Universidad de Concepción
Dirección de Postgrado
Facultad de Farmacia - Programa de Ciencias y Tecnología Analítica

Desarrollo de una plataforma científica tecnológica para estudiar el proceso de clarificación de vinos orientada a disminuir la prevalencia de agentes clarificantes con potencial alergénico.

Tesis para optar al grado de doctor en Ciencias y Tecnología Analítica

JESSY PAVÓN PÉREZ
CONCEPCIÓN-CHILE
2019

Profesor Guía: Mario Aranda Bustos
Profesor Co-guia: Karem Henriquez Aedo
Dpto. de Ciencias y Tecnología de los Alimentos, Facultad de Farmacia
Universidad de Concepción

INDICE DE CONTENIDO

INDICE DE FIGURAS

INDICE DE TABLAS

RESUMEN

La caseína y la ovoalbúmina son usadas habitualmente como agentes clarificantes durante el proceso de elaboración del vino, con el objetivo de promover interacciones con compuestos no deseados como algunos polifenoles y taninos. Este tipo de proteínas pueden desencadenar reacciones alérgicas en individuos susceptibles, por tanto su presencia en vinos puede ser un riesgo para la salud humana, especialmente cuando su presencia no está reportada o indicada en la etiqueta. Es por esto que la Unión Europea estableció a través de su Directiva 2003/89/EC, actualizada en el año 2012 Directiva 2012/579/EC que las concentraciones mayores a 0.25 mg L^{-1} deben ser declaradas en la etiqueta. De ahí la necesidad de contar con técnicas con un alto poder de resolución que proporcionen la información necesaria para el análisis de estas proteínas en vino.

En la presente tesis doctoral se demuestra el enorme poder que tiene la cromatografía líquida acoplada a la espectrometría de masa para la identificación y cuantificación de péptidos de caseína y ovoalbúmina en vinos. La extracción de las proteínas desde la matriz vino se realizó combinando el uso de membranas de ultracentrifugación y precipitación con solventes orgánicos. En particular el análisis se desarrolló a partir de la estrategia de trabajo *bottom-up*. El paso limitante de esta metodología son los largos tiempos de digestión enzimática que se requieren (12-24 horas). Debido a que varios factores (tiempo, temperatura, relación concentración enzima:proteína, etc) pueden afectar el rendimiento de la digestión tríptica la estrategia más eficiente es aplicar herramientas qumiométricas para su optimización. Se empleó un Diseño Central Compuesto para optimizar la digestión con tripsina aplicando una temperatura de 37^{o}C y energías del infrarrojo, microondas y ultrasonido. Cada método fue evaluado a partir de SDS-PAGE y del % de sequence coverage (SQ) arrojado por la base de datos Mascot. En todos los casos fue posible disminuir los tiempos de digestión y aumentar su rendimiento.

Para la determinación de los péptidos resultantes fue desarrollado, implementado y validado un método UHPLC-DAD-ESI-MS/MS el cual en conjunto con la comparación de los resultados teóricos arrojados por las bases de datos Swiss-Prot y Mascot permitió la identificación y cuantificación de un péptido marcador para la α-caseína, β-caseína y la ovoalbúmina en 60 muestras comerciales de vinos chilenos, empleando como estándar interno un péptido marcado isotópicamente. De las muestras analizadas 17 presentaron

concentraciones de estas proteínas en un valor cercano o superior a lo regulado por la OIV, por consiguiente se hace necesario establecer normativas que normalicen su uso en Chile.

Con la finalidad de lograr una mayor identificación de péptidos marcadores de caseína y ovoalbúmina en los vinos se desarrolló un método UHPLC-ESI-Q/TOF, identificándose y cuantificándose 5 péptidos para α-caseína, 4 para β-caseína, uno para κ-caseína y 3 para ovoalbúmina. Esto evidencia las ventajas de emplear analizadores de masa híbridos de alta resolución en los análisis proteómicos.

La separación e identificación de los péptidos marcadores en vinos se realizó también mediante HPTLC-ESI-MS. El uso de esta herramienta analítica, permitió detectar de manera rápida dos bandas características de la β-caseína y ovoalbúmina.

Explorando alternativas para reducir o evitar el uso de estos clarificantes se realizó un estudio químico-analítico utilizando bentonita, tierras de diatomea, caseína y ovoalbúmina. Se evaluó su influencia sobre el color, turbidez, antocianinas y polifenoles del vino. En aras de no añadir al vino agentes exógenos se evaluó también la capacidad de eliminar caseína y ovoalbúmina por una membrana de ultracentrifugación con flujo cruzado y tamaño de poro de 10kDa. En vinos tratados con estas proteínas no se encontraron péptidos característicos luego de ser sometidos al proceso de filtración.

CAPITULO I: Introducción y objetivos

1. INTRODUCCION

1.1 Vino

La vinificación es un proceso ancestral basado en la transformación del mosto en vino por la acción de diferentes microorganismos presentes en la uva. Actualmente las prácticas básicas de vinificación no difieren mucho a las empleadas por los egipcios, griegos y romanos, diferenciándose principalmente en la incorporación de tecnología y mejoras en las etapas críticas como son los procesos fermentativos (Bartowsky, 2009).

Hoy en día el vino es producido en diferentes países, siendo los más reconocidos los producidos por Francia, España y Estados Unidos. Chile se encuentra entre los 10 países principales productores de vino con cerca de 12.9 mhL producidos durante el 2018 (Organisation Internationale de la Vigne et du Vin (OIV), 2018) lo que es equivalente al 4% del volumen producido a nivel mundial. Chile ocupa el cuarto lugar en el ranking de países exportadores de vino con 966.4 millones de litros exportados en el 2018, lo que representa aproximadamente 1502.2 millones de doláres (ODEPA, 2018). Estas cifras avalan la importancia económica del sector vitivinicultor y por consiguiente su importancia social (empleos directos e indirectos), siendo aún más relevante para un país exportador como Chile. La calidad del vino chileno es indiscutida y reconocida internacionalmente, su *¨flavor¨* es característico, siendo considerado muchas veces un producto de categoría *¨premium¨*. Teniendo en cuenta el actual estatus del vino chileno y los aspectos económicos y sociales que representan, se hace imprescindible evitar y/o controlar la presencia de sustancias que afecten negativamente la calidad del vino y por consiguiente la industria. Para esto es necesario perfeccionar entre otros, el proceso de clarificado del vino, eligiendo aquellos clarificantes que brinden mejores resultados y no sean perjudiciales a la salud.

1.2 Proceso de clarificación de vinos

Al culminar el proceso de fermentación maloláctica el vino apenas descubado se presenta turbio, lo cual se debe a partículas en suspensión, fragmentos de tejido vegetal, borras, levaduras o cristales de bitartrato de potasio mantenidos en el líquido debido al movimiento generado por el desprendimiento del CO_2. Cuando termina la fermentación las partículas más gruesas sedimentan en primer momento y luego las más pequeñas provocando así la autoclarificación del vino. Esta sedimentación de las partículas constituyentes del turbio se

debe por una parte a su tamaño y peso, y por otra, a la resistencia que ofrece la viscosidad del vino a la caída de ellas. La autoclarificación del vino suele por lo general ser lenta y requerir períodos largos de tiempo (González-Neves, Favre, & Gil, 2014). Uno de los inconvenientes que se presentan es que a la limpidez momentánea, pueden seguirle nuevos enturbiamientos o insolubilizaciones que empañan la brillantez del vino, lo cual es una característica física fundamental del producto. Existen entonces dos problemas, lograr la limpidez del vino y lograr que se mantenga durante todo el tiempo de almacenamiento (en tanques o en botellas) y en las diferentes condiciones de almacenamiento (luz, temperatura, aire, etc) (Martínez-Lapuente, Guadalupe, & Ayestarán, 2017). Los enturbiamientos de los vinos ya terminados se deben a las siguientes causas: reinicio de la fermentación en vinos con azúcares residuales, alteraciones microbiológicas y enzimáticas, coagulación de sustancias en estado coloidal preexistentes en el vino, o compuestos que toman ese estado por reacciones químicas e insolubilizaciones de algunas sustancias en estado cristalino (Togores, 2003). Por lo cual es necesario entonces conocer, entender y prevenir los factores de inestabilidad fisicoquímica y biológica del vino. La limpidez y la estabilidad se logran por procedimientos físicos como frío, filtración, centrifugación, trasiegos. Sin embargo estos procedimientos por si solos no garantizan la estabilidad del vino y no evitan la formación de depósitos una vez embotellado (Martínez-Lapuente, et al., 2017). Solamente permiten extraer o eliminar partículas causantes de la turbidez y microorganismos (estabilidad biológica). Por lo cual se emplea también un procedimiento químico, la clarificación, en la cual los procesos químicos y coloidales involucrados permiten lograr la estabilidad fisicoquímica deseada. *"La clarificación artificial o provocada consiste en la introducción al vino de determinadas sustancias de naturaleza coloidal, las que, floculando, aumentan su tamaño y se depositan en el fondo de las vasijas, arrastrando con ellas (por adsorción y en parte por acción mecánica) las partículas dispersas en el vino"* (Galiotti, 2016). De esta manera, en poco tiempo, el vino se vuelve límpido. Estas sustancias permiten además corregir pequeños defectos organolépticos como el color, el aroma y determinados sabores indeseados como el amargo, la astringencia, etc (Urkiaga, De Las Fuentes, Acilu, & Uriarte, 2002).

Debido a que la intensidad o tonalidad del color de un vino, así como su transparencia o brillo son características organolépticas que predisponen a la aceptación o al rechazo del

producto se hace necesario entonces medir mediante técnicas analíticas estas características, lo que permite evaluar la eficiencia del proceso de clarificación del mismo.

Dentro de los compuestos responsables del enturbiamiento del vino se encuentran los compuestos fenólicos, principalmente los de mayor peso molecular como los taninos. La presencia excesiva de estos puede provocar entre otros efectos, la oxidación y la astringencia del vino lo cual no es deseable para los consumidores. Igualmente favorecen la formación de sedimentos, a través de la formación de complejos poliméricos entre polisacáridos, azúcares, iones metálicos, y las proteínas (Cassano, Conidi, & Tasselli, 2015). Por lo cual otros de los análisis que se pueden realizar para verificar la eficiencia del proceso de clarificación es la determinación del contenido de polifenoles y capacidad antioxidante, ya que los compuestos fenólicos se relacionan fuertemente con la capacidad antioxidante del vino.

1.3 Metodologías analíticas para el análisis de la eficiencia del proceso de clarificación

1.3.1 Medida de la turbidez del vino

La turbidez del vino es el resultado de la dispersión de la luz por partículas sólidas dispersas en el medio líquido (Mutanen, Räty, Gornov, Lehtonen, Peiponen, & Jaaskelainen, 2007; Organisation Internationale de la Vigne et du Vin (OIV), 2000). Se puede medir usando un nefelómetro con el detector fijado en un ángulo de 90º con respecto al haz de luz incidente. Se expresa en unidades de turbidez nefelométricas (NTU) y tiene un rango de 0 a 10000 NTU (Mutanen, et al., 2007). En un rango de valores de turbidez menores o iguales a 30 NTU se disminuye el riesgo de sobrefiltración, posiblemente responsable último de la retención y eliminación de coloides que son aconsejables conservar en el seno del vino (Mutanen, et al., 2007). Algunos autores han reportado también la determinación de la turbidez midiendo espectrofotométricamente la absorbancia a 650 nm (Cosme, Ricardo-da-Silva, & Laureano, 2008). En la tabla 1 (El Rayess, et al., 2011) se muestra la relación entre la turbidez y el aspecto del vino.

Tabla 1. Relación entre turbidez y aspecto del vino.

	Brillantez	Opalescencia
Vino blanco	< 1.1NTU	> 4.4NTU
Vino rosado	< 1.4NTU	> 5.8NTU
Vino tinto	< 2.0NTU	> 8.0NTU

1.3.2 Medida del color del vino

El color es un atributo del vino que es relativamente fácil de evaluar y generalmente está asociado a su calidad, así es como los vinos de un buen año de cosecha suelen tener un color más profundo (Jackson, Timberlake, Bridle, & Vallis, 1978). Se han reportado diversos métodos de análisis para evaluar el color, la Organización Internacional de la Viña y el Vino establece dos formas de medir las características cromáticas del producto final. Una primera vía plantea medir la intensidad del color y el matiz del vino determinando su absorbancia espectrofotométricamente a longitudes de onda de 420, 520 y 620 nm (Organisation Internationale de la Vigne et du Vin (OIV), 2001). La intensidad del color será la suma de todas las absorbancias mientras que el matiz es la relación entre la absorbancia medida a 420 nm y 520 nm. Una segunda vía es la determinación de las características cromáticas del vino según la CIELab (Organisation Internationale de la Vigne et du Vin (OIV), 2006) (Comisión Internacional de la Luz). Este es un método espectrofotométrico que tiene por objeto definir el proceso de medición y cálculo de las características cromáticas de los vinos y otras bebidas derivadas a partir de los componentes tricromáticos X, Y y Z, tratando de imitar los observadores reales en lo que respecta a sus sensaciones al color (Organisation Internationale de la Vigne et du Vin (OIV), 2006). Otros autores (Gutiérrez, Lorenzo, & Espinosa, 2005) han reportado la Cromatografía Líquida de Alta Eficiencia (HPLC) acoplada a detectores Ultravioleta-Visible (UV-Vis) y arreglo de diodo (DAD) (Jayprakasha, Patil, & Pellati), esta técnica contrariamente a las descritas anteriormente permite hacer un análisis detallado de todos los pigmentos presentes en el vino.

1.3.3 Determinación de antocianinas monoméricas

Las antocianinas son los principales compuestos responsables de la coloración del vino, se encuentran principalmente en la piel de la uva y brindan una amplia gama de colores desde naranja hasta púrpura (Ju, et al., 2019). Las condiciones de cultivo y las variadas formas de elaboración hacen que el contenido de antocianinas y otros pigmentos en el vino sean muy diferentes. La composición y el contenido de antocianinas juegan un rol muy importante en la estabilidad del color del vino (Li & Moore, 2006), por lo cual en cierta medida su cuantificación contribuye a determinar la calidad de este parámetro. Considerando que el pH es uno de los principales factores que afecta la estabilidad del medio la cuantificación de antocianinas se puede realizar a partir del método de diferencial de pH (Giusti & Wrolstad, 2001). Este es un método espectrofotométrico que se basa en la transformación estructural de las antocianinas con el cambio de pH (pH 1 predomina la forma de oxonio coloreada y pH 4.5 la forma hemicetal incolora). De esta forma se puede medir rápidamente el total de antocianinas monoméricas presentes en la muestra. HPLC acoplado a un detector de arreglo de diodo (Jayprakasha, et al.) puede ser empleada para la separación, identificación y cuantificación individual de las antocianinas presentes en el vino (Ju, et al., 2019).

1.3.4 Determinación de la capacidad antioxidante del vino

No existe un único compuesto fenólico responsable de la actividad antioxidante del vino, sino que ésta se explica por el conjunto de todos ellos. Se dispone de metodologías espectrofotométricas que permiten la determinación de estos compuestos por agrupaciones estructurales como el ensayo de polifenoles totales mediante el método de Folin-Ciocalteu que otorga una aproximación de los posibles polifenoles, pero presenta algunos inconvenientes, como la interferencia que pueden ejercer los azucares reductores en la identificación de compuestos. Este método se basa en la capacidad de los fenoles de reaccionar con agentes oxidantes, ya que el reactivo Folin-Ciocalteu contiene molibdato y tungsteno sódico, los cuales reaccionan con cualquier tipo de fenol, formando complejos fosfomolíbdico-fosfotúngstico. La transferencia de electrones a pH básico reduce los complejos formados en óxidos cromógenos de color azul intenso de tungsteno y molibdeno, siendo este color proporcional a la cantidad de compuestos fenólicos presentes

originalmente en la muestra (Turkmen, Sari, & Velioglu, 2006). También se cuenta con ensayos de capacidad antioxidante, lo cuales desde el punto de vista de las reacciones químicas involucradas se pueden clasificar en dos categorías:

1. Ensayos basados en la reacción de transferencia de un átomo de hidrógeno (HAT).
2. Ensayos basados en la reacción de transferencia de un electrón (ET).

Los ensayos ET miden la capacidad que tiene un antioxidante para transferir un electrón y reducir un compuesto, estas reacciones suelen ser lentas y dependen en gran parte del pH. Dentro de estos se encuentra el ensayo FRAP (poder antioxidante de reducción del hierro), ensayo TEAC (capacidad antioxidante equivalente del trólox) entre otros.

Los ensayos HAT, en cambio, monitorean la cinética de reacción por competencia, ya que generalmente se compone por un generador de radical sintético, un compuesto oxidable, y la molécula antioxidante que se quiere evaluar. Un ejemplo de este tipo de ensayo es el ensayo ORAC (capacidad de absorción de radicales de oxígeno). En este los radicales peroxilos se forman a partir 2,2′-azo-bis(2-amidinopropano)-dihidrocloruro (AAPH) en medio acuoso para reaccionar con un sustrato que puede ser fluoresceína o rojo de pirogalol (PGR). Generalmente la mezcla se compone de la muestra, control y estándar (TROLOX) y se incuba a 37ºC antes de agregar el AAPH. Este ensayo es capaz de medir todo el periodo de tiempo de la reacción y no como otros métodos que solo miden el periodo lag o de latencia, siendo que algunos compuestos no muestran un claro periodo lag. Para evitar la subestimación de la capacidad antioxidante y tener en cuenta los efectos potenciales de los productos antioxidantes secundarios, el ensayo ORAC sigue la reacción durante períodos prolongados, por ejemplo, ≥30 min. Es particularmente útil para las muestras que a menudo contienen varios ingredientes y tienen una cinética de reacción complejas (Karadag, Ozcelik, & Saner, 2009). A pesar que estos tipos de ensayos presentan interesantes características de aproximación, no cumplen el objetivo de identificar, separar y cuantificar los compuestos, para lo cual se requiere de técnicas instrumentales más avanzadas, entre las que destaca la cromatografía. El empleo de un detector DAD junto con HPLC permite la rápida y simultánea separación, identificación y cuantificación de los distintos compuestos fenólicos según sus propiedades espectrales y su orden de elución sin necesidad de aislamientos previos. Se trata de una herramienta analítica que permite la separación y

cuantificación de la inmensa mayoría de estas especies y por tanto observar su evolución en las diferentes etapas de elaboración de los vinos (Lu & Luthria, 2014).

1.4 Clarificantes usados en los vinos

Durante las etapas que están involucradas en el proceso de elaboración del vino se utiliza una cantidad considerable de energía, de clarificantes y de agentes filtrantes que favorecen su limpidez final. Dentro de los métodos físicos para clarificar se encuentran aquellos que involucran la utilización de membranas. Saxena y colaboradores (Saxena, Tripathi, Kumar, & Shahi, 2009) describen una membrana como una interfase heterogénea que actúa como barrera entre especies iónicas y moleculares presentes en los líquidos o gases que se ponen en contacto con ambas superficies de la membrana. Dentro de las ventajas que ofrece la filtración con membranas se encuentra que no requiere el uso de aditivos y se puede realizar isotérmicamente a bajas temperaturas. Dentro de la industria vitivinícola se han utilizado membranas de nanofiltración (García-Martín, et al., 2010), ultrafiltración (Gonçalves, Fernandes, & Pinho, 2001) y microfiltración (Ulbricht, Ansorge, Danielzik, König, & Schuster, 2009). En todos estos casos el proceso que se utiliza para operar la membrana es el gradiente de presión que se establece durante la separación y la diferencia más notable que existe entre estos distintos tipos de membranas radica en su tamaño de poro (figura 1) el cual determina la selectividad y la permeabilidad de la membrana. Su principal ventaja es que permite realizar la clarificación, la estabilización microbiológica y filtración estéril en un solo y continuo paso de manera automática. Sin embargo es el proceso de ensuciamiento de la membrana lo cual disminuye su vida útil y disminuye su productividad. Por lo cual en cada caso hay que tener presente cuales son los principales mecanismos involucrados en este proceso así como los compuestos responsables del mismo los cuales son polifenoles, polisacáridos y proteínas (Güell & Davis, 1996).

Para cada aplicación es importante la elección apropiada de la membrana y del módulo, para obtener una eficiencia satisfactoria del proceso de separación. Las propiedades claves que determinan el comportamiento de una membrana son su selectividad y densidad de flujo, estabilidad mecánica, química y térmica bajo las condiciones de operación. Los módulos de membrana que permiten una producción eficiente desde el punto de vista de los

costos se caracterizan por su alta densidad de empaque, buen control del ensuciamiento y bajos costos de operación y mantenimiento.

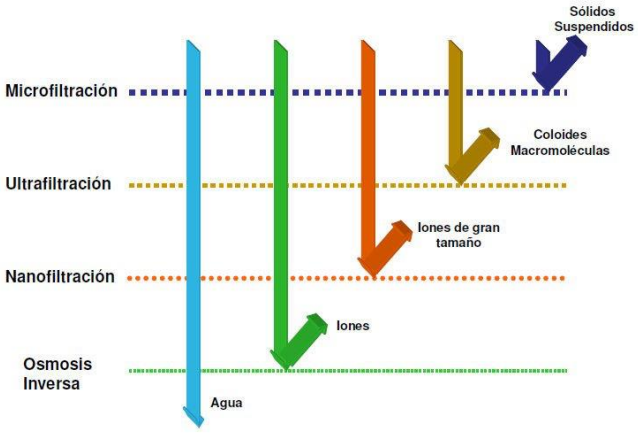

Figura 1. Capacidad de separación de cada tipo de membrana de acuerdo a su tamaño de poro (Adaptado de (Programme, 1997))

- Membranas de nanofiltración: Las membranas de nanofiltración poseen por lo general un tamaño de poro de 0.5 a 10 nm (Salehi, 2014) reteniendo el paso de moléculas de pequeño tamaño entre los 300-500 Da e iones mono y divalentes, por lo general se emplean presiones entre 10-30 bar (Mohammad, Teow, Ang, Chung, Oatley-Radcliffe, & Hilal, 2015). En la industria vitivinícola se usa un gradiente de presión para transportar el mosto de la uva a través de la membrana produciéndose así la separación del permeato de los solutos (Salehi, 2014). Este sistema de filtración se ha empleado para concentrar el vino logrando reducir alrededor de 5 al 20 por ciento de agua, alcanzando un enriquecimiento del mismo en taninos (Ferrarini, Versari, & Galassi, 2001). De la misma forma se ha logrado obtener un vino con bajo contenido alcohólico sin perder sus características organolépticas (Catarino, 2011). Dentro de las principales desventajas de este tipo de membrana se encuentra su alta tendencia a ensuciarse lo cual disminuye su vida útil (Van der Bruggen, Mänttäri, & Nyström, 2008), además involucran un gasto de energía elevado debido a las altas presiones a las que trabaja (Fane, Tang, & Wang, 2011).
- Membranas de ultrafiltración: Estas membranas poseen un tamaño de poro que oscila desde 1 hasta 100 nm, por lo que son empleadas para eliminar bacterias, coloides y macromoléculas (Fane, Tang, & Wang, 2011). Al igual que en las membranas de

nanofiltración para la separación se usa una diferencia de presión que permite que los solutos de menor tamaño pasen a través de la membrana y los de mayor tamaño queden retenidos en la superficie (Cassano & Basile, 2011). Debido a su estructura porosa y asimétrica pueden ser operadas a presiones entre 1 y 10 bar y son altamente permeables aumentando así su eficiencia en la separación. Generalmente se construyen de materiales poliméricos, derivados de celulosa y cerámica siendo estas las menos resistentes (Cassano, et al., 2015). Este tipo de membrana ha demostrado ser eficiente en la eliminación de las bacterias presentes en el vino llegando a obtener un vino con buena calidad en cuanto a claridad, aroma y transparencia (Shu-Sen, Al-Lian, Ling-Ling, & De-Kuan, 1989).

- Membranas de microfiltración: Las membranas de microfiltración tienen un tamaño de poro que va desde los 50 hasta los 10000 nm por lo cual poseen una elevada permeabilidad y pueden funcionar en un rango de presiones entre 1 y 10 bar (Fane, et al., 2011). Se emplean para eliminar compuestos con tamaño de partícula superior a su tamaño de poro como bacterias o sólidos suspendidos. Los tamaños de poro de membrana que más se utilizan en el procesamiento del vino son de 0.1 a 0.22 µm para vino blanco y de 0.2 µm para vino tinto (Urkiaga, et al., 2002).
- Filtración con flujo cruzado: En la actualidad una de las técnicas más empleada es la filtración con flujo cruzado (CF) (El Rayess, et al., 2011). Contrariamente a la filtración frontal clásica donde el flujo es forzado a pasar perpendicularmente a la superficie filtrante, en la filtración con flujo cruzado, la solución, pasa tangencialmente a través de la superficie de un filtro (Figura 2). Esta filtración tangencial significa que la muestra fluye paralela al filtro y se recicla varias veces a través de un depósito. Esto hace que la misma alimentación barra continuamente la superficie del medio filtrante, y disminuye así drásticamente el depósito e incrustación de solutos en la membrana, se enlentece entonces el proceso de ensuciamiento de la membrana, alargando su vida útil y disminuyendo los costos. Igualmente permite procesar volúmenes mayores de alimentación en forma continua o batch, mejorando la eficiencia del proceso de separación. En el proceso de elaboración del vino además de poder utilizarse en la etapa final de clarificado, esta técnica también puede sustituir la gran cantidad de pasos que requiere lograr el asentamiento del mosto por decantación en la elaboración del vino blanco o eliminar las sucesivas filtraciones para disminuir la turbidez del mosto en el vino tinto (El Rayess, et al., 2011). Sin embargo, a

pesar del progreso logrado, el desarrollo a gran escala de la tecnología de la filtración en flujo cruzado todavía se ve obstaculizado por las barreras tecnológicas y económicas inducidas por el ensuciamiento de la membrana.

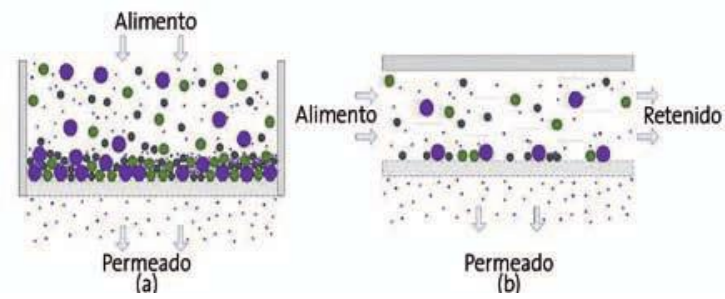

Figura 2. Filtración convencional o perpendicular (a) y tangencial (b) (Adaptado de (García Pacheco & García-Calvo, 2011)

Como ya se había mencionado anteriormente las sustancias empleadas en el proceso de clarificación de vinos se denominan clarificantes. Estas se agrupan en dos categorías: de origen mineral y orgánico. Dentro de las sustancias de origen mineral más empleadas se encuentran la bentonita y las Tierras de diatomea (Lira, Rodríguez-Bencomo, Salazar, Orriols, Fornos, & López, 2015).

- Bentonita: La bentonita es un mineral de origen volcánico, existen diversos tipos en dependencia de su localización, por lo que es comercializada con diferentes tamaños de partícula, capacidad adsorbente y pureza. Por estas razones la naturaleza de la bentonita utilizada en el proceso de fabricación del vino afecta su capacidad de remover sustancias y su eficiencia oenológica (Dordoni, Galasi, Colangelo, De Faveri, & Lambri, 2015). Son arcillas caracterizadas por un elevado contenido de montmorillonita y su estructura cristalina determina una distribución de cargas superficiales, de manera tal que en suspensión acuosa, la superficie de las capas elementales se cargan negativamente, mientras que en los bordes de las mismas se forman zonas cargadas positivamente. Tiene una carga neta negativa al pH del vino, interactúa electrostáticamente con las proteínas del vino cargadas positivamente induciendo así su floculación (Lambri, Dordoni, Giribaldi, Violetta, & Giuffrida, 2012). La forma más usada dentro del proceso de clarificación del vino es la sódica la cual tiene mejor capacidad de unión a las proteínas naturales del vino. Existe como pequeñas placas las cuales cuando se hidratan se separan en forma de suspensión

coloidal con una amplia superficie de contacto (300–900 m^2/g), propiciando su poder adsorbente y floculante lo cual es aprovechado en su función como clarificante. Su principal ventaja es su bajo costo y que no se requiere equipamiento especial para su incorporación al vino. No obstante, la clarificación con bentonita es un proceso que afecta la calidad sensorial del vino ya que remueve los compuestos deseables y los no deseables porque no es un adsorbente específico. Al eliminar gran parte de los proteínas, el vino pierde estructura y untuosidad (Ledoux, Dulau, & Dubourdieu, 1992). Además afecta seriamente el aroma del vino, ya que las proteínas son fijadores de aromas y al ser eliminadas arrastran con ellas parte de los mismos (Guillou, Aleixandre, García, & Lizama, 1998). La dosis a utilizar deben ser estimadas en forma particular para cada vino, de manera que se asegure la estabilización proteica causando el menor impacto en el vino (Tabilo-Munizaga, et al., 2014). De igual manera su contenido de sílica cristalina hace que tenga un impacto sobre la salud de las personas que se encuentran expuestas por largos períodos de tiempo, pudiendo causar, entre otras, enfermedades pulmonares (Saxena, et al., 2009). Su uso tiene un impacto medioambiental pues los restos de bentonita son eliminados del vino por filtración con tierras de diatomea. Por estas razones hoy en día toma mayor atención el desarrollo de prácticas que sean económicamente viables y sostenibles para lograr la limpidez del vino manteniendo su calidad.

- Tierras de diatomea: La diatomita es una roca sedimentaria constituida esencialmente por cáscaras silíceas de diatomeas fósiles y es un coadyuvante de filtración de los mostos y de los vinos (Flower, 2013). Para ser utilizada en enología esta roca es partida, secada, molida, depurada por lavado y calcinada a alta temperatura (950 a 1100°C). Es utilizada en estado pulverulento entre 5 y 40 micrones y se presenta bajo el aspecto de un polvo rosado para los productos calcinados, o blanco en el caso de los productos calcinados y activados. Debido al tamaño de sus partículas y a su composición, puede causar cáncer pulmonar a sujetos que la inhalen prolongada o repetidamente en ambientes de trabajo. Por esto, una vez que la tierra se ha saturado luego del filtrado, presenta un serio problema para su disposición final.

Dentro de los clarificantes de origen orgánico destacan proteínas de diversos orígenes.

- Proteína de colágeno de pescado: Estas proteínas son extraídas de diferentes especies, sin embargo principalmente desde hace varios años son obtenidas a partir de los

esturiones. Al pH del vino presenta un punto isoeléctrico de 5.5 y carga neta positiva por lo cual atrae electrostáticamente a las partículas presentes en el vino con carga negativa, especialmente a los taninos (Rankine, 1996). Es especialmente recomendado para clarificar vinos blancos ya que a penas interactúa con los compuestos fenólicos responsables del color.

- Gelatina: Es una proteína soluble del tipo albúmina producto de la ebullición prolongada de algunos tejidos de animales (principalmente bovinos y porcinos) como huesos, piel y tendones. Su punto isoeléctrico depende del animal que proviene y por tanto su interacción con los taninos del vino principalmente. Es usada generalmente para reducir la astrigencia de los vinos tintos, puestos que en los vinos blancos deja un residuo que provoca turbidez en el mismo (Margalit, 2004).

La aparición de la encefalopatía espongiforme bovina (EEB) generó un considerable interés en la sustitución de proteínas de origen animal en el procesamiento de alimentos y en el uso alternativo de proteínas de origen vegetal (González-Neves, et al., 2014). Diversos autores (Marchal, Marchal-Delahaut, Lallement, & Jeandet, 2002; Meistermann & Pinsun, 2018) reportan el uso de una amplia variedad de preparaciones comerciales de proteínas derivadas de plantas de soja, gluten de trigo, arroz, papa, lupino o maíz para fines enológicos .

Dentro de las proteínas animales destacan la proteína de la leche o caseína y la principal proteína del huevo (ovoalbúmina) por los buenos resultados alcanzados en la clarificación del vino y por ser económicas. Existen dos tipos de proteínas lácteas, las caseínas, que representan el 80% y las proteínas de suero. Estos dos tipos de proteínas se separan por precipitación isoeléctrica, a pH 4.6 (Swaisgood, 1993). Bajo la denominación de caseínas se incluyen cuatro tipos de cadenas polipeptídicas, αs1-caseína (αs1-CN), αs2-caseína (αs2-CN), β-caseína (β-CN) y κ-caseína (κ-CN). Las secuencias de sus estructuras primarias son complejas y muy distintas a las de otras proteínas, debido al gran contenido de prolina y a las regiones ácidas que incluyen los residuos de fosfoserina (Creamer & MacGibbon, 1996). Debido a su diferente distribución de las cargas y a su hidrofobicidad, las caseínas se unen entre sí formando micelas, compuestas por un 92% de proteína y un 8% de sales inorgánicas, principalmente fosfato cálcico. Las principales proteínas presentes en el huevo son la ovoalbúmina (54%), ovotransferrina (12%), ovomucoide (11%), ovomucina (3.5%), y lisozima (3.4%) (Deckwart, et al., 2014). De estas la ovoalbúmina es usada desde hace

muchos años en el proceso de clarificado del vino tinto. El mecanismo por el cual la caseína y la ovoalbúmina propician la clarificación no está del todo dilucidado. Rankine et al en el año 1996 (Rankine, 1996) plantearon que este mecanismo puede estar dado por interacciones electrostáticas. Producto de la reacción de las proteínas con los taninos del vino estas pasan de ser coloides hidrófilos de carga positiva (al pH del vino) en coloides hidrófobos negativos, resultando esto en su floculación, es decir en la asociación de partículas entre ellas y a la formación de flóculos que se ensamblan y precipitan El complejo tanino proteína está en función de numerosos factores (pH, temperatura, concentración de proteína y de taninos). Son estables en solución límpida y precipitan en presencia de cationes metálicos que neutralizan su carga. Lo cual se traduce en aparición de turbidez. Las proteínas que no reaccionan con los taninos, pueden combinarse con las partículas en suspensión o en solución coloidal que están cargadas negativamente (la mayoría de las partículas productoras de turbios en los vinos poseen cargas negativas), produciéndose la floculación mutua de los dos coloides (neutralización de cargas). Estos complejos son removidos del vino por decantación, centrifugación y filtración. Sin embargo la completa eliminación de estos agentes clarificantes no siempre es segura y es posible que se encuentren en pequeñas cantidades en los vinos lo cual puede ser un riesgo para los consumidores susceptibles a estos tipos de proteínas (Tolin, et al., 2012).

Los estudios que existen acerca del empleo de proteínas en vinos como clarificantes no se pueden extrapolar a otras muestras debido a que las prácticas de clarificación son muy diversas y no están estandarizadas. Estudios realizados demuestran que un 26% de los vinos producidos en Francia son clarificados con caseína y un 11% con ovoalbúmina (EFSA Panel on Dietetic Products, 2011). Mientras que en Alemania un 20% de los vinos blancos son clarificados con caseína y un 2% (EFSA Panel on Dietetic Products, 2011) de los vinos tintos producidos son clarificados con ovoalbúmina. En Chile no existen reportes de investigaciones al respecto y las prácticas seguidas para la clarificación son variadas. Por lo general los estudios de proteínas alergénicas en vinos van enfocados a su detección e identificación y no así a la cuantificación. Mattarozzi et al (Mattarozzi, Milioli, Bignardi, Elviri, Corradini, & Careri, 2014) analizaron 20 vinos comerciales italianos arrojando límites de detección de 0.2 $\mu g\ mL^{-1}$ y 1.6 $\mu g\ mL^{-1}$ para la caseína y la ovoalbúmina respectivamente, rango dentro del cual se encuentra los valores permitidos por la OIV (0.25

mg/L). Valores entre 0.1-0.3 mg mL^{-1} de caseinato de potasio han sido encontrados en vinos italianos (Monaci., Losito, Palmisano, Godula, & Visconti, 2011). Lo cual evidencia la utilización de estas proteínas en el proceso de elaboración del vino en ocasiones fuera de los límites establecidos.

1.5 Proteínas alergénicas en vinos

Las enfermedades causadas por alimentos son conocidas desde hace muchos años y aún entre los expertos existen desacuerdos en cuanto a su nomenclatura o clasificación. Algunas reacciones de hipersensibilidad pueden estar relacionadas con los alimentos pero no son mediadas por la inmunoglobulina E (IgE) por lo cual en este caso son consideradas reacciones de hipersensibilidad no alérgicas (Weber, Steinhart, & Paschke, 2007). Usualmente las reacciones que no involucran una respuesta inmune son denominadas intolerancias alimenticias y pueden ocurrir por ejemplo cuando existe una falta en el organismo de alguna enzima necesaria para digerir un componente alimenticio en particular. En cambio, una alergia alimentaria es una reacción de hipersensiblidad iniciada por mecanismos inmunológicos específicos, respuestas inmunitarias mediadas por IgE resultantes de la ingestión de alimentos específicos, cuando el sistema inmunológico del cuerpo reacciona a sustancias inocuas en ciertos alimentos (Figura 3). La alergia puede ser mediada por anticuerpos o mediadas por células, los que pueden provocar en el sistema inmunológico de individuos sensibles el comienzo de síntomas clínicos perjudiciales.

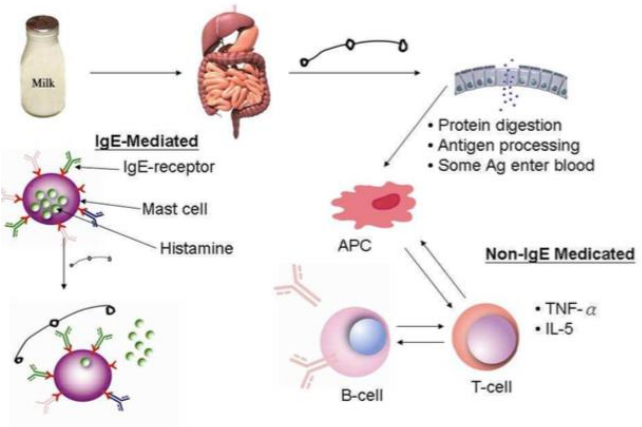

Figura 3. Mecanismo inmune de la alergia a la leche. Adaptado de (Ho, Chan, & Lee, 2014).

Desde la pasada década el interés en esta área ha ido en aumento debido a un marcado incremento a las alergias a los alimentos entre la población, y al potencial de algunos alérgenos de desencadenar reacciones inmunológicas las cuales pueden ser desde leves hasta graves que pongan en peligro la vida humana. Las proteínas presentes en los alimentos alergénicos son numerosas y heterogéneas, con un variado poliformismo genético resultando en muchas variantes para cada proteína. A menudo las modificaciones post-translacionales y cambios en la conformación tridimensional de las proteínas pueden ocurrir como consecuencia del procesamiento de los alimentos afectando la alergenicidad total (Sabato, et al., 2011). Aunque alrededor de 180 proteínas alergénicas presentes en los alimentos han sido identificadas, solo una pequeña cantidad concurren en algunos alimentos, como en la leche de bovinos, huevos, cacahuates, soja, trigo y pescados (Poms, 2013). Hasta la fecha no hay cura para las alergias a los alimentos por lo que las personas alérgicas deben privarse de ingerir estos alimentos, aunque tengan pequeñas cantidades de proteínas alergénicas.

El uso de caseína y ovoalbúmina en el proceso de clarificación del vino resulta casi de forma inevitable en la presencia de remanentes de estas sustancias. Esto sumado a la no declaración de la presencia de éstas en las etiquetas de las botellas, produce que personas alérgicas a estas proteínas se encuentren expuestas a sufrir posibles reacciones alérgicas. Según reportes de la Autoridad Europea de Seguridad Alimentaria alrededor del 0.3% de la población mundial es alérgica a la ovoalbúmina y del 0.5 al 1.0% es alérgica a la caseína (Efsa Panel on Dietetic Products Nutrition and Allergies, 2011). Por esta razón la Unión Europea adoptó la Directiva 2003/89/EC y su última versión Directiva 2012/579/EC, las cuales contienen una lista de las sustancias alergénicas incluyendo el huevo y sus derivados y la leche (European Commision (EU), 2012). Este documento establece la obligación de declarar la presencia de estas proteínas en la etiqueta del vino (Figura 4) cuando sus concentraciones sean superiores a 0.25 mg L^{-1}, límite descrito en la resolución OIV/COMEX/ 502/2012 (Organisation Internationale de la Vigne et du Vin (OIV), 2012).

Aunque un porcentaje pequeño de la población adulta mundial es alérgica a estas proteínas y a las concentraciones encontradas en vinos no se ha reportado anafilaxia, estudios realizados evidencian el desarrollo de reacciones indeseables en algunos individuos. En un estudio en vinos australianos se utilizó un panel de cinco pacientes alérgicos al huevo y uno

a la leche para estudiar el potencial alergénico de la caseína y la ovoalbúmina en este tipo de bebida (Rolland, et al., 2006). Aunque no se detectaron reacciones anafilácticas o síntomas graves atribuibles al consumo de vino con estas proteínas, fueron observadas tres reacciones clínicas leves. Casos de alergia derivadas del consumo de vino han sido particularmente reportados en el área del Mediterráneo. Sin embargo no se ha podido establecer una asociación directa de estas reacciones adversas con la presencia de proteínas alergénicas en el vino, ya que también pueden estar dadas por intolerancias a compuestos como sulfitos o histamina (Weber, et al., 2007).

Figura 4. Modelo de etiqueta establecido por la UE para declarar la presencia de proteínas del huevo o de la leche en los vinos.

Siguiendo las directrices de la EU para el etiquetado de proteínas alergénicas en el vino otros países también han establecido marcos regulatorios. En Australia y Nueva Zelanda es obligatorio declarar la presencia de estas proteínas en el vino (FSANZ, 2015). Estados Unidos implementó una declaración obligatoria de alérgenos en la Ley de Etiquetado de Alérgenos Alimentarios y Protección del Consumidor del 2004 (Food and Drugs Administration (FDA), 2004) Este documento establece la obligación de declarar los 8 mayores alérgenos alimentarios en caso de que sean empleados como clarificantes del vino.

A pesar de la creación de las directivas para la protección de los consumidores alérgicos, se necesitan métodos adecuados para la detección de estos alérgenos en vinos, poca información concerniente a este tipo de análisis ha sido reportada y aún se necesitan métodos precisos y exactos.

1.6 Métodos de análisis de proteínas en vino

1.6.1 Ensayo por Inmunoabsorción Ligado a Enzimas

Dentro de los métodos más utilizados para el análisis de proteínas en vino se encuentra el Ensayo por Inmunoabsorción Ligado a Enzimas (Jorge, et al.) (ELISA) el cual es el método aceptado por la OIV (Resolución OIV/OENO/427/2010). En general este método presenta buena sensibilidad, es fácil y rápido de realizar pero tiene como inconveniente la presencia de interferentes de la matriz (Monaci, Nørgaard, & van Hengel, 2010). Este problema tiene una particular relevancia en vinos y sobre todo en vinos tintos donde el alto contenido de polifenoles interactúan con las proteínas y anticuerpos, provocando problemas en el desarrollo del método para la detección de proteínas alergénicas (Patrick, Hans, & Angelika, 2009). Por otra parte, en este tipo de método la adsorción de los alérgenos en la matriz sólida que sirve de soporte al análisis, puede alterar los epítopes de las proteínas, comprometiendo su reconocimiento por los anticuerpos, perjudicando así la sensibilidad del ensayo (Kaul, et al., 2007).

1.6.2 Espectrometría de masa

Con el objetivo de superar los inconvenientes que presenta el análisis por ELISA, la espectrometría de masa (MS) ha comenzado a ser utilizada para la detección directa de alérgenos en vinos. Gracias a la sensibilidad de este método se pueden detectar las trazas de ovoalbúmina y caseína que contiene el vino y es posible la identificación de las proteínas alergénicas independientemente de su estructura (Monaci & Visconti, 2009). Contrariamente al análisis por ELISA la MS es un método de detección directa y además permite detectar múltiples alérgenos en un solo análisis lo que permite reducir los tiempos (Heick, Fischer, & Popping, 2011). El adelanto de esta técnica analítica y de la bioinformática ha posibilitado los avances en el análisis de proteínas desde diversas

matrices alimentarias, abriéndose así todo un campo de investigación de la proteómica en alimentos.

1.7 Proteómica

El término proteoma proviene de las palabras PROTEÍnas y genOMA, fue empleado por primera vez en Siena por Marc Wilkins en la primera conferencia mundial sobre proteómica celebrada en 1994 (Wilkins, et al., 1996). La proteómica es un enfoque experimental que encripta la información contenida en las secuencias genómicas en términos de la estructura, función y control de procesos biológicos. No solo incluye un conocimiento estructural y funcional de las proteínas sino también el estudio de las modificaciones, interacciones, localización y cuantificación de las proteínas (Rabilloud, Chevallet, Luche, & Lelong, 2010).

La MS ha jugado un papel fundamental en la investigación proteómica, los analizadores de masas han demostrado ser de gran alcance para el análisis de proteínas y péptidos derivados de estas. Técnicas de fraccionamiento se utilizan comúnmente para purificar la proteína bajo investigación antes del análisis por MS (proteínas intactas). Alternativamente, el extracto puede ser sometido a digestión proteolítica y al análisis por MS-tandem después de haber sido los péptidos resultantes separados por alguna técnica separativa. Mediante el uso de MS/MS, una secuencia completa de estos péptidos de interés es a menudo lograda. Para identificarlos, los espectros de masa obtenidos se comparan contra las bases de datos de proteínas utilizando algoritmos de búsqueda (Switzar, Nicolardi, Rutten, Oberstein, Aartsma-Rus, & van der Burgt, 2016).

1.7.1 Estrategias de trabajo en proteómica

Dos diferentes estrategias en el estudio de la proteómica se pueden emplear, estas se conocen como *bottom-up* y *top-down*. El uso de una o de otra depende del nivel de información que se necesite obtener (Ivanov, et al., 2015). La figura 5 ilustra el flujo de trabajo general empleado en el análisis de alimentos a través de la proteómica.

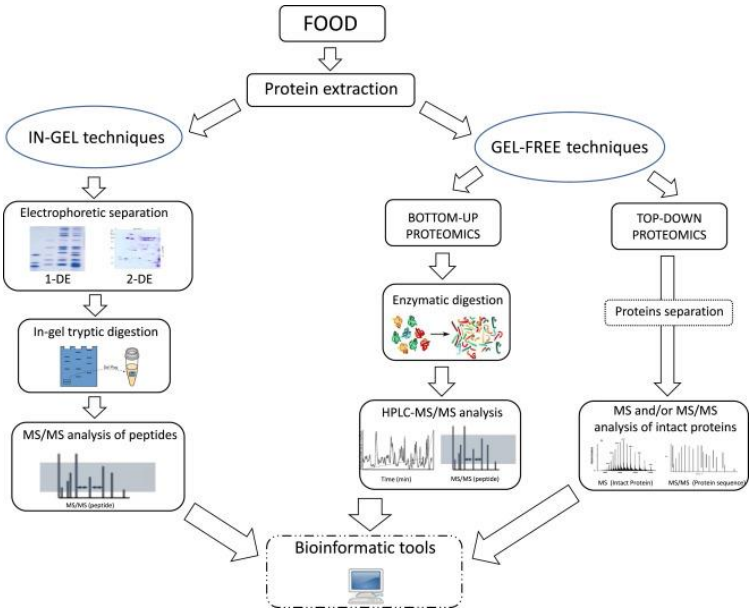

Figura 5. Flujo de trabajo general basado en el análisis de alérgenos por espectrometría de masa. Adaptado de (Pali-Schöll, Verhoeckx, Mafra, Bavaro, Clare Mills, & Monaci, 2019)

1.7.1.1 Bottom-up

Con esta estrategia las proteínas de interés son convertidas en péptidos por acción de enzimas proteolíticas, siendo la más empleada la tripsina, y los fragmentos de péptidos resultantes se analizan por MS (Pandey & Mann, 2000). Esta estrategia se divide en dos rutas, si el fraccionamiento se realiza antes (a nivel de la proteína) o después de la digestión enzimática (a nivel de péptido). Las técnicas más antiguas involucran la utilización de la electroforesis bidimensional, donde las proteínas son separadas por su punto isoeléctrico y por su peso molecular, por lo que pueden ser extraídas del gel y digeridas en péptidos que se analizan posteriormente por MS. Las técnicas más actuales involucran la digestión enzimática en solución de las proteínas y el fraccionamiento de los péptidos resultantes por espectrometría de masas, los cuales son previamente separados por HPLC. Cuando la capacidad del pico cromatográfico es insuficiente se puede emplear cromatografía líquida multidimensional en combinación intercambio catiónico fuerte y fase inversa (Gallardo, Ortea, & Carrera, 2013). Un primer enfoque de esta metodología involucra la identificación de la huella digital de cada péptido por MS (Peptide mass fingerprint) lo que permite crear un set de péptidos único para cada proteína y sus masas ser utilizadas como huella digital para identificar la proteína original. Un segundo enfoque está basado en la información

obtenida de la disociación de uno o más péptidos con lo que se puede conocer la huella digital de cada fragmento de péptido (Peptide fragment fingerprint) (Bogdanov & Smith, 2005). En la estrategia bottom-up la digestión proteolítica de las proteínas ocurre inmediatamente después de su extracción de la matriz y un alto número de péptidos se espera que se formen, lo cual requiere de un buen método de separación. Esta vía de trabajo ha sido preferida durante muchos años por numerosos autores para el análisis de proteínas alergénicas en diferentes matrices alimentarias (Zhang & Jian, 2014). Ha permitido determinar proteínas alergénicas del maní en diferentes matrices alimentarias (Careri, et al., 2007). Así como identificar proteínas de la leche en vino (Monaci, Losito, Palmisano, & Visconti, 2010).

1.7.1.2 Top down

Este enfoque involucra la caracterización de péptidos producidos por la fragmentación de las proteínas intactas directamente dentro del espectrómetro de masa, lo cual evita el paso de digestión enzimática. Esta estrategia es posible debido al adelanto y a la resolución de los nuevos analizadores de masa. Permite revelar la identidad de las modificaciones post-translacionales (PTMs). La sensibilidad de este método puede estar empañada por la alta complejidad de los espectros de masas obtenidos caracterizados por múltiples cargas. Theberge et al (Theberge, Dikler, Heckendorf, Chui, Costello, & McComb, 2015) emplearon esta estrategia de trabajo para caracterizar las distintas variantes de hemoglobina mediante MALDI (Desorción / ionización láser asistida por matriz)-MS. Con esta estrategia de trabajo se ha logrado identificar el mayor conjunto de datos de proteoma bacteriano reportado hasta la fecha, identificando 5700 proteoformas de *Escherichia coli*. Esto a través de una plataforma de separación multidimensional combinando la cromatografía de exclusión y la cromatografía líquida de fase inversa y el prefraccionamiento de las proteínas con electroforesis capilar asociado a MS en tándem (McCool, et al., 2018). Ambas estrategias de trabajo mencionadas tienen sus debilidades y fortalezas, sin embargo, la edad de oro de la proteómica podría desvanecerse con el advenimiento de las nuevas tecnologías ómicas, como la metabolómica, que arrojan nueva luz sobre lo que está sucediendo dentro de los organismos vivos en tiempo real. *Bottom-up* permite la identificación de miles de proteínas y brinda información indirecta, basada en el

análisis de péptidos, por lo que en ocasiones no proporciona una cobertura completa de la secuencia proteica ya que tiene como limitante la capacidad de examinar sitios específicos de mutaciones y modificaciones postraduccionales. En contraste a esto, con la estrategia de trabajo top-down puede ser dilucidado el 100% de la estructura de las proteínas incluidas las diferentes isoformas y las PTMs. Sin embargo esta última presenta como desventaja con respecto a la primera que se necesitan espectrómetros de masa y técnicas lo suficientemente potentes como para lograr la fragmentación de las proteínas intactas por lo que se limita entonces a proteínas de pequeño tamaño. El desarrollo de nuevas estrategias como *top-down* para lograr un análisis de proteínas completo y directo presenta un gran desafío tecnológico, pero no insuperable, y beneficiaría enormemente a la comunidad científica.

