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Implementación de una plataforma analítica HPTLC-bioensayo-MS de efecto dirigido para la identificación de compuestos bioactivos en chirimoya (*Annona cherimola* Mill.)

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

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TABLAS DE CONTENIDO

ÍNDICE

ÍNDICE DE FIGURAS ..... **viii**

ÍNDICE DE TABLAS ..... **xi**

RESUMEN ..... **xii**

CAPÍTULO I: *Introducción y objetivos* **1**

1. INTRODUCCIÓN **2**

1.1 Compuestos bioactivos y alimentos funcionales 2

1.1.1 Fuente de compuestos bioactivos 3

1.1.2 Experiencia Chilena de compuestos bioactivos y alimentos funcionales 4

1.2 Chirimoya 6

1.2.1 Aspectos comerciales y agrícolas 6

1.2.2 Composición química y antecedentes de bioactividad 7

1.2.2.1 Actividad antioxidante 8

1.2.2.2 Otras actividades biológicas 9

1.2.2.3 Estudios sobre otras especies de *Annona* 11

1.3 Técnicas analíticas para la evaluación de bioactividad e identificación de compuestos bioactivos 12

1.3.1 Cromatografía acoplada a bioensayos 14

1.3.2 Aplicaciones de análisis de efecto dirigido mediante HPTLC-bioensayo 16

1.3.2.1 HPTLC-bioensayo para compuestos antioxidantes 17

1.3.2.2 HPTLC-bioensayo para la detección de inhibidores enzimáticos 19

1.3.3 Identificación y elucidación estructural en HPTLC-bioensayo 20

1.3.4 HPTLC acoplado a espectrometría de masas (HPTLC-MS) 22

1.4 Metodologías extractivas de compuestos bioactivos 25

1.4.1 Extracción asistida por ultrasonido (UAE) 26

1.4.2 Extracción mediante fluidos supercríticos (SFE) 26

1.4.3 Extracción mediante líquidos presurizados (PLE) 27

Referencias 28

2. HIPÓTESIS Y OBJETIVOS **34**

2.1 Planteamiento del problema 34

2.2 Hipótesis 35

2.3 Objetivos 35

2.3.1 Objetivo general 35
2.3.2 Objetivos específicos 35
3. ESTRATEGIA ANALÍTICA 36
3.1 Desarrollo de una metodología HPTLC-bioensayo-MS para inhibidores de acetilcolinesterasa (AChE) y α-glucosidasa. 36
3.2 Evaluación de una metodología HPTLC-DPPH-MS para la identificación de compuestos antioxidantes y cuantificación de la actividad antioxidante. 37
3.3 Extracción de compuestos bioactivos mediante fluidos supercríticos. 37
CAPÍTULO II: *Resultados y Discusión* 38
1. DETECTION AND IDENTIFICATION OF ACETYLCHOLINESTERASE INHIBITORS IN *Annona cherimola* Mill. BY EFFECT-DIRECTED ANALYSIS USING THIN-LAYER CHROMATOGRAPHY-BIOASSAY-MASS SPECTROMETRY 39
ABSTRACT 41
1. INTRODUCTION 42
2. MATERIAL AND METHODS 43
2.1 Reagents and solvents 43
2.2 Sample preparation 43
2.3 Chromatography 44
2.4 HPTLC-AChE bioassay 44
2.5 Chemical identification of AChE inhibitors 44
2.6 Mass spectrometry identification of AChE inhibitors 45
3. RESULTS AND DISCUSSION 46
3.1 HPTLC-bioassay 46
3.2 Evaluation of AChE inhibitory activity of cherimoya extracts 46
3.3 Identification of AChE inhibitors in cherimoya peel 48
ACKNOWLEDGMENTS 53
REFERENCES 54
2. AN IMPROVED METHOD FOR A FAST SCREENING OF α-GLUCOSIDASE INHIBITORS IN CHERIMOYA FRUIT (*Annona cherimola* Mill.) APPLYING EFFECT-DIRECTED ANALYSIS VIA HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY-BIOASSAY-MASS SPECTROMETRY 56
Abstract 58
1. Introduction 59
2. Materials and methods 60

2.1 Reagents, chemical and solvents 60
2.2 Sample preparation 60
2.3 Chromatography 61
2.4 EDA-HPTLC-α-glucosidase bioassay 61
2.5 Chemical identification of AGIs 62
2.6 Mass spectrometry identification of AGIs 62
3. Results and discussion 63
3.1 Development of HPTLC-bioassay method 63
3.2 Evaluation of α-glucosidase inhibitory activity of cherimoya extracts 64
3.3 Identification of α-glucosidase inhibitor compounds in cherimoya 65
4. Conclusions 71
Acknowledgements 71
References 72
3. EVALUATION OF THREE ANALYTICAL METHODOLOGIES FOR AN *IN SITU* QUANTIFICATION OF THE ANTIOXIDANT CAPACITY OF CHERIMOYA EXTRACTS USING A HPTLC- DPPH ASSAY 75
ABSTRACT 76
1. Introduction 77
2. Materials and methods 78
2.1 Reagents, chemical and solvents 78
2.2 Sample preparation 78
2.3 Chromatography 79
2.4 HPTLC-DPPH assay 79
2.5 Digital image processing 80
2.6 Mass spectrometry identification of antioxidant compound 80
3. Results and discussion 81
3.1 Evaluation methods of the antioxidant capacity in HPTLC-DPPH assay 81
3.2 Mass spectrometry identification of antioxidant compounds 85
4. Conclusions 87
References 89
4. SUPERCRITICAL FLUID EXTRACTS FROM CHERIMOYA BY-PRODUCTS AS A PROMISING SOURCE OF BIOACTIVE ALKALOIDS 91
ABSTRACT 93
1. Introduction 94

2. Material and methods 95
2.1 Materials and reagents 95
2.2 Samples and pre-treatment 96
2.3 Supercritical fluid extraction procedure 97
2.4 Total alkaloids content 98
2.5 Experimental design 98
2.6 Determination of antioxidant activity 99
2.7 Determination of total phenolic content 99
2.8 Acetylcholinesterase (AChE) inhibition assay 100
2.9 α-glucosidase inhibition assay 100
2.10 UHPLC-ESI-MS analysis 101
3. Results and discussion 101
3.1. Evaluation of sample pre-treatment 101
3.2 Optimization of SFE conditions 102
3.2.1 Co-solvent evaluation 102
3.2.2 Experimental design 103
3.3 Chemical and functional characterization of extracts 106
3.3.1 Alkaloids content 106
3.3.2 Antioxidant Capacity 106
3.3.3 AChE inhibitory activity 108
3.3.4 α-glucosidase inhibitory activity 108
3.3.5 Preliminary quantification of bioactive alkaloids 110
4. Conclusions 112
Acknowledgements 113
References 114

CAPÍTULO IV: *Conclusiones finales* **118**

ÍNDICE DE FIGURAS

CAPÍTULO I: ***Introducción y objetivos***

1. INTRODUCCIÓN

Figura 1. Esquema que ejemplifica las etapas de un aislamiento de compuestos bioactivos guiado por bioensayos. (Adaptado de: Montalvão et al. (2014)). 13

Figura 2. Esquema general de un análisis HPTLC-bioensayo de efecto dirigido. (Adaptado de: Weiss et al. (2017)). 16

Figura 3. Resumen de los principales HPTLC-bioensayos, agrupados como ensayos microquímicos, bioquímicos y biológicos. (Adaptado de: Morlock (2018)). 18

Figura 4. Flujo de trabajo que puede ser aplicado para obtener una identificación y caracterización de las moléculas bioactivas (Adaptado de: Yüce and Morlock (2016)). 21

Figura 5. Interfaz TLC-MS basada en elución. (A) flujo del solvente en la cabeza de elución. (B) Placa cromatográfica luego de la elución mediante una cabeza de elución circular y oval. (C) Equipo TLC-MS Interface CAMAG® disponible comercialmente. (Adaptado de: Morlock and Schwack (2010a)). 23

Figura 6. Análisis de compuestos químicos sobre la superficie de una placa TLC mediante (A) ELDI-MS, (B) DESI-MS y (C) DART-MS. (Adaptado de Cheng and Shiea (2015)). 24

3. ESTRATEGIA ANALITICA

Figura 1. Esquema resumen de la estrategia analítica aplicada. 36

CAPITULO II: ***Resultados y Duscusión***

1. DETECTION AND IDENTIFICATION OF ACETYLCHOLINESTERASE INHIBITORS IN *Annona cherimola* Mill. BY EFFECT-DIRECTED ANALYSIS USING THIN-LAYER CHROMATOGRAPHY-BIOASSAY-MASS SPECTROMETRY

FIGURE 1. HPTLC chromatograms of methanolic and DCM extracts from cherimoya peel on silica gel 60 F_{254} plates using a mobile phase composed of chloroform-methanol-ethyl acetate (80:14:6 v/v/v). HPTLC-AChE bioassay photo-documented under white light of DCM (A) and methanolic extract (B); photo-documentation at 366 nm-fluorescence (C) and 254 nm-UV (D); selected bands marked with soft pencil for

elution to MS via TLC/MS interface (E) and ESI-MS spectra of selected bands (F and G). 48
FIGURE 2. Chemical structures of AChEi found in cherimoya peel. 49
FIGURE 3. ESI-MS/MS spectra of AChEi found in cherimoya peel. Product ions of anonaine *m/z* 266 $[M-H]^+$ (A); xylopine *m/z* 296 $[M-H]^+$ (B); and glaucine *m/z* 356 $[M-H]^+$ (C). 51
FIGURE 4. UHPLC/DAD chromatograms and UV spectra of inhibitory compounds extracted from HPTLC plate: anonaine and xylopine (A) and glaucine (B). 52
2. AN IMPROVED METHOD FOR FAST SCREENING OF α-GLUCOSIDASE INHIBITORS IN CHERIMOYA FRUIT (*Annona cherimola* Mill.) BY EFFECT-DIRECTED ANALYSIS-HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY-BIOASSAY-MASS SPECTROMETRY
Fig. 1. HPTLC chromatograms of peel and cherimoya seed extracts on silica gel F_{254} plates. Detection of AGIs via HPTLC-bioassay photo-documented under white light, 254 nm-UV and 366 nm-fluorescence and HPTLC-ESI-MS mass spectra of selected bands. 66
Fig. 2. Chemical structure of proposed phenolamides as AGIs. 67
Fig. 3. UHPLC-DAD-ESI-MS/MS analysis of AGIs: N-*trans* feruloyl tyramine (AGI-P2 and AGI-S1), found in cherimoya peel and seeds. ESI-MS/MS spectrum in positive mode at -40V of parent ion *m/z* 314 $[M-H]^+$ (A); and UHPLC/DAD chromatogram and UV spectra of AGI N-*trans* and *cis*-feruloyl tyramine (B). 68
Fig. 4. UHPLC-DAD-ESI-MS/MS analysis of AGI: N-*trans*-feruloyl phenethylamine (AGI-P1) found in cherimoya peel. ESI-MS/MS spectra in positive mode at -40V of parent ion *m/z* 298 $[M-H]^+$ (A); and UHPLC/DAD chromatogram and UV spectrum of N-*trans*-feruloyl phenethylamine (B). 69
Fig. 5. UHPLC-DAD-ESI-MS/MS analysis of AGI: N-*trans*-*p*-coumaroyl tyramine (AGI-S2) found in cherimoya seeds. ESI-MS/MS spectra in positive mode at -40V of parent ion *m/z* 284 $[M-H]^+$ (A); and UHPLC/DAD chromatogram and UV spectra of AGI N-*trans* and *cis*-*p*-coumaroyl tyramine (B). 70
3. EVALUATION OF THREE ANALYTICAL METHODOLOGIES FOR AN IN SITU QUANTIFICATION OF THE ANTIOXIDANT CAPACITY OF CHERIMOYA EXTRACTS USING A HPTLC- DPPH ASSAY.

