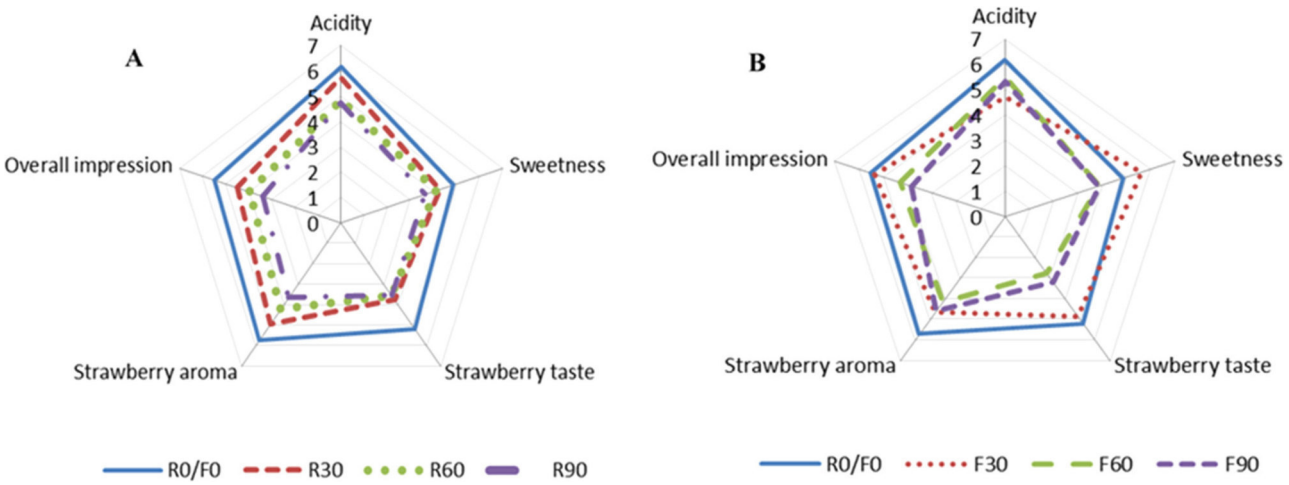

Figure 3. Sensory analysis: spider charts of samples (A) stored at room temperature and (B) stored at refrigeration conditions.

(phloridzin and quercetin glucuronide). All of these data are shown Table S1.

PCA was carried out to explore the effects of storage time and temperature on the nonanthocyanin phenolic compound content profile (Figure 1). The matrix had 18 samples × 24 quantified nonanthocyanin phenolic compounds. The two principal components (factors 1 and 2) accounted for 86.54% of the variability of the original data. PCA showed clustering of the samples into four main groups, illustrated in Figure 1A. It was observed that samples belonging to the same storage period are very close to each other, which generate four well-separated clusters, thus highlighting the effect of time. Interestingly, R_{90} and R_{60} were very separately located from the other samples (Figure 1A). The cluster presented in the middle of the Cartesian plane includes samples R_{30}, F_{60}, and F_{90}, which indicates that storage under refrigeration has a preservative effect on nonanthocyanin phenolic composition. The last cluster consists of initial samples (represented by F_0), F_{15}, F_{30}, and R_{15} with high weights on factor 1, as determined by their high content of procyanidin B1 (**17**); procyanidin trimer (**18**) and (+)-catechin (**1**). In addition, Figure 1B shows the variables separated into two groups: on the right, all compounds that experience a decrease; on the left, those that increased their concentrations during storage time at two temperature conditions. In all cases, the effect was greater at room temperature than at refrigerated conditions; specifically the composition remains virtually unchanged until day 15 of storage at room temperature and day 30 under refrigerated conditions.

Influence of Storage Conditions in Antioxidant Activity. Figure 2, left and right panels, represent antioxidant activity by ORAC and DPPH, respectively. No significant differences were observed for ORAC results, but DPPH results were statistically different ($p < 0.05$). At room temperature, there has been observed a progressive increase until 60 days of storage and, then, it decreased. At refrigeration conditions, the trend was similar.

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This result was in accordance with that reported before in alcoholic fermented mulberries.[18] With the fermentation process hydroxycinamic acids increased while the progressive growing in the content of protocatechuic acid indicated that there existed a degradation of anthocyanins generating another compound with antioxidant activity.

A similar behavior was reported before in the analysis of antioxidant activity in solutions of phenolic compounds.[38] When the storage time was prolonged, the antioxidant activity decreased and so maximum value was observed (Figure 2, right, maximum at 60 days and then decreased). This increase in the overall antioxidant activity value could be probably explained by the formation of oligomers from free polyphenols as (+)-catechin as its content decreased as shown in Table 3.

Sensory Analysis. The panel carried out 12 triangle tests to differentiate samples with different storage times and 6 triangle tests to differentiate samples stored at different temperatures. In general, sensory differences were perceived from 30 days of storage on at room temperature, whereas the panel could not establish differences between refrigerated samples whatever the storage time was.

On the other hand, the panel could not differentiate samples stored during 30 days at different temperatures. However, differences between samples stored at 4 or 27−30 °C for 60 days could be perceived, and even larger differences were perceived for samples stored for 90 days.

The sensory profile of the beverage was built using the marks given for each attribute by the panel. Figure 3 displays the spider charts for the samples stored at room temperature (Figure 3A) and samples stored in refrigeration conditions (Figure 3B). The attributes (overall impression, strawberry aroma, and strawberry taste) have highest marks in refrigeration conditions, and the lowest marks were in samples stored at room temperature. Additionally, the higher the time of storage is, the lower overall impression scores are. These results should be taken into account to establish the shelf life of the beverage.

In conclusion, four new compounds were identified with HRMS technique in samples of fermented strawberry beverage: aromadendrin hexoside, phloretin 2′-*O*-xylosyl glucoside, dihydroferulic acid 4-*O*-glucuronide, and kaempferol hexosyl-hexoside.

Nonanthocyanin phenolic composition underwent fewer changes under refrigeration than at room temperature, although 10 compounds declined when they were stored mainly at room temperature. However, our results show that 14 compounds [ellagic acid, ellagic acid hexose, *p*-coumaric acid, *p*-coumaroylquinic acid, apigenin, apigenin pentose, eriodictyol glucose, kaempferol, kaempferol glucuronide, protocatechuic acid, brevifolin carboxylic acid, (+)-taxifolin, galloylquinic acid, and apigenin-7-*O*-glucose] increased during storage.

Strawberry fermented beverage storage period should not exceed 30 days at room temperature (27−30 °C) or 60 days at refrigeration conditions (4 °C). Gluconic fermentation is an alternative process to prevent fruit waste while elaborating a glucose-free product that contains bioactive compounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b05617.

Table S1, concentrations of compounds whereas not part of PCA analysis; Table S2, variable contributions; Figure S1, MS chromatograms; Figure S2 and S3 fragmentation patterns of compounds **6** and **18** (PDF)

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Notes

The authors declare no competing financial interest.

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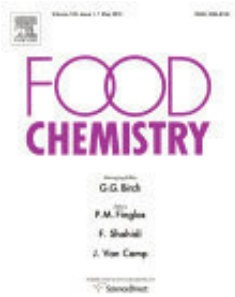

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Determination of hydroxytyrosol produced by winemaking yeasts during alcoholic fermentation using a validated UHPLC–HRMS method

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ABSTRACT

Hydroxytyrosol (HT) is a phenolic compound of recognized bioactivity that has been described in wines but little is known about its origin. This work demonstrates that yeast involved in wine making, i.e. *Saccharomyces cerevisiae* strains and the non-*Saccharomyces Torulaspora delbrueckii*, can synthesise HT, as this compound was identified in the intracellular media of three strains by means of a developed and validated UHPLC–HRMS method with LOQ and LOD of 0.108 and 0.035 ng mL^{-1} respectively. Controlled fermentations were performed with different varieties of grapes (*Corredera*, *Moscatel*, *Chardonnay*, *Palomino fino*, *Sauvignon Blanc*, *Vijiriega*, and *Tempranillo*) and synthetic must. The *Saccharomyces cerevisiae* strain QA23 was the most efficient producer of HT from tested yeasts. On the other hand, the grape variety influences HT wine concentrations. Furthermore, the maximum concentration of HT is reached between the fourth and sixth day of fermentation. This work reveals that yeasts have a great potential for the production of HT.

1. Introduction

Hydroxytyrosol (HT) 2-(3, 4-dihydroxy-phenyl) ethanol (3, 4-DHPEA), is a higher alcohol (phenyl ethyl alcohol), found in extra-virgin olive (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012) as well as in fermented beverages such as wine (Bordiga et al., 2016). The most common synthesis pathway includes the hydroxylation of its immediate precursor, tyrosol, in the Ehrlich pathway (Fig. 1). This is in turn produced from tyrosine by yeasts during alcoholic fermentation (AF), as follows: (1) transamination of tyrosine; (2) decaboxylation of *p*-hydroxyphenylpyruvate by pyruvate decarboxylase; (3) reduction of *p*-hydroxyphenylaldehyde by alcohol dehydrogenase (ADH) (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008; Pineiro, Cantos-Villar, Palma, & Puertas, 2011; Satoh, Tajima, Munekata, Keasling, & Lee, 2012). Consequently, Tyrosol and HT could be considered secondary metabolites produced from tyrosine by some yeast strains by means of a transformation of amino acids (Garrido & Borges, 2013) during alcoholic fermentation (Zhu et al., 2011). On the one hand, the content of HT appears to be related mainly to the nitrogen content in musts during alcoholic fermentation while on the other, it is well known that *Saccharomyces cerevisiae* can use tyrosine and tryptophan as a source of cellular nitrogen. Fusel alcohol such as tyrosol, HT and tryptophol respectively are the main products of its catabolism (Bordiga et al., 2016). This suggests that the final contents of HT and tyrosol in wine could be influenced by microbial activity during alcoholic fermentation (Romboli, Mangani, Buscioni, Granchi, & Vincenzini, 2015).

As a bioactive compound, HT has been object of study in many research reports. Some studies have demonstrated that HT is a potent stimulator of mitochondrial biogenesis in retinal epithelial cells that contribute to eye health (Zhu et al., 2010) as well as having anticarcinogenic (Roleira et al., 2015), cardioprotective (Mnafgui et al., 2015), antidiabetic, and neuroprotective qualities (Fernández-Mar et al., 2012; Marhuenda et al., 2016; Rigacci & Stefani, 2016). Furthermore, the EFSA (European Food Safety Authority), has admitted a claim on these healthy effects based on the protection of LDL particles from oxidative damage and maintenance of normal blood HDL-cholesterol concentrations, as these effects were demonstrated after the consumption of extra-virgin olive oil due to its high content in HT (European Food Safety Authority [EFSA] Panel on Dietetic Products Nutrition and Allergies [NDA], 2011).

To identity, quantify, and elucidate the occurrence of HT, different

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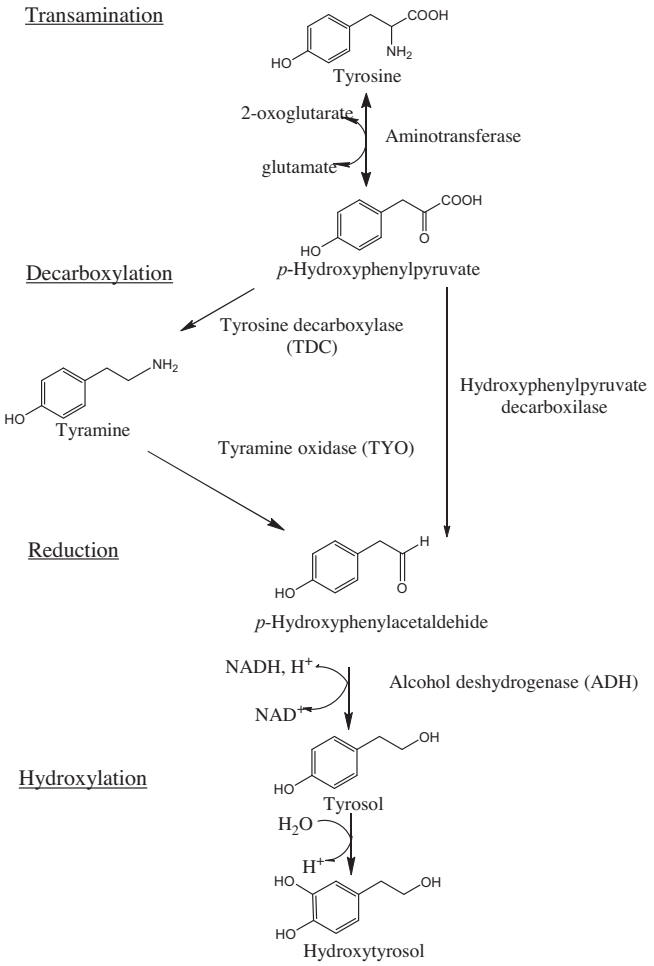

Fig. 1. Ehrlich pathway for the production of hydroxytyrosol.

analytical methods have been used in various food matrices. Thus HT has been analysed by: gas and liquid chromatography; capillary electrophoresis in wines (Piñeiro et al., 2011); nuclear magnetic resonance in herbal medicine products (Lemonakis, Gikas, Halabalaki, & Skaltsounis, 2013). Associated techniques have also been used, such as: HPLC with GC in olive mill wastewaters (Allouche, Damak, Ellouz, & Sayadi, 2004); HPLC with fluorescence in grape leaves (Vrhovsek et al., 2012); HPLC with DAD and with MS in wines (Boselli, Minardi, Giomo, & Frega, 2006). The use of HRMS associated with UHPLC has been used before in herbal medicinal products in the determination of HT (Lemonakis, Skaltsounis, Tsarbopoulos, & Gikas, 2016). This latter technique is a powerful tool to unequivocally identify and quantify compounds in different matrices, and therefore it may be useful in trying to elucidate the origin and evolution of HT in wines.

The aim of the present work is to develop and validate an UHPLC–HRMS method to assess HT and to investigate the origin of its occurrence in wines. For this purpose, the role of different strains of winemaking yeasts on the occurrence of HT in wines is studied during alcoholic fermentation (AF). Finally, the main objective is to examine the production of HT by yeasts through the evidence that the determination of this compound in the intracellular compartment provides when using a validated HRMS method coupled with UHPLC.

2. Materials and methods

2.1. Reagents and materials

HT standard (98%) was purchased from Chengdu Biopurify Phytochemicals Ltd. (Wenjiang Zone, Chengdu, Sichuan, China), HPLC-grade methanol was acquired from Merck (Darmstadt, Germany) and HPLC-grade formic acid from Panreac (Barcelona, Spain).

2.2. Yeast strains

The experiments on white musts were performed with the commercial wine yeast strain Enartis Ferm Aroma White (Enartis). In the fermentation of *Tempranillo* must and synthetic must, three different commercial strains: were used *S. cerevisiae* Lalvin YSEO QA23® (Lallemand), *S. cerevisiae* Red Fruit RF® (Enartis) and *T. delbrueckii* TD291 Biodiva™ (Lallemand).

2.3. Samples

2.3.1. Grapevine

Vines were grown in a typical soil-type (*albariza)* with a plant density of 3600 vines/ha. The grapes used in the fermentations belonged to an experimental cultivar located in the Rancho de la Merced (IFAPA, Jerez de la Frontera, Spain), and were from seven varieties as follows: *Corredera*, *Moscatel*, *Chardonnay*, *Palomino fino*, *Sauvignon Blanc*, *Vijiriega*, and *Tempranillo*.

The degree of ripeness was followed weekly during the maturation process (data not shown). Grapes were harvested at their stage of optimum maturity in August 2015.

2.3.2. Alcoholic fermentation procedure

2.3.2.1. Musts of white grapes. Grapes were harvested at optimum ripening conditions. Subsequently, they were destemmed, crushed, and pressed. Then pectolitic enzymes (2.5 mL h L^{-1}, Enartis ZYM, Italy) and SO_2 (Sulfosol, Sepsa-Enartis) were added into the must. After 24 h at 4 °C, the must was dejuiced and placed in a 100-L steel vessel. Alcoholic fermentation (AF) was carried out and monitored in vessels by yeasting (Aroma White, Italy) at 18 °C. AF was considered completed when the concentration of residual sugars was lower than 3 g L^{-1}.

2.3.2.2. Musts of Tempranillo grapes. Tempranillo grapes at optimum ripeness were harvested manually in 18 kg plastic boxes; they were in good sanitary conditions and were transported to the experimental winery. Musts were produced using a pneumatic press with pectolitic enzymes (3 mL h L^{-1}, Enartis ZYM, Italy) and 40 mg L^{-1} of sulphur dioxide (SO2) (Sepsa- Enartis) were added. Musts were placed in 15 stainless steel vats of 10-L capacity.

Five different methods of inoculation were used: (1) CTQA, with *Saccharomyces cerevisae* QA23 yeast strain; (2) CTRF, with *Saccharomyces cerevisae* RF yeast strain; (3) SIQA23, sequential inoculation first with commercial non-*Saccharomyces* strain *Torulaspora delbrueckii* TD291 and later when the density had decreased by 15 points just after the start of the AF with *S. cerevisiae* QA23; (4) SIRF, sequential inoculation first with commercial non-*Saccharomyces* strain *Torulaspora delbrueckii* TD291 and when density had decreased by 15 points just after the start of the AF with *S. cerevisiae* RF; and (5) SP, spontaneous fermentation without any inoculation using commercial yeasts.

2.3.3. Intracellular samples

Six alcoholic fermentations were performed in synthetic must with a sugar content of 100 g L^{-1} fructose and 100 g L^{-1} glucose and amino acids (purity ≥ 99 %) (Riou, Nicaud, Barre, & Gaillardin, 1997) with three strains of yeast (QA23, RED FRUIT and *Torulaspora delbrueckii*). The must was sterilized with bottle-top vacuum filters (Nalgene PES membrane). Each Erlenmeyer flask with 750 mL of SM was inoculated with 10^6 cell mL^{-1} and capped with taps equipped with a capillary to release carbon dioxide. The fermentation was monitored by weighing the flasks daily before and after sampling.

Table 1A
Sampling and mean of reducing sugars content at the end of fermentation in different variety of grapes.

Grape variety	Palomino fino	Vijiriega	Corredera	Moscatel	Sauvignon Blanc	Chardonay
Days of fermentation	7	10	11	11	13	15
Reducing Sugars (g L^{-1})	2.00	1.30	2.33	1.47	1.55	1.95

Table 1B
Sampling days and mean of the content of reducing sugars at the end of fermentation in different inoculations ways.

Fermentation	CTQA23	CTRF	ISQA23	ISRF	Spontaneous
Days of fermentation	13	13	14	15	13
Reducing Sugars (g L^{-1})	0.64	1.02	0.56	0.84	1.2

2.4. Sampling

Samples were taken every day from inoculation until the end of AF. Samples were collected and stored at −80 °C until the analysis. The end of AF for each grape variety was different and, consequently, each fermentation lasted a different number of days. The end of AF was reached when the sugars were almost all consumed (lower 3 g L^{-1}). Table 1 shows the time of the fermentation process and the concentrations of sugars at the end of alcoholic fermentation in white grapes (Table 1a). Table 1b shows the time of fermentation and the concentration of sugars (approximately 10.9 °Be) at the end of fermentation of the 5 fermentation methods for *Tempranillo* grapes.