El campo del análisis de la proteómica está cambiando debido a la complejidad que brinda el proteoma, el amplio rango de abundancia de proteínas, la existencia de múltiples formas de las proteínas debido a las modificaciones post-translacionales y a la dificultad de cuantificar el contenido absoluto de las proteínas. Algunos de estos desafíos se pueden superar con técnicas complementarias a la proteómica, las cuales involucran en general 3 pasos básicos: preparación de la muestra, análisis por espectrometría de masa y búsqueda en base de datos (Zhao, et al., 2016).

1.8 Metodología de trabajo seguida en el análisis proteómico

1.8.1 Preparación de la muestra

La preparación de la muestra es el paso crítico dentro del análisis proteómico y es el que más tiempo requiere. Generalmente involucra procesos de reducción y alquilación de las proteínas, para lograr así romper su estructura tridimensional y obtener más puntos de cortes durante la digestión enzimática. El paso de reducción se realiza con mercaptoetanol o ditiotreitol (DTT) los cuales convierten los enlaces disulfuros en grupos sulfihídrilos libres. Luego con agentes alquilantes (iodoacetamida (IAM) o acrilamida) estos grupos sulfihídrilos libres se convierten en derivados carboximetílicos impidiéndose así que la proteína vuelva a plegarse. El paso de digestión enzimática tiene como objetivo generar un grupo de péptidos con una masa adecuada para el análisis por MS. La digestión proteolítica se lleva a cabo con diferentes endoproteasas, siendo la más utilizada la tripsina por su especificidad de cortar las proteínas en el enlace amida del carbono terminal de la lisina y la

arginina. Igualmente genera péptidos de un tamaño entre 500-2500 Da adecuados para su análisis por MS. En aquellos casos en que la proteína a analizar tiene en su secuencia poca cantidad de aminoácidos lisina o arginina o el pH del medio es incompatible con la tripsina es necesario emplear otro tipo de endoproteasa. Ejemplo de esto es la pepsina la cual a pesar de ser poco específica es resistente a pH fuertemente ácidos y permite digestiones altamente reproducibles. Al igual que la tripsina y la quimotripsina es efectiva para digestiones en gel (Giansanti, Tsiatsiani, Low, & Heck, 2016). La principal desventaja de ese tipo de enzimas poco específicas es que se obtienen muchos péptidos o con un tamaño muy pequeño, lo cual resulta en espectros de masa muy complejos con dificultad para establecer una correcta identificación de la proteína de interés. Las endoproteasas Arg-C, Asp-N, Glu-C y Lys-C son altamente específicas y en muchos casos son utilizadas como alternativas a la tripsina. Entre ellas Lys-C es la menos específica pero tiene la ventaja de que puede ser utilizada bajo condiciones denaturantes severas como altas temperaturas y altas concentraciones de urea. La quimotripsina corta específicamente en los enlaces peptídicos formados por residuos aromáticos de los aminácidos tirosina, fenilalanina y triptófano. Tiene la desventaja de que resulta en péptidos de tamaño más grandes que los resultantes de la digestión con tripsina (Switzar, Giera, & Niessen, 2013).

Este proceso de digestión enzimática se puede hacer tanto en gel como en solución (Jorge, et al., 2007). La cantidad de enzima a añadir es un aspecto clave en los ensayos proteómicos. Si la concentración de enzima con respecto a la de proteína en la muestra es muy pequeña, la digestión puede no ser completa e incluso puede no encontrarse el péptido marcador. Por otra parte si es muy elevada puede ocurrir la autolisis de la tripsina generándose una gran cantidad de péptidos enzimáticos que interfieren en el análisis (Capelo, et al., 2009). Es por esto que es fundamental optimizar la relación enzima:proteína. La digestión proteolítica es el paso que más tiempo requiere dentro de la preparación de la muestra. Normalmente se realiza durante la noche (12-16 horas) a 37°C, aunque se han reportado tiempos de digestión de 24 horas debido a la heterogeneidad, cantidad y complejidad de las proteínas de la muestra. Estos largos tiempos no garantizan éxito en los resultados ni reproducibilidad en los mismos. Diferentes técnicas se han desarrollado para acelerar el proceso de digestión (Hustoft, Reubsaet, Greibrokk, Lundanes, & Malerod, 2011). Estas técnicas pueden ser agrupadas en aquellas que emplean

temperatura (calor, radiación infrarroja y digestión asistida por microondas), digestión inmovilizada (filtración asistida en la preparación de la muestra (FASP) y tripsina inmovilizada en microrreactores) y un grupo de métodos donde se agrupan otras formas de acelerar la digestión (ultrasonido y efecto de los solventes) (Hustoft, et al., 2011).

1.8.2 Métodos para acelerar la digestión proteolítica

- Calor: El uso de calor fue la primera herramienta que se utilizó para realizar la digestión enzimática. La temperatura óptima sugerida para realizar la digestión enzimática con tripsina es de 37°C, como se mencionó anteriormente. Sin embargo Havlis et al (Havliš, Thomas, Šebela, & Shevchenko, 2003) mostraron que modificando la tripsina mediante metilación su autolisis decrece y la actividad catalítica es óptima entre los 50-60°C. Y aunque esta puede ser una alternativa prometedora para obtener mejores resultados aún no ha sido ensayada en muestras complejas. Además de que el uso de tripsina modificada aumenta los costos de los análisis.

- Digestión asistida por radiación infrarroja: La energía del infrarrojo (IR) incrementa la excitación de las moléculas, lo cual favorece la interacción de la enzima con los enlaces peptídicos de la proteína (Wang, Zhang, Yang, & Chen, 2008). Este método se ha empleado en el análisis de mioglobina y albúmina de suero bovino (Hustoft, et al., 2011), logrando disminuir los tiempos de digestión a cinco minutos. Otros estudios (Hustoft, et al., 2011) no encontraron diferencias entre la digestión tríptica a 37ºC y la digestión con IR de albumina de suero bovino (BSA), lisozima de huevo de gallina, citocromo C y α-lactoalbúmina bovina. Una de las principales desventajas para el empleo de esta técnica son las cantidades tan pequeñas de proteínas que se emplean en los análisis proteómicos, además de la dificultad que resulta el control de la temperatura. Por lo cual se hace necesario continuar con estos estudios.

- Digestión asistida por microondas: La energía del microonda es capaz de acelerar la digestión enzimática debido a la rotación de las moléculas bipolares lo que induce la perturbación de la estructura tridimensional de la proteína y la digestión podría facilitarse mediante la exposición de las regiones de la proteína previamente no expuestas a la enzima proteolítica (Chen, Li, Lin, Wei, Du, & Ruan, 2014). La digestión asistida por microondas fue introducida por Pramanik en el 2002 (Pramanik, et al., 2002) como una herramienta

para aumentar la velocidad de la digestión enzimática de las proteínas. Para esto utilizó tripsina como enzima y temperaturas de microonda de 37, 45 y 55°C, con lo cual logró aumentar la temperatura de reacción y acelerar el proceso de digestión en 10 minutos. Sin embargo en otros estudios no se han encontrado diferencias en el rendimiento de la digestión obtenido utilizando microondas y la digestión convencional (Hustoft, et al., 2011). Esta contradicción hace necesario la continuidad de los estudios en cuanto a la utilización de la energía de microondas para acortar los tiempos de la digestión enzimática.

- FASP: En la preparación de la muestra asistida por filtración (FASP), la enzima no está directamente inmovilizada dentro de un dispositivo de ultrafiltración, sino que se añade disuelta dentro del filtro (Wisniewski, Zougman, Nagaraj, & Mann, 2009). El tubo de ultrafiltración actúa como un ¨reactor proteómico¨ donde ocurre la remoción del detergente, el cambio del buffer y las modificaciones químicas que tienen lugar durante la digestión. Por otra parte se ha visto que el dispositivo no es capaz de eliminar todo el detergente (habitualmente el dodecil sulfato de sodio) lo cual interfiere en el análisis por LC-MS (Hustoft, et al., 2011).

- Tripsina inmovilizada en reactores: La inmovilización de enzimas en reactores es una técnica que se utiliza desde 1950, sin embargo no es hasta las últimas décadas que este método se ha venido desarrollando. Columnas donde la tripsina es inmovilizada en un soporte sólido de alta densidad (sílica químicamente modificada) han sido introducidas entre otros por la empresa Sigma-Aldrich. Se plantea que de esta forma se acorta el tiempo de digestión a 15 minutos y se reducen los fragmentos productos de la autolisis de la tripsina. Las enzimas proteolíticas se pueden enlazar covalentemente o ser adsorbidas en diferentes portadores, como compuestos inorgánicos como la sílica y materiales órganicos que sean biocompatibles como la poliacrilamida. Este tipo de reactores parecen ser la técnica más utilizada y desarrollada en un futuro para acelerar la digestión enzimática. Consume poca cantidad de reactivos, tiene poca contribución de los fragmentos productos de la autolisis de la tripsina y tiene la posibilidad de ser acoplada en línea al análisis por LC-MALDI y LC-ESI (Ionización por electrospray). Šlechtova et al (Šlechtová, Gilar, Kalíková, Moore, Jorgenson, & Tesařová, 2017) diseñaron un prototipo de reactor para tripsina inmovilizada en una columna de UHPLC rellenada de sílica con tamaño de partícula de 5 µm y de poro 300 Å capaz de resistir presiones por encima 15000 psi y

acoplado directamente al UHPLC. Lograron la digestión de citocromo C en 4.2 segundos sin embargo observaron la presencia de cortes perdidos de la enzima por lo cual se necesitan velocidades de digestión mayores. Los autores plantean la necesidad de optimizar el tamaño de partícula del relleno, la temperatura, el solvente orgánico a emplear entre otros factores. Diferentes estrategias se han utilizado (Hustoft, et al., 2011) como portadores con tamaños de partícula diferentes, materiales monolíticos, tubos capilares abiertos y membranas donde inmovilizar la enzima, todo lo cual lleva a pensar que este será el método clave para avanzar en la preparación de la muestra en el campo de la proteómica. Sin embargo materiales con una fuerza mecánica mayor, una superficie de contacto más amplia, con una alta capacidad de carga de la enzima y mejor biocompatibilidad son necesarios. La principal desventaja asociada al uso de reactores está en el costo comercial de estos. Una preparación de estos reactores en el laboratorio requiere de personal con experiencia en la inmovilización de enzimas dentro de soportes ya que aún no existen métodos que permitan realizar este paso de manera automática.

- Otras vías de digestión:
- Digestión asistida por ultrasonido: El mecanismo por el cual la energía del ultrasonido acelera la digestión aún no está del todo dilucidado. Al parecer está relacionado con un incremento de la difusión como consecuencia del fenómeno de cavitación y el calentamiento (Kadam, Tiwari, Álvarez, & O'Donnell, 2015). El rompimiento causado por la cavitación produce grandes fuerzas mecánicas de cizallamiento que dan como resultado una degradación de la estructura de la proteína y la apertura de grupos hidrófilos. Este fenómeno estructural aumenta la solubilidad de la proteína facilitando la interacción enzima-sustrato (proteína) (Santos, et al., 2007). Su principal ventaja es que puede ser utilizada durante todo el proceso de digestión, acelera los pasos de reducción y de alquilación. El principal inconveniente de esta técnica es que requiere un equipamiento especial. Actualmente el equipo más común dentro de los laboratorios es el baño de ultrasonido sin embargo la energía brindada por este no es suficiente para lograr la digestión de las proteínas en menos tiempo que lo métodos de digestión convencionales. Los sonoreactores son los más efectivos obteniéndose con este un mayor número de péptidos mejorando así la eficiencia de la digestión (Capelo, et al., 2009).

- Efecto de los solventes: Los solventes orgánicos como el acetonitrilo pueden causar una denaturalización parcial de las proteínas lo cual favorece el proceso de digestión enzimática (Gupta & Roy, 2004), pudiendo disminuir así los tiempos de digestión incluso a una hora.

Como se ha planteado muchas de estas técnicas utilizadas para acelerar el proceso de digestión deben ser validadas y correctamente evaluadas ya que con muchas de ellas los resultados no son completos, reproducibles o confiables.

1.8.3 Separación y purificación

Un segundo paso que involucra un análisis proteómico es la separación y purificación de los péptidos. Los métodos tradicionales para generar un mapa de péptidos involucran el fraccionamiento de mezclas complejas de péptidos obtenidos a partir de la digestión proteolítica de la proteína. Este fraccionamiento se puede realizar por varias técnicas entre otras se destacan: la electroforesis en gel de policrilamida (SDS-PAGE), la cromatografía en capa fina de alta eficiencia (HPTLC) y cromatografía líquida de alta eficiencia en fase inversa (RP-HPLC) (Han, Aslanian, & Yates, 2008). En muchos casos cuando se utiliza SDS-PAGE la digestión enzimática de la proteína se puede realizar sobre el propio gel. Se requiere entonces remover los posibles contaminantes antes de realizar el análisis por espectrometría de masa. La cantidad de proteína detectada depende entre otros factores del peso molecular de la proteína, del tamaño del gel, por lo general mediante esta técnica se pueden detectar entre 5 y 10 ng de proteína por banda. En el caso específico del análisis de proteínas alergénicas en vino, donde las cantidades a detectar son muy pequeñas, se requieren técnicas más exactas y sensibles para el análisis de los péptidos como es la espectrometría de masas. El desarrollo de la cromatografía líquida (LC) revolucionó la velocidad y separación de los péptidos. LC ofrece una buena reproducibilidad, selectividad y versatilidad debido principalmente al gran número de mecanismos de separación disponibles (cromatografía de reparto, de intercambio iónico, exclusión, y de adsorción). Permite la separación de compuestos de bajo y alto peso molecular, con un amplio rango de polaridad y de diferentes propiedades ácido-base. RP-HPLC es el método más empleado para la separación de péptidos productos de la digestión enzimática sin embargo también se reporta el uso de la cromatografía de exclusión y de intercambio iónico. La alta versatilidad

de fases móviles a utilizar, de materiales de empaque, la posibilidad de emplear temperatura para mejorar la eficiencia en la separación, así como la posibilidad de acople de esta técnica a la MS han hecho que en los últimos años sea la técnica más empleada para la separación de péptidos. Los adelantos tecnológicos han permitido el acoplamiento de HPTLC a los espectrómetros masa, lo cual ha favorecido el uso de esta técnica para el análisis de péptidos pequeños e hidrófilos. HPTLC también permite analizar múltiples muestras en paralelo, salvar la separación en una placa cromatográfica para la posterior detección y cuantificación de los compuestos. HPTLC acoplada a un espectrómetro de masa con ionización por desorción con electrospray (DESI) fue empleado para separar y posteriorermente identificar péptidos resultantes de digestión enzimática con tripsina de citocromo C y mioglobina (P Pasilis, Kertesz, J Van Berkel, Schulz, & Schorcht, 2008). La capacidad que tiene esta técnica cromatográfica para ser acoplada a diversos métodos de detección como colorimétricos, anticuerpos o más recientemente aptámeros que están diseñados para interaccionar con otras proteínas (Morschheuser, et al., 2016) ha abierto nuevos horizontes en su aplicación a la proteómica.

1.8.4 Análisis por espectrometría de masas

Muchos péptidos y proteínas contienen sitios básicos capaces de protonarse por lo cual son analizables por MS. Para el análisis por ESI los analitos son ionizados directamente desde una solución y para la ionización por MALDI desde un estado sólido: en consecuencia el manejo de la muestra es diferente en ambos métodos. En ESI-MS la muestra es introducida al espectrómetro en un flujo constante por lo cual esta técnica es ideal para ser acoplada a HPLC. Es la combinación online de ESI con LC la técnica que ha tenido un gran impacto en proteómica (Cordwell, Nouwens, & Walsh, 2001). Con esta tecnología es posible la separación de los péptidos y la determinación de su masa molecular, simultáneamente. Además, este acoplamiento hace posible obtener la secuencia aminoacidica de cada péptido. La ionización por electrospray produce múltiples iones cargados los cuales pueden ser analizados con analizadores de masa convencionales como triple quadrupolo (QQQ) y/o analizador de tiempo de vuelo (TOF) (Hustoft, et al., 2011). ESI es más utilizado si se requiere realizar el análisis por MS/MS, ya que es capaz de generar péptidos con doble y triple carga los que brindan espectros con mayor calidad e información.

Para MALDI-MS el analito es co-cristalizado con una matriz que absorbe la radiación láser y promueve la ionización. Los materiales ideales para la matriz en el caso del anális de péptidos o proteínas son los ácidos aromáticos como el sinapico o el 2,4-dihidroxibenzoico. En muchos casos cuando la digestión de las proteínas se realiza sobre un gel de SDS-PAGE, el análisis de los péptidos se realiza sobre el mismo gel con el empleo de MALDI-TOF. Este método es mejor si no se desea hacer una separación cromatográfica previa, tiene buena sensibilidad y no es relativamente robusto frente a la presencia de contaminantes. La ionización por MALDI generalmente asigna solo una carga a los péptidos o proteínas en consecuencia el rango de *m/z* a analizar por el analizador de masa debe ser mucho mayor que en ESI por lo cual MALDI se usa fundamentalmente acoplado a un TOF (D. C. M. Rolland, Lim, & Elenitoba-Johnson, 2019)

Los límites de detección obtenidos por ESI o MALDI dependen de varios factores como, la naturaleza de la muestra, su preparación, pureza y el instrumento usado para la determinación. Para péptidos y proteínas se han reportado límites de detección de femtomoles, picomoles, incluso attomoles (Martin, Shabanowitz, Hunt, & Marto, 2000).

Otra técnica compatible con el análisis de mapas de péptidos a partir de pequeñas cantidades de proteínas es la combinación online de electroforesis capilar-ionización por electrospray-espectrometría de masas (CE-ESI-MS). La electroforesis capilar brinda una alta resolución en la separación y se puede complementar a RP-HPLC. La concentración de una gran cantidad de analito dentro de un pequeño volumen de aplicación requerido en la electroforesis capilar mejora los límites de detección (Wigand, Tenzer, Schild, & Decker, 2009). El principal inconveniente de esta técnica es el desarrollo de una interfase adecuada y estable para acoplar el análisis por electroforesis capilar al espectrómetro de masa.

Los analizadores de masa más empleados en proteómica son: cuadrupolo (Q), trampa de iones (IT), TOF y Orbitrap, cada uno de ellos tiene sus ventajas y debilidades. El analizador de IT por si solo permite deteminar la masa de un péptido determinado y su secuencia por MS/MS. Es robusto y sensible (en el orden de los picomoles) pero presenta en comparación con el resto de los analizadores baja resolución (1000-1500) y baja exactitud (100-1000 mg L^{-1}). El TOF por si solo permite lograr una resolución entre 10000 y 20000 con una exactitud entre 5 y 50 mg L^{-1} y sensibilidad de femtomoles. Con el advenimiento de los espectrómetros híbridos de alta resolución se ha logrado mejorar la sensibilidad y la

posibilidad de detectar un gran número de alérgenos en un solo análisis. Por ejemplo el híbrido Q-TOF permite realizar la identificación de múltiples péptidos en una sola corrida cromatográfica con una gran resolución (10000-40000), una exactitud de 5-50 mg L^{-1} y una sensiblidad de attomoles (Cunsolo, Muccilli, Saletti, & Foti, 2014). Las innovaciones y la versatilidad del analizador de masa de trampa de iones con celda dual ha permitido identificar los iones más intensos en análisis no dirigidos (Monaci, De Angelis, Montemurro, & Pilolli, 2018). De la misma manera el híbrido LTQ-Orbitrap el cual combina IT para acumulación, almacenamiento y fragmentación de los iones, con detección de masa exacta de alta resolución (Orbitrap), produce una sensitividad (femtomoles) que rivaliza la de los espectrómetros de masas triple cuadrupolo y una resolución 50000-500000) que sobrepasa los instrumentos Q-TOF con una exactitud menor a 5 mg L^{-1} (Cunsolo, et al., 2014).

Típicamente la cuantificación de proteínas alergénicas se ha realizado a partir seguir una transición ión padre/ión hijo de un péptido marcador de la proteína, mediante el empleo del método Monitoreo de Reacciones Múltiple (MRM) usando un analizador de masa QQQ. Este presenta una resolución de 1000, una exactidud entre 100 y 1500 mg L^{-1} y una sensiblidad de attomoles. En el primer cuadrupolo se selecciona el ión precursor o ión padre, el segundo cuadrupolo actúa como celda de colisión en donde el ión precursor se fragmenta y el tercer cuadrupolo aisla el ión producto o ión hijo adecuado. El espectro MS/MS del péptido producto debe ser interpretado en términos de la secuencia aminoacídica, la nomenclatura de los distintos iones resultantes de la fragmentación en la celda de colisión de toda la cadena central peptídica y de las cadenas laterales de aminoácidos ha sido bien documentada por varios autores (Ashcroft, 2003). Si la carga permanece en el fragmento *N*-terminal el ion es denominado “a”, “b”, o “c” dependiendo de qué enlace particular se escinde en la cadena peptídica. Por el contrario, si, después de los fragmentos peptídicos la carga permanece en el fragmento *C*-terminal los iones son llamados “x”, “y”, o “z”.

Para la cuantificación un péptido marcado isotópicamente con la misma secuencia que el péptido marcador puede ser empleado como estándar interno y así obtener resultados más confiables. El empleo del estándar interno permite desestimar toda la variabilidad relacionada desde la eficiencia de la digestión, el rendimiento de la extracción y reducir el

impacto de las posibles difrencias en el proceso de ionización, asumiendo que todo el proceso es idéntico tanto para la muestra como para el estándar. Sin embargo son pocos los trabajos reportados con el uso de este tipo de estándar principalmente debido a su alto precio en el mercado (Newsome & Scholl, 2013).

En general la MS de alta resolución ofrece muchos beneficios sobre la convencional (Makarov & Scigelova, 2010) y son muchas sus aplicaciones en el campo de identificar y cuantificar alérgenos en matrices alimentarias (Tabla 2). Entre otras tiene la ventaja de recopilar todos los espectros de masa en alta resolución proporciona así mayor información sobre la identidad y la estructura química de un componente alimentario. Esto permite hacer un análisis no dirigido, y la identificación de numerosos marcadores de alérgenos simultáneamente sin información preliminar.

1.8.5 Búsqueda en bases de datos

El paso final en el análisis de proteínas es la búsqueda en la base de datos con los espectros de masa adquiridos y con los datos obtenidos del análisis de MS en tándem. Los pasos previos a la obtención de los datos (preparación de la muestra, instrumento utilizado, etc) son de tan vital importancia que algunos autores (McHugh & Arthur, 2008) señalan que cerca del 90% de los espectros de masas obtenidos mediante LC-MS/MS no se pueden identificar en bases de datos debido a la mala calidad de los espectros. Esto puede estar dado por la presencia de contaminantes en la muestra, mezclas de muestras muy complejas, o digestiones y fragmentaciones incompletas. De ahí el desafío de lograr una correcta interpretación de los resultados obtenidos por el software de identificación de proteínas. Para identificar proteínas en base a la información obtenida por MS/MS se han elaborado diferentes algoritmos bioinformáticos como Mascot, Sequest, PeptidesSearch, Comet, X!tandem entre otros (Dresen, Weinmann, & Wurst, 2004). En estas bases de datos el patrón de fragmentación de los péptidos observado experimentalmente es comparado con el patrón de fragmentación calculado teóricamente. Se identifica la secuencia aminoacídica de cada péptido medido en el espectro de masa a partir de las aparecidas en la base de datos. Las bases de datos generan un *hit* que conjuntamente con un *score* representan el grado de concordancia entre los resultados experimentales y los teóricos. Otra función de evaluación ampliamente utilizada es "*coverage*". Dado que cada pico representa un fragmento del

péptido, "*coverage*" es la proporción de la proteína cubierta por estos fragmentos correspondientes. La asociación entre los péptidos identificados con la proteína de la cual provienen es un paso crítico y de gran dificultad ya que muchos péptidos son comunes a muchas proteínas. De ahí la importancia de contar con equipos y softwares que permitan la identificación de la proteína a partir de los espectros de masa de los péptidos fragmentados.
El factor limitante en todas las herramientas de identificación de proteínas es la distinción entre falsos positivos y falsos negativos. Es esencial lograr el menor número de falsos positivos durante la identificación de la proteína, ya que la identificación equivocada puede llevar a pérdida de tiempo y recursos. Igualmente es deseable identificar tantas proteínas como sea posible para obtener el máximo beneficio de los datos experimentales. Una de las dificultades en el uso de estas bases de datos es la falta de métodos que evalúen de manera global la calidad de los datos obtenidos. Usualmente estas herramientas están asociadas a una sola plataforma y a un solo espectrómetro de masas. Los distintos motores de búsqueda de estas bases de datos no producen resultados idénticos, ya que se basan en diferentes algoritmos, lo que hace que la comparación e integración de los resultados de diferentes experimentos sea dificultosa. Además, la búsqueda requiere de mucho tiempo y trabajo computacional. Inevitablemente, el investigador debe seleccionar manualmente muchos de los parámetros para la búsqueda, una gran proporción de los cuales puede que no sean relevantes para el experimento en particular. Aún no existe un consenso sobre cómo se pueden informar y comparar los resultados de las identificaciones de proteínas. Algunas revistas de proteómica ya han introducido estándares para el informe de identificación. Entre otros: información de respaldo que detalle el uso de todos los pasos de procesamiento, diseño experimental, versiones de software y bases de datos, y todos los parámetros utilizados en la búsqueda; *coverage* y / o *score*; valores de *P*; justificación de la evidencia para las identificaciones realizadas en péptidos individuales, para una proteína particular dentro de una familia de proteínas, o proteínas identificadas en otra especie; y múltiples réplicas para análisis complejos (Chandramouli & Qian, 2009) (McHugh, et al., 2008).

Tabla 2. Resumen de los métodos de análisis por MS para detectar alérgenos en productos alimenticios.

Referencia	Ingrediente alérgenico	Proteína	Producto alimenticio	Fuente de ionización	Analizador MS híbrido	LOD/LOQ
Monaci et al (Monaci, Losito, et al., 2010)	Caseínato	αS1-caseína, β-caseína	Vino blanco	ESI	Q-TOF	LOD: 50 μg mL^{-1}
Monaci et al (Monaci, Nørgaard, et al., 2010)	Leche en polvo	αS1-caseína, β-caseína, BSA	Galletas	ESI	Q-TOF	LOD: 100 μg g^{-1}
Tolin et al (Tolin, et al., 2012)	Huevo	Ovoalbúmina, ovotransferrina, lisozima, ovomicina	Vino tinto	ESI	Q-TOF	LOD: 50 μg mL^{-1}
Newsome et al (Newsome, et al., 2013)	Leche desnatada en polvo	αS1-caseína	Galletas	ESI	Q-TRAP	LOQ: 3 μg g^{-1}
Costa et al (Costa, Ansari, Mafra, Oliveira, & Baumgartner, 2014)	Avellana	Cor a 8, Cor a 9, Cor a 11	Chocolate	ESI	Q-TRAP	LOD: 1ng mL^{-1} LOQ: 2-10 ng mL^{-1}
Ji et al (Ji, et al., 2017)	β-lactoalbúmina, β-lactoglobulina, αS1-caseína	β-lactoalbúmina, β-lactoglobulina, αS1-caseína	Galletas, pasteles	ESI	Q-TOF	LOD: 0.2-0.39 μg mL^{-1} LOQ: 0.48 μg mL^{-1}
Inman et al (Inman, Groves, McCullough, Quaglia, & Hopley, 2018)	Almendra	Prunin	Comino, paprika	ESI	Q-TRAP	No determinado
Heick et al (Heick, et al., 2011)	Huevo, leche en polvo descremada, avellana, almendra, maní, nuez, soya	Ovoalbúmina, αS1-caseína, 11 S globulina, prunin, Ara h 1, glicina	Pan	ESI	Q-TRAP	LOD: 3-70 μg g^{-1}
Hoffmann et al (Hoffmann, Münch, Schwägele, Neusüß, & Jira, 2017)	Pera, lupino, soya	Conglutina β2, convicilina, glicinina G2	Carne	ESI	Q-TRAP	LOD: 2-5 μg g^{-1}
Huschek et al (Huschek, Bönick, Löwenstein, Sievers, & Rawel, 2016)	Soya, semillas de sésamo, lupino	Gly m6, Ses i6, β-conglutina	Galletas, pan, harina de trigo	ESI	Q-TRAP	LOQ: 10-50 μg g^{-1}
Parker et al (Parker, et al., 2015)	Huevo en polvo, leche descremada, harina de maní	Lisozima, ovoalbúmina, αS1-caseína, β-lactoglobulina, Ara h 1, h2 y h3	Barra de cereal, muffin	ESI	Q-TRAP	No determinado
Sealey-Voyksner et al (Sealey-Voyksner, Zweigenbaum, & Voyksner, 2016)	Maní, almendra, nuez, marañon, avellana, pistacho, coco	No especificado	Galletas, pasteles, pastas, sopas, pures, barra de cereal	ESI	Q-TOF	LOD: 0.1 μg g^{-1}
Gomma et al (Gomaa & Boye, 2015)	Caseinato, soya	αS2-, β-, κ-caseínas, glicina G1, β-conglicina	Galletas	Nano ESI	Q-TOF	LOD: 10 μg g^{-1}
Montowska et al (Montowska & Fornal, 2018)	Soya, leche, huevo	αS1-caseína, β-lactoglobulina, glicinina, β-conglicina, ovotransferrina, lisozima C	Productos de carne de ave	Nano ESI	Q-TOF	No determinado
Boo et al (Boo, Parker, & Jackson, 2018)	Huevo en polvo, leche descremada, maní	Ovoalbúmina, lisozima C, β-lactoglobulina, αS1-caseína, Ara h 1, h2 y h3	Galletas	Nano ESI	Q-TRAP	LOQ: 5 μg g^{-1}
Pilolli et al (Pilolli, De Angelis, & Monaci, 2018)	Huevo en polvo, leche descremada, maní	Gal d 2, Bos d 5, Bos d 9, Gly m 5, Gly m 6, Ara h 1, Cor a9	Calletas	ESI	Q-ORBITRAP	LOD: 4-14 μg g^{-1} LOQ: 12-50 μg g^{-1}

2. HIPOTESIS y OBJETIVOS

En base a los antecedentes planteados y discutidos en la sección anterior, se plantea la siguiente hipótesis para la presente Tesis:

2.1 Hipótesis:

Analítica

Las actuales metodologías químico-analíticas de vanguardia permiten determinar el contenido de los agentes clarificantes alergénicos caseína y ovoalbúmina en vinos.

Tecnológica

El estudio químico del proceso de clarificación de vinos permite establecer alternativas tecnológicas que disminuyan el contenido o eviten el uso de caseína y ovoalbúmina como agentes clarificantes en vinos.

2.2 Objetivo General

Desarrollar una plataforma científica tecnológica para estudiar el proceso de clarificación de vinos orientada a disminuir la prevalencia de agentes clarificantes con potencial alergénico.

2.3 Objetivos específicos

2.3.1 Desarrollar una metodología analítica que permita la extracción y digestión simultánea de caseína y ovoalbúmina desde la matriz vino.

2.3.2 Establecer una metodología analítica aplicando cromatografía líquida de ultra alta eficiencia acoplada a espectrometría de masas que permita una cuantificación inequívoca de caseína y ovoalbúmina en vinos.

2.3.3 Realizar un estudio químico sobre la efectividad de diferentes alternativas tecnológicas que reduzcan o eviten el uso de clarificantes proteicos en vino.

3. ESTRATEGIA ANALITICA

La estrategia analítica para dar cumplimiento al objetivo general, los específicos detallados en la sección precedente, se puede dividir en etapas, las cuales se ilustran en la figura 6.

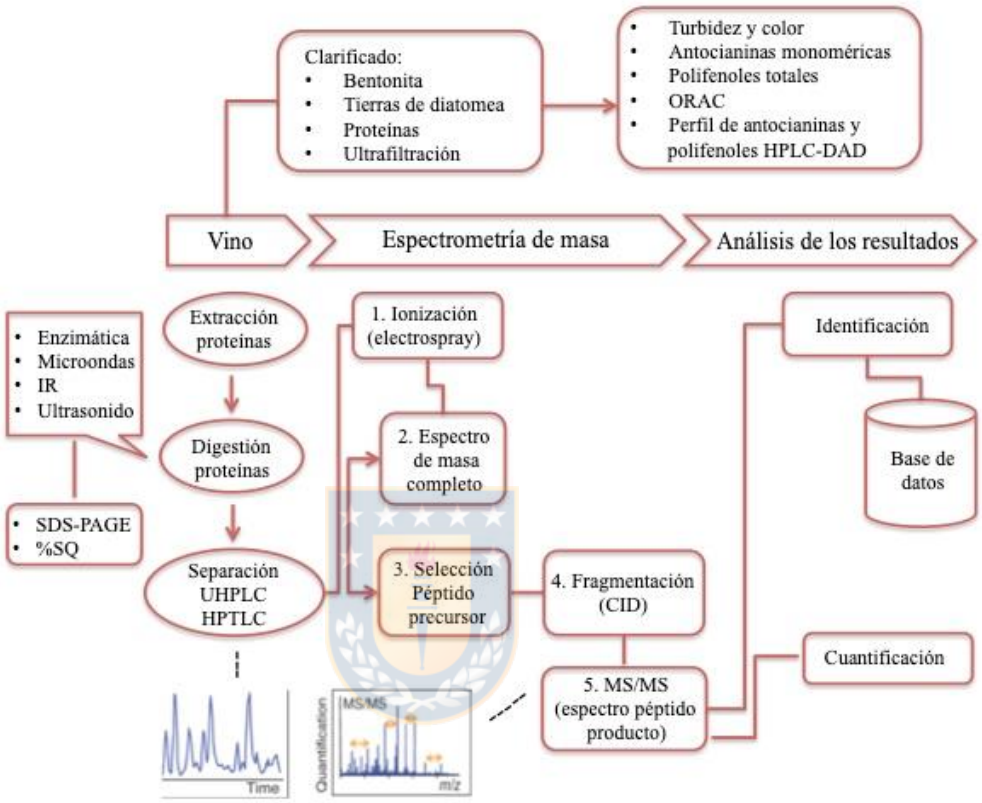

Figura 6. Estrategia analítica propuesta.

3.1 Desarrollo de una metodología de extracción de las proteínas desde la matriz vino

Una metodología analítica que permite la extracción y digestión simultánea de caseína y ovoalbúmina desde la matriz vino fue desarrollada. La extracción fue realizada utilizando tubos de ultracentrifugación con membranas de tamaño de poro 10kDa. Se ensayó como solvente de extracción una mezcla de acetona: ácido tricloroácetico sin embargo no se obtuvieron buenos resultados. Por lo que se probó adicionar 8 volumenes de 15% de ácido tricloroácetico en etanol por dos horas en hielo. En estas condiciones se logró la

precipitación de las proteínas desde el vino y el sobrenadante fue desechado. Las metodologías empleadas y los resultados obtenidos se explicitan en el Capítulo II sección 1.

3.2 Implementación y desarrollo de diferentes métodos de digestión

Se ensayaron cuatro métodos de digestión de las proteínas, para cada caso se empleó vino tinto libre de agentes clarificantes y tripsina como enzima proteolítica. Utilizando técnicas quimiométricas los métodos ensayados fueron optimizados tomando como factores el tiempo de digestión y la relación enzima: proteína. Se optimizaron la digestión convencional con calor a 37^{o}C, con energía proveniente del IR, con microondas y ultrasonido. La eficiencia de cada método fue evaluada a partir del % de sequence coverage, del análisis por SDS-PAGE con tinción de plata y mediante LC- MS/MS. Los análisis realizados, las metodologías implementadas y los resultados obtenidos se explicitan en el Capítulo II sección 2.

3.3 Análisis de las proteínas digeridas en vino mediante Ensayo de inmunoabsorción ligado a enzima (ELISA)

Se cuantificó caseína y ovoalbúmina utilizando un kit de ELISA RIDASCREEN FAST CASEIN y RIDASCREEN FAST EGG PROTEIN. Para esto se empleó vino tinto no clarificado con estas proteínas y sobrecargado con una concentración de 1.2 mg L^{-1}. Se analizaron dos muestras de vinos comerciales chilenos en los cuales, mediante LC-ESI-MS/MS estas proteínas ya habían sido cuantificadas, con la finalidad de comprobar la fiabilidad de emplear estos kits en el análisis de estas proteínas en las concentraciones tan bajas en las que se encuentran en el vino.

3.4 Análisis de las proteínas digeridas por UHPLC/MS-MS y por HPTLC-MS

Los péptidos resultantes de la digestión se identificaron y cuantificaron mediante UHPLC/MS-MS empleando un espectrométro de masa triple cuadrupolo. Para la identificación los espectros de masa fueron sometidos a análisis con las bases de datos Uniprot Swissprot y Mascot. Con este método fueron analizadas 60 muestras de vinos chilenos entre vinos blancos y tintos. El método fue validado según las recomendaciones de la International Conference of Harmonization (*ICH)* y la cuantificación se realizó con un

péptido marcado isotópicamente como estándar interno. Estos resultados al igual que los obtenidos mediante el análisis por ELISA son explicitados en el Capítulo II sección 3.

Con el objetivo de encontrar un mayor número de péptidos marcadores de caseína y ovoalbúmina en las muestras de vinos, estas también se analizaron con un espectrómetro de masa de alta resolución híbrido Q-TOF. La metodología seguida y los resultados alcanzados son explicitados en el Capítulo II sección 5.

Para la separación e identificación de los péptidos se implementó además una metodología en cromatogafía en capa fina acoplada a un espectrómetro de masa QQQ. Las metodologías implementadas y los resultados obtenidos son explicitados en el Capítulo II sección 4.

3.5 Estudio químico sobre la efectividad de diferentes alternativas tecnológicas que reduzcan o eviten el uso de clarificantes proteicos en vinos

Se ensayaron varios agentes clarificantes como alternativas al empleo de proteínas alergénicas. Para esto se empleó una muestra de vino blanco y vino tinto chileno no sometidos a ningún proceso de clarificación. Para evaluar la eficiencia de cada agente clarificante se midió turbidez, intensidad de color, antocianinas monoméricas, polifenoles totales, ORAC, perfil de antocianinas y polifenoles por HPLC-DAD, en cada caso se utilizó una muestra testigo sin tratar. Los tratamientos estudiados fueron bentonita, tierras de diatomea, caseína y ovoalbúmina, cada uno se añadió en las concentraciones recomendadas por los productores. Igualmente se sometieron las muestras de vino a un proceso de clarificación con membranas de ultrafiltración y se analizó la capacidad de esta membrana de eliminar caseína y ovoalbúmina a partir de vinos clarificados con estas proteínas. Las metodologías y los resultados obtenidos se explicitan en el Capítulo II sección 6.

4. REFERENCIAS

Ashcroft, A. (2003). Protein and Peptide Identification: The Role of Mass Spectrometry in Proteomics. *Nat. Prod. Rep, 20*(2), 202-215.

Bartowsky, E. J. (2009). Bacterial spoilage of wine and approaches to minimize it. *Lett Appl Microbiol, 48*(2), 149-156.

Bogdanov, B., & Smith, R. D. (2005). Proteomics by FTICR mass spectrometry: top down and bottom up. *Mass Spectrom Rev, 24*(2), 168-200.

Boo, C. C., Parker, C. H., & Jackson, L. S. (2018). A Targeted LC-MS/MS Method for the Simultaneous Detection and Quantitation of Egg, Milk, and Peanut Allergens in Sugar Cookies. *J AOAC Int, 101*(1), 108-117.

Capelo, J. L., Carreira, R., Diniz, M., Fernandes, L., Galesio, M., Lodeiro, C., Santos, H. M., & Vale, G. (2009). Overview on modern approaches to speed up protein identification workflows relying on enzymatic cleavage and mass spectrometry-based techniques. *Anal Chim Acta, 650*(2), 151-159.

Careri, M., Costa, A., Elviri, L., Lagos, J. B., Mangia, A., Terenghi, M., Cereti, A., & Garoffo, L. P. (2007). Use of specific peptide biomarkers for quantitative confirmation of hidden allergenic peanut proteins Ara h 2 and Ara h 3/4 for food control by liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem, 389*(6), 1901-1907.

Cassano, A., & Basile, A. (2011). 20 - Membranes for industrial microfiltration and ultrafiltration. In *Advanced Membrane Science and Technology for Sustainable Energy and Environmental Applications*, (pp. 647-679): Woodhead Publishing.

Cassano, A., Conidi, C., & Tasselli, F. (2015). Clarification of pomegranate juice (Punica Granatum L.) by hollow fibre membranes: analyses of membrane fouling and performance. *J. Chem. Technol. Biotechnol, 90*(5), 859-866.

Catarino, M. (2011). Dealcoholizing wine by membrane separation processes. *Innov food sci emerg,12*, 330-337.

Chandramouli, K., & Qian, P. (2009). Proteomics: Challenges, Techniques and Possibilities to Overcome Biological Sample Complexity. *Hum Genomics Proteomics, 2009: 239204*

Chen, Z., Li, Y., Lin, S., Wei, M., Du, F., & Ruan, G. (2014). Development of continuous microwave-assisted protein digestion with immobilized enzyme. *Biochem. Biophys. Res. Commun, 445*(2), 491-496.

Cordwell, S. J., Nouwens, A. S., & Walsh, B. J. (2001). Comparative proteomics of bacterial pathogens. *Proteomics, 1*(4), 461-472.

Cosme, F., Ricardo-da-Silva, J. M., & Laureano, O. (2008). Interactions between protein fining agents and proanthocyanidins in white wine. *Food Chem, 106*(2), 536-544.

Costa, J., Ansari, P., Mafra, I., Oliveira, M. B., & Baumgartner, S. (2014). Assessing hazelnut allergens by protein- and DNA-based approaches: LC-MS/MS, ELISA and real-time PCR. *Anal Bioanal Chem, 406*(11), 2581-2590.

Creamer, L. K., & MacGibbon, A. K. H. (1996). Some recent advances in the basic chemistry of milk proteins and lipids. *Int. Dairy J, 6*(6), 539-568.

Cunsolo, V., Muccilli, V., Saletti, R., & Foti, S. (2014). Mass spectrometry in food proteomics: a tutorial. *J Mass Spectrom, 49 (9),*768-84.

Deckwart, M., Carstens, C., Webber-Witt, M., Schäfer, V., Eichhorn, L., Schröter, F., Fischer, M., Brockow, K., Christmann, M., & Paschke-Kratzin, A. (2014). *Impact of wine manufacturing practice on the occurence of fining agents with allergenic potential* (Vol. 31).

Dordoni, R., Galasi, R., Colangelo, D., De Faveri, D. M., & Lambri, M. (2015). Effects of fining with different bentonite labels and doses on colloidal stability and colour of a Valpolicella red wine. *J. Food Sci. Technol, 50*(10), 2246-2254.

Dresen, S., Weinmann, W., & Wurst, F. M. (2004). Forensic confirmatory analysis of ethyl sulfate--a new marker for alcohol consumption--by liquid-chromatography/electrospray ionization/tandem mass spectrometry. *J Am Soc Mass Spectrom, 15*(11), 1644-1648.

EFSA Panel on Dietetic Products, N. a. A. N. (2011). Scientific Opinion related to a notification from the International Organisation of Vine and Wine (OIV) on ovalbumin/egg white to be used in the manufacture of wine as clarification processing aids. *EFSA Journal, 10*, 2385.

EFSA Panel on Dietetic Products Nutrition and Allergies. (2011). Scientific Opinion related to a notification from the International Organisation of Vine and Wine (OIV) on casein/caseinate/milk products to be

used in the manufacture of wine as clarification processing aids pursuant to Article 6, paragraph 11 of Directive 2000/13/EC – for permanent exemption from labelling. *EFSA Journal, 9*(10), 2384.

El Rayess, Y., Albasi, C., Bacchin, P., Taillandier, P., Raynal, J., Mietton-Peuchot, M., & Devatine, A. (2011). Cross-flow microfiltration applied to oenology: A review. *J MembraneSci, 382*(1), 1-19.

European Commision (EU). (2012). Commission implementing regulation 579/2012. *Official Journal of the European Union, L1*(71), 4-7.

Fane, A. G., Tang, C. Y., & Wang, R. (2011). 4.11 - Membrane Technology for Water: Microfiltration, Ultrafiltration, Nanofiltration, and Reverse Osmosis A2 - Wilderer, Peter. In *Treatise on Water Science*, (pp. 301-335). Oxford: Elsevier.

Ferrarini, R., Versari, A., & Galassi, S. (2001). *A preliminary comparison between nanofiltration and reverse osmosis membranes for grape juice treatment* (Vol. 50).

Flower, R. J. (2013). DIATOM METHODS | Diatomites: Their Formation, Distribution, and Uses. In, (pp. 501-506).

Food and Drugs Administration (Food and Drugs Administration (FDA)) (2004) Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA). https://www.fda.gov/downloads/Food/GuidanceRegulation/UCM179394.pdf. Accessed 15.03.18

FSANZ (2015) Australia New Zealand food standards code. Commonwealth of Australia. http://www.foodstandards.gov.au/code/Pages/default.aspx. . Accessed 15.03.18

Galiotti, H. (2016) Clarificación. http://campus.fca.uncu.edu.ar/pluginfile.php/22761/mod_resource/content/1/Clase%20Clarificaci%C3%B3n%20de%20Vinos.pdf.

Gallardo, M. J., Ortea, I., & Carrera, M. (2013). Proteomics and its applications for food authentication and food-technology research. *TrAC Trends Anal Chem, 52*, 135–141.

García Pacheco, R., & García-Calvo, E. (2011). Estado del arte del ensuciamiento, limpieza y modificación de la superficie de membranas de filtración para el tratamiento de disoluciones acuosas. In, (pp. 17-41).

García-Martín, N., Pérez-Magariño, S., Ortega-Heras, M., González-Huerta, C., Mihnea, M., González-SanJosé, M. L., Palacio, L., Prádanos, P., & Hernández, A. (2010). *Sugar reduction in musts with nanofiltration membranes to obtain low alcohol-content wines* (Vol. 76).

Giusti, M. M., & Wrolstad, R. E. (2001). Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy. *Current Protocols in Food Analytical Chemistry, 00*(1), F1.2.1-F1.2.13.

Gomaa, A., & Boye, J. (2015). Simultaneous detection of multi-allergens in an incurred food matrix using ELISA, multiplex flow cytometry and liquid chromatography mass spectrometry (LC-MS), *Food Chem, 175*, 585-592.

Gonçalves, F., Fernandes, C., & Pinho, M. (2001). *White wine clarification by micro/ultrafiltration: Effect of removed colloids in tartaric stability* (Vol. 22).

González-Neves, G., Favre, G., & Gil, G. (2014). Effect of fining on the colour and pigment composition of young red wines, *Food Chem, 157*, 385-392.

Güell, C., & Davis, R. H. (1996). Membrane fouling during microfiltration of protein mixtures.*J Membrane Sci, 119*(2), 269-284.

Guillou, C., Aleixandre, J., García, M., & Lizama, V. (1998). Clarification influence upon sensorial and analytical characteristics of Muscat dry wine. *J Int Sci Vigne Vin, 32*(2), 8.

Gupta, M. N., & Roy, I. (2004). Enzymes in organic media. Forms, functions and applications. *Eur Biochem, 271*(13), 2575-2583.

Gutiérrez, I. H., Lorenzo, E. S.-P., & Espinosa, A. V. (2005). Phenolic composition and magnitude of copigmentation in young and shortly aged red wines made from the cultivars, Cabernet Sauvignon, Cencibel, and Syrah. *Food Chem, 92*(2), 269-283.

Han, X., Aslanian, A., & Yates, J. R., 3rd. (2008). Mass spectrometry for proteomics. *Curr Opin Chem Biol, 12*(5), 483-490.

Havliš, J., Thomas, H., Šebela, M., & Shevchenko, A. (2003). Fast-Response Proteomics by Accelerated In-Gel Digestion of Proteins. *Anal Chem, 75*(6), 1300-1306.

Heick, J., Fischer, M., & Popping, B. (2011). First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. *J Chromatogr A, 1218*(7), 938-943.

Ho, M., Chan, J. S.-L., & Lee, T. H. (2014). Guideline for the diagnosis and management of cow´s milk protein allergy (CMPA) in Hong Kong. In).

Hoffmann, B., Münch, S., Schwägele, F., Neusüß, C., & Jira, W. (2017). A sensitive HPLC-MS/MS screening method for the simultaneous detection of lupine, pea, and soy proteins in meat products. *Food Control, 71*, 200-209.

Huschek, G., Bönick, J., Löwenstein, Y., Sievers, S., & Rawel, H. (2016). Quantification of allergenic plant traces in baked products by targeted proteomics using isotope marked peptides. *LWT - Food Sci Technol, 74*, 286-293.

Hustoft, H. K., Reubsaet, L., Greibrokk, T., Lundanes, E., & Malerod, H. (2011). Critical assessment of accelerating trypsination methods. *J Pharm Biomed Anal, 56*(5), 1069-1078.

Inman, S. E., Groves, K., McCullough, B., Quaglia, M., & Hopley, C. (2018). Development of a LC-MS method for the discrimination between trace level Prunus contaminants of spices. *Food Chem, 245*, 289-296.

Ivanov, M., Levitsky, L., Lobas, A., Tarasova, I., Pridatchenko, M., Zgoda, V., Moshkovski, S., Mitulović, G., & Gorshkov, M. (2015). Peptide identification in "shotgun" proteomics using tandem mass spectrometry: Comparison of search engine algorithms. *J Anal Chem, 70*(14), 1614-1619.

Jackson, M. G., Timberlake, C. F., Bridle, P., & Vallis, L. (1978). Red wine quality: Correlations between colour, aroma and flavour and pigment and other parameters of young Beaujolais. *J Sci Food Agric, 29*(8), 715-727.

Jayprakasha, G. K., Patil, B. S., & Pellati, F. (2014). Instrumental Methods for the Analysis and Identification of Bioactive Molecules. In *ACS Symposium Series*, vol. 1185 (pp. 0): American Chemical Society.

Ji, J., Zhu, P., Pi, F., Sun, C., Sun, J., Jia, M., Ying, C., Zhang, Y., & Sun, X. (2017). Development of a liquid chromatography-tandem mass spectrometry method for simultaneous detection of the main milk allergens. *Food Control, 74*, 79-88.

Jorge, I., Casas, E. M., Villar, M., Ortega-Pérez, I., López-Ferrer, D., Martínez-Ruiz, A., Carrera, M., Marina, A., Martínez, P., Serrano, H., Cañas, B., Were, F., Gallardo, J. M., Lamas, S., Redondo, J. M., García-Dorado, D., & Vázquez, J. (2007). High-sensitivity analysis of specific peptides in complex samples by selected MS/MS ion monitoring and linear ion trap mass spectrometry: Application to biological studies. *J Mass Spectrom, 42*(11), 1391-1403.

Ju, Y.-l., Yang, B.-h., He, S., Tu, T.-y., Min, Z., Fang, Y.-l., & Sun, X.-y. (2019). Anthocyanin accumulation and biosynthesis are modulated by regulated deficit irrigation in Cabernet Sauvignon (Vitis Vinifera L.) grapes and wines. *Plant Physiol Bioch, 135*, 469-479.

Kadam, S. U., Tiwari, B. K., Álvarez, C., & O'Donnell, C. P. (2015). Ultrasound applications for the extraction, identification and delivery of food proteins and bioactive peptides. *Trends Food Sci Tech, 46*(1), 60-67.