Figure 1. Chromatograms of the calibration curve, diluted peel extract (1), peel extract (2) and seed extract (3); under UV light at 360 nm before to the derivatization (A), DPPH assay (B) and after of image processing (C). 82
Figure 2. The results obtained from HPTLC-DPPH assay to quantify the antioxidant activity using image processing with ImageJ program (A), absorbance mode densitometry (*normal scan*) + image processing (B), and fluorescence mode densitometry (*inverse scan*) (C)..... 83

4. SUPERCRITICAL FLUID EXTRACTS FROM CHERIMOYA BY-PRODUCTS AS A PROMISING SOURCE OF BIOACTIVE ALKALOIDS

Fig. 1. Schematic diagram of the supercritical extraction system. 97
Fig. 2. Total alkaloid content and global yield of cherimoya peel extracts obtained by different extraction conditions. (A) Comparison between freeze drying and heat drying using ultrasound assisted extraction. (B) Comparison between different extraction condition using heat drying and SFE. 102
Fig. 3. (A) Standardized Pareto chart for the total alkaloids content showing the effects of factors on the response. (B) Response surface plot for the response optimization showing the effect of temperature and pressure with a co-solvent percentage constant at 15%. 105
Fig. 4. Representative UHPLC-ESI-MS chromatogram with selected-ion monitoring (SIM) from the quantitative determination of alkaloids in peel extract. 111

ÍNDICE DE TABLAS

CAPÍTULO I: ***Introducción y objetivos***

1. INTRODUCCIÓN

Tabla 1. Resumen de la capacidad antioxidante en chirimoya determinada por diferentes metodologías. 10

Tabla 2. Resumen de ensayos para la determinación de compuestos con actividad antioxidante. 10

CAPÍTULO II: ***Resultados y Discusión***

3. EVALUATION OF THREE ANALYTICAL METHODOLOGIES FOR AN IN SITU QUANTIFICATION OF THE ANTIOXIDANT CAPACITY OF CHERIMOYA EXTRACTS USING A HPTLC- DPPH ASSAY.

Table 1. Calibration curves of the quantification methods applied for HPTLC-DPPH assay. 84

Table 2. Scavenging activity against DPPH radical (μg CAE*/g sample) of peel and seed cherimoya extract..... 85

Table 3. Molecular mass (as m/z values) founded in each band with antioxidant activity and proposed compounds 87

4. SUPERCRITICAL FLUID EXTRACTS FROM CHERIMOYA BY-PRODUCTS AS A PROMISING SOURCE OF BIOACTIVE ALKALOIDS

Table 1. Experimental runs for a central composite design with the corresponding responses. 104

Table 2. Evaluation of alkaloids content, total phenols and in vitro bioactivity[a] of extracts obtained by an optimized SFE process of different varieties of peel and leaf cherimoya. 109

Table 3. Alkaloids content in leaves and peel cherimoya extracts obtained by SFE and quantification by UHPLC-DAD-ESI-MS with SIM mode[a]. 112

RESUMEN

El chirimoyo/a (*Annona cherimola* Mill.) es un árbol pequeño cultivado en diferentes países incluido Chile. Este árbol produce un fruto conocido como chirimoya que ha sido largamente utilizado en medicina tradicional. Algunas actividades biológicas beneficiosas para la salud humana han sido demostradas mediante ensayos *in vitro*, sin embargo son pocos los estudios que individualizan los compuestos que ejercen la bioactividad. El estudio de los componentes bioactivos de la chirimoya permitiría descubrir moléculas con potencial terapéutico para enfermedades crónicas no transmisibles, otorgando de esta manera un valor agregado a la fruta y subproductos generados. Para la determinación de compuestos bioactivos fue desarrollada e implementada una plataforma analítica HPTLC-bioensayo-MS de efecto dirigido que permite la determinación e identificación *in situ* de compuestos bioactivos mediante un flujo de trabajo simple y directo, que permite un *screening* general de la composición de piel, pulpa y semilla de chirimoya. El uso de esta herramienta analítica, permitió detectar en la piel de chirimoya tres compuestos con actividad inhibitoria sobre la enzima acetilcolinesterasa, que luego fueron analizados mediante HPTLC-MS y UHPLC-DAD-MS/MS. Los compuestos fueron identificados como anonaina, glaucina, y un tercer candidato como xilopina, siendo por primera vez reportada la capacidad inhibitoria de estos alcaloides. Aplicando el mismo sistema, tres fenolamidas presentes en piel y semilla fueron detectadas e identificadas como inhibidores de α-glucosidasa, las cuales corresponden a *N-trans*-feruloil tiramina, *N-trans-p*-coumaroil tiramina y *N-trans*-feruloil fenetilamina, siendo primera vez que se reporta esta bioactividad así como la presencia de este último en chirimoya. Los compuestos antioxidantes presentes en extracto de piel y semilla fueron detectados vía ensayo HPTLC-DPPH, y la capacidad antioxidante fue cuantificada a partir del mismo ensayo mediante procesamiento de imagen digital usando el software ImageJ. Los compuestos que presentaron una mayor actividad, expresada como equivalentes de ácido cafeico, fueron estudiados directamente mediante un análisis TLC-MS y clasificados preliminarmente como como acetogeninas y fenolamidas. De esta manera, el ensayo HPTLC-DPPH-MS resultó en una metodología completa que permite detectar, cuantificar e identificar compuestos antioxidantes, estableciendo la aplicabilidad del procesamiento de imagen digital sobre otras metodologías HPTLC-bioensayo.

El trabajo realizado logró establecer también un extracto de piel de chirimoya con destacada actividad inhibitoria de AChE, α-glucosada y capacidad antioxidante, obtenido mediante

una metodología optimizada de extracción por fluidos supercríticos. La presencia de algunos de los compuestos, caracterizados en la plataforma analítica, fue cuantificada mediante un análisis UHPLC-ESI-MS. Adicionalmente, hojas de diferentes variedades de chirimoya fueron evaluados con la misma metodología, estableciendo su potencial como fuente de alcaloides inhibidores de AChE. En resumen, la aplicación de la plataforma analítica permitió evaluar el potencial de sub-productos de la agroindustrial, piel y semilla, como fuente de compuestos bioactivos con potencial terapéutico.

CAPÍTULO I: *Introducción y objetivos*

1. INTRODUCCIÓN

1.1 Compuestos bioactivos y alimentos funcionales

Los fitoquímicos comprenden una familia de compuestos de amplia variedad estructural e importante potencial como compuestos bioactivos, por lo que han sido empleados extensamente en la industria farmacéutica, nutracéutica y cosmética (Yahya et al. 2018). En la naturaleza los fitoquímicos están presentes como mezclas, siendo por tanto de una composición heterogénea. Actualmente, importantes propiedades biológicas han sido adscritas a este grupo de compuestos, dentro de las cuales destacan actividad antioxidante, anticancerígena, antimicrobiana, antiproliferativa, cardio- y neuroprotectora, hipoglicemiante entre otras (Neri-Numa et al. 2018). De esta manera, los compuestos bioactivos se han convertido en sustancias relevantes para la salud humana, y el estudio de sus propiedades podría ser de utilidad para conservar un buen estado de salud o en el tratamiento de enfermedades. Cuando los compuestos bioactivos están contenidos de manera natural en frutas y vegetales, pueden ser consumidos de manera directa, mientras que su extracción y aislamiento permitiría adicionarlos en diferentes productos alimenticios o desarrollar nutracéuticos. De esta forma, dependiendo de su presentación y forma de consumo, es posible establecer las categorías de alimentos funcionales, ingredientes funcionales y nutracéuticos.

Bajo estos antecedentes, el estudio de compuestos bioactivos y desarrollo de alimentos funcionales tiene como pilar fundamental, aunque no exclusivo, disminuir el riesgo de padecer enfermedades crónicas no transmisibles (ECNT). Se estima que estas enfermedades son responsables de un 71.0% del total de muertes a nivel mundial, equivalente a 41.0 millones de muertes por año, siendo las enfermedades cardiovasculares las que más aportan (17.9 millones cada año), seguidas del cáncer, enfermedades respiratorios y diabetes tipo 2 (Organización Mundial de Salud 2018). En Chile es posible observar la misma tendencia, las ECNT son la principal causa de muerte, entre éstas las enfermedades cardiovasculares (ECV) y los tumores malignos dan cuenta de más de la mitad de las muertes con un 27.1% y 25.8% respectivamente en el año 2011, siendo catalogadas por el Ministerio de Salud de Chile (2015) como el mayor problema de salud en Chile. Actualmente se estimada que el 51.5% de la población tiene un riesgo cardiovascular moderado-alto, un 40.1% síndrome metabólico, 27.6% hipertensión arterial y 12.3% diabetes mellitus (Ministerio de Salud de Chile 2018).

1.1.1 Fuente de compuestos bioactivos

Las fuentes naturales de compuestos bioactivos las constituyen las plantas, animales, microrganismos y organismos marinos de diversos origines geográficos (Alamgir 2017). De esta manera, la naturaleza se ha convertido en una importante fuente de agentes bioactivos, y se estima que más de un tercio de las nuevas moléculas terapéuticas aprobadas por la FDA (Food and Drug Administration) son de origen natural, y de éstas un cuarto provienen de plantas. En la actualidad la fracción total de productos naturales ha disminuido, sin embargo, los derivados de productos naturales semi-sintéticos han aumentado (Patridge et al. 2016). Dentro de las fuentes naturales, las plantas poseen un alto contenido en compuestos bioactivos, y han sido utilizadas por décadas para desarrollar preparaciones cosmecéuticas y con fines medicinales. De este modo, la planta entera o parte de ella, así como secreciones y exudados, han sido consideradas una importante fuente de agentes terapéuticos como por ejemplo opioides, efedrina, digoxina, etc. (Alamgir 2017; Yahya et al. 2018).

Una importante parte de la planta son los frutos, que suelen consumirse por su aporte nutricional, y además son considerados una buena fuente de compuestos bioactivos como flavonoides, ácidos fenólicos, caratenoides fitoestrógenos y estilbenos, que promueven un buen estado de salud. El consumo de frutas y verduras, dado los compuestos funcionales que presenta, disminuye el riesgo de padecer algunas enfermedades/condiciones como hipertensión, obesidad, enfermedad coronaria y riesgo de accidente cerebrovascular, cáncer en general, enfermedades oculares, asma y osteoporosis. Se estima que se han identificado más de 5000 fitoquímicos individuales en frutas, verduras y granos, pero un gran porcentaje de ellos sigue sin ser identificados (Yalcin and Çapar 2017).