2.4.1. Intracellular metabolite extraction

Samples of the intracellular compartment were collected at the second day of fermentation in a volume corresponding to 10^9 cells mL^{-1}. Immediately, they were subjected to a cold glycerol-saline quenching (Villas-Bôas & Bruheim, 2007), were stored at −80 °C until the extraction process was conducted. The intracellular extraction was performed following the method reported by Smart et al. with minor modifications (Smart, Aggio, Van Houtte, & Villas-Bôas, 2010). To the cell pellets, 2.5 mL of cold methanol-water solution (50% [v/v], −30 °C) were added, mixed for 1 min and then frozen at −80 °C. The samples were subjected to two cycles of freeze-thaw (thaw in an ice bath for 4 min; then were frozen at −80 °C for 30 min). After the last cycle, they were subjected to sonication for 1 min in an ice bath using an ultrasonic liquid processor (Sonicator Sonoplus HD 2070, Bandelin electronic GmbH & Co. KG, Berlin, Germany). Afterwards, the samples were centrifuged at 36086*g* for 20 min at −20 °C using a refrigerated centrifuge (Sorvall LYNK 6000, ThermoFisher scientific, Waltham, MA USA). Another 2.5 mL of cold methanol-water was added to the pellet and then centrifuged; the supernatants were collected, pooled, and stored at −80 °C until analysed.

2.5. Sample clean up

Samples were cleaned up as previously reported by Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, and Garcia-Parrilla (2011) with the following modifications: C18 SPE cartridges (Variant, Agilent) were conditioned with 2 mL of methanol and 2 mL of milliQ water. An aliquot of 500 µL of sample was loaded followed by a washing step with 2 mL of a 10% v/v methanol solution. The analytes were eluted with 1 mL of methanol; afterwards solvents were evaporated until dryness at 34 °C, 2000 rpm during 6 h with a vacuum concentrator (HyperVAC-LITE, GYOZEN, Korea). Then samples were reconstituted with 167 µL of methanol/water 10% v/v and stored at −20 °C until analysis.

2.6. UHPLC/HRMS parameters

The analysis was carried out in a UHPLC Dionex Ultimate 3000 system (Thermo Fisher Scientific (Bremen, Germany) all devices were controlled by Chromeleon Xpress Software. The column used was a ZORBAX RRHDSB-C18 (2.1 × 100 mm, 1.8-µm particle size) with a guard column (2.1 × 5 mm, 1.8-µm particle size). Column and guard column were purchased from Agilent Technologies (Waldbronn, Germany). The separation was performed using column temperature of 40 °C, a flow of 0.5 mL min^{-1}, and injection volume of 5 µL. The chromatographic conditions consisted of two phases (A) aqueous formic solution 0.1%, and (B) solution 0.1% of formic acid in methanol and the gradient was programmed as follows: 95% A, 5% B (0–1 min); 0% A, 100% B (1–8.5 min); 95% A, 5% B (8.6–10 min).

A target MS^2 in negative mode with a heated ionization source HESI was selected using the transition 153 → 123 HCD 100.00 in order to both identify and quantify. The main HRMS parameters were heater and capillarity temperature (400–275 °C respectively), spray voltage 3.0 kV; flow rates of sheath gas and auxiliary gas (65, 25 arbitrary units, respectively). Other parameters of HRMS methods were normalized collision energy (NCE) 40; S-lens RF 50% and mass resolving power (RP) 70,000 FWHM.

2.7. Statistical analysis

Statistical analyses were performed by means of Statistica software (StatSoft, 2013). One-way analysis of variance (ANOVA) and Tukey's HSD (honest significant difference) test were assessed to test significant differences at $p < 0.05$. Additionally, principal component analysis (PCA) was used for data analysis

3. Results and discussion

3.1. Method validation

The validation procedure was carried out following different international guides (Aoac, 1998; FDA, 2012) to establish parameters, such as: detection (LOD) and quantification (LOQ) limits, precision, linearity, recovery, matrix effects, and effects of solid-phase extraction. As a blank, a fermentation sample was used after cleaned using SPE, because it bears a greater similarity to the matrix than to the must without fermentation.

The linearity, LOD, and LOQ were experimentally determined by the injection of 11 solutions in the fermentation medium cleaned using SPE, as explained in Section 2.4, because it reproduces matrix characteristics better than does the solvent (methanol/water 10% v/v). Calibration standards of HT were prepared for each analytical batch and three replicates were determined at 11 concentrations (1000, 500, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 ng mL^{-1}) with 4 degrees of linearity. The detection limits were calculated based on the standard deviation of the response (σ) and the slope (Ich, 2005). Calibrations curves with their slope (*S*), intercept and correlation coefficient (*r*) were calculated by plotting the peak area vs. the concentration of the standards using Statististica software version 12 (StatSoft, 2013).

$$LOD = 3.3\,\sigma\,S^{-1} LOQ = 10\,\sigma\,S^{-1}$$

The results indicated linearity by a curve with $r^2 = 0.9995$ with LOD 0.035 ng mL^{-1} and LOQ 0.108 ng mL^{-1}. These results improved the limits reached before by Bordiga et al. in wines using HPLC-PDA-MS/MS (LOD 4 ng mL^{-1}–LOQ 11 ng mL^{-1}) (Bordiga et al., 2016) probably because they determined several compounds simultaneously

Table 2A
Parameters of calibration curves. LDR (linear dynamic range), LOD (limit of detection), LOQ (limit of quantitate).

	LDR	LOD	LOQ	Curve		
				(Slope)	(Offset)	R^2
Matrix-spiked calibration	0.079–1011.00	0.03	0.01	950135	−5070483	0.9991
Solvent calibration (methanol 10%)	0.079–1011.00	0.03	0.08	72224	−355656	0.9991

Table 2B
Accuracy and repetitivity.

	Intra-day (%RSD)	Inter-day (%RSD)	Accuracy (%RE)
Low concentration	1.6	0.5	−1
High concentration	1.4	0.5	−1

and our method focused on HT.

The intermediate precision was calculated measuring standard deviation (RSD) in a set of two concentrations (0.1–1 ng mL^{-1}) for 5 days with 5 replicates per concentration. Repeatability was assessed during a working session with 5 replicates per concentration. The data for intermediate precision and repeatability are shown in Table 2.

The matrix effect was tested in a clean-up matrix by spiking with standard solution in 10 concentrations: 0.079, 0.158, 0.316, 0.632; 1.264, 5.056, 20.224, 80.896, 404.48, and 1011 ng mL^{-1}.The slopes resulting from the spiked matrix and calibration solutions (methanol 10% v/v) in the linear range were used to evaluate the matrix effect. The relation between the slopes was defined as (slope in solvent/slope in the spiked matrix)*100 and expressed as %ME (Trufelli, Palma, Famiglini, & Cappiello, 2011). The resulting value was 1.05%, which is considered an irrelevant value, as the calibration curves in the solvent and matrix spiked are very similar. Fig. 1 of Supplementary material shows the results.

Recovery was calculated from the spiked matrix at five different concentrations ranging from 1.2 to 1011.2 ng mL^{-1}. The results were from 116 to 58% within the recommended values (40–120%) for concentrations ranging from 1 to 1000 ng mL^{-1} (Gustavo González & Ángeles Herrador, 2007). Fig. 2 of Supplementary material shows the results.

SPE was used as a cleaning technique because it allowed the removal of different components that could be interfering with mass analysis. A solid-phase extraction (SPE) approach has been tested in order to avoid overestimation and sub-estimation of the quantity. Three different solutions were prepared with a concentration LOQ, LOQ + 50% and 3LOQ in methanol/water 10% v/v, which were analysed after the SPE clean-up procedure (Gasperotti, Masuero, Guella, Mattivi, & Vrhovsek, 2014). The results showed an extraction efficacy of 96.7%, 86.3%, and 143.2%, respectively. On the other hand, to evaluate the amount of analyte that is dragged in the washing step, the solutions were analysed and the HT contents were under LOQ limits, indicating the clean-up procedure was efficient.

3.2. Intracellular HT

Hydroxytyrosol is a phenolic compound that could be formed from a degradation or transformation from other polyphenolic structures present in wines (i.e. anthocyanins, (Motilva et al., 2016). Likewise it is formed from oleopurein degradation in olive oil (Charoenprasert & Mitchell, 2012). Nonetheless, different synthesis of HT has been proposed. For instance, the metabolism in humans involves a pathway starting from dopamine, which is transformed by the monoaminoxidase to give 3,4-dihydroxyphenylacetaldehyde that can be reduced by the aldehyde reductase to HT (Pérez-Mañá et al., 2015). Additionally, the Ehrlich pathway shown in Fig. 1 relates the amino acid metabolism with HT synthesis. However, up to now, no direct evidence of this synthesis by yeast could be determined. To demonstrate that it is a metabolite formed by yeast, we analysed the intracellular media of the yeast, as this could unequivocally demonstrate its origin apart from others that might happen. Fig. 2 shows three mass chromatograms of the biomass of the strains QA, RF and *T. delbrueckii* taken at day 2 of the alcoholic fermentations of synthetic must; each of these strains were analysed in duplicate. A total of six samples were analysed and HT quantified as follows: 8.6 ± 2.7 ng mL^{-1} in the intracellular media of QA at day 2 of fermentation; 106.2 ± 35.1 ng mL^{-1} in the intracellular media of RF at day 2; and 16.1 ± 2.3 ng mL^{-1} in the intracellular media of *T. delbrueckii* at day 2 of fermentation. These results demonstrate the production of HT by the strains studied, conferring yeast with a high potential as a producer of this bioactive compound.

3.3. Fermentations in Tempranillo must

Three fermentations with different inoculation processes were performed with the *Tempranillo* variety. As shown in Fig. 3A, the production of HT was influenced by the yeast strain involved in the fermentation process. The higher concentrations of HT were observed at day 5 in CTQA and at day 3 in CTRF, ranging between 400 and 235 ng mL^{-1}, respectively. Only in the case of CTQA, was the maximum HT achieved at the moment when the reducing sugars were totally consumed. When

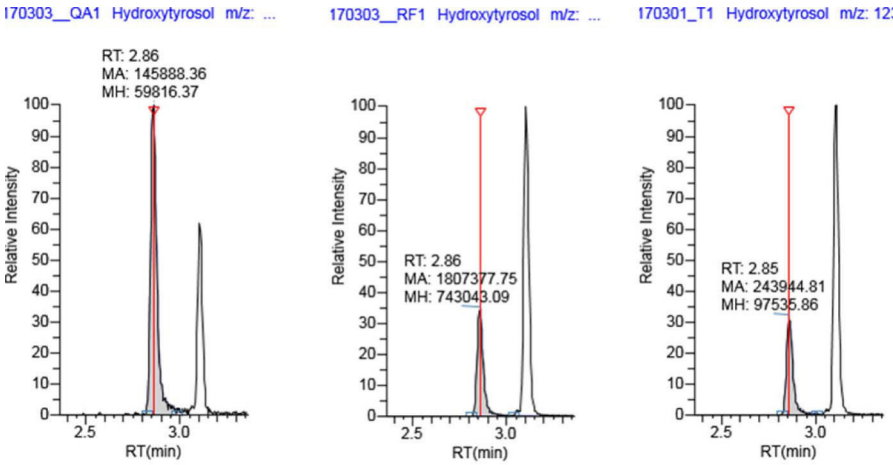

Fig. 2. TIC chromatogram of HT of intracellular samples of second day of fermentation. On the left the QA sample, in the middle RF and on the right *T. delbrueckii*.

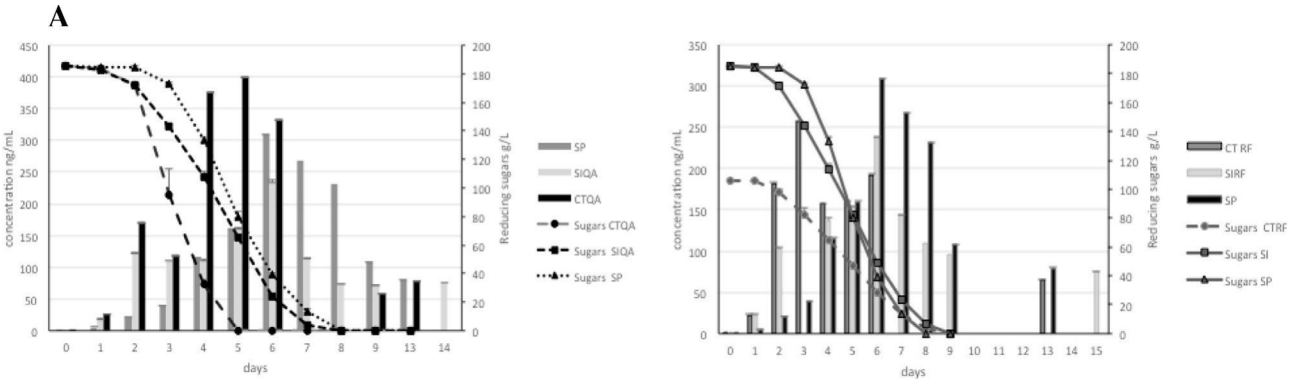

Fig. 3A. Effects of two yeast strains (QA23 on the left and RF on the right) on the production of hydroxytyrosol, and time course reducing sugar in the fermentation of natural must of *Tempranillo* grapes. Different forms of inoculation were used: SI (sequential inoculation). SP (spontaneous fermentation). CTQA (control fermentation with *Saccharomyces cerevisiae* QA23); CTRF (control fermentation with RED FRUIT).

sequential fermentation was performed, lower concentrations of HT were found, as can be seen when comparing CTQA, QA and *T delbrueckii,* (41.3%; Fig. 3A left). Moreover, when SP and SIQA were compared, SP fermentation was found to show higher concentrations (24.3%) over the other fermentations. On the other hand, Fig. 3A (right) shows RF fermentations and it can be observed that concentration values in SIRF (8.51%) were lower than in CTRF. In addition, the content of HT in SP fermentation reached values 23.8% higher than the concentration in SIRF and CTRF (16.7%). Therefore, our results imply that when *T. delbrueckii* was used in the fermentations, the HT was in a lower concentration. Romboli et al. made a similar observation in sequential fermentation with another non-*Saccharomyces* strain, *C zemplinina* (Romboli et al., 2015). These researchers reported concentrations of 18.4 mg L^{-1} for HT + tyrosol in wines produced by *S. cerevisiae* alone, and concentrations of 5.8 mg L^{-1} of HT + tyrosol in wines produced by sequential inoculation with *C. zemplinina* and *S. cerevisiae* Sc1.

Fig. 3B shows the score plots of PCA analysis displaying on the right side the samples that were separated considering the methods of inoculation. The samples CTQA and CTRF presented the same location while the samples of sequential inoculation were separated from each other. Spontaneous fermentation was located on the bottom-left quadrant, possibly related to the influence of concentrations of days 7 and 8, which appear in the same place in the projection of cases due to their high concentrations of SP samples.

3.4. Fermentations in must of six white varieties of grapes

To study the effect of different white grapes on HT production during alcoholic fermentation, musts made from six white grape varieties were analysed after alcoholic fermentation by *S. cerevisiae* strain Aroma White. The grapes varieties were *Corredera*, *Moscatel*, *Chardonnay*, *Sauvignon Blanc*, *Palomino Fino* and *Vijiriega*, and the results are represented in a bar graph together with reducing sugars (Fig. 4A). All varieties followed a similar trend, a progressive increase until the highest concentration was reached at the fifth day (173, 159, 167, 288, 89 and 238 ng mL^{-1}, respectively) except for the variety *Chardonnay*, which presented a slight delay, and reached the highest concentration one day later (185 ng mL^{-1}). The maximum contents of HT were determined when the value of reducing sugars ranged from 53% to 35% of the initial concentration. After this point, the concentrations decreased, falling to the previous values achieved on the 3rd and 4th days (no significant differences $p < 0.05$). In fact, all these results show that the time course of hydroxytyrosol production by the AROMA WHITE strain was affected by the grape-must composition and, consequently, by the duration of the fermentation. These results agree well with those reported before by Romboli et al. (2015), who correlated high amounts of HT with the slowness in the fermentation process. In fact, the longer the fermentation lasted, the higher the HT concentration, as with the fermentations of *Sauvignon Blanc*.

Fig. 4B plots the PCA analysis, representing on the left the days of the fermentation process. The data are grouped into four clusters, two smaller for the first two days (day 1and 2) and other of last days (12, 13, 14, and 15), located on the right side of the plot. The biggest cluster

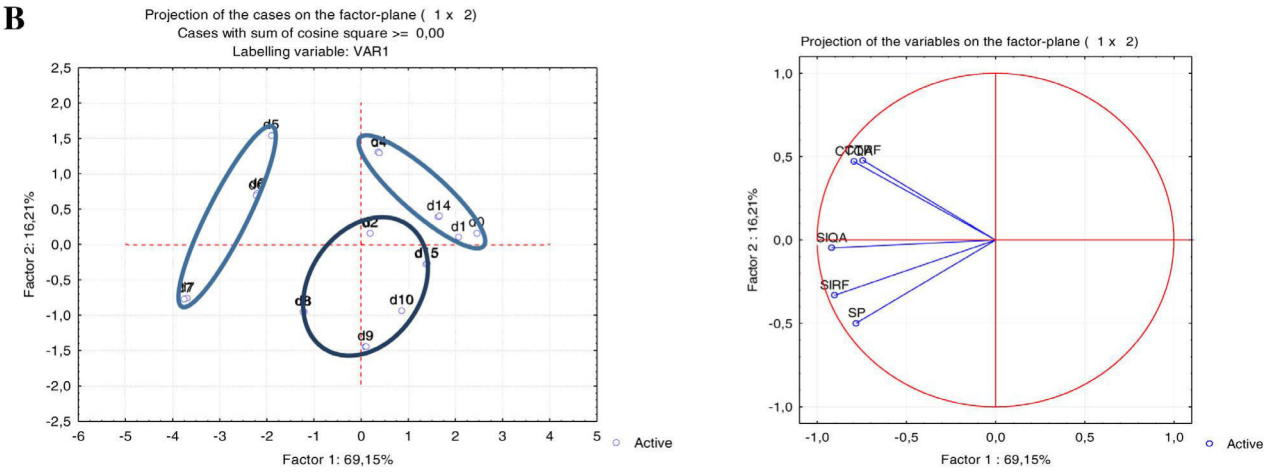

Fig. 3B. Score plot of PCA analysis, on the left side, considering days of fermentation and on the right side considering different methods of inoculation.