Karadag, A., Ozcelik, B., & Saner, S. (2009). *Review of Methods to Determine Antioxidant Capacities* (Vol. 2).

Kaul, S., Luttkopf, D., Kastner, B., Vogel, L., Holtz, G., Vieths, S., & Hoffmann, A. (2007). Mediator release assays based on human or murine immunoglobulin E in allergen standardization. *Clin Exp Allergy, 37*(1), 141-150.

Lambri, M., Dordoni, R., Giribaldi, M., Violetta, M. R., & Giuffrida, M. G. (2012). Heat-unstable protein removal by different bentonite labels in white wines. *LWT - Food Sci Technol, 46*(2), 460-467.

Ledoux, V., Dulau, L., & Dubourdieu, D. (1992). *Interprétation de l'amélioration de la stabilité protéique des vins au cours de l'élevage sur lies* (Vol. 26).

Li, H., & Moore, S. (2006). Security evaluation at design time against optical fault injection attacks. In *IEE Proceedings - Information Security*, vol. 153 (pp. 3-11).

Lira, E., Rodríguez-Bencomo, J. J., Salazar, F. N., Orriols, I., Fornos, D., & López, F. (2015). Impact of Bentonite Additions during Vinification on Protein Stability and Volatile Compounds of Albariño Wines. *J Agric Food Chem, 63*(11), 3004-3011.

Lu, Y., & Luthria, D. (2014). Influence of Postharvest Storage, Processing, and Extraction Methods on the Analysis of Phenolic Phytochemicals. In *Instrumental Methods for the Analysis and Identification of Bioactive Molecules*, vol. 1185 (pp. 3-31): American Chemical Society.

Makarov, A., & Scigelova, M. (2010). Coupling liquid chromatography to Orbitrap mass spectrometry. *J Chromatogr A, 1217*(25), 3938-3945.

Marchal, R., Marchal-Delahaut, L., Lallement, A., & Jeandet, P. (2002). Wheat gluten used as a clarifying agent of red wines. *J Agric Food Chem, 50*(1), 177-184.

Margalit, Y. (2004). *Concepts in wine technology* (1 ed.). San Francisco, Calif: Wine Appreciation Guild.

Martin, S. E., Shabanowitz, J., Hunt, D. F., & Marto, J. A. (2000). Subfemtomole MS and MS/MS Peptide Sequence Analysis Using Nano-HPLC Micro-ESI Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. *Anal Chem, 72*(18), 4266-4274.

Martínez-Lapuente, L., Guadalupe, Z., & Ayestarán, B. (2017). Effect of egg albumin fining, progressive clarification and cross-flow microfiltration on the polysaccharide and proanthocyanidin composition of red varietal wines. *Food Res Int, 96*, 235-243.

Mattarozzi, M., Milioli, M., Bignardi, C., Elviri, L., Corradini, C., & Careri, M. (2014). Investigation of different sample pre-treatment routes for liquid chromatography–tandem mass spectrometry detection of caseins and ovalbumin in fortified red win. *Food Control, 38*, 82-87.

McCool, E. N., Lubeckyj, R. A., Shen, X., Chen, D., Kou, Q., Liu, X., & Sun, L. (2018). Deep Top-Down Proteomics Using Capillary Zone Electrophoresis-Tandem Mass Spectrometry: Identification of 5700 Proteoforms from the Escherichia coli Proteome. *Anal Chem, 90*(9), 5529-5533.

McHugh, L., & Arthur, J. (2008). Computational methods for protein identification from mass spectrometry data. *PLoS Comput Biol, 4 (2)*.

Meistermann, E., & Pinsun, M. (2018). *Treatment with different fining agents of white musts from spoiled wine grapes* (Vol. 6).

Mohammad, A. W., Teow, Y. H., Ang, W. L., Chung, Y. T., Oatley-Radcliffe, D. L., & Hilal, N. (2015). Nanofiltration membranes review: Recent advances and future prospects. *Desalination, 356*, 226-254.

Monaci, L., De Angelis, E., Montemurro, N., & Pilolli, R. (2018). Comprehensive overview and recent advances in proteomics MS based methods for food allergens analysis. *TrAC Trend Anal Chem, 106*, 21-36.

Monaci, L., Losito, I., Palmisano, F., & Visconti, A. (2010). Identification of allergenic milk proteins markers in fined white wines by capillary liquid chromatography-electrospray ionization-tandem mass spectrometry. *J Chromatogr A, 1217*(26), 4300-4305.

Monaci, L., Nørgaard, J. V., & van Hengel, A. J. (2010). Feasibility of a capillary LC/ESI-Q-TOF MS method for the detection of milk allergens in an incurred model food matrix. *Anal Methods, 2*(7), 967-972.

Monaci, L., & Visconti, A. (2009). Mass spectrometry-based proteomics methods for analysis of food allergens. *Trac-Trend Anal Chem, 28*(5), 581-591.

Monaci., Losito, I., Palmisano, F., Godula, M., & Visconti, A. (2011). Towards the quantification of residual milk allergens in caseinate-fined white wines using HPLC coupled with single-stage Orbitrap mass spectrometry. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 28*(10), 1304-1314.

Montowska, M., & Fornal, E. (2018). Detection of peptide markers of soy, milk and egg white allergenic proteins in poultry products by LC-Q-TOF-MS/MS. *LWT - Food Sci Technol, 87*, 310-317.

Morschheuser, L., Wessels, H., Pille, C., Fischer, J., Hünniger, T., Fischer, M., Paschke-Kratzin, A., & Rohn, S. (2016). HPTLC-aptastaining - Innovative protein detection system for high-performance thin-layer chromatography. *Sci Rep, 6*, 26665-26665.

Mutanen, J., Räty, J., Gornov, E., Lehtonen, P., Peiponen, K.-E., & Jaaskelainen, T. (2007). Measurement of Color, Refractive Index, and Turbidity of Red Wines. *American J Enol Vitic, 58*(Han, et al.), 387.

Newsome, G. A., & Scholl, P. F. (2013). Quantification of allergenic bovine milk alpha(S1)-casein in baked goods using an intact (1)(5)N-labeled protein internal standard. *J Agric Food Chem, 61*(24), 5659-5668.

ODEPA (2018) Una mirada al mercado vitivinícola nacional e internacional. https://www.odepa.gob.cl/wp-content/uploads/2018/02/vino2018.pdf.

Organisation Internationale de la Vigne et du Vin (OIV) (2000) Compendium of International Methods of Analysis–OIV Wine turbidity. http://www.oiv.int/public/medias/2476/oiv-ma-as2-08.pdf. Accessed 15.03.18

Organisation Internationale de la Vigne et du Vin (OIV) (2001) Compendium of International Methods of Analysis–OIV Chromatic Characteristics. http://www.oiv.int/public/medias/2475/oiv-ma-as2-07b.pdf. Accessed 18.03.18

Organisation Internationale de la Vigne et du Vin (OIV) (2006) Compendium of International Analysis of Methods - OIV Chromatic Characteristics. http://www.oiv.int/public/medias/2478/oiv-ma-as2-11.pdf. Accessed 21.03.18

Organisation Internationale de la Vigne et du Vin (OIV) (2012) Revision of the limit of detection and limit of quantiifcation related to potentially allergenic residues of finning agent proteins in wine. http:// www.oiv.int/public/medias/2154/oiv-comex-502-2012-en.pdf. Accessed 15.03.18

Organisation Internationale de la Vigne et du Vin (OIV) (2018) Datos de la coyuntura vitivinícola mundial http://www.oiv.int/public/medias/6305/oiv-comunicado-de-prensa-los-datos-de-la-coyuntura-vitivin-c.pdf. Accessed 3.01.19

P Pasilis, S., Kertesz, V., J Van Berkel, G., Schulz, M., & Schorcht, S. (2008). Using HPTLC/DESI-MS for peptide identification in 1D separations of tryptic protein digests. *Anal Bioanal Chem, 391*(1), 317-324.

Pali-Schöll, I., Verhoeckx, K., Mafra, I., Bavaro, S. L., Clare Mills, E. N., & Monaci, L. (2019). Allergenic and novel food proteins: State of the art and challenges in the allergenicity assessment. *Trends Food Sci Tech, 84*, 45-48.

Pandey, A., & Mann, M. (2000). Proteomics to study genes and genomes. *Nature, 405*(6788), 837-846.

Parker, C. H., Khuda, S. E., Pereira, M., Ross, M. M., Fu, T. J., Fan, X., Wu, Y., Williams, K. M., DeVries, J., Pulvermacher, B., Bedford, B., Zhang, X., & Jackson, L. S. (2015). Multi-allergen Quantitation and the Impact of Thermal Treatment in Industry-Processed Baked Goods by ELISA and Liquid Chromatography-Tandem Mass Spectrometry. *J Agric Food Chem, 63*(49), 10669-10680.

Patrick, W., Hans, S., & Angelika, P. (2009). Determination of the Bovine Food Allergen Casein in White Wines by Quantitative Indirect ELISA, SDS−PAGE, Western Blot and Immunostaining. *J Agric and Food Chem, 57*(18), 8399-8405.

Pilolli, R., De Angelis, E., & Monaci, L. (2018). In house validation of a high resolution mass spectrometry Orbitrap-based method for multiple allergen detection in a processed model food. *Anal Bioanal Chem, 410*(22), 5653-5662.

Poms, R. (2013). MoniQA's contribution towards a global harmonisation of foodstuff quality and safety assessment and monitoring strategies. *Qual Assur Saf crop,, 5*(1), 3-6.

Pramanik, B. N., Mirza, U. A., Ing, Y. H., Liu, Y.-H., Bartner, P. L., Weber, P. C., & Bose, A. K. (2002). Microwave-enhanced enzyme reaction for protein mapping by mass spectrometry: a new approach to protein digestion in minutes. *Protein science : a publication of the Protein Society, 11*(11), 2676-2687.

Programme, E. T. B. P. (1997). *Cost-effective Membrane Technologies for Minimising Wastes and Effluents*: Environmental Technology Best Practice Programme.

Rabilloud, T., Chevallet, M., Luche, S., & Lelong, C. (2010). Two-dimensional gel electrophoresis in proteomics: Past, present and future. *J Proteomics, 73*(11), 2064-2077.

Rankine, B. (1996). *Making Good Wine A Manual of Winemaking Practice for Australia and New Zealand.* New Zealand.

Rolland, D. C. M., Lim, M. S., & Elenitoba-Johnson, K. S. J. (2019). Mass spectrometry and proteomics in hematology. *Semin Hematol, 56*(1), 52-57.

Rolland, J. M., Apostolou, E., Deckert, K., de Leon, M. P., Douglass, J. A., Glaspole, I. N., Bailey, M., Stockley, C. S., & O'Hehir, R. E. (2006). Potential food allergens in wine: double-blind, placebo-controlled trial and basophil activation analysis. *Nutrition, 22*(9), 882-888.

Sabato, V., J van Hengel, A., J De Knop, K., M Verweij, M., Hagendorens, M., Bridts, C., Clerck, L., Schiavino, D., Stevens, W., & Ebo, D. (2011). Human Basophils: A Unique Biological Instrument to Detect the Allergenicity of Food. *Journal of investigational allergology & clinical immunology: official organ of the International Association of Asthmology (INTERASMA) and Sociedad Latinoamericana de Alergia e Inmunología, 21* 179-184.

Salehi, F. (2014). Current and future applications for nanofiltration technology in the food processing. *Food Bioprod Process, 92*(2), 161-177.

Santos, H. M., Rial-Otero, R., Fernandes, L., Vale, G., Rivas, M. G., Moura, I., & Capelo, J. L. (2007). Improving sample treatment for in-solution protein identification by peptide mass fingerprint using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J Proteome Res, 6*(9), 3393-3399.

Saxena, A., Tripathi, B. P., Kumar, M., & Shahi, V. K. (2009). Membrane-based techniques for the separation and purification of proteins: An overview. *Adv Colloid Interfac, 145*(1), 1-22.

Sealey-Voyksner, J., Zweigenbaum, J., & Voyksner, R. (2016). Discovery of highly conserved unique peanut and tree nut peptides by LC-MS/MS for multi-allergen detection. *Food Chem, 194*, 201-211.

Shu-Sen, W., Al-Lian, L., Ling-Ling, W., & De-Kuan, K. (1989). Ultrafiltration of rice wine for removal of bacteria. *J Membrane Sci, 44*(2), 245-252.

Šlechtová, T., Gilar, M., Kalíková, K., Moore, S. M., Jorgenson, J. W., & Tesařová, E. (2017). Performance comparison of three trypsin columns used in liquid chromatography. *J Chromatogr A, 1490*, 126-132.

Swaisgood, H. (1993). *Review and Update of Casein Chemistry* (Vol. 76).

Switzar, L., Nicolardi, S., Rutten, J. W., Oberstein, S. A., Aartsma-Rus, A., & van der Burgt, Y. E. (2016). In-Depth Characterization of Protein Disulfide Bonds by Online Liquid Chromatography-Electrochemistry-Mass Spectrometry. *J Am Soc Mass Spectrom, 27*(1), 50-58.

Switzar, L., Giera, M. & Niessen, W. (2013). Protein digestion: an overview of the available techniques and recent developments. *J Proteome Res, 12 (3),* 1067-77.

Tabilo-Munizaga, G., Gordon, T. A., Villalobos-Carvajal, R., Moreno-Osorio, L., Salazar, F. N., Perez-Won, M., & Acuna, S. (2014). Effects of high hydrostatic pressure (HHP) on the protein structure and thermal stability of Sauvignon blanc wine. *Food Chem, 155*, 214-220.

Theberge, R., Dikler, S., Heckendorf, C., Chui, D. H., Costello, C. E., & McComb, M. E. (2015). MALDI-ISD Mass Spectrometry Analysis of Hemoglobin Variants: a Top-Down Approach to the Characterization of Hemoglobinopathies. *J Am Soc Mass Spectrom, 26*(8), 1299-1310.

Togores, H. (2003). *Tratado de Enología* (Vol. I y II). Madrid: Mundi-Prensa.

Tolin, S., Pasini, G., Curioni, A., Arrigoni, G., Masi, A., Mainente, F., & Simonato, B. (2012). Mass spectrometry detection of egg proteins in red wines treated with egg white. *Food Control, 23*(1), 87-94.

Turkmen, N., Sari, F., & Velioglu, Y. S. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin–Ciocalteu methods. *Food Chem, 99*(4), 835-841.

Ulbricht, M., Ansorge, W., Danielzik, I., König, M., & Schuster, O. (2009). Fouling in microfiltration of wine: The influence of the membrane polymer on adsorption of polyphenols and polysaccharides. *Sep Purif Technol, 68*, 335-342.

Urkiaga, A., De Las Fuentes, L., Acilu, M., & Uriarte, J. (2002). Membrane comparison for wine clarification by microfiltration. *Desalination, 148*(1), 115-120.

Van der Bruggen, B., Mänttäri, M., & Nyström, M. (2008). Drawbacks of applying nanofiltration and how to avoid them: A review. *Sep Purif Technol, 63*(2), 251-263.

Wang, S., Zhang, L., Yang, P., & Chen, G. (2008). Infrared-assisted tryptic proteolysis for peptide mapping. *Proteomics, 8*(13), 2579-2582.

Weber, P., Steinhart, H., & Paschke, A. (2007). Investigation of the Allergenic Potential of Wines Fined with Various Proteinogenic Fining Agents by ELISA. *J Agric Food Chem, 55*(8), 3127-3133.

Wigand, P., Tenzer, S., Schild, H., & Decker, H. (2009). Analysis of Protein Composition of Red Wine in Comparison with Rosé and White Wines by Electrophoresis and High-Pressure Liquid Chromatography−Mass Spectrometry (HPLC-MS). *J Agric Food Chem, 57*(10), 4328-4333.

Wilkins, M. R., Sanchez, J.-C., Gooley, A. A., Appel, R. D., Humphery-Smith, I., Hochstrasser, D. F., & Williams, K. L. (1996). Progress with Proteome Projects: Why all Proteins Expressed by a Genome Should be Identified and How To Do It AU - Wilkins, Marc R. *Biotechnol Genet Eng Reviews, 13*(1), 19-50.

Wisniewski, J. R., Zougman, A., Nagaraj, N., & Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nat Methods, 6*(5), 359-362.

Zhang, S., & Jian, W. (2014). Recent advances in absolute quantification of peptides and proteins using LC-MS. *Rev Anal Chem, 33*(1), 31-47.

Zhao, Y., Chang, C., Qin, P., Cao, Q., Tian, F., Jiang, J., Li, X., Yu, W., Zhu, Y., He, F., Ying, W., & Qian, X. (2016). Mining the human plasma proteome with three-dimensional strategies by high-resolution Quadrupole Orbitrap Mass Spectrometry. *Anal Chim Acta, 904*, 65-75.

CAPITULO II: Resultados y Discusión

CAPITULO II SECCION 1

Title: Mass spectrometry determination of fining-related allergen proteins in Chilean wines.

Jessy Pavón-Pérez[a], Karem Henriquez-Aedo[a,b], Mario Aranda[a,b]*

[a] Laboratory of Advanced Research on Foods and Drugs, Department of Food Science and Technology, Faculty of Pharmacy, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile.

[b] Laboratory of Functional Foods, Center for Biotechnology, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile.

*Corresponding author: Dr. Mario Aranda, Laboratory of Advanced Research on Foods and Drugs, Department of Food Science and Technology, Faculty of Pharmacy, University of Concepcion, Barrio Universitario s/n Concepcion, Chile.

Email: maranda@udec.cl; maranda@gmx.net.

Telephone: +56-41-2203032. Fax: +56-41-2210568

Formato manuscrito aceptado el 10 de diciembre del 2018 en Food Analytical Methods.

DOI: https://doi.org/10.1007/s12161-018-01416-0

Abstract

Casein (milk powder) and ovalbumin (egg-white powder) are often used as wine fining agents with the objective to promote interactions with undesirable compounds, e.g. polymeric phenols. This kind of proteins may trigger allergic reactions in susceptible individuals; therefore, their occurrence in wines could become a human health risk, moreover when their presence is not reported. The objective of this work was to establish a mass spectrometry method to determine casein and ovalbumin in Chilean wines. To the best of our knowledge the present work reports for the first time the presence of these proteins in Chilean wines. Proteins were extracted combining the use of ultrafiltration membranes and protein precipitation with organic solvents. Thereafter, proteins were digested with trypsin during 7 hours with a 1:10 enzyme protein ratio. Chromatographic separation was carried out on a Kinetex XB C_{18} (100 x 4.6 mm, 2.6 µm) column set at 35ºC using an acidified (0.1% v/v formic acid) mobile phase composed of ultrapure water (A) and acetonitrile (B). For a highly selective evaluation, quantification was carried out by mass spectrometry applying multiple reaction monitoring (MRM). Validation was established according to the International Conference Harmonization guidelines. Calibration data (n=6) fitted a linear regression model with determination coefficients (R^2) higher than 0.99. Repeatability (n=6) and intermediate precision (n=3) showed RSD values ≤1.36% and ≤1.53%, respectively. Recovery (n=3) at three levels ranged from 86.40 to 106.98%. Detection and quantification limits ranged from 4.70 to 8.50 µg L^{-1} and 10 to 20 µg L^{-1}, respectively. Twenty Chilean wine samples were analyzed, eighteen samples showed quantifiable levels, from which three samples presented a total casein and ovalbumin concentration (0.24-0.26 mg L^{-1}) closer or higher to the European limit for mandatory labelling (0.25 mg L^{-1}).

Keywords: Casein; ovalbumin; mass spectrometry; wines, fining agents

1. Introduction

Wine grape production is a very dynamic sector of Chile's economy in terms of productivity, exports and employment. Chile is located within the ten principal worldwide wine producers with over 10 mhL produced during 2016, which are equivalent to almost 4% of the world's produced volume. It ranks in the fourth position of largest wine exporter with 9.1 mhL exported in 2016, which represents almost €1.7 billion euros (Organisation Internationale de la Vigne et du Vin (OIV) 2017). Chilean wines have international recognition due to its particular sensorial and chemical profiles, both extensively investigated (Bridi et al. 2014; Mendoza et al. 2011). During winemaking, some fining agents are used with the aim of promoting interactions with undesirable compounds, especially polymeric phenols and polyphenols (Dordoni et al. 2015; Losito et al. 2013; Restani et al. 2014). Commonly, caseinate or milk powders are used for white wine fining, and ovalbumin or egg-white powders proteins are used for red wines (González-Neves et al. 2014). The use of allergenic proteins in wines and their unlabeled presence in the final product has been subject of debate in the last years due to the health risk for susceptible consumers. Thus, its determination in wines is highly desirable to ensure consumer's health safety. For this reason the European Union (EU) implemented the Directive 89/2003/EC and its latest version 579/2012/EC, which contain a list of allergenic substances including egg and milk derivatives (European Commission (EU) 2012). This document indicates the obligation of declaring the presence of egg and milk proteins when the concentration in wines is higher than the analytical limit described in OIV/COMEX/502/2012 resolution (Organisation Internationale de la Vigne et du Vin (OIV) 2012). The OIV official method for egg and milk derivatives determination in wines is based on enzyme-linked immunosorbent assay (ELISA), which present detection and quantification limits of 0.25 mg L^{-1} and 0.50 mg L^{-1}, respectively. Immunoassays analysis are simple and fast (Kerkaert et al. 2010) but present some drawbacks and limitations. In red wines the high content of polyphenols could cause detection problems due its interaction with proteins and antibodies (Patrick et al. 2009). Also, allergens adsorption to solid matrix could produce some kind of alteration of protein epitopes modifying its recognition or accessibility, thus, impairing the assay sensitivity (Kaul et al. 2007). Due to that, proteomic analysis by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Mainente et al. 2014) and

liquid chromatography (LC) (Mattarozzi et al. 2014) have become valued options, moreover considering that proteins and peptides separation is a key-step for this kind of analysis. 2D-PAGE is a labor-intensive technique with some limitations in detecting hydrophobic and alkaline proteins (Chambery et al. 2009). This technique has not achieved an important automatization, forcing to researchers to search others alternatives for protein separation. In this scenario systems like liquid chromatography coupled to mass spectrometry (LC-MS) has become the most suitable technique for protein analysis. Currently, the techniques-of-choice to determine food allergen proteins are LC–electrospray ionization (ESI) and Matrix-Assisted Laser Desorption/Ionization (MALDI), both in tandem mass spectrometry (LC-ESI or MALDI-MS/MS). These techniques offer several advantages, including multi-allergen detection and unambiguous identification and characterization of food allergen (Cristina et al. 2016). Due to the diversity of molecular weights and the different peptides and proteins properties, there is not an unique workflow suitable for all LC-ESI or MALDI-MS/MS analyses (Zhang and Jian 2014). Proteomic analysis can be performed applying two different strategies, top down and bottom up. The first one involves direct analyzes of intact proteins without prior digestion (Monaci and Visconti 2009), for which the most convenient and used ionization technique is MALDI. In bottom-up approach, identification is performed comparing MS/MS spectra of protein digestion products (peptides) against protein-sequence databases (Monaci and Visconti 2009). This is the most preferred strategy to perform proteomics studies because the obtained spectra are much easier to interpret than those obtained by top-down. For quantitative purposes bottom-up experimental workflow is generally based on multiple reaction monitoring (MRM) detection of selected parent and fragmentation ions (Mattarozzi et al. 2014). The present work reports a fast and highly selective method to determine casein and ovalbumin in wines using LC separation coupled to ESI-triple quadrupole (TQ) mass spectrometry. To the best of our knowledge this work reports for the first time the evaluation of fining-related allergen proteins in Chilean wines. Prior to LC/MS/MS analysis, proteins are commonly extracted by ultrafiltration and then digested using trypsin enzyme. Several sample preparation approaches have been reported with the aim to reduce the analysis time (Careri et al. 2007; Gallardo et al. 2013; Heick et al. 2011; Monaci et al. 2014). In particular, protein digestion is considered the bottleneck of bottom-

up proteomics analyses due to the long time required for digestion (12-24h). Trypsin is the most used enzyme because it exclusively cleaves basic residues to arginine and lysine (Loziuk et al. 2013), generating peptides with an adequate mass range, which facilitate the sequence detection. In order to reduce digestion time, the present work optimized trypsin digestion conditions applying a central composite design based on reported digestion conditions (Calleri et al. 2005; Chiva et al. 2014; Fremout et al. 2010) and optimized protocols (Proc et al. 2010; Walmsley et al. 2013).

2. Material and methods

2.1 Reagents, standards and samples

Formic acid (98-100%), trichloroacetic acid (TCA, >99%), ammonia solution (>25%), ammonium hydrogen carbonate (NH_4HCO_3, 99%), ethanol absolute, LC-grade acetonitrile and Amicon ultra-centrifugal filter devices (10 kDa cut-off membrane) were purchased from Merck (Darmstadt, Germany). Casein from bovine milk, albumin from chicken egg white, iodoacetamide (IAM, >99%), DL- dithiothreitol (DTT, >99 %) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Trypsin from bovine pancreas was obtained from BDH Biochemicals (Poole, England). Buffered solutions and mobile phase were prepared with ultrapure water (18.2 MΩ cm) produced by means of Simplicity system from Millipore (Bedford, MA, USA). Casein and ovalbumin stock solutions were prepared in 50 mM NH_4HCO_3 (pH 7.8) for a given concentration of 12 mg L^{-1}. Standard solutions were obtained by aliquot dilution from stock solution. All solutions stored refrigerated were stable for at least twenty days. A total of 20 Chilean commercial wine samples without allergen labelling were analyzed. All samples were purchased directly from supermarkets and specialized stores without contact with wineries.

2.2 Sample preparation

Allergen proteins were extracted and purified from wine samples following the method proposed by Mattarozzi et al. (2014) with slight modifications. Briefly, 12.5 mL of wine were centrifuged using a Hettich (Tuttlingen, Germany) centrifuge at 5433 x *g* for 40 min at 20ºC into 10 kDa cut-off membrane ultrafiltration tube, previously conditioned with 10 mL of distilled water. 2.5 mL were recovered from which proteins were precipitated for 2 h on

ice after addition of eight volumes of ethanol: TCA (15%w/v), pH 4.5. Thereafter, the mixture was centrifuged at 8981 x *g* for 10 min at 4ºC using a Hettich refrigerated centrifuge. Ethanol: TCA residues were evaporated under a gentle flow of nitrogen. The obtained pellet was solubilized in 1 mL of 50 mM NH_4HCO_3, pH 7.8. Trypsin digestion of both, sample and standards, was performed after proteins reduction and alkylation. The first one was carried out by addition of 12 µL of 100 mM DTT and incubation at 95ºC for 5 min. After cooling down, the cysteine SH-groups, formed during the reduction step, were irreversibly alkylated adding 24 µL of freshly prepared aqueous solution of 100 mM IAM, the mixture was left stand protected from light for 15 min at room temperature. Enzymatic digestion was performed adding 4 µL of trypsin solution (0.5 mg mL^{-1} in 50 mM of NH_4HCO_3, pH 7.8) obtaining an enzyme: protein ratio of 1:10, the mixture was incubated at 37ºC for 7 h. The reaction was stopped by addition of 10 µL of formic acid (0.1%v/v) aqueous solution. All digested samples and standards were filtered through 13 mm PVDF syringe filter (0.22 µm) before LC injection.

2.3 Liquid chromatography – tandem mass spectrometry

For LC-MS analyses a Shimadzu (Kyoto, Japan) Nexera X2 UHPLC system consisted of: LC-30AD pump, DGU-20A5R degassing unit, SIL-30AC autosampler, CTO-20AC column oven, CBM-20A communication module, SPD-M20A diode array detector (DAD) and LCMS-8030 triple quadrupole (TQ) mass spectrometer (MS) was used. The system was controlled by Shimadzu LabSolution 5.8 software. Peptides separation was carried out on Phenomenex (Torrance, CA, USA) Kinetex XB Core-Shell C_{18} column (100 mm x 4.6 mm, 2.6 µm), connected to a guard column of the same chemistry, both thermostated at 35ºC, using an acidified (0.1% v/v formic acid) mobile phase composed of ultrapure water (A) and acetonitrile (B). The following gradient program was applied at a flow rate of 0.6 mL min^{-1}: 0–19 min 10–40% B, 19–20 min 40–10% B, with 5 min for column conditioning. MS analysis was performed in positive mode using the following conditions: ESI voltage 4.5 kV; collision energy -30.0 V for casein and -40.0 V to ovalbumin; nebulizer gas (N_2) 3 L min^{-1}, desolvation gas (N_2) 18 L min^{-1}; desolvation line temperature 250ºC and heat block temperature 400ºC. Full scan spectra were acquired from *m/z* 100 to 1000. All ions with signal intensity greater than 100 counts (range 10^1 to 10^6) and charge state +2 (or

unknown charge (+1 to +3)) were submitted to fragmentation and product ion scan. MS/MS data were searched in SwissProt online database using Mascot Peptide Mass Fingerprint (Matrix Science Ltd., London, UK) as a bioinformatic tool applying the following stringent criteria: i) only one missed cleavage was allowed for trypsin digestion; ii) cysteine carbamidomethylation as fixed amino acid modification; and iii) variable modifications: methionine oxidation, and threonine and tyrosine phosphorylation. For MRM method, the most intense signals from the product ion spectra were chosen for MRM transitions, i.e. for α-casein 634.6→991.8, for β-casein 390.9→258.5 and for ovalbumin 929.5→1116.5.

2.4 Statistical analysis

Data were evaluated using descriptive statistics [mean, standard deviation (SD) and relative standard deviation (RSD)]. Peptides calibrations were established applying linear regression analysis. Calibrations with and without matrix were compared using F-test. All above tests were done with a significance level (α) of 0.05 using GraphPad (San Diego, CA, USA) Prism 6.0 software. Central composite design was prepared and analyzed by means of Statgraphics Centurion XV version 15.1.02 software (Rockville, MD, USA) and SAS (Marlow, Buckinghamshire, England) JMP 8 statistical software.

3. Results and discussion

3.1 Selection of marker peptides

Selection of marker peptides is one of the key steps to develop quantitative MS-based method. In this work peptide and fragment markers were established using pure standard and red wine samples (free of allergen proteins) spiked with 100 mg $L^{-1}$of each protein (caseins and ovalbumin). Both were digested with trypsin using general conditions and studied/analyzed by LC-MS/MS. As expected, several *m/z* signals were observed along the entire mass chromatogram. To limit the exploration, the search was based on already published data (Fremout et al. 2010; Heick et al. 2011; Losito et al. 2013; Mattarozzi et al. 2014; Monaci et al. 2011; Monaci et al. 2010; Monaci et al. 2014; Patrick et al. 2009; Tolin et al. 2012a) finding the following parents (precursor) ions: YLGYLEQLLR (*m/z* 634.6; +2, t_R 15.1 min), VLPVPQK (*m/z* 390.9; +2, t_R 4.0 min), and ELINSWVESQTNGIIR (*m/z* 929.5; +2, t_R 13.5 min) for α-casein, β-casein, and ovalbumin, respectively. After

establishing precursor ions, a MS/MS approach was performed in product ion scan mode using the LC-MS/MS conditions described in section 2.3. Fragment ions selection from each precursor was carried out applying the same criteria indicated in section 2.3 plus the following aspects: i) matching with precursor peptides already reported in literature (Heick et al. 2011; Mattarozzi et al. 2014; Monaci et al. 2014; Tolin et al. 2012a), and ii) quality of product ion spectra matches (Matrix Science Ltd., London, UK). This approach allowed the selection of casein and ovalbumin peptides and fragment ions present in every digestion and fragmentation. Thus, it was possible to define peptide markets (precursor and fragment ions) to detect α-casein, β-casein, and ovalbumin in MRM mode. Figure 1 shows the product ion spectrum of YLGYLEQLLR peptide from α-casein after fragmentation into collision-induced dissociation cell set up at -35.0 V. Following the early describe criteria, the two most abundant and stable fragments from the product ion spectrum were chosen for each peptide (protein), one for qualitative/confirmatory and one for quantitative purposes (Table 1). Thus, the MRM transitions established for quantification were *m/z* 634.6→991.8 for α-casein, *m/z* 390.9→258.5 for β-casein and m/z 929.5→1116.5 for ovalbumin.

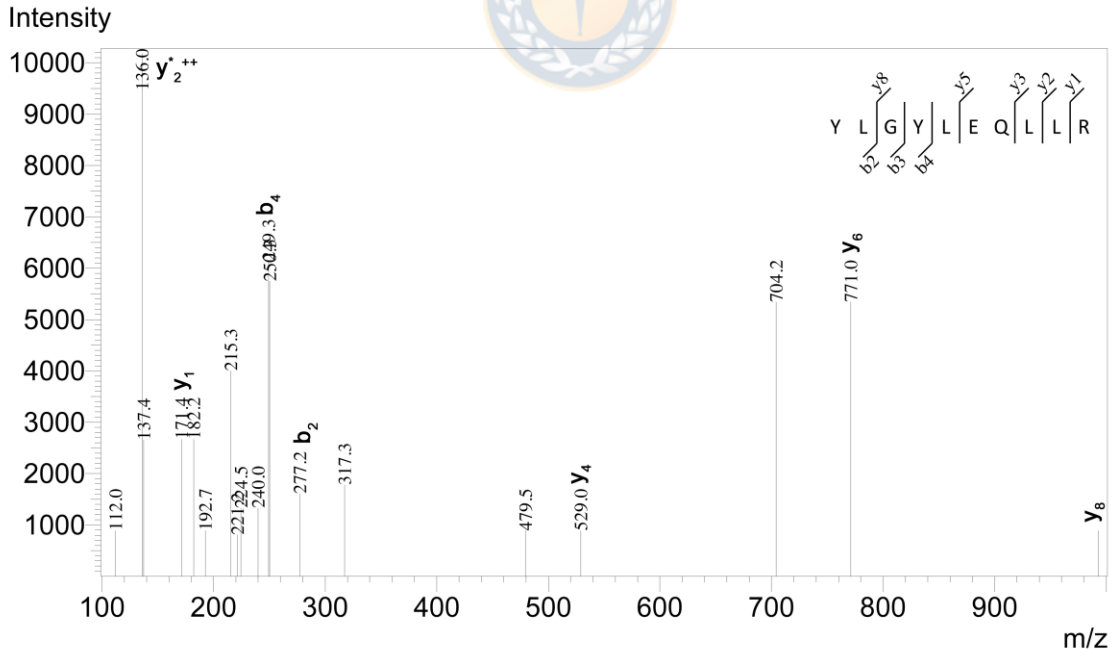

Figure 1. Product ion spectrum of α-casein peptide YLGYLEQLLR (m/z 634.6)

Table 1. MRM transitions for α, β-casein and ovalbumin determination in wines

Protein	Precursor ion sequence (*m/z*; charge state; MW [Da])	Product ion sequence (*m/z*; charge state)
α-casein	YLGYLEQLLR (*m/z* 634.6; +2; 1267.2)	GYLEQLLR (*m/z* 991.8; +1)[a,b]
		LEQLLR (*m/z* 771.4; +1)[c]
β-casein	VLPVPQK (*m/z* 390.9; +2; 779.8)	QK (*m/z* 258.25; +1)[a,b]
		PVPQK (*m/z* 284.6; +2)[c]
Ovalbumin	ELINSWVESQTNGIIR (*m/z* 929.5; +2; 1857.9)	VESQTNGIIR (*m/z* 1116.5; +1) [a,b]
		ESQTNGIIR (*m/z* 1017.5; +1)[c]

[a] Most consistent MS/MS transition.
[b] *m/z* transition monitored for quantification.
[c] *m/z* transition monitored for confirmation.

3.2 Optimization of enzymatic digestion

A face-centered central composite design with two central points was selected to optimize the enzymatic digestion. This type of design was chosen because it has a high efficiency with a reduced number of experiments (Dejaegher and Vander Heyden 2011). The optimization was done for each marker peptide in MRM mode. Since detection capability is one of the most relevant aspect for wine allergen proteins determination, peak area was chosen as the critical variable (response) looking for lower detection limits. From the factors that possibly affect the critical variable, two were chosen: digestion time (X_1), and enzyme to protein ratio (X_2). Considering previous experiments and the intervals described in literature, the following ranges were established for digestion time (2.0 to 12.0 h), and enzyme to protein ratio (1:10 to 1:100), resulting in an experimental plan with 10 runs (Table 2).

Table 2. Experimental runs for a central composite design with the corresponding responses (means) for each MRM transition (peak area)

	Factors		Responses (peak area, *m/z*)*		
Runs	Time (h)	Enzyme: protein ratio	634.6→991.8	390.9→258.25	929.5→1116.5
1	2	1:10	675.00±4.59	1954.00±2.47	23.00±2.12
2	7	1:20	740.00±0.35	2945.25±0.70	263.32±7.42
3	12	1:10	391.00±2.82	2175.00±3.53	47.00±3.88
4	7	1:10	845.12±4.24	4604.00±1.76	188.25±1.06
5	12	1:20	233.00±2.82	1350.00±0.35	56.00±3.18
6	2	1:20	493.25±1.41	1546.25±3.18	104.15±4.94
7	7	1:100	704.00±5.30	2059.00±0.70	98.00±4.24
8	2	1:100	311.00±0.00	1458.00±1.06	167.00±2.47
9	7	1:20	598.00±0.00	2764.75±0.36	261.15±3.18
10	12	1:100	152.70±1.41	863.00±1.06	43.00±1.41

*mean± standard deviation (*n*=2)

All experiments were randomly conducted in duplicate (*n*=2) in order to minimize the effects of uncontrolled factors. All experiments were performed using a red wine sample (blank), free of allergen proteins, obtained from a winery that not uses casein or ovalbumin as fining agents. Wine sample was spiked with 1.2 mg L^{-1} of each protein and then submitted to the different digestion conditions according to the experimental plan. An analysis of variance (ANOVA) with a significance level of 0.05 was carried out to determine which experimental factors significantly affect the yields (peak area) obtained for each MRM transitions. According to the observed results, digestion time affected to α-casein (P=0.01) yields; enzyme to protein ratio affected to α and β-casein (P=0.01 and P=0.02); and digestion time (quadratic coefficient) affected to α-casein (P=0.002), β-casein (P=0.008) and ovalbumin (P=0.04) yields. For all cases, lack of fit test for quadratic models showed P values higher than 0.05, and adjusted R^2>75%, which is adequate in terms of model suitability (Derringer 1980). The corresponding digestion yields obtained for each MRM transitions for each experimental run are shown in Table 2. Using the individual optimum, a multiple response optimization was done in order to determine the optimal conditions for all responses (desirability conditions). β-casein and ovalbumin shared the same optimal conditions, while α-casein showed an optimal yield value with a digestion time of 6 h. The response surface plots for each transition are shown in Figure 2. Optimal trypsin digestion conditions to simultaneously determine α-casein, β-casein, and ovalbumin in wines were 7 h as digestion time and 1:10 as enzyme to protein ratio. The optimum digestion time is ca. 42% lower than the most reported conditions (12 h) (Fremout et al. 2010; Heick et al. 2011; Mattarozzi et al. 2014; Tolin et al. 2012a). Regarding trypsin to protein ratio, it is clear that enzyme concentration is crucial to accomplish adequate digestion yields, and therefore, higher concentration might result in higher digestion yields, however this concentration must be controlled because autolysis could occurs decreasing enzyme activity (Hustoft et al. 2011). This phenomenon can be avoided using a "modified trypsin" which is manufactured to provide maximum specificity through reductive methylation of lysine residues, yielding a highly active and stable molecule that is extremely resistant to autolytic digestion (Finehout et al. 2005). This makes the enzyme very attractive for proteomic analyzes, however its price is much higher than the traditional one. In this case, the optimum enzyme-to-substrate ratio for maximum yields and therefore

highest signal intensities was found to be the highest enzyme ratio assayed (1:10). Higher enzyme levels were not assayed to avoid autolysis and because the obtained signals were more than suitable for allergen protein determination in wines (ca. 60 times lower than OIV limit). Under these optimal conditions the percentage of sequence coverage (SQ) increased from 18% to values above 23% for each marker peptide, which is adequate in terms of yield of proteolytic digestion (Hustoft et al. 2011).

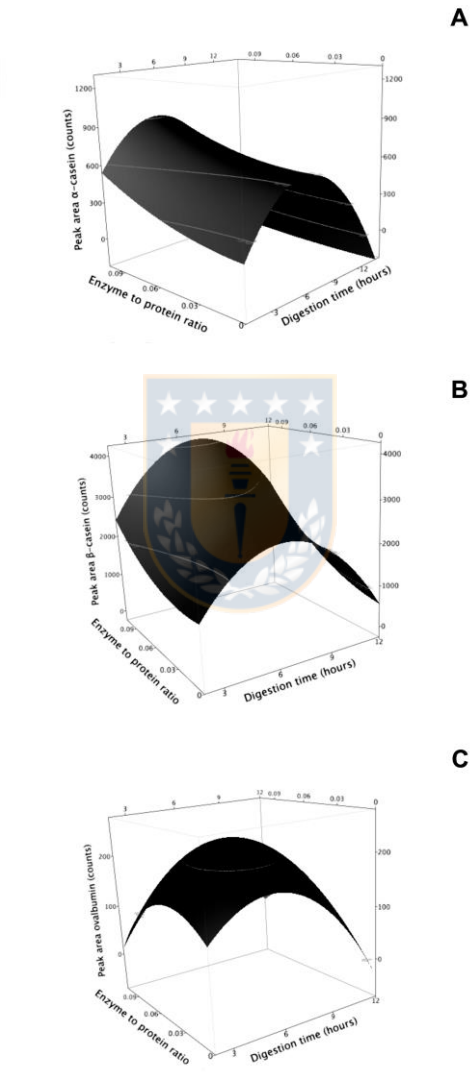

Figure 2. Response surface plots for α-casein (a), β-casein (b), and ovalbumin (c) transitions

3.3 Method Validation

The optimized method was validated following the International Conference on

Harmonization recommendations (International Conference on Harmonisation (ICH) 2005). Calibration curves of all proteins were established with and without wine matrix in order to evaluate a possible matrix effect. Since slopes were not statistically different ($P > 0.05$) calibrations were established with pure standards at six levels in triplicate from 0.10 to 1.20 mg L^{-1}. Calibrations data fit a linear regression model with determination coefficients (R^2) =0.99 for all proteins. Method precision was evaluated through repeatability and intermediate precision. Repeatability was studied injecting in sextuplicate (*n*=6) a spiked wine sample (0.25 mg L^{-1}) showing RSDs values from 0.51% to 1.36%. Intermediate precision was determined measuring in triplicate a spiked wine sample (0.25 mg L^{-1}) during three different days (*n*=3), showing RSDs values from 0.48% to 1.53%. Method accuracy was determined via recovery evaluation. Wine samples spiked at three levels (0.15, 0.25, 0.50 mg L^{-1}) were prepared and analyzed daily during three days. All proteins showed recovery yields between 86.40 % to 106.98 % with RSD values <2.80%. Detection and quantification limits were calculated using signal-to-noise ratios (S/N) of 3 and 10 respectively. Considering the use of a core-shell column and an injection volume of 50 µL, the detection and quantification limits ranged from 4.7 to 8.5 µg L^{-1} and 10.0 to 20.0 µg L^{-1}, respectively. Comparatively, this optimized method showed similar validation results than others methods for allergen proteins determination in wines using LC-MS/MS. Linear range (0.10-1.20 mg L^{-1}) is lower than reported by Mattarozzi et al. (2014) (50-200 mg L^{-1}) and Chen et al. (2015) (1-100 mg L^{-1}). Recovery results (>80%) are similar to those reported by Chen et al. (2015) (98.8 to 106.7%), Mattarozzi et al. (2014) (82 to 105%) and Losito et al. (2013) (80 to 85%). In terms of precision RSDs values from 0.51% to 1.36% are slightly lower than reported by Mattarozzi et al. (2014) (10% to 19%) and Chen et al. (2015) (3.9% to 8.9%). Detection and quantification limits were lower than those reported by Restani et al. (2014) using ELISA method (LOD 0.15 mg mL^{-1}; LOQ 0.38 mg mL^{-1}); and Mattarozzi et al. (2014) (LOD=0.01 to 0.80 mg L^{-1} and LOQ = 0.03 to 2.00 mg L^{-1}). Regarding analysis time, some improvements were incorporated to decrease the required time to separate the proteins under study, i.e. shorter and lower particle size core-shell column, higher flow rates, and gradient program adjustments. Thus, a complete separation was achieved in less than 20 min (Figure 3), which is slightly lower than the reported by Heick et al. (2011)-30 min- and Mattarozzi et al. (2014)-25 min-, and at least 65% lower

than the most published analysis time (60 min). Losito et al. (2013) developed a LC-ESI-IT-MS method for caseinate quantification in Italian commercial white wines in 75 min runs. Monaci et al. (2010) identified allergen milk proteins markers in fined white wines by capillary LC-ESI-MS/MS with 60 min runs. Tolin et al. (2012a) detected egg proteins in red wines by LC-MS method with 50 min runs and Wang et al. (2016) published a corn peptides identification method based on HPLC-diode array detection and HPLC-ESI-MS/MS that required ca. 80 min runs.

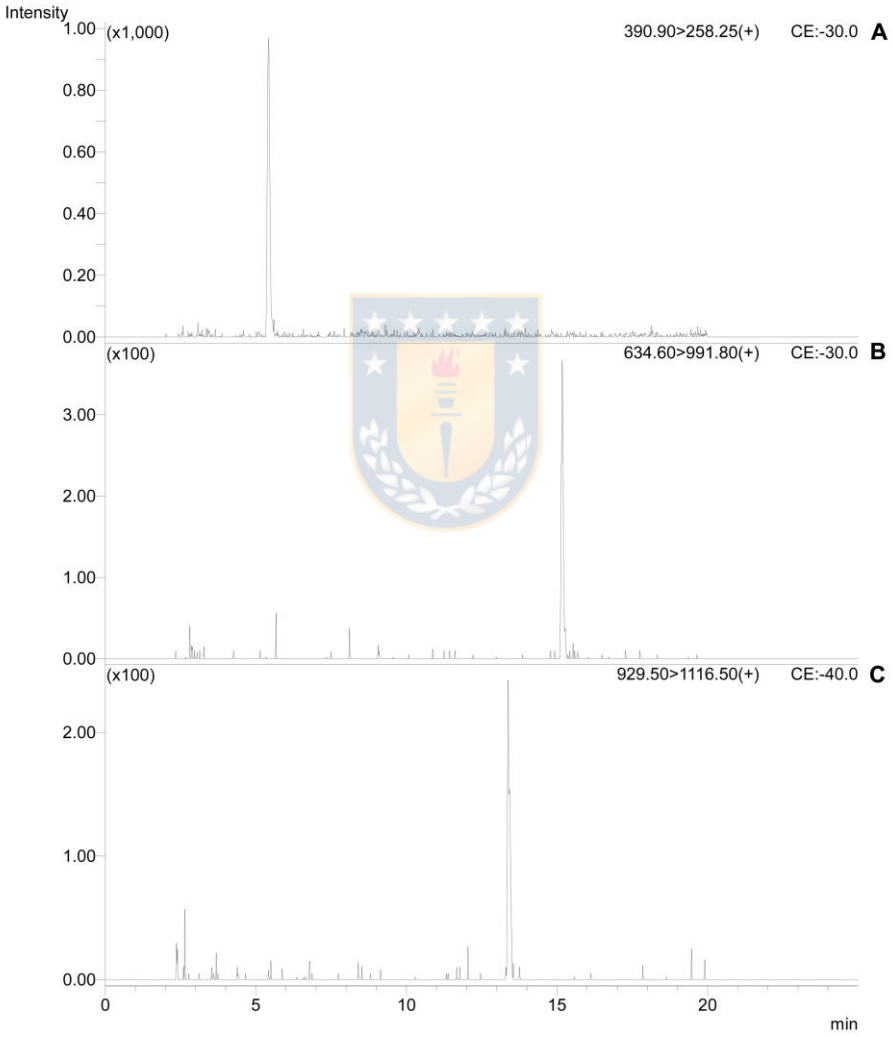

Figure 3. LC-ESI-MS/MS chromatograms of Cabernet Sauvignon sample analyzed in MRM mode for β-casein (a), α-casein (b), and ovalbumin (c) detection

3.4 Samples analysis

As indicated above, allergen proteins detection in wines by ELISA (OIV official method) presents some limitations that impair the assay sensitivity. Contrarily, the proposed LC-MS/MS method is highly sensitive capable of detecting concentrations as low as 0.01 mg L^{-1} (Table 3).

Table 3. Allergen proteins content (mg L^{-1}) in Chilean wine samples

	Wine samples	Valley	Year[a]	α-casein	β-casein	Ovalbumin	Total
1	S. Blanc	Leyda	2016	0.06±0.001	0.03±0.000	Traces	0.09
2	S. Blanc	Rapel	2015	0.11±0.000	0.09±0.001	0.04±0.002	0.24
3	S. Blanc	Central	2015	0.14±0.002	0.10±0.002	ND	0.24
4	S. Blanc	Maipo	2016	0.05±0.002	0.03±0.000	ND	0.08
5	C. Sauvignon	Central	2015	0.03±0.002	0.01±0.000	Traces	0.04
6	C. Sauvignon	Maipo	2015	0.03±0.002	0.01±0.000	0.02±0.001	0.06
7	C. Sauvignon	Maipo	2013	0.01±0.000	Traces	0.11±0.003	0.12
8	C. Sauvignon	Central	2015	ND	Traces	Traces	Traces
9	C. Sauvignon	Colchagua	2015	ND	ND	0.06±0.006	0.06
10	C. Sauvignon	Central	2014	ND	ND	0.09±0.000	0.09
11	C. Sauvignon	Central	2013	ND	ND	0.09±0.006	0.09
12	C. Sauvignon	Central	2014	Traces	Traces	ND	Traces
13	Carménère	Colchagua	2016	0.05±0.001	0.03±0.001	Traces	0.08
14	Carménère	Colchagua	2015	0.02±0.000	0.05±0.001	ND	0.07
15	Carménère	Central	2015	0.01±0.001	0.01±0.000	0.03±0.003	0.06
16	Carménère	Curicó	2014	0.08±0.002	0.06±0.001	0.02±0.002	0.17
17	Merlot	Maipo	2015	0.03±0.000	0.02±0.000	0.21±0.080	0.26
18	Merlot	Maipo	2013	0.01±0.001	0.01±0.000	0.02±0.002	0.04
19	Merlot	Curicó	2015	0.05±0.005	0.04±0.002	Traces	0.09
20	Merlot	Rapel	2014	0.02±0.000	0.02±0.000	0.02±0.002	0.06

Two independent extractions from each sample were analyzed in duplicate, results are expressed as mean ± standard deviation.
ND: not detected (<LOD)
[a]Year indicated in the label. Traces: (>LOD; <LOQ).