En la actualidad, muchas variedades naturales de frutas y verduras cultivadas como parte de la agricultura, han sido estudiadas para una mejor comprensión de sus componentes valiosos desde el punto de vista nutricional y funcional, lo cual ha llevado a una revitalización de su cultivo. En este proceso se ha requerido de tecnología e innovación, además de incorporar la ciencia, la tecnología y la nutrición. Además del consumo directo de fruta, la detección, identificación y extracción de compuestos bioactivos a partir sus de diferentes secciones, podría permitir el desarrollo de alimentos e ingredientes funcionales, así como nutracéuticos, o su estudio como moléculas plantillas para el desarrollo de fármacos. En este aspecto los desechos y subproductos agro-industriales se han convertido en interesantes fuentes de estas moléculas, los cuales se pueden emplear para generar nuevos productos con valor agregado. Es sabido que la industria de procesamiento de

frutas es una de las que más genera subproductos y desechos, en parte por el aumento de la producción y el consumo de frutas a nivel mundial (Lai et al. 2017). Particularmente los subproductos provenientes del procesamiento de las frutas son ricos en polifenoles, proteínas, vitaminas, alcaloides, ácidos grasos esenciales, compuestos con actividad antimicrobiana etc. (Balasundram et al. 2006; Lai et al. 2017). Por ejemplo, la industria de procesamiento de pomelos, limones, naranjas, mandarinas y otros cítricos, producen grandes cantidades de subproductos y desechos en forma de semillas, cáscara y pulpa, todo lo cual puede representar hasta un 50.0% de la fruta cruda procesada (Silvan and Martinez-Rodriguez 2019). Estudios demuestran que la cáscara y semillas de los cítricos son ricos en polifenoles y flavonoides, lo cual ha fomentado por ejemplo el estudio de la capacidad antimicrobiana de estos compuestos (Gorinstein et al. 2001). Los subproductos y desechos frutas tropicales o exóticas también han demostrado ser una fuente de compuestos bioactivos. La piel de mango destaca por su alto poder antioxidante y su contenido de ácido gálico y quercetina (6.0 y 4.5 mg por gramo de muestra fresca respectivamente). Así también altos valores de ambos compuestos fueron encontrados en semillas (37.3 mg de ácido gálico y 36.0 mg de quercetina por gramo de muestra fresca) (Ayala-Zavala et al. 2011). De este modo, la creciente cantidad de residuos producidos por la industria alimentaria hace necesario crear nuevas formas de reciclaje, desarrollando nuevas tecnologías para el tratamiento de residuos. Así también desarrollar métodos de extracción eficientes que permitan emplear el potencial de los subproductos alimenticios como fuente de compuestos bioactivos.

1.1.2 Experiencia Chilena de compuestos bioactivos y alimentos funcionales

A nivel mundial los consumidores están cada vez más conscientes de su autocuidado y buscan en el mercado alimentario productos que contribuyan a su salud y bienestar. A pesar que no hay una clara categorización comercial de los alimentos funcionales, sí se puede establecer que las empresas que los producen presentan una acelerada expansión mundial. La situación en Chile respecto a estos alimentos muestra signos de un exitoso mercado, un claro ejemplo es el caso de los berries nativos. De acuerdo a lo informado en el proyecto “Productos agroindustriales ricos en antioxidantes a base de berries nativos”, de la Fundación para la Innovación Agraria (FIA) (2009) del Ministerio de Agricultura, la importancia de estas frutas ha aumentado principalmente por los efectos beneficiosos que han demostrado y que ayudarían a reducir el riesgo de enfermedades, lo que se ha traducido en una mayor demanda y consumo, satisfaciendo las nuevas necesidades de los

consumidores. De acuerdo a algunos reportes económicos, la exportación de berries chilenos ha crecido en promedio 20.0% anual en la última década, llegando a US$ 896.0 millones el año pasado. Así, nuestro país se ubica como el tercer exportador a nivel mundial, sólo detrás de España y Estados Unidos (Bertoni and Namur 2016). La funcionalidad de los berries ha debido ser demostrada en diferentes estudios que han observado como por ejemplo una alta capacidad antioxidante y contenido de polifenoles en maqui (*Aristotelia chilensis*), que se asocia con posibles beneficios para la salud como efectos antiinflamatorios, antidiabéticos (Rojo et al. 2012), cardioprotectores (Brauch et al. 2017), actividad anti-aterogénica (Miranda-Rottmann et al. 2002), inhibición de la adipogénesis (Schreckinger et al. 2010), e inhibición del metabolismo de carbohidratos (Rubilar et al. 2011). También un alto poder antioxidante fue determinado en el fruto y hojas de murtilla (*Ugni molinae*) como resultado de su alto contenido de polifenoles, además se determinó cierta actividad antimicrobiana (López de Dicastillo et al. 2017; Rubilar et al. 2006; Shene et al. 2009) y efecto analgésico *in vitro* fue determinado solo en hoja de murtilla (Delporte et al. 2007). Por último, el calafate (*Barberis microphylla*) ha demostrado ser el fruto con mayor capacidad antioxidante, incluso mayor al maqui (Speisky et al. 2012), debido a la presencia de polifenoles como antocianinas, derivados de ácidos hidroxicinámicos y flavonoles (Bustamante et al. 2018; Ruiz et al. 2010).

Respecto a la investigación de compuestos bioactivos en residuos y subproductos de la agroindustria, algunos antecedentes han sido reportados. Procianidinas han sido extraídas y aisladas desde semilla de uva, orujo de uva y corteza de pino, y su influencia demostrada sobre la inducción de apoptosis de células de cáncer de colon y células de melanoma. Por su parte, los extractos de orujo de uva que contienen proantocianidinas mostraron un potencial cardioprotector. Los extractos de las partes verdes de murta y maqui resultaron ser ricas en flavonoles (kaempferol, miricetina, rutina, entre otros) y las frutas ricas en antocianinas, además de mostrar actividad antihemolítica e inhibición de la α-amilasa (Sineiro et al. 2012).

De la experiencia de los berries nativos y el potencial de los subproductos agroindustriales, se pueden destacar aspectos importantes de su éxito, como el rol fundamental que juega la alianza empresa e investigación académica para revitalizar un producto y otorgarle un valor agregado a su cultivo. Estos productos logran satisfacer a los consumidores que buscan beneficios funcionales de los alimentos. Es así como otras frutas han sido cultivadas en Chile por décadas y se desconocen sus potenciales beneficios, restándose de un importante valor agregado y competitividad dentro del sector. Este es el caso de la

chirimoya, fruta del centro-norte del país que podría ser revitalizada mediante estudios de funcionalidad.

1.2 Chirimoya

La chirimoya/o (*Annona cherimola* Mill.) es un árbol pequeño integrante del género *Annona* que pertenece a la familia *Annonaceae.* Es desarrollado en áreas subtropicales y es originario de Sudamérica particularmente en el sur de Ecuador y Norte de Perú (Pinto et al. 2005). El fruto conocido como chirimoya es de tamaño irregular con un peso que va de 200 a 700.0 g, su pulpa es blanquecina y se describe con un sabor excepcional, jugosa y aromática, una mezcla entre dulzura y leve acidez parecido a una mezcla de plátano, maracuyá, papaya y piña, y que es utilizada para la producción de sabrosas cremas, helados y su jugo se mezcla con jugo de naranja o limón. En su interior contiene semillas duras y negras y la superficie de la fruta es suave en algunas variedades, en otras contiene pequeñas protuberancias, la piel es delicada y fina (Anaya-Esparza et al. 2017; Loizzo et al. 2012).

1.2.1 Aspectos comerciales y agrícolas

El principal productor de chirimoya es España, y su cultivo se desarrolla principalmente en las costas de Granada y Málaga, también llamada 'Costa Tropical', siendo las principales variedades 'Fino de Jete' y 'Campas' (García-Salas et al. 2015). Otros países productores son Perú, Chile, Ecuador, Portugal, Australia, México y Estados Unidos. En los últimos años, la chirimoya ha ido ganando importancia en el mercado internacional, y para sus productores se ha convertido un fruto de alto valor económico. Además, se considera que la chirimoya tiene un gran potencial para la producción comercial, especialmente para los mercados de exportación. Eventualmente, la producción podría convertirse en una gran industria en varios países (Anaya-Esparza et al. 2017). En el año 1997 Chile se ubicó dentro de los tres mayores productores de chirimoya, antecedido por España y Perú, sin embargo el cultivo de chirimoya ha ido disminuyendo desde aquellos años (Pinto et al. 2005), siendo una de las causas los requerimientos climáticos para su cultivo. El chirimoyo es una especie subtropical por lo que requiere de condiciones climáticas con una alta humedad relativa y libre de heladas, pudiendo ser producido en la Zona Central y Centro Norte, entre la región de Atacama y la Metropolitana (Oficina de Estudios y Políticas Agrarias 2018). Respecto a las variedades cultivadas en dichas regiones destacan principalmente chirimoya 'Bronceada' y 'Concha lisa' (Manríquez et al. 2014).

El área de cultivo del chirimoyo en Chile es reducida, siendo catalogado como frutal menor. De acuerdo a la Oficina de Estudios y Políticas Agrarias (2018) la superficie cultivada el año 2011 fue 405.0 ha, disminuyendo con respecto al año 2011 (523.0 ha). El cultivo de la chirimoya se distribuyen principalmente en la IV Región, con una superficie cultivada correspondiente al 70.0% de la superficie total país, siendo la ciudad de Coquimbo (132.0 ha) y La Serena (99.0 ha) las principales ciudades de cultivo de esta fruta. Respecto a la producción, la IV Región comercializó 3734.0 ton el año 2018 (Oficina de Estudios y Políticas Agrarias 2018) siguiendo la tendencia de disminución, ya que el año 2011 se informó una producción de 4475.0 ton (Oficina de Estudios y Políticas Agrarias and Centro de Información de Recursos Naturales 2011). El destino de la producción de chirimoya es principalmente el mercado interno con un 80.0%, le sigue la exportación con un 13.9% y un 6.1% destinado a la agroindustria. El destino de exportación fue Estados Unidos, con un volumen de exportación de 284.0 ton con un valor cercano a los US$ 1.4 millones el año 2018 (Oficina de Estudios y Políticas Agrarias 2018).

1.2.2 Composición química y antecedentes de bioactividad

Actualmente la chirimoya ha tenido especial atención por las potenciales propiedades medicinales de diferentes partes de la planta y fruta. Además, el contenido en compuestos bioactivos, principalmente compuestos fenólicos, en los residuos y sub-productos de la agroindustria de la chirimoya podría ser mayor que en la parte comestible de la fruta (Díaz-de-Cerio et al. 2018). En la medicina tradicional, diferentes partes de la planta han sido usadas para el tratamiento de variados desordenes. Las semillas de chirimoya tradicionalmente trituradas se utilizaron como insecticida, principalmente en el tratamiento de piojos e infecciones cutáneas parasitarias. Extractos de hojas han sido utilizados en diarrea, como antiparasitario y trastornos respiratorios. Por su parte, el fruto tendría propiedades antimicrobianas e insecticidas, y ha sido usado para el tratamiento del dolor de estómago y úlceras pancreáticas. También el consumo de pulpa de chirimoya puede contribuir a la prevención de diferentes enfermedades asociadas con el estrés oxidativo (Albuquerque et al. 2016; Anaya-Esparza et al. 2017; Jamkhande et al. 2017). Estas actividades biológicas son atribuibles a la presencia de compuestos bioactivos que incluyen alcaloides, lignanos, amidas, acetogeninas, purinas, esteroles, tocoferoles, fosfolípidos, flavonoides, taninos, fitoesteroles, luteína, β-caroteno, vitamina C, y compuestos fenólicos entre otros (Anaya-Esparza et al. 2017).

A pesar del actual interés que ha generado la chirimoya y los subproductos de su procesamiento, la información sigue siendo escasa, considerando que principalmente los actuales reportes solo se enfocan en la identificación de compuestos sin evaluar directamente la bioactividad de éstos. Recientemente, dos reportes han identificado variados compuestos bioactivos en cáscara, pulpa y semilla de chirimoya cultivada en España (García-Salas et al. 2015; García-Salas et al. 2016). Un trabajo similar fue realizado por Santos et al. (2016) determinando la composición lipofílica y fenólica de pulpa de chirimoya cultivada en Portugal. Mientras que Díaz-de-Cerio et al. (2018) han descrito los principales componentes de las hojas de chirimoya. Por otro lado, los estudios sobre bioactividad de la chirimoya y sus componentes son limitados, al contrario de otras especies del genero *Annona*. Sin embargo, hay cierta información que muestra los potenciales efectos beneficios para la salud y una posible categorización de esta fruta como un alimento funcional.