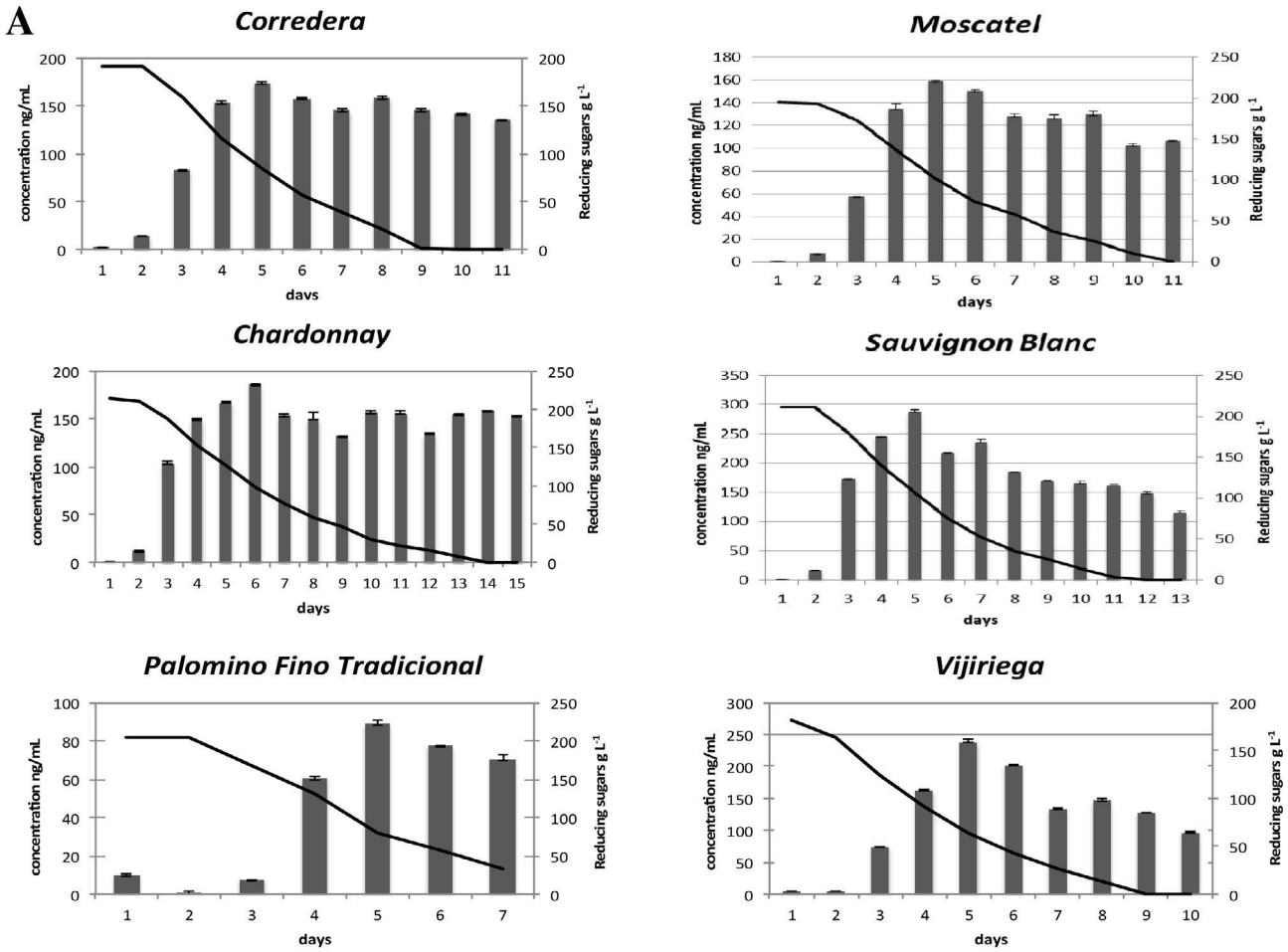

Fig. 4A. Time course of HT and consumption of reducing sugars in alcoholic fermentation in natural must of six different grape varieties.

is in the middle of the plot, corresponding to the interval from the 8th to the 11th days and day 3. On the left, the cluster corresponds to the interval from days 4 to 7, which are those with the highest concentrations. Fig. 4B displays the projection of the varieties of grapes treated as variables (Fig. 4B right). However, the Chardonnay variety is located at the bottom of the projection, probably being influenced by the location of the intermediate cluster (3, 8, 9, 10, and 11) days in which the HT content remained practically unchanged (159–134 ng mL^{-1}). Although further studies are required to understand how the chemical composition of the must influences the HT concentration as well as the role that this compound exerts on the yeast, it is clear from our results that the strains studied synthesise HT and therefore this compound is found in wines.

4. Conclusions

As we know, this is the first study available in which HT has been identified and quantified in an intracellular compartment of *Saccharomyces* (QA23, RF) and Non-*Saccharomyces* (*Torulaspora delbrueckii*). This could be accomplished thanks to a validated HRMS

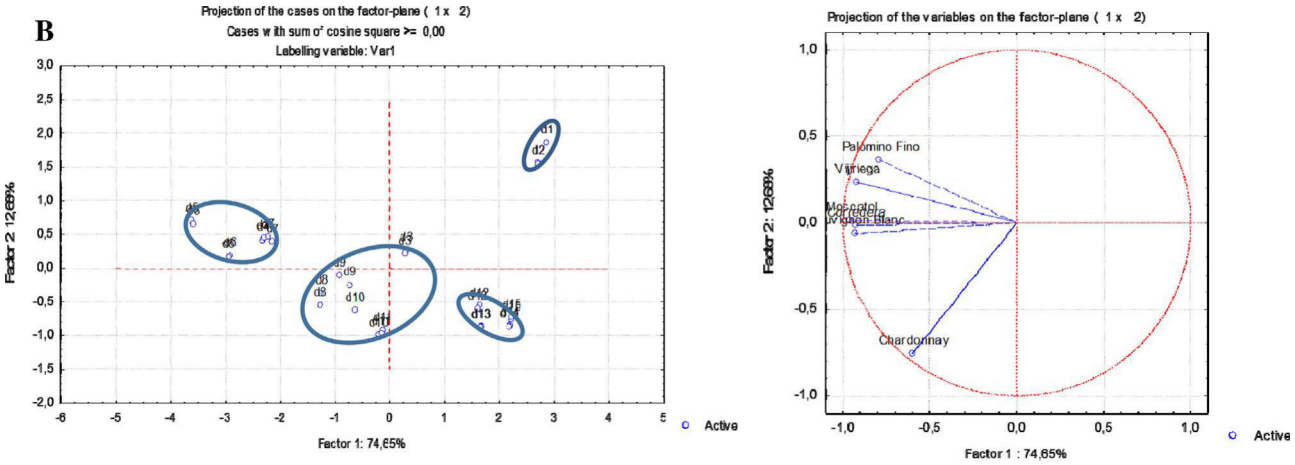

Fig. 4B. Score plot of PCA analysis, on the left side considering days of fermentation and on the right considering the varieties of grapes using in the elaboration of musts.

method developed specifically to diminish LOD and LOQ.

Furthermore, we ascertained that the strain is a crucial factor that influences the production of HT in wines. *Saccharomyces cerevisiae* (QA23) was a more efficient producer of HT than RF in grape musts. Sequential fermentations involving the non-*Saccharomyces* yeast, *T. delbrueckii,* adversely affect the content of HT.

Additionally, the composition of grape must affect the HT concentration. *Sauvignon Blanc* and *Vijiriega* were grape varieties from which AROMA WHITE produced higher concentrations at 5th day of alcoholic fermentation. In summary, HT was a bioactive compound produced by yeast strain that can be modulated both by the involved strain and by the composition of the must.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.09.072.

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Saccharomyces cerevisiae and *Torulaspora delbrueckii* Intra- and Extra-Cellular Aromatic Amino Acids Metabolism

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Supporting Information

ABSTRACT: Tryptophan, phenylalanine, and tyrosine play an important role as nitrogen sources in yeast metabolism. They regulate biomass production and fermentation rate, and their catabolites contribute to wine health benefits and sensorial character through the yeast biotransformation of grape juice constitutes into biologically active and flavor-impacting components. A UHPLC-MS/MS method was applied to monitor 37 tryptophan/phenylalanine/tyrosine yeast metabolites both in extra- and intracellular extracts produced by the fermentation of two *Saccharomyces cerevisiae* strains and one *Torulaspora delbrueckii*. The results shed light on the intra- and extra-cellular metabolomic dynamics, by combining metabolic needs, stimuli, and signals. Among others, the results indicated (a) the production of 2-aminoacetophenone by yeasts, mainly by the two *Saccharomyces cerevisiae*; (b) the deactivation and/or detoxification of tryptophol via sulfonation reaction; and (c) the deacetylation of N-acetyl tryptophan ethyl ester and N-acetyl tyrosine ethyl ester by producing the corresponding ethyl esters.

KEYWORDS: *nitrogen metabolism, alcoholic fermentation, sulfonation, Torulaspora delbrueckii, tryptophan, tyrosine, aromatic amino acids*

INTRODUCTION

Over several millennia, yeasts have transformed grape must into wine using a biotechnological process, where principally sugars turn to alcohol.[1] To achieve this, yeasts use the nutrients present in the medium for growth and in parallel produce metabolites, which lead to a final hydro-alcoholic solution of special flavor and pleasant aroma.[2]

The winemaking yeast *Saccharomyces cerevisiae* can grow by consuming nitrogen from different sources, such as purine and pyrimidine bases, ammonium, urea, amino acids, and small peptides. However, yeast growth, fermentation rate, and biomass yield depend on both quantity and nature of the nitrogen source(s) available, with low nitrogen concentrations leading to low biomass production and slow and/or stuck fermentations.[3,4] On the other hand, in recent years non-*Saccharomyces* yeasts have received an increased interest in wine science/making. In fact, recent studies demonstrated that co-fermentations and sequential-fermentations of non-*Saccharomyces* with *Saccharomyces cerevisiae* yeasts could produce higher quality wines with an enhanced aromatic profile.[5,6] Therefore, non-*Saccharomyces* yeasts are no longer considered purely a source of microbial spoilage. However, non-*Saccharomyces* strains when used as pure cultures in alcoholic fermentation have limited fermentation aptitudes, such as a low fermentation capacity, low fermentation rate, and low resistance to SO_2.[6] One more concern about synergetic fermentations is that the result is more unpredictable, so metabolomic studies are necessary to better understand them. *Torulaspora delbrueckii* is probably the most popular non-*Saccharomyces* yeast in winemaking and one of the first to be commercially released.[7] This yeast was suggested for the fermentations of low-sugar and -acid level musts[8] and has been demonstrated to produce lower volatile acidity levels.[9]

The sources of nitrogen in alcoholic fermentation are classified into three groups depending on the order in which they are consumed.[10] Lysine belongs to the first group (named prematurely consumed). Phenylalanine (PHE) belongs to the early consumed group (second group), and tyrosine (TYR) and tryptophan (TRP) belong to the late consumed group.[10] The essential amino acid TRP and its related indolic metabolites (Figure 1)[11,12] are widely studied due to their biological and pharmacological activities. Metabolites with proved biological activity[13,14] such as melatonin (MEL), serotonin (SER), tryptophol (TOL), kynurenic acid (KYNA), kynurenine (KYN), and indole acetic acid (IAA) are related to TRP yeast's metabolism pathway (Figure 1)[12]. MEL was initially classified as an animal neurohormone[14] but later was found to have multiple functions and to be present in many medicinal plants, foodstuffs, and yeasts.[15−18] The auxin IAA has been reported as a molecule capable of stimulating the developmental transition from the vegetative yeast to the

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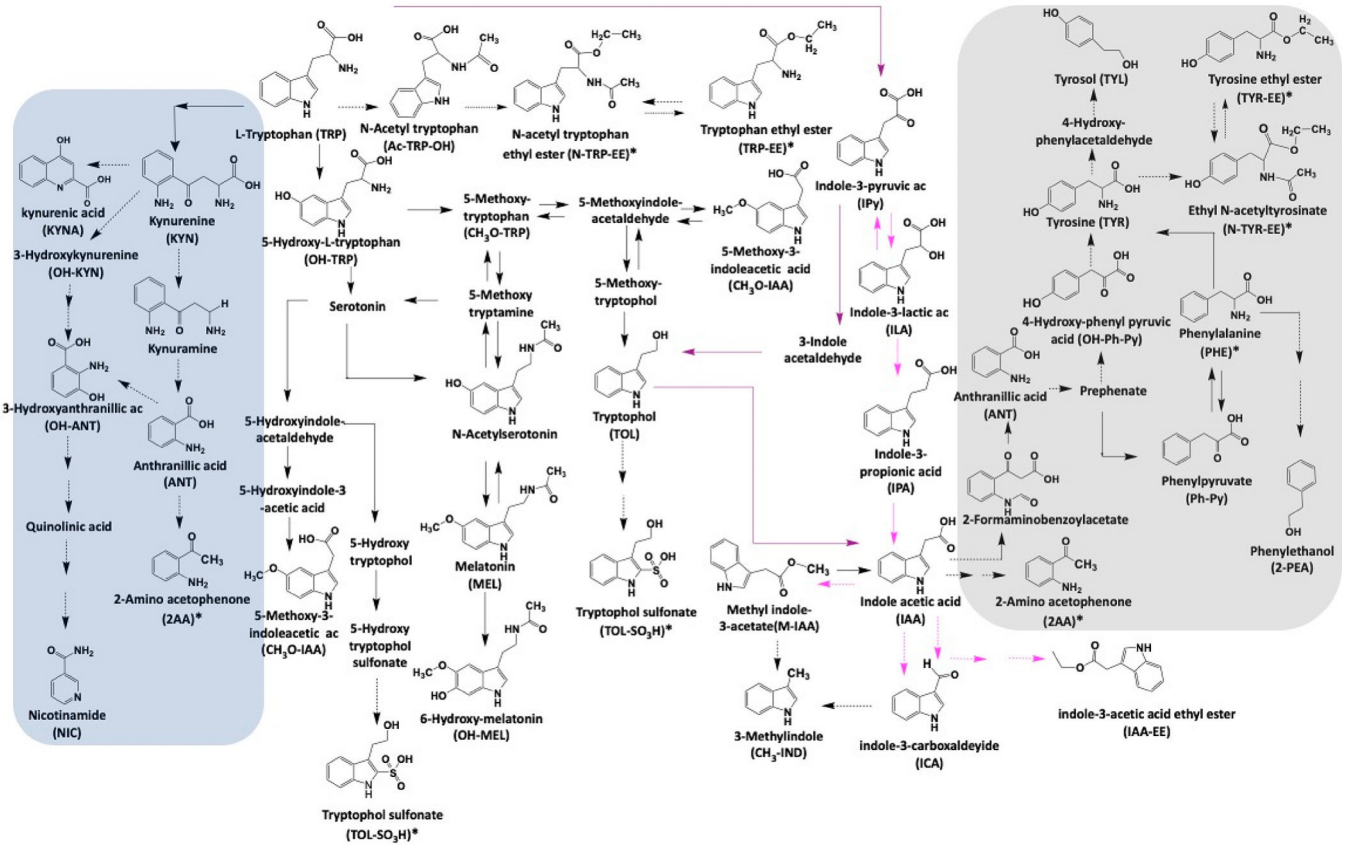

Figure 1. Scheme of proposed pathway of tryptophan (TRP), tyrosine (TYR), phenylalanine (PHE), and related compounds. Pink arrows indicate the compounds enclosed in a cluster of heat-maps of extracellular samples. The box on the left encloses the kynurenine pathway. The box on the right encloses the reactions of compounds related to TYR and PHE metabolism. The compounds with * could also be products of chemical reaction(s).

filamentous form. IAA inhibits growth at high concentrations and induces filamentation and adhesion at low concentrations, acting as a signal to modulate population growth.[19]

Via the Ehrlich pathway, the amino acids TRP, PHE, and TYR produce the aromatic higher alcohols TOL, phenylethanol, and tyrosol (TYL), respectively. The biosynthesis of these compounds is positively correlated with ethanol stress-tolerant yeasts that have an enhanced expression of genes related to TRP metabolism.[20] These compounds, as autoinducers, are able to transmit information about the population density and the amount of available nitrogen.[14,21] Excessive concentration of these higher alcohols can result in a strong, pungent smell and taste, whereas optimal levels have a positive impact, producing wines with a flowery character.[22] Moreover, through the TRP pathway, yeast contributes to wine aroma directly by biotransforming odorless metabolites into flavor-active compounds, such as methyl mercaptan and indole[20], and indirectly via chemical reactions during wine aging, since the indolic metabolites are putative precursors of other aromatic substances, like 2-aminoacetophenon (2AA;[11] Figure 1). In addition, indoles can react with the SO_2, when added to wine for protection against oxygen and microorganisms, delivering sulfonated metabolites and effecting wine shelf life and a metabolic fingerprint.[23]

PHE and TYR catabolism during fermentation, which is better known with respect to TRP, between others produces the aromatic ester 2-phenyl acetate (related to rose, honey, and flowery odors), phenyl acetic acid (Ph-AA), *p*-OH-phenyl acetic acid, phenyl-pyruvate, and *p*-OH-phenyl-pyruvate.[24]

Most metabolomic research relies on the analysis of yeast extracellular metabolites, while the studies on intracellular metabolic changes are relatively less. However, the possibility to follow the behavior of metabolites during the fermentation process, in parallel for both intracellular and extracellular media, is useful in order to gain knowledge of the signaling and the metabolomic reaction network. Such experimental design could help us improve the nutritional value and quality of wine—and other fermented food.

The goal of this work was to use a high throughput targeted UHPLC−MS/MS method to monitor metabolites related to aromatic amino acid metabolism (Figure 1) and to provide novel information on the aromatic amino acid intra- and extracellular metabolism during the alcoholic fermentation in synthetic must of two *Saccharomyces cerevisiae* and one *Torulaspora delbrueckii* yeast strain.