Applying this optimized and validated method, twenty Chilean commercial wine samples without allergen labelling were analyzed. All wine samples labels described the wine type/category, i.e. young (without aging) or reserve (higher quality and/or aged), cv. (one grape variety represents at least 75% of the blend), harvest year and geographical origin (denomination of origin). According to that, fifteen samples were reserve and five young wines, belonging to six valleys and fourteen wineries. Eight samples were Cabernet Sauvignon, four Sauvignon Blanc, four Carménère and four Merlot. Two independent extractions from each sample were analyzed in duplicate. All samples, except two, showed quantifiable levels of allergen proteins, the level found ranged from non-detected to 0.21

mg L^{-1} (Table 3). One Merlot sample (1/20; 5%), showed a total concentration of casein and ovalbumin higher than the limit recommended by the European Community (0.25 mg L^{-1}) and other two showed very close values (0.24 mg L^{-1}). Even when a reduced number of samples were analyzed, the percentage of positive samples was high (18/20; 90%), which demonstrated the prevalent use of casein and ovalbumin as finning agent in Chilean wineries. This result is much higher than international reports. Lifrani et al. (2009) found residual contents of ovalbumin and casein in 11% (44/400) of commercially available French wines analyzed. Hoxey et al. (2013) reported detectable levels of casein (from 0.27 to 5 mg/L) in 0.6% (3/521) of Australian wines sampled. Rolland et al. (2008) found detectable concentrations of ovalbumin (1 μg L^{-1}) in 1.3% (2/153) of Australian wines. Tolin et al. (2012b) informed the presence of casein and ovalbumin in 32% (8/25) of Italian commercial wines evaluated. Currently, Chilean sanitary authorities have not yet established an official limit for allergen proteins content in wines, hence, casein and ovalbumin determination is not a common assay in wineries or in national reference laboratories. Therefore, the information obtain with this study could be useful in terms of food safety, decreasing the possible health risk for sensible consumers, and thereby preventing future sanitary problems that can negatively change the current worldwide status of Chilean wine. Even when clarification and filtration processes are commonly applied after fining process, residues of both proteins at ng or μg L^{-1} levels are still present as it was demonstrated in the present study. This could become a serious problem for sensitive individuals that consume wines without allergen labelling since the lowest observed adverse effect level is ranged from 100 to 200 μg of allergen protein (Stockley and Johnson 2015). Further, others allergen related compounds such as biogenic amines (e.g. histamine and tyramine) could be present in Chilean wine increasing the possibilities to suffer some kind of adverse effects in sensitive consumers (Henríquez-Aedo et al. 2012; Pineda et al. 2012). Finally, attention should be paid considering also the Chilean per capita wine consumption of 23.5 liters per year (Henríquez-Aedo et al. 2012).

4. Conclusions

This work showed for the first time the evaluation of allergen proteins in Chilean wines, including the analysis of white and red wines from four different grape varieties produced

by fourteen wineries. The enzymatic digestion time was reduced in 42%, which together with shorter chromatographic analysis (20 min), resulted in an important reduction in the analysis time required for casein and ovalbumin determination in wines. This highly selective method was capable of detecting casein and ovalbumin at ppb (µg L^{-1}) levels, which is more than 60 times lower than the European and OIV limit. Method suitability for casein and ovalbumin quantification in wines was demonstrated through validation results. Regarding allergen proteins presence in Chilean wines, from twenty samples analyzed, 90% showed quantifiable levels (>0.01 mg L^{-1}) and one showed a total concentration of casein and ovalbumin higher than the limit recommended by the European Community (0.25 mg L^{-1}). According to results it seems appropriate that Chilean sanitary authorities established an official limit for mandatory labelling of allergen proteins in wines in order to avoid a toxicological risk.

Acknowledgments

This work is part of Jessy Pavón Pérez thesis to obtain the degree of Doctor in Science and Analytical Technology from the University of Concepcion, Chile. Authors want to thank to the National Commission of Scientific and Technological Research (CONICYT) of the Chilean Government for the doctoral scholarship granted. This work was financially supported by National Fund for Scientific & Technological Development (FONDECYT), projects N°1171857, FONDEQUIP 130209 and University of Concepcion.

Compliance with Ethical Standards

Funding: this study was funded by the National Fund for Scientific and Technological Development (FONDECYT) project No1171857 and by the Fund for Scientific and Technological Equipment (FONDEQUIP) project No 130209.

Conflict of Interest: Jessy Pavon, Karem Henríquez-Aedo and Mario Aranda declare that they have no conflict of interest.

Ethical Approval: this article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: not applicable.

References

Bridi R, Lobato S, Lopez-Alarcon C, Lissi E (2014) Influence of the growing region, quality classification and harvest year on the ORAC (Oxygen Radical Absorbance Capacity) index of Chilean Cabernet Sauvignon red wines. Cienc Investig Agrar 41:395-402 doi:10.4067/s0718-16202014000300012

Calleri E et al. (2005) Trypsin-based monolithic bioreactor coupled on-line with LC/MS/MS system for protein digestion and variant identification in standard solutions and serum samples. J Proteome Res 4:481-490 doi:10.1021/pr049796h

Careri M et al. (2007) Use of specific peptide biomarkers for quantitative confirmation of hidden allergenic peanut proteins Ara h 2 and Ara Ill 3/4 for food control by liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 389:1901-1907 doi:10.1007/s00216-007-1595-2

Chambery A, del Monaco G, Di Maro A, Parente A (2009) Peptide fingerprint of high quality Campania white wines by MALDI-TOF mass spectrometry. Food Chem 113:1283-1289 doi:https://doi.org/10.1016/j.foodchem.2008.08.031

Chen Q et al. (2015) Quantification of bovine beta-casein allergen in baked foodstuffs based on ultra-performance liquid chromatography with tandem mass spectrometry. Food Addit Contam 32:25-34 doi:10.1080/19440049.2014.990994

Chiva C, Ortega M, Sabido E (2014) Influence of the Digestion Technique, Protease, and Missed Cleavage Peptides in Protein Quantitation. J Proteome Res 13:3979-3986 doi:10.1021/pr500294d

Cristina L et al. (2016) Validation of a mass spectrometry-based method for milk traces detection in baked food. Food Chem 199:119-127 doi:https://doi.org/10.1016/j.foodchem.2015.11.130

Dejaegher B, Vander Heyden Y (2011) Experimental designs and their recent advances in set-up, data interpretation, and analytical applications. J Pharm Biomed Anal 56:141-158 doi:10.1016/j.jpba.2011.04.023

Derringer GyRS (1980) Simultaneous Optimization of Several Response Variables. J Qual Technol 12:214-219

Dordoni R, Galasi R, Colangelo D, De Faveri DM, Lambri M (2015) Effects of fining with different bentonite labels and doses on colloidal stability and colour of a Valpolicella red wine. Int J Food Sci Tech 50:2246-2254 doi:doi:10.1111/ijfs.12875

European Commission (EU) (2012) Commission implementing regulation 579/2012. Official Journal European Union L171:4-7

Finehout EJ, Cantor JR, Lee KH (2005) Kinetic characterization of sequencing grade modified trypsin. Proteomics 5:2319-2321 doi:10.1002/pmic.200401268

Fremout W, Dhaenens M, Saverwyns S, Sanyova J, Vandenabeele P, Deforce D, Moens L (2010) Tryptic peptide analysis of protein binders in works of art by liquid chromatography–tandem mass spectrometry. Anal Chim Acta 658:156-162 doi:http://dx.doi.org/10.1016/j.aca.2009.11.010

Gallardo JM, Ortea I, Carrera M (2013) Proteomics and its applications for food authentication and food-technology research. Trends Analyt Chem 52:135-141 doi:http://dx.doi.org/10.1016/j.trac.2013.05.019

González-Neves G, Favre G, Gil G (2014) Effect of fining on the colour and pigment composition of young red wines. Food Chem 157:385-392 doi:http://dx.doi.org/10.1016/j.foodchem.2014.02.062

Heick J, Fischer M, Pöpping B (2011) First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. J Chromatogr A 1218:938-943 doi:http://dx.doi.org/10.1016/j.chroma.2010.12.067

Henríquez-Aedo K, Vega M, Prieto-Rodríguez S, Aranda M (2012) Evaluation of biogenic amines content in chilean reserve varietal wines. Food Chem Toxicol 50:2742-2750 doi:http://dx.doi.org/10.1016/j.fct.2012.05.034

Hoxey L, Stockley C, Wilkes E, Johnson D (2013) What's in a label? How science is helping winemakers to respond to new EU rules concerning allergens in wine. Wine & Viticulture Journal 4:38-41

Hustoft HK, Reubsaet L, Greibrokk T, Lundanes E, Malerod H (2011) Critical assessment of accelerating trypsination methods. J Pharm Biomed Anal 56:1069-1078 doi:http://dx.doi.org/10.1016/j.jpba.2011.08.013

International Conference on Harmonisation (ICH) (2005) ICH Harmonised tripartite guideline validation of analytical procedures: text and, methodology Q2 (R1).

Kaul S, Luettkopf D, Kastner B, Vogel L, Hoeltz G, Vieths S, Hoffmann A (2007) Mediator release assays based on human or murine immunoglobulin E in allergen standardization. Clin Exp Allergy 37:141-150 doi:10.1111/j.1365-2222.2006.02618.x

Kerkaert B, Mestdagh F, De Meulenaer B (2010) Detection of hen's egg white lysozyme in food: Comparison between a sensitive HPLC and a commercial ELISA method. Food Chem 120:580-584 doi:https://doi.org/10.1016/j.foodchem.2009.10.027

Lifrani A, Dos Santos J, Dubarry M, Rautureau M, Blachier F, Tome D (2009) Development of animal models and sandwich-ELISA tests to detect the allergenicity and antigenicity of fining agent residues in wines. J Agric Food Chem 57:525-534 doi:10.1021/jf8024584

Losito I, Introna B, Monaci L, Minella S, Palmisano F (2013) Development of a Method for the Quantification of Caseinate Traces in Italian Commercial White Wines Based on Liquid Chromatography-Electrospray Ionization-Ion Trap-Mass Spectrometry. J Agric Food Chem 61:12436-12444 doi:10.1021/jf4034909

Loziuk PL, Wang J, Li QZ, Sederoff RR, Chiang VL, Muddiman DC (2013) Understanding the Role of Proteolytic Digestion on Discovery and Targeted Proteomic Measurements Using Liquid Chromatography Tandem Mass Spectrometry and Design of Experiments. J Proteome Res 12:5820-5829 doi:10.1021/pr4008442

Mainente F, Zoccatelli G, Lorenzini M, Cecconi D, Vincenzi S, Rizzi C, Simonato B (2014) Red wine proteins: Two dimensional (2-D) electrophoresis and mass spectrometry analysis. Food Chem 164:413-417 doi:https://doi.org/10.1016/j.foodchem.2014.05.051

Mattarozzi M, Milioli M, Bignardi C, Elviri L, Corradini C, Careri M (2014) Investigation of different sample pre-treatment routes for liquid chromatography–tandem mass spectrometry detection of caseins and ovalbumin in fortified red wine. Food Control 38:82-87 doi:http://dx.doi.org/10.1016/j.foodcont.2013.10.015

Mendoza L, Matsuhiro B, Aguirre MJ, Isaacs M, Sotes G, Cotoras M, Melo R (2011) Characterization of phenolic acids profile from Chilean red wines by High-Performance Liquid Chromatography. J Chil Chem Soc 56:688-691

Monaci L, Losito I, Palmisano F, Godula M, Visconti A (2011) Towards the quantification of residual milk allergens in caseinate-fined white wines using HPLC coupled with single-stage Orbitrap mass spectrometry. Food Addit Contam 28:1304-1314 doi:10.1080/19440049.2011.593191

Monaci L, Losito I, Palmisano F, Visconti A (2010) Identification of allergenic milk proteins markers in fined white wines by capillary liquid chromatography–electrospray ionization-tandem mass spectrometry. J Chromatogr A 1217:4300-4305 doi:http://dx.doi.org/10.1016/j.chroma.2010.04.035

Monaci L, Pilolli R, De Angelis E, Godula M, Visconti A (2014) Multi-allergen detection in food by micro high-performance liquid chromatography coupled to a dual cell linear ion trap mass spectrometry. J Chromatogr A 1358:136-144 doi:10.1016/j.chroma.2014.06.092

Monaci L, Visconti A (2009) Mass spectrometry-based proteomics methods for analysis of food allergens. Trends Analyt Chem 28:581-591 doi:10.1016/j.trac.2009.02.013

Organisation Internationale de la Vigne et du Vin (OIV) (2012) Revision of the limit of detection and limit of quantiifcation related to potentially allergenic residues of finning agent proteins in wine

Organisation Internationale de la Vigne et du Vin (OIV) (2017) State of the vitiviniculture world market-April 2017.

Patrick W, Hans S, Angelika P (2009) Determination of the Bovine Food Allergen Casein in White Wines by Quantitative Indirect ELISA, SDS-PAGE, Western Blot and Immunostaining. J Agric Food Chem 57:8399-8405 doi:10.1021/jf9013982

Pineda A, Carrasco J, Pena-Farfal C, Henriquez-Aedo K, Aranda M (2012) Preliminary evaluation of biogenic amines content in Chilean young varietal wines by HPLC. Food Control 23:251-257 doi:10.1016/j.foodcont.2011.07.025

Proc JL et al. (2010) A Quantitative Study of the Effects of Chaotropic Agents, Surfactants, and Solvents on the Digestion Efficiency of Human Plasma Proteins by Trypsin. J Proteome Res 9:5422-5437 doi:10.1021/pr100656u

Restani P et al. (2014) Collaborative Interlaboratory Studies for the Validation of ELISA Methods for the Detection of Allergenic Fining Agents Used in Wine According to the Criteria of OIV Resolution 427-2010 Modified by OIV-Comex 502-2012. Food Anal Methods 7:706-712 doi:10.1007/s12161-013-9741-2

Rolland JM, Apostolou E, de Leon MP, Stockley CS, O'Hehir RE (2008) Specific and Sensitive Enzyme-Linked Immunosorbent Assays for Analysis of Residual Allergenic Food Proteins in Commercial Bottled Wine Fined with Egg White, Milk, and Nongrape-Derived Tannins. J Agric Food Chem 56:349-354 doi:10.1021/jf073330c

Stockley CS, Johnson DL (2015) Adverse food reactions from consuming wine. Aust J Grape Wine Res 21:568-581 doi:10.1111/ajgw.12171

Tolin S, Pasini G, Curioni A, Arrigoni G, Masi A, Mainente F, Simonato B (2012a) Mass spectrometry detection of egg proteins in red wines treated with egg white. Food Control 23:87-94 doi:10.1016/j.foodcont.2011.06.016

Tolin S, Pasini G, Simonato B, Mainente F, Arrigoni G (2012b) Analysis of commercial wines by LC-MS/MS reveals the presence of residual milk and egg white allergens. Food Control 28:321-326 doi:10.1016/j.foodcont.2012.05.015

Walmsley SJ, Rudnick PA, Liang YX, Dong Q, Stein SE, Nesvizhskii AI (2013) Comprehensive Analysis of Protein Digestion Using Six Trypsins Reveals the Origin of Trypsin As a Significant Source of Variability in Proteomics. J Proteome Res 12:5666-5680 doi:10.1021/pr400611h

Wang C, He H, Zhang J-l, Li X, Ma Z-l (2016) High performance liquid chromatography (HPLC) fingerprints and primary structure identification of corn peptides by HPLC-diode array detection and HPLC-electrospray ionization tandem mass spectrometry. J Food Drug Anal 24:95-104 doi:http://dx.doi.org/10.1016/j.jfda.2015.05.005

Zhang S, Jian W (2014) Recent advances in absolute quantification of peptides and proteins using LC-MS. Rev Anal Chem 33:31-47 doi:10.1515/revac-2013-0019

CAPITULO II SECCION 2

Title: Chemometric optimization of trypsin digestion method applying infrared, microwave and ultrasound energies for determination of fining-related allergen proteins in wines

Jessy Pavón-Pérez[a], Karem Henriquez-Aedo[a,b], Ricardo Salazar[c], Miguel Herrero[d], Mario Aranda[a,b]*

[a] Laboratory of Advanced Research on Foods and Drugs, Department of Food Science and Technology, Faculty of Pharmacy, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile.

[b] Laboratory of Functional Foods, Center for Biotechnology, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile.

[c] Laboratory of Environmental Electrochemistry, Department of Chemistry of Materials, Faculty of Chemistry and Biology, University of Santiago de Chile, Santiago, Chile.

[d] Foodomics Laboratory, Institute of Food Science Research (CIAL, CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain

*Corresponding author: Tel.:+56-41-2203032; fax: +56-41-2210568. E-mail: maranda@udec.cl, maranda@gmx.net.

Formato manuscrito en revisión enviado a la revista LWT Food Science and Technology.

Abstract

A chemometric optimization of trypsin digestion applying infrared, microwave and ultrasound energies for fining-related allergen proteins determination in wines was carried out. A face-centered central composite design was used to investigate the impact of digestion time and trypsin protein ratio over digestion yields, which were evaluated by mass spectrometry through peptides peak area (transitions) in multiple reaction monitoring mode. Comparing these techniques, the most effective one was microwave followed by ultrasound and infrared. Ultrasound-assisted digestion was chosen considering sample preparation easiness. This technique was capable of reducing protein digestion time from 7h to 3 min (140-fold reduction), with adequate percentages of sequence coverage (>23%). If non-optimized digestion time is considered, the reduction was *ca.* 240-fold, from 12h to 3 min. The proposed methodology allowed the detection of fining-related allergen proteins in wines below the current limit of 0.25 mg L^{-1}.

Keywords: Casein; ovalbumin; microwave, ultrasound, infrared; mass spectrometry

1. Introduction

In winemaking process different kind of fining agents are commonly used with the objective of interacting/eliminating undesirable compounds like polymeric phenols and polyphenols (Losito, Introna, Monaci, Minella, & Palmisano, 2013; Restani et al., 2014). Caseinate or milk powders are traditionally used for white wine fining, and ovalbumin or egg-white powders are used for red wines (González-Neves, Favre, & Gil, 2014). This enological practice might leave behind certain amount of these allergenic proteins in wines, which can be a health risk for susceptible consumers, especially when their presence is unlabeled in the final product. Due to this food safety issue, the European Union (El Rayess et al., 2011) implemented the Directive 89/2003/EC and its latest version 579/2012/EC, which contains a list of allergenic substances including egg and milk derivatives (European Commission (EU), 2012). This Directive indicates the obligation of declaring the presence of egg and milk proteins when the concentration in wines is higher than the analytical limit described in OIV/COMEX/502/2012 resolution (Organisation Internationale de la Vigne et du Vin (OIV), 2012). Currently, this limit is 0.25 mg L^{-1}, based on enzyme-linked immunosorbent assay (Vogt, Kupfer, Vogel, & Niessen, 2016), which is a simple and fast immunoassay (Rolland, Apostolou, De Leon, Stockley, & O'Hehir, 2008) although presents some drawbacks and limitations (Pavón-Pérez, Henriquez-Aedo, & Aranda, 2019). Considering this issue, proteomic analysis by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Mainente et al., 2014) and liquid chromatography (LC) (Mattarozzi et al., 2014) have become valued options; moreover, proteins and peptides separation is a key-step. 2D-PAGE is a labor-intensive technique with some limitations in detecting hydrophobic and alkaline proteins (Chambery, del Monaco, Di Maro, & Parente, 2009). Furthermore, this technique has not accomplished an important automatization, forcing researchers to search for others alternatives for protein separation. In this scenario, liquid chromatography coupled to mass spectrometry (LC-MS) has become the most suitable technique for proteomic analysis. This hyphenated technique offers several advantages, including multi-allergen detection and unambiguous identification and characterization of food allergens (Cristina et al., 2016). Proteomic analysis can be performed applying two different strategies: *top down* or *bottom up* approaches. Within the first one, intact proteins are analyzed without previous digestion (Monaci & Visconti,

2009), whereas in the second, an enzymatic digestion before LC-MS analysis is required, which is based on MS/MS spectra comparison of protein digestion products (peptides) against protein-sequence databases (Monaci & Visconti, 2009). *Bottom-up* is the most preferred strategy to perform proteomics quantitative studies (Chiva, Ortega, & Sabidó, 2014) because the obtained spectra are much easier to interpret than those obtained by *top-down* approaches. However, sample preparation for *bottom-up* analysis is an important limitation since requires complex and time-consuming procedures with many different steps, which must be carefully done to achieve reliable results (Rial-Otero et al., 2007). Protein digestion is mostly carried out using trypsin enzyme that exclusively cleaves basic residues of arginine and lysine (Loziuk et al., 2013) generating peptides with an adequate mass range (7-9 amino acids) for mass spectra analysis. Unfortunately, trypsin digestion time (12 to 24 hours) is incompatible with high-throughput protein analysis (Wang, Zhang, Yang, & Chen, 2008) being considered the bottleneck of *bottom up* analysis (Reddy, Hsu, Hu, & Ho, 2010). In this respect, several efforts have been done to reduce digestion time, for example an enzymatic reactor with different kinds of supports was developed (Jiang et al., 2012), such as magnetic particles (Li, Teoh, Gooding, Selomulya, & Amal, 2010), silica (Liu, Hu, Zhang, & Lu, 2013), polymer particles (Yuan et al., 2009) and monolithic materials (Y. Chen et al., 2011; Rivera & Messersmith, 2012). Other sample preparation techniques like microwave (Z. Chen et al., 2014; Birendra N. Pramanik et al., 2002; Reddy et al., 2010), infrared (Wang et al., 2008) and ultrasound have been assayed due to their advantages compared with traditional methods. Microwave-assisted (MA) digestion is capable of accelerating enzymatic cleavage by rotation of bipolar molecules inducing a perturbation of protein three-dimensional structure exposing protein regions previously inaccessible for proteolytic enzyme (Z. Chen et al., 2014; Joergensen & Thestrup, 1995). Reddy et al (Reddy et al., 2010) studied how different solvents, reaction times, enzyme to protein molar ratios, and microwave temperatures affected digestion processes of myoglobin, lysozyme, cytochrome *c*, ubiquitin, ribonuclease A, α-casein, albumin and transferrin. They concluded that all these proteins could be digested into peptides within 30 min at 60ºC under microwave irradiation. Infrared (IR)-assisted digestion is based on the use of radiation as a heat source, which presents a high molecular penetration ability (Capelo et al., 2009; Wang et al., 2008). Ultrasound-assisted (UA) digestion has shown an

important effectiveness in shortening processing time (Kadam, Tiwari, Álvarez, & O'Donnell, 2015). UA mechanism is not completely explained but it seems to be related to an increase in diffusion rates as a consequence of cavitation phenomenon and heating (Santos et al., 2007). Cavitation crushing produces large mechanical shearing forces resulting in a degradation of protein structure and hydrophilic groups opening. This structural phenomenon increases the protein solubility facilitating the enzyme-substrate (protein) interaction (Kadam et al., 2015). UA has been applied using different type of sources, e.g. sonoreactor, ultrasound probes and ultrasound bath (Dominguez-Vega, Garcia, Crego, & Marina, 2010; Rial-Otero et al., 2007). The main difference among them is energy intensity. There are not well-established standard protocols for trypsin digestion, parameters such as time and the enzyme to substrate ratio vary widely depending on the laboratory. Enzyme to protein ratios typically used in proteomics experiments range from 1:100 to as high as 1:2.5 (Loziuk et al., 2013). Since several factors can affect trypsin digestion yields, the most efficient approach is the application of chemometric tools. Thus, the objective of this work was to carry out a chemometric optimization of trypsin digestion method applying infrared, microwave and ultrasound energies for fining-related allergen proteins determination in wines. To the best of our knowledge, this is the first report about chemometric optimization of trypsin digestion method applying these techniques for α-casein, β-casein and ovalbumin determination in wines.

2. Materials and Methods

2.1 Reagents, standards and samples

Formic acid (98-100%), trichloroacetic acid (TCA, >99%), ammonia solution (>25%), ammonium hydrogen carbonate (NH_4HCO_3, 99%), sodium hydroxide, absolute ethanol, acetic acid (100%), silver nitrate ($AgNO_3$, extra pure), formaldehyde solution (37%), citric acid, sequencing modified trypsin from bovine pancreas and LC-grade acetonitrile were purchased from Merck (Darmstadt, Germany). Casein from bovine milk, albumin from chicken egg white, iodoacetamide (IAM, >99%), DL- dithiothreitol (DTT, >99 %), sodium dodecyl sulfate (SDS, >99%), 2-mercaptoethanol (98%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Bis-Acrylamide (>99%), Acrylamide (>99.9%), Tris buffer (1 mol L^{-1}, pH 8.8 and pH 6.8), ammonium persulfate, tetramethylethylenediamine (TEMED,

>99%), were bought from Winkler (Santiago, Chile). Precision Plus Protein All Blue Prestained Protein Standards, 4x Laemmli sample buffer were purchased from Bio-Rad (California, USA). Buffer solutions and mobile phases were prepared with ultrapure water (18.2 MΩ cm) produced by means of Simplicity system from Millipore (Bedford, MA, USA). 12 mg L^{-1} casein and ovalbumin stock solutions were prepared in 0.05 mol L^{-1} NH_4HCO_3 (pH 7.8). All standard solutions were obtained by aliquot dilution from stock solution.

2.2 Sample preparation

Sample preparation was carried out following the method proposed by Pavon-Perez et al (Pavón-Pérez et al. 2019). Briefly, 12.5 mL of red wine sample (blank), free of allergen proteins obtained from a winery that not uses casein or ovalbumin as fining agents, were spiked with 1.2 mg L^{-1} of each protein. Spiked sample was centrifuged using a Hettich (Tuttlingen, Germany) refrigerated centrifuge at 5433 x *g* for 40 min at 20ºC into 10 kDa cut-off membrane ultrafiltration tube (Amicon Merck Millipore), previously conditioned with 10 mL of distilled water. After centrifugation, 2.5 mL were obtained from which proteins were precipitated on ice for 2 h after addition of eight volumes of ethanol: TCA (15%w/v). Thereafter, the mixture was centrifuged at 8981 x *g* for 10 min at 4ºC using a Hettich refrigerated centrifuge. Ethanol: TCA residues were evaporated under a gentle flow of nitrogen without heat. Pellet was solubilized in 1 mL of 0.05 mol L^{-1} NH_4HCO_3, pH 7.8. Trypsin digestion of both, sample and standards, was performed after proteins reduction and alkylation. The first one was carried out by addition of 12 µL of 0.10 mol L^{-1} DTT and incubation for 5 min at 95ºC. After cooling down, cysteine SH-groups, formed during the reduction step, were irreversibly alkylated by addition of 24 µL of aqueous solution of 0.10 mol L^{-1} IAM (freshly prepared), the mixture was left stand protected from light for 15 min at room temperature. Enzymatic digestion was performed adding 4 µL of trypsin solution (0.5 mg mL^{-1}, in 0.05 mol L^{-1} of NH_4HCO_3, pH 7.8) obtaining a trypsin: protein ratio of 1:10, the mixture was incubated according to the digestion method under study. Reaction was stopped adding 10 µL of aqueous solution of formic acid (0.1%v/v). All digested samples and standards were filtered through a 13mm PVDF syringe filter (0.22 µm) before gel electrophoresis (GE) and LC analysis.

2.3 Trypsin digestion conditions

UA- and IR-assisted protein digestion procedures were carried out in Brand (Wertheim, Germany) 1.5 mL microcentrifuge tubes. Each digestion time and trypsin: protein ratios condition was prepared according to the experimental plan established via chemometric tools. IR-assisted digestions were performed in a home-made IR oven according to Wang et al (Wang et al. 2008). UA digestion experiments were performed in a BiosLab (Santiago, Chile) ultrasound bath model SB-5200D, which provides indirect sonication with a frequency of 40 kHz with programmable temperature. MA digestion conditions were assayed on Milestone (Inc. Shelton, CT, USA) Ethos UP with contact-less temperature and pressure sensors, applying a microwave power of 400W and the lowest programmable temperature (41^{o}C).

2.4 Gel electrophoresis

One-dimensional SDS PAGE was used to evaluate each digestion method following the conditions reported by Laemmli (Laemmli 1970) with slight modifications. Briefly, GE was carried out on a vertical chamber from Biotec (Santiago, Chile) using a Bio-Rad (Bio-Rad, Hercules, CA) PowerPac 1000 power supply set at 100 V (constant) for 120 min at room temperature. Prior to application onto 14% polyacrylamide gels, standards (1 mg L^{-1} of casein and ovalbumin in 0.05 mol L^{-1} of NH_4HCO_3, pH 7.8) were mixed at 1:4 ratio with 4x Laemmli Sample buffer (0.0625 mol L^{-1} Tris-HCl pH 6.8, 10% v/v glycerol, 1% lithium dodecyl sulphate (LDS), and 0.005% bromophenol blue) and heated for 4 min at 95 °C. 10 µL of this mixture were loaded onto the gel. After the electrophoretic run, gels were silver stained according to the method of Blum et al (Helmut et al. 1987) with slight modifications. Briefly, gels were incubated for one hour into fixer solution composed of ethanol: water: acetic acid (3:6:1 v/v/v); washed with water for at least 30 min and incubated for 20 min in a silver staining solution. Gel washed step was carried out three times with water during 10 min and then it was developed with a mixture of citric acid, formaldehyde and water (about 2-3 min) under gently mixing until the protein bands get dark stained.

2.5 Liquid chromatography – tandem mass spectrometry

Following the method proposed by Pavon-Perez et al (Pavón-Pérez et al. 2019), LC-MS analysis was performed on Shimadzu (Kyoto, Japan) Nexera X2 UHPLC system consisted of: LC-30AD pump, DGU-20A5R degassing unit, SIL-30AC autosampler, CTO-20AC column oven, CBM-20A communication module, SPD-M20A diode array detector and LCMS-8030 triple quadrupole (TQ) mass spectrometer. The system was controlled by the LabSolution 5.8 software. Peptides separation was carried out on a Phenomenex (Torrance, CA, USA) Kinetex XB Core-Shell C_{18} column (100 mm x 4.6 mm, id. 2.6 µm), thermostated at 35ºC, using a mobile phase composed of ultrapure pure water (A) and acetonitrile (B) both with 0.1 % (v/v) formic acid. The following gradient program was applied at a flow rate of 0.6 mL min $^{-1}$: 0–19 min, 10–40% B; 19–20 min, 40–10% B; with 5 min for column conditioning. MS analysis was performed using ESI in positive mode using the following conditions: ESI voltage, 4.5 kV; collision energy, -30.0 V for casein and -40.0 V for ovalbumin; nebulizer gas (N_2), 3 L min^{-1}; desolvation gas (N_2), 18 L min^{-1}; desolvation line temperature, 250ºC; heat block temperature, 400ºC. Full scan spectra were acquired from *m/z* 100 to 2000. In a previous work in our laboratory, the two most abundant and stable fragments from product ion spectrum were established for each peptide (protein), one for qualitative/confirmatory and one for quantitative purposes (Pavón-Pérez et al. 2019). MRM transitions defined as variable response for each different digestion method were *m/z* 634.6→991.8 for α-casein, *m/z* 390.9→258.5 for β-casein and *m/z* 929.5→1116.5 for ovalbumin.

2.6 Statistical analysis

Data were evaluated using descriptive statistics [mean, standard deviation (SD) and relative standard deviation (RSD)]. Peptides calibrations were established applying linear regression analysis. Calibrations with and without matrix were compared using F-test. All above tests were done with a significance level (α) of 0.05 using GraphPad (San Diego, CA, USA) Prism 6.0 software. Central composite design was prepared and analyzed by means of SAS (Marlow, Buckinghamshire, England) JMP 8 statistical software.

3. Results

3.1 Optimization of trypsin digestion conditions

As described above, enzymatic digestion is considered the bottleneck of *bottom-up* analysis because enzyme cleavage requires *ca*. 12-24 hours. In this scenario, the present work evaluated the use of modern techniques to reduce trypsin digestion time. Each method efficiency, repeatability and reliability were evaluated by SDS-PAGE, percentage of sequence coverage (%SQ) and peak area of each MRM transitions (α-casein 634.6→991.8, β-casein 390.9→258.5, ovalbumin 929.5→1116.5, Figure 1).

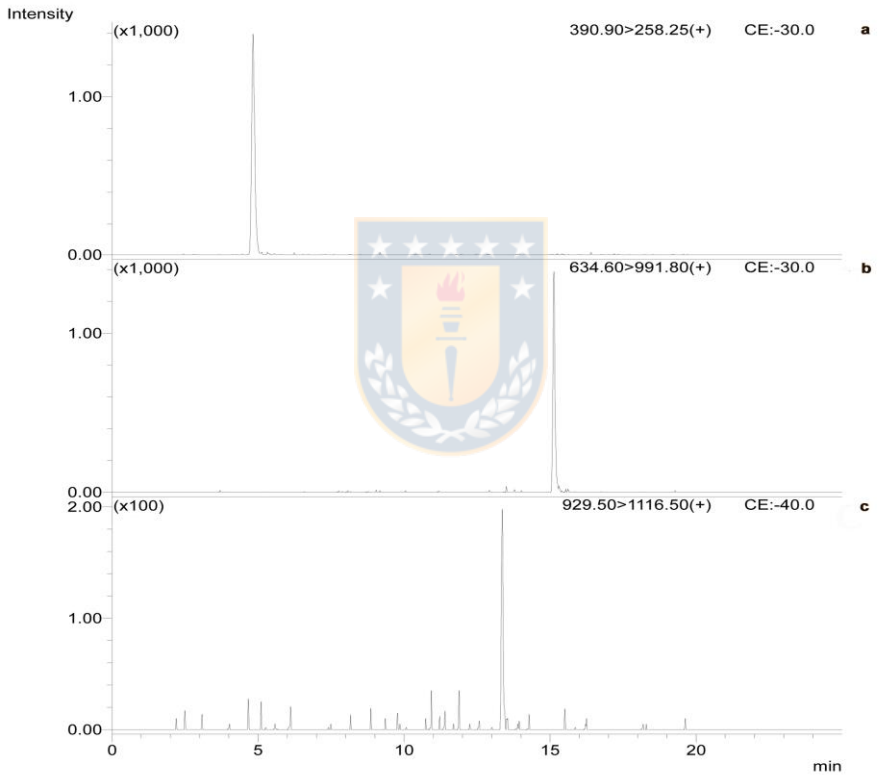

Figure 1. LC-ESI-MS/MS chromatograms in MRM mode of red wine sample (blank) spiked with 1.2 mg L^{-1} of each protein using optimal UA digestion conditions for β-casein (a), α-casein (b), and ovalbumin (c) detection.

In the case of SDS-PAGE, the evaluation of UA-, MA- and IR-assisted protein digestion methods was carried out using a mixture of 1mg L^{-1} of casein and ovalbumin standard, because this technique could suffer certain level of interference in presence of red wines

polyphenols; and using silver stained gels may show a high background and low bands resolution (Vogt et al. 2016). Sequence coverage (%SQ) is commonly used to evaluate protein digestion efficiency (Shevchenko and Shevchenko 2001) and its value is easily obtained from a database like Mascot. Incomplete digestion generated longer peptides with possible uncleaved sites. UA-, MA- and IR-assisted protein digestion conditions were individually optimized applying a face-centered central composite design with two central points. This optimization was performed considering each MRM transition. Since one of the most relevant aspect of wine allergen proteins evaluation is method's detection capability, peak area was chosen as the critical variable (response) looking for lower detection limits. Among the factors that possibly affect the critical variable, two were chosen: digestion time (X_1), and enzyme to protein ratio (X_2). The later considering that lower or higher amounts of trypsin may cause an incomplete digestion or auto-cleavage. In our previous report, (Pavón-Pérez et al. 2019) traditional digestion conditions were optimized reducing digestion time from 12 to 7 hours using a 1:10 enzyme to protein ratio. Thus, the following ranges were established for digestion time (3.0 to 30.0 min), and enzyme to protein ratio (1:10 to 1:100). An experimental plan of 10 different digestion conditions (for each method assayed) were randomly conducted in duplicate (n=2) in order to minimize the effects of uncontrolled factors. All experiments were performed using red wine sample (blank), free of allergen proteins, obtained from a winery that not uses casein or ovalbumin as fining agents. Wine sample was spiked with 1.2 mg L^{-1} of each protein and then submitted to the different digestion conditions according to the experimental plan. An analysis of variance (ANOVA) with a significance level of 0.05 was carried out to determine which experimental factors significantly affect the yields (peak area) measured via MRM transitions.

3.2 Optimization of IR assisted digestion

Experimental results showed that digestion time (quadratic coefficient) affected α-casein (P=0.0009) and β-casein (P=0.04) yields. For all cases lack of fit test for quadratic models showed P values higher than 0.05, and adjusted R^2>75% which is adequate in terms of model suitability (Derringer and Suich 1980). The corresponding digestion yields obtained for each MRM transition for each experimental run are shown in Table 1.

Table 1. Experimental runs for a central composite design for IR-assisted digestion with the corresponding responses (means) for each MRM transition (peak area)

	factors		responses (peak area, *m/z*)*		
	time (min)	enzyme: protein ratio	634.6→991.8	390.9→258.25	929.5→1116.5
1	16.5	1:20	691.00±1.50	2175.00±4.24	47.00±2.08
2	30.0	1:10	61.00±0.35	335.00±0.35	65.00±2.70
3	3.0	1:10	152.00±2.12	863.00±2.88	63.00±2.82
4	16.5	1:20	106.00±1.70	402.00±2.47	26.00±3.88
5	30.0	1:100	675.00±2.94	2954.00±3.59	23.00±2.82
6	3.0	1:20	34.00±2.51	98.00±2.82	28.00±3.30
7	16.5	1:10	134.00±2.21	679.00±0.50	47.00±2.98
8	3.0	1:20	848.00±1.41	6319.00±4.78	118.00±1.41
9	30.0	1:20	29.00±2.47	196.00±3.53	19.00±1.50
10	16.5	1:100	470.00±3.18	1017.00±0.35	62.00±1.50

*mean± standard deviation (*n*=2)

Using the individual optimum, a multiple response optimization was done in order to determine the optimal conditions for all responses (desirability conditions). Response surface plots for each transition are shown in Figure 2.

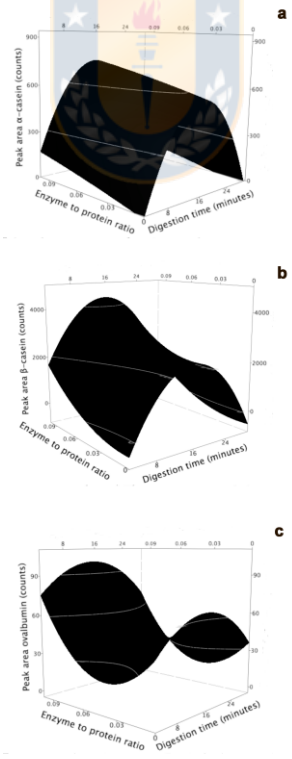

Figure 2. Response surface plots for α-casein (a), β-casein (b), and ovalbumin (c) transitions obtained with IR assisted digestion.

The optimal IR-assisted digestion conditions to simultaneously determine α-casein, β-casein, and ovalbumin in wines were 16 min as digestion time and 1:10 enzyme to protein ratio. In view of results, this technique accomplished deficient enzymatic digestion yields, signals intensities from MRM were very low for each transition; in stained SDS-PAGE gel bands from digested proteins were not clearly observed (Suplementary data, Figure 1A); and %SQ values were only 5, 4 and 3 for α-casein, β-casein and ovalbumin, respectively.

3.3 Optimization of microwave assisted digestion

MA digestion was assayed considering its capability of favoring fast protein cleavage even at very lower amount. Hahn et al (Hahn et al. 2009) demonstrated that MA protocol ensured fast and reproducible digestion yields at low protein concentrations (ca. 10 µg mL^{-1}). Zhao et al (Zhao et al. 2016) used microwave energy to accelerate enzymatic digestion of bovine serum albumin, b-lactoglobulin, and cytochrome c, reducing 15-fold the analysis time compared with traditional in-solution method (1 h vs. 15 h). According to experimental results, protein trypsin ratio affected α-casein (P=0.005), β-casein (P=0.02) and ovalbumin (P=0.01) yields, while digestion time only affected α-casein yield (P=0.002). For all cases lack of fit test for quadratic models showed P values higher than 0.05, and adjusted R^2>90% which is adequate in terms of model suitability (Derringer and Suich 1980). The corresponding digestion yields obtained for each MRM transition for each experimental run are shown in Table 2.

Table 2. Experimental runs for a central composite design for microwave-assisted digestion with the corresponding responses (means) for each MRM transition (peak area)

	factors		responses (peak area, *m/z*)*		
	time (min)	enzyme: protein ratio	634.6→991.8	390.9→258.25	929.5→1116.5
1	30.0	1:20	3252.00±3.18	9245.00±2.12	178.00±1.41
2	3.0	1:100	2555.00±2.47	10877.00±1.73	108.00±1.00
3	3.0	1:20	3384.00±4.24	11218.00±2.82	175.00±1.50
4	30.0	1:100	1273.00±1.00	9631.00±2.62	110.00±1.41
5	3.0	1:10	3815.00±3.18	13303.00±2.38	221.00±2.82
6	16.5	1:100	1391.00±1.76	10136.00±1.70	145.00±4.27
7	30.0	1:10	3265.00±3.30	10050.00±2.06	185.00±1.70
8	16.5	1:20	2985.00±2.12	10351.00±2.75	171.00±2.98
9	16.5	1:10	4098.00±1.29	10649.00±0.50	174.00±1.70
10	16.5	1:20	2690.00±4.24	10419.00±0.95	166.00±2.44

*mean± standard deviation (*n*=2)

A multiple response optimization based on each individual optimum was established in order to determine the optimal conditions for all responses. Response surface plots for each transition are shown in Figure 3. Optimal trypsin digestion conditions to simultaneously determine α-casein, β-casein, and ovalbumin in wines were as follow: 3 min as digestion time and 1:10 enzyme to protein ratio. This digestion time is ca. 140-fold (99%) lower than the time obtained previously for our working group (7 h) (Pavón-Pérez et al. 2019); 3 times lower (70%) than the one (10 min) reported by Pramanik et al (Pramanik et al. 2002) for several types of proteins; and ca. 2-fold lower than the one published by Wu et al (Wu et al. 2015) for myoglobin (horse), BSA (bovine), and α- casein (bovine milk) digestion. MRM results were concordant with stained SDS-PAGE gel, which showed a large number of bands corresponding to the peptides from digested proteins (Suplementary data, Figure 1 B). %SQ were also adequate observing values of 25, 24 and 21 for α-casein, β-casein, and ovalbumin, respectively.

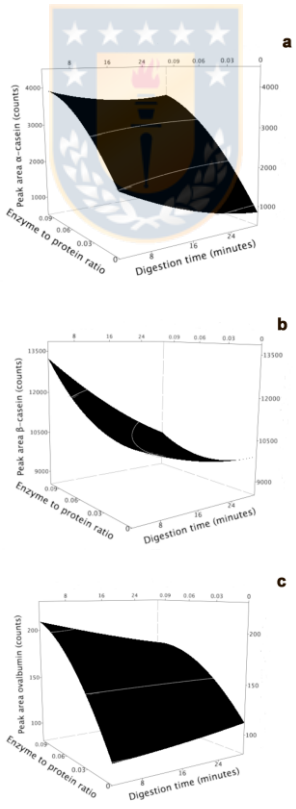

Figure 3. Response surface plots for α-casein (a), β-casein (b), and ovalbumin (c) transitions obtained with MA digestion.

3.4 Optimization of ultrasound-assisted digestion

UA digestion has emerged as a powerful strategy for proteomics analysis because drastically reduces the time required for protein digestion (Rial-Otero et al. 2007). Santos et al (Santos et al. 2007) studied three ultrasound energy sources to speed-up sample preparation for in-solution protein identification by peptide mass fingerprint using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Digestion time of α-lactalbumin, bovine serum albumin and albumin from hen egg white was reduced from hours (12/24h) to 15 min. Dominguez-Vega et al (Dominguez-Vega et al. 2010) accomplished soybean protein digestion in one minute using an ultrasound probe. This clearly evidences the efficiency of this type of energy for proteins digestion. UA digestion applying the conditions described in section 2.3. According to model results, protein trypsin ratio significantly affected β-casein (P=0.01) and ovalbumin (P=0.03) yields, whereas digestion time significantly modified α-casein (P=0.005) and β-casein (P=0.002) yields. For all cases lack of fit test for quadratic models showed P values higher than 0.05, and adjusted R^2>90% which is adequate in terms of model suitability (Derringer and Suich 1980). Corresponding digestion yields obtained for each MRM transition for each experimental run are shown in Table 3.

Table 3. Experimental runs for a central composite design for ultrasound-assisted digestion with the corresponding responses (means) for each MRM transition (peak area)

	factors		responses (peak area, *m/z*)*		
	time (min)	enzyme: protein ratio	634.6→991.8	390.9→258.25	929.5→1116.5
1	30.0	1:10	233.00±0.35	1350.00±4.59	36.00±1.06
2	3.0	1:100	760.00±2.12	2823.00±2.82	58.00±2.47
3	3.0	1:10	1000.00±3.18	9018.00±0.70	105.00±0.35
4	16.5	1:100	311.00±11.31	1478.00±5.65	167.00±0.70
5	16.5	1:20	493.00±1.76	2546.00±7.42	94.00±0.00
6	16.5	1:10	598.00±2.47	2764.00±0.70	51.00±0.70
7	30.0	1:20	173.00±8.38	877.00±6.71	47.00±1.06
8	3.0	1:20	845.00±2.82	4604.00±0.35	48.00±5.31
9	30.0	1:100	173.00±0.00	862.00±1.06	176.00±2.75
10	16.5	1:20	110.00±4.94	1555.00±2.12	107.00±5.25

*mean± standard deviation (*n*=2)

Based on individual optimum, a multiple response optimization was calculated in order to determine the optimal conditions for all responses (desirability conditions). Response

surface plots for each transition are shown in Figure 4. Optimal trypsin digestion conditions to simultaneously determine α-casein, β-casein, and ovalbumin in wines were 3 min as digestion time and 1:10 enzyme to protein ratio. As expected, MRM results were consistent with stained SDS-PAGE gel, which showed a large number of bands in the lanes corresponding to the products (peptides) of digested proteins (Suplementary data, Figure 2). %SQ values were appropriated showing values of 25, 26 and 23 for α-casein, β-casein, and ovalbumin, respectively.

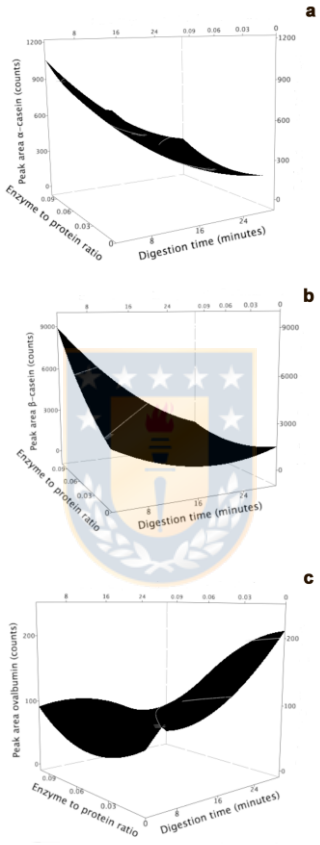

Figure 4. Response surface plots for α-casein (a), β-casein (b), and ovalbumin (c) transitions obtained with UA digestion.

4. Conclusions

In conclusion, a face-centered central composite design was used to optimize UA-, MA- and IR-assisted protein digestion conditions. To the best of our knowledge this work reports for the first time the use of these techniques for α-casein, β-casein and ovalbumin determination in wines. All optimized methods allowed the reduction of digestion time as

well as an increase on trypsin yields. According to results obtained via MRM peptide marker (transitions), the most effective technique was MA follow by UA and IR. Taking in to account the simplicity of sample preparation procedure, UA was defined as technique of choice. This technology allowed the reduction of protein digestion time from 7h to 3 min (140-fold reduction), without compromising %SQ. Further, compared with non-optimized conventional digestion time, the reduction was ca. 240-fold, from 12h to 3 min.

Acknowledgments

This work is part of Jessy Pavón Pérez thesis to obtain the degree of Doctor in Science and Analytical Technology from the University of Concepcion, Chile. Authors want to thank to the National Commission of Scientific and Technological Research (CONICYT) of the Chilean Government for the doctoral scholarship granted.

Funding

This work was supported by the National Fund for Scientific and Technological Development (FONDECYT) project No1171857 and by the Fund for Scientific and Technological Equipment (FONDEQUIP) project No 130209.

Disclosure statement

Jessy Pavon, Karem Henríquez-Aedo, Ricardo Salazar, Miguel Herrero and Mario Aranda declare that they have no conflict of interest.

References

Capelo, J. L., Carreira, R., Diniz, M., Fernandes, L., Galesio, M., Lodeiro, C., et al. (2009). Overview on modern approaches to speed up protein identification workflows relying on enzymatic cleavage and mass spectrometry-based techniques. *Analytical Chemical Acta, 650*(2), 151-159. doi: 10.1016/j.aca.2009.07.034

Chambery, A., del Monaco, G., Di Maro, A., & Parente, A. (2009). Peptide fingerprint of high quality Campania white wines by MALDI-TOF mass spectrometry. *Food Chemistry, 113*(4), 1283-1289. doi: https://doi.org/10.1016/j.foodchem.2008.08.031

Chen, Y., Wu, M., Wang, K., Chen, B., Yao, S., Zou, H., et al. (2011). Vinyl functionalized silica hybrid monolith-based trypsin microreactor for on line digestion and separation via thiol-ene "click" strategy. *Journal of Chromatography A, 1218*(44), 7982-7988. doi: https://doi.org/10.1016/j.chroma.2011.09.002

Chen, Z., Li, Y., Lin, S., Wei, M., Du, F., & Ruan, G. (2014). Development of continuous microwave-assisted protein digestion with immobilized enzyme. *Biochemical Biophysical Research Communications, 445*(2), 491-496. doi: https://doi.org/10.1016/j.bbrc.2014.02.025

Chiva, C., Ortega, M., & Sabidó, E. (2014). Influence of the Digestion Technique, Protease, and Missed Cleavage Peptides in Protein Quantitation. *Journal of Proteome Research, 13*(9), 3979-3986. doi: 10.1021/pr500294d

Cristina, L., Elena, A., Davide, C., Marzia, G., Lucia, D., Cristiano, G., et al. (2016). Validation of a mass spectrometry-based method for milk traces detection in baked food. *Food Chemistry, 199*, 119-127. doi: https://doi.org/10.1016/j.foodchem.2015.11.130

Derringer, G., & Suich, R. (1980). Simultaneous Optimization of Several Response Variables. *Journal of Quality Technology, 12*(4), 214-219. doi: 10.1080/00224065.1980.11980968

Dominguez-Vega, E., Garcia, M. C., Crego, A. L., & Marina, M. L. (2010). First approach based on direct ultrasonic assisted enzymatic digestion and capillary-high performance liquid chromatography for the peptide mapping of soybean proteins. *Journal of Chromatography A, 1217*(42), 6443-6448. doi: 10.1016/j.chroma.2010.08.027

El Rayess, Y., Albasi, C., Bacchin, P., Raynal, J., Taillandier, P., Mietton-Peuchot, M., et al. (2011). Cross-flow microfiltration applied to oenology: A review. *Journal of Membrane Science, 382*, 1-19. doi: 10.1016/j.memsci.2011.08.008

European Commission (EU). (2012). Commission implementing regulation 579/2012. *Official Journal European Union, L171*, 4-7.