1.2.2.1 Actividad antioxidante

Las investigaciones sobre chirimoya hacen referencia principalmente a la determinación y caracterización de su actividad y capacidad antioxidante mediante estudios *in vitro*. La actividad antioxidante puede ser evaluada por diferentes metodologías, entre las que se encuentra FRAP (Ferric-reducing antioxidant power), ORAC (Oxygen radical absorbing capacity), DPPH (2,2-diphenyl-1-picrylhydrazyl) y ABTS (2,2′-azinobis(3-ethylbenzothiazolline-6-sulfonic acid). Las diferentes metodologías son útiles para una aproximación del efecto antioxidante, sin embargo, no parece haber consenso para concluir el método más conveniente o estándar. Los diferentes ensayos difieren en su mecanismo de generación de radicales libres y en su capacidad para reducir las moléculas (antioxidantes) en la muestra variando la expresión de los resultados, haciendo difícil la comparación de resultados, sumado a esto los extractos son obtenidos en diferentes solventes variando los tenores de extracción (Anaya-Esparza et al. 2017; Karadag et al. 2009). Los valores reportados de la actividad antioxidante en chirimoya son descritos en la Tabla 1, donde se observa que no solo la pulpa ha sido estudiada, sino también piel y semillas, siendo en general la piel la que tiene mayor actividad antioxidante. A pesar que es difícil hacer una comparación entre estos resultados, sí se puede establecer de manera general que cada parte del fruto tiene cierta actividad antioxidante (Karadag et al. 2009).

Otra forma de determinar la capacidad antioxidante es cuantificando el contenido de compuestos antioxidantes. Entonces los compuestos son agrupados de acuerdo a sus

características estructurales, y es así como algunos ensayos determinan la cantidad de polifenoles totales, más específicamente flavonoides totales, antocianidinas, carotenoides, entre otros. Del mismo modo que los ensayos anteriormente nombrados, éstos tienen la desventaja que la expresión de los resultados es variable, al igual que el método extractivo, lo que hace difícil comparar los resultados. Un resumen de los valores publicados se muestra en la Tabla 2. De los estudios reportados, Vasco et al. (2008) comparó los resultados de chirimoya con otros frutos, ubicándola sobre de la frutilla, y por debajo de la mora. Loizzo et al. (2012) correlacionan la capacidad antioxidante con la cantidad de polifenoles y acetogeninas, atribuyendo la actividad a los polifenoles. De manera general, se puede establecer que la piel de chirimoya es la que posee una mayor cantidad de compuestos con actividad antioxidante. Estos parámetros pueden ser utilizados como una aproximación de la funcionalidad y capacidad antioxidante de esta fruta.

A pesar de los promisorios resultados de los ensayos químicos, es necesario conocer la biodisponibilidad en el momento de la ingesta y su capacidad sobre organismos vivos. Al respecto, Gupta-Elera et al. (2011) ha establecido un aumento de la capacidad antioxidante de algunas líneas celulares al ser expuestas a extractos de pulpa, piel y jugo, dicho incremento depende de la capacidad de absorción de los extractos. También el extracto de pulpa disminuiría entre un 60.0 a 70.0% la peroxidación lipídica de la membrana de eritrocito y aumenta la vialidad celular entre un 22.0 a 25.0% de linfocitos periféricos (Barreca et al. 2011).

1.2.2.2 Otras actividades biológicas

Además de las características antioxidantes, los extractos de chirimoya han mostrado otras actividades biológicas. El extracto de hoja demostró una disminución de la permeación de colesterol a través de células similares a la barrera intestinal, Caco-2, disminuyendo en un 47.0% la absorción de colesterol al organismo, además de cierta inhibición de la hidroximetilglutaril coenzima A (HMG-CoA) reductasa, actividad atribuida a la rutina (Falé et al. 2013). Las semillas de chirimoya han mostrado una actividad citotóxica selectiva sobre líneas de células tumorales, identificando como responsables a algunas acetogeninas como anomolina, amocherimolina y cherimolaciclopeptido A, B, C y D (Kim et al. 2001; Quispe-Mauricio et al. 2009; Wélé et al. 2005; Wélé et al. 2004; Woo et al. 1999). El aceite esencial obtenido de pulpa, flores y hojas de chirimoya demostró que inhiben moderadamente el desarrollo de algunas bacterias gram positivas, gram negativas y un hongo, atribuido principalmente a la presencia de terpenos (Ríos et al. 2003).

Tabla 1. Resumen de la capacidad antioxidante en chirimoya determinada por diferentes metodologías.

Solvente de Extracción	Parte Utilizada	FRAP	ORAC	DPPH	ABTS	Ref.
Metanol:H_2O	Pulpa	20.0 µmol ET/g	--	23.0 µmol ET/g	40.0 µmol ET/g	Vasco et al. (2008)
Metanol Etanol DMFA	Pulpa	1.8-2.8 µM ET	--	--	22.9 - 37.6 µM ET	Barreca et al. (2011)
H_2O	Jugo Piel Pulpa		98.1 ET/100g 74.7 ET/100g 6.0 ET/100g			Gupta-Elera et al. (2011)
Etanol	Pulpa Piel	52.8 µM Fe(II)/g 68.3 µM Fe(II)/g		57.7 µg/mL 72.2 µg/mL	3.6 ET 4.4 ET	Loizzo et al. (2012)
Etanol	Pulpa Piel Semilla			1.0 - 4.6 mg/mL 0.2 - 0.4 mg/mL 3.2 - 4.2 mg/mL		Albuquerque et al. (2016)

ET/g: capacidad antioxidante en equivalentes TROLOX por gramo de muestra fresca
DMFA: solvente dimetilformamida

Tabla 2. Resumen de ensayos para la determinación de compuestos con actividad antioxidante.

Solvente de Extracción	Parte Utilizada	Polifenoles Totales	Flavonoides Totales	Vitamina C	Vitamina E	Ref.
Metanol:H_2O	Pulpa	323.0 mg EAG/100 g	--	4.0 – 6.0 mg/100 g	--	Vasco et al. (2008)
Metanol Etanol DMFA	Pulpa	5.8-6.8 µM EAG (98.5 mg EAG/100 g)	3.5-5.7 µM ECat	--	--	Barreca et al. (2011)
Etanol	Jugo-Pulpa-Piel	14.6-12.6 mg EAC/100 g	8.2-3.8 EQue/100 g	--	--	Loizzo et al. (2012)
Etanol	Pulpa Piel Semilla	3.1-12.0 mg EAG/100 g 17.0-19.1 mg EAG/100 g 3.4-4.2 mg EAG/100 g	1.3-1.5 mg EEpic/100 g 33.0-44.9 mg EEpic/100 g 3.1-6.8 mg EEpic/100 g	2.1-6.7 mg/100 g 4.2-5.2 mg/100 g 1.5-2.0 mg/100 g	0.2 - 0.7 mg/100 g 0.5 - 0.6 mg/100 g	Albuquerque et al. (2016)

EAG: equivalentes ácido gálico / EAC: equivalentes ácido cafeico / EEpic: equivalentes epicatequina

1.2.2.3 Estudios sobre otras especies de *Annona*

Existen otras tres especies de importancia comercial perteneciente al género *Annona* que han sido estudiadas más extensamente. Sin embargo estas especies no pueden ser nombradas como chirimoya, ya que poseen otros nombres comunes (Pareek et al. 2011).

***Annona reticulata*:** su nombre común es "*custard apple*". Se han estudiado los extractos de hoja, raíz y semillas. Los extractos indican cierta actividad antipirética, antimicrobiana y actividad citotóxica sobre algunas líneas celulares. En ratones muestran cierta actividad antihiperglicémica, antiulcerosa y antinociceptiva. También se describe una acción antioxidante y cicatrizante (Jamkhande and Wattamwar 2015; Jamkhande et al. 2014).

***Annona muricata*:** comúnmente llamada '*soursop*'. Su actividad se ha descrito similar a la especie anterior; el extracto de piel muestra actividad antiparasitaria atribuida a las acetogeninas, mientras que los extractos de hojas muestran actividad antibacteriana y la corteza una marcada reducción de cicatrices. Estudios *in vitro* además demuestran una actividad antiviral (Herpes simplex 1) (Gajalakshmi et al. 2012) y citotoxicidad sobre líneas celulares de carcinoma hepático (Liaw et al. 2002).

***Annona squamosa*:** es la especie más estudiada y comúnmente se denomina "*sugar apple*". Se reportan estudios *in vitro* e *in vivo* de sus diferentes partes. Los extractos de tallo tendrían cierta actividad analgésica y antiinflamatoria en ratas (Chavan et al. 2010), inhibición de la agregación plaquetaria en conejos (Yang et al. 2002) y acción inmunomoduladora (Soni et al. 2012). Los extractos de hojas muestran un alto contenido de polifenoles, alta capacidad antioxidante, moderada acción antimicrobiana *in vitro* (Alkhawalidy and Hossain 2015; Gupta et al. 2005), y acción hipoglicémica y antidiabética en ratas y conejos (El-Chaghaby et al. 2014). Las semillas muestran actividad antitumoral, antimicrobiana y citotoxicidad selectiva atribuible a las acetogeninas (Chen et al. 2012a; Chen et al. 2012b; Mukhlesur Rahman et al. 2005), algunos polifenoles encontrados tendrían acción contra el hipertiroidismo en ratones y protección hepática (Panda and Kar 2007). En pulpa se determinó una alta capacidad antioxidante *in vitro,* atribuibles a las altas cantidades de compuestos fenólicos y flavonoles (Nandhakumar and Indumathi 2013).

A pesar de los variados estudios que se han reportado de las diferentes especies, no se ha logrado identificar claramente él o los compuestos responsable de la actividad antioxidante

o biológica de los extractos de chirimoya. Por lo general, el enfoque para el estudio de los compuestos bioactivos es primero evaluar la actividad biológica de un extracto para posteriormente identificar los componentes responsables de dicha actividad. En esta segunda etapa, de identificación y elucidación estructural, se enfrenta a un laborioso escenario debido a los múltiples componentes que pueden estar presentes en el extracto y posterior evaluación de la actividad de cada uno de los constituyentes, resultando en una tarea que requiere un largo tiempo y un elevado costo. Ante este laborioso escenario, se proponen metodologías analíticas de efecto dirigido con separación previa que permitan una identificación más rápida, precisa y directa de los compuestos y la actividad que éstos poseen. En estos ensayos el orden del análisis es diferente, debido que la separación y la evaluación de la actividad se realizan simultáneamente, para posteriormente aislar e identificar solamente los constituyentes que tienen la actividad. Una vez identificados los compuestos se puede evaluar la metodología extractiva más conveniente que permita obtener una alta concentración de compuestos bioactivos.

1.3 Técnicas analíticas para la evaluación de bioactividad e identificación de compuestos bioactivos

Los compuestos bioactivos en el reino vegetal son variados, entre los que se encuentran vitaminas, alcaloides, esteroles, polisacáridos, ácidos grasos y compuestos fenólicos. Los ensayos *in vitro* para determinar cierta actividad biológica, también llamados bioensayos, son esenciales para el descubrimiento de nuevos compuestos bioactivos que sean utilizados directamente o que aporten estructuras plantillas para el desarrollo de nuevos compuestos con fines medicinales. Un enfoque comúnmente utilizado para la obtención de compuestos bioactivos es el aislamiento dirigido por bioensayos, que consta de una serie de etapas que pueden que lo convierten en un laborioso proceso, como se esquematiza en la Figura 1 (Montalvão et al. 2014).