MATERIALS AND METHODS

Reagents and Materials. All chemicals used in this study were of the highest purity grade available and purchased from Sigma-Aldrich (Madrid, Spain or Milan, Italy) and Cymit Quimica S.L. Barcelona, Spain (Supporting Information Table S1), unless otherwise stated. Sulfonated tryptophol was prepared as previously described.[25]

Yeast Strains. The experiments on synthetic musts were performed with the three different commercial wine yeast

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Table 1. UHPLC−ESI−MS/MS Conditions for Quantification and Identification of Metabolites and Internal Standards (IS; n.q.: not quantified)

#	metabolite (abbreviation)	RT (min)	ESI mode	cone voltage (V)	quantifier MRM (collision energy)	qualifier MRM (collision energy)	LOQ ($\mu g\ L^{-1}$)	QCex $\mu g\ L^{-1}$ (%RSD)	QCin $\mu g\ L^{-1}$ (%RSD)
1	nicotinamide (NIC)	1.50	+	28	123→ 79(14)	123→95(14)	4.2	14.81 (9)	5.73 (10)
2	5-OH-tryptophan (OH-TRP)	2.16	+	14	221→162(18)	221→133(24)	3.9	n.q.	n.q.
3	5-CH_3O-tryptophan (CH_3O-TRP)	2.16	+	14	235→176(18)	235→148(24)	10.50	n.q.	n.q.
4	kynurenine (KYN)	2.37	+	14	209→146(16)	209→136(12)	16.40	n.q.	n.q.
5	phenylalanine (PHE)	2.65	+	16	166→120(12)	1661→03(24)	44.90	130 (12)	70 (9)
6	3-nitrotyrosine (IS)	2.76	+	16	227→181(14)	227→117(24)	3.90	7300 (9)	5740 (4)
7	4-OH-phenyl pyruvic ac (OH-Ph-Py)	2.79	+	12		181→106(20)	1185.90	44100 (25)	n.q.
			−	8	179→106(14)				
8	3-OH-anthranilic acid (OH-ANT)	3.01	+	12	154→79(24)	154→108(22)	3.20	n.q.	n.q.
9	tryptophan (TRP)	3.07	+	12	205→146(18)	205→117(26)	11.20	1170 (20)	190 (3)
10	tyrosine ethyl ester (TYR-EE)	3.08	+	18	210→136(14)	210→90(26)	13.00	n.q.	n.q.
11	N-acetyl serotonin (N-SER)	3.27	+	16	219→160(16)	219→132(26)	9.10	n.q.	n.q.
12	tyrosol (TYL)	3.37	+	24	139→102(18)	139→93(12)	36.00	n.q.	n.q.
13	tryptophol sulfonate (TOL-SO_3H)	3.41	+	8	240→160(22)	240→130(28)	4.2	100 (20)	n.q.
14	kynurenic ac (KYNA)	3.46	+	22	190→143(14)	190→116(32)	10.30	54.58 (31)	n.q.
15	4-OH-phenyl acetic ac (OH-Ph-AA)	3.54	+	12	153→107(8)	153→76(24)	1262.33	n.q.	n.q.
16	tryptophan methyl ester (TRP-ME)	3.70	+	12	219→144(30)	219→160(18)	24.06	n.q.	n.q.
17	phenyl pyruvic ac (Ph-Py)	3.97	+	10		164→90(20)	43.20	n.q.	n.q.
			−	14	163→90(10)				
18	indole pyruvic ac (IPy)	4.21	+	16	204→130(22)	204→158(12)	0.77	n.q.	n.q.
19	anthranilic ac (ANT)	4.23	+	12	138→92(22)	138→64(26)	9.40	n.q.	n.q.
20	tryptophan ethyl ester (TRP-EE)	4.62	+	14	233→174(14)	233→159(20)	27.36	n.q.	n.q.
21	phenyl lactic acid (Ph-LA)	4.80	−	14	165→103(16)	165→119(16)	208.50	850 (12)	n.q.
22	3-indole lactic acid (ILA)	4.80	+	18	206→117(20)	206→160(10)	0.45	58.36 (4)	20.64 (28)
23	N-acetyl-L-tyrosine ethyl ester (N-TYR-EE)	4.99	+	14	253→136(22)	253→178(12)	1.70	1.75 (9)	n.d.
24	indole carboxaldehyde (ICA)	5.13	+	22	146→118(14)	146→90(24)	0.09	236.78 (2)	12.35 (8)
25	melatonin (MEL)	5.20	+	16	233→174(14)	233→159(28)	0.65	n.q.	n.q.
26	5-CH_3O-indole acetic acid (CH_3O-IAA)	5.30	+	18	206→160(18)	206→145(28)	0.16	3.60 (9)	12.21 (12)
27	tryptophol (TOL)	5.56	+	14	162→144(20)	162→127(22)	71.00	5840 (3)	80 (4)
28	3-indole acetic acid (IAA)	5.58	+	18	176→102(28)	176→130(12)	0.52	9.31 (5)	74.30 (4)
29	phenyl acetic acid (Ph-AA)	5.62	+	12	137→90(12)	137→64(30)	166.90	n.q.	n.q.
30	2-aminoacetophenone (2AA)	5.68	+	20	136→117(20)	136→42(16)	0.15	1.20 (16)	n.d.
31	indole (IND)	6.90	+	30	118→118(16)	118→90(18)	0.19	n.q.	n.q.
32	3-indole propionic acid (IPA)	7.12	+	12	190→130(14)	190→54(20)	0.19	n.q.	n.q.
33	N-acetyl tryptophan ethyl ester (N-TRP-EE)	7.61	+	18	275→159(22)	275→201(12)	0.50	0.21 (25)	n.d.
34	3-indole butyric acid (IBA)	7.78	+	18	204→130(22)	204→144(22)	0.08	2.58 (26)	n.d.
35	methyl-indole acetic acid (M-IAA)	7.80	+	14	190→130(10)	190→103(32)	2.80	3.59 (9)	1.22 (13)
36	3-CH_3-indole (CH_3−IND)	8.06	+	34	132→103(20)	132→76(22)	6.60	n.q.	n.q.
37	3-indole acetic acid ethyl ester (IAA-EE)	8.11	+	14	204→130(14)	204→102(36)	0.06	3.00 (12)	3.91 (3)
38	3-ethyl-indole carboxylic acid (E-ICa)	8.15	+	16	190→118(18)	190→162(12)	0.18	16.28 (10)	1.48 (37)

strains: *S. cerevisiae* Lalvin YSEO QA23 (Lallemand; QA), *S. cerevisiae* Red Fruit RF (Enartis; RF), and *T. delbrueckii* TD291 Biodiva (Lallemand; Td).

Alcoholic Fermentation. Three alcoholic fermentations were performed in a 3.5 pH synthetic must (SM), prepared as per Riou et al.[26] with slight modifications (Supporting Information Table S2 shows in detail the must composition and nitrogen sources). Briefly, glucose and fructose were adjusted at 100 g L^{-1} each, while the concentration of the rest of the compounds was maintained unaltered.[26] The *Saccharomyces cerevisiae* QA23 and RED FRUIT and the *Torulaspora delbrueckii* were used separately for the three fermentations. To obtain the most reliable results possible and capture the random biological variation, six biological replicates of each

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fermentation were considered (biological variability). SM was sterilized with bottle top vacuum filters (Nalgene PES membrane). Each Erlenmeyer flask with 750 mL of SM was inoculated with 10^6 cell mL^{-1} and capped with taps equipped with a capillary to release carbon dioxide. All Erlenmeyer flasks were stored in an orbital incubator at 28 °C during the period of the experiment. To monitor the fermentation, the flasks were weighed daily before and after sampling. Cell growth and evolution were determined by measuring the optical density (DO) at 600 nm every sampling day. Enzymatic kits (Megazyme International, Ireland) were used to assay the reducing sugars and primary amino nitrogen (PAN) content in extracellular media.

Extracellular Metabolite Extraction. A volume of sample corresponding to 10^9 cells of each Erlenmeyer flask was taken at days 2, 5, and 15. The volume was adjusted according to the number of cells accounted with a Neubauer chamber and the DO value. Immediately, the samples were centrifuged, at 5992*g* for 3 min at 4 °C, in order to separate the cells from extracellular contents. The extracellular samples were collected and stored at −80 °C until the analysis.

Intracellular Metabolite Extraction. Cells were pelleted by centrifugation at 5992*g* for 3 min at 4 °C twice with distilled water to wash them. The washed cells were subject to a cold glycerol saline quenching procedure, the focus of which was to stop cellular metabolism and avoid the turnover of metabolites by stopping the enzymatic activity, maintaining the *in vivo* metabolite concentrations at a constant level. The procedure used followed the one reported earlier by Villas-Bôas and Bruheim.[27] For the quenching, the pellets were resuspended with 1 mL of distilled water and transferred to a precooled centrifuge tube containing 4 mL of a cold-quenching solution [3:2 (v/v) glycerol/saline solution (saline solution 0.9 wt %/vol NaCl/water)] maintained at −23 °C in a refrigerated bath (ethylene glycol as cryo fluid). Next, the solution was homogenized, returned to the cold bath for 5 min, and centrifuged at 36 086*g* for 20 min at −20 °C (Sorvall LYNK 6000, Thermo Fisher Scientific, Waltham, MA USA). After removing the supernatant, the pellets were resuspended in the same volume of cold glycerol, and the process was repeated once again. The quenched pellets were stored at −80 °C until the extraction. The extraction of the intracellular metabolites was performed following the method reported by Smart et al. with minor modifications.[28] To the cell pellets, 2.5 mL of cold methanol−water solution (50% (v/v), −30 °C) was added, vortexed for 1 min, and then frozen at −80 °C. Each sample was subjected to two cycles of freeze−thaw (frozen at −80 °C for 30 min and then thawed in an ice bath for 4 min followed by 1 min of shaking with a vortex). After the last cycle, it was sonicated for 1 min in an ice bath using an ultrasonic liquid processor (Sonicator Sonoplus HD 2070, Bandelin Electronic GmbH & Co. KG, Berlin, Germany). Subsequently, the solution was centrifuged at 36 086*g* for 20 min at −20 °C, and the supernatant was collected. Another 2.5 mL of cold methanol−water was added to the pellets, and then the extraction was repeated from the cycles of freeze−thaw. The supernatants were collected, pooled, and stored at −80 °C until solid phase extraction.

Sample Treatment/Preparation. All extracellular samples and intracellular extracts were cleaned up as previously reported by Rodriguez-Naranjo et al.[17] with the following modifications. Briefly, C18 SPE cartridges (1 g, Variant, Agilent) were conditioned with 2 mL of methanol and 2 mL of milli Q water. An aliquot of 1.5 mL of extracellular sample or 2.5 mL of intracellular sample was loaded, followed by a washing step with 2 mL of a 10% v/v methanol solution. The analytes were eluted with 1.5 mL of methanol, and methanol was then evaporated until dryness by using a vacuum concentrator at 34 °C and 896*g* for 6 h (HyperVAC-LITE, GYOZEN, Korea). Each sample was reconstituted with 300 μL of 9.37 mg/L of 3-nitrotyrosine (internal standard) in 10% methanol/water v/v and immediately analyzed.

UHPLC-MS/MS Instrumental Analysis. The analysis was carried out in a Waters Acquity UHPLC (Milford, Massachusetts, USA) using the MassLynx MS software, according to Arapitsas et al.[25] with slight modifications. Separation was performed in order to separate 37 metabolites and an internal standard (3-nitrotyrosine). For the analysis, a Waters Acquity column (Milford, Massachusetts, USA), HSS T3 (2.1 × 150 mm, 1.8 μm particle size), was used. Mobile phase A was water with 0.1% formic acid; mobile phase B was methanol with 0.1% formic acid. The gradient was programmed as follows: 95% A, 5% B (0 min); 80% A, 20% B (1.5 min); 65% A, 35% B (2 min); 55% A, 45% B (6 min); 100% B (8−10 min); 95% A, 5% B (10.1−13 min). The column was kept at 40 °C. The flow rate was 0.4 mL min^{-1}. The injection volume was 2 and 10 μL in order to allow the quantitation as a function of the concentration of metabolites. The injection volume of 10 μL was used to quantitate all compounds except TRP and phenyl pyruvic acid (Ph-Py), where the 2 μL injection volume analysis was used. The MS analysis was performed with a Waters Xevo TQ (Milford, Massachusetts, USA) triple quadrupole mass spectrometer. Each metabolite was directly infused in the MS system in negative and positive modes with a 50/50 v/v of the two phases A and B, in order to optimize the detection process. The two most abundant fragments were selected for each metabolite, one as a quantifier (most abundant) and the other as a qualifier ion, using the MRM (multiple reaction monitoring) method for selective quantification. Table 1 shows, for each metabolite, the MRM parameters, the retention time, and the standard deviation (RSD) for the extracellular and intracellular QC (quality control) injections.

Statistical Analysis. Statistical analyses were performed using Statistica software[29] version 12 and MetaboAnalyst.[30] Statistical significance between groups was tested by analysis of ANOVA variance and Tukey's HSD (honest significant difference) test. ANOVA and Tukey's were assessed to test significant differences at $p < 0.05$. The posthoc analysis was used in conjunction with the ANOVA to test which means were significantly different between analyzed groups of samples (Supporting Information Figures S1−S6). The multivariate statistical analysis and heat-maps were made by using the Metaboanalyst web platform, where (a) missing values were estimated by replacing them with a small value (half of the minimum positive value in the original data), (b) no sample normalizations were applied, (c) no data transformation was applied, and (d) autoscaling (mean-centered and divided by the standard deviation of each variable) was applied.[30] For the PCA plots, the SIMCA-P software was used by inserting the Metaboanalyst table in order to have scaling and missing values estimation (Umetrics AB, Malmö, Sweden).

RESULTS

The development of the UHPLC-MS/MS method was based on previous works[25,31,32] where the adequacy of LC-MS

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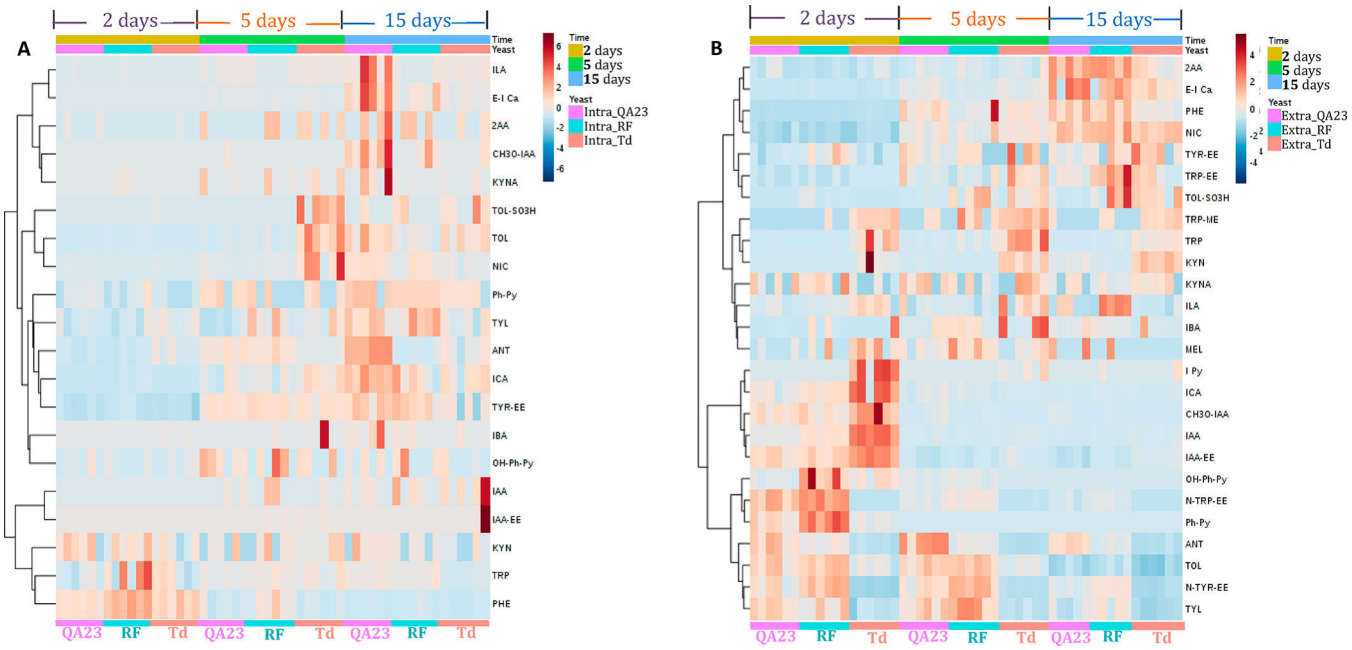

Figure 2. Clustered heat-maps of the metabolites measured separately for the intracellular (A) and extracellular (B) samples of the three yeast strains (the *Saccharomyces cerevisiae* strains QA23 and RF and the *Torulaspora delbrueckii* (Td)) at three time points of the fermentation process (2, 5, and 15 days). TOL, tryptophol; NIC, nicotinamide; TYR-EE, tyrosine ethyl ester; TYL, tyrosol; IAA-EE, indole acetic acid ethyl ester; PHE, phenylalanine; OH-Ph-Py, OH-phenyl-pyruvic acid; N-TRP-EE, N-acetyl-tryptophan ethyl ester; Ph-Py, phenyl-pyruvic acid; KYNA, kynurenic acid; N-TYR-EE, N-acetyl-tyrosine ethyl ester; ANT, anthranilic acid; CH3O-IAA, methoxy-indole acetic acid; IPY, indole pyruvic acid; IAA, indole acetic acid; ICA, indole carboxaldehyde; 2AA, 2-aminoacetophenone; E-ICA, ethylindole carboxaldehyde; ILA, indole lactic acid; TRP-EE, tryptophan ethyl ester; TOL-SO_3H, sulfonated tryptophol; TRP-ME, tryptophan methyl ester; TRP, tryptophan; KYN, kynurenine; IBA, indole butyric acid; MEL, melatonin.

systems for the analysis of tryptophan metabolites was proved. As described above, only slight modifications were made in order to adapt the method to the current experimental design. Table 1 shows the basic instrumental parameters of the 38 analytes (37 metabolites and the IS) included in the method and selected in order to cover the metabolic pathways of Figure 1. In detail, they included the major metabolites of TRP catabolism: tryptophan ethyl ester (TRP-EE), tryptophan methyl ester (TRP-ME), N-acetyl tryptophan ethyl ester (N-TRP-EE), TOL, 5-OH-tryptophan (OH-TRP), 5-methoxy-tryptophan (CH_3O-TRP), SER, N-acetyl serotonin (N-SER), MEL, indole pyruvic acid (IPy), indole lactic acid (ILA), IAA, 5-methoxy-indole acetic acid (CH_3O-IAA), methylindole acetic acid (CH_3-IAA), indole acetic acid ethyl ester (IAA-EE), ethylindole carboxylic acid (E-ICa), 2AA, indole carboxaldehyde (ICA), indole proprionic acid (IPA), indole butyric acid (IBA), KYN, KYNA, nicotinamide (NIC), anthranilic acid (ANT), and 3-OH-anthranilic acid (OH-ANT); catabolites of the amino acid PHE: 4-OH-phenyl pyruvic acid (OH-Ph-Py), 4-OH-phenyl acetic acid (OH-Ph-AA), Ph-Py, phenyl lactic acid (Ph-LA), and phenyl acetic acid (Ph-AA); and, finally, the catabolites of the amino acid TYR: tyrosine ethyl ester (TYR-EE), N-acetyl-tyrosine ethyl ester (N-TYR-EE), and TYL. As expected, the qualitative characteristics of the applied method were comparable with previous methods.[25,31,32] On the basis of our former experience in high-throughput targeted analysis,[33] to control the robustness of the LC-MS system and its signal stability, (a) 3-nitrotyrosine was added as an internal standard, (b) the order of sample injection was randomized, and (c) a QC sample was injected every 10 real sample injections (instrumental variability). The QC samples were separately prepared as a pooled mix of all extracellular (QC_{ex}) and intracellular (QC_{in}) samples. Finally, three technical replicates, of each sample, were injected. In order to verify instrumental accuracy and stability, 33 injections of the QC were carried out as part of the analysis method, through the measure of concentrations of almost all metabolites of interest.[33] Table 1 displays the values of standard deviation (RSD) as a measure of the variation corresponding to intracellular and extracellular QC injections. The results were in accordance with our previous experience.[33] The internal standard RSD was 9% for extracellular QC injections and 4% for intracellular QC injections; these values are in accordance with AOAC (Association of Official Analytical Chemists) requirements.