González-Neves, G., Favre, G., & Gil, G. (2014). Effect of fining on the colour and pigment composition of young red wines. *Food chemistry, 157*, 385-392. doi: 10.1016/j.foodchem.2014.02.062

Hahn, H. W., Rainer, M., Ringer, T., Huck, C. W., & Bonn, G. K. (2009). Ultrafast Microwave-Assisted In-Tip Digestion of Proteins. *Journal of Proteome Research, 8*(9), 4225-4230. doi: 10.1021/pr900188x

Helmut, B., Hildburg, B., & J., G. H. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *ELECTROPHORESIS, 8*(2), 93-99. doi: doi:10.1002/elps.1150080203

Jiang, B., Yang, K., Zhao, Q., Wu, Q., Liang, Z., Zhang, L., et al. (2012). Hydrophilic immobilized trypsin reactor with magnetic graphene oxide as support for high efficient proteome digestion. *Journal of Chromatography A, 1254*, 8-13. doi: https://doi.org/10.1016/j.chroma.2012.07.030

Joergensen, L., & Thestrup, H. N. (1995). Determination of amino acids in biomass and protein samples by microwave hydrolysis and ion-exchange chromatography. *Journal of Chromatography A, 706*(1), 421-428. doi: https://doi.org/10.1016/0021-9673(94)01107-P

Kadam, S. U., Tiwari, B. K., Álvarez, C., & O'Donnell, C. P. (2015). Ultrasound applications for the extraction, identification and delivery of food proteins and bioactive peptides. *Trends Food Science and Technology, 46*(1), 60-67. doi: https://doi.org/10.1016/j.tifs.2015.07.012

Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature, 227*, 680. doi: 10.1038/227680a0

Li, D., Teoh, W. Y., Gooding, J. J., Selomulya, C., & Amal, R. (2010). Functionalization Strategies for Protease Immobilization on Magnetic Nanoparticles. *Advance Functional Materials, 20*(11), 1767-1777. doi: doi:10.1002/adfm.201000188

Liu, M., Hu, Y., Zhang, Y., & Lu, H. (2013). Mechanism exploration of adsorption-immobilized enzymatic reactor using polymer-coated silica microbeads. *Talanta, 110*, 101-107. doi: https://doi.org/10.1016/j.talanta.2013.02.021

Losito, I., Introna, B., Monaci, L., Minella, S., & Palmisano, F. (2013). Development of a method for the quantification of caseinate traces in Italian commercial white wines based on liquid chromatography-electrospray ionization-ion trap-mass spectrometry. *Journal of Agricultural and Food Chemistry, 61*(50), 12436-12444. doi: 10.1021/jf4034909

Loziuk, P. L., Wang, J., Li, Q., Sederoff, R. R., Chiang, V. L., & Muddiman, D. C. (2013). Understanding the Role of Proteolytic Digestion on Discovery and Targeted Proteomic Measurements Using Liquid Chromatography Tandem Mass Spectrometry and Design of Experiments. *Journal of Proteome Research, 12*(12), 5820-5829. doi: 10.1021/pr4008442

Mainente, F., Zoccatelli, G., Lorenzini, M., Cecconi, D., Vincenzi, S., Rizzi, C., et al. (2014). Red wine proteins: Two dimensional (2-D) electrophoresis and mass spectrometry analysis. *Food Chemistry, 164*, 413-417. doi: https://doi.org/10.1016/j.foodchem.2014.05.051

Mattarozzi, M., Milioli, M., Bignardi, C., Elviri, L., Corradini, C., & Careri, M. (2014). Investigation of different sample pre-treatment routes for liquid chromatography–tandem mass spectrometry detection of caseins and ovalbumin in fortified red wine. *Food Control, 38*, 82-87. doi: https://doi.org/10.1016/j.foodcont.2013.10.015

Monaci, L., & Visconti, A. (2009). Mass spectrometry-based proteomics methods for analysis of food allergens. *Trends in Analytical Chemistry, 28*(5), 581-591. doi: 10.1016/j.trac.2009.02.013

Revision of the limit of detection and limit of quantiifcation related to potentially allergenic residues of finning agent proteins in wine (2012).

Pavón-Pérez, J., Henriquez-Aedo, K., & Aranda, M. (2019). Mass Spectrometry Determination of Fining-Related Allergen Proteins in Chilean Wines. *Food Analytical Methods, 12*(4), 827-837. doi: 10.1007/s12161-018-01416-0

Pramanik, B. N., Mirza, U. A., Ing, Y. H., Liu, Y.-H., Bartner, P. L., Weber, P. C., et al. (2002). Microwave-enhanced enzyme reaction for protein mapping by mass spectrometry: a new approach to protein digestion in minutes. *Protein Society, 11*(11), 2676-2687. doi: 10.1110/ps.0213702

Reddy, P. M., Hsu, W. Y., Hu, J. F., & Ho, Y. P. (2010). Digestion completeness of microwave-assisted and conventional trypsin-catalyzed reactions. *Journal of the American Society for Mass Spectrometry, 21*(3), 421-424. doi: 10.1016/j.jasms.2009.11.006

Restani, P., Uberti, F., Tarantino, C., Ballabio, C., Gombac, F., Bastiani, E., et al. (2014). Collaborative Interlaboratory Studies for the Validation of ELISA Methods for the Detection of Allergenic Fining Agents Used in Wine According to the Criteria of OIV Resolution 427–2010 Modified by OIV–Comex 502–2012. *Food Analytical Methods, 7*(3), 706-712. doi: 10.1007/s12161-013-9741-2

Rial-Otero, R., Carreira, R. J., Cordeiro, F. M., Moro, A. J., Fernandes, L., Moura, I., et al. (2007). Sonoreactor-based technology for fast high-throughput proteolytic digestion of proteins. *Journal of Proteome Research, 6*(2), 909-912. doi: 10.1021/pr060508m

Rivera, J. G., & Messersmith, P. B. (2012). Polydopamineo, F. M., Moro, A. J., Fernandes, L., Moura, I., et al. (2007). Sonoreactor-based tech*Journal of Separation Science, 35*(12), 1514-1520. doi: doi:10.1002/jssc.201200073

Rolland, J. M., Apostolou, E., De Leon, M. P., Stockley, C. S., & O'Hehir, R. E. (2008). Specific and sensitive enzyme-linked immunosorbent assays for analysis of residual allerqenic food proteins in commercial bottled wine fined with egg white, milk, and nongrape-derived tannins. [Article]. *Journal of Agricultural and Food Chemistry, 56*(2), 349-354. doi: 10.1021/jf073330c

Santos, H. M., Rial-Otero, R., Fernandes, L., Vale, G., Rivas, M. G., Moura, I., et al. (2007). Improving sample treatment for in-solution protein identification by peptide mass fingerprint using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Journal of Proteome Research, 6*(9), 3393-3399. doi: 10.1021/pr0702518

Shevchenko, A., & Shevchenko, A. (2001). Evaluation of the Efficiency of In-Gel Digestion of Proteins by Peptide Isotopic Labeling and MALDI Mass Spectrometry. *Analytical Biochemistry, 296*(2), 279-283. doi: https://doi.org/10.1006/abio.2001.5321

Vogt, E. I., Kupfer, V. M., Vogel, R. F., & Niessen, L. (2016). A novel preparation technique of red (sparkling) wine for protein analysis. *EuPA Open Proteomics, 11*, 16-19. doi: https://doi.org/10.1016/j.euprot.2016.03.001

Wang, S., Zhang, L., Yang, P., & Chen, G. (2008). Infrared-assisted tryptic proteolysis for peptide mapping. *Proteomics, 8*(13), 2579-2582. doi: 10.1002/pmic.200800086

Wu, S., Zhang, H., Yang, K., Ma, J., Liang, Z., Zhang, L., et al. (2015). A rapid protein sample preparation method based on organic-aqueous microwave irradiation technique. *Science China Chemistry*, *58*(3), 526-531. doi: 10.1007/s11426-014-5163-2

Yuan, H., Zhang, L., Hou, C., Zhu, G., Tao, D., Liang, Z., et al. (2009). Integrated Platform for Proteome Analysis with Combination of Protein and Peptide Separation via Online Digestion. *Analytical Chemistry*, *81*(21), 8708-8714. doi: 10.1021/ac900310y

Zhao, Q., Fang, F., Wu, C., Wu, Q., Liang, Y., Liang, Z., et al. (2016). imFASP: An integrated approach combining in-situ filter-aided sample pretreatment with microwave-assisted protein digestion for fast and efficient proteome sample preparation. *Analytical Chimica Acta*, *912*, 58-64. doi: 10.1016/j.aca.2016.01.049

Capítulo II Sección 2 Suplementary material

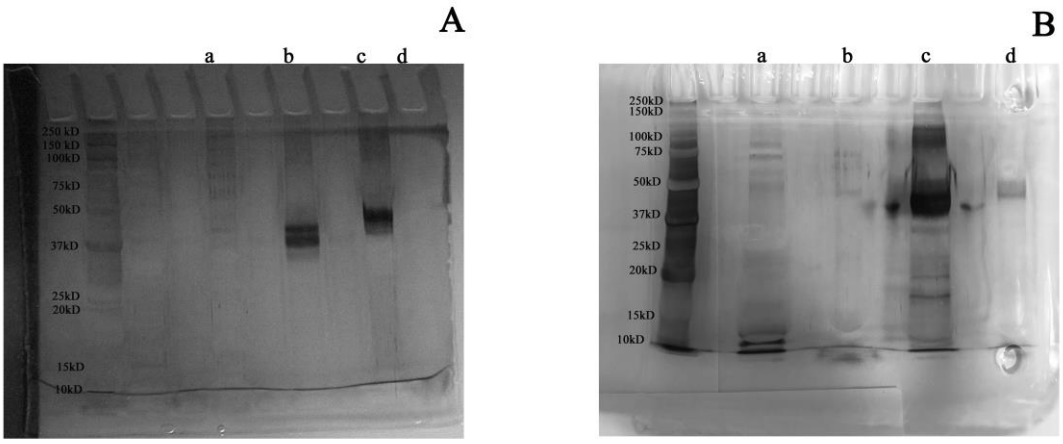

Figure SI-1. Bands in stained SDS-PAGE gel for native standard of casein (A-a) and ovalbumin (A-c), and digest of casein (A-b) and ovalbumin (A-d) with IR energy as digestion accelerated method. Native standard of casein (B-b) and ovalbumin (B-d), and digest of casein (B-a) and ovalbumin (B-c) with microwave energy as digestion accelerated method.

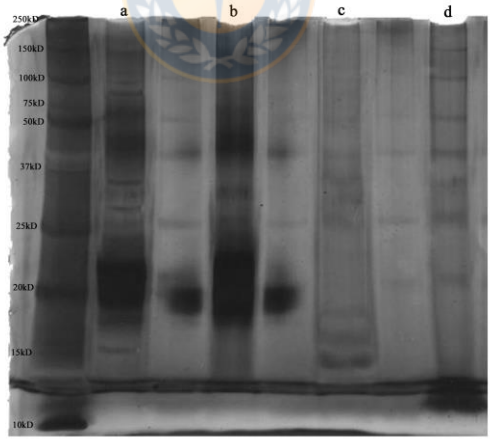

Figure SI-2 Bands in stained SDS-PAGE gel for native standard of ovalbumin (b) and casein (c), and digest of ovalbumin (a) and casein (d) with US energy as digestion accelerated method.

CAPITULO II SECCION 3

Title: Occurrence of allergen proteins in wines from Chilean market.

Jessy Pavón-Pérez[a], Karem Henriquez-Aedo[a,b], Miguel Herrero[c] Mario Aranda Bustos[a,b]*

[a] Laboratory of Advanced Research on Foods and Drugs, Department of Food Science and Technology, Faculty of Pharmacy, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile. Email: jpavon@udec.cl

[b] Laboratory of Functional Foods, Center for Biotechnology, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile. Email: karhenri@udec.cl

[c] Foodomics Laboratory, Institute of Food Science Research (CIAL, CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain

Abstract

Casein and ovalbumin are used in winemaking process as fining agents. They may trigger allergic reactions in susceptible individuals; therefore, their occurrence in wines could become a human health risk, moreover when their presence is not reported. The objective of this work was to determine the occurrence of casein and ovalbumin in Chilean wines. Proteins were digested applying a ultrasound-assisted method and quantified by tandem mass spectrometry using isotope labelled peptide as internal standard. Ultrasound bath, 3 minutes and 1:10 enzyme protein ratio were the conditions selected for proteins digestion. Repeatability and intermediate precision showed RSD values <1.67% and <1.95%, respectively. Recoveries ranged from 89.75 to 115.09%. Detection and quantification limits ranged from 5.6 to 8.4 µg L^{-1} and 10 to 20 µg L^{-1}, respectively. Sixty Chilean wine samples were analyzed, fixty-six samples showed quantifiable levels, from which seventeen samples presented a total casein and ovalbumin concentration (0.24-0.30 mg L^{-1}) closer or higher to the European limit for mandatory labelling (0.25 mg L^{-1}).

1. Introduction

Wine industry is a very important sector of Chile's economy in terms of productivity, exports and employment. Chilean wine possess international recognition due to its particular sensorial and chemical profiles, both extensively investigated (Bridi, Lobato, López-Alarcón, & Lissi, 2014). Fining agents are used in winemaking process, with the principal objective to remove undesirable compounds like polymeric phenols and polyphenols (Losito, Introna, Monaci, Minella, & Palmisano, 2013). Caseinates or milk powerds and ovalbumin or egg white powders are proteins employed for this purpose. Even when these allergenics proteins are removed from the wine after the finnig process, low concentrations of these can be found in the final product. Thus, its unlabeled presence in commercial product has been subject of debate in the last years due to the risk to susceptible consumers. Therefore, the determination at low concentration levels is highly desirable to ensure consumer health security (D'Amato, Kravchuk, Bachi, & Righetti, 2010). The European Union implemented the Directives 89/2003/EC and its latest version 579/2012/EC, which contain a list of allergenic substances including egg and milk derivatives (European Commission (EU), 2012). In this document is established that wines with concentration higher than the analytical limits defined by the Organisation Internationale de la Vigne et du Vin (OIV) in its resolution OIV/COMEX/502/2012 (Organisation Internationale de la Vigne et du Vin (OIV), 2012) should be declared. The OIV official method for egg and milk derivatives analysis in wines is the enzyme-linked immunosorbent assay (Vogt, Kupfer, Vogel, & Niessen, 2016), which achieved detection and quantification limits of 0.25 mg L^{-1} and 0.50 mg L^{-1}, respectively. (Hoxey, Stockley, Wilkes, & Johnson, 2013) reported detectable levels of casein (from 0.27 to 5 mg L^{-1}) in 3 of 521 Australian wines sampled. (Rolland, Apostolou, De Leon, Stockley, & O'Hehir, 2008) found detectable concentrations of ovalbumin (1 µg L^{-1}) in Australian wines. (Tolin, Pasini, Simonato, Mainente, & Arrigoni, 2012) informed the presence of casein and ovalbumin in 32% (8/25) of Italian commercial wines evaluated. Currently, in Chile there is no a normative that regulate the use of these proteins as fining wine, hence, casein and ovalbumin determination is not a common assay in wineries or in national reference laboratories. The objective of this work was to determine the occurrence of casein and ovalbumin in Chilean wines.

ELISA analysis are simple and fast (Mattarozzi, et al., 2014) but in red wines the high content of polyphenols interact with proteins and antibodies, causing detection problems (Patrick, Hans, & Angelika, 2009) and due to the adsorption of allergens to solid matrix that supports the analysis, protein epitopes may suffer some alterations modifying its recognition or accessibility impairing the assay sensitivity (Kaul, et al., 2007). For that liquid chromatography coupled to mass spectrometry have become a value option. Usually in proteomic analysis proteins are subjected to proteolytic cleavage by chemical hydrolysis or enzymatic digestion before the analysis by mass spectrometry (Reddy, Hsu, Hu, & Ho, 2010). This digestion process is considered the bottleneck of *bottom up* analysis because conventional proteolytic digestion methods typically involve several hours (12 to 14 hours) (Reddy, et al., 2010). Trypsin is the most used enzyme due to it cleaves exclusively at basic residues to arginine and lysine (Loziuk, et al., 2013) which generates peptides with an ideal mass range (7-9 amino acids) for mass spectrum interpretation. The typical time of tryptic digestion is as long as 12 h being incompatible with high-throughput protein identification (Wang, Zhang, Yang, & Chen, 2008). Ultrasound energy has been previously used to shorten the digestion time. The mechanism is not completely understood yet, it appears to be related to an increase in diffusion rates as consequence of the cavitation phenomena and heating (Santos, et al., 2007). Ultrasound bubble cavitation causes crushing, which results in large mechanical shearing forces, which degrade protein structure and open up hydrophilic groups. This opening up of structure increases the protein solubility and allows the enzyme to more easily bind with the protein substrate (Kadam, Tiwari, Álvarez, & O'Donnell, 2015). To evaluate the occurrence of casein and ovalbumin in Chilean wines, the present work reports a fast proteolytic cleavage applying ultrasound-assisted digestion method and for a realiable determination, quantification was carried out by tandem mass spectrometry using isotope labelled peptide as internal standard.

2. Materials and Methods

2.1 Reagents, standards and samples

Formic acid (98-100%), trichloroacetic acid (TCA, >99%), ammonia solution (>25%), ammonium hydrogen carbonate (NH_4HCO_3, 99%), ethanol absolute p.a, acetic acid (100%), (37%), sequencing modified trypsin from bovine pancreas and LC-grade

acetonitrile were purchased from Merck (Darmstadt, Germany). Casein from bovine milk, albumin from chicken egg white, iodoacetamide (IAM, >99%), DL- dithiothreitol (DTT, >99 %), were acquired from Sigma-Aldrich (St. Louis, MO, USA). Buffered solutions and mobile phases were prepared with ultrapure water (18.2 MΩ cm) produced by means of Simplicity system from Millipore (Bedford, MA, USA) Stable isotope-labelled [$^{13}C_5$, ^{15}N]-valine (V*) and [$^{13}C_6$, ^{15} N]-leucine (L*), VL*PV*PQK (IS) was synthesized and purchased from GenScript (Hon Kong, China). 12 mg L^{-1} casein and ovalbumin stock solution and IS solution (100 mg L^{-1}) was prepared in 50 mM NH_4HCO_3 (pH 7.8). Standard solutions were obtained by aliquot dilution from stock solution

2.2 Sample preparation

Allergen proteins from wine samples were extracted and purified following the method proposed by Mattarozzi et al (Mattarozzi, et al., 2014) with slight modifications. Briefly, 12.5 mL of wine were spiked with 10 µl IS solution (100 mg L^{-1} in 50 mM NH_4HCO_3) and centrifuged at 5433 x *g* for 40 min at 20 ^{o}C into 10 kDa cut-off membrane ultrafiltration tube (Amicon Merck Millipore), previously conditioned with 10 mL of distilled water. 2.5 mL were obtained from which proteins were precipitated on ice for 2 h after addition of eight volumes of ethanol: TCA (15%w/v), pH 4.5. Thereafter, the mixture was centrifuged at 8981 x *g* for 10 min at 4ºC using a Hettich (Tuttlingen, Germany) refrigerated centrifuge. The ethanol: TCA residues were evaporated under a gentle flow of nitrogen without heat. The obtained pellet was solubilized in 1 mL of 50 mM NH_4HCO_3, pH 7.8. Trypsin digestion of both, sample and standards, was performed after proteins reduction and alkylation. The first was carried out by addition of 12 µL of 100 mM DTT and incubation at 95ºC for 5 min. After cooling down, the cysteine SH-groups, formed during the reduction step, were irreversibly alkylated adding 24 µL of freshly prepared aqueous solution of 100 mM IAM, the mixture was left stand for 15 min at room temperature protected from light. Enzymatic digestion was performed adding 4 µL of trypsin solution (0.5 mg mL^{-1}, in 50 mM of NH_4HCO_3, pH 7.8) for obtaining a trypsin: protein ratio of 1:10, the mixture was incubated at 37ºC for 3 min in ultrasound bath. The reaction was stopped adding 10 µL of formic acid (0.1%v/v) aqueous solution. All digested samples and

standards were filtered through a 13mm PVDF syringe filter (0.22 µm) before ELISA and LC analysis.

A total of 60 Chilean commercial wine samples without allergen labelling were analyzed. All samples were purchased directly from supermarkets and specialized stores without contact with wineries.

2.3 Ultrasound-assisted digestion

Previously an optimized trypsin digestion method with ultrasound energy of the proteins in study was developed in our laboratory. The protein and trypsin mixtures were placed in transparent eppendorff tubes in ultrasound bath model SB-5200D from BiosLabChile (Santiago, Chile). This device provides indirect sonication, working at 40 kHz of frequency with the capacity for controling the temperature at 37 ºC. The digestion was performed for 3 min at 37 ºC.

2.4 ELISA test

Wines were tested for the presence of casein and ovalbumin using the RIDASCREENFAST Casein ELISA (R-Biopharm AG, Germany) following the test kit description (LOQ=0.5 mg L^{-1}, LOD=0.12 mg L^{-1}) and egg white proteins using the RIDASCREENFAST Ei/Egg ELISA (R-Biopharm AG) according to the leaflet (LOQ=0.5 mg L^{-1} and LOD=0.10 mg L^{-1} whole egg powder). It is a sandwich ELISA where wells of the microtiter strips are first coated with the specific anti-casein or ovalbumin antibody; after incubation with the wine sample a secondary anti-casein/ovalbumin antibody conjugated with to peroxidase is added to form a sandwich. The detection of peptides takes place by adding Substrate/Chromogen solution. The enzyme conjugate converts the chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement was made photometrically in plate multilector Tecan Infinite M200 Pro (Männedorf, Suiza) at 450 nm. According to manufacturers specifications 1 mL of wine can be extracted with 9 mL diluted Allergen extraction buffer and peptides concentration in the sample can directly be read from the standard curve.

2.5 Liquid chromatography – tandem mass spectrometry

For LC-MS analyses a Shimadzu (Kyoto, Japan) Nexera X2 UHPLC system consisted of: LC-30AD pump, DGU-20A5R degassing unit, SIL-30AC autosampler, CTO-20AC column oven, CBM-20A communication module, SPD-M20A diode array detector (DAD) and LCMS-8030 triple quadrupole (TQ) mass spectrometer. The system was controlled by the LabSolution 5.8 software. Peptides separation was carried out on Phenomenex (Torrance, CA, USA) Kinetex XB Core-Shell C_{18} column (100 mm x 4.6 mm, id. 2.6 µm), thermostated at 35ºC, using a mobile phase composed of ultrapure pure water (A) and acetonitrile (B) both with 0.1 % (v/v) formic acid. The following gradient program was applied at a flow rate of 0.6 mL min $^{-1}$: 0–19 min 10–40% B, 19–20 min 40–10% B, followed by 5 min for column conditioning. MS analysis was performed in ESI positive mode using the following conditions: ESI voltage 4.5 kV; collision energy -30.0 V for casein and -40.0 V to ovalbumin; nebulizer gas (N_2) 3 L min^{-1}, desolvation gas (N_2) 18 L min^{-1}; desolvation line temperature 250ºC and heat block temperature 400ºC. Full scan spectra were acquired from *m/z* 100 to 1000. In previous works in our laboratory following the early describe criteria, the two most abundant and stable fragments from the product ion spectrum were chosen for each peptide (protein), one for qualitative/confirmatory and one for quantitative purposes (Table 1). Thus, the MRM transitions established for quantification were *m/z* 634.6→991.8 for α-casein, *m/z* 390.9→258.5 for β-casein and *m/z* 929.5→1116.5 for ovalbumin and for SI *m/z* 397.3→220.3 recommended by the manufacturer.

Table 1. MRM transitions for α, β-casein and ovalbumin determination in wines

Protein	Precursor ion sequence (*m/z*; charge state; MW [Da])	Product ion sequence (*m/z*; charge state)
α-casein	YLGYLEQLLR (*m/z* 634.6; +2; 1267.2)	GYLEQLLR (*m/z* 991.8; +1)[a,b] LEQLLR (*m/z* 771.4; +1)[c]
β-casein	VLPVPQK (*m/z* 390.9; +2; 779.8)	QK (*m/z* 258.25; +1)[a,b] PVPQK (*m/z* 284.6; +2)[c]
Ovalbumin	ELINSWVESQTNGIIR (*m/z* 929.5; +2; 1857.9)	VESQTNGIIR (*m/z* 1116.5; +1) [a,b] ESQTNGIIR (*m/z* 1017.5; +1)[c]
IS	VL*PV*PQK (*m/z* 397.3; +2; 792.97)	VP (*m/z* 220.3)[b]

[a] Most consistent MS/MS transition. [b] *m/z* transition monitored for quantification. [c] *m/z* transition monitored for confirmation.

2.6 Statistical analysis

Data were evaluated using descriptive statistics [mean, standard deviation (SD) and relative standard deviation (RSD)]. Peptides calibrations were established via linear regression analysis with area ratios. Calibrations with and without matrix were compared using F-test. All above tests were done with a significance level (α) of 0.05 using GraphPad (San Diego, CA, USA) Prism 6.0 software.

3. Results and discussion

3.1 ELISA

For ELISA test six wine samples were employed. One Cabernet Sauvignon sample and one Sauvignon Blanc free from allergen related proteins, both spiked with 2.5 mg L^{-1} of casein and ovalbumin standards were analyzed. Also, two samples of commercial wine were analyzed, one Sauvignon Blanc and one Merlot, previously quantified by LC-MS/MS in our laboratory, with casein and ovalbumin concentrations of 0.20 mg L^{-1} and 0.21 mg L^{-1} respectively. Samples digestion was performed with ultrasound energy for three minutes at 37ºC with 1:10 trypsin protein ratio. Even when the LOD of the commercially ELISA test used were 0.12 mg L^{-1} and 0.10 mg L^{-1} for casein and ovalbumin, in commercial wines no concentrations of proteins were detected. Probably matrix components interfere during the ELISA reactions, in contrast with UHPLC-MS/MS method already implemented in our laboratory.

3.2 Method Validation

Arsene et al (Arsene, et al., 2008) in 2008 demonstrated the efficiency of stable isotope-labelled protein like IS because it was always added into the sample at the first step of sample preparation and theoretically could normalise all variation during the whole experimental process. As a consequence of the high molecular weight of the proteins this strategy has a high cost. The use of smaller isotope-labelled peptide homologue of the signature peptide has been more employed. Its proper selection and the moment at which it will be added are decisive to obtain good results. Lutter et al (Lutter, Parisod, & Weymuth, 2011) designed an IS homologue of the signature peptide, which was added after the tryptic digestion. Zhang et al (Zhang, et al., 2012) designed peptide containing a similar amino acid sequence to the signature peptide in the middle with the extension of several amino

acids from the C- and N-end homologue to the original sequence. This IS was added prior to sample extraction and the tryptic digestion to correct any loss of analytes during sample pre-treatment. Since this IS relied on tryptic digestion to be converted, any change in the digestion reaction would affect the IS in the same way as the target analyte. All variations in the tryptic digestion reaction could be normalised by this IS. Therefore, such IS not only could normalise the variation during the tryptic digestion reaction but also could be used as an indicator for the presence of trypsin inhibitor (Chen, et al., 2015). However, the longer the amino acid sequence the more expensive is the IS. Therefore, in this work we chose as IS a stable isotope-labelled peptide (VL*PV*PQK), which has the same amino acid sequence of bovine β-casein signature peptide (VLPVPQK). The use of IS should normalize the variation of the ionization during the MS detection and correct for the loss of analytes during the sample pre-treatment process. Also, due to both peptides (isotope-labeled peptide and the native peptide), has the same chemical properties, they should exhibit identical behaviors during LC and electrospray ionization processes and be separated by their differences in mass, thus enabling accurate quantification.

The optimized method was validated following the International Conference on Harmonization recommendations (International Conference on Harmonisation (ICH), 2005). For each peptide analyzed by LC-MRM/MS, the peak area was normalized to that of the internal standard peptide, thus, a complete separation was achieved in less than 20 min (Figure 1).

Calibration curves of all proteins were established with and without wine matrix in order to evaluate a possible matrix effect. Since slopes were not statistically different ($P > 0.05$) calibrations were established with pure standards at six levels in triplicate from 0.10 to 1.20 mg L^{-1}. Calibrations data fit a linear regression model with determination coefficients (R^2) =0.99 for all proteins. Method precision was evaluated through repeatability and intermediate precision. Repeatability was studied injecting in sextuplicate (n=6) a spiked wine sample (0.25 mg L^{-1}) showing RSDs values from 0.39% to 1.67%. Intermediate precision was determined measuring in triplicate a spiked wine sample (0.25 mg L^{-1}) during three different days (n=3), showing RSDs values from 0.03% to 1.95%. Method accuracy was determined via recovery evaluation.

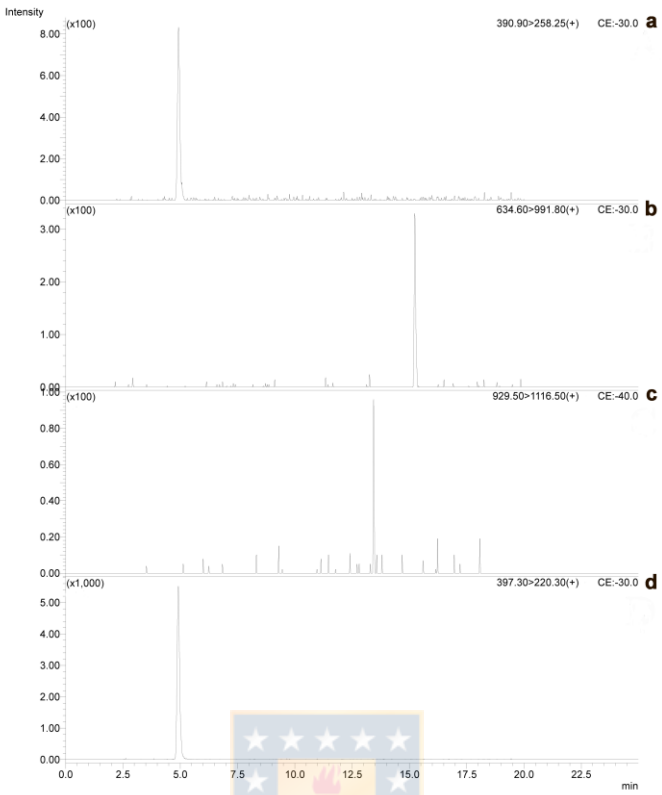

Figure 1. LC-ESI-MS/MS chromatograms of Merlot sample analyzed in MRM mode for β-casein (a), α-casein (b), ovalbumin (c) and internal standard (d) detection.

Wine samples spiked at three levels (0.15, 0.25, 0.50 mg L^{-1}) were prepared and analyzed daily during three days. All proteins showed recovery yields between 89.75 % to 115.09 % with RSD values <5.84%. Detection and quantification limits were calculated using signal-to-noise ratios (S/N) of 3 and 10 respectively. Considering the use of a core-shell column and an injection volume of 50 µL, the detection and quantification limits ranged from 5.6 to 8.4 µg L^{-1} and 10.0 to 20.0 µg L^{-1}, respectively. Comparatively, this optimized method showed similar validation results than others methods for allergen proteins determination in foods using internal standard and LC-MS/MS. Linear range (0.10-1.20 mg L^{-1}) is lower than reported by Chen et al (Chen, et al., 2015) (1-10 mg L^{-1} and 10-100 mg L^{-1}) for bovine β-casein quantification in baked foodstuffs. Recovery results (>85%) are similar to those reported by (Chen, et al., 2016) for β-casein quantification in human milk (99.5 to 100.3%) and (Zhang, et al., 2014) for bovine lactoferrin quantification in dairy products (87.8 to

104.7%). In terms of precision RSDs values from 0.03% to 1.95% are slightly lower than reported by (Zhang, et al., 2014) which reported values from 1.9% to 7.10%) and (Ippoushi, Sasanuma, Oike, Kobori, & Maeda-Yamamoto, 2016) with values from 10.5% to 13.9%. Detection and quantification limits were lower than those reported by (Restani, et al., 2014) using ELISA method (LOD 0.15 mg mL^{-1}; LOQ 0.38 mg mL^{-1}); and (Mattarozzi, et al., 2014) (LOD=0.01 to 0.80 mg L^{-1} and LOQ = 0.03 to 2.00 mg L^{-1}).

3.3 Samples analysis

As indicated above, allergen proteins detection in wines by ELISA (OIV official method) presents some limitations that impair the assay sensitivity. Contrarily, the proposed LC-MS/MS method is highly sensitive capable of detecting concentrations as low as 0.01 mg L^{-1} (Table 2). Employing ultrasound energy to accelerate the enzymatic digestion, and applying this optimized and validated method, sixty Chilean commercial wine samples without allergen labelling were analyzed. All wine samples labels described the wine type/category, i.e. young (without aging) or reserve (higher quality and/or aged), cv. (one grape variety represents at least 75% of the blend), harvest year and geographical origin (denomination of origin). According to that, twenty five samples were reserve and thirty five young wines, belonging to eight valleys and twenty four wineries. Eighteen samples were Cabernet Sauvignon, nine Sauvignon Blanc, eleven Carménère, thirteen Merlot, theree Chardonnay, three Syrah, one Malbec, one Rosé and one formed by a mixture of strains (Cabernet Sauvignon 50%, Carménère 30% and Syrah 20%). Two independent extractions from each sample were analyzed in duplicate. All samples, except four, showed quantifiable levels of allergen proteins, the level found ranged from non-detected to 0.30 mg L^{-1} (Table 2). Six Merlot samples (6/60; 10%), four Cabernet Sauvignon samples (4/60; 6%), three Sauvignon Blanc (3/60; 5%), two Carménère (2/60; 3%), one Malbec (1/60; 1%), and one sample formed by a mixture of strains (1/60; 1%) showed a total concentration of casein and ovalbumin higher than the limit recommended by the European Community (0.25 mg L^{-1}). The percentage of positive samples was high (56/60; 93%), which demonstrated the prevalent use of casein and ovalbumin as finning agent in Chilean wineries. This result is much higher than international reports. (Lifrani, et al., 2009) found residual contents of ovalbumin and casein in 11% (44/400) of commercially available

French wines analyzed. (Hoxey, et al., 2013) reported detectable levels of casein (from 0.27 to 5 mg/L) in 0.6% (3/521) of Australian wines sampled. (Rolland, et al., 2008) found detectable concentrations of ovalbumin (1 μg L^{-1}) in 1.3% (2/153) of Australian wines. (Tolin, et al., 2012) informed the presence of casein and ovalbumin in 32% (8/25) of Italian commercial wines evaluated.

Table 2. Allergen proteins content (mg L^{-1}) in Chilean wine samples

	Wine samples	Valley	Year[b]	α-casein	β-casein	Ovalbumin	Total
1	S. Blanc	Colchagua	2016	0.02±0.00	0.02±0.00	ND	0.04
2	S. Blanc	Rapel	2015	0.08±0.00	0.04±0.00	ND	0.11
3	S. Blanc	Curicó	2016	0.05±0.00	0.03±0.00	ND	0.08
4	S. Blanc	Leyda	2016	0.05±0.00	0.03±0.00	ND	0.09
5	S. Blanc	Central	2015	0.03±0.00	0.03±0.01	ND	0.07
6	S. Blanc	Central	2011	0.19±0.06	0.08±0.01	ND	0.27
7	S. Blanc	Central	2014	0.19±0.01	0.06±0.00	ND	0.25
8	S. Blanc	Maipo	2016	0.22±0.01	0.05±0.00	ND	0.27
9	S. Blanc	Maule	2015	0.03±0.00	0.03±0.00	ND	0.06
10	Chardonay	Maipo	2016	0.05±0.01	0.04±0.00	ND	0.09
11	Chardonay	Rapel	2016	0.04±0.00	0.03±0.00	ND	0.08
12	Chardonay	Casa Blanca	2012	0.05±0.01	0.03±0.01	ND	0.09
13	Syrah	Maipo	2015	0.06±0.01	0.05±0.00	ND	0.11
14	Syrah	Central	2015	0.07±0.01	0.04±0.00	ND	0.11
15	Syrah	Maipo	2015	0.06±0.01	0.04±0.01	ND	0.10
16	Merlot	Central	2014	0.01±0.00	Traces	0.19±0.04	0.21
17	Merlot	Central	2016	0.02±0.00	Traces	0.25±0.04	0.27
18	Merlot	Central	2016	0.03±0.00	Traces	0.25±0.01	0.28
19	Merlot	Central	2016	0.01±0.00	Traces	0.28±0.03	0.30
20	Merlot	Maipo	2013	0.08±0.00	0.04±0.00	ND	0.11
21	Merlot	Central	2015	0.02±0.00	Traces	0.19±0.01	0.21
22	Merlot	Rapel	2014	0.20±0.03	0.08±0.01	ND	0.28
23	Merlot	Maipo	2015	0.16±0.01	0.12±0.01	Traces	0.28
24	Merlot	Central	2015	0.03±0.000	0.06±0.06	0.04±0.00	0.13
25	Merlot	Central	2014	0.06±0.00	0.04±0.00	ND	0.10
26	Merlot	Curicó	2015	0.22±0.01	0.07±0.00	ND	0.29
27	Merlot	Cochalgua	2014	ND	ND	ND	---
28	Merlot	Rapel	2014	Traces	Traces	ND	---
29	Malbec	Central	2016	0.17±0.02	0.09±0.01	ND	0.26
30	Rosé	Central	2016	0.09±0.01	0.05±0.01	ND	0.14
31	Carménère	Curicó	2014	0.15±0.02	0.08±0.01	ND	0.23
32	Carménère	Central	2015	0.02±0.01	Traces	0.05±0.01	0.07
33	Carménère	Peumo	2015	0.02±0.00	Traces	ND	0.02
34	Carménère	Cochalgua	2016	0.09±0.01	0.10±0.01	ND	0.19
35	Carménère	Rapel	2012	0.09±0.00	0.06±0.00	ND	0.15
36	Carménère	Central	2015	0.05±0.01	0.07±0.01	ND	0.12
37	Carménère	Colchagua	2015	0.08±0.01	0.05±0.00	ND	0.13
38	Carménère	Maipo	2016	0.05±0.00	0.05±0.00	0.19±0.02	0.28
39	Carménère	Maipo	2016	0.07±0.01	0.05±0.00	ND	0.12
40	Carménère	Central	2016	ND	ND	ND	---
41	Carménère	Maipo	2014	0.16±0.01	0.11±0.00	Traces	0.26
42	C.Sauvignon	Central	2016	0.09±0.00	0.06±0.00	Traces	0.15
43	C.Sauvignon	Central	2016	0.11±0.01	0.07±0.01	ND	0.18

	Wine samples	Valley	Year[b]	α-casein	β-casein	Ovalbumin	Total
44	C. Sauvignon	Curicó	2013	0.09±0.01	Traces	0.08±0.05	0.17
45	C. Sauvignon	Central	2013	0.08±0.00	0.06±0.00	Traces	0.14
46	C. Sauvignon	Central	2014	0.08±0.00	0.05±0.00	Traces	0.13
47	C. Sauvignon	Maipo	2013	0.05±0.00	0.05±0.00	ND	0.10
48	C. Sauvignon	Central	2016	0.06±0.00	0.05±0.00	ND	0.10
49	C. Sauvignon	Maule	2016	0.09±0.01	0.05±0.00	ND	0.14
50	C. Sauvignon	Maipo	2013	0.07±0.01	0.04±0.00	ND	0.11
51	C. Sauvignon	Central	2016	0.14±0.02	0.06±0.01	ND	0.20
52	C. Sauvignon	Central	2016	0.08±0.02	0.06±0.01	ND	0.14
53	C. Sauvignon	Colchagua	2015	0.15±0.00	0.06±0.01	0.05±0.01	0.27
54	C. Sauvignon	Colchagua	2015	0.11±0.01	0.06±0.00	0.10±0.00	0.27
55	C. Sauvignon	Bio Bio	2015	0.07±0.01	0.04±0.01	ND	0.11
56	C. Sauvignon	Curicó	2014	0.15±0.03	0.09±0.07	ND	0.24
57	C. Sauvignon	Colchagua	2016	0.19±0.02	0.07±0.00	ND	0.26
58	C. Sauvignon	Colchagua	2015	0.21±0.00	0.08±0.01	ND	0.29
59	C. Sauvignon	Central	2015	ND	Traces	Traces	---
60	C.Sauvignon 50% Carménère 30% Syrah 20%	Maule	2016	0.16±0.01	0.06±0.00	0.03±0.00	0.25

Two independent extractions from each sample were analyzed in duplicate, results are expressed as mean ± standard deviation.
ND: not detected (<LOD)
[a]Year indicated in the label. Traces: (>LOD; <LOQ).

4. Conclusions

The present work showed for the first time the occurrence of casein and ovalbumin in Chilean wines. Trypsin digestion was performed applyin UA method reducing digestion time to only 3 min. A novel LC-MS/MS method was developed and validated to detect and quantify these allergen related proteins using an stable-isotope peptide as IS, wich could correct the a possible changes during sample preparation and ionization. This highly selective method was capable of detecting casein and ovalbumin at ppb (μg L^{-1}), which is more than 60 times lower than the European and OIV limit. Method suitability for casein and ovalbumin quantification in wines was demonstrated through validation results. Regarding allergen proteins presence in Chilean wines, from sixty samples analyzed, 93% showed quantifiable levels (>0.01 mg L^{-1}) and seventeen showed a total concentration of casein and ovalbumin higher than the limit recommended by the European Community (0.25 mg L^{-1}). According to results it seems appropriate that Chilean sanitary authorities established an official limit for mandatory labelling of allergen proteins in wines in order to avoid a toxicological risk.

Acknowledgments

This work is part of Jessy Pavón Pérez thesis to obtain the degree of Doctor in Science and Analytical Technology from the University of Concepcion, Chile. Authors want to thank to the National Commission of Scientific and Technological Research (CONICYT) of the Chilean Government for the doctoral scholarship granted.

Funding

This work was supported by the National Fund for Scientific and Technological Development (FONDECYT) project No1171857 and by the Fund for Scientific and Technological Equipment (FONDEQUIP) project No 130209.

References

Arsene, C. G., Ohlendorf, R., Burkitt, W., Pritchard, C., Henrion, A., O'Connor, G., Bunk, D. M., & Guttler, B. (2008). Protein quantification by isotope dilution mass spectrometry of proteolytic fragments: cleavage rate and accuracy. *Analitical Chemistry, 80*, 4154-4160.

Bridi, R., Lobato, S., López-Alarcón, C., & Lissi, E. (2014). Influence of the growing region, quality classification and harvest year on the ORAC (Oxygen Radical Absorbance Capacity) index of Chilean Cabernet Sauvignon red wines. *Ciencia e investigación agraria, 41*, 395-402.

Chen, Q., Zhang, J., Ke, X., Lai, S., Li, D., Yang, J., Mo, W., & Ren, Y. (2016). Simultaneous quantification of α-lactalbumin and β-casein in human milk using ultra-performance liquid chromatography with tandem mass spectrometry based on their signature peptides and winged isotope internal standards. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1864*, 1122-1127.

Chen, Q., Zhang, J., Ke, X., Lai, S., Tao, B., Yang, J., Mo, W., & Ren, Y. (2015). Quantification of bovine β-casein allergen in baked foodstuffs based on ultra-performance liquid chromatography with tandem mass spectrometry. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 32*, 25-34.

D'Amato, A., Kravchuk, A. V., Bachi, A., & Righetti, P. G. (2010). Noah's nectar: the proteome content of a glass of red wine. *Journal of Proteomics, 73*, 2370-2377.

European Commission (EU). (2012). Commission implementing regulation 579/2012. *Official Journal European Union, L171*, 4-7.

Hoxey, L., Stockley, C., Wilkes, E., & Johnson, D. (2013). What's in a label? How science is helping winemakers to respond to new EU rules concerning allergens in wine. *Wine & Viticulture Journal, 4*, 38-41.

International Conference on Harmonisation (ICH). (2005). ICH Harmonised tripartite guideline validation of analytical procedures: text and, methodology Q2 (R1). In.

Ippoushi, K., Sasanuma, M., Oike, H., Kobori, M., & Maeda-Yamamoto, M. (2016). Absolute quantification of Pru av 2 in sweet cherry fruit by liquid chromatography/tandem mass spectrometry with the use of a stable isotope-labelled peptide. *Food Chemistry, 204*, 129-134.

Kadam, S. U., Tiwari, B. K., Álvarez, C., & O'Donnell, C. P. (2015). Ultrasound applications for the extraction, identification and delivery of food proteins and bioactive peptides. *Trends in Food Science & Technology, 46*, 60-67.

Kaul, S., Luttkopf, D., Kastner, B., Vogel, L., Holtz, G., Vieths, S., & Hoffmann, A. (2007). Mediator release assays based on human or murine immunoglobulin E in allergen standardization. *Clinical Experimental & Allergy, 37*, 141-150.

Lifrani, A., Dos Santos, J., Dubarry, M., Rautureau, M., Blachier, F., & Tome, D. (2009). Development of animal models and sandwich-ELISA tests to detect the allergenicity and antigenicity of fining agent residues in wines. *Journal of Agricultural and Food Chemistry, 57*, 525-534.

Losito, I., Introna, B., Monaci, L., Minella, S., & Palmisano, F. (2013). Development of a method for the quantification of caseinate traces in Italian commercial white wines based on liquid chromatography-electrospray ionization-ion trap-mass spectrometry. *Journal of Agricultural and Food Chemistry, 61*, 12436-12444.

Loziuk, P. L., Wang, J., Li, Q., Sederoff, R. R., Chiang, V. L., & Muddiman, D. C. (2013). Understanding the Role of Proteolytic Digestion on Discovery and Targeted Proteomic Measurements Using Liquid Chromatography Tandem Mass Spectrometry and Design of Experiments. *J Proteome Res, 12*, 5820-5829.

Lutter, P., Parisod, V., & Weymuth, H. (2011). Development and validation of a method for the quantification of milk proteins in food products based on liquid chromatography with mass spectrometric detection. *J AOAC Int, 94*, 1043-1059.

Mattarozzi, M., Milioli, M., Bignardi, C., Elviri, L., Corradini, C., & Careri, M. (2014). Investigation of different sample pre-treatment routes for liquid chromatography–tandem mass spectrometry detection of caseins and ovalbumin in fortified red wine. *Food Control, 38*, 82-87.

Organisation Internationale de la Vigne et du Vin (OIV). (2012). Revision of the limit of detection and limit of quantifcation related to potentially allergenic residues of finning agent proteins in wine

Patrick, W., Hans, S., & Angelika, P. (2009). Determination of the bovine food allergen casein in white wines by quantitative indirect ELISA, SDS-PAGE, Western blot and immunostaining. *Journal of Agricultural and Food Chemistry, 57*, 8399-8405.

Reddy, P. M., Hsu, W. Y., Hu, J. F., & Ho, Y. P. (2010). Digestion completeness of microwave-assisted and conventional trypsin-catalyzed reactions. *J Am Soc Mass Spectrom, 21*, 421-424.

Restani, P., Uberti, F., Tarantino, C., Ballabio, C., Gombac, F., Bastiani, E., Bolognini, L., Pavanello, F., & Danzi, R. (2014). Collaborative Interlaboratory Studies for the Validation of ELISA Methods for the Detection of Allergenic Fining Agents Used in Wine According to the Criteria of OIV Resolution 427–2010 Modified by OIV–Comex 502–2012. *Food Analytical Methods, 7*, 706-712.

Rolland, J. M., Apostolou, E., De Leon, M. P., Stockley, C. S., & O'Hehir, R. E. (2008). Specific and sensitive enzyme-linked immunosorbent assays for analysis of residual allerqenic food proteins in commercial bottled wine fined with egg white, milk, and nongrape-derived tannins. *Journal of Agricultural and Food Chemistry, 56*, 349-354.

Santos, H. M., Rial-Otero, R., Fernandes, L., Vale, G., Rivas, M. G., Moura, I., & Capelo, J. L. (2007). Improving sample treatment for in-solution protein identification by peptide mass fingerprint using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J Proteome Res, 6*, 3393-3399.

Tolin, S., Pasini, G., Simonato, B., Mainente, F., & Arrigoni, G. (2012). Analysis of commercial wines by LC-MS/MS reveals the presence of residual milk and egg white allergens. *Food Control, 28*, 321-326.

Vogt, E. I., Kupfer, V. M., Vogel, R. F., & Niessen, L. (2016). A novel preparation technique of red (sparkling) wine for protein analysis. *EuPA Open Proteomics, 11*, 16-19.

Wang, S., Zhang, L., Yang, P., & Chen, G. (2008). Infrared-assisted tryptic proteolysis for peptide mapping. *Proteomics, 8*, 2579-2582.

Zhang, J., Lai, S., Cai, Z., Chen, Q., Huang, B., & Ren, Y. (2014). Determination of bovine lactoferrin in dairy products by ultra-high performance liquid chromatography–tandem mass spectrometry based on tryptic signature peptides employing an isotope-labeled winged peptide as internal standard. *Anal Chim Acta, 829*, 33-39.

Zhang, J., Lai, S., Zhang, Y., Huang, B., Li, D., & Ren, Y. (2012). Multiple reaction monitoring-based determination of bovine alpha-lactalbumin in infant formulas and whey protein concentrates by ultra-high performance liquid chromatography-tandem mass spectrometry using tryptic signature peptides and synthetic peptide standards. *Anal Chim Acta, 727*, 47-53.

CAPITULO II SECCION 4

Title: Determination of fining-related allergen proteins in wines by High Performance Thin Layer Chromatography/Electrospray Ionization-Mass spectrometry

Jessy Pavón[1], Karem Henriquez-Aedo[1,2], Mario Aranda Bustos[1,2*]

[1] Laboratory of Advanced Research on Foods and Drugs, Department of Food Science and Technology, Faculty of Pharmacy, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile.

[2] Center for Biotechnology, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile.

Abstract

Casein (milk powder) and ovalbumin (egg-white powder) are employed during winemaking process like fining agents. This kind of proteins may trigger allergic reactions in susceptible individuals, therefore their presence in wines is a human health risk, especially when their presence is not reported or indicated on the label. The objective of this work was to develop a high performance thin layer chromatography mass spectrometry method to determine casein and ovalbumin levels in Chilean wine. For tryptic peptide separation an HPTLC silica gel 60F $_{254}$ glass plates was derivatized with fluorescamine and triethylamine and then peptides were visualized by fluorescence at 366/>400 nm. Full scan and tandem mass spectra were acquired in separated and underivatized plates, for peptide ions. Search database was used and sequence coverages determined. The method was applied in two Chilean wine samples allowing the identification of characteristic peptides of casein (0.05 mg L^{-1}) and ovalbumin (0.11 mg L^{-1}), concentrations previously determined

Keywords: Casein; ovalbumin; mass spectrometry; wines; HPTLC

1. Introduction

Wine grape production is a very important sector of Chile's economy in terms of productivity, exports and employment. Chile is the ten principal worldwide wine producer with over 12,9 mhl produced during 2018 which is equivalent to almost 4% of the world's produced volume [1]. Chilean wine possess international recognition due to its particular sensorial and chemical profiles, both extensively investigated [2]. Milk and egg proteins are often used in winemaking process as finning agents. They promote the interactions with undesirable compounds especially polymeric phenols and polyphenols, allowing their partial remotion through precipitation [3]. Caseinate or milk powders are used for white wine fining, and ovalbumin or egg-white powders proteins are used for red wines. The use of allergenic proteins in wines and their unlabeled presence in the final product has been subject of debate in the last years because involve a health risk for susceptible consumers. Thus, its determination is highly desirable to avoid any risk for consumer health [4]. For this reason the European Union (EU) adopted Directive 2003/89/EC and its latest version Directive 2012/579/EC which contain a list of allergenic substances including egg and milk derivatives [5]. The Organization Internationale de la Vigne et du Vin (OIV) establishes in its Resolution OIV/COMEX/502/2012 [6] that casein or ovalbumin levels higher than 0.25 mg L^{-1} must be declared on the labels. OIV official method is based on enzyme-linked immunosorbent assay [7] [8]. This immunoassay is simple and fast [9] but presents some drawbacks and limitations, for example in red wines, the high content of polyphenols interact with proteins and antibodies, causing detection problems [10] and due to the adsorption of allergens to solid matrix that supports the analysis, protein epitopes may suffer some alterations modifying its recognition or accessibility impairing the assay sensitivity [11]. In this scenario, reliable methods like liquid chromatography and gel electrophoresis gain much attention for protein/peptides determination in wines. In both cases, protein analysis can be performed applying two approaches, i.e. *top down* and *bottom up.* Top down involves direct analysis of intact proteins without prior digestion [12] and in *bottom-up* strategy, protein identification is based on measurements of a set of protein-digestion products (peptides).