Los métodos de separación, identificación y cuantificación requieren ser altamente sensibles y selectivos, lo cual no resulta fácil debido a la gran cantidad de compuestos que puede contener una matriz. Posterior a una extracción exhaustiva se dispone principalmente de metodologías espectrofométricas que permiten la determinación de bioactividad de los extractos, como Folin-Ciocalteu, ORAC, DPPH, FRAP, y ABTS (Yingjian and Devanand 2014). También otros bioensayos de *screening* son utilizados para evaluar el efecto de los compuestos bioactivos sobre microorganismos y enzimas, los cuales pueden ser utilizados para extractos o compuestos puros (Montalvão et al. 2014). A pesar

que estos 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.

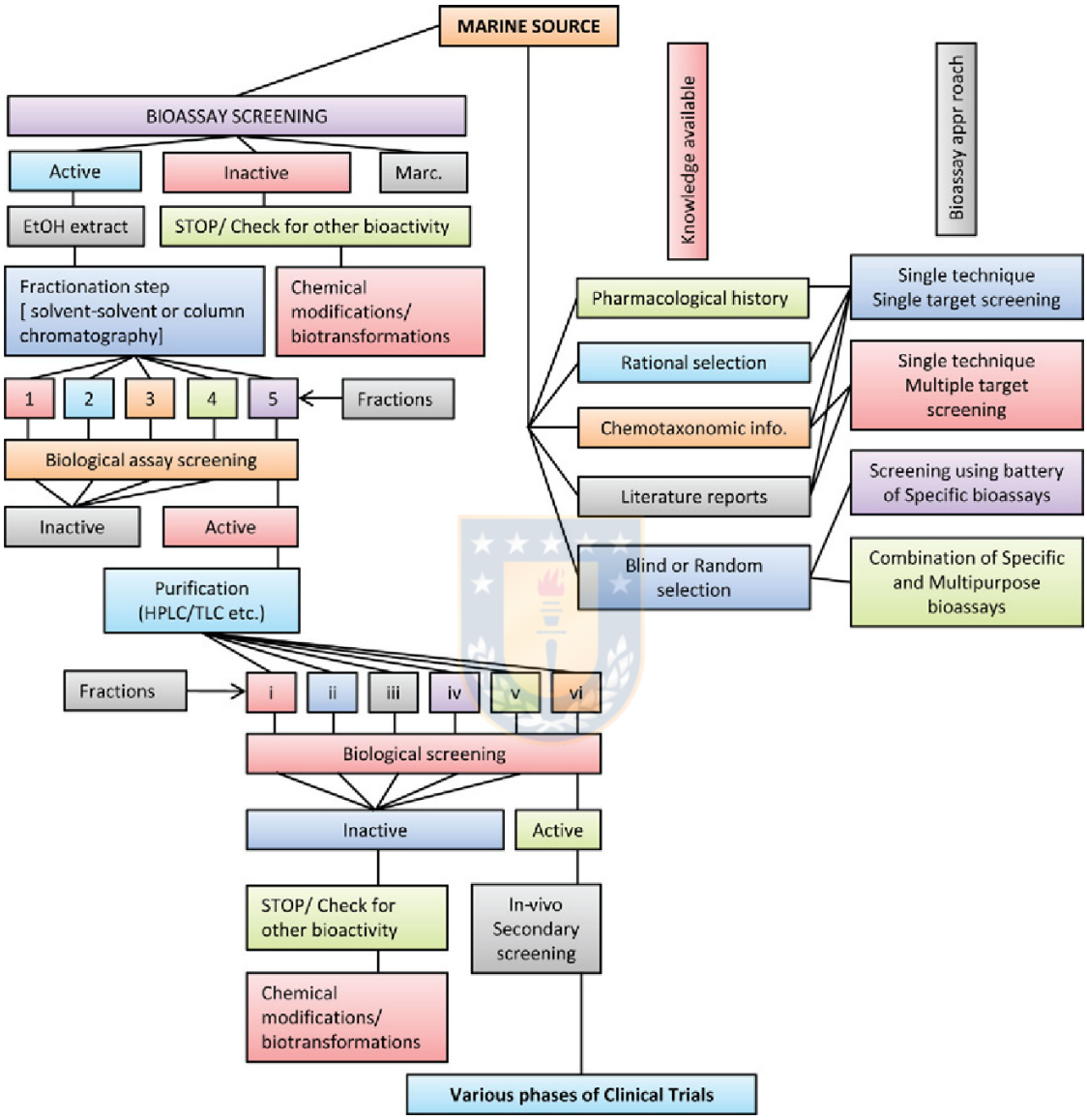

Figura 1. Esquema que ejemplifica las etapas de un aislamiento de compuestos bioactivos guiado por bioensayos. (Adaptado de: Montalvão et al. (2014)).

Los diferentes tipos de cromatografías como la líquida (HPLC), en capa fina (TLC/HPTLC) y gaseosa (GC) son ampliamente usadas para la separación, identificación y cuantificación. La técnica cromatográfica más utilizada es HPLC, por sobre TLC y GC. Estos métodos de separación son comúnmente acoplados con detectores espectrofotométricos UV/Vis,

fluorescencia, electroquímicos, espectrómetros de masas (MS) y resonancia magnética nuclear (NMR). Las detecciones por MS y NMR son comúnmente usadas para la elucidación estructural más que para cuantificación. Usualmente los compuestos son purificados mediante cromatografía en columna y luego son analizados mediante NMR, siendo utilizada hoy en día para la confirmación estructural de fitoquímicos, principalmente cuando son compuestos minoritarios. Estas dos últimas técnicas se complementan de buena manera, mientras MS otorga información respecto a la masa molecular del compuesto, NMR puede dilucidar la conformación estructural (Yingjian and Devanand 2014).

El análisis de bioactividad y compuestos bioactivos mediante HPLC y GC puede ser difícil y en general las muestras necesitan un tratamiento exhaustivo. Primero se realiza un arduo trabajo de separación e identificación para luego estudiar o corroborar su bioactividad mediante ensayos biológicos, lo que se traduce en un largo periodo de tiempo y utilización de recursos (Figura 1).

De las tres técnicas cromatográficas la menos utilizada es TLC o HPTLC a pesar del alto potencial e impacto de esta técnica. Posee ciertas características que la hacen una técnica muy ventajosa al momento de la determinación de compuestos bioactivos. Entre las ventajas destaca la gran flexibilidad pudiendo variar o modificar cada una de las etapas en la determinación, la posibilidad de analizar y detectar varias muestras simultáneamente aplicando diversos detectores sin necesidad de realizar un nuevo desarrollo cromatográfico. En el caso de compuestos bioactivos tiene una mayor facilidad para integrar la detección de actividad biológica *in situ* acoplando ensayos químicos o enzimáticos, disminuyendo considerablemente el tiempo y costo de otras técnicas analíticas, luego de la biodetección puede acoplarse a detectores que permitan obtener información molecular y estructural (Taha et al. 2015).

1.3.1 Cromatografía acoplada a bioensayos

Los alimentos presentan miles de compuestos químicos de naturaleza desconocida, y por lo general no se cuenta con la disponibilidad de estándares comerciales de mucho de ellos, adicionalmente cada compuesto puede ejercer una bioactividad diferente, por lo tanto el estudio de compuestos bioactivos debe definir claramente su objetivo de estudio. Una perspectiva que define la bioactividad son los ensayos de efecto dirigido (EDA). Esta aproximación permite el análisis de una bioactividad específica y diferentes muestras de

manera más simple. Esto asociado a un sistema cromatográfico, permite individualizar un grupo de compuestos o moléculas (Weiss et al. 2017).

Los bioensayos comunes, realizados sobre microplacas, cubetas o placas Petri (ensayo de actividad microbiana), pueden detectar y en ocasiones cuantificar la bioactividad, pero no pueden identificar cuáles son los compuestos responsables. Por ello se hace necesario combinar los bioensayos con cromatografía, enlazando directamente el compuesto con la bioactividad e identificarlo individualmente. La combinación de EDA y cromatografía conduce a respuestas específicas en un contexto global y una gama de compuestos potencialmente relevantes, o sea todos los compuestos que indican una actividad en el bioensayo, pueden ser identificados, por lo tanto, miles de compuestos presentes son reducidos a unos pocos. Independiente de las características estructurales de los compuestos bioactivos, que en ocasiones dificultan su detección por técnicas espectrofotométricas, estos podrán ser detectados ya que lo importante es si presentan bioactividad (Morlock 2014; Weiss et al. 2017).

Los sistemas *online* más sofisticados son llamativos para el desarrollo de bioensayos, pero son complejos y si la cadena de análisis se interrumpe todo el análisis debe detenerse. En este aspecto HPTLC es poco considerado y los sistemas de análisis de flujo continuo, como HPLC presentan inconvenientes que hacen difícil acoplarla a bioensayos. Algunos inconvenientes de la asociación HPLC-bioensayo es el constante lavado que debe ser realizado luego de un análisis con desinfectantes y posterior remoción de éste (Morlock 2014). Una incompatibilidad aún más importante en ensayos enzimáticos es la utilización de solventes orgánicos en la fase móvil, de acuerdo Ingkaninan et al. (2000) un 10.0% de acetonitrilo inhibió cerca del 90.0% de la actividad de la enzima acetilcolinesterasa. Desde el punto de vista instrumental la instalación es laboriosa y compleja, y solo se pueden realizar un análisis por separación cromatográfica.

A pesar de ser poco considerada, los desafíos son menos críticos para acoplar la HPTLC con bioensayos de efecto dirigido, reconociéndose como la opción más racional. El formato abierto, *offline* y planar HPTLC no genera las desventajas que los sistemas basados en columnas. Un *screening* respecto a los componentes relevantes puede ser realizado en una sola placa con menos esfuerzo y manejo de datos. Luego de una separación cromatográfica convencional el solvente de la fase móvil es evaporado, y las placas son sumergidas o atomizadas en soluciones enzimáticas o microbiológicas. Posterior a la incubación la actividad es visualizada por lo general a través de una reacción colorimétrica. A pesar que el tiempo de incubación puede ser bastante largo, las bandas en la placa son lo

suficientemente definidas como para permitir una adecuada cualificación. El sistema planar abierto es ajustable a diferentes detectores, y en el caso de HPTLC los módulos son completamente automatizados en sus componentes unitarios. Un sistema HPTLC-bioensayo puede ser utilizado en cualquier momento para cualquier proyecto sin costos adicionales, es decir puede ser utilizado por más de un usuario realizando diferentes análisis, mientras que un sistema HPLC necesita exclusividad para realizar una tarea específica. Además, posee la gran ventaja que luego del desarrollo cromatográfico el solvente orgánico contenido en la fase móvil es removido por evaporación, quedando retenidos los componentes en la placa pudiendo evitando la incompatibilidad con la enzima o los microorganismos. En paralelo a la placa usada en el bioensayo, otras placas pueden ser desarrolladas y utilizadas para estudiar y dilucidar sus estructuras mediante reacciones de derivatización y técnicas espectroscópicas, o bien extraer las bandas activas para un análisis por espectrometría de masa de alta resolución (HRMS) o NMR (Morlock 2014). Una aproximación general del flujo de trabajo de una metodología HPTLC-bioensayo de efecto dirigido es esquematizado en la Figura 2 (Weiss et al. 2017).