Before we get into a detailed discussion, it is worth emphasizing that the metabolite production/consumption between the second and fifth day of the fermentation should be predominant due to the yeast's activity, as a period of growth and cellular development. On the other hand, during the period between the fifth and 15th day, the metabolic changes should be mainly the outcome of chemical reactions and macromolecule degradation (without excluding yeast activity), since the alcoholic fermentation has terminated (Supporting Information Figure S7). Normally, the fermentations in synthetic media conclude in shorter periods than normal grape must fermentations.[26]

From the 37 metabolites of Table 1, 20 were detected and quantified in the intracellular samples and 26 in the extracellular samples. In comparison to the extracellular samples, the intracellular samples profile was characterized by the predominance of the amino acids PHE, NIC, and TYR-EE and the higher alcohols TOL and TYL (Supporting Information Figure S8).

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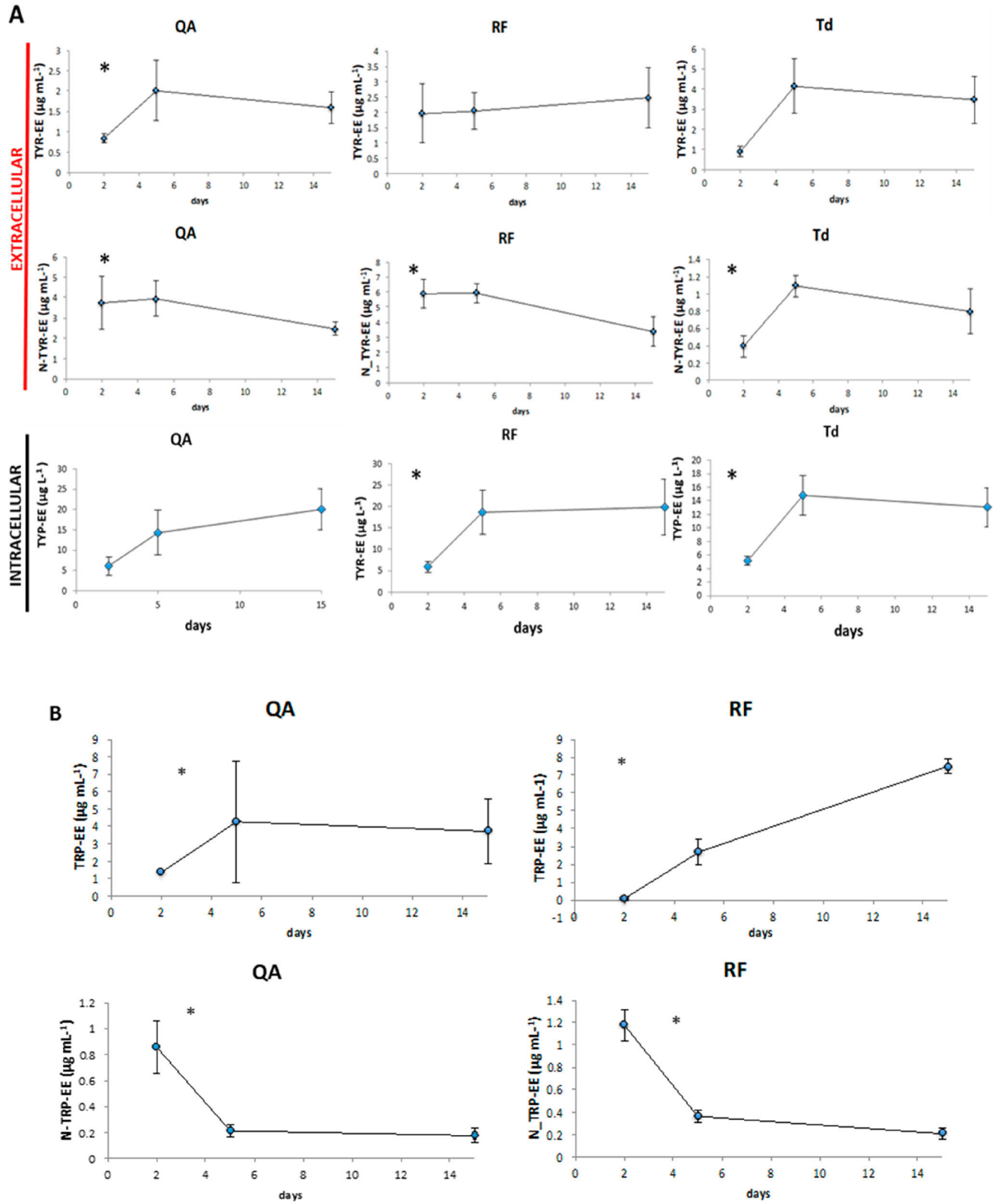

Figure 3. Kinetics of the deacetylation process: (A) N-acetyl tyrosine ethyl ester (N-TYR-EE) and (B) N-acetyl tryptophan ethyl ester (N-TRP-EE). *Statistically significant differences $p < 0.05$. QA means *Saccharomyces cerevisiae* Lalvin YSEO QA23 (Lallemand); RF means *Saccharomyces cerevisiae* Red Fruit RF (Enartis); and Td means *Torulaspora delbrueckii* TD291 Biodiva (Lallemand).

Intracellular Metabolic Profile. Figure 2A provides a detailed picture concerning the intracellular behavior of the various metabolites with respect to the sampling time and the yeast strain. The highest concentrations of TRP and PHE were observed on the second day in intracellular samples for RF fermentations. They could be the result of the amino acid transporter permease activity, which facilitates the inclusion of these two amino acids in the intracellular space.[10] During the

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early phases of fermentation, TRP and PHE are not used as nitrogen sources in the first instance. Quiros et al.[34] reported that yeasts initially consume lysine and later other amino acids such as alanine, tryptophan, or tyrosine. Intracellularly, TRP and PHE were the only metabolites whose concentrations were higher at the first sampling point and then decreased, while the concentration of most of the others metabolites increased. On the other hand, TOL and TYL showed major concentrations on the fifth and/or 15th day. TOL in QA and Td samples reached concentrations between 137−1995 $\mu g\ L^{-1}$ and 106−2869 $\mu g\ L^{-1}$, respectively. The highest value of concentrations of TYL ranged between 68 and 206 $\mu g\ L^{-1}$ in RF fermentations, the only case with significant differences (Supporting Information Figures S9−S13). Such behavior, and thus the consumption of the amino acids TRP and PHE and the production of the higher alcohols TOL and TYL, could be explained by the Ehrlich pathway metabolism (Figure 1).[2] The amino acid catabolism serves as a nitrogen source to the cell, while the higher alcohols produced accomplish several biological functions as described in the Introduction.[14,21]

Extracellular Metabolic Profile. According to the heat-map hierarchical clustering of Figure 2B, a group of metabolites included IPy, ICA, CH_3O-IAA, IAA, and IAA-EE. Interestingly, the same metabolites were also clearly connected in the metabolomic pathways described in Figure 1 (pink arrows). All these compounds appeared in higher concentrations on the second day of extracellular samples of Td, while the same trend was seen in extracellular samples on the second day in RF.

The hierarchical cluster analysis (Figure 2B) then pointed out a second group of metabolites, which included two subgroups: (a) OH-Ph-Py, Ph-Py, and N-TRP-EE and (b) ANT, TOL, N-TYR-EE, and TYL. Chemically, this group comprised the N-acetyl ethyl esters of the amino acids tryptophan and tyrosine (N-TRP-EE and N-TYR-EE), the two phenyl-pyruvic acid metabolites (OH-Ph-Py and Ph-Py), two aromatic alcohols (TYL and TOL), and anthranilic acid (ANT). They all exhibited a similar behavior in extracellular samples of the *Saccharomyces cerevisiae* strains, having their higher concentration on the second day and then decreasing. This reduction of the concentration was faster for the first subgroup, OH-Ph-Py, Ph-Py, and N-TRP-EE. The amino benzoic acid ANT has a role as an environmental cue that modulates and induces morphological transitions in *Saccharomyces* and other fungi.[19] In addition, this compound was also related with OH-Ph-Py and Ph-Py through the phenylalanine pathway (Figure 1, right box). The N-acetyl derivatives of tryptophan and tyrosine, N-TRP-EE and N-TYR-EE, disclosed the opposite trend with respect to their analogues, TRP-EE and TYR-EE, most probably due to a deacetylation enzymatic process, as reported by Kradolfer et al.[35], which intervenes in the production of TOL from TRP (Figure 3).

These two last metabolites, TRP-EE and TYR-EE, clustered together with the sulfonated tryptophol ($TOL\text{-}SO_3H$) according to the hierarchical cluster analysis of Figure 2B, showing the highest concentration at 5 and 15 days. According to the graphs of Figure 3, the three yeasts had slightly different behavior. The decrease of the two N-acetyl esters (N-TRP-EE and N-TYR-EE) and the increase of their corresponding ethyl esters (TRP-EE and TYR-EE) was statistically significant for QA. RF demonstrated a more pronounced effect for the TRP derivatives, since TYR-EE did not show statistical significance. The third yeast, the non-*Saccharomyces* Td, showed statistically significant changes only for the TYR derivatives. The sulfonation of TOL was lately discovered as a reaction occurring in bottled white wines in the presence of O_2 and added SO_2. Here, we report for the first time that this reaction can also occur in the context of an alcoholic fermentation where the presence of SO_2 can be only attributed to the yeast sulfur metabolism. As for the O_2, the reducing sugars were already consumed on the fifth day, with only a little remaining in Td (Supporting Information Figure S7), which leads to the absence of CO_2 formation, as a result of limited amounts of O_2 being able to enter into the fermentation flask through the capillary between the fifth and the 15th day, favoring the sulfonation of TOL.

A group of metabolites clustered close to the previous one in extracellular metabolome, thus presenting a similar trend, enclosed 2AA, E-ICa, PHE, and NIC. 2AA in extracellular samples increased significantly through time, with concentrations ranging between 0.3−1.2 $\mu g\ L^{-1}$ in QA and RF and 0.31−0.61 $\mu g\ L^{-1}$ in Td samples (Figure 2 and Supporting Information Figures S9−S13). Therefore, the two *Saccharomyces cerevisiae* yeasts were able to produce a higher amount of 2AA than the non-*Saccharomyces* yeast. 2AA can be chemically produced by oxidative degradation of IAA and KYN, as suggested by Hoenicke et al.[36] (Figure 1). However, 2AA can also be produced enzymatically from ANT.[11]

Finally, the last cluster of the first section of the hierarchical analysis of extracellular samples (Figure 2B) contained TRP-ME, TRP, KYN, KYNA, ILA, IBA, and MEL, which was almost similar to the last one in the heat maps of all samples (Supporting Information Figure S8). In most intracellular samples, MEL had lower concentrations than the LOQ (Limit of Quantification), and it was therefore difficult to follow the evolution though the sampling time. In extracellular samples, MEL was detected on all sampling days, but its concentrations were lesser than the LOQ. On the 15th day in QA extracellular samples, the concentration of MEL was higher, and the content of reducing sugars was low (Supporting Information Figure S7). These results are consistent with those reported by Rodriguez-Naranjo et al.,[37] who concluded that the *S. cerevisiae var. bayanus* QA strain had the ability to produce MEL (Figure 1) when the reducing sugars were totally consumed. Other compounds included in this cluster were KYN and KYNA related to the TRP metabolism via kynurenine pathway (described in *Saccharomyces cerevisiae*[12,18]). KYN reached concentrations ranging between 0.34−1.58 $\mu g\ L^{-1}$ in QA and 0.39−0.54 $\mu g\ L^{-1}$ in RF, the two cases with significant differences for extracellular samples belonging to the fifth and 15th days.

DISCUSSION

The possibility to follow the intra- and extra-cellular behavior of the metabolomic changes in parallel during fermentation offers great advantages. The sets of information obtained are furthermore complementary to each other and help to extract a more complete view of the yeast metabolism. Even though the culture medium influences the intracellular metabolism, cells have the ability to adapt rapidly to minimal changes through very fast turnover mechanisms. Generally, the metabolite concentration in the cell is lower than the extracellular environment. The design of the present study did not follow the industrial condition in all respects, since fermentations did not take place in grape must, and *Torulaspora delbrueckii* fermentations were achieved without the coinoculation of a

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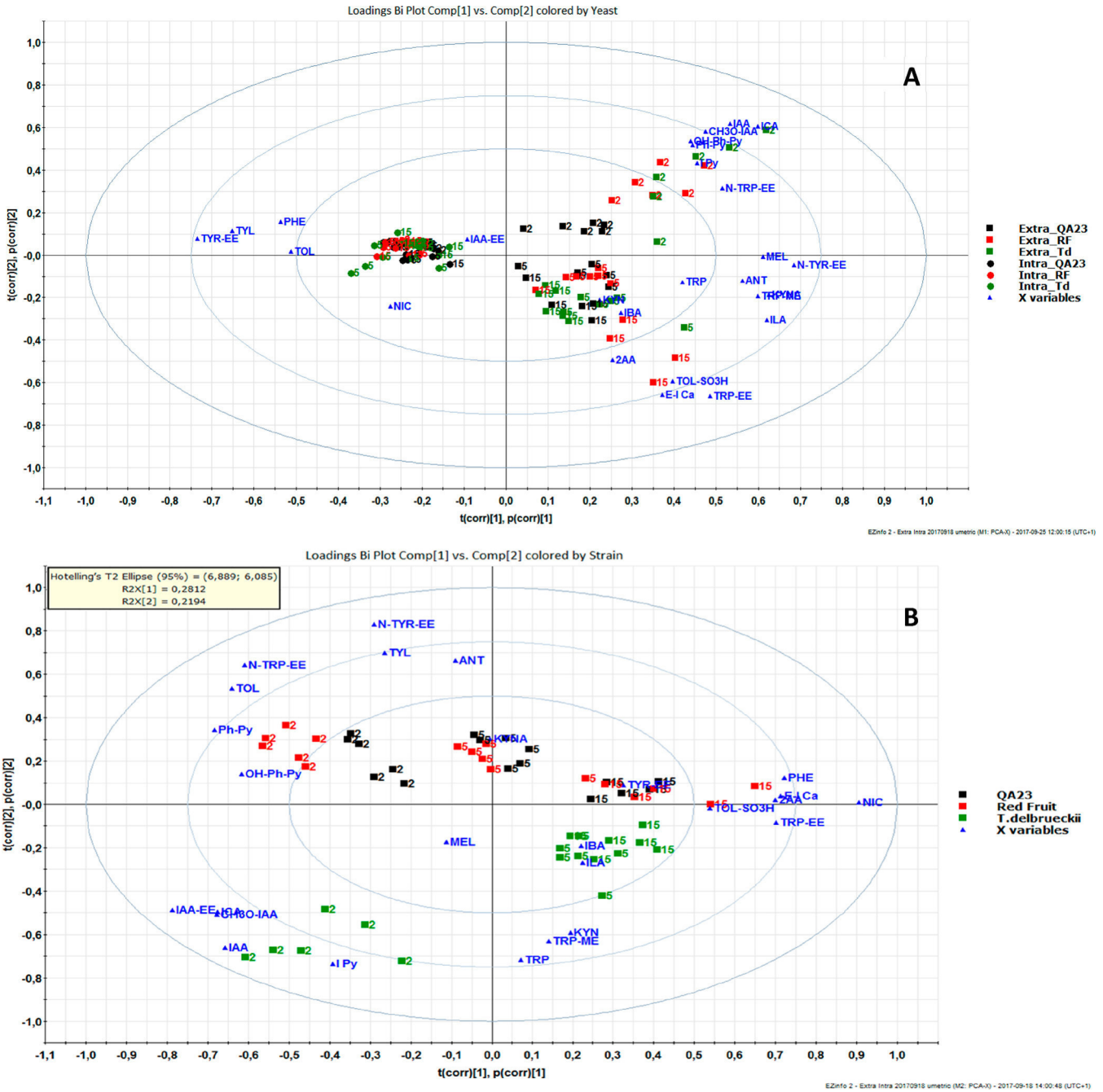

Figure 4. PCA biplots, including both PCA plot (samples) and plots of loadings (metabolites), based on the measured metabolites produced by the three yeast strains (the *Saccharomyces cerevisiae* strains QA23 and RF and the *Torulaspora delbrueckii* (Td) at three time points of the fermentation process (2, 5, and 15 days)). The loadings/metabolites are labeled in blue letters. Numbers indicate the time in days. (A) Includes both extracellular (left side) and intracellular samples (right side). (B) Based only on the extracellular samples. TOL, tryptophol; NIC, nicotinamide; TYR-EE, tyrosine ethyl ester; TYL, tyrosol; IAA-EE, indole acetic acid ethyl ester; PHE, phenylalanine; OH-Ph-Py, OH-phenyl-pyruvic acid; N-TRP-EE, N-acetyl-tryptophan ethyl ester; Ph-Py, phenyl-pyruvic acid; KYNA, kynurenic acid; N-TYR-EE, N-acetyl-tyrosine ethyl ester; ANT, anthranilic acid; CH_3O-IAA, methoxy-indole acetic acid; IPY, indole pyruvic acid; IAA, indole acetic acid; ICA, indole carboxyladehyde; 2AA, 2-aminoacetophenone; E-ICA, ethylindole carboxyladehyde; ILA, indole lactic acid; TRP-EE, tryptophan ethyl ester; TOL-SO_3H, sulfonated tryptophol; TRP-ME, tryptophan methyl ester; TRP, tryptophan; KYN, kynurenine; IBA, indole butyric acid; MEL, melatonin.

Saccharomyces cerevisiae strain. However, such exploratory steps and experimental designs like this are crucial in order to have highly controlled processes, easily replicated by excluding the must variability, and manage to monitor each yeast metabolism separately.

The PCA graph of Figure 4A shows the loading bi plot including both samples and compounds and summarizes the behavior of the metabolic profile between intra- and extracellular metabolism and the different time points. In fact, component 1 separated the intracellular samples located on the left from the extracellular samples on the right. On the other hand, component 2 separated the extracellular samples by days, where the second day samples were located on the upper right side of the PCA and the fifth and 15th day samples were

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located on the right-hand bottom of the plot. As for the heat-map of Supporting Information Figure S8, here, too, the intracellular sample metabolic profile was characterized by TYL and TOL, IAA-EE, TYR-EE, PHE, and NIC. The grouping of the intracellular samples in one dense cluster, with respect to the extracellular samples, was further proof of the intracellular metabolism plasticity and of the ability of the yeast to maintain a tightly regulated composition in a drastically changing environment.