Two-dimensional polyacrilamide gel electrophoresis (2D-PAGE) allows the separation of proteoforms and it has became therefore in one of the most used techniques in proteomics

[13]. However, it is a time consuming technique and proteins/peptides extraction from PAGE is difficult [14]. In this scenario systems like liquid chromatography coupled to mass spectrometry (LC-MS) has become the most suitable technique for protein analysis. The most used ionization sources for MS analysis are Electrospray Ionization and Matrix-Assisted Laser Desorption/Ionization (MALDI) in tandem mass spectrometry (ESI or MALDI-MS/MS). Both techniques offer severals advantages, including multi-allergen detection and unambiguous identification and characterization of food allergens [15]. In *top down* analysis the most convenient and used ionization technique is MALDI. Its main disadvantage lies in the complexity of the mass spectrum. In *bottom-up* approach protein is broken up into peptides, either by chemical or enzymatic digestion identification [16] and the resulting peptides are subjected generally to ESI/tandem-MS analysis after separation. To identify these peptides, mass spectra are matching against protein-sequence databases using search algorithms [12]. Because the spectra obtained by *bottom-up* proteomics are much easier to interpret than those obtained by *top-down* this method is the most used to perform proteomics studies [17]. Separation of protein or peptides with LC is fast, and the sensitivity and resolution of these technique are high [13]. Online coupling HPLC to mass spectrometer as detection method and the development of bioinformatic tools makes the identification of proteins relatively easy. But the main disadvantage of LC is the large amount of solvent that can be consumed [18] and high–performance thin-layer chromatography (HPTLC) could be an alternative technique for proteins or peptides analysis. Thin layer methods have the ability to separate several samples in parallel, save a separation on a TLC plate, and to detect small and hydrophilic peptides [18]. HPTLC also allows the possibility to change separation conditions easily by varying mobile and stationary phases [13]. Moreover with HPTLC is possible to analyze one chromatogram by a simple staining with one or more different coloring reagents, use of bioactivity-guided, effect-directed analysis or hyphenations with MS [13] [19-21] and miniaturization of these techniques also are considered [22]. All these characteristics have made planar chromatography most attractive for research in proteomics, in addition to the possibility of coupling this technique to MS.

In the present research, a fast and highly selective method using a HPTLC separation combined with ESI-triple quadrupole (QQQ) mass spectrometry for unequivocal

simultaneous identification of β - casein and ovalbumin is described. To the best of our knowledge this work reports for the first time the evaluation of fining-related allergen proteins in Chilean wines.

2. Materials and methods

2.1 Reagents

2-propanol, pyridine, ammonia solution (> 25%), triethylamine, acetone, formic acid (98-100%), trichloroacetic acid (TCA, > 99%), ethanol absolute, and HPLC grade acetonitrile and HPTLC silica gel 60F $_{254}$ glass-backed plates (10 cm x 10 cm) were purchased from Merck (Darmstadt, Germany). Ammonium hydrogen carbonate (NH_4HCO_3, 99%), casein from bovine milk, albumin from chicken egg white, iodoacetamide (IAM, > 99%), DL-dithiothreitol (DTT, > 99%), and fluorescamine were acquired from Sigma (St. Louis, MO, USA). Trypsin from bovine pancreas was from BDH Biochemicals (Poole, England). Buffered solutions and mobile phases were prepared with Ultrapure water (18.2 MΩ cm) produced by means of Simplicity system from Millipore (Bedford, MA, USA). Casein and ovalbumin stock solutions were prepared in 50 mM NH_4HCO_3 (pH 7.8) for a given concentration of 12 mg mL^{-1}. Standard solutions were prepared by aliquot dilution from the stock solution. All solutions stored refrigerated were stable for at least twenty days.

2.2 Samples preparation

Sample preparation was carried out following the method proposed by Mattarozzi et al. [9] with slight modifications. Briefly, 12.5 mL of wine were centrifuged using a Hettich (Tuttlingen, Germany) centrifuge at 5433 x *g* for 40 min at 20ºC into 10 kDa cut-off membrane ultrafiltration tube, previously conditioned with 10 mL of distilled water. 2.5 mL were recovered from which proteins were precipitated for 2 h on ice after addition of eight volumes of ethanol: TCA (15%w/v), pH 4.5. Thereafter, the mixture was centrifuged at 8981 x *g* for 10 min at 4ºC using a Hettich refrigerated centrifuge. Ethanol: TCA residues were evaporated under a gentle flow of nitrogen. The obtained pellet was solubilized in 1 mL of 50 mM NH_4HCO_3, pH 7.8. Trypsin digestion of both, sample and standards, was performed after proteins reduction and alkylation. The first one was carried out by addition of 12 µL of 100 mM DTT and incubation at 95ºC for 5 min. After cooling down, the

cysteine SH-groups, formed during the reduction step, were irreversibly alkylated adding 24 µL of freshly prepared 100 mM aqueous solution of IAM, the mixture was left stand protected from light for 15 min at room temperature. Enzymatic digestion was performed adding 4 µL of trypsin solution (0.5 mg mL^{-1} in 50 mM of NH_4HCO_3, pH 7.8) obtaining an enzyme: protein ratio of 1:10, the mixture was incubated at 37ºC for 7 h. The reaction was stopped by addition of 10 µL of formic acid (0.1%v/v) aqueous solution. All digested samples and standards were filtered through a 13mm PVDF syringe filter (0.22 µm) before HPTLC application.

2.3 Thin layer chromatography

Samples and standard solutions were applied on HPTLC silica gel 60F $_{254}$ glass plates (10cm x 10cm) by means of CAMAG (Muttenz, Switzerland) automatic TLC sampler applicator 4 (ATS 4) equipped with a spray-on band applicator with a 25-µL syringe using the following settings band length 6.0 mm, band velocity 10 mm s^{-1}, distance between tracks 3.0 mm, first application x axis: 12.0 mm, application y axis: 10.0 mm for 10 tracks/plate. Spaces between tracks were automatically adjusted according to the number of samples and the plate size. Solvent systems were developed based on recent literature [18-23]. Chromatographic separation was carried out inside of flat-bottomed chamber using a mixture of 2-propanol/pyridine/ammonia/water (39:34:10:26, v/v/v/v) as mobile phase up to a migration distance of 80 mm. Both, samples and standards were applied at least in duplicate dividing the HPLTC plate in two sections: the first section was used for derivatization with fluorescamine and the second one for mass spectrometry analysis.

2.4 Post–chromatographic derivatization

Dried plates were immersed for 1s into fluorescamine solution (0.02% in acetone) and dried 10 minutes at room temperature. Thereafter plates were immersed again for 1s into triethylamine solution (10% in acetone) and dried at room temperature for 10 minutes. Visualization, evaluation and documentation were performed by means of DigiStore2 photodocumentation system (CAMAG) in fluorescence mode at 366/>400 nm. All instruments were controlled via WinCats software 1.4.2 Planar Chromatography Manager (CAMAG).

2.5 HPTLC-ESI-MS/MS

For mass spectra acquisition, selected bands were eluted from HPTLC plate to MS by means of CAMAG TLC-MS interface assembled with oval elution head (4.0 x 2.0 mm) using a acetonitrile at a flow rate of 0.2 mL min^{-1} for 60 seconds. MS analysis was performed in Shimadzu (Kyoto, Japan) LCMS 8030 triple quadrupole mass spectrometer with electrospray ionization [15] source operated with the following conditions: ESI in positive mode, capillary voltage 3.0 kV, nebulizing gas (N2) 3 L min^{-1}, drying gas (N2) 15 L min^{-1}, DL temperature 250ºC, and block temperature 400ºC. Mass spectra were acquired in full scan mode between *m/z* values of 100 and 1000. Plate background signals were subtracted for each analysis. Data were acquired and recorded by Shimadzu LabSolution software version 5.51. All ions with signal intensity greater than 100 counts (range 10^1 to 10^6) and charge state +2 (or unknown charge (+1 to +3)) were submitted to fragmentation and a product ion scan. MS/MS data were searched in online version of SwissProt database using Mascot Peptide Mass Fingerprint (Matrix Science Ltd., London, UK) applying the following stringent criteria: i) only one missed cleavage was allowed for trypsin digestion; ii) fixed amino acid modification was cysteine carbamidomethylation; and iii) variable modifications were methionine oxidation, and threonine and tyrosine phosphorylation. For confirmatory purposes precursor and fragment ions reported for β-casein 742.5→625.2 and ovalbumin 761.6→1036.5 [21, 24, 25] were monitored. Plate background signals were also recorded to obtain information about matrix/background.

3. Results and discussion

3.1 HPTLC-FLD detection of casein and ovalbumin peptides in wine

A correct selection of solvents is crucial for liquid chromatographic separation systems, and due to the number of peptides formed after enzymatic cleavage, solvents combination and their proportions is almost unique for each type of protein. The mixture 2-butanol/pyridine/ammonia/water (39:34:10:26, v/v/v/v) was firstly used in this work as mobile phase, however, separation time required to achieve a migration distance of 80 mm was *ca.* two hours. In order to decrease analysis time, mobile phase viscosity was reduced using 2-propanol instead of 2-butanol. With this change analysis time reduced to 60 min for a migration distance of 80 mm. HPTLC offers the possibility of various post-

chromatographic derivatization procedures [17]. First, peptide detection was carried out by derivatization with 0.5% (w/v) ninhydrin in 2-propanol. Casein and ovalbumin peptides at 0.01, 0.10 and 1.00 mg mL^{-1} levels appear as violet bands on white background, but at those levels only a faint color was observed. Thus, considering that both proteins residues can be found at ng or μg L^{-1} levels in wines, it was decided to use fluorescamine for post-chromatographic derivatization. This reagent reacts with nitrogen-containing side chains of proteins or peptides to form highly fluorescent compounds [26]. It is 10 to 100 times more sensitive in detecting primary amino groups than the ninhydrin reaction [27]. Panchagnula et al. [28] reported a detection limit as low as 4 ng for phosphopeptide analysis using fluorescamine. In this work the derivatization with fluorescamine allowed to observe a greater number of bands more intense than those observed with ninihydrin, which proves the advantages of fluorescamine to detect lower concentrations The chemical change occurring in the molecules after derivatization process affects the detection of the molecular mass of the compounds of interest. It is this reason that it was decided to carry out the analysis by MS in underivatized plates. In order to identify the bands, a derivatized plate was used as reference.

3.2 Selection of marker peptides

In proteomic analysis one of the key step for development of MS-based method is an adequate selection of marker peptides. For wine allergen is necessary to define precursor and fragment ions that allows an unequivocal identification and accurate quantification. To establish peptide and fragment markers, three levels (0.01, 0.10 and 1.00 mg mL^{-1}) of casein and ovalbumin standard were digested using trypsin general conditions and then studied/analyzed by HPTLC-FLD and HPTLC-ESI-MS/MS. After separation one plate section was derivatized with fluorescamine and triethylamine, observing a well resolved separation of peptides (Figure 1), similar to those reported by other authors [18, 23].

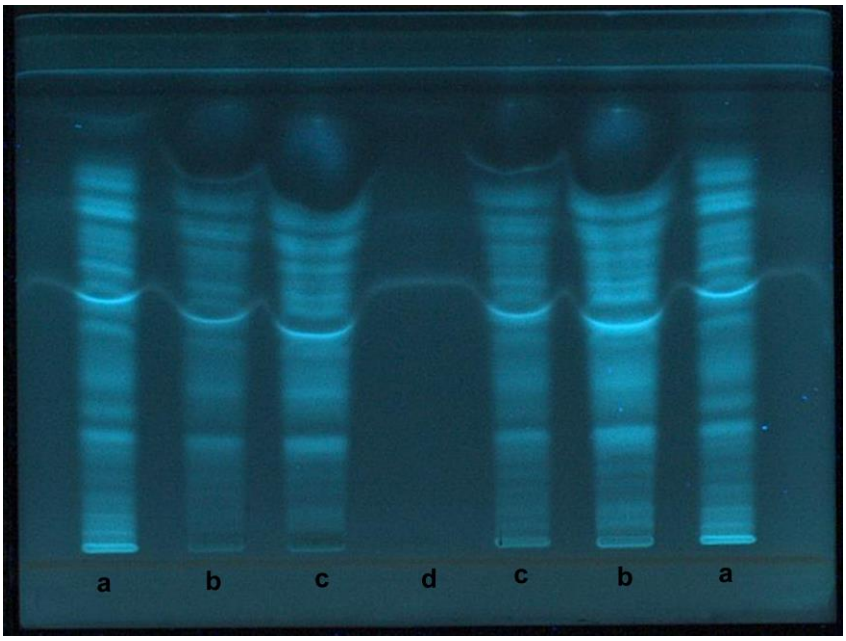

Figure 1. HPTLC chromatogram after fluorescamine derivatization showing peptides produced by casein and ovalbumin standards trypsin cleavage. a) 1.00 mg mL^{-1}, b) 0.1 mg mL^{-1}, c) 0.01 mg mL^{-1} and d) blank.

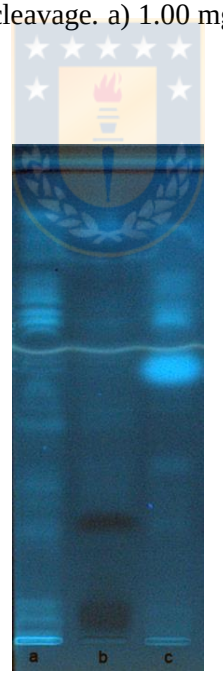

Figure 2. HPTLC chromatogram after fluorescamine derivatization showing peptides produced by a) casein 1.00 mg mL^{-1}, b) bovine serum albumin 1.00 mg mL^{-1} and c) ovalbumin 1.00 mg mL^{-1} standards trypsin cleavage

Figure 2 shows the separation pattern of peptides of casein and ovalbumin separately, bovine serum albumin was used as a protein control. In the separation of the peptide

mixture from casein and ovalbumin, a greater number of bands were observed than in the separation of the peptides of both proteins separately. To the best of our knowledge, this is the first time that peptides from ovalbumin digestion are analyzed by HPTLC.

ESI-MS analysis were performed using the underivatized plate section acquiring full-scan mass spectra of 1.00 mg mL^{-1} casein and ovalbumin standards bands. As expected, several *m/z* signals were observed along the entire mass chromatograms. To limit the exploration, an oriented search based on already published data [7, 24, 29] was carried out finding the following parents (precursor) ions, GPFPIIV (*m/z* 742.5; +1) and YPILPEYLQCVK (*m/z* 764.6; +2) for β-casein and ovalbumin, respectively (Figure 3). For α-casein was not possible to found any marker peptide this could be due to a poor digestion or for the presence of sodium adducts of fluorescamine (*m/z* 301.05) with high intensity, which decrease the overall protonated molecule signal. In HPTLC/MS, analyte elution from the plate to MS is a critical step. As can be observed in figure 3, both identified peptides showed higher migration distances (R_f values). Instead, those with smaller R_f showed lower mass signal intensities. This phenomenum could be explained by peptides chemical composition, those with smaller R_f values are more hydrophilic (polar) than peptides with higher R_f values, and therefore, they are more strongly associated with hydrophilic stationary phase (silica). This adsorption and/or ionic interaction difficult peptides desorption, thus, a fraction of peptide amount present in chromatographic band finally reach the ESI source, therefore smaller mass signal intensities are observed.

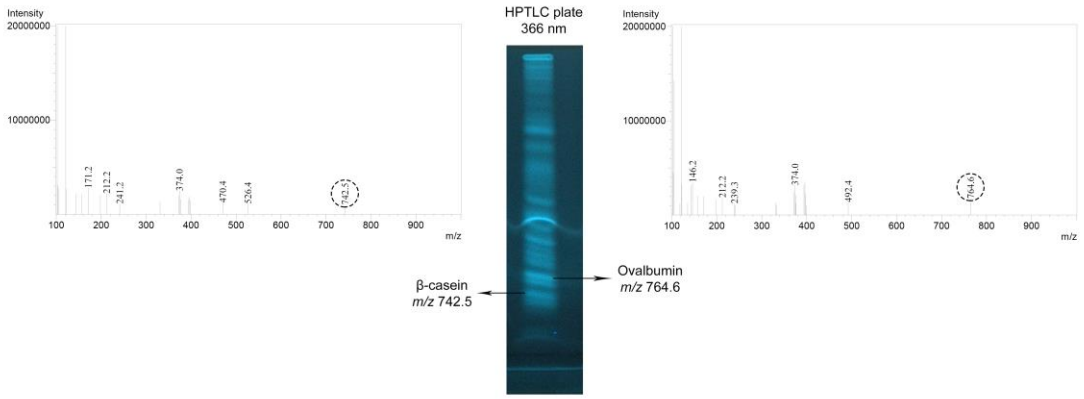

Figure 3. HPTLC-MS spectra of β-casein and ovalbumin standard 1.00 mg L^{-1} with bands characteristic of *m/z* 742.5 and 764.6 (their sequence are indicated in Table 1)

After established the precursor ions, a MS/MS approach was performed applying product ion scan mode using the HPTLC-ESI-MS/MS conditions described in section **2.5**. Fragment ions selection was carried out applying the same criteria indicate in section **2.5** and other relevant aspects like: i) match with precursor peptides already reported in literature [7, 9, 21, 30] and ii) quality of product ion spectra matches with Mascot Peptide Mass Fingerprint. Figure 4 shows the product ion spectrum of GPFPIIV peptide from β-casein after fragmentation into collision-induced dissociation cell set up at -35.0 V.

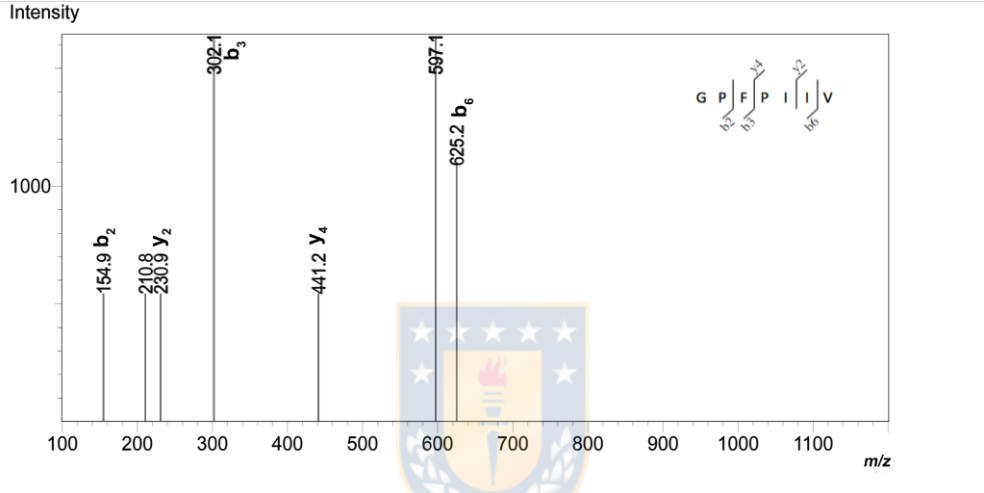

Figure 4. Product ion spectrum for the peptide GPFPIIV of β-casein, *m/z* 742.5

Following the early describe criteria, the more stable fragment from the product ion spectrum were chosen for each peptide (protein) for qualitative/confirmatory purposes (Table 1).

Table 1. Precursor and fragment ions for β-casein and ovalbumin determination in wines

Protein	Precursor ion sequence (*m/z*; charge state; mass (Da))	Product ion sequence (*m/z*)
β-casein	GPFPIIV (*m/z* 742.5; +1; 741.5)	GPFPII (*m/z* 625.2)
Ovalbumin	YPILPEYLQCVK (*m/z* 764.6; +2; 1523.2)	PEYLQCVK (*m/z* 1036.5)

Thus, precursor and fragment ions defined for an inequivocal detection of β-casein and ovalbumin were *m/z* 742.5→625.2 and *m/z* 764.6→1036.5, respectively. These marker peptides match with those reported by Monaci [7, 25] using LC-ESI-MS/MS, which evidences the feasibility of HPTLC for the analysis of these proteins. Under these

conditions it was possible to achieve a percentage of sequence coverage (SQ) of ca. 23% for β-caseine. SQ represents the percentage of the protein sequence covered by identified peptides, so a high value of this parameter means that a larger number of peptides is to match a larger percentage of the protein sequence. However there is no standard acceptable value of SQ, generally % of SQ greater than 22% is considerated adequate in terms of proteolytic digestion yield [31]. For ovalbumin % SQ was 11%, this lower value could be maybe explained due to the ovalbumin higher molecular weight, which influences the peptides size and ionization efficiency. However, the analysis of HPTLC only with the visualization of peptides corresponding to casein and ovalbumin, allowed identifying the presence of these proteins in wine. In this case the analysis by MS is only for the confirmation, which shortens the analysis time and costs.

3.3 Method applicability

Method applicability was determined evaluating two Chilean wine samples without allergen labelling. All wine samples labels described the category, young (without aging) and reserve (higher quality and/or aged), as well as also the harvest year and their geographical origin (Table 2). The concentrations of casein and ovalbumin in these samples were determined previously in our laboratory by UHPLC-MS/MS. In Cabernet sample ovalbumin was 0.11 mg L^{-1} and the concentration of β-casein in Carménère was 0.05 mg L^{-1}. By HPTLC in Cabernet Sauvignon sample was only identified the precursor and fragment ions for ovalbumin and in Carménère sample the precursor and fragment ions for β-casein (Table 2). This demonstrates the ability of HPTLC to detect and quickly identify low concentrations of casein and ovalbumin in wines. HPTLC-ESI-MS/MS analysis of underivatized plate section was first performed in full scan mode with the aim of identifying the precursor peptides and then with the information obtained trough MASCOT from the MS spectrum in product ion scan mode the marker peptides (precursor and fragment ions) were identified for each protein.

The use of HPTLC-ESI-MS/MS technique for proteins analysis offers a broad range of advantages and the obtained results are comparable with those achieved with LC-MS techniques. Similar results have been obtained with other desorption/ionization approaches, Bakry et al [26] employed matrix-assisted laser desorption / ionization (MALDI) for

analysis proteins and peptides. However, currently, ESI is preferred because produces multiply charged peptides providing more information than singly charged peptides in the MS/MS spectrum [32]. Pasilis et al. [33] employed HPTLC/DESI-MS (Desorption electrospray ionization mass spectrometry) for peptide identification in one dimension (1D) separations of tryptic digests of cytochrome c and myoglobin. The present report demonstrated the applicability of HPTLC-ESI-MS/MS for the detection of allergens proteins in wines. Nevertheless, MS signals from peptides located in low R_f bands should be improved in order to improve %SQ, which also allows the detection of other proteins isoforms like alpha-casein.

Table 2. Precursor and fragment ions found in wine samples.

Sample	Valley	Vintage	Precursor and fragment ions (protein)
Cab Sauvignon (Reserve)	Maipo	2013	764.6→1036.5 (ovalbumin)
Carménère (Young)	Colchagua	2015	742.5→625.2 (β-casein)

4. Conclusions

This work demonstrated the feasibility of HPTLC coupled to mass spectrometry for the identification of peptides resulting from the enzymatic digestion of allergenic proteins used like finning during winemaking process. The derivatization with fluorescamine allowed the visualization of multiple bands resulting from the enzymatic proteins digestion in low concentrations. In this way in a quick and simple way it was possible to identify milk and egg proteins in wine. For the confirmation of the presence of these proteins the analysis by MS was useful. However, it is necessary to improve the technique in order to obtain higher %SQ values for ovalbumin. As well as identifying a greater number of characteristic peptides for both proteins, to validate the proposed method and thus use it to quantify proteins in wines. In our knowledge this is the first time that HPTLC-ESI-MS/MS is used for the identification of casein and ovalbumin in Chilean wines.

Acknowledgments

This work is part of Jessy Pavón Pérez thesis to obtain the degree of Doctor in Science and Analytical Technology from the University of Concepcion, Chile. Authors want to thank to the National Commission of Scientific and Technological Research (CONICYT) of the

Chilean Government for the doctoral scholarship granted. This work was financially supported by National Fund for Scientific & Technological Development (FONDECYT), projects N°1171857, FONDEQUIP 130209 and University of Concepcion.

References

[1] OIV Organisation Internationale de la Vigne et du Vin, State of the vitiviniculture world market-April. http://www.oiv.int/public/medias/5287/oiv-noteconjmars2017-en.pdf (Accessed 15 March 2018)
[2] R. Bridi, S. Lobato, C. Lopez-Alarcon, E. Lissi, Influence of the growing region, quality classification and harvest year on the ORAC (Oxygen Radical Absorbance Capacity) index of Chilean Cabernet Sauvignon red wines, Ciencia E Investigacion Agraria. 41 (2014) 395-402.
[3] I. Losito, B. Introna, L. Monaci, S. Minella, F. Palmisano, Development of a Method for the Quantification of Caseinate Traces in Italian Commercial White Wines Based on Liquid Chromatography-Electrospray Ionization-Ion Trap-Mass Spectrometry, Journal of Agricultural and Food Chemistry. 61 (2013) 12436-12444.
[4] A. D'Amato, A.V. Kravchuk, A. Bachi, P.G. Righetti, Noah's nectar: The proteome content of a glass of red wine, Journal of Proteomics. 73 (2010) 2370-2377.
[5] E. Commission., Commission implementing regulation 579/2012, Official Journal European Union. L171 (2012) 4-7.
[6] OIV Organization Internationale de la Vigne et du Vin, Revision of the limit of detection and limit of quantification related to potentially allergenic residues of fining agent proteins in wine. OIV-COMEX 502-2012. http://www.oiv.int/en/technical-standards-and-documents/resolutions-of-the-oiv/comex-resolutions (Accessed 15 March 2018)
[7] L. Monaci, R. Pilolli, E. De Angelis, M. Godula, A. Visconti, Multi-allergen detection in food by micro high-performance liquid chromatography coupled to a dual cell linear ion trap mass spectrometry, Journal of Chromatography A. 1358 (2014) 136-144.
[8] OIV Organisation Internationale de la Vigne et du Vin, Organisation Internationale de la Vigne et du Vin, Criteria OIV-MA-AS315-23. http://www.oiv.int/oiv/info/enmethodesinternationalesvin (Accessed 29 November 2018)
[9] M. Mattarozzi, M. Milioli, C. Bignardi, L. Elviri, C. Corradini, M. Careri, Investigation of different sample pre-treatment routes for liquid chromatography–tandem mass spectrometry detection of caseins and ovalbumin in fortified red wine, Food Control. 38 (2014) 82-87.
[10] W. Patrick, S. Hans, P. Angelika, Determination of the Bovine Food Allergen Casein in White Wines by Quantitative Indirect ELISA, SDS-PAGE, Western Blot and Immunostaining, Journal of Agricultural and Food Chemistry. 57 (2009) 8399-8405.
[11] S. Kaul, D. Luttkopf, B. Kastner, L. Vogel, G. Holtz, S. Vieths, A. Hoffmann, Mediator release assays based on human or murine immunoglobulin E in allergen standardization, Clinical and Experimental Allergy. 37 (2007) 141-150.
[12] L. Monaci, A. Visconti, Mass spectrometry-based proteomics methods for analysis of food allergens, TrAC Trends in Analytical Chemistry. 28 (2009) 581-591.
[13] S.K.G. Grebe, R.J. Singh, Clinical peptide and protein quantification by mass spectrometry (MS), TrAC Trends in Analytical Chemistry. 84 (2016) 131-143.
[14] J. Biller, L. Morschheuser, M. Riedner, S. Rohn, Development of optimized mobile phases for protein separation by high performance thin layer chromatography, Journal of Chromatography A. 1415 (2015) 146-154.
[15] E. Becerra-Martínez, E. Florentino-Ramos, N. Pérez-Hernández, L. Gerardo Zepeda-Vallejo, N. Villa-Ruano, M. Velázquez-Ponce, F. García-Mendoza, A.E. Bañuelos-Hernández, 1H NMR-based metabolomic fingerprinting to determine metabolite levels in serrano peppers (Capsicum annum L.) grown in two different regions, Food Research International. 102 (2017) 163-170.
[16] C.K. Faeste, H.T. Ronning, U. Christians, P.E. Granum, Liquid Chromatography and Mass Spectrometry in Food Allergen Detection, Journal of Food Protection. 74 (2011) 316-345.
[17] A. Tholey, A. Becker, Top-down proteomics for the analysis of proteolytic events - Methods, applications and perspectives, Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 1864 (2017) 2191-2199.
[18] J.F. Emory, M.J. Walworth, G.J. Van Berkel, M. Schulz, S. Minarik, Direct analysis of reversed-phase high-performance thin layer chromatography separated tryptic protein digests using a liquid microjunction surface sampling probe/electrospray ionization mass spectrometry system, European Journal of Mass Spectrometry. 16 (2010) 21-33.
[19] B. Hu, G.Z. Xin, P.K. So, Z.P. Yao, Thin layer chromatography coupled with electrospray ionization mass spectrometry for direct analysis of raw samples, Journal of Chromatography A. 1415 (2015) 155-160.

[20] G.E. Morlock, I. Klingelhofer, Liquid Chromatography-Bioassay-Mass Spectrometry for Profiling of Physiologically Active Food, Analytical Chemistry. 86 (2014) 8289-8295.
[21] S.P. Pasilis, V. Kertesz, G.J. Van Berkel, M. Schulz, S. Schorcht, HPTLC/DESI-MS imaging of tryptic protein digests separated in two dimensions, Journal of Mass Spectrometry. 43 (2008) 1627-1635.
[22] S. Hage, G.E. Morlock, Bioprofiling of Salicaceae bud extracts through high-performance thin-layer chromatography hyphenated to biochemical, microbiological and chemical detections, Journal of Chromatography A. 1490 (2017) 201-211.
[23] T.T. Habe, G.E. Morlock, Miniaturization of Instrumental Planar Chromatography with Focus on Mass Spectrometry, Chromatographia. 79 (2016) 797-810.
[24] J. Heick, M. Fischer, B. Pöpping, First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry, Journal of Chromatography A. 1218 (2011) 938-943.
[25] L. Monaci, I. Losito, F. Palmisano, M. Godula, A. Visconti, Towards the quantification of residual milk allergens in caseinate-fined white wines using HPLC coupled with single-stage Orbitrap mass spectrometry, Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment. 28 (2011) 1304-1314.
[26] R. Bakry, G.K. Bonn, D. Mair, F. Svec, Monolithic porous polymer layer for the separation of peptides and proteins using thin-layer chromatography coupled with MALDI-TOF-MS, Analytical Chemistry. 79 (2007) 486-493.
[27] L. Morschheuser, S. Mükusch, M. Riedner, H. Seitz, S. Rohn, High-performance thin-layer chromatography as a fast screening tool for phosphorylated peptides, Journal of Chromatography B. 1008 (2016) 198-205.
[28] V. Panchagnula, A. Mikulskis, L. Song, Y. Wang, M. Wang, T. Knubovets, E. Scrivener, E. Golenko, I.S. Krull, M. Schulz, H. Heinz Emil, W.F. Patton, Phosphopeptide analysis by directly coupling two-dimensional planar electrochromatography/thin-layer chromatography with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Journal of Chromatography A. 1155 (2007) 112-123.
[29] Q. Chen, J.S. Zhang, X. Ke, S.Y. Lai, B.H. Tao, J.C. Yang, W.M. Mo, Y.P. Ren, Quantification of bovine beta-casein allergen in baked foodstuffs based on ultra-performance liquid chromatography with tandem mass spectrometry, Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment. 32 (2015) 25-34.
[30] S. Tolin, G. Pasini, B. Simonato, F. Mainente, G. Arrigoni, Analysis of commercial wines by LC-MS/MS reveals the presence of residual milk and egg white allergens, Food Control. 28 (2012) 321-326.
[31] S. Tolin, G. Pasini, A. Curioni, G. Arrigoni, A. Masi, F. Mainente, B. Simonato, Mass spectrometry detection of egg proteins in red wines treated with egg white, Food Control. 23 (2012) 87-94.
[32] L. Sokolova, I. Wittig, H.D. Barth, H. Schagger, B. Brutschy, U. Brandt, Laser-induced liquid bead ion desorption-MS of protein complexes from blue-native gels, a sensitive top-down proteomic approach, Proteomics. 10 (2010) 1401-1407.
[33] S.P. Pasilis, V. Kertesz, G.J. Van Berkel, M. Schulz, S. Schorcht, Using HPTLC/DESI-MS for peptide identification in 1D separations of tryptic protein digests, Analytical and Bioanalytical Chemistry. 391 (2008) 317-324.

CAPITULO II SECCION 5

Title: QQQ and Q-TOF liquid chromatography mass spectrometry analysis of casein and ovalbumin peptides in wine.

Jessy Pavón-Pérez[a], Gerardo Álvarez Rivera[b], Miguel Herrero Calleja[b], Alejandro Cifuentes[b], Karem Henriquez-Aedo[a,c], Mario Aranda[a,c]*

[a] Laboratory of Advanced Research on Foods and Drugs, Department of Food Science and Technology, Faculty of Pharmacy, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile.

[b] Foodomics Laboratory, Institute of Food Science Research (CIAL, CSIC), Nicolás Cabrera, 9, 28049 Madrid, Spain

[c] Laboratory of Functional Foods, Center for Biotechnology, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile.

Abstract

One of the most popular and widely used technique for proteomics analysis is mass spectrometry on triple quadrupole (QQQ) using Multiple reaction monitoring (MRM) mode. However, recent advances in quadrupole-time-of-flight analyzer (Q-TOF) have shown that this technique could be an useful tool for quantitative analysis of food proteins. In this work, to the best of our knowledge, is reported for the first time an analytical comparison of QQQ and Q-TOF performance for casein and ovalbumin identification and quantification in wines. Technique performance were evaluated analyzing fifteen wine samples spiked with labelled isotope peptide as internal standard. Results of Q-TOF were comparable to QQQ. The first one operating on full and a product ion scan was superior in terms of number of identified marker peptides. Otherwise, the latter, operating in MRM mode, was superior in terms of detection and quantification limits and precision for both proteins. Calibrations data fit a linear regression model with determination coefficients (R^2) =0.99 for all proteins. LOD (S/N = 3) and LOQ (S/N = 10), using a core-shell column and an injection volume of 2 µL, ranged from 0.01 to 0.08 mg L^{-1} and 0.02 to 0.10 mg L^{-1}, respectively. All samples, with both mass analyzers showed quantifiable levels of allergen proteins, the level found for each protein/transition ranged from non-detected to 0.81 mg L^{-1} Overall, the results of this study suggest that Q-TOF may represent a valid alternative in order to identify as many peptides as possible. While triple quadrupole, for its exact quantification.

Keywords: Casein; ovalbumin; Q-TOF; QQQ; wines, fining agents

1. Introduction

Caseinate or milk powder and ovalbumin or egg powder are proteins commonly used in winemaking process for wine clarification [1]. Formation of tannin-protein aggregates or protein-polyphenols complexes has made possible the use of these proteins as finning agents. This type of binding reduce also off-flavor ingredients that may affect wine taste and colour. Although that fining agents are usually removed from wine during winemaking process, residues of these proteins are often found unlabeled in commercial wines [1-3]. Even when low concentrations of casein and ovalbumin are not capable of inducing anaphylaxis, mild allergic reactions in sensitive individuals have been observed [4]. For this reason the European Union implemented the Directive 89/2003/EC [5] and its latest version 579/2012/EC, which contain a list of allergenic substances including egg and milk derivatives. This document indicates the obligation of declaring the presence of egg and milk proteins when the concentration in wines is higher than the analytical limit described in OIV/COMEX/502/2012 resolution (0.25 mg L^{-1}) [6]. Enzyme-linked immunosorbent assay is the official method recommended by the OIV [7] for egg and milk derivatives determination in wines. However, in red wines the high polyphenols content could cause detection problems due its interaction with proteins and antibodies [8]. Also, allergens adsorption to solid matrix could produce some kind of alteration of protein epitopes modifying its recognition or accessibility, thus, impairing the assay sensitivity [9] [10]. Due to that mass spectrometry has become an attractive strategy for proteomics research of food allergens proteins via peptides analysis (*Bottom-up*) or intact proteins (*Top-down*) [11]. Liquid chromatography coupled to triple quadrupole (LC-QQQ-MS/MS) operated in multiple reaction monitoring (MRM) mode with stable isotope labelled internal standard [12] has been widely used for protein quantification due to its high sensitivity and selectivity. Subfemtomole detection levels are possible to achieve because their high signal to noise ratio [13]. However, this technique using MRM mode is limited in the number of compounds that can simultaneously be monitored [14] and only allows targeted analysis [15]. In this scenario liquid chromatography coupled to high resolution mass spectrometry (HR-MS), such as LC time-of-flight (TOF) MS, operating in full scan mode (untargeted) has become an excellent alternative for screening and structural elucidation of most food allergen proteins. Improvements made in sensitivity and dynamic range of this type of mass

analyzer have made satisfactory for quantitative analysis of several allergens present in food samples at trace levels [16]. Hybrid equipment like Q-TOF present a high resolution and fast acquisition speed [17], and provides high-resolution collision induced dissociation MS/MS spectra similar to QQQ-MS [18]. Thus, Q-TOF equipment can achieve a similar performance to MRM for quantitative analysis. For quantitative analysis using hybrid Q-TOF MS, peptide full scan MS/MS spectra of selected precursor ions are obtained first, then peptide fragments observed in the high-resolution MS/MS data are selected to obtain quantitative results. High-resolution extracted ion chromatograms from each transition are combined to yield quantitative information of target peptide (called pseudo-MRM MS) [12]. The objective of the present work was to perform an analytical comparison of QQQ and Q-TOF performance for casein and ovalbumin identification and quantification in wines.

2. Materials and methods

2.1 Reagents, standards and samples

Formic acid (98-100%), trichloroacetic acid (TCA, >99%), ammonia solution (>25%), ammonium hydrogen carbonate (NH_4HCO_3, 99%), ethanol absolute p.a., acetic acid (100%), sequencing modified trypsin from bovine pancreas and LC-grade acetonitrile were purchased from Merck (Darmstadt, Germany). Casein from bovine milk, albumin from chicken egg white, iodoacetamide (IAM, >99%), DL- dithiothreitol (DTT, >99 %), were acquired from Sigma-Aldrich (St. Louis, MO, USA). Buffered solutions and mobile phases were prepared with ultrapure water (18.2 MΩ cm) produced by means of Simplicity system from Millipore (Bedford, MA, USA) Stable isotope-labelled [$^{13}C_5$,^{15}N]-valine (V*) and [$^{13}C_6$,^{15}N]-leucine (L*), VL*PV*PQK, internal standard (IS), was synthesizedand purchased from GenScript (Hon Kong, China). 12 mg L^{-1} casein and ovalbumin stock solution and IS solution (100 mg L^{-1}) was prepared in 50 mM NH_4HCO_3 (pH 7.8). Standard solutions were obtained by aliquot dilution from stock solution.

2.2 Samples preparation

Allergen proteins from wine samples were extracted and purified following the method proposed by Mattarozzi et al [2] with slight modifications. Briefly, 12.5 mL of wine were

spiked with 10 µL IS solution (100 mg L^{-1} in 50 mM NH_4HCO_3) and centrifuged at 5433 x *g* for 40 min at 20ºC into 10 kDa cut-off membrane ultrafiltration tube (Amicon Merck Millipore), previously conditioned with 10 mL of distilled water. 2.5 mL were obtained from which proteins were precipitated on ice for 2 h after addition of eight volumes of ethanol: TCA (15%w/v), pH 4.5. Thereafter, the mixture was centrifuged at 8981 x *g* for 10 min at 4ºC using a Hettich (Tuttlingen, Germany) refrigerated centrifuge. The ethanol: TCA residues were evaporated under a gentle flow of nitrogen without heat. The obtained pellet was solubilized in 1 mL of 50 mM NH_4HCO_3, pH 7.8. Trypsin digestion of both, sample and standards, was performed after proteins reduction and alkylation. The first was carried out by addition of 12 µL of 100 mM DTT and incubation at 95ºC for 5 min. After cooling down, the cysteine SH-groups, formed during the reduction step, were irreversibly alkylated adding 24 µL of freshly prepared aqueous solution of 100 mM IAM, the mixture was left stand for 15 min at room temperature protected from light. Enzymatic digestion was performed adding 4 µL of trypsin solution (0.5 mg mL^{-1}, in 50 mM of NH_4HCO_3, pH 7.8) for obtaining a trypsin: protein ratio of 1:10, the mixture was incubated at 37ºC for 3 min in ultrasound bath, model SB-5200D from BiosLabChile (Santiago, Chile). This device provides indirect sonication, working at 40 kHz of frequency with the capacity for controlling the temperature at 37 ºC. The reaction was stopped adding 10 µL of formic acid (0.1%v/v) aqueous solution. All digested samples and standards were filtered through a 13mm PVDF syringe filter (0.22 µm) before LC analysis. A total of 15 Chilean commercial wine samples without allergen labelling and previously analyzed in our laboratory by LC QQQ MS/MS were evaluated. All samples were purchased directly from supermarkets and specialized stores without contact with wineries

2.3 Apparatus

2.3.1 LC-QQQ-MS/MS

LC separations were performed on a Shimadzu (Kyoto, Japan) Nexera X2 UHPLC system consisted of: LC-30AD pump, DGU-20A5R degassing unit, SIL-30AC autosampler, CTO-20AC column oven, CBM-20A communication module, SPD-M20A diode array detector (DAD) and LCMS-8030 triple quadrupole (QQQ) mass spectrometer. The system was controlled by the LabSolution 5.8 software. Peptides separation was carried out on

Phenomenex (Torrance, CA, USA) Kinetex XB Core-Shell C_{18} column (100 mm x 4.6 mm, id. 2.6 µm), thermostated at 35ºC, using a mobile phase composed of ultrapure pure water (A) and acetonitrile (B) both with 0.1 % (v/v) formic acid. The following gradient program was applied at a flow rate of 0.6 mL min $^{-1}$: 0–19 min 10–40% B, 19–20 min 40–10% B, followed by 5 min for column conditioning. MS analysis was performed in ESI positive mode using the following conditions: ESI voltage 4.5 kV; collision energy -30.0 V for casein and -40.0 V to ovalbumin; nebulizer gas (N_2) 3 L min^{-1}, desolvation gas (N_2) 18 L min^{-1}; desolvation line temperature 250ºC and heat block temperature 400ºC. Full scan spectra were acquired from *m/z* 100 to 1000. All ions with signal intensity greater than 100 counts (range 10^1 to 10^6) and charge state +2 (or unknown charge (+1 to +3)) were submitted to fragmentation and product ion scan. MS/MS data were searched in SwissProt online database using Mascot Peptide Mass Fingerprint (Matrix Science Ltd., London, UK) as a bioinformatic tool applying the following stringent criteria: i) only one missed cleavage was allowed for trypsin digestion; ii) cysteine carbamidomethylation as fixed amino acid modification; and iii) variable modifications: methionine oxidation, and threonine and tyrosine phosphorylation. In previous works in our laboratory following the early described criteria, the two most abundant and stable fragments from the product ion spectrum were chosen for each peptide (protein), one for qualitative/confirmatory and one for quantitative purposes (Table 1). Thus, the MRM transitions established for quantification were *m/z* 634.6→991.8 for α-casein, *m/z* 390.9→258.5 for β-casein and *m/z* 929.5→1116.5 for ovalbumin and for IS *m/z* 397.3→220.3 recommended by the manufacturer.

2.3.2 LC-QTOF-MS/MS

UHPLC separations were carried out using an Agilent (Santa Clara, CA, USA) 1290 system coupled to an Agilent 6540 Q/TOF MS equipped with an orthogonal electrospray ionization (ESI) source. Separation was carried out using the same conditions described for UHPLC-QQQ-MS in Section 2.3.1. MS analysis was performed with nebulizer pressure of 40 psig, ESI voltage 4.5 kV; drying gas (N_2) 8 L min^{-1}; drying gas temperature 275 °C; sheath gas temperature, 300 °C; sheath gas, 7.5 L min^{-1}; skimmer voltage, 45 V; fragmentor voltage 110 V. Collision energy was set at 30 eV for casein and 40 eV for

ovalbumin. Operation, acquisition and data analysis were carried out by means of Agilent MassHunter Acquisition Software Ver. B.05.01. Accurate mass measurements were obtained applying ion correction techniques using reference masses at *m/z* 121.0509 (protonated purine) and 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine or HP-921] in positive ion mode. Full scan data were collected in TOF scan mode from 100 to 1700 *m/z* at 5 spectra s^{-1}. Matching of identified *m/z* peaks from LC-QTOF/MS with molecular masses of peptides identified by *bottom-up* analysis, was performed using PeptideMass tool from ExPasy (http://web.expasy.org/peptide_mass/) [19]. This tool allows the generation of theoretical peptide masses from known proteins. All matching signals (*m/z*) with intensity greater than 100 counts (range 10^1 to 10^6) and charge state +2 (or unknown charge (+1 to +3)) were submitted to fragmentation and product ion scan. From the resulting mass chromatograms MS and MS/MS data were extracted. mzML data files from MS/MS were studied in SwissProt online database using Mascot MS/MS Ions Search (Matrix Science Ltd., London, UK) as a bioinformatic tool applying the following stringent criteria: i) only one missed cleavage was allowed for trypsin digestion; ii) cysteine carbamidomethylation as fixed amino acid modification; and iii) variable modifications: methionine oxidation, and threonine and tyrosine phosphorylation. Mass tolerances for MS and MS/MS were 50 ppm and 0.6 Da, respectively. For evaluation in MRM mode, the most intense and reproducible signals from product ion spectra of each target peptide were chosen for pseudo MRM transitions (Table 2).

2.4 Statistical analysis

Data were evaluated using descriptive statistics [mean, standard deviation (SD) and relative standard deviation (RSD)]. Peptides calibrations were established applying linear regression analysis. All above tests were done with a significance level (α) of 0.05 using GraphPad (San Diego, CA, USA) Prism 6.0 software.

3. Results and Discussion

In previous work [20] from our laboratory the maker peptides for α-casein, β-casein and ovalbumin (table 1) were established to quantify these proteins in Chilean wines by LC-QQQ-MS/MS working in MRM mode.

Table 1. QQQ-MRM transitions for α, β-casein and ovalbumin determination in wine

Protein	Precursor ion sequence (*m/z*; charge state; MW [Da])	Product ion sequence (*m/z*; charge state; fragment type)
α-casein	YLGYLEQLLR (*m/z* 634.6; +2; 1267.2)	GYLEQLLR (*m/z* 991.8; +1; y_8^{+1})[a,b]
		LEQLLR (*m/z* 771.4; +1; y_6^{+1})[c]
β-casein	VLPVPQK (*m/z* 390.9; +2; 779.8)	QK (*m/z* 258.25; +1; y_2^{*})[a,b]
		PVPQK (*m/z* 284.6; +2; y_5^{+2})[c]
Ovalbumin	ELINSWVESQTNGIIR (*m/z* 929.5; +2; 1857.9)	VESQTNGIIR (*m/z* 1116.5; +1; y_{10}^{+1}) [a,b]
		ESQTNGIIR (*m/z* 1017.5; +1; y_9^{+1})[c]

[a] Most consistent MS/MS transition. [b] *m/z* transition monitored for quantification. [c] *m/z* transition monitored for confirmation.

The accelerated digestion method was previously optimized in our laboratory applying a face-centered central composite design with two central points. This optimization was performed considering each MRM transition. Since one of the most relevant aspect of wine allergen proteins evaluation is method's detection capability, peak area was chosen as the critical variable (response) looking for lower detection limits. Among the factors that possibly affect the critical variable, two were chosen: digestion time (X_1), and enzyme to protein ratio (X_2). Based on individual optimum; a multiple response optimization was calculated in order to determine the optimal conditions for all responses (desirability conditions). Optimal trypsin digestion conditions to simultaneously determine α-casein, β-casein, and ovalbumin in wines were 3 min as digestion time and 1:10 enzyme to protein ratio using ultrasound bath as described in **2.2**. Detection and quantification limits were calculated using signal-to-noise ratios (S/N) of 3 and 10 respectively, which considering the use of a core-shell column and an injection volume of 50 µL, ranged from 5.6 to 8.4 µg L^{-1} and 10.0 to 20.0 µg L^{-1}, respectively. Although this methodology showed low LOD and LOQ, it was possible to identify a few marker peptides for casein and ovalbumin. Even no marker peptide for kappa casein could be identified.

3.1 Selection of marker peptides in LC-QTOF-MS

The first step in the typical workflow development of a MS based method by bottom up strategy for wine allergen detection is the proper selection of peptide marker which must have excellent stability, sensitivity and greater abundance. In this work the first step was the search in UniProtKB for protein identifier and in silico generation of the tryptic peptides for casein and ovalbumin with the search in PeptideMass tool according to section **2.3.2**. This search generated about 200 candidate marker peptides for both proteins. The

second step in the workflow, was matching of identified *m/z* peaks from full scan data acquired in LC-QTOF/MS with theorical molecular masses of peptides identified by PeptideMass. As expected for QTOF MS in scan mode, several *m/z* signals were observed form which an important number matched with those *m/z* found *in silico* by PeptideMass tool from ExPasy. This in vitro validation allowed identifying and selecting about 40 peptides with intensity greater than 100 counts (Figure 1). From these results in vitro validation targeted high resolution MS/MS according to the described methodology in section **2.3.2** about 20 fragment marker peptides with reproducible and high MS/MS spectral quality were selected (Figure 2).

Fragment markers were established using pure standard and red wine samples (free of allergen proteins) spiked with 100 mg L^{-1} of each protein (caseins and ovalbumin) plus 10 μL of IS. Both were digested with trypsin using general conditions and studied/analyzed by LC-MS/MS. Thirteen peptides (Table 2) from trypsin digestion of casein and ovalbumin were finally defined, they showed the same charged state distribution and corresponding molecular weight with the theoretical tryptic cleavage products. Once the specific marker peptides are identified to limit the exploration, all those fragments with higher intensity were submitted to Product Ion Scan and the resulting *m/z* signals were matched with Swiss-prot database according to the conditions described in section 2.3. In our previous work by LC-QQQ-MS/MS it was not possible to identify κ-casein peptide, but using LC-QTOF-MS, κ-casein sequence SPAQILQWQVLSNTVPAK (*m/z* 990.5493; +2, t_R 15.1 min) parent/precursor ion was clearly observed (Figure 3). This finding agree with Monaci et al report [1]. It was also possible to identify more parent/precursor ion for α-casein, β-casein and ovalbumin than those found by LC-QQQ. In the next step in the workflow, the same transitions established by LC-QQQ for target proteins and IS with a high MS intensity were employed for pseudo-MRM quantitation (table 2) and analyzed in LC-QTOF analysis. IS sequence (VL*PV*PQK) is a stable isotope-labeled analog of signature peptide of β-casein sequence identified by LC-QQQ. Mass transition 397.2705→220.1761 was followed according to manufacturer recommendations.

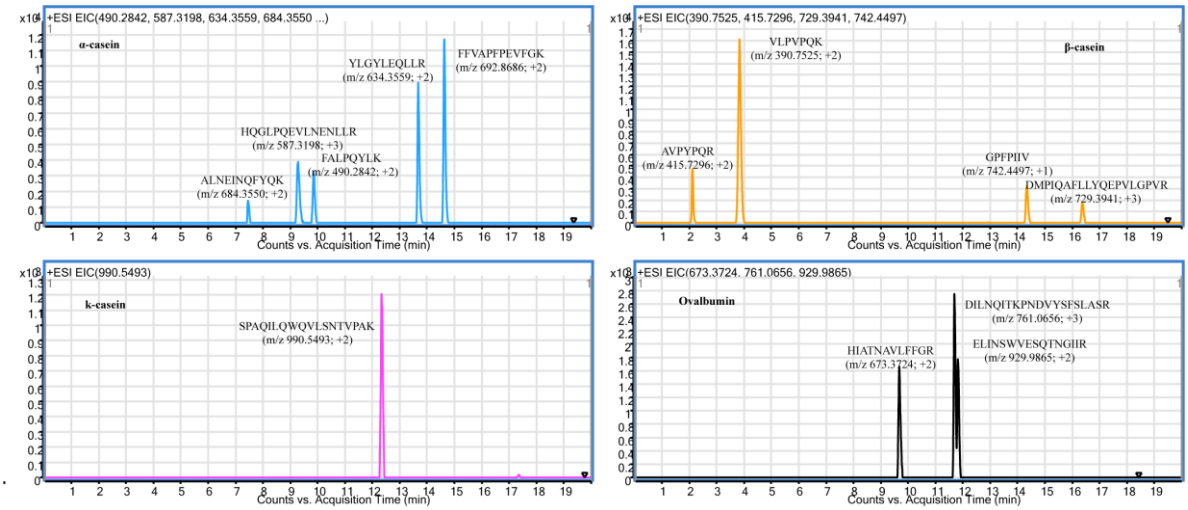

Figure 1. Some MS chromatograms of in vitro validation of candidate peptides.