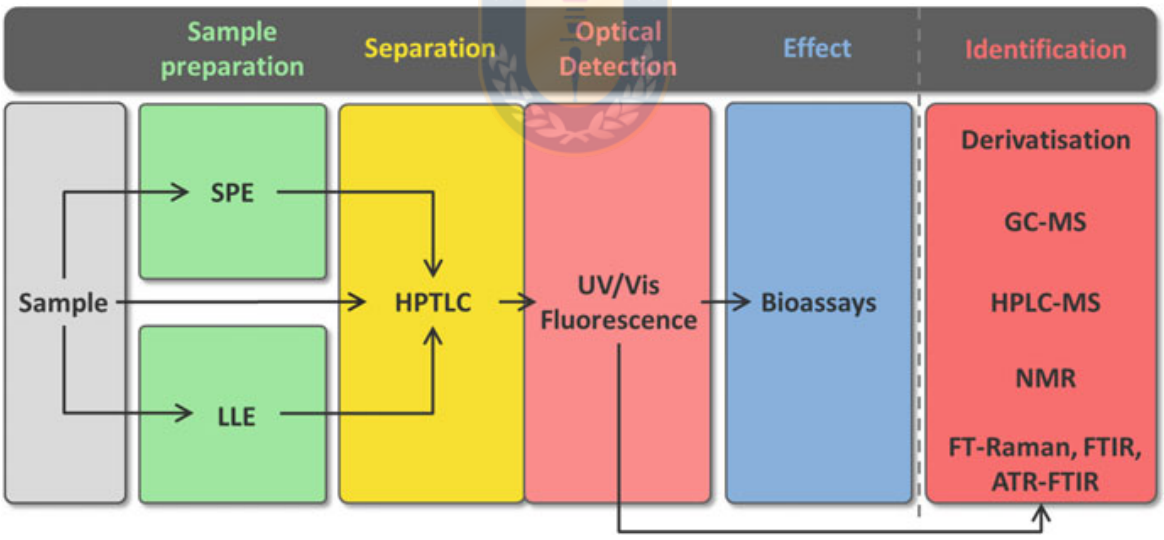

Figura 2. Esquema general de un análisis HPTLC-bioensayo de efecto dirigido. (Adaptado de: Weiss et al. (2017)).

1.3.2 Aplicaciones de análisis de efecto dirigido mediante HPTLC-bioensayo

Como se ha descrito, cuando HPTLC se combina con método de detección biológicos y químicos, se convierte en una técnica eficaz y económica para el estudio de extractos vegetales. Cuando HPTLC se acopla a una detección biológica se denomina HPTLC-

bioautografía, mientras que cuando se utiliza una detección química se nombra como TLC-autografía, dos categorías que normalmente se unen y se nombran indistintamente como TLC-bioensayo (Marston 2011). Este tipo de análisis se ha convertido en una potente herramienta para una rápida identificación de compuestos bioactivos en extractos botánicos, y cuando se asociada a MS permite la identificación de nuevos compuestos. Con esto es posible cambiar el laborioso y costoso enfoque de estudiar la bioactividad sólo después del aislamiento y la identificación de los compuestos. Esta metodología ha tenido varias aplicaciones en donde destacan ensayos para determinar la capacidad antioxidante, actividad enzimática y actividad antimicrobiana, los que se resumen y clasifican en la Figura 3, además se establece que es una técnica poco explorada, pero que ha tenido importantes avances llegando hoy en día a utilizar células para la detección de compuestos tóxicos (Morlock 2014; Morlock 2018).

A continuación, se realiza una breve reseña de los tipos de ensayos reportados de importancia para el desarrollo de este trabajo basado principalmente en compuestos con potencial terapéutico, se excluye la determinación de capacidad antimicrobiana. Cabe destacar que cada uno de estos ensayos se realiza posterior al desarrollo cromatográfico en placa.

1.3.2.1 HPTLC-bioensayo para compuestos antioxidantes

Los antioxidantes son importantes en los sistemas biológicos por contrarrestar los efectos del estrés oxidativo que son parte de variados desordenes como aterosclerosis, diabetes mellitus, inflamación crónica, desordenes neurodegenerativos y ciertos tipos de cánceres, por eso es de gran interés la cuantificación de antioxidantes y la determinación de la capacidad antioxidante de los alimentos (Karadag et al. 2009).

Las características que presenta un análisis por HPTLC son tremendamente ventajosas, siendo además muy útiles en la detección e identificación de antioxidantes naturales. La mezcla de compuestos primero es separada sobre la placa HPTLC y luego es sumergida o atomizada con soluciones de DPPH o ABTS, de esta manera las bandas con capacidad antioxidante serán detectadas, acotando significativamente el número de compuestos de la mezcla. Mediante el siguiente paso se puede identificar los compuestos bioactivos acoplando un detector MS (Cieśla et al. 2015).

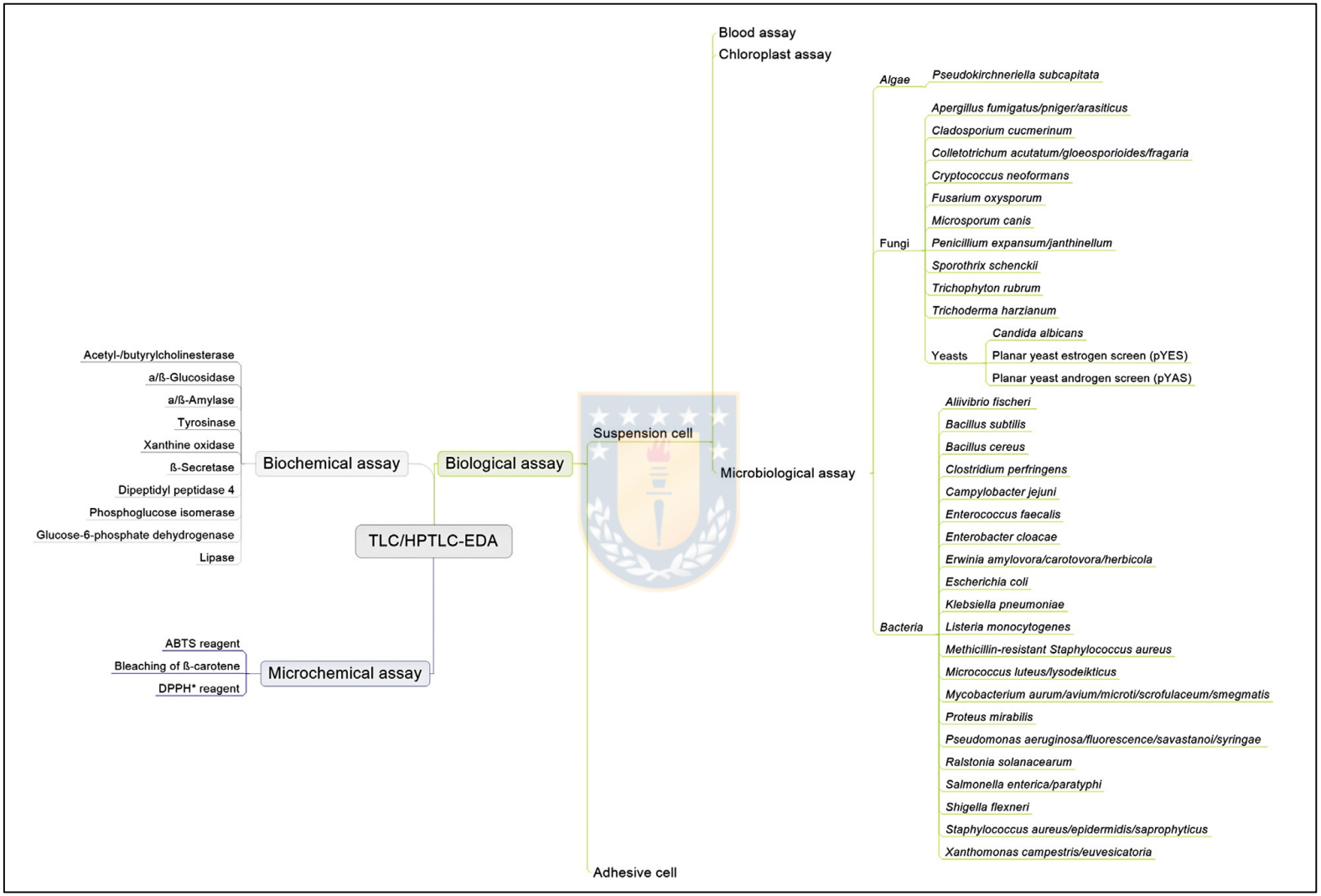

Figura 3. Resumen de los principales HPTLC-bioensayos, agrupados como ensayos microquímicos, bioquímicos y biológicos. (Adaptado de: Morlock (2018)).

El ensayo utilizando DPPH es uno de los más utilizados en HPTLC, el radical DPPH otorga un color violeta, y la estabilización del radical por el compuesto antioxidante permite que este color cambie a amarillo pálido. Este ensayo se ha vuelto la primera opción en HPTLC, reportándose en varios estudios sobre extractos vegetales y frutas (Cieśla et al. 2012; Cieśla et al. 2015; Wojtanowski and Mroczek 2018). Incluso la actividad antioxidante ha podido ser evaluada mediante el procesamiento de imagen digital, permitiendo una aproximación cuantitativa (Ibrahim et al. 2018; Olech et al. 2012).

1.3.2.2 HPTLC-bioensayo para la detección de inhibidores enzimáticos

Dentro de los ensayos de actividad enzimática, que comúnmente se realizan en microplacas, solo unos pocos han sido transferidos a una metodología mediante HTPLC, por lo que existe un tremendo potencial en escalar diversos ensayos y aumentar el espectro de detección de compuestos bioactivos. Estos ensayos destacan una de las mayores ventajas de HPTLC, los solventes orgánicos utilizados en la fase móvil pueden ser removidos de manera que no influyen en la actividad enzimática. Luego de evaporar el solvente la placa es sumergida o atomizada en una solución enzimática y una solución de sustrato que impregna la placa, para seguir con una incubación, generalmente a 37.0ºC, sin que la placa se seque y posteriormente se lleva a cabo una derivatización o reacción reveladora de actividad que permita diferenciar la zona con inhibición enzimática (Cieśla et al. 2015). Entre los ensayos reportados para evaluar la actividad enzimática, como se resume en la Figura 3, para este trabajo toman vital importancia los que involucran la detección de compuestos bioactivos que aporten al tratamiento de enfermedades crónicas no transmisibles. En este sentido destacan los ensayos desarrollados para la detección de inhibición de α-glucosidasa y acetilcolinesterasa.

Inhibición de acetilcolinesterasa: los inhibidores de esta enzima podrían contribuir disminuyendo la progresión de la enfermedad de Alzheimer, que se caracteriza por una disminución del neurotransmisor acetilcolina. La biodetección a través de HPTLC ha sido reportada principalmente bajo dos métodos: utilizando el reactivo de Ellman y la reacción de diazotización (Cieśla et al. 2015; Marston 2011). El uso del reactivo de Ellman tiene el inconveniente que otorga bajo contraste entre las zonas de inhibición y el fondo, siendo la diazotización la más utilizada. En el método reportado por Marston et al. (2002) la placa desarrollada y seca es atomizada con una solución de enzima acetilcolinesterasa o butirilcolineterasa e incubada durante 20 min a 37.0ºC, luego es atomizada con una solución

que contiene el sustrato y el revelador, acetato de 1-naftilo y sal de Fast Blue B respectivamente. Luego de 1 a 2 min los componentes bioactivos se observan como bandas incoloras (blancas) sobre un fondo purpura.