The PCA biplot of Figure 4B provided a complementary visualization of the data with regard to Figure 2B, summarizing the extracellular behavior of samples and metabolites. This PCA plot is not a zoom of the Figure 4A PCA plot, since only the data of the extra-cellular samples were used to produce it. The first component separated the sampling points (2, 5, and 15 days), while the second component separated Td from the two *S. cerevisiae* yeasts. The combination of the various data offered the possibility to put forward several remarks as far as the yeast metabolism and some chemical reactions are concerned. The amino acids TRP and PHE, together with the "kind of amino acid" KYN,[3839] were consumed in order to produce several metabolites belonging to the metabolic pathways shown in Figure 1. Through the Ehrlich pathway, TRP produced intracellularly the quorum-sensing TOL, which was released in the extracellular environment. However, TOL was not accumulated but reacted with SO_2 and delivered TOL-SO_3H. Next to this TRP mechanism, we could also speculate that IAA was first produced in the cell, then released in the media, then later chemically sulfonated and finally broken down to 2AA, even though the presence of 2AA in intracellular samples indicates a possible direct production of yeast metabolism. Indeed, 2AA can also be enzymatically synthesized from ANT, through the KYN pathway of TRP (Figure 1).[11] 2AA is directly associated with the wine fault called "untypical ageing off-flavor,"[36] and as far as we know this is the first time that it was detected as an intracellular metabolite. Further research in 2AA intracellular biosynthesis should be of great importance for wine production, especially for yeast choice. By taking into consideration that the known chemical production of 2AA passes through the sulfonation of IAA, and that IAA and TOL have the same indolic skeleton, we could postulate that TOL-SO_3H chemical breakdown could also deliver 2AA or other similar metabolites. TOL-SO_3H and 2AA clustered together with other final products in both the Figure 2B heat-map and Figure 4B PCA plot, whereas mainly TOL and IAA and secondly KYN were characterized better in the first day fermentation samples. According to the manufacturer's specifications, QA and Td produce low SO_2 quantities (3−4 mg L^{-1}) and RF produces medium quantities (8−20 mg L^{-1}). For the extracellular samples, our results could be explained by these specifications, since RF demonstrated the highest concentration in TOL-SO_3H (Figure 2B) compared to the other two yeasts. As far as the intracellular samples are concerned, the high amounts of TOL on the fifth day of Td fermentations could be the reason for the high amount of TOL-SO_3H quantified in the same samples (Figure 2A).

Figure 3 shows that, while during fermentation (and/or postfermentation) the concentration of N-TYR-EE and N-TRP-EE decreased, the concentration of TYR-EE and TRP-EE meanwhile increased. This could be an indication that the ethyl esters of TRP and TYR did not occur by the esterification of the free amino acids, but through the deacetylation of the N-acetyl ethyl esters of the amino acid. In addition, the process of deacetylation of N-TYR-EE may occur inside the cell, since TYR-EE was measured in intracellular samples. Such a biosynthetic path seems valid only for the two *Saccharomyces cerevisiae* strains where also the extracellular TRP levels were much lower (Figures 2 and 3). N-TYR-EE participates in the regulation of the TRP synthesis in yeasts, as a tryptophan synthase inhibitor,[40,41] and both PCA (Figure 4B) and hierarchical cluster analysis (Figure 2B) grouped N-TYR-EE and N-TRP-EE together with the quorum sensing metabolites TYL and TOL. On the other hand, the deacetylated TYR-EE and TRP-EE clustered together with other final products (i.e., TOL-SO_3H and 2AA), where they are in higher concentration as measured at the last sampling point (Figure 2). Generally, metabolite sulfonation and deacetylation are mechanisms of deactivation and/or detoxification.[42,43] This could be an indication that N-TYR-EE and N-TRP-EE play a strategic role in the yeast mechanism, machinery, and regulation and thus influence the fermented food quality. However, further experiments are necessary to validate such a hypothesis.

The clustering of NIC and PHE with other final products of the yeast metabolism possibly attested to a *de novo* synthesis (Figures 1, 2B, and 4B).[44] The confirmation of such a hypothesis was further enhanced by the intracellular behavior of NIC and PHE (Figure 4A).

Finally, MEL is located near the center, between the second and fifth day samples (Figure 4B). There are some hypotheses that could explain these findings: (i) MEL plays a role as growth signal, the production of this indole amine may be correlated with a yeast-growth phase.[37] (ii) There is a possibility that MEL binds to a specific protein that establishes an equilibrium between consumed and free MEL.[45] (iii) MEL is an intermediate metabolite, which is rapidly transformed through deacetylation, like N-TRP-EE and N-TYR-EE. Some of the deacetylation pathway metabolites were included in the method (5-methoxy tryptophan and 5-methoxyindole acetic acid), but their concentrations were below the quantification threshold. This possibility is the most likely due to this pathway being described in fish, mammals, and the pineal glands of both *Anolis carolinensis* and *Sceloporus jarrooi*,[43] in yeast metabolism.[18,46] However, we need to underline that the quantified concentrations of MEL were close to the LOQ.

In conclusion, this study focused on elucidating a comprehensive status of yeast metabolism by combining an intra- and extra-cellular metabolomic data set of tryptophan/phenylalanine/tyrosine metabolism and by using a fast and sensitive LC-MS analytical method able to measure 38 analytes in 13 min. In total, the concentrations of 26 metabolites were monitored (26 extra- and 20 intracellular) during the alcoholic fermentation of three winemaking yeast strains, two *Saccharomyces cerevisiae* and a non-*Saccharomyces* one, specifically *Torulaspora delbrueckii*. The extracellular extract appeared to be richer both in terms of concentration and number of metabolites, and the intracellular fluid was characterized by the predominance of the alcohols TOL and TYL and the amino acids PHE and NIC. Generally, during the fermentation, the amino acids PHE and TRP decreased in the intracellular compartment and increased in the extracellular medium. Such behavior is in agreement with previous studies reporting that inside cells vacuoles exist as reservoirs of amino acids, the contents having increased gradually in a logarithmic phase and decreased thereafter, as cells reached a stationary phase.[47] Generally, the catabolism of the nitrogenous compounds (i.e.,

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amino acids) by the yeasts is crucial, as it serves directly and indirectly for the biosynthetic process of the cell function.

To the best of our knowledge, this is the first time that 2AA and TOL-SO_3H have been detected and quantified in intracellular samples, which indicates that they could be produced by yeast. *Torulaspora delbrueckii* demonstrated a capacity to produce minor amounts of 2AA relative to the two *Saccharomyces cerevisiae* yeasts, which should be a positive characteristic.

Some findings indicated the deactivation/detoxification of metabolites like (a) the quorum sensing high alcohol TOL by sulfonation to TOL-SO_3H and (b) the regulators N-TYR-EE and N-TRP-EE by deacetylation to TYR-EE and TRP-EE. Other findings pointed out a release of PHE mainly due to a cellular lysis to *denovo* synthesis of the amino acid PHE. In addition, the activation of the KYN pathway was measured during the yeast fermentation, especially for the non-*Saccharomyces Torulaspora delbrueckii.*

These new facts could help us to better understand and monitor how yeasts may modify, enrich, and benefit the nutrition value and sensorial character of several foodstuffs. Further experiments are necessary to validate such results in grape must media and to explore the metabolomic dynamics in coinoculated alcoholic fermentations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b01844.

(Table S1) Standards; (Table S2) composition of synthetic must; (Table S3) calibration curve information; (Figures S1−S6) ANOVA analysis of QA/RF/Td intracellular/extracellular samples; (Figure S7) graphic representation of variability of reducing sugar consumption through sampling time of the six folds of the three strain of yeast studied; (Figure S8) clustered heatmap of the measured metabolites including both extracellular and intracellular samples of the three yeast strains (the *Saccharomyces cerevisiae strains* QA and RF, and the *Torulaspora delbrueckii* Td) at three time points of the fermentation process (2, 5, and 15 days); (Figures S9−S13) kinetics of selected compounds of the QA/RF/Td intracellular/extracellular samples (PDF)

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U.V., M.C.G.-P., A.M.T., and M.A.A.F. conceived and designed the experiments. M.A.A.F., P.A., and E.F.-C. performed the experiments. M.A.A.F. and P.A. analyzed the data. All authors interpreted the data and wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

(NIC) Nicotinamide; (OH-TRP) 5-OH-tryptophan; (CH3O-TRP) 5-CH3O-tryptophan; (KYN) kynurenine; (PHE) phenylalanine; (TYR) tyrosine; (IS) 3-nitrotyrosine (internal standard); (OH-Ph-Py) 4-OH-phenyl pyruvic ac; (OH-ANT) 3-OH-anthranilic acid; (TRP) tryptophan; (TYR-EE) tyrosine ethyl ester; (N-SER) N-acetyl serotonin; (TYL) tyrosol; (TOL-SO3H) tryptophol sulfonate; (KYNA) kynurenic ac; (OH-Ph-AA) 4-OH-phenyl acetic ac; (TRP-ME) tryptophan methyl ester; (Ph-Py) phenyl pyruvic acid; (IPy) indole pyruvic ac; (ANT) anthranilic ac; (TRP-EE) tryptophan ethyl ester; (Ph-LA) phenyl lactic acid; (ILA) 3-indole lactic acid; (N-TYR-EE) N-acetyl-L-tyrosine ethyl ester; (ICA) indole carboxaldehyde; (MEL) melatonin; (CH3O-IAA) 5-CH3O-indole acetic acid; (CH3-IND) methylindole; (TOL) tryptophol; (IAA) 3-indole acetic acid; (Ph-AA) phenyl acetic acid; (2AA) 2-aminoacetophenone; (IND) indole; (IPA) 3-indole propionic acid; (N-TRP-EE) N-acetyl tryptophan ethyl ester; (IBA) 3-indole butyric acid; (M-IAA) methyl-indole acetic acid; (CH3-IND) 3-CH3-indole; (IAA-EE) 3-indole acetic acid ethyl ester; (E-ICa) 3-ethyl-indole carboxylic acid; (QA) QA23 *Saccharomyces cerevisiae* strain; (RF) RED FRUIT *Saccharomyces cerevisiae* strain; (Td) *Torulaspora delbrueckii* strain; (SM) synthetic must; (PAN) primary amino nitrogen; (MRM) multiple reaction monitoring; (TQ) triple quadrupole; (SPE) solid phase extraction; (UHPLC-MS/MS) ultrahigh resolution liquid chromatography associated with mass spectrometry; (RSD) standard deviation; (MS) mass spectrometry analysis; (QC) quality control; (AOAC) Association of Official Analytical Chemists; (LOQ) limit of quantification; (LOD) limit of detection; (DO) optical density

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Title: Efficiency of three intracellular extraction methods in the determination of metabolites related to tryptophan and tyrosine in winemaking yeast's metabolism by LC-HRMS

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Efficiency of three intracellular extraction methods in the determination of metabolites related to tryptophan and tyrosine in winemaking yeast's metabolism by LC-HRMS

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ABSTRACT

Yeast nitrogen metabolism produces metabolites, whose origin in wines has scarcely been studied, with an important biological and organoleptic role. The present work focuses on comparing three intracellular extraction methods in order to elucidate efficiency of extraction while measuring the effect of temperature upon the integrity of the compounds related to the metabolism of tryptophan and tyrosine by yeast.

Two UHPLC/HRMS methods to measure 16 metabolites were developed and validated. The validation provided optimum values of LOD ($7.4{\cdot}10^{-6}$ to 0.1 μg L^{-1}), of LOQ ($2{\cdot}10^{-5}$ to 0.02 μg L^{-1}) of precision (11–0.5% RSD) and repeatability (12–0.5% RSD). The removal of interfering molecules enabled matrix effects to be kept at low levels.

The results pointed out that the low-temperature methods were more effective, providing better precision for 16 metabolites. The high-temperature extraction method may yield false enhanced compounds concentrations since they originate in cell wall macromolecules degradation.

1. Introduction

Tryptophan (TRP) is the precursor of such compounds as Melatonin (MLT), Serotonin (5-HT), Kynurenic acid (KYNA), Kynurenine (KYN), all of which have important biological activities and health implications (Davis & Liu, 2015). MLT was originally known as indolamine pineal hormone in mammals, while simultaneously being widely-distributed in many organism of the animal and plant kindoms to 'in unicellular (Sprenger, Hardeland, Fuhrberg, & Han, 1999) and pluricellular, animal and plant kindoms (Hardeland, Pandi-Perumal, & Cardinali, 2006). It has recently been demonstrated that MLT could be synthesised by yeast during alcoholic fermentation (Rodriguez-Naranjo, Torija, Mas, Cantos-Villar, & Garcia-Parrilla, 2012). There are other TRP-related molecules of interest, such as KYN and KYNA, involved in the kynurenine pathway (Hardeland, Tan, & Reiter, 2009). This pathway accounts for the catabolism of ~99% of the TRP ingested, which is used by mammals for protein synthesis, but could also be involved in nicotinamide adenine dinucleotide synthesis. This catabolic route of TRP is also shared by yeast (Kucharczyk, Zagulski, Rytka, & Herbert, 1998; Panozzo et al., 2002). Both KYN and KYNA are connected to indoleamines and TRP by pyrrole ring cleavage reactions, enzymatically catalysed by indole-amine 2, 3-dioxigenase (IDO). These reactions were first demonstrated for tryptamine and 5-HT and later also for MLT (Hardeland et al., 2009).

Compounds such as Tryptophol (TOL), Tryptophan ethyl ester (TRP-EE), N-acetyl tryptophan ethyl ester and Indole acetic acid (IAA) are related to TRP yeast metabolism and have been detected in wines (Fernández-Cruz, Álvarez-Fernández, Valero, Troncoso, & García-Parrilla, 2017). TOL, a higher alcohol produced by the Ehrlich pathway, the most relevant TRP metabolism pathway in yeast, has a signaling

Abbreviations: 5MIAA, 5-methoxy indole acetic acid; 5HIAA, 5-hydroxy indole acetic acid; 5HTP, 5-hydroxy tryptophan; IAA, 3-indole acetic acid; KYNA, kynurenic acid; KYN, kynurenine; TRP, tryptophan; TEE, tryptophan ethyl ester; TYR, tyrosine; MLT, melatonin; N-TEE, N-acetyl tryptophan ethyl ester; IS, 3-nitrotyrosine; 5-HT, serotonine; TOL, tryptophol; HT, hydroxytryptophol; TYL, tyrosol; HRMS, High Resolution Mass Spectrometry; SM, synthetic must; H, boiling ethanol extraction; LT, freezing temperature extraction; MT, refrigeration temperature extraction; SPE, solid phase extraction; MFS, Matrix Free Sample response; PES, Post-Extracted spiked Sample response; ME, matrix effect

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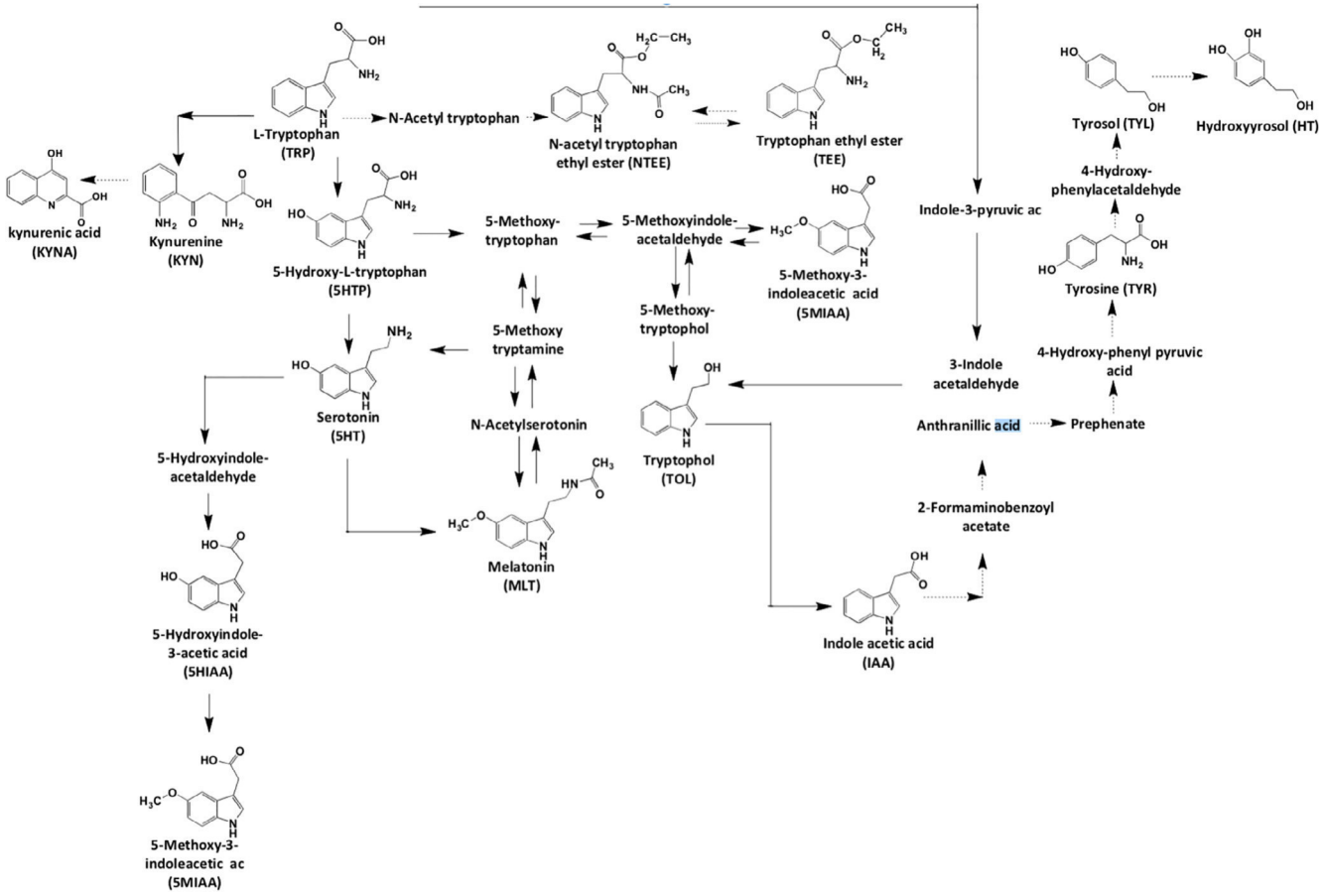

Fig. 1. Compounds in the analytical method and their relationship to MLT/L-TRP and TYR metabolism. Compounds included in the quantitate method appear with structural formula.

role, as does its counterpart Tyrosol (TYL), produced from Tyrosine (TYR). Both TOL and TYL are compounds capable of transmitting information concerning population density and the amount of nitrogen available (Martín-Cabrejas, Aguilera, Benítez, & Reiter, 2017). In addition, Hydroxytyrosol (HT), is a product of TYL hydroxylation with important reported health effects (European Food Safety Authority (EFSA) Panel on Dietetic Products Nutrition and Allergies (NDA), 2011). *Saccharomyces cerevisiae* has recently been demonstrated to produce HT as a TYL derivative (Álvarez-Fernández, Fernández-Cruz, Cantos-Villar, Troncoso, & García-Parrilla, 2018). Fig. 1 displays possible pathways involving aromatic amino acid derivatives described in yeast (KEGG: Encyclopaedia of Genes and Genomes (Kanehisa & Bork, 2003).