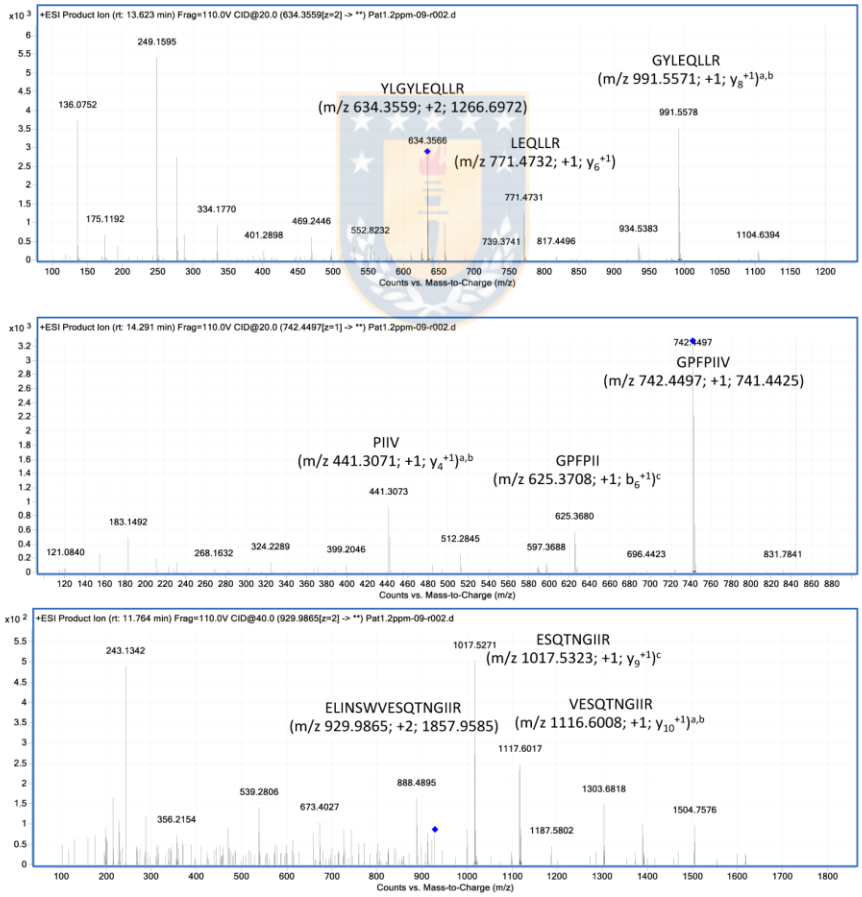

Figure 2. Targeted HR-MS/MS (Full MS/MS) for precursor peptide of α-casein (*m/z* 634.3559), β-casein (*m/z* 742.4497) and ovalbumin (*m/z* 929.9865)

Table 2. QTOF-precursor ion sequence/product ion sequence for α, β, κ-casein and ovalbumin determination in wines

Protein	Precursor ion sequence (*m/z*; charge state; MW [Da])	Product ion sequence (*m/z*; charge state; fragment type)
α-casein	YLGYLEQLLR (*m/z* 634.3559; +2; 1266.6972)	GYLEQLLR (*m/z* 991.5571; +1; y_8^{+1})[a,b]
		LEQLLR (*m/z* 771.4732; +1; y_6^{+1})[c]
	FFVAPFPEVFGK (*m/z* 692.8686; +2; 1383.7227)	FGK (*m/z* 351.2027; +1; y_3^{+1})[a]
		PEVFGK (*m/z* 920.4876; +1, y_8^{+1})[c]
		FF (*m/z* 295.1441; +1; b_2^{+1})[c]
		FFV (*m/z* 394.2125; b+1; b_3^{+1})[c]
	HQGLPQEVLNENLLR (*m/z* 587.3198; +3; 1758.9377)	HQGLPQE (*m/z* 790.3842; +1; b_7^{+1})[a]
		HQGLPQEVLN (*m/z* 758.4155; +1; y_6^{+1})
		HQGLPQEV (*m/z* 445.2300; +2; b_8^{+2})
	FALPQYLK (*m/z* 490.2842; +2; 978.5538)	PQYLK (*m/z* 648.3709; +1; y_5^{+1})[a]
		FA (*m/z* 219.1121; +1; b_2^{+1})[c]
	ALNEINQFYQK (*m/z* 684.3550; +2; 1366.6881)	INQFYQK (*m/z* 940.4868; +1; y_7^{+1})[a]
		AL (*m/z* 185.1298; +1; b_2^{+1})[c]
		NQFYQK (*m/z* 827.4058; +1; y_6^{+1})[c]
		ALN (*m/z* 299.1723; +1; b_3^{+1})[c]
β-casein	VLPVPQK (*m/z* 390.7525; +2; 779.4905)	QK (*m/z* 258.1448; +1; y_2^{*})[a,b]
		PVPQK (*m/z* 284.6763; +2; y_5^{+2})[c]
	AVPYPQR (*m/z* 415.7296; +2; 829.4446)	PQR (*m/z* 400.2303; +1; y_3)[a]
		PYPQR (*m/z* 660.3464; +1; y_5^{+1})[c]
	GPFPIIV (*m/z* 742.4497; +1; 741.4425)	PIIV (*m/z* 441.3071; +1; y_4^{+1})[a]
		GPFPII (*m/z* 625.3708; +1; b_6^{+1})[c]
	DMPIQAFLLYQEPVLGPVR (*m/z* 729.3941; +3; 2185.1605)	PVLGPVR (*m/z* 737.4869; +1; y_7^{+1})[a]
		PIQAFLLQEPVLGPVR (*m/z* 962.0500; +2; y_{17}^{*+2})[c]
κ-casein	SPAQILQWQVLSNTVPAK (*m/z* 990.5493; +2; 1979.0840)	PAK (*m/z* 315.207; +1; y_3^{+1})[a,b]
		LSNTVPAK ((*m/z* 829.4778; +1; y_8^{+1})[c]
		SNTVPAK (*m/z* 716.3937; +1; y_7^{+1})[c]
Ovalbumin	ELINSWVESQTNGIIR (*m/z* 929.9865; +2; 1857.9585)	VESQTNGIIR (*m/z* 1116.6008; +1; y_{10}^{+1})[a,b]
		ESQTNGIIR (*m/z* 1017.5323; +1; y_9^{+1})[c]
		EL (*m/z* 243.1339; +1; b_2^{+1})[c]
	HIATNAVLFFGR (*m/z* 673.3724; +2; 1344.7303)	ATNAVLFFGR (*m/z* 1095.5946; +1; y_{10}^{+1})[a]
		HI (*m/z* 251.1503; +1; b_2^{+1})[c]
	DILNQITKPNDVYSFSLASR (*m/z* 761.0656; +3; 2280.1750)	YSFSLASR (*m/z* 930.4681; +1; y_8^{+1})[a]
		DI (*m/z* 229.1176; +1; b_2^{+1})[c]

[a] Most consistent MS/MS parent/precursor ion. [b] *m/z* parent/precursor ion monitored for quantification. [c] *m/z* parent/precursor ion monitored for confirmation.

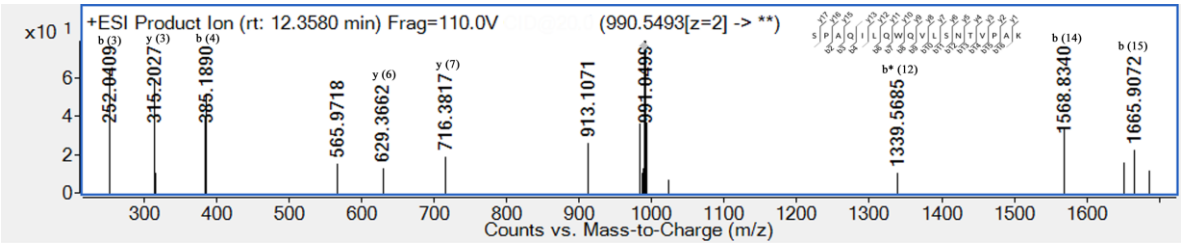

Figure 3. Product ion spectrum of κ-casein peptide SPAQILQWQVLSNTVPAK (*m/z* 990.5493)

3.2 Analytical figures of merit

Some of precursor ion sequences/product ion sequences in our knowledge this is the first time reported. For α-casein *m/z* 587.3198→*m/z* 790.3842, *m/z* 684.3550→*m/z* 185.1298, *m/z* 490.2842→*m/z* 219.1121, from β-casein *m/z* 729.3941→*m/z* 962.0500 and from ovalbumin *m/z* 761.0656→*m/z* 229.1176. This demonstrates the versatility of Q-TOF-MS/MS instruments as powerful tools for targeted and non-targeted analysis and the great possibilities offered in identifying a large number of genuine peptides in a single analysis.

For each peptide analyzed by pseudo-MRM quantitation calibrations were established at six levels from 0.10 to 1.20 mg L^{-1} (*n*=3) relating peptides and IS peak areas with peptides concentration. Calibrations data fit a linear regression model with determination coefficients (R^2) =0.99 for all proteins (table 3). LOD (S/N = 3) and LOQ (S/N = 10), using a core-shell column and an injection volume of 2 µL, ranged from 0.01 to 0.08 mg L^{-1} and 0.02 to 0.10 mg L^{-1}, respectively. LOD and LOQ obtained by QQQ-MS were lower than those observed by QTOF Table 3. Figure 4 shows a comparison of calibration curves established with QTOF-MS and QQQ-MS data. Even when these results demonstrate that pseudo-MRM by hybrid Q-TOF MS provides an accurate and reliable evaluation of peptides present at low concentration, as expected, QQQ working in MRM showed a higher sensitivity (slope) and less dispersion. Since slopes were statistically different (P<0.0001) quantitation should be performed using a QQQ mass analyzer in MRM mode. This results are opposite to those reported by Kim et al [12] they obtained lower LOD and LOQ with QTOF than with QQQ. This could be explained by matrix complexity and/or the lineal range used.

3.3 Samples analysis

As indicated above, allergen proteins detection in wines by ELISA (OIV official method) presents some limitations that impair the assay sensitivity. Contrarily, the proposed LC-QQQ-MS/MS and LC-QTOF-MS/MS method are highly sensitive and capable of detecting concentrations as low as 0.01 mg L^{-1} (Supplementary material). All wine samples labels described the wine type/category, i.e. young (without aging) or reserve (higher quality and/or aged), cv. (one grape variety represents at least 75% of the blend), harvest year and geographical origin (denomination of origin). According to that, six samples were reserve

and nine young wines, belonging to four valleys and seven wineries. Three samples were Cabernet Sauvignon, three Sauvignon Blanc, one Carménère, five Merlot, one Malbec, one Syrah and one blended wine (Cabernet Sauvignon 50%, Carménère 30% and Syrah 20%). Two independent extractions from each sample were analyzed in duplicate. All samples, with both mass analyzers showed quantifiable levels of allergen proteins, the level found for each protein/transition ranged from non-detected to 0.81 mg L^{-1} (Table 5). Five Merlot samples (5/15; 33.3%), three Cabernet Sauvignon samples (3/15; 20%), three Sauvignon Blanc (3/15; 20%), one Carménère (1/15; 6.6%), one Malbec (1/15; 6.6%), one Syrah (1/15; 6.6%) and one blended sample (1/15; 6.6%) showed a total concentration of casein and ovalbumin higher than the limit recommended by the European Community (0.25 mg L^{-1}). Samples quantification by pseudo-MRM mode with QTOF spectrometer showed similar results that with QQQ, however there are some transitions were not possible to be quantified by QTOF because its LOQ was higher than the one obtained by QQQ. Nevertheless, QTOF technique allowed the identification of a greater number of marker peptides than those obtained by LC-QQQ-MS/MS. These results demonstrated the applicability of QTOF mass analyzer for peptides detection and identification and QQQ for peptide accurate and sensitive quantification. Even when the LOD and LOQ were higher than those found by QQQ-MS/MS, were lower than some international reports. The number of identified marker peptides for both proteins in one single analysis in real wine samples were higher than some international reports. The detection and quantification limits were lower than those reported by Monaci et al [1] using the same method (HPLC-ESI-QTOF) (LOD=50 mg L^{-1}) for α and β-casein in white wine fortified with this protein. Also the authors reported only the identification of 3 marker peptides for casein. In 2011 Monaci et al [21] reported for the analysis of casein marker peptides in white wine with an HPLC-Orbitrap, LOD from 0.15 mg L^{-1} to 0.50 mg L^{-1} and LOQ from 0.5 to 3 mg L^{-1} values higher than those found in this work. Tolin et al [3] identified 4 marker peptides for ovalbumin in red wine with HPLC-QTOF technique but LOD (50 mg L^{-1}) was at least 98% greater than those found in this work. Monaci et al in 2013 [22] detected several marker peptides for casein, ovalbumin and lysozyme in one single analysis employing an HPLC-Orbitrap, but the LOD and LOQ reported were ten times higher than those achieved in this work. Mattarozzi et al [2] used the liquid cromatography coupled online with an linear ion

trap for the analysis of potential residuals of ovalbumin and caseins added to red wines. They reported low LOD (0.01 mg L^{-1} to 0.80 mg L^{-1}) and LOQ (0.03 mg L^{-1} to 2.0 mg L^{-1}) but they found 4 marker peptides for casein and two for ovalbumin. LOD and LOQ found in this work are similar to those reported by Pilolli et al [23] (0.01 mg L^{-1} and 0.03 mg L^{-1} respectively) with an Linear Ion Trap Mass Spectrometer. However they found three marker peptides for ovalbumin in white and rosé wine (ca 77% less than in this work).

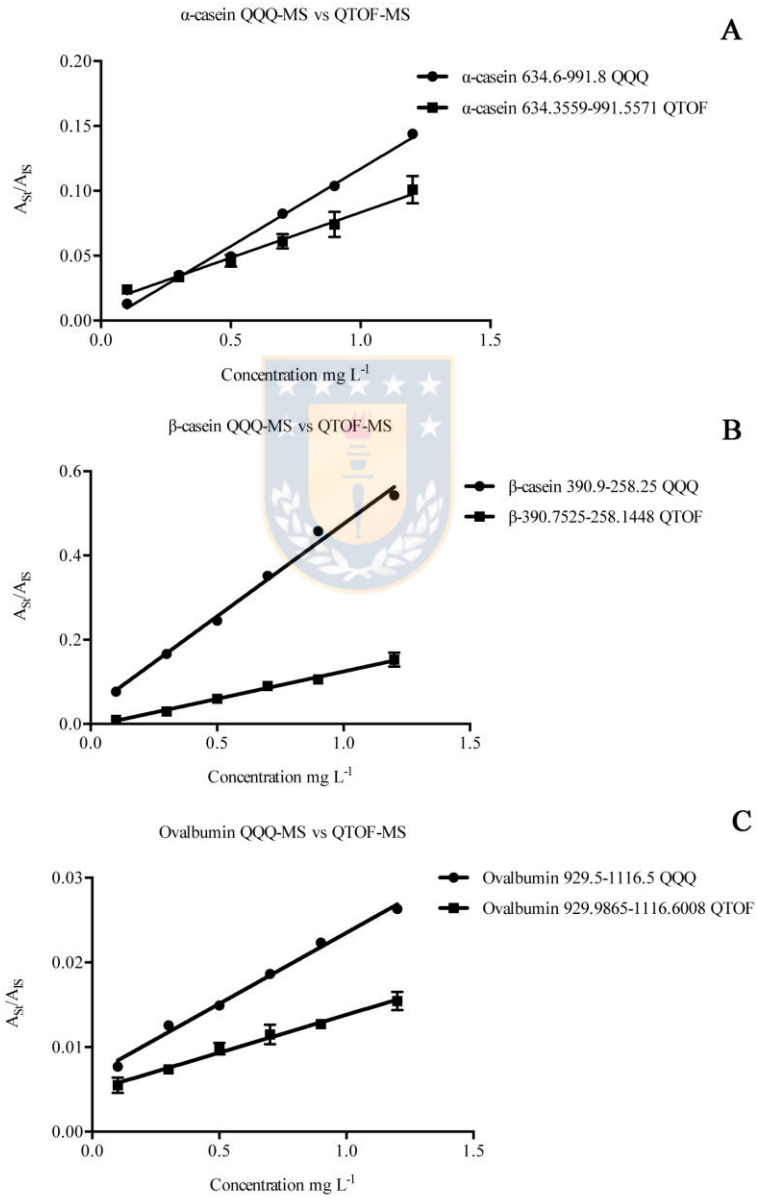

Figure 4. Calibration curves of α-casein, β-casein and ovalbumin by QTOF MS and QQQ MS

Table 3. Summary of calibrations data by QTOF MS and QQQ MS

Protein		Regression equation [ab] y = ax±SD + b		Goodness of fit[c] R^2		LOD[d] (mg L^{-1})		LOQ[e] (mg L^{-1})	
		QTOF	QQQ	QTOF	QQQ	QTOF	QQQ	QTOF	QQQ
α-casein	634.3559 →991.5571	y=0.0699x±0.0035+0.0134±0.0025	y=0.1195x±0.005-0.002±0.004	0.9903	0.9925	0.03	0.006	0.10	0.02
	692.8686→351.2027	y=0.3019x±0.0145-0.0263±0.0104		0.9908		0.01		0.04	
	587.3198→790.3842	y=0.8100x±0.0407-0.1068±0.0292		0.9900		0.03		0.10	
	490.2842→648.3709	y=0.1442x±0.0070+0.0411±0.0050		0.9929		0.01		0.02	
	684.3530→940.4868	y=0.0696x±0.0022-0.0069±0.0017		0.9970		0.06		0.10	
β-casein	390.7525→258.1448	y=0.1300x±0.0048-0.0053±0.0035	y=0.4386x±0.02+0.036±0.01	0.9945	0.9917	0.04	0.006	0.10	0.01
	415.7296→400.2303	y=0.1109x±0.0049+0.0022±0.0035		0.9923		0.03		0.10	
	742.4497→441.3071	y=0.5747x±0.0202-0.0587±0.0145		0.9951		0.01		0.06	
	729.3941→737.4869	y=0.1222x±0.0068+0.0349±0.0054		0.9906		0.08		0.10	
κ-casein	990.5493→315.2027	y=0.1375x±0.0064-0.0003±0.0046		0.9914		0.03		0.10	
Ovalbumin	929.9865→1116.6008	y=0.0089x±0.0004+0.0049±0.0003	y=0.0168x±0.0007+0.007±0.0005	0.9919	0.9923	0.01	0.008	0.10	0.02
	673.3724→1095.5946	y=0.1191x±0.0044-0.0069±0.0032		0.9944		0.05		0.10	
	761.0656→930.4681	y=0.1833x±0.0091-0.0227±0.0065		0.9902		0.04		0.10	

[a] n=3, three injections for each level
[b] a represent slope and b intercept
[c] R^2: determination coefficient
[d] S/N=3
[e] S/N=1

4. Conclusions

In this work a pseudo MRM analysis method to quantify casein and ovalbumin peptides in Chilean wines using hybrid Q-TOF MS was developed. Using a common bottom-up approach, an analytical comparison between QQQ and Q-TOF performance for casein and ovalbumin identification and quantification in wines was performed. Q-TOF analysis in full scan and product ion scan modes allowed the identification of a higher number of marker peptides than those observed by QQQ, including the identification of *k*-casein, which had not been achieved by QQQ. Pseudo-MRM analysis using hybrid Q-TOF MS, based on the full scan MS/MS data and the simultaneous monitoring of multiple transitions from fragment ions provided lower sensitivity for quantitation than MRM analysis using QQQ MS, therefore it is possible to conclude that QTOF mass analyzer is the technique of choice for non-targeted peptides detection and identification and QQQ for peptides quantification.

Acknowledgments

This work is part of Jessy Pavón Pérez thesis to obtain the degree of Doctor in Science and Analytical Technology from the University of Concepcion, Chile. Authors want to thank to the National Commission of Scientific and Technological Research (CONICYT) of the Chilean Government for the doctoral scholarship granted. This work was financially supported by National Fund for Scientific & Technological Development (FONDECYT), projects N°1171857, FONDEQUIP 130209 and University of Concepcion.

References

[1] L. Monaci, I. Losito, F. Palmisano, A. Visconti, Identification of allergenic milk proteins markers in fined white wines by capillary liquid chromatography–electrospray ionization-tandem mass spectrometry, J. Chromatogr. A. 1217 (2010) 4300-4305.
[2] M. Mattarozzi, M. Milioli, C. Bignardi, L. Elviri, C. Corradini, M. Careri, Investigation of different sample pre-treatment routes for liquid chromatography–tandem mass spectrometry detection of caseins and ovalbumin in fortified red wine, Food Control. 38 (2014) 82-87.
[3] S. Tolin, G. Pasini, A. Curioni, G. Arrigoni, A. Masi, F. Mainente, B. Simonato, Mass spectrometry detection of egg proteins in red wines treated with egg white, Food Control. 23 (2012) 87-94.
[4] J.M. Rolland, E. Apostolou, K. Deckert, M.P. de Leon, J.A. Douglass, I.N. Glaspole, M. Bailey, C.S. Stockley, R.E. O'Hehir, Potential food allergens in wine: double-blind, placebo-controlled trial and basophil activation analysis, Nutrition (Burbank, Los Angeles County, Calif.). 22 (2006) 882-888.
[5] European Commission (EU), Commission implementing regulation 579/2012, Off. J. Eur. Union. L171 (2012) 4-7.
[6] OIV, Revision of the limit of detection and limit of quantification related to potentially allergenic residues of finning agent proteins in wine, Organisation Internationale de la Vigne et du Vin, Paris, 2012
[7] Organisation Internationale de la Vigne et du Vin, Criteria OIV-MA-AS315-23. http://www.oiv.int/oiv/info/enmethodesinternationalesvin (noviembre 29)
[8] W. Patrick, S. Hans, P. Angelika, Determination of the bovine food allergen casein in white wines by quantitative indirect ELISA, SDS-PAGE, Western blot and immunostaining, J. Agric. Food. Chem. 57 (2009) 8399-8405.
[9] S. Kaul, D. Luttkopf, B. Kastner, L. Vogel, G. Holtz, S. Vieths, A. Hoffmann, Mediator release assays based on human or murine immunoglobulin E in allergen standardization, Clin. Exp. Allergy. 37 (2007) 141-150.
[10] M. Gavage, K. Van Vlierberghe, C. Van Poucke, M. De Loose, K. Gevaert, M. Dieu, P. Renard, T. Arnould, N. Gillard, Selection of egg peptide biomarkers in processed food products by high resolution mass spectrometry, J. Chromatogr. A. (2018).
[11] F. Calderón-Celis, L. Cid-Barrio, J.R. Encinar, A. Sanz-Medel, J.J. Calvete, Absolute venomics: Absolute quantification of intact venom proteins through elemental mass spectrometry, J Proteomics. 164 (2017) 33-42.
[12] K.H. Kim, Y.H. Ahn, E.S. Ji, J.Y. Lee, J.Y. Kim, H.J. An, J.S. Yoo, Quantitative analysis of low-abundance serological proteins with peptide affinity-based enrichment and pseudo-multiple reaction monitoring by hybrid quadrupole time-of-flight mass spectrometry, Anal. Chim. Acta. 882 (2015) 38-48.
[13] P. Herrero, N. Cortés-Francisco, F. Borrull, J. Caixach, E. Pocurull, R.M. Marcé, Comparison of triple quadrupole mass spectrometry and Orbitrap high-resolution mass spectrometry in ultrahigh performance liquid chromatography for the determination of veterinary drugs in sewage: benefits and drawbacks, J. Mass Spectrom. 49 (2014) 585-596.
[14] S. Saito-Shida, T. Hamasaka, S. Nemoto, H. Akiyama, Multiresidue determination of pesticides in tea by liquid chromatography-high-resolution mass spectrometry: Comparison between Orbitrap and time-of-flight mass analyzers, Food Chem. 256 (2018) 140-148.
[15] R. Bąchor, P. Mielczarek, M. Rudowska, J. Silberring, Z. Szewczuk, Sensitive detection of charge derivatized peptides at the attomole level using nano-LC-ESI–MRM analysis, Int. J. of Mass Spectrom. 362 (2014) 32-38.
[16] M. Mezcua, O. Malato, J.F. Garcia-Reyes, A. Molina-Diaz, A.R. Fernandez-Alba, Accurate-mass databases for comprehensive screening of pesticide residues in food by fast liquid chromatography time-of-flight mass spectrometry, Anal. Chem. 81 (2009) 913-929.
[17] G.L. Andrews, B.L. Simons, J.B. Young, A.M. Hawkridge, D.C. Muddiman, Performance characteristics of a new hybrid quadrupole time-of-flight tandem mass spectrometer (TripleTOF 5600), Anal. Chem. 83 (2011) 5442-5446.
[18] J. Singh, G.K. Jayaprakasha, B.S. Patil, An optimized solvent extraction and characterization of unidentified flavonoid glucuronide derivatives from spinach by UHPLC-HR-QTOF-MS, Talanta. 188 (2018) 763-771.
[19] M.R. Wilkins, I. Lindskog, E. Gasteiger, A. Bairoch, J.-C. Sanchez, D.F. Hochstrasser, R.D. Appel, Detailed peptide characterization using PEPTIDEMASS – a World-Wide-Web-accessible tool, ELECTROPHORESIS. 18 (1997) 403-408.

[20] J. Pavón-Pérez, K. Henriquez-Aedo, M. Aranda, Mass Spectrometry Determination of Fining-Related Allergen Proteins in Chilean Wines, Food Analytical Methods. 12 (2019) 827-837.
[21] L. Monaci, I. Losito, F. Palmisano, M. Godula, A. Visconti, Towards the quantification of residual milk allergens in caseinate-fined white wines using HPLC coupled with single-stage Orbitrap mass spectrometry, Food Addit Contam Part A. 28 (2011) 1304-1314.
[22] L. Monaci, I. Losito, E. De Angelis, R. Pilolli, A. Visconti, Multi-allergen quantification of fining-related egg and milk proteins in white wines by high-resolution mass spectrometry, Rapid Commun. Mass Spectrom. 27 (2013) 2009-2018.
[23] R. Pilolli, R. Chaudhari, F. Palmisano, L. Monaci, Development of a mass spectrometry immunoassay for unambiguous detection of egg allergen traces in wines, Anal Bioanal Chem. 409 (2017) 1581-1589.

Capítulo II Sección 5 Suplemmentary material

Table SI-1. Allergen content (mg L^{-1}) in Chilean wine samples

	Wine samples	Valley	Year[a]	α-casein		β-casein		κ-casein	Ovalbumin	
				m/z 634.3559	634.3559 →991.5571	m/z 390.7525	390.7525→258.1448	m/z 990.5493	m/z 929.9865	929.9865→1116.6008
				QTOF	QQQ	QTOF	QQQ	QTOF	QTOF	QQQ
1	S. Blanc	Central	2011	0.27±0.08	0.19±0.05	Traces	0.08±0.01	ND	ND	ND
2	S. Blanc	Leyda	2016	0.19±0.03	0.19±0.01	0.10±0.03	0.06±0.00	ND	ND	ND
3	S. Blanc	Maipo	2016	0.20±0.00	0.22±0.01	Traces	0.05±0.01	ND	ND	ND
4	Merlot	Central	2016	Traces	0.02±0.00	Traces	Traces	0.13±0.03	ND	0.25±0.04
5	Merlot	Central	2016	Traces	0.02±0.00	Traces	Traces	Traces	ND	0.25±0.01
6	Merlot	Central	2016	Traces	0.01±0.00	ND	Traces	ND	0.13±0.00	0.28±0.03
7	Merlot	Rapel	2014	0.11±0.04	0.20±0.03	Traces	0.08±0.01	0.18±0.01	ND	ND
8	Merlot	Maipo	2015	0.17±0.04	0.16±0.01	0.15±0.01	0.12±0.01	ND	ND	Traces
9	Malbec	Central	2016	0.16±0.01	0.17±0.02	Traces	0.09±0.01	Traces	ND	ND
10	Carménère	Maipo	2016	Traces	0.05±0.00	Traces	0.05±0.00	ND	0.24±0.05	0.19±0.03
11	C. Sauvignon	Central	2015	ND	ND	Traces	Traces	ND	ND	Traces
12	C. Sauvignon	Colchagua	2015	0.15±0.02	0.15±0.00	Traces	0.06±0.01	ND	ND	0.05±0.01
13	C. Sauvignon	Colchagua	2015	0.15±0.02	0.11±0.01	0.11±0.01	0.06±0.00	Traces	0.24±0.04	0.10±0.00
14	Syrah	Central	2015	Traces	0.07±0.01	Traces	0.04±0.00	ND	ND	ND
15	C. Sauvignon Carménère Syrah	Maule	2016	0.21±0.02	0.16±0.01	Traces	0.06±0.00	0.12±0.01	Traces	0.03±0.00

Two independent extractions from each sample were analyzed in duplicate, results are expressed as mean ± standard deviation.
ND: not detected (<LOD)
[a]Year indicated in the label. Traces: (>LOD; <LOQ)

CAPITULO II SECCION 6

Title: Impact of fining agents on colour and composition of white and red wines: effect of cross-flow ultrafiltration on casein and ovalbumin elimination.

Jessy Pavón-Pérez[a], Karem Henriquez-Aedo[a,b], Rodrigo Borquez[c], Mario Aranda[a,b]*

[a] Laboratory of Advanced Research on Foods and Drugs, Department of Food Science and Technology, Faculty of Pharmacy, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile.

[b] Laboratory of Functional Foods, Center for Biotechnology, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile.

[c] Department of Chemical Engineering, Faculty of Engineering, University of Concepcion, Barrio Universitario s/n, Concepcion, Chile.

Abstract

This work reports the effect of bentonite, diatomaceous earth, casein and ovalbumin fining agents on oenological parameters, polyphenols and anthocyanins composition of red and white wines. Colour and turbidity were evaluated by spectrophotometry and polyphenols and anthocyanins were analyzed by spectrophotometry and HPLC-UV/Vis. The results showed that all treatments affected wines quality parameters. The most remarkable effects on phenolic composition were produced by bentonite and protein fining agents. Bentonite has the major impact under anthocyanins composition in red wine and casein and ovalbumin under polyphenol levels in white and red wine respectively. The use of casein at 1 g L^{-1} and ovalbumin at 0.1 g L^{-1} followed by cross-flow ultrafiltration caused a significant reduction of colour and turbidity in wines. The ultrafiltration membranes allowed removed the traces of casein and ovalbumin present in wines.

*Keywords**:** Fining, colour, anthocyanins, polyphenols, filtration, wine*

1. Introduction

One of the principal problems in wine industry is haze formation (Jaeckels et al., 2017). Turbidity in the final product is not desired by consumers affecting sales and therefore causing economic losses for wineries (Van Sluyter et al., 2015). Wine limpidity must be maintained during storage time under different conditions of light, aeration, temperature, etc (Martinez-Lapuente, Guadalupe, & Ayestaran, 2017). Therefore, it is important to achieve the physicochemical stability of wine before bottling. During winemaking wine stabilization and limpidity is progressively obtained by natural phenomena that produce undesirable compounds precipitation. Since this natural clarification is a very slow process, different compounds or techniques have been assayed to accelerate the interaction between wine components (Gustavo González-Neves, Favre, & Gil, 2014). In this scenario many fining agents have been employed and the type of stabilizing methods depends on the kind of haze. Grape proteins, polysaccharides polymerized and phenolic compounds are the main responsible for the haze in wine. Among fining agents, bentonite seems to be the best alternative to remove proteins (Van Sluyter et al., 2015). It is a mineral with cation exchange properties, hence, proteins can be adsorbed on bentonite surface and then precipitate due to its positive charges (Huertas, Carretero, Delgado, Linares, & Samper, 2001). However, bentonite is not a specific adsorber, molecules with beneficial characteristics for wine aroma and colour could be adsorbed affecting the wine quality (Vincenzi, Panighel, Gazzola, Flamini, & Curioni, 2015). For example, Patil et al (Patil, Kaur, & Sharma, 2012) reported an important reduction of wine colour intensity due to the use of bentonite. Filtration on diatomaceous earth is another widely used fining agent, mainly for microbial stabilization. However, some adverse effects have limited its use, e.g. skin irritation and dryness and eye irritation (Rice et al., 2001). Proteins such as gelatin, isinglass, casein and ovalbumin are commonly used like fining agents (Maury, Sarni-manchado, & Cheynier, 2018) (Cosme, Ricardo-da-Silva, & Laureano, 2008). Casein and ovalbumin are the most employed ones, its activity is based on the interaction with polyphenolic compounds inducing their precipitation preventing haze formation (Maury et al., 2018). Protein related fining agents can reduce the astringency and bitterness of wine due to its interaction with tannins, or proanthocyanidins, particularly with highly polymerized tannins (Karamanidou, Kallithraka, & Hatzidimitriou, 2011). Casein and

ovalbumin are food-related allergen proteins and part of the amount used for fining could remain in wine after treatment. Since this kind of proteins may trigger allergic reactions in susceptible individuals; their occurrence in wines could become a human health risk, moreover when their presence is not reported. Therefore, it is very important to identify and quantify these proteins in wine. Physical methods are an alternative to wine clarification without adding any exogenous substance. Filtration during winemaking process provides limpidity and microbiological stabilization of wines (Martinez-Lapuente et al., 2017). However, this process alone does not guarantee the physicochemical stabilization of wine, because cannot prevent the formation of hazes and precipitates after bottling. Cross-flow ultrafiltration is a technique that involves a one-step procedure, and can substitute the conventional filtration of progressive clarification. Cross-flow ultrafiltration has the advantage of combining clarification, microbial stabilization and sterile filtration in one single and continuous step (El Rayess et al., 2016). However, Ulbricht et al (Ulbricht, Ansorge, Danielzik, König, & Schuster, 2009) reported the negative effects of wine polysaccharides and polyphenols on the permeation flux, especially by adsorption of these molecules on membrane materials. Then, it is very important to experimentally compare all possible fining agents in order to find the most suitable for wine clarification. In the present work white and red wines clarified using the following fining agents: bentonite; (b) diatomaceous earth; (c) casein and ovalbumin; and (d) cross-flow ultrafiltration. The effect of all these techniques on wine quality parameters, i.e. colour, turbidity, anthocyanin, and polyphenols composition were evaluated. Further, the cross-flow ultrafiltration capacity of removing casein and ovalbumin residues was also determined.

2. Materials and Methods

2.1 Reagents, standards and samples

Formic acid (98-100%), hydrochloric acid (37%), potassium chloride (KCl, 99%), sodium acetate ($CH_3CO_2Na \cdot 3H_2O$, 99%), sodium hydrogen carbonate ($NaHCO_3$, ≥ 99.7%), ethanol absolute p.a (100%), sodium dihydrogen phosphate anhydrous (NaH_2PO_4, ≥99.9%), disodium hydrogen phosphate (Na_2HPO_4 H_2O, ≥99%), trichloroacetic acid (TCA, >99%), ammonia solution (>25%), ammonium hydrogen carbonate (NH_4HCO_3, 99%), Folin-Ciocalteu phenol reagent (2 M), and LC-grade acetonitrile and methanol were purchased

from Merck (Darmstadt, Germany). Iodoacetamide (IAM, >99%), DL- dithiothreitol (DTT, >99 %), sequencing modified trypsin from bovine pancreas, pyrogallol red, 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH, 97%), (±)-6-Hydroxy-2,5,7,8-tetramethylchromano-2-carboxylic acid (TROLOX, 97%), gallic acid (97.5–102.5%), quercetin (≥ 95%), benzoic acid (≥ 99.5%), *p*-coumaric acid (≥ 98%), kaempferol (≥ 90%), vanillic acid (≥ 97%), caffeic acid (≥ 98%), resveratrol (≥ 99%) and myricetin (≥ 99%) were obtained from Sigma-Aldrich (St. Louis. MO, USA). Anthocyanin standards cyanidin 3-O-glucoside chloride (99%), delphinidin 3-glucoside chloride (99%) and malvidin 3-glucoside chloride (99%) were purchased from Zeus Atenea Spa (Santiago, Chile). Ammonium formate (≥ 99%) was purchased from Fluka (Munich, England). Bentonite was a kind donation from the Neira winery (Región de Bío-Bío, Chile), diatomaceous earth (calcined power) was from Sigma-Aldrich, casein (Caseimix) and ovalbumin were obtained from Industrias Vinicas SA (Santiago, Chile). Buffer solutions and mobile phases were prepared with ultrapure water (18.2 MΩ cm) produced by means of Simplicity system from Millipore (Bedford, MA, USA).

2.2 Wine treatments

Clarification trials were performed using a white and red wine samples free of any kind of fining process. Each type of wine was clarified with the four fining agents using the corresponding untreated wine as a control for each assay. The dosage employed of each fining agent were the usually ones used in wineries (Table 1), they were prepared following manufacturers recommendations. In case of bentonite a 48 h hydration was required previous its used. Fining treatments were performed in duplicate on 150 mL of each wine under constant agitation a room temperature for 48 h. Thereafter, supernatants were filtered through filter paper Nº4, Whatman (NJ, USA) and analyzed in triplicate.

Table 1. Concentration of fining agents evaluated in this study

Fining agents	Control	Concentration 1 (g hL^{-1})	Concentration 2 (g hL^{-1})	Concentration 3 (g hL^{-1})
Bentonite	0	50 (TBA)	100 (TBB)	150 (TBC)
Earth diatomaceous	0	25 (TTA)	50 (TTB)	100 (TTC)
Casein	0	30 (TCA)	65 (TCB)	100 (TCC)
Ovalbumin	0	8 (TOA)	9 (TOB)	10 (TOC)

2.3 Analysis of turbidity of wines

Turbidity was evaluated by measuring the optical density at 650 nm in a Jasco (Easton, USA) V530 UV–visible spectrophotometer using quartz cells of 1-cm path length, before and after centrifugation as described by Feuillat et al (Feuillat & Bergeret, 1966).

2.4 Colour

Wine samples were centrifuged for 3 min at 3000 rpm before spectrophotometric analysis according to Gonzáles-Neves et al (Gustavo González-Neves et al., 2014). Measurements were carried out using a Jasco V530 UV–visible spectrophotometer, employing quartz cells with a 1-cm path length. The intensity of red wine colour is given by the sum of absorbencies at 420, 520 and 620 nm ("Organisation Internationale de la Vigne et du Vin. Compendium of International Methods of Analysis–OIV. Chromatic Characteristics," 2009) and hue was calculated as the ratio between absorbance at 420 and 520 nm (Karamanidou et al., 2011). The intensity of white wine colour is given only by the absorbance at 420nm.

2.5 Determination of total monomeric anthocyanin content

Total monomeric anthocyanins content (TAC) was determined through pH differential method (Socaciu, 2008). Wine sample was divided in two portions, one diluted with potassium chloride buffer (0.025 M, pH 1) and the other one in sodium acetate buffer (0.4 M, pH 4.5), both 4-fold diluted with buffer and pH adjusted with 0.2 M HCl. After 15 min of equilibration at room temperature, the absorbance of both portions were determined at 520 nm and 700 nm using Infinite 200 Pro microplate reader (Tecan, Gröedig, Austria). Total monomeric anthocyanin pigment concentration, was calculated as cyanidin-3-glucoside equivalents:

Anthocyanin pigment (cyanidin-3-glucoside equivalents, mg/L) =

$$\frac{A \; x \; MW \; x \; DF \; x \; 10^3}{\varepsilon \; x \; l}$$

where A = (A 520nm – A 700nm) pH 1.0 – (A 520nm – A 700nm) pH 4.5;

MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu);

DF = dilution factor established;

l = pathlength in cm; ε = 26900 molar extinction coefficient, in L mol^{-1} cm^{-1}, for cyd-3-glu;

10^3 = factor for conversion from g to mg.

2.6 Determination of total phenols content

Total phenolic content was determined by the Folin-Ciocalteu assay based on the work of Speisky et al (Speisky, Lopez-Alarcon, Gomez, Fuentes, & Sandoval-Acuna, 2012). Briefly, on 96-well plate, 20 μL of standard or sample, 115 μL of 0.2 N Folin-Ciocalteu aqueous solution and 115 μL of 60 g L^{-1} sodium carbonate were mixed. Solution was incubated at 37ºC for 90 min before absorbance measure at 725 nm. A calibration was established with gallic acid (20, 40, 60, 80, 100, 200, 400 and 600 μg mL^{-1}), using ethanol as blank. Sample background absorption was determined using 115 μL of water instead of Folin-Ciocalteu reagent. All standards and samples were analyzed in triplicate and the results were expressed as milligrams of gallic acid equivalents per liter of sample (mg GAE L^{-1}).

2.7 Determination of antioxidant activity

Antioxidant capacity of wines was determined by a microplate-based oxygen radicals absorbance capacity (ORAC) assay using pyrogallol red (PGR) following the method reported by Ortiz et al (Ortiz et al., 2012) with slight modifications. Briefly, on a 96-well microplates the following reaction mixture was mixed for a final volume of 250 μL: 50 μL of blank, sample (100-fold diluted with sodium phosphate buffer) or Trolox solutions, 150 μL of 75 mM sodium phosphate buffer (pH 7.4) and 25 μL of PGR (5 μM final concentration). The mixture was preincubated for 30 min at 37°C, before the addition of AAPH solution (25 μL; 10 mM, final concentration), previously incubated at 37ºC. The microplate was immediately placed in Tecan Infinite 200 Pro microplate reader, automatically shaken, and PGR absorbance (A) was read and recorded at 540 nm every 60 s for 180 min. A calibration was established in triplicate (*n*=3) with Trolox (50, 100, 150, 200, 300, 400 and 500 μM). Positive control was PGR and AAPH using methanol instead

of the antioxidant solution; negative control was just PGR without AAPH and background was measured using 50 µL of sample and 200 mL sodium phosphate buffer. Results were expressed as mM Trolox equivalents for red wine and as µM Trolox equivalents for white wine.

2.8 Analysis of polyphenols by HPLC/UV

Polyphenols were analyzed by HPLC/UV according to Lambert et al (Lambert et al., 2015) with slight modifications. Briefly, wine samples were 5-fold diluted with MeOH:H_2O (80:20 v/v). Polyphenols stock solution was prepared in MeOH: H_2O (80:20 v/v) for a given concentration of 100 mg L^{-1}. Working solutions were established at six levels from 1 mg L^{-1} to 10 mg L^{-1} were prepared by stock solution dilution. All solutions kept refrigerated at 4ºC were stable for at least seven days. Polyphenols analysis was carried out using a Shimadzu (Kyoto, Japan) Prominence HPLC system, composed by: LC-20AT binary pump, DGU-20A5R degassing unit, SIL-20AC autosampler, CBM-20A communication module, CTO-20AC column oven and SPD-20A UV-Vis detector, all controlled by means of LabSolutions software (version 5.51). Chromatography was performed on Kinetex XB-C_{18} (4.6 x 150 mm; 5 µm) column connected to guard-column of the same chemistry, both set at 40ºC. A binary mobile phase composed acidified acetonitrile (0.1% v/v formic acid, solvent A) and 10 mM ammonium formate pH 3 (solvent B), was applied using the following gradient program at a flow rate of 0.5 mL min^{-1}: 0-3 min, 18-20% (A); 3-4 min, 20-30% (A); 4-6 min, 30% (A) (isocratic step); 6-10 min, 30-80% (A); 10-14 min, 80% (A) (isocratic step); 14-16 min, 80-18% (A); and 16-20 min, 18% for column conditioning. Detection was done at 280 nm.

2.9 Analysis of Anthocyanins by HPLC/Vis

Anthocyanins were analyzed by HPLC/Vis, according to the International Organisation of Vine and Wine (OIV. Organization Internationale de la Vigne et du Vin, 2007), with slight modifications. Briefly, anthocyanins stock solution was prepared in MeOH: ACN (1% formic acid); 1:1 v/v for a given concentration of 100 mg L^{-1}. Working solutions were prepared by stock solutions dilution. All solutions kept refrigerated at 4ºC were stable for at least seven days. Delphinidin calibration was prepared at six levels in a range from 1 mg L^{-}

^{1} to 10 mg L^{-1}, and malvidin and cyanidin curve range from 10 mg L^{-1} to 100 mg L^{-1} at six levels. Anthocyanins analysis was carried out using a Shimadzu (Kyoto, Japan) Prominence HPLC system, composed by: LC-20AT binary pump, DGU-20A5R degassing unit, SIL-20AC autosampler, CBM-20A communication module, CTO-20AC column oven and SPD-20A UV-Vis detector, all controlled by means of LabSolutions software (version 5.51). Chromatography was performed on Kinetex XB-C_{18} (4.6 x 150 mm; 5 µm) column connected to guard-column of the same chemistry, both set at 40ºC. A binary mobile phase is composed of H_2O: formic acid: ACN (87:10:3 v/v/v solvent A) and a mixture of H_2O: formic acid: ACN (40:10:50 v/v/v solvent B) was used applying the following gradient program at flow a rate of 0.8 mL min^{-1}: 0-15 min, 94-70% (A); 15-30 min, 70-50% (A); 30-35 min, 50-40% (A); 35-41 min, 40-94%, (A); and 41-45 min, 94% for column conditioning. Detection was done at 518 nm.

2.10 Filtration of wine with membranes

2.10.1 Filtration Units

Ultrafiltration assay was carried out according to the parameters defined by Pandolfi in 2008 (Pandolfi P, 2008). One cross-flow unit Alfa Laval DSS LabUnit M10 (Rudeboksvägen, Sweden) equipped with a flat plate configuration module for 4 flat sheet membranes generating a filtering surface of 0.0336 m^2 was used. The unit consisted of a positive displacement pump with frequency modulator, heat exchanger of concentric tubes and manometers at the entrance and exit of the module. Filtration was performed using an Alfa Laval GRM 2.0 polysulfone membrane with a nominal diameter of 2.0 µm and a nominal cut-off pore size of 10 kDa. Tangential filtration velocity (60 Hz) and intramembrane pressure (6.75 m s^{-1}) were kept constant. The temperature was maintained between 20 and 25 ºC, using the heat exchanger fed with potable water. For each assay one liter of red or white wine free of fining process was used.

2.10.2 Elimination of protein fining agents via ultrafiltration

In addition to analyzing the influence of ultrafiltration over some wine characteristics (colour, turbidity and hue), its ability of removing casein and ovalbumin remains was also studied. One liter of each wine was clarified with casein (100 g hL^{-1}) and ovalbumin (10 g

hL^{-1}) and then filtered. The corresponding untreated wines were used as a control in each assay. Analysis of protein residues was carried out by UHPLC-ESI-MS/MS according to Pavón et al (Pavón, Henriquez, & Aranda, 2019). Briefly, 12.5 mL of wine were centrifuged at 5433 x *g* for 40 min at 20 ºC into 10 kDa cut-off membrane ultrafiltration tube (Amicon Merck Millipore), previously conditioned with 10 mL of distilled water. 2.5 mL were obtained from which proteins were precipitated on ice for 2 h after addition of eight volumes of ethanol: TCA (15%w/v), pH 4.5. Thereafter, the mixture was centrifuged at 8981 x *g* for 10 min at 4ºC using a Hettich (Tuttlingen, Germany) refrigerated centrifuge. Ethanol: TCA residues were evaporated under a gentle flow of nitrogen without heat. The obtained pellet was solubilized in 1 mL of 50 mM NH_4HCO_3, pH 7.8. Trypsin digestion of both, sample and standards, was performed after proteins reduction and alkylation. The first was carried out by addition of 12 µL of 100 mM DTT and incubation at 95ºC for 5 min. After cooling down, the cysteine SH-groups, formed during the reduction step, were irreversibly alkylated adding 24 µL of freshly prepared aqueous solution of 100 mM IAM, the mixture was left stand for 15 min at room temperature protected from light. Enzymatic digestion was performed adding 4 µL of trypsin solution (0.5 mg mL^{-1}, in 50 mM of NH_4HCO_3, pH 7.8) for obtaining a trypsin: protein ratio of 1:10, the mixture was incubated at 37ºC for 3 min in ultrasound bath. The reaction was stopped adding 10 µL of formic acid (0.1%v/v) aqueous solution. All digested samples and standards were filtered through a 13mm PVDF syringe filter (0.22 µm) before LC analysis.

2.10.3 Liquid chromatography – tandem mass spectrometry

For LC-MS analyzes a Shimadzu (Kyoto, Japan) Nexera X2 UHPLC system consisted of: LC-30AD pump, DGU-20A5R degassing unit, SIL-30AC autosampler, CTO-20AC column oven, CBM-20A communication module, SPD-M20A diode array detector (DAD) and LCMS-8030 triple quadrupole (TQ) mass spectrometer. The system was controlled by the LabSolution 5.8 software. Peptides separation was carried out on Phenomenex (Torrance, CA, USA) Kinetex XB Core-Shell C_{18} column (100 mm x 4.6 mm, id. 2.6 µm), thermostated at 35ºC, using a mobile phase composed of ultrapure pure water (A) and acetonitrile (B) both with 0.1 % (v/v) formic acid. The following gradient program was applied at a flow rate of 0.6 mL min^{-1}: 0–19 min 10–40% B, 19–20 min 40–10% B,

followed by 5 min for column conditioning. MS analysis was performed in ESI positive mode using the following conditions: ESI voltage 4.5 kV; collision energy -30.0 V for casein and -40.0 V to ovalbumin; nebulizer gas (N_2) 3 L min^{-1}, desolvation gas (N_2) 18 L min^{-1}; desolvation line temperature 250ºC and heat block temperature 400ºC. Full scan spectra were acquired from *m/z* 100 to 1000. In previous works in our laboratory following the early describe criteria, the most abundant and stable fragment from the product ion spectrum were chosen for each peptide (protein). Thus, the transitions established for identification were *m/z* 634.6→991.8 for α-casein and *m/z* 929.5→1116.5 for ovalbumin.

3. Results and discussion

3.1 Turbidity, colour and hue of wines

As can be seen in Table 2 all fining treatments decreased wine turbidity, the extension of this reduction was dependent of the type and fining agent dosage, these results are concordant with previous reports (Cosme et al., 2008; Maury et al., 2018; Oberholster, Carstens, & du Toit, 2013). Among fining agents, bentonite had the highest impact on turbidity and red wine colour (Figure 1), it was observed a reduction from 80.5% to 93.9% and from 76.6% to 80.1%, respectively. Turbidity decrease was doses dependant, while for colour change was not observed a significant difference among bentonite doses. Simultaneous with colour intensity decrease a hue increase was observed (0.87 to 0.94). These results are similar to reported by González-Neves et al (Gustavo González-Neves et al., 2014). In agree with Stankovic et al (Stankovic, Jovic, Zivkovic, & Pavlovic, 2012) report, bentonite produces a significantly ($p<0.05$) decrease of anthocyanin content, which are the molecules responsible of wine colour (Table 2). This effect could be explained by electrostatic interaction with all compounds carrying a positive net charge at wine pH. Therefore, in addition to proteins adsorption, bentonite also removes other positively charged molecules as anthocyanins (Gustavo González-Neves et al., 2014) (Pocock, Salazar, & Waters, 2011).

Table 2. The chromatic parameters (CI, turbidity and hue), monomeric anthocyanins, total polyphenol index and antioxidant activity of control and treated red wines[a].