Inhibición de α-glucosidasa: los compuestos que inhiben la enzima α-glucosidasa podrían potencialmente ser usados como co-ayudantes en el tratamiento de la diabetes mellitus tipo 2, en el tratamiento de la obesidad, como antivirales, antibacterianos o antimetastásicos. El método espectrofotométrico de elección para detectar inhibidores de la α-glucosidasa y β-glucosidasa es utilizando *p*-nitrofenil-α(β)-D-glucopiranósido como sustrato, que otorga un color amarillo por la formación de *p*-nitrofenol, sin embargo al ser implementado en HPTLC las bandas inhibitorias son pobremente visibles (Marston 2011). Simões-Pires et al. (2009) realiza una modificación cambiando el sustrato por 2-naftil-α-D-glucopiranósido, que se caracteriza por ser más estable al pH y otorga un mayor contraste al reaccionar con sal de Fast Blue B como revelador. Luego de la separación de las bandas, la placa es atomizada con una solución enzimática de α-glucosidasa a pH 7.5 es incubada por 60 min a temperatura ambiente en un ambiente húmedo. Luego para la detección de la actividad enzimática la placa es atomizada con una mezcla del sustrato y revelador. La escisión del sustrato genera como producto 2-naftol que al reaccionar con la sal produce un color purpura como fondo de placa, mientras que los compuestos con actividad inhibitoria no generan ningún color observándose como bandas del color de la placa (blanco).

1.3.3 Identificación y elucidación estructural en HPTLC-bioensayo

Una vez estudiada la presencia de compuestos bioactivos mediante HPTLC-bioensayo, la siguiente etapa en este flujo de trabajo analítico es la identificación y caracterización estructural de los compuestos responsables de la bioactividad. Generalmente la combinación de una técnica de separación e identificación, para obtener una caracterización eficiente de las moléculas, está dado por HPTLC combinada a espectrometría de masas de alta resolución (HRMS) y resonancia magnética nuclear (NMR). En este sentido HPLC ha recibido poca relevancia analítica, sin embargo, una de las principales ventajas de esta técnica es la fácil adaptación a varios sistemas de detección, es así como se han empleado múltiples espectrómetros. Normalmente HPTLC se acopla a detectores UV/Vis y fluorescencia (FLD), a lo cual se ha sumado la obtención de información mediante espectroscopia infrarroja (FTIR y ATR), y espectroscopia de Raman de superficie mejorada (SERS). Sin embargo, el acoplamiento de MS y HRMS

mediante el desarrollo de interfaces TLC-MS ha sido de gran interés para la identificación de compuestos, además de poder sumar un análisis por NMR (Morlock and Schwack 2010b)

Está claro que un análisis de efecto dirigido mediante HPTLC-bioensayo apunta directamente a los compuestos bioactivos que se encuentran retenidos en placa. De este modo, comienza una secuencia de posibles etapas que conducen a la identificación y caracterización molecular de los compuestos bioactivos, como se esquematiza en la Figura 4 (Yüce and Morlock 2016).

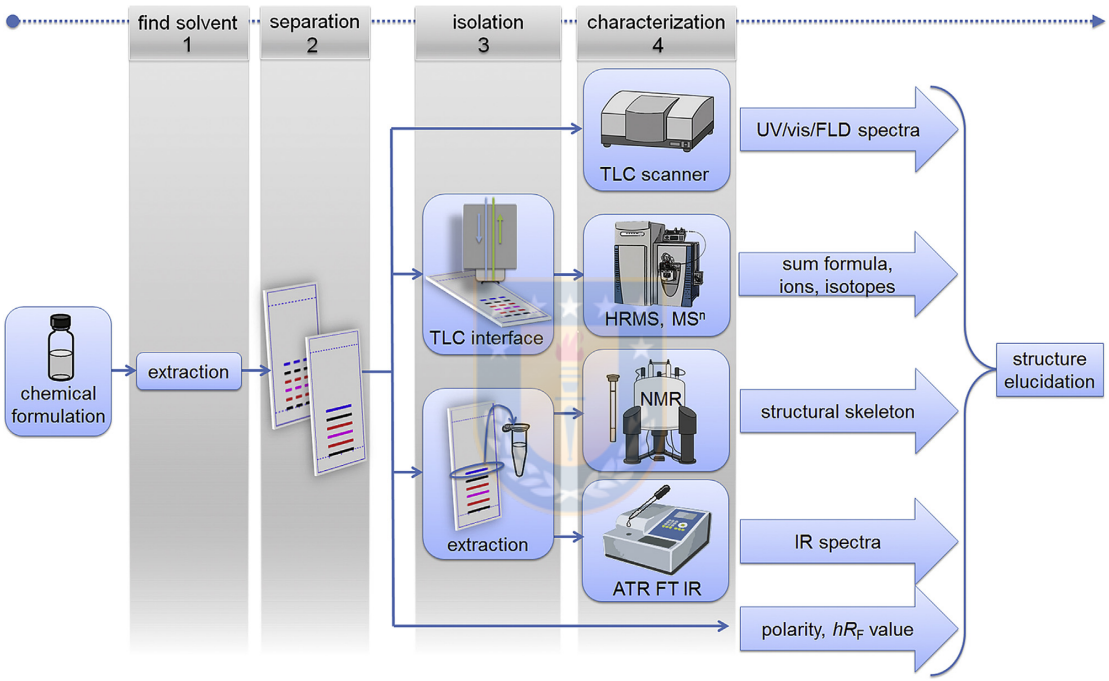

Figura 4. Flujo de trabajo que puede ser aplicado para obtener una identificación y caracterización de las moléculas bioactivas (Adaptado de: Yüce and Morlock (2016)).

Para la etapa identificación y elucidación la placa no debe ser sometida al bioensayo para evitar posibles reacciones y modificación de la estructura original. Inicialmente, las muestras separadas en la placa pueden ser sometidas a detección por UV/Vis/FLD pudiendo capturar imágenes y obtener información estructural básica. Una aproximación de polaridad de los compuestos bioactivos puede ser estimada sobre la base de información del desarrollo cromatográfico y la distancia de migración en la placa (R_F). Opcionalmente se puede realizar una reacción de derivatización para obtener información de los grupos funcionales presentes o conocer la familia estructural del compuesto (polifenoles, azúcares, alcaloides,

etc.) Esta información obtenida es importante y ayuda a direccionar la identificación, sin embargo, es evidente que la unión de HPTLC con MS/HRMS y NMR permite una visión más profunda de la identidad y la estructura de los compuestos estudiados. En este campo analítico, el acoplamiento de la cromatografía planar a la espectrometría de masas es de gran interés, ya que permite el acceso directo de la muestra en condiciones ambientales, y permite un análisis rápido, reproducible, de alto rendimiento, preciso y barato (Morlock 2018). Para llevar a cabo un análisis por NMR, generalmente se escala la cromatografía analítica a una cromatografía planar preparativa (PLC) bajo las mismas condiciones de separación usadas en el HPTLC-bioensayo. Las largas bandas son identificadas y desorbidas con un solvente adecuado para alcanzar una concentración adecuada para el análisis (Azadniya and Morlock 2018; Móricz et al. 2018b). Una alternativa, es el uso de la interfaz TLC-MS, que permite eluir directamente la banda de interés para su posterior análisis por MS, HRMS, MS/MS, ATR-FTIR o NMR (Yüce and Morlock 2016).

1.3.4 HPTLC acoplado a espectrometría de masas (HPTLC-MS)

El acoplamiento entre HPTLC y MS ha requerido un mayor esfuerzo que para técnicas que utilizan columnas, y la asociación se ha logrado realizar mediante interfaces basadas en elución o desorción. En estos análisis el sistema MS entrega información más clara con una menor cantidad interferentes de la matriz, ya que solo las zonas de compuestos activos se transfieren. Este minimalismo en el registro de datos relevantes solamente, facilita el almacenamiento y manejo de datos, evita los retos actuales de big data (Yüce and Morlock 2016).

Respecto a las técnicas basadas en la elución varios han sido los intentos por acoplar de manera exitosa dos fases diferentes, por un lado, una placa cromatográfica plana y estática, y por otro lado una fase en flujo como son las interfaces de ionización por electrospray (ESI), ionización química a presión atmosférica (APCI) y fotoionización a presión atmosférica (APPI). Dentro de estas investigaciones en la última década se ha destacado la versatilidad alcanzada por interfaces basadas en cabezal de elución (*elution head-based interfaces*), llegando a una interfaz mejorada disponible en el mercado desde el 2009 (TLC-MS interface CAMAG). Esta interfaz permite eluir con precisión; operativamente la cabeza de elución de la interfaz es ubicada herméticamente sobre la banda seleccionada para luego eluir aplicando flujo de solvente constante que permite conducir el analito a la fuente de ionización, como se ilustra en la Figura 5 (Morlock and Schwack 2010a).

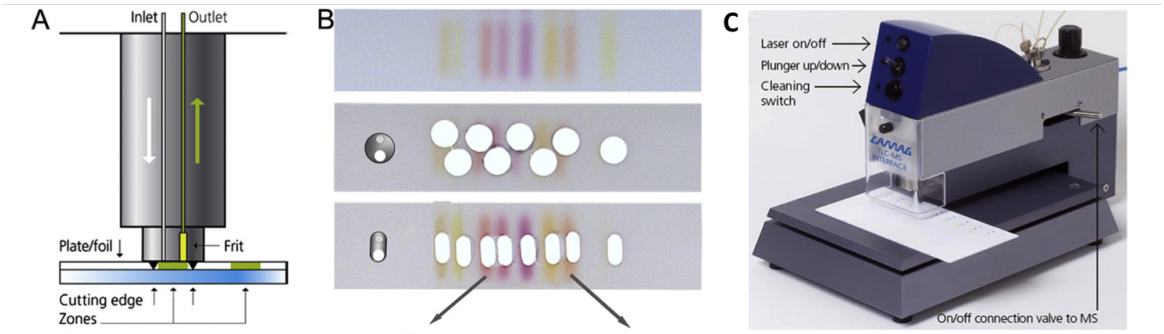

Figura 5. Interfaz TLC-MS basada en elución. (A) flujo del solvente en la cabeza de elución. (B) Placa cromatográfica luego de la elución mediante una cabeza de elución circular y oval. (C) Equipo TLC-MS Interface CAMAG® disponible comercialmente. (Adaptado de: Morlock and Schwack (2010a)).

Además, esta interfaz TLC-MS ha permitido la integración de una columna HPLC en la línea de salida de la interfaz que permite dar una mayor selectividad antes que llegue a la fuente de ionización, en caso que más de un compuesto estuviese presente en la zona activa (Morlock and Schwack 2010b). Incluso se ha reportado un análisis 2D, HPTLC/HPLC, donde la línea de salida de la interfaz es conectada a una columna HPLC-C_{18} seguido de un detector de arreglo de diodos (DAD) y ESI-TOF-MS (Mroczek 2016). Actualmente, una segunda interfaz de elución se encuentra disponible, Plate Express® TLC-MS interface, desarrollado por la empresa Advion (Azadniya and Morlock 2018).

Para el desarrollo de interfaces basadas en la desorción del analito se han utilizado bombardeos de átomos, luz láser, ionización por electrospray y corriente de gas. Entre los primeros intentos destacan el desarrollo de interfaces de bombardeo rápido de átomos (FAB) y el bombardeo de iones (SIMS), en los cuales solo se puede obtener información de una zona acotada al impacto de los átomos reduciendo la capacidad de detección y resolución, realizando esfuerzos por mejorar esta desventaja. Los métodos basados en láser son más numerosos y destaca la desorción por luz láser como la desorción/ionización láser asistida por matriz (MALDI), mucho más versátil y sensibles que los métodos anteriores, sin embargo, es limitado por requerimiento de incorporación post-cromatográfica de la matriz sobre la placa MALDI. Mediante modificaciones para la mejora de los sistemas de desorción se ha llegado a interfaces como ELDI (desorción/ionización por láser asistido por electrospray) que se basa en la utilización de laser UV pulsado de alta energía que ayuda a desorber las moléculas a presión atmosférica, DESI (desorción por ionización con electrospray) con lo que se obtuvo altas intensidades de las señales, pero los efectos de

difusión de algunos solventes causan disminución de la intensidad, y DART (análisis directo en tiempo real) basado en la desorción por corriente de gas en un estado excitado (meta-estable) que puede ser helio o nitrógeno, que apunta hacia la muestra y los iones formados son guiados al sistema de vacío logrando una resolución de 1 mm entre bandas cromatográficas. En la Figura 6 se esquematiza el funcionamiento de algunas interfaces desarrolladas (Cheng and Shiea 2015; Morlock and Schwack 2010a).