MLT has been reported to be an amphiphilic molecule present in low concentrations in many of the food samples in which it has been studied. In fruits and plants, for instance, it was quantified at concentrations of ng g^{-1}; in beverages in amounts of μg g^{-1} and pg mL^{-1} (Iriti & Varoni, 2012). As a result, in order to analyse such low concentrations, techniques such as ELISA and HRMS (High Resolution Mass Spectrometry) have been used (Fernández-Cruz et al., 2017; Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos-Villar, & Garcia-Parrilla, 2011). A sample treatment involving a concentration step is, however, generally required.

Several extraction methods for enhancing analytical performance have been reported in the literature: extractions with 10% sodium carbonate in bananas (Dubbels et al., 1995) or extractions with methanol and C_{18} cartridges used in many fruit substrates (Johns, Johns, Porasuphatana, Plaimee, & Sae-Teaw, 2013) and in wine (Fernández-Cruz et al., 2017; Rodriguez-Naranjo et al., 2011). In addition, immune affinity purification was used in biological samples. This system consists of sorbent-bound specific antibodies for MLT extraction with optimal results. However, as the antibodies are specific, they will only work for this compound (Van den Ouweland & Kema, 2012). The success of the intracellular extraction method depends directly on the microorganism's cell envelope structure and on the chemical nature of the target metabolites. Many intracellular extraction methods have been proposed. The most popular, boiling ethanol, has been in use for many years. This method is useful for extracting water-soluble intracellular metabolites such as ɤ-aminobutyric acid, aspartic acid, glycine, and lactic acid. It does, however, have a poor recovery yield for metabolites such as phosphorylated metabolites, nucleotides and tricarboxylic acids (Pinu, Villas-Boas, & Aggio, 2017). The most widely-used intracellular extraction methods were compared for detecting various yeast metabolites such as amino acids, sugars, sugar phosphates, sugar alcohols and peptides (Villas-Boas, Jesper Højer-Pedersen, Mats Akesson, 2005). Following a comparative test of four different methods (boiling ethanol, freeze–thaw cycles, pure methanol and pure methanol coupled to sonication) with metabolomic analysis, Duportet concluded that one of the principal factors influencing intracellular extraction was the differential composition of metabolites and that it was not possible to eliminate the influence of the biological matrix (Duportet, Aggio, Carneiro, & Villas-Bôas, 2012). A method combining freezing/thawing cycles with ultrasound would, therefore, be worth trying, since the mechanical methods could have a great potential for enhancing intracellular metabolites extraction, particularly non-polar compounds (Canelas et al., 2009).

In addition, and due to the fact that the extraction process entailed the breaking of cell walls, it would not be surprising that the resulting extract contained the main compounds of the cell walls, phospholipids (PLs) and proteins. As they are known to exert significant interference

on the analysis by reverse phase chromatographic methods coupled with mass spectrometry, it is advisable to remove them. Therefore, PLs and proteins removal prior to analysis could be the most effective way of reducing the matrix effects from the endogenous cellular extracts (Carmical & Brown, 2016). By these means, introducing a sample clean-up filtration would be recommended (Reinholds, Pugajeva, Perkons, & Bartkevics, 2016). By coating silica with zirconia, this filtration has the advantage of high sorbent selectivity. First, the organic solvent precipitates the proteins and then the PLs are retained through interactions between the phosphate moiety of phospholipids (Lewis base) and zirconium oxide (Lewis acid) coated on the silica surface. Purifying phospholipids and proteins precipitation as a clean-up method instead of SPE (solid phase extraction), avoids the loss of metabolites traditionally associated with conventional SPE (Reinholds et al., 2016).

The purpose of this work is to compare the efficiency of three procedures for the intracellular extraction of TRP- and TYR-related compounds from yeast metabolism in order to establish the optimal conditions to obtain results that faithfully reflect reality. The selected methods were the classic boiling ethanol and two methods involving freeze-thaw cycles associated with ultrasound at two different temperatures of centrifugation in order to verify the effect of temperature upon extraction efficiency. The determinations were performed using two validated UHPLC-MS methods capable of quantifying 15 compounds and internal standard, one in positive and other in negative ionization mode. These determinations were performed after a sample preparation that implied the protein and PLs removal.

As they assess these metabolites more accurately with the aim of exploring and improving knowledge of the metabolic pathway, the results of this work could be useful to researchers in the field of microbial metabolomics.

2. Materials and methods

2.1. Reagents and materials

The chemicals used in this work were of the highest grade of purity and purchased from Sigma-Aldrich (Madrid, Spain), Chengdu Biopurify Phytochemicals Ltd. (Wenjiang Zone, Chengdu-Sichuan, China), Merck (Darmstadt, Germany) and VWR International Eurolab S.L. (Barcelona, Spain), unless otherwise stated (Supplementary Electronic Material Table S1).

2.2. Alcoholic fermentation procedure

The alcoholic fermentations were performed in synthetic must (SM), prepared according to Riou, Nicaud, Barre, and Gaillardin (1997), but with slight modifications (Supplementary Electronic Material Table S2 shows the must composition in detail). Fructose and glucose were added as a carbon source (100 g L^{-1} each). The other compounds were unmodified: amino acids (purity ≥ 99%), vitamins and anaerobic factors (Riou et al., 1997). The SM was sterilised with bottle-top vacuum filters (Nalgene PES membrane) after adjusting the pH to 3.5 with NaOH. Each Erlenmeyer flask was filled with 750 mL of SM. Inoculation was performed with 10^6 cell mL^{-1} and the Erlenmeyer flasks were then capped with tops equipped with a capillary for releasing carbon dioxide. The fermentations were due to the action of *Saccharomyces cerevisiae* QA23 strains of yeast in six replicates in order to consider biological variability. Lallemand (Blagnac, France) supplied the *Saccharomyces cerevisiae* QA23 strains. The flasks were weighed before and after the samples were taken in order to monitor fermentation.

2.3. Intracellular extraction methods

2.3.1. Cold glycerol quenching procedure

On day two of fermentation, a sample volume containing 10^9 cells (previously counted with an automated Invitrogen Countess Cells counter) was taken from each Erlenmeyer flask. The samples were immediately centrifuged at 4500 rpm for 3 min at 4 °C to separate the cells from extracellular media. Cells were pelleted twice by centrifugation at 4500 rpm for 3 min at 4 °C with distilled water to wash them in order to prevent contaminations from the extracellular metabolites.

The washed cells were subjected to a cold glycerol saline quenching procedure in order to stop enzymatic activity, maintaining the *in vivo* metabolite concentrations at a constant level. The procedure used was that reported by Villas-Bôas et al. (Villas-Bôas & Bruheim, 2007).

The pellet was re-suspended with 1 mL of distilled water and transferred into a pre-cooled centrifuge tube containing 4 mL of cold-quenching solution [3:2 (vol/vol) glycerol: saline solution] maintained at −23 °C in a refrigerated bath using ethylene glycol as cryo fluid. The solution was homogenised and returned to the cold bath for 5 min. The treated samples were centrifuged at 36,086 *g* for 20 min at −20 °C (Sorvall LYNK 6000, Thermo Fisher Scientific, Waltham, MA USA). The supernatant was removed and the pellet was then re-treated with 2.5 mL of cold washing solution [1:1 (vol/vol) glycerol/saline solution] maintained at −20 °C. They were then centrifuged under the same conditions. The supernatant was then discarded and the pellet was stored at −80 °C until extraction. Fig. 2A shows a general vision of the whole procedure (Villas-Boas, 2007).

2.3.2. Boiling ethanol extraction (H)

The boiling ethanol extraction procedure was performed following the method reported by Gonzalez et al. (Gonzalez, François, & Renaud, 1997). Three mL of N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) ethanol boiling solution (absolute ethanol buffered with 70 mM HEPES) and a volume of methanol internal standard (3-nitrotyrosine) solution to obtain a final concentration of 0.08 μM were added to the cell pellet, and incubated at 80 °C for 3 min. After cooling in an ice bath for 3 min, the solution was evaporated until dry at 34 °C, and 2000 rpm with a vacuum concentrator (Hyper VAC-LITE, GYOZEN, Korea). The residue was re-suspended to a final volume of 3 mL with ultrapure Milli-Q water and was then centrifuged for 10 min at 15,543*g* and 4 °C. The supernatant was collected and stored at −80 °C, until clean up. Fig. 2A shows a general vision of the whole procedure (Villas-Boas, 2007).

2.3.3. Freezing temperature extraction (LT)

Intracellular extraction was performed following the method reported by Smart, Aggio, Van Houtte, and Villas-Bôas (2010) with some modifications (Smart et al., 2010). 2.5 mL of cold methanol-water solution (50% (v/v), −30 °C) was added to the cell pellet, together with a volume of IS methanolic solution to obtain a final concentration of 0.08 μM. Afterwards, the sample was mixed for 1 min with vortex and then frozen at −80 °C. The samples were subjected to two freeze-thaw cycles (frozen at −80 °C for 30 min and then thawed in an ice bath for 4 min). After the last cycle they were subjected to sonication for 1 min in an ice bath using an ultrasonic liquid processor (Sonicator Sonoplus HD 2070, Bandelin electronic GmbH & Co. KG, Berlin, Germany). The samples were then centrifuged at 36,086*g* for 20 min at −20 °C. Another aliquot of 2.5 mL of cold methanol-water was added to the pellet and the same process, starting at the freeze-thaw cycles was repeated; the supernatants were collected, pooled and subsequently stored at −80 °C until clean up. The aim of this kind of extraction process is to render the cell walls more permeable to organic solvents and thus enable intracellular metabolites to be released, while preserving their chemical integrity (Smart et al., 2010). Fig. 2A shows a general vision of the whole procedure (Villas-Boas, 2007).

2.3.4. Refrigeration temperature extraction (MT)

This method follows the same procedure as the above, but with a change in the centrifugation temperature. After two cycles of freezing-thawing and one min of ultrasound, the samples were centrifuged at

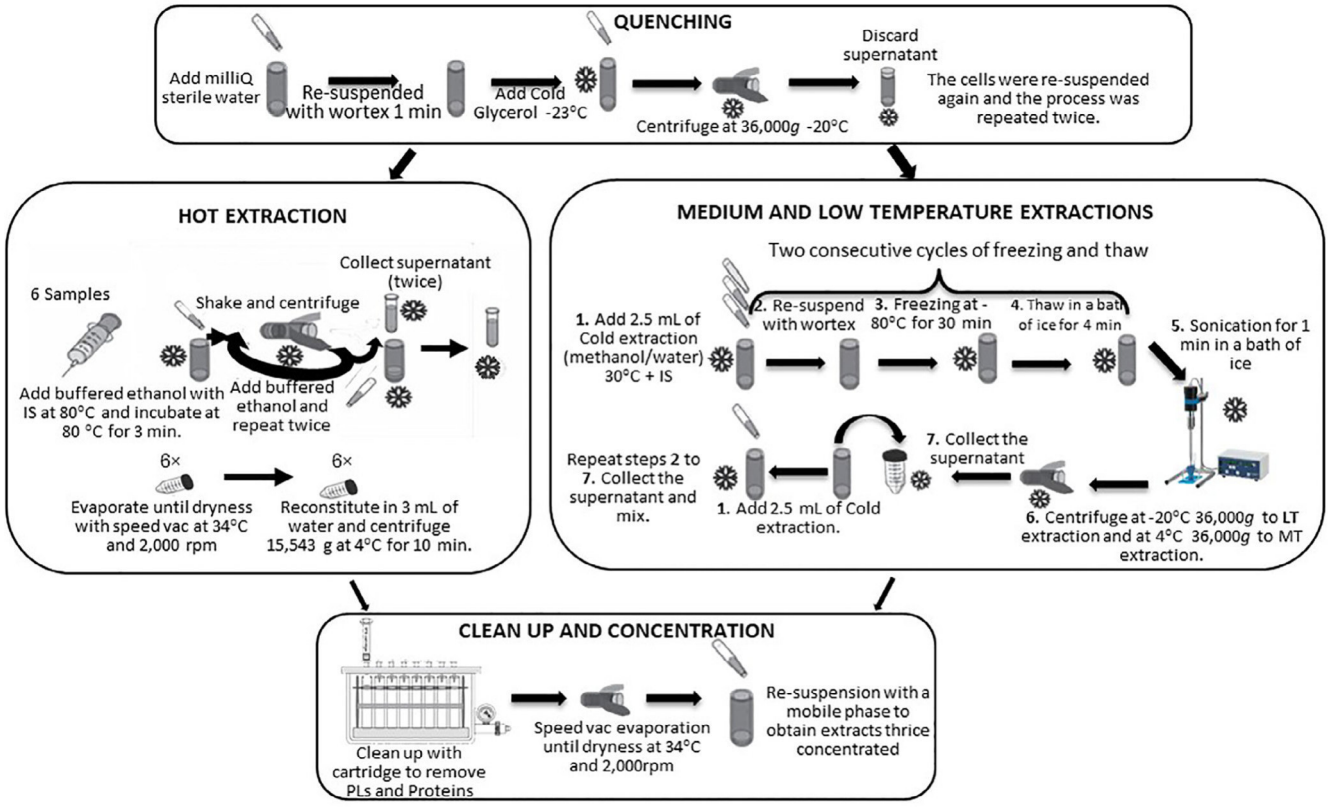

Fig. 2. Box plot of variability of 3-nitrotyrosine (IS) in the three extraction methods studied: Boiling ethanol (H), Freezing-thaw method at −20 °C (LT) and Freezing-thaw method at 4 °C (MT).

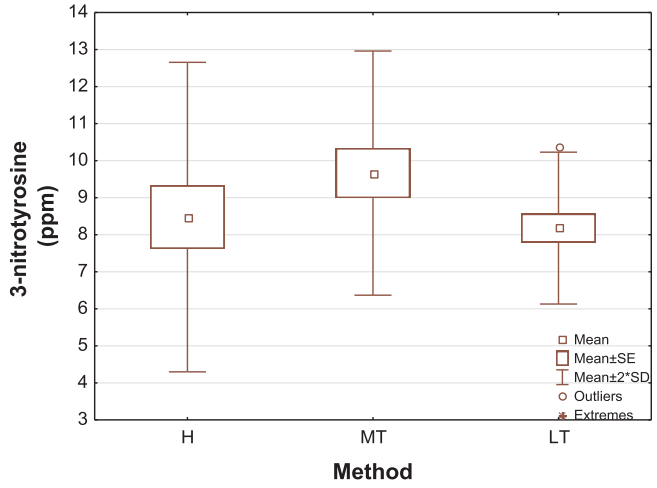

Fig. 2. (*continued*)

36,086*g* for 20 min at 4 °C. Subsequently, the same procedure as that described above was performed. Finally, the extracts were stored at −80 °C until clean up. Fig. 2A shows a general vision of the whole procedure (Villas-Boas, 2007).

2.3.5. Clean-up and concentration procedure

After every treatment described above, the samples were cleaned up using 1 mL zirconia-coated Phree™ cartridge in order to avoid the presence of phospholipids and proteins due to cell wall fragmentation (Carmical & Brown, 2016; Van Der Rest et al., 1995). The procedure was performed in four steps, following the manufacturer's protocol. When the filtration process had finished, the samples were speed vac evaporated to dryness at 34 °C and at 2000 rpm, to be later re-suspended with a mobile phase in order to obtain thrice-concentrated extracts. Fig. 2A shows all of the sample preparation steps in parallel (Villas-Boas, 2007).

2.4. UHPLC/HRMS analysis

Analysis was performed in an UHPLC Dionex Ultimate 3000 system (Thermo Fisher Scientific-Bremen, Germany) consisting of a binary pump, cooling autosampler, online vacuum degasser, and column oven, coupled to a hybrid Quadrupole-Orbitrap Q Exactive Mass Spectrometer (Thermo Fisher Scientific-Bremen, Germany). The analysis column used was a Zorbax RRHDSB-C18 (2.1 × 5 mm, 1.8 µm particle size) with a guard column (2.1×, 1.8 µm particle size), both purchased from Agilent Technologies (Waldbronn, Germany). The separation conditions are: column temperature set to 40 °C with a flow of 0.5 mL min^{-1}, and an injection volume of 5 µL. The chromatographic conditions for analysis in positive ionization mode consisted of two phases (A) aqueous formic acid solution 0.1%, and (B) methanolic formic acid solution 0.1%. The gradient was programmed as follows: 95% A (0 − 1 min), 0% A (1–12 min), 95% A (12.1–15 min). Electrospray positive ionisation mode was applied with the source parameters set as follows: capillary voltage at 0.1 V; sheath gas flow rate 60.13; aux gas flow rate 24.85 and sweep gas flow rate 0.07, all in arbitrary units. To quantify in positive ionization mode, a full scan was used, with the most abundant transition as the confirming ion. To quantify compounds similar to 5-HT, the most important product ion was used, due to the fact that in this type of compound the protonated molecule may be too unstable and impossible to detect. For analysis, a window of 30 s and lens of 50 was used. Table 1 shows MS parameters, retention time and standard deviation values (% RSD) as a measure of the intra- and inter-day variation of each metabolite measured, based on quality control samples.

In the negative ionization mode analysis, phase (A) was aqueous

Table 1
UHPLC–HRMS conditions for quantifying and identifying compounds and internal standards (IS).

Compound (acronyms)	RT (min)	ESI mode	Quantifier	Confirming ion	LOQ ($\mu g\ L^{-1}$)	LOD ($\mu g\ L^{-1}$)	Intra day (% RSD)	Inter day (% RSD)
tyrosine (TYR)	0.84	+	182.0811	182.0811 → 165.0546	0.05	0.02	0.92	0.62
serotonine (5-HT)	0.87	+	160.0756	177.1022 → 160.0756	0.02	0.08	5.08	0.84
5-hydroxy tryptophan (5HTP)	1.26	+	221.0920	221.0920 → 204.0655	0.03	0.01	5.03	9.60
kynurenine (KYN)	1.50	+	209.0921	209.0920 → 192.0655	0.04	0.01	1.60	2.04
hydroxytyrosol (HT)	2.03	−	153.0629 → 123.0440	153.0629 → 109.2840	0.10	0.03	0.86	2.25
3-nitrotyrosine (IS)	2.42	+	227.0662	227.0662 → 181.0604	0.02	0.01	0.89	0.86
tyrosol (TYL)	2.85	−	137.0680 → 119.0502	137.0680 → 108.0217	0.39	0.13	1.55	1.58
tryptophan (TRP)	3.00	+	205.0971	205.0971 → 188.0706	0.12	0.04	1.58	1.34
5-hydroxy indole acetic acid (5HIAA)	3.83	+	192.0655	192.0655 → 146.0600	0.40	0.13	1.15	1.50
tryptophan ethyl ester (TEE)	4.84	+	233.1284	233.1284 → 216.1019	0.16	0.05	2.56	0.97
kynurenic acid (KYNA)	4.91	+	190.0498	190.0498 → 162.0549	2.20E-05	7.40E-06	12.49	11.56
5-methoxy indole acetic acid (5MIAA)	5.70	+	206.0811	206.0811 → 160.0754	0.07	0.02	0.48	0.94
3-indole acetic acid (IAA)	5.76	+	176.0706	176.0706 → 130.0651	0.08	0.02	3.87	9.40
tryptophol (TOL)	5.76	+	162.0913	162.0913 → 144.0807	0.10	0.03	0.55	0.92
melatonin (MLT)	5.79	+	233.1284	233.1284 → 174.0913	0.11	0.04	1.13	0.50
N-acetyl tryptophan ethyl ester (N-TEE)	6.91	+	275.1390	275.1390 → 201.1024	0.08	0.03	1.88	1.90

Three technical replicates were analised to calculate LOD and LOQ, for the others parameters two technical replicates were analysed.

acetic solution 0.2% and (B) acetonitrile, while the chromatographic gradient was set as follows: 95% (A) (0–1 min), 0% (A) (1–8.5 min), 95% (A) (8.6–10 min). The electrospray negative ionisation mode was performed with the source parameters set as follows: capillary voltage at 0.1 V; sheath gas flow rate 60.15; aux gas flow rate 25.03 and sweep gas flow rate 0.04, all in arbitrary unit. The analysis was performed, considering both qualifier and quantifier ions in order to perform data-dependent scanning as the quantifying method. All samples were analysed in duplicate and in randomised sequences. Table 1 shows the MS parameters.