			CI[b]	Turbidity[c]	Hue[d]	Total monomeric anthocyanins[e]	Total polyphenols[f]	Antioxidant capacity (mMol)[g]
Red wine	Bentonite	TBA	0.98 ± 0.01 b	0.16 ± 0.01 b	0.87 ± 0.00 b	40.60 ± 0.90 b	216.50 ± 53.41 a	39.77 ± 1.66 a b
		TBB	0.92 ± 0.02 b	0.08 ± 0.01 c	0.76 ± 0.08 c	40.50 ± 2.82 b	201.70± 12.52 a	22.30 ± 0.45 b c
		TBC	0.83 ± 0.01 c	0.05 ± 0.00 c	0.94 ± 0.02 b	39.70 ± 2.93 b	193.90 ± 1.71 a	14.50 ± 0.36 c
	Diatomaceous earth	TTA	3.64 ± 0.64 d	0.24 ± 0.01 d	1.14 ± 0.14 d	90.00 ± 2.62 c e	87.50± 6.79 b	50.60± 5.52 a
		TTB	3.67 ± 0.65 d e	0.21 ± 0.01 d	1.14 ± 0.14 d	74.60 ± 2.57 d	82.30 ± 2.47 b	38.90 ± 6.86 a
		TTC	3.80 ± 0.64 e	0.39 ± 0.00 e	1.18 ± 0.19 d	47.40 ± 6.68 b	78.40 ± 2.64 b	13.80 ± 4.13 d
	Casein	TCA	4.27 ± 0.68 a	0.60 ± 0.03 f	1.33 ± 0.36 a	119.70 ± 3.12 a	92.40 ± 3.91 b	32.00 ± 7.78 b
		TCB	4.17 ± 0.69 a f	0.53 ± 0.00 g	1.32 ± 0.34 a	113.90 ± 0.04 a	82.40 ± 0.29 b	27.80 ± 4.56 b
		TCC	4.04 ± 0.70 f g	0.50 ± 0.04 h	1.31 ± 0.33 a	87.70 ± 1.40 c	65.90 ± 2.88 b	19.70 ± 6.66 b
	Ovalbumin	TOA	4.49 ± 0.32 h	0.76 ± 0.01 i	1.29 ± 0.32 a f	99.70 ± 7.79 e	100.90 ± 9.23 b	22.60 ± 1.71 b
		TOB	3.96 ± 0.23 g	0.53 ± 0.03 g	1.21 ± 0.22 d f	68.40 ± 8.69 d	68.80 ± 6.15 b	20.80 ± 0.75 b
		TOC	3.74 ± 0.03 e	0.48 ± 0.04 h	1.02 ± 0.03 e	37.90 ± 5.00 b	41.60 ± 11.4 b	10.00 ± 5.17 b
	Control		4.18 ± 0.01 a, f	0.82 ± 0.04 a	1.33 ± 0.36 a	113.60 ± 1.40 a	221.10 ± 89.03 a	55.70 ± 29.1 a

[a] Average of the three measurements ± standard deviation (SD). Different letters within the same column indicate statistical differences according to Tukey HSD test ($p < 0.05$).
[b] CI, color intensity as sum of absorbances at 420, 520, and 620 nm
[c] Turbidity as absorbance at 650 nm
[d] Hue, A420/A520
[e] mg L^{-1}, cyanidin-3-glucoside equivalents
[f] Total polyphenols in mg L^{-1} of gallic acid.
[g] mM Trolox equivalent

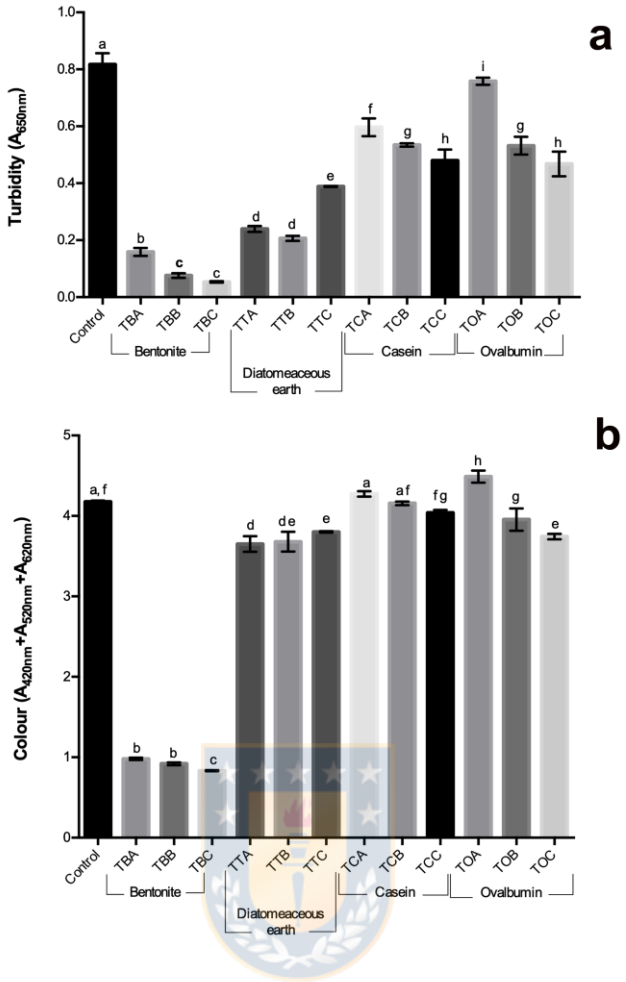

Figure 1. Turbidity (a) and colour (b) after treatment of red wine with fining agents. Control= wine without fining treatment; TBA= fining with bentonite at 50 g hL^{-1}; TBB= fining with bentonite at 100 g hL^{-1}; TBC= fining with bentonite at 150 g hL^{-1}; TTA= fining with diatomaceous earth at 25 g hL^{-1}; TTB= fining with diatomaceous earth at 50 g hL^{-1}; TTC= fining with diatomaceous earth at 100 g hL^{-1}; TCA= fining with casein at 30 g hL^{-1}; TCB= fining with casein at 65 g hL^{-1};TCC= fining with casein at 100 g hL^{-1}; TOA= fining with ovalbumin at 8 g hL^{-1}; TOB= fining with ovalbumin at 9 g hL^{-1}; TOC= fining with ovalbumin at 10 g hL^{-1}. Values are means ± SD (n = 3). Different letters represent means significantly different at $p < 0.05$

The second fining agent with the greatest impact on colour and turbidity was diatomaceous earth, followed by casein and finally ovalbumin. Protein fining agents had the same behavior, with the highest dose used, the reduction of both parameters, was higher. However, with diatomeaceous earth an opposite result was observed, the reduction of turbidity and colour was greater at the lowest dose 70.7% to 52.4% and 12.9% to 10.0%

respectively. This result may be due to the higher dose of this fining it may have been interference in the absorbance measurement.

Table 3 shows the results for turbidity and colour in white wine. All fining treatments significantly ($p<0.05$) decreased the turbidity and colour with respect to control except bentonite at the lowest dose (50 g hL^{-1}). White wine colour (expressed as the absorbance at 420 nm) had the biggest reduction with casein (31.0%), similar results were reported by Cosme et al (33.0%) (Cosme et al., 2008). This is could be related with total polyphenols decrease since casein was the fining agent that most reduced this kind of compounds (table 3). Cosme et al explained associated this colour reduction with casein action on flavonoid levels. Turbidity was measured at 650 nm following the report of Feulliat et al (Feuillat & Bergeret, 1966), however, at this wavelength the absorbance observed was quite small (table 3), which may introduced some errors in the measurements.

Table 3. The chromatic parameters (CI, turbidity and hue), total polyphenol index and antioxidant activity of control and treated white wines[a].

			CI[b]	Turbidity[c]	Total Polyphenols[d]	Antioxidant capacity (μM)[e]
White wine	Bentonite	TBA	0.19 ± 0.00 b	0.07 ± 0.01 a	112.80 ± 3.66 b d	1020.30 ± 34.02 a b c
		TBB	0.15 ± 0.01 c	0.05 ± 0.03 b	102.60 ± 3.59 b c	821.80 ± 35.30 a b c
		TBC	0.10 ± 0.00 d	0.04 ± 0.00 c	99.90 ± 9.12 c e	854.10 ± 135.10 a b
	Diatomaceous earth	TTA	0.09 ± 0.00 d e	0.04 ±0.00 c	114.60 ± 9.27 d	955.40 ± 1.85 c d e
		TTB	0.09 ± 0.00 d	0.04 ± 0.00 c	102.60 ± 4.73 c e	737.30 ± 19.40 f g
		TTC	0.09 ± 0.01 d e	0.04 ± 0.01 c	99.90 ± 5.23 e f	336.90 ± 236.20 d f e
	Casein	TCA	0.07 ± 0.00 e f	0.05 ± 0.01 d	58.50 ± 7.21 g	534.30 ± 12.22 g h
		TCB	0.06 ± 0.00 f	0.04 ± 0.00 d e	55.50 ± 8.98 g	307.10 ± 13.24 h
		TCC	0.06 ± 0.00 f	0.04 ± 0.02 d	51.60 ± 1.22 g	100.10 ± 2.85 e i
	Ovalbumin	TOA	0.18 ± 0.01 b	0.04 ± 0.00 e	117.20 ± 5.60 a d	569.00 ± 37.63 f g i
		TOB	0.09 ± 0.00 d	0.05 ± 0.03 b	108.10 ± 5.77 b c d	511.60 ± 12.61 f g i
		TOC	0.10 ± 0.00 d e	0.03 ± 0.00 c	82.80 ± 8.37 f	412.60 ± 13.49 f g i
	Control		0.27± 0.04 a	0.06 ± 0.01 a	123.70 ± 4.49 a	1078.50 ± 5.82 a

[a] Average of the three measurements ± standard deviation (SD). Different letters within the same column indicate statistical differences according to Tukey HSD test ($p < 0.05$).
[b] CI, color intensity as absorbance at 420 nm
[c] Turbidity as absorbance at 650 nm
[d] Total polyphenols in mg L^{-1} of gallic acid.
[e] μM Trolox equivalent

3.2 Effect of the fining agents on monomeric antochyanins, total polyphenols and antioxidant capacity

The highest removal level of monomeric antochyanins in red wine was observed with bentonite treatment (64.6%), followed by ovalbumin (31.3%), diatomaceous earth (29.3%) and casein (5.71%). The remaining amounts of monomeric anthocyanins present (35.0%),

were consistent with those reported by González-Neves et al (34.7%) at bentonite dose of 50 g hL^{-1} (G. González-Neves & Gil, 1998). Fining with bentonite significantly diminished the anthocyanin contents of red wine (table 2) which is related to the impact that bentonite causes in colour intensity decrease. This effect was not dose dependent since no statistical differences were observed between the different doses assayed ($p>0.05$). Anthocyanins content were slightly decreased using diatomaceous earth, ovalbumin and casein at the highest dose (100 g hL^{-1}). These results are concordant with the effect observed on colour. Total polyphenol content of red wines was generally diminished by fining agents, except with bentonite, which did not show a significantly affect over polyphenol concentration. Ovalbumin had the most important effect decreasing total polyphenols level between 54.4% and 18.8% (Table 2), this effect could be explained by the interaction of protein fining agents with wines tannins. In fact, this kind of fining agents are mostly employed by its ability to complex tannins. Several authors have reported a correlation between antioxidant activity and total polyphenol content (Di Majo, La Guardia, Giammanco, La Neve, & Giammanco, 2008; Ghanem et al., 2017). This relation was also observed with the use of fining agents, antioxidant activity was higher in wine treated with bentonite (lower removal of polyphenols). Bentonite (150 g hL^{-1}) and diatomaceous earth (100 g hL^{-1}) had no statistically different ($p > 0.05$) which contrasted with egg albumin and casein. Treatment with egg albumin and casein significantly decreased the antioxidant capacity (82.0% - 60.0% and 64.1% - 43.0% respectively) compared with untreated wine. As indicated above, this effect is associated with the interaction with polyphenols. Levels of antioxidant capacity expressed as mM Trolox equivalents after fining are in agree with those reported by Bridi et al (Bridi, Lobato, López-Alarcón, & Lissi, 2014) for Chilean wines.

All treatments decreased the total polyphenols content in white wine, except diatomaceous earth and ovalbumin (at high concentrations). In white wines casein had the most important effect regarding polyphenol levels reduction (52.7%-58.3%), without significant difference between doses (Table 3). Casein is composed of hydrophobic and hydrophilic amino acids, resulting in an amphiphilic character, thus, micelle-forming properties (Webber-Witt et al., 2015). The fining effects of this protein are attributed to this bipolar behavior. Particularly, the hydrophobic regions can interact with phenolic compounds, and significantly reduces the total polymeric phenolic content (Webber-Witt et al., 2015). ORAC results were

consistent with those obtained for total polyphenols, the lowest values of antioxidant capacity were observed with casein (Table 3, figure 2). Ovalbumin was the second fining agent with high impact on antioxidant capacity reduction (47.2% - 61.8%). These results are similar to the ones observed in red wine assays, corroborating that protein fining agents are the most important agents for polyphenolic compounds reduction.

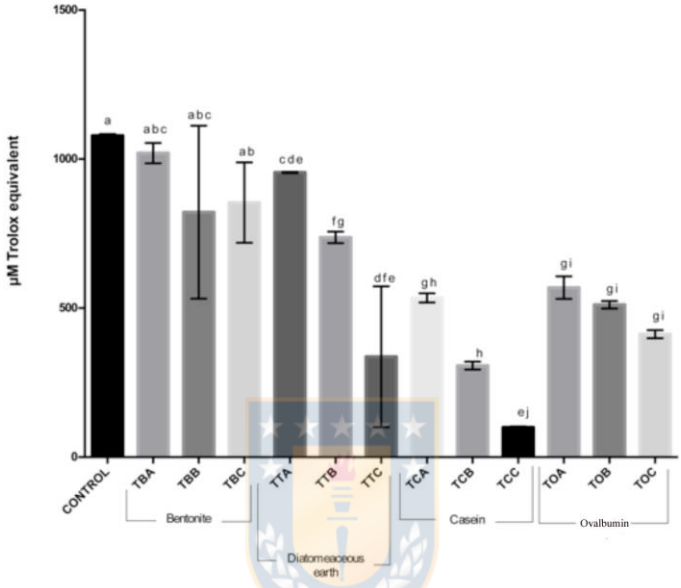

Figure 2. Variation of antioxidant capacity expressed as µM of TROLOX equivalents, in relation to their content in control wine after treatment of white wine with fining agents. Control= untreated wine; TBA= fining with bentonite at 50 g hL^{-1}; TBB= fining with bentonite at 100 g hL^{-1}; TBC= fining with bentonite at 150 g hL^{-1}; TTA= fining with diatomaceous earth at 25 g hL^{-1}; TTB= fining with diatomaceous earth at 50 g hL^{-1}; TTC= fining with diatomaceous earth at 100 g hL^{-1}; TCA= fining with casein at 30 g hL^{-1}; TCB= fining with casein at 65 g hL^{-1};TCC= fining with casein at 100 g hL^{-1}; TOA= fining with ovalbumin at 8 g hL^{-1}; TOB= fining with ovalbumin at 9 g hL^{-1}; TOC= fining with ovalbumin at 10 g hL^{-1}. Values are means ± SD (n = 3). Different letters represent means significantly different at $p< 0.05$

3.3 Anthocyanins and polyphenols composition in wine by HPLC/Uv/Vis

In agree with previous reports (Ghanem et al., 2017), malvidin-3-glucoside was the major anthocyanin present in untreated wine followed by delphinidin-3-glucoside and cyanidin-3-glucoside. Among fining agents, bentonite had the highest impact on the individual anthocyanins content in wines. Its concentration had an important impact on the decrease of

anthocyanins levels. The additions of bentonite affected more significantly the amounts of anthocyanins based on delphinidin and malvidin, all treatments decreased the cyanidin content, although the effect was significantly the decrease was not so great. Of the three anthocyanins studied delphinidin was the most retained by the bentonite. In agree with the reported by González- Neves et al (Gustavo González-Neves et al., 2014), who stated that bentonite was more related to the more polar anthocyanins. No statistical differences were observed between the different doses, according to a Tukey test ($p > 0.05$). The anthocyanins concentration is show in Table 4, ovalbumin was the second fining agents to reduce the delphinidin (32.9% - 71.1%) and malvidin (61.9% - 72.2%) contens. Diatomaceous earth and casein at their smaller doses do not have a statistically significant difference ($p > 0.05$) in delphinidin content with the control wine. At higher doses they show statistical differences, however they do not achieve the same decrease as bentonite or ovalbumin. These fining agents decreased the malvidin content significantly but not in an important way. Figure 3 shows the variations in delphinidin and malvidin contents of the clarified wines in relation to their content in the respective control wines.

Table 4. Monomeric individual anthocyanins of control and treated red wines[a].

			Delphinidin -3 glc (mg L^{-1})	Cyanidin -3-glc (mg L^{-1})	Malvidin -3-glc (mg L^{-1})
Red wine	Bentonite	TBA	1.52 ± 0.03 b	2.61 ± 0.00 b	36.90 ± 0.08 b
		TBB	1.05 ± 0.01 d	2.43 ± 0.00 c d	26.90 ± 0.13 c
		TBC	1.00 ± 0.01 d	2.04 ± 0.02 d e f	17.20 ± 0.00 d
	Diatomaceous earth	TTA	3.97 ± 0.19 a	2.44 ± 0.06 c	75.10 ± 2.33 e
		TTB	2.45 ± 0.04 e f	2.15 ± 0.01 e	46.60 ± 0.44 f
		TTC	1.37 ± 0.04 c	1.92 ± 0.00 f	27.30 ± 1.06 c
	Casein	TCA	4.04 ± 0.04 a	2.52 ± 0.08 b c	85.60± 1.46 g
		TCB	3.61 ± 0.03g	2.38 ± 0.01 c	66.40 ± 0.65 h
		TCC	2.47 ± 0.03 e f	2.17 ± 0.00 e	46.70 ± 0.87 f
	Ovalbumin	TOA	2.67 ± 0.05 e	2.56 ± 0.01 b d	55.50 ± 1.00 i
		TOB	2.20 ± 0.01 f	2.18 ± 0.19 e	45.90 ± 0.46 f
		TOC	1.15 ± 0.06 d c	1.96 ± 0.00 f	26.40 ± 1.01 c
	Control		3.98 ± 0.06 a	3.37 ± 0.00 a	96.90 ± 0.00 a

[a] Average of the three measurements ± standard deviation (SD). Different letters within the same column indicate statistical differences according to Tukey HSD test ($p < 0.05$).

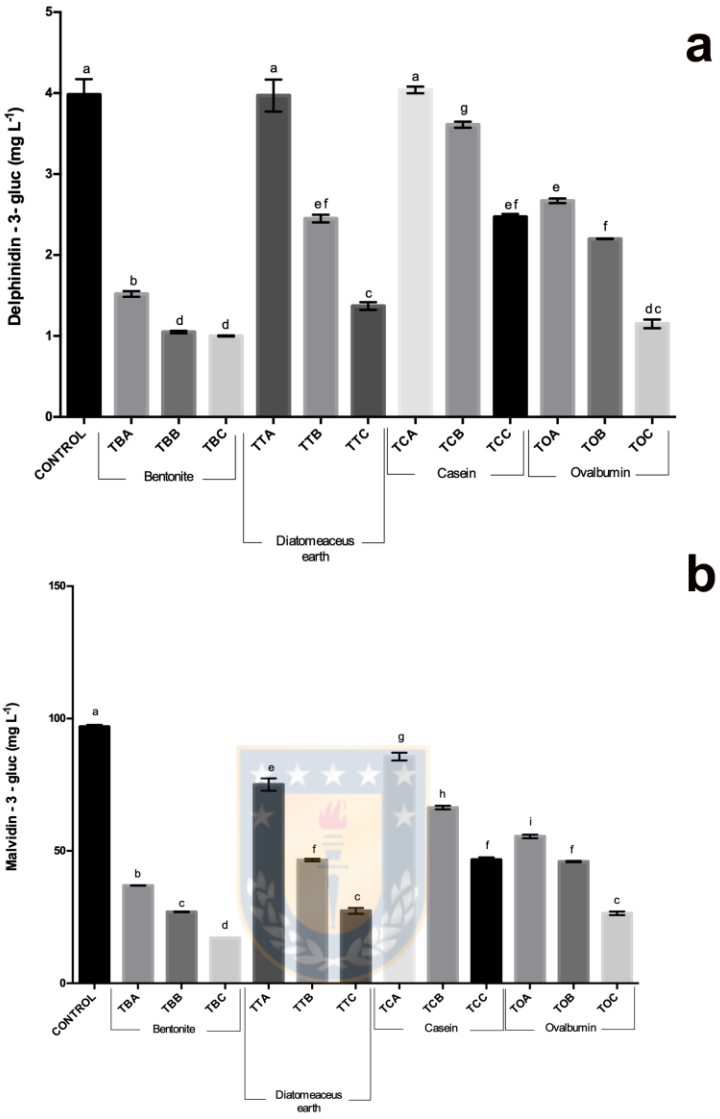

Figure 3. Variation (mg L^{-1}) in delphinidin (A) and malvidin (B) contents of the clarified red wine in relation to their content in control wines after fining. Control= unfinned wine; TBA= fining with bentonite at 50 g hL^{-1}; TBB= fining with bentonite at 100 g hL^{-1}; TBC= fining with bentonite at 150 g hL^{-1}; TTA= fining with diatomaceous earth at 25 g hL^{-1}; TTB= fining with diatomaceous earth at 50 g hL^{-1}; TTC= fining with diatomaceous earth at 100 g hL^{-1}; TCA= fining with casein at 30 g hL^{-1}; TCB= fining with casein at 65 g hL^{-1}; TCC= fining with casein at 100 g hL^{-1}; TOA= fining with ovalbumin at 8 g hL^{-1}; TOB= fining with ovalbumin at 9 g hL^{-1}; TOC= fining with ovalbumin at 10 g hL^{-1}. Values are means ± SD (n = 3). Different letters represent means significantly different at $p < 0.05$

As can be seen on table 5, polyphenol content in red wines was generally diminished by fining agents. Concentrations of gallic acid, caffeic acid and resveratrol in untreated wine, were similar at those reported by Ghanem et al (Ghanem et al., 2017). Casein and ovalbumin had the highest impact on the individual polyphenols content. Benzoic acid, p-coumaric acid, myricetin, resveratrol, quercetin and kaempferol were the main polyphenols removed by casein and ovalbumin. Even in some cases at the highest doses they removed it below quantification levels. This result can be explained by polyphenols to proteins binding affinity, which increases with their molecular size (Ozdal, Capanoglu, & Altay, 2013). Gallic acid was the principal polyphenol removed by all fining agents, reaching a removal level of 96.0% at casein dose of 100 g hL^{-1}. Benzoic acid, quercetin and kaempferol were the polyphenols quantified in white wine. Benzoic acid was little affected by the fining agents and kaempferol was the principal polyphenol removed mainly by bentonite and diatomaceous earth (Table 6).

3.4 Effect of cross-flow filtration under wine fining with casein and ovalbumin.

Filtration does not guarantee the physico-chemical stabilization of wine by itself, which is why it is used in combination with fining agents. Nowadays, enologists enhance the stabilization and limpidity of crude wines by using technologies such as centrifugation, dead-end filtration and of fining agents (Martinez-Lapuente et al., 2017). Industry recognized that best practice filtration methods should be used to remove insoluble protein fining agents (Webber-Witt et al., 2015).

White and red wines submitted to cross-flow ultrafiltration alone or combined with casein and ovalbumin treatment showed a color intensity decrease of 37.6% and 86.0%, respectively (figure 4). In the case of red wine this decrease in colour intensity was accompanied by an increase of hue values. In red wines membrane filtration reduced turbidity in 16.3% while its combination with fining agents produced a reduction of 83.7%. A similar behavior was observed on white wines, filtration reduced wine turbidity in 92.7% and the combination of both techniques produced a reduction of 98.2%. Thus, both techniques were able to reduce significantly wine turbidity ($p < 0.05$), however, its jointly use had a more pronounced effect on wine turbidity reduction.

Table 5. Individual polyphenols of control and treated red wines[a]

			Gallic acid (mg L^{-1})	Caffeic acid (mg L^{-1})	Vanillic acid (mg L^{-1})	p-coumaric acid (mg L^{-1})	Benzoic acid (mg L^{-1})	Myricetin (mg L^{-1})	Resveratrol (mg L^{-1})	Quercetin (mg L^{-1})	Kaempferol (mg L^{-1})
Red wine	Bentonite	TBA	6.76 ± 0.56 b	7.23 ± 0.15 b	8.57 ± 1.41 b	8.54 ± 0.22 a	8.05 ± 0.49 a	4.21 ± 0.22 b d	8.51 ± 0.51 a b	7.20 ± 1.25 b	3.53 ± 0.57 b
		TBB	5.12 ± 0.00 c e	6.43 ± 0.07 c	5.21 ± 0.03 b c d	7.86 ± 0.06 a	7.70 ± 0.01 a	1.13 ± 0.00 c	7. 56 ± 0.01 b	4.14 ± 0.04 c	2.61 ± 0.00 c
		TBC	3.30 ± 0.13 d	4.91 ± 0.02 d	4.71 ± 0.16 c	6.66 ± 0.34 b	6.24 ± 0.26 b	1.09 ± 0.06 c	6.33 ± 0.35 c	2.93 ± 0.01 c	1.06 ± 0.04 d
	Diatomaceous earth	TTA	9.29 ± 0.04 f	7.91 ± 0.01 a	8.36 ± 0.08 b	8.32 ± 0.16 a	5.04 ± 0.09 c	7.04 ± 0.74 a	7.52 ± 0.20 b e	7.92 ±0.87 b d	1.98 ± 0.10 c e
		TTB	9.34 ± 0.25 f	7.51 ± 0.76 a b	8.34 ± 0.25 b	6.91 ± 1.11 b	4.79 ± 0.46 c	6.28 ± 1.31 a e	6.46 ±1.35 c e	9.49 ± 2.59 d	1.41 ± 3.13 d e
		TTC	8.78 ± 0.10 f	6.36 ± 0.16 c	3.21 ± 0.07 d c e	4.77 ± 0.06 c	4.86 ± 0.01 c	5.62 ± 0.48 e f	4.28 ± 0.11 d	4.13 ± 0.19 c	1.64 ± 0.13 d e
	Casein	TCA	8.61 ± 0.09 f	2.54 ± 0.29 e g	8.33 ± 0.02 b	1.38 ± 0.13 d	1.05 ± 0.08 d	4.64 ± 0.06 b f	Detected	Detected	Detected
		TCB	3.02 ± 0.22 d g	3.43 ± 0.04 f	6.00 ± 0.72 b c	1.76 ± 0.03 d	1.25 ± 0.00 d	2.25 ± 0.03 c g	Detected	Detected	Detected
		TCC	1.71 ± 0.12 g	2.12 ± 0.00 e	0.99 ± 0.09 e	Detected	Detected	2.00 ± 0.07 c g	Detected	Detected	Detected
	Ovalbumin	TOA	9.35 ± 0.14 f	2.26 ± 0.08 e	6.54 ± 0.07 b c	Detected	Detected	2.93 ± 0.51 d g	Detected	Detected	Detected
		TOB	5.77 ± 0.02 b e	2.30 ± 0.11 e	4.79 ± 0.09 c	Detected	Detected	Detected	Detected	Detected	Detected
		TOC	3.17 ± 0.11 d	3.01 ± 0.03 g f	3.55 ± 0.16 c e	Detected	Detected	Detected	Detected	Detected	Detected
	Control		42.80 ± 0.07 a	7.71 ± 0.00 a	12.60 ± 0.02a	8.46 ± 0.07 a	8.14 ± 0.08 a	6.92 ± 0.10 a	8.57 ± 0.00 a	12.60 ± 0.02 a	4.21 ± 0.00 a

[a] Average of the three measurements ± standard deviation (SD). Different letters within the same column indicate statistical differences according to Tukey HSD test ($p < 0.05$). Detected: < 1 mg L^{-1}

Table 6. Individual polyphenols of control and treated white wines[a]

			Benzoic acid (mg L^{-1})	Quercetin (mg L^{-1})	Kaempferol (mg L^{-1})
White wine	Bentonite	TBA	1.99 ± 0.16 b	1.00 ± 0.01 b	1.09 ± 0.01
		TBB	1.24 ± 0.01 c	Detected	Detected
		TBC	Detected	Detected	Detected
	Diatomaceous earth	TTA	1.77 ± 0.05 b	1.12 ± 2.31 c	Detected
		TTB	1.16 ± 0.07 c	Detected	Detected
		TTC	Detected	Detected	Detected
	Casein	TCA	2.04 ± 0.01 b d	1.30 ± 0.01 d	1.17 ± 0.00
		TCB	1.39 ± 0.02 c	1.08 ± 0.01 e	Detected
		TCC	Detected	Detected	Detected
	Ovalbumin	TOA	5.13 ± 0.07 a	1.26 ± 0.00 f	1.44 ± 0.00
		TOB	3.86 ± 0.08 e	Detected	1.30 ± 0.00
		TOC	2.38 ± 0.08 d	Detected	1.18 ± 0.04
	Control		5.34 ± 0.00 a	1.23 ± 0.00 a	1.35 ± 0.00

[a] Average of the three measurements ± standard deviation (SD). Different letters within the same column indicate statistical differences according to Tukey HSD test ($p < 0.05$). Detected: < 1 mg L^{-1}

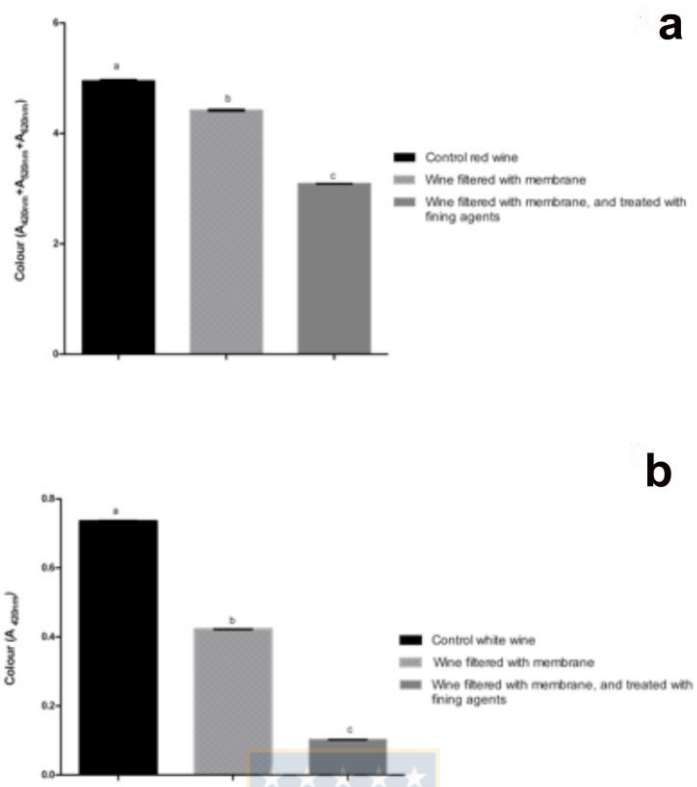

Figure 4. Colour of red (a) and white (b) wine after treatment with casein and ovalbumin and cross-flow ultrafiltration. Control= unfinned wine; TBA= fining with bentonite at 50 g hL^{-1}; TBB= fining with bentonite at 100 g hL^{-1}; TBC= fining with bentonite at 150 g hL^{-1}; TTA= fining with diatomaceous earth at 25 g hL^{-1}; TTB= fining with diatomaceous earth at 50 g hL^{-1}; TTC= fining with diatomaceous earth at 100 g hL^{-1}; TCA= fining with casein at 30 g hL^{-1}; TCB= fining with casein at 65 g hL^{-1};TCC= fining with casein at 100 g hL^{-1}; TOA= fining with ovalbumin at 8 g hL^{-1}; TOB= fining with ovalbumin at 9 g hL^{-1}; TOC= fining with ovalbumin at 10 g hL^{-1}. Values are means ± SD (n = 3). Different letters represent means significantly different at $p < 0.05$

Casein and ovalbumin may trigger allergic reactions in susceptible individuals, therefore, their occurrence in wines could become a human health risk, moreover when their presence is not reported. Thus, their complete elimination after wine fining is essential to avoid food safety issues. In this sense it was studied the capacity of cross-flow ultrafiltration membrane to eliminate casein and ovalbumin residues. Applying tandem mass spectrometry, it was evaluated the presence of this kind of molecules after ultrafiltration, for that, first wine fined with casein and ovalbumin but without ultrafiltration was analyzed by tandem mass spectrometry in multiple reaction monitoring (MRM) mode in order to

detect marker peptides resulted from tryptic digestion of casein (YLGYLEQLLR-GYLEQLLR *m/z* 634.6→991.8 and ovalbumin (ELINSWVESQTNGIIR-VESQTNGIIR *m/z* 929.5→1116.5) (Figure 5). Then, the same wine samples were submitted to ultrafiltration process and the analuzed by MRM mass spectrometry. As can be observed in Figure 6, the presence of both marker peptides was not detected, proving the capability of cross-flow ultrafiltration of removing both proteins residues. The main advantage of cross-flow filtration is it capacity of removing allergen-related proteins without the need of adding other substance that may affect wine quality.

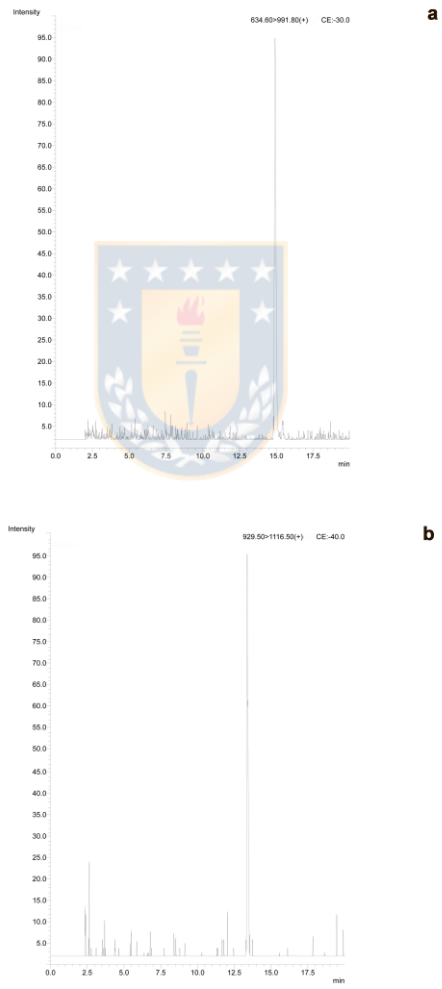

Figure 5. LC-ESI-MS/MS chromatograms of white (a) and red (b) wine samples fining with casein and ovalbumin respectively. Analyzed in MRM mode for α-casein (a), and ovalbumin (b) detection

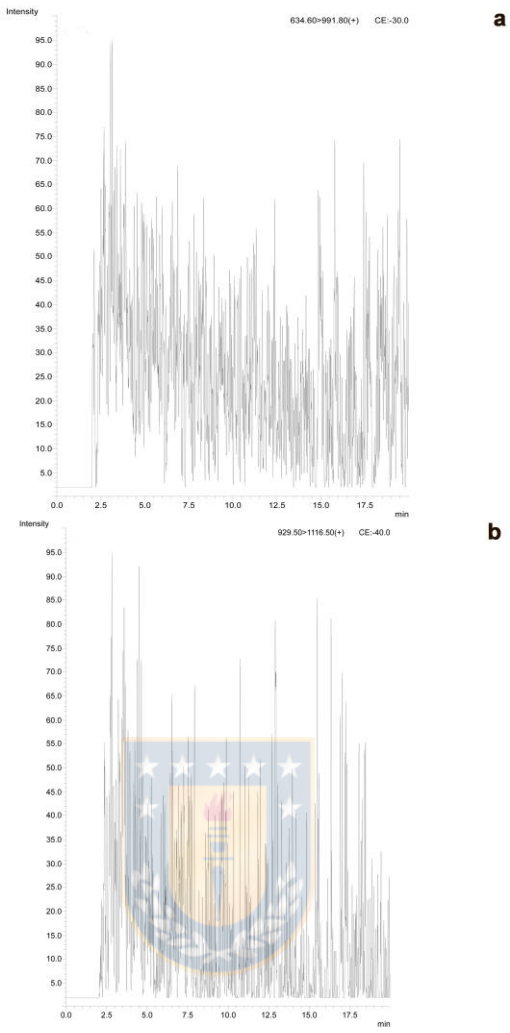

Figure 6. LC-ESI-MS/MS chromatograms of white (a) and red (b) wine samples fining with casein and ovalbumin followed by cross-flow ultrafiltration respectively. Analyzed in MRM mode for α-casein (a), and ovalbumin (b) detection

4. Conclusions

All treatments improved wine limpidity, and showed different effects over polyphenolic composition, colour and wine turbidity. In red wine the most remarkable effect was observed on anthocyanins composition with bentonite addition, which reduced considerably the colour intensity. In white wines the most relevant effect over colour intensity and wine turbidity was produced applying casein. Polyphenol content was not affected in the same magnitude by all fining agentsdier decreased significantly in all wines clarified by protein fining agents, casein in white and ovalbumin in red wines. Cross-flow ultrafiltration

together with casein and ovalbumin produced a higher reduction on colour intensity and wine turbidity than both techniques separately. Therefore, the use of 10kDa ultrafiltration membrane after casein and ovalbumin treatment fully accomplish the fining goals in terms of wine stability and food safety because simultaneously remove allergen-related proteins residues.

Acknowledgments

This work is part of Jessy Pavón Pérez thesis to obtain the degree of Doctor in Science and Analytical Technology from the University of Concepcion, Chile. Authors want to thank to the National Commission of Scientific and Technological Research (CONICYT) of the Chilean Government for the doctoral scholarship granted. This work was financially supported by National Fund for Scientific & Technological Development (FONDECYT), projects N°1171857, FONDEQUIP 130209 and University of Concepcion.

References

Bridi, R., Lobato, S., López-Alarcón, C., & Lissi, E. (2014). Influence of the growing region, quality classification and harvest year on the ORAC (Oxygen Radical Absorbance Capacity) index of Chilean Cabernet Sauvignon red wines. *Ciencia e investigación agraria*, *41*, 395-402.

Cosme, F., Ricardo-da-Silva, J. M., & Laureano, O. (2008). Interactions between protein fining agents and proanthocyanidins in white wine. *Food Chemistry*, *106*(2), 536-544. doi: https://doi.org/10.1016/j.foodchem.2007.06.038

Di Majo, D., La Guardia, M., Giammanco, S., La Neve, L., & Giammanco, M. (2008). The antioxidant capacity of red wine in relationship with its polyphenolic constituents. *Food Chemistry*, *111*(1), 45-49. doi: https://doi.org/10.1016/j.foodchem.2008.03.037

El Rayess, Y., Manon, Y., Jitariouk, N., Albasi, C., Mietton-Peuchot, M., Devatine, A., & Fillaudeau, L. (2016). Wine clarification with Rotating and Vibrating Filtration (RVF): Investigation of the impact of membrane material, wine composition and operating conditions. *Journal of Membrane Science, vol. 513*, pp. 47-57. doi: 10.1016/j.memsci.2016.03.058

Feuillat, M., & Bergeret, J. (1966). Determination de la limpidite des mouts et vins. In I. J. Blouin (Ed.), *Manuel Pratique d'Analyse des Mouts et des Vins.* Gironde.

Ghanem, C., Taillandier, P., Rizk, M., Rizk, Z., Nehme, N., Souchard, J. P., & El Rayess, Y. (2017). Analysis of the impact of fining agents types, oenological tannins and mannoproteins and their concentrations on the phenolic composition of red wine. *LWT - Food Science and Technology*, *83*, 101-109. doi: https://doi.org/10.1016/j.lwt.2017.05.009

González-Neves, G., Favre, G., & Gil, G. (2014). Effect of fining on the colour and pigment composition of young red wines. *Food Chemistry*, *157*, 385-392. doi: https://doi.org/10.1016/j.foodchem.2014.02.062

González-Neves, G., & Gil, G. (1998). Modificaciones del color y la composición fenólica de un vino tinto tannat por efecto de diversos tratamientos de clarificación. *Panorama Vitivinícola*, *5*(1), 34-37.

Huertas, F. J., Carretero, P., Delgado, J., Linares, J., & Samper, J. (2001). An Experimental Study on the Ion-Exchange Behavior of the Smectite of Cabo de Gata (Almeria, Spain): FEBEX Bentonite. *J Colloid Interface Sci*, *239*(2), 409-416. doi: 10.1006/jcis.2001.7605

Jaeckels, N., Tenzer, S., Meier, M., Will, F., Dietrich, H., Decker, H., & Fronk, P. (2017). Influence of bentonite fining on protein composition in wine. *LWT*, *75*, 335-343. doi: https://doi.org/10.1016/j.lwt.2016.08.062

Karamanidou, A., Kallithraka, S., & Hatzidimitriou, E. (2011). Fining of red wines: effects on their analytical and sensory parameters. *Journal international des sciences de la vigne et du vin 45*(1).

Lambert, M., Meudec, E., Verbaere, A., Mazerolles, G., Wirth, J., Masson, G., . . . Sommerer, N. (2015). A High-Throughput UHPLC-QqQ-MS Method for Polyphenol Profiling in Rose Wines. *Molecules*, *20*(5), 7890-7914. doi: 10.3390/molecules20057890

Martinez-Lapuente, L., Guadalupe, Z., & Ayestaran, B. (2017). Effect of egg albumin fining, progressive clarification and cross-flow microfiltration on the polysaccharide and proanthocyanidin composition of red varietal wines. *Food Research International*, *96*, 235-243. doi: 10.1016/j.foodres.2017.03.022

Maury, C., Sarni-manchado, P., & Cheynier, V. (2018). Highlighting protein fining residues in a model red wine. *Food Chemistry*. doi: https://doi.org/10.1016/j.foodchem.2018.11.141

Oberholster, A., Carstens, L. M., & du Toit, W. J. (2013). Investigation of the effect of gelatine, egg albumin and cross-flow microfiltration on the phenolic composition of Pinotage wine. *Food Chemistry*, *138*(2), 1275-1281. doi: https://doi.org/10.1016/j.foodchem.2012.09.128

HPLC-Determination of nine major anthocyanins in red and rosé wine. (2007).

. Organisation Internationale de la Vigne et du Vin. Compendium of International Methods of Analysis–OIV. Chromatic Characteristics. (2009) Retrieved December 17 2018, from http://www.oiv.int/public/medias/2475/oiv-ma-as2-07b.pdf

Ortiz, R., Antilen, M., Speisky, H., Aliaga, M. E., Lopez-Alarcon, C., & Baugh, S. (2012). Application of a microplate-based ORAC-pyrogallol red assay for the estimation ofantioxidant capacity: First Action 2012.03. *J AOAC Int*, *95*(6), 1558-1561.

Ozdal, T., Capanoglu, E., & Altay, F. (2013). A review on protein–phenolic interactions and associated changes. *Food Research International*, *51*(2), 954-970. doi: https://doi.org/10.1016/j.foodres.2013.02.009

Pandolfi P. (2008). *Aplicación de la tecnología de membranas en el proceso de vinificación.* University of Concepcion, Concepcion, Chile.

Patil, S., Kaur, M., & Sharma, H. K. (2012). Effect of incorporation of mahua extract, fining agent and ageing on the quality characteristics of red wine. *Indian journal of microbiology*, *52*(3), 406-410. doi: 10.1007/s12088-012-0249-z

Pavón, J., Henriquez, K., & Aranda, M. (2019). Mass Spectrometry Determination of Fining-Related Allergen Proteins in Chilean Wines. *Food Analytical Methods*. doi: https://doi.org/10.1007/s12161-018-01416-0

Pocock, K. F., Salazar, F. N., & Waters, E. J. (2011). The effect of bentonite fining at different stages of white winemaking on protein stability. *Australian Journal of Grape and Wine Research*, *17*(2), 280-284. doi: doi:10.1111/j.1755-0238.2011.00123.x

Rice, F. L., Park, R., Stayner, L., Smith, R., Gilbert, S., & Checkoway, H. (2001). Crystalline silica exposure and lung cancer mortality in diatomaceous earth industry workers: a quantitative risk assessment. *Occup Environ Med*, *58*(1), 38-45.

Socaciu, C. (2008). *Food Colorants.* Boca Raton: CRC Press.

Speisky, H., Lopez-Alarcon, C., Gomez, M., Fuentes, J., & Sandoval-Acuna, C. (2012). First web-based database on total phenolics and oxygen radical absorbance capacity (ORAC) of fruits produced and consumed within the south Andes region of South America. *J Agric Food Chem*, *60*(36), 8851-8859. doi: 10.1021/jf205167k

Stankovic, S., Jovic, S., Zivkovic, J., & Pavlovic, R. (2012). Influence Of Age On Red Wine Colour During Fining With Bentonite And Gelatin. *International Journal of Food Properties*, *15*(2), 326-335. doi: 10.1080/10942912.2010.487625

Tschiersch, C., Pour Nikfardjam, M., Schmidt, O., & Schwack, W. (2010). *Degree of hydrolysis of some vegetable proteins used as fining agents and its influence on polyphenol removal from red wine* (Vol. 231).

Ulbricht, M., Ansorge, W., Danielzik, I., König, M., & Schuster, O. (2009). Fouling in microfiltration of wine: The influence of the membrane polymer on adsorption of polyphenols and polysaccharides. *Separation and Purification Technology*, *68*(3), 335-342. doi: https://doi.org/10.1016/j.seppur.2009.06.004

Van Sluyter, S. C., McRae, J. M., Falconer, R. J., Smith, P. A., Bacic, A., Waters, E. J., & Marangon, M. (2015). Wine protein haze: mechanisms of formation and advances in prevention. *J Agric Food Chem*, *63*(16), 4020-4030. doi: 10.1021/acs.jafc.5b00047

Vincenzi, S., Panighel, A., Gazzola, D., Flamini, R., & Curioni, A. (2015). Study of Combined Effect of Proteins and Bentonite Fining on the Wine Aroma Loss. *Journal of Agricultural and Food Chemistry*, *63*(8), 2314-2320. doi: 10.1021/jf505657h

Webber-Witt, M., Deckwart, M., Carstens, C., Eichhorn, L., Brockow, K., Schaefer, V., . . . Christmann, M. (2015). Effect of Various Treatments and Filtration Methods on Eliminating Residues of Casein-based Fining Agents from Wine. *American Journal of Enology and Viticulture*, *66*(3), 388. doi: 10.5344/ajev.2015.14122

CAPITULO III: Conclusiones finales

Conclusiones

El trabajo de investigación de esta tesis doctoral tuvo como base el desarrollo de metodologías químico analíticas para analizar la presencia de proteínas alérgenicas en vino chilenos. Considerando los resultados descritos anteriormente es posible concluir lo siguiente:

1. Una metodología extractiva de caseína y ovoalbúmina desde vino fue desarrollada con el empleo de membranas de ultracentrifugación de 10kDa y precipitación con una mezcla de ácido tricloroacético y etanol.

2. La optimización de la digestión convencional con tripsina mediante un Diseño Central Compuesto, permitió aumentar el rendimiento de la digestión y disminuir el tiempo de digestión en un 42%. Lo cual junto con los cortos tiempos de separación cromatográfica resultó en una importante disminución de los tiempos de análisis para caseína y ovoalbúmina en vinos.

3. Se desarrolló un método UHPLC-ESI-QQQ-MS/MS que permitió la identificación y cuantificación de péptidos marcadores de caseína y ovoalbúmina en 20 muestras comerciales de vinos chilenos, con condiciones optimas del voltaje de la fuente de ionización y la energía de la celda de colisión para cada proteína, lo cual en conjunto con herramientas bioinformáticas de búsquedas en bases de datos, permitieron identificar péptidos marcadores para la α-caseína, la β-caseína y la ovoalbúmina. El método fue validado según las recomendaciones de la *ICH* lo que permitió detectar y cuantificar los péptidos en modo MRM en niveles de μg L^{-1} lo que es 60 veces menor al límite establecido por la OIV. Bajo estas condiciones 20 muestras fueron analizadas, de las cuales el 90 % presentó valores cuantificables de estas proteínas y una presentó concentraciones superiores a 0.25 mg L^{-1}.

4. Se implementaron tres métodos de digestión enzimática rápidos de caseína y ovoalbúmina con tripsina desde la matriz vino empleando energía proveniente del IR, del microondas y con ultrasonido. La optimización de las condiciones de digestión con el empleo de técnicas quimiométricas de diseño de experimentos permitió reducir los tiempos de digestión de 420 min (7 h) a 3 minutos para microondas y ultrasonido y a 15 minutos para IR. La evaluación de cada método a partir del análisis por SDS-PAGE arrojó la aparición de varias bandas correspondientes a los péptidos obtenidos con microondas y

ultrasonido sin comprometer el % de SQ arrojado del análisis con la base de datos Mascot, comportamiento que no fue posible replicar con energía del IR, principalmente debido a la imposibilidad de lograr un estricto control de la temperatura. Teniendo en cuenta la disponibilidad de equipamiento en el laboratorio, el método implementado para la digestión final de las proteínas fue el uso de ultrasonido.

5. Una metodología HPTLC-ESI-MS complementaria para el análisis de los péptidos fue desarrollada e implementada. La asociación de esta metodología con las herramientas bioinformáticas permitió separar e identificar de manera rápida un péptido marcador de la β-caseína en vino chileno variedad *Carménère* y en vino chileno variedad *Cabernet Sauvignon* un péptido de ovoalbúmina, presentándose como una promisoria metodología analítica.

6. El empleo de un péptido marcado isotópicamente como estándar interno permitió la identificación y cuantificación preliminar de las proteínas en 60 muestras de vinos chilenos comerciales mediante el monitoreo de los péptidos marcadores anteriormente establecidos a través de UHPLC-ESI-QQQ-MS/MS. El método fue igualmente validado encontrándose en 17 muestras de las analizadas concentraciones de caseína y ovoalbúmina superiores al límite establecido por la OIV. De esa manera la metodología demostró ser adecuada para la determinación de contenido alergénico en vinos.

7. La compracion entre dos espectrómetros de masas permitió establecer la complementaridad de ambos. El uso de un espectrómetro de masa híbrido de alta resolución Q-TOF acoplado a UHPLC permitió la identificación exacta de un mayor número de péptidos marcadores para ambas proteínas en vinos. Incluyendo la identificación de un péptido marcador de κ-caseína lo cual no se pudo lograr con el empleo de un QQQ. Sin embargo, los LOD y LOQ obtenidos con el QQQ fueron alrededor de 10 veces menores que los obtenidos con el Q-TOF. Lo cual evidencia la factibilidad de emplear un espectrómetro de masa de alta resolución para hacer una identificación exhaustiva de manera dirigida y no dirigida de los posibles péptidos marcadores y el uso de un QQQ para lograr una cuantificación exacta de los mismos.

8. La determinación de caseína y ovoalbúmina en vinos chilenos se desarrolló por ELISA, considerando que este es el método oficial aceptado por la OIV. No fue posible detectar o cuantificar estas proteínas en vinos chilenos variedad *Sauvignon Blanc* y *Cabernet*

Sauvignon con concentraciones de caseína y ovoalbúmina de 0.20 mg L^{-1} y 0.21 mg L^{-1} cuantificadas anteriormente mediante UHPLC-ESI-QQQ-MS/MS.

9. Tras el estudio en profundidad de varias estrategias para disminuir o evitar el uso de agentes clarificantes alergénicos se determinó que la bentonita afecta más significativamente el color y los niveles de antocianinas en vino, mientras que la caseína y ovoalbúmina el contenido de polifenoles. El uso de una membrana de ultracentrifugación de 10kDa de tamaño de poro y en flujo cruzado fue capaz de eliminar los restos de caseína y ovoalbúmina. El uso conjunto de agentes clarificantes proteicos y de métodos físicos de clarificación como las membranas permite obtener un vino con mejores características visuales como color y turbidez. El empleo de las membranas en este caso contribuye igualmente a eliminar los restos de posibles proteínas alergénicas dañinas para aquellos individuos susceptibles.