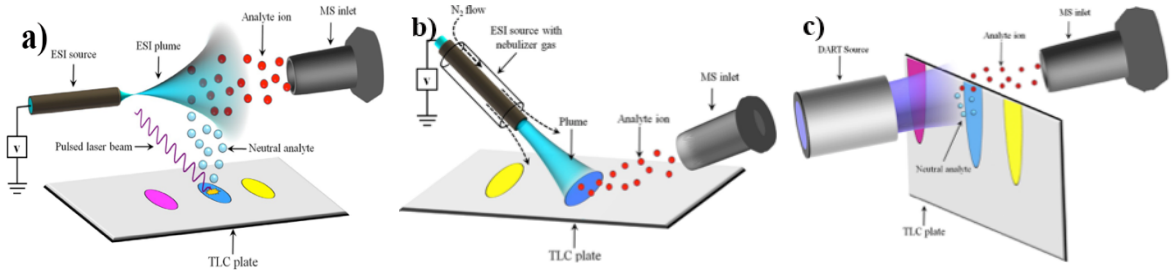

Figura 6. Análisis de compuestos químicos sobre la superficie de una placa TLC mediante (A) ELDI-MS, (B) DESI-MS y (C) DART-MS. (Adaptado de Cheng and Shiea (2015)).

De las interfaces basadas en la desorción de analito, DART-MS es actualmente la de mayor protagonismo, siendo utilizada acoplada a los análisis de efecto dirigido mediante HPTLC-bioensayo para la identificación de compuestos de interés (Móricz et al. 2018a; Móricz et al. 2018b). En DART-MS, la corriente de gas de la fuente de DART interactúa directamente con los componentes de la placa HPTLC sin distorsión de la forma del punto. Pero sin duda, su más importante ventaja radica en la posibilidad de realizar un análisis MS directamente *in situ* desde la placa donde se realizó el bioensayo, es decir, en presencia de microorganismos, medio de bioensayo o sustrato enzimático. Sin embargo, la sensibilidad es menor ya que solo se transfiere la cantidad del analito presente en la capa superior de la placa, por tanto, se necesita acoplar a un analizador de MS potente capaz de detectar bajo los nanogramos por banda (Häbe et al. 2018; Morlock 2018).

En resumen, el desarrollo de equipamiento que permiten el acoplamiento entre HPTLC y los espectrómetros de masa han dado un importante impulso al análisis de efecto dirigido, transformándose en uno de los pilares fundamentales de estas metodologías de análisis de efecto dirigido mediante HPTLC. El uso de la interfaz TLC-MS basada en la elución resulta ser más versátil, ya que permite eluir directamente al espectrómetro, eluir sobre un vial para un posterior análisis por técnicas complementarias (NMR o ATR-FTIR) y conectarla directamente sobre un sistema HPLC/UHPLC. Además, tiene mayor sensibilidad y no requiere un analizador de masa potente.

1.4 Metodologías extractivas de compuestos bioactivos

En general, el flujo de trabajo de análisis de efecto dirigido mediante HPTLC-Bioensayo-UV/Vis/FLD/MS/HRMS está altamente optimizado. La preparación de la muestra es simple, incluso puede utilizarse sin tratamiento, lo que permite tener una amplia visión sobre su composición. Sin embargo, es importante considerar que después de la detección, identificación y caracterización de los compuestos bioactivos, la siguiente etapa comprende la extracción de los compuestos biológicamente activos desde frutas o subproductos, considerando que estos pueden tener un gran valor para la industria cosmética, nutracéutica y farmacéutica. Por estos motivos es importante buscar una metodología que presente una alta eficiencia extractiva, y además considerar un enfoque global teniendo en cuenta aspectos económicos y ambientales (Etxabide et al. 2018).

Dentro de una estrategia general de recuperación de compuestos bioactivos desde fuentes vegetales generalmente se consideran cinco etapas: pretratamiento macroscópico, separación de micro y macromoléculas, extracción, purificación y aislamiento y formación de productos. La extracción ha sido considerada una de las etapas más importantes, lo que ha llevado a evaluar muchas metodologías para la obtención de compuestos específicos. La extracción con disolventes convencionales ha sido la más empleada, la cual está basada en el poder de disolución de diferentes solventes y en ocasiones la aplicación de altas temperaturas, promoviendo la transferencia de masa entre las diferentes fases del sistema. Dentro de este grupo se encuentran la extracción por maceración, convencional líquido-líquido, y sólido-líquido con Soxhlet. Sin embargo, los principales inconvenientes son el uso de grandes volumenes de disolventes orgánicos que pueden ser tóxicos para la salud humana y el medio ambiente, además del alto consumo de energía y tiempo del proceso. Por estos motivos, actualmente se realiza una nueva búsqueda de técnicas ecológicas, seguras y de bajo costo operativo para superar las desventajas de los métodos convencionales. En este aspecto, estas técnicas pueden ser clasificadas como técnicas no convencionales o emergentes, entre las que se encuentran: extracción subcrítica y supercrítica, extracción de líquidos presurizados, extracción asistida por microondas, extracción asistida por ultrasonido, extracción asistida por enzimas, extracción por cavitación a presión negativa, extracción por homogenización a altas presión y extracción por descompresión instantánea controlada, entre otros (Etxabide et al. 2018; Renard 2018). Para el desarrollo de este trabajo se otorga una mayor importancia a la extracción asistida por ultrasonido, por su accesibilidad respecto a equipamiento, y extracciones a presión y temperatura modificada, como la extracción por líquidos presurizados y fluidos

supercríticos. Las metodologías que serán aplicadas tienen como objetivo realizar una aproximación respecto a qué método presenta las mejores perspectivas para ser desarrollado a futuro.

1.4.1 Extracción asistida por ultrasonido (UAE)

Técnica aplicada principalmente sobre tejidos biológicos como es el caso de la chirimoya. El ultrasonido produce un fenómeno de cavitación acústica que implica mecanismos independientes o combinados entre fragmentación, erosión, capilaridad, detexturación y sonoporación. Todos estos procesos causan ruptura celular, alta penetración de solventes y reducción del tamaño de partículas, aumentando la eficiencia de extracción y reduciendo el tiempo del proceso. Para la extracción de compuestos bioactivos se trabaja principalmente en una frecuencia de onda que varía entre 20 y 40 kHz, y se reporta su aplicación para la extracción de polifenoles, flavonoides, flavonoles, azúcares, minerales y carotenoides, polisacáridos, entre otros (Moreira et al. 2019; Wijngaard et al. 2012).

1.4.2 Extracción mediante fluidos supercríticos (SFE)

La SFE es una de las técnicas más empleadas basada en el uso de fluidos comprimidos. Corresponde a una técnica verde que permite llevar a cabo la extracción a bajas temperaturas, disminuyendo la degradación térmica, evita la oxidación y el uso disolventes orgánicos tóxicos. Este proceso de extracción corresponde a una operación de transferencia de materia llevada a cabo en condiciones de presión y temperatura superiores al punto crítico del disolvente, el cual alcanza propiedades similares a los líquidos (baja tensión superficial) y a su vez propiedades similares a los gases (difusividad elevada y baja viscosidad), lo que favorece la extracción. El principal solvente usado en SFE es el CO_2, cuyo punto supercrítico es de 73.8 bar y 30.9 ºC, además es de bajo costo, amigable con el medio ambiente y generalmente considerado seguro. El CO_2 es un solvente apolar por tanto puede disolver compuestos apolares o de muy baja polaridad, como aceites, grasas y ceras. Sin embargo, su polaridad se puede modificar mediante la adición de co-solventes, que permitan dar una mayor polaridad como metanol o etanol. Los fluidos supercríticos tienen un buen grado de selectividad modificando sus parámetros como presión y temperatura, además posterior a la descomprensión del fluido se produce la separación instantánea entre solvente principal (CO_2) y el analito (Etxabide et al. 2018; Herrero et al. 2010). La optimización de las condiciones operativas de una SFE mejora el rendimiento extractivo y recuperación de compuestos bioactivos. Las principales variables a modificar

son temperatura, presión, tiempo, tipo y porcentaje de co-solventes, y tamaño de muestra. De este modo, SFE ha sido ampliamente utilizada para valorar los subproductos de la industria alimentaria, como carotenoides desde subproductos del tomate (piel y orujo); y para la obtención de compuestos bioactivos desde fuentes vegetales, como compuestos fenólicos, alcaloides, aceites esenciales, fitoesteroles, terpenoides, etc. (Herrero et al. 2010)

1.4.3 Extracción mediante líquidos presurizados (PLE)

Junto con SFE, PLE, conocida también como extracción acelerada por solventes, extracción con solventes presurizados o extracción con solventes subcríticos, ha sido empleada para extraer compuestos polifenólicos como flavonoides, ácidos fenólicos y antocianinas, desde una gran variedad de fuentes vegetales. Esta metodología permite aplicar temperaturas sobre el punto de ebullición del solvente debido a la aplicación de altas presiones, lo que permite incrementar la transferencia de masa y flujo de extracción, traduciéndose en una mayor capacidad de solubilizar analitos, mayor difusión, mejorar disrupción soluto-matriz y disminuye la viscosidad del solvente. La extracción de fitoquímicos se lleva a cabo en un corto periodo de tiempo (5 a 20 min) empleando temperatura (40.0 a 200.0ºC), presión (5.0 a 20.0 MPa) y un solvente adecuado (agua, metanol, etilacetato) (Etxabide et al. 2018; Wijngaard et al. 2012).

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2. HIPÓTESIS Y OBJETIVOS

2.1 Planteamiento del problema

Los compuestos bioactivos de origen natural son actualmente bien valorados por su posible aplicación en la promoción de la salud y prevención de desórdenes fisiológicos. El potencial uso terapéutico de los compuestos bioactivos de origen natural tiene importantes antecedentes, por ejemplo, la fisostigmina y galantamina son principios activos usados para el tratamiento de la enfermedad de Alzheimer, provienen de las plantas *Physostigma venenosum* y *Galanthus woronowii* respectivamente. Entonces los compuestos bioactivos representan una perspectiva real, pero no exclusiva, para disminuir la alta prevalencia de enfermedades crónicas no transmisibles (ECNT).

Para la determinación de compuestos bioactivos se pueden utilizar diferentes metodologías. Los bioensayos *in vitro* comunes pueden detectar una bioactividad, pero no pueden identificar cuáles son los compuestos responsables. Considerando que actualmente no es suficiente determinar la actividad de un extracto, sino identificar los compuestos químicos que la poseen, cromatografía planar tiene la ventaja de realizar una separación previa al bioensayo haciendo la identificación un trabajo más acotado, sin necesidad de compuestos de referencia. Conociendo ésta y otras ventajas descritas que presenta la técnica HPTLC con biodetección es posible desarrollar metodologías analíticas capaces de determinar e identificar compuestos con potencial actividad biológica de manera directa y simultánea en un corto periodo de tiempo. La importancia del desarrollo de esta metodología radica en que es fácilmente reproducible y repetible, de manera que la identificación de la bioactividad no está estrictamente ligada al extracto de una especie sino a cualquier extracto que posea la bioactividad y pueda ser identificada en el ensayo. Es así como una vez desarrollada la metodología podrá ser aplicada en frutas de diferentes orígenes geográficos y no exclusivamente sobre la que se implementó el método, alcanzando en parte una universalidad analítica, siendo un importante aporte al desarrollo científico.

Este trabajo determinó compuestos bioactivos orientados a