2.5. Statistical analysis

Statistical analyses were performed using MetaboAnalyst 3.0 (Xia & Wishart, 2011) and Statistica software (Migut, Jakubowski, & Stout, 2014). Statistical significance ($p < 0.05$) between groups was tested by ANOVA analysis of variance and Tukey's HSD (honest significant difference) test (Fig. S1 Supplementary Material). The heat-map was produced as a multivariate statistical analysis where the data were auto-scaled and missing values were replaced by very low values. Given these circumstances, MetaboAnalyst 3.0 software was used for the PCA plots.

3. Results and discussion

3.1. Validation method and matrix effects

The validation procedure was performed following the AOAC guidelines (AOAC, 1998). The parameters measured were as follows: detection and quantification limits (LOD, LOQ), precision, linearity, recovery, matrix effect and extraction procedure efficiency via the internal standard (3-nitrotyrosine) (Fig. 2B). Table 1 shows the LOD and LOQ calculated for 15 compounds and the internal standard, based on the standard deviation of the response (σ) and the slope of the calibration curve. The linearity data are shown in Table S3 (Supplementary Electronic Material). The precision was measured using QC (quality control) prepared as a pool of all samples. In order to measure instrumentational stability and accuracy, QC samples were injected every four injections in duplicate, in positive and negative ionization mode. The measure of precision was expressed as intraday and interday (% RSD) data (Table 1) (Ehrhardt, Arapitsas, Stefanini, Flick, & Mattivi, 2014). In addition, all values were highly satisfactory and ranged from 0.48 to 12.49, matching the AOAC requirements with regard to their concentration levels (Gustavo González & Ángeles Herrador, 2007).

The matrix effect was examined by comparing the MS/MS response (peak areas) of an analyte at two concentrations spiked post-extraction into a sample extract (PES) to the MS/MS response of the same analyte at the same two concentrations in the neat mobile phase solution (MFS). Following the sample preparation procedure, the samples (PES and MFS) were thrice-concentrated and re-suspended with methanol 0.1% formic acid (FA) for positive analysis. For the negative analysis, they were re-suspended with acetonitrile with 5% acetic acid. A previous clean-up sample was used as a blank. The matrix effect was calculated using the following equation:

$$\%\ \text{Matrix Effect}(\%ME) = ((PES/MFS) - 1) \times 100$$

where MFS is the Matrix Free Sample response and PES is the Post-Extracted spiked Sample response.

This value is also known as absolute ME. A suppression or enhancement is considered acceptable if the matrix effect ranges from −20% to 20%. Percentage values of ME higher than 20% or lower than −20% indicate a strong matrix effect (Álvarez & Madrid, 2016). Table 2 shows the recovery and matrix effect values for every analyte in the method.

3.2. Comparison of extraction efficiency

As previously described in materials and methods, all the extraction procedures consisted of several steps, which may involve substantial metabolite losses, before the sample was injected. In order to assess this technical variability, a known concentration of IS (3-nitrotyrosine) was added at the beginning of the sample preparation process. 3-nitrotyrosine was selected as IS due to its structural similarities, its stability characteristics in the UHPLC-MS method, its low cost and the fact that before it was added, it was not present in the samples. Indeed, the recovery values RE (%) obtained after the intracellular extraction process with the three methods were 46% (± 2.08) in boiling ethanol extraction, 53% (± 1.64) in MT and 45% (± 1.02) in LT extraction. Fig. 2 shows the variability plot of 3-nitrotyrosine comparing the three methods. The data were analysed by ANOVA/Tukey's HSD test and no significant differences were detected between the three methods. Moreover, taking into account that the role of the IS mimics the compounds analysed as closely as possible, and the fact that the procedure could affect all the analytes in the same proportion, correction with IS enables this technical variation to be corrected. As a result and due to the abundance of IS (Smart et al., 2010), the analyte concentrations in samples have to be normalised.

The efficiency of the extraction methods was defined as a method's

Table 2
Matrix effects parameters. ME (%) entity means that ME (%) > 100 = there is a signal enhancement and if ME (%) < 100 = a signal suppression (Hoenicke et al., 2001).

Metabolite (acronims)	Conc ($\mu g\ L^{-1}$)	Recovery (%)	Matrix effect
5-methoxy indole acetic acid (5MIAA)	247	89.22	−10.77
5-hydroxy indole acetic acid (5HIAA)	247	126.95	24.88
	4	104.62	6.99
5-hydroxy tryptophan (5HTP)	247	100.77	1.11
	4	132.81	15.56
3-indole acetic acid (IAA)	247	92.42	11.87
	1	120.64	15.46
kynurenic acid (KYNA)	247	110.06	−1.75
	4	89.69	−10.3
kynurenine (KYN)	247	100.08	0.67
	4	92.78	−7.21
tryptophan (TRP)	247	99.41	−0.59
	4	136.38	36.38
tryptophan ethyl ester (TEE)	247	93.91	−6.08
	4	92.90	−7.1
tyrosine (TYR)	247	166.88	−7.23
	1	61.25	−22.14
melatonin (MLT)	247	89.32	−10.68
	4	117.86	17.86
N-acetyl tryptophan ethyl ester (N-TEE)	247	82.69	−15.08
	1	82.65	−19.07
3-nitrotyrosine (IS)	247	95.40	−4.59
serotonine (5-HT)	247	97.34	−0.75
	1	64.26	−21.03
tryptophol (TOL)	247	101.21	1.54
hydroxytryptophol (HT)	60	129.84	−3.61
	0.5	106.93	9.49
tyrosol (TYL)	200	105.62	16.03
	0.5	120.32	−0.22

Three technical replicates were analised of each concentration.

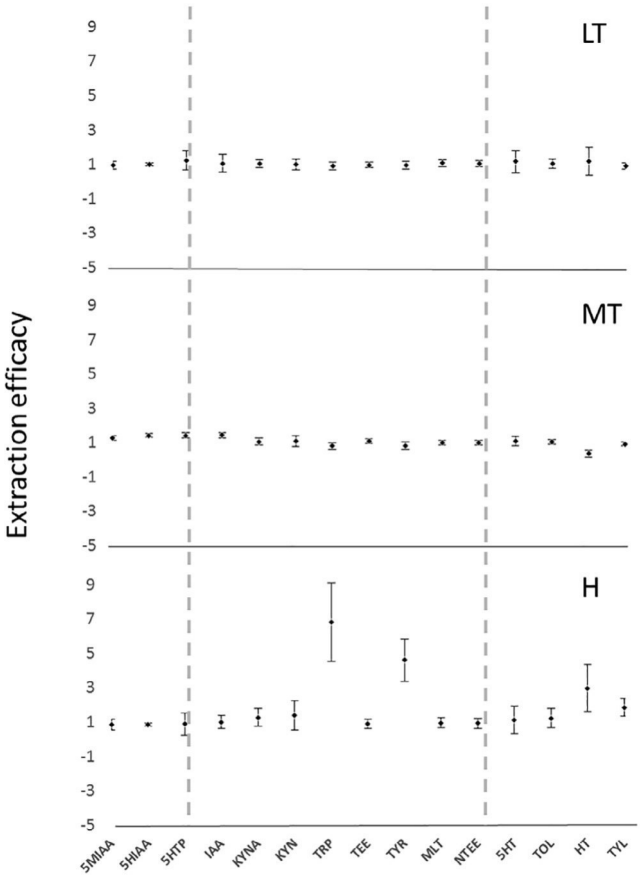

Fig. 3. Comparative extraction efficiencies for 15 metabolites and for each extraction method. Data are means and standard deviations of normalised data by median of six replicates (see Results and Discussion for definitions of efficiency). Dashed grey lines are for guidance.

ability to release analytes from the cells (Canelas et al., 2009). In certain situations, the extraction efficiency could be wrongly assessed. Such examples are when the substrate is converted into intermediates or when macromolecules are subsequently broken down to produce smaller molecules. In the these situations, such molecules have disappeared and would be impossible to quantify. This misleading situation can be counteracted by the efficiency measurement, calculated against the median of each analyte's concentrations. The efficiency factor was defined as:

efficiency vs median = x/median

These efficiency factor values thus calculated will depend upon the extraction method used (Canelas et al., 2009). Fig. 3 shows the extraction plots for the normalised efficiencies (efficiency factors) of 15 compounds included in the work. The efficiency profiles plotted as efficiency factors against metabolites for all extraction methods were very similar, with values near 1 for most metabolites. The H extraction profile showed a clear discrimination between two amino acids [L-TRP (> 2 standard deviation) and TYR (> 1 standard deviation)] compared with all remaining metabolites. There is a possibility that the temperature of the boiling ethanol may break the proteins down, releasing amino acids and increasing the measured concentrations, thus yielding incorrect efficiency values. On the other hand, the two phenolic compounds TYL (> 0.5 standard deviation) and HT (> 1 standard deviation) presented higher efficiency than the other compounds and other methods, but lower efficiency than the two aromatic amino acids.

As can be observed, the two low-temperature extraction methods, LT and MT presented a similar profile. The LT extractions profile was noticeably the flatter (Fig. 3) indicating excellent reproducibility, with the advantage of preserving the integrity of the metabolites. This avoids false positive results and provides an exact vision of the intracellular content. We reached a similar conclusion with regard to the PCA analysis (Fig. S2), where the samples corresponding to the low-temperature extraction methods were close to each other and distant from the hot extraction method samples.

Fig. 4 shows a heat map that provides an overview of the data set with hierarchical clustering. The overall results of the quantification of each metabolite were represented for every extraction and the six replications were expressed in $\mu g\ L^{-1}$. The first cluster, enclosing TRP, TYR, HT and TYL, all had higher concentrations in H extraction. The concentrations values measured in each method (H, MT, and LT) were as follows: 387 – 45 – 51 $\mu g\ L^{-1}$ for TRP; 966 – 170 – 198 $\mu g\ L^{-1}$ for TYR; 24 – 3 – 9 $\mu g\ L^{-1}$ for HT and finally 144 – 72 – 70 $\mu g\ L^{-1}$ for TYL. As can be observed, the differences between the H and LT methods were outstanding. Indeed, in the case of TRP, the factor was as high as eight. As stated earlier with regard to the high extraction efficiency, it seems plausible to suggest that the high levels were the result of decomposing macromolecules, such as proteins. This observation matched similar conclusions reported earlier by Canelas et al. (Canelas et al., 2009). These authors suggested that the hydrolysis of as little as 0.2% of the cell protein would be sufficient to explain the higher percentages of amino acids in extractions using hot water. It was conceivable that the high TYL and HT concentrations might have a similar explanation, reflecting the fact that they could be the product of macromolecule degradation. The exact biomass components generated by thermal hydrolysis are, however, still to be elucidated.

A second cluster contained KYN, KYNA, and TOL. These presented higher concentrations (163, 3 and 164 $\mu g\ L^{-1}$) in H extraction for the three compounds, respectively, but with a remarkable variability (101,

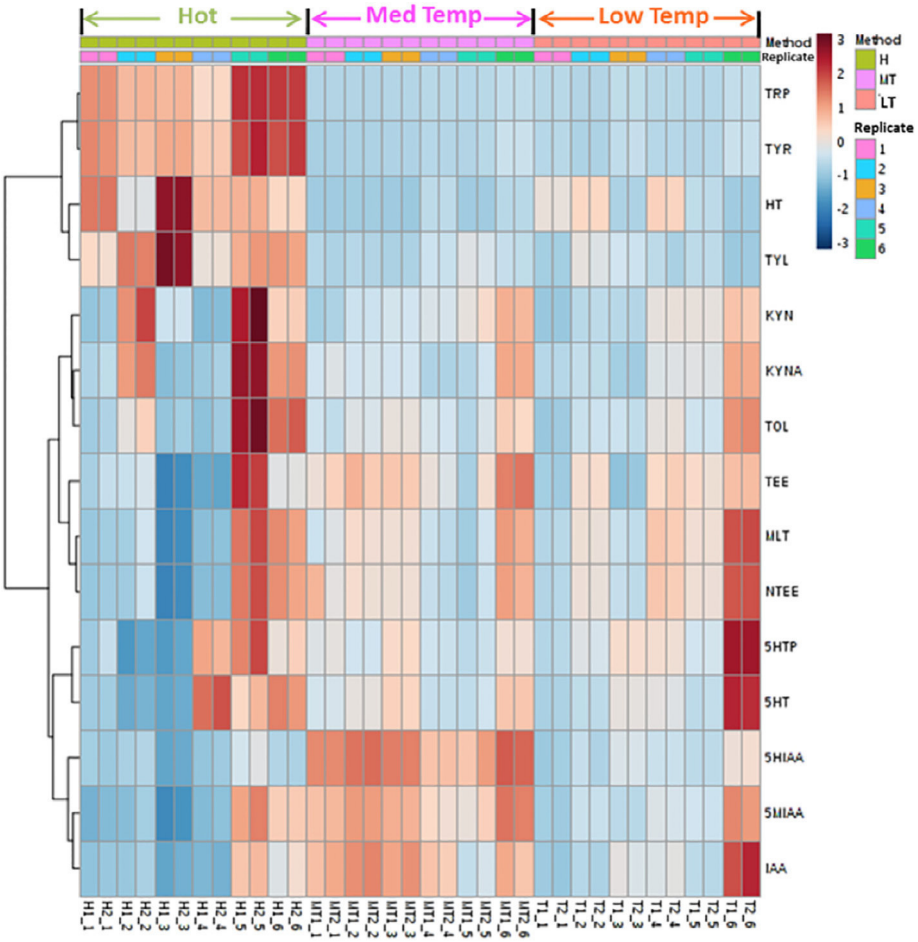

Fig. 4. Heat map, representing concentrations expressed in $\mu g\,L^{-1}$ related to the method; boiling ethanol (Hot), freezing thaw at 4 °C (Medium Temperature), freezing thaw at −20 °C (Low temperature).

1 and 75 deviations respectively). With the two low-temperature methods, KYN presented the same concentration and low deviation (0.59 for MT and 0.55 for LT). For KYNA 114 $\mu g\,L^{-1}$ ± 37 with the MT extraction and 127 $\mu g\,L^{-1}$ ± 36 with LT, and for TOL 140 $\mu g\,L^{-1}$ ± 35 with the MT and 140 $\mu g\,L^{-1}$ ± 17 with LT extraction.

The third cluster, comprising TEE, MLT and NTEE, had very similar results in the three extractions. The concentrations were 3 $\mu g\,L^{-1}$ on average for TEE, the same for the three methods, for MLT (27, 28 and 30 $\mu g\,L^{-1}$) and in case of NTEE 9 $\mu g\,L^{-1}$ with H extraction and 10 $\mu g\,L^{-1}$ with two low-temperature extraction methods. The values were closer to each other, but variability was better with low-temperature extraction than with H extraction, especially in MLT (8, 5 and 4 for each method respectively).

Finally, the last two clusters contained 5HTP, 5-HT, 5HIAA, 5MIAA and IAA, all of which had the highest concentrations (101, 3, 22, 358 and 14 $\mu g\,L^{-1}$, respectively) in MT extractions with better reproducibility (47, 1, 2, 40 and 3 deviation, respectively). With H, however, the concentrations obtained were 83, 3, 14, 243 and 8 $\mu g\,L^{-1}$ with large deviations of 55, 2, 1.5, 95 and 4, respectively, with the sole exception of 5-HT which had the same concentration in all cases, but better variability in MT extraction. The compounds included in these two clusters have indolic structures in common and exhibited lower concentrations in H than in MT extraction. This might be due to the high temperatures which may produce degradation, as reported earlier for IAA (Su et al., 2017). The low deviations with the two low-temperature methods indicate that, in contrast with Canelas et al. (Canelas et al., 2009), enzymatic activity stopped. This is probably due to the fact that cold glycerol quenching was more efficient than the pure methanol quenching at −40 °C method that they used (Canelas et al., 2008).

It is interesting to highlight that the MLT concentrations obtained with low -temperature methods, ranging from 28 to 30 $\mu g\,L^{-1}$, are significantly higher than other previously-reported values in extracellular media – even with an identical yeast strain (Fernández-Cruz, Álvarez-Fernández, Valero, Troncoso, & García-Parrilla, 2016; Fernández-Cruz et al., 2017). Moreover, the detection and quantification of MLT and 5-HT in the intracellular media was an advance in our knowledge of the metabolism of *S. cerevisiae*. Indeed, the changes introduced in the sample preparation and in the analytical method could help the research community, since there are few references related to producing these two compounds by yeast (Mas et al., 2014).

4. Conclusions

This study demonstrated how the extraction method temperature could affect the extraction efficiency of 15 tryptophan – and tyrosine-related metabolites by developing two validated and sensitive UHPLC/MS analytical methods.

The proposed sample preparation scheme was based on removing phospholipids and proteins in order to enable good values for matrix effects, indicating that the possible impact of ion enhancement or ion suppression were small and unable to produce distortive behaviour on the quantification process.

As far as we know, this was the first time melatonin was quantified in the intracellular media in normal conditions of growth at 48 h of fermentation, providing an exact concentration value. This fact stresses

that the *Saccharomyces cerevisiae* strain of yeast QA23 will definitely produce melatonin. Moreover, this work showed that low-temperature intracellular extraction methods are more suitable for studying melatonin and its related compounds.

It is obvious that extraction efficiency depends on the metabolites' characteristics. For the metabolites analysed in this work, extractions at low temperatures had a better efficiency and more satisfactory repeatability values than extraction methods using boiling ethanol. The low deviations indicate that, contrary to the results obtained by other authors, the enzymatic activity was stopped. Higher temperatures raise the risk of overestimating some metabolites – possibly due to macromolecules hydrolysis. Due to the small differences between both low-temperature extractions methods, MT extraction can be used without incurring a greater risk of loss in comparison with LT and, furthermore, the extraction process of these bioactive compounds has the advantage of using more affordable equipment.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.05.198.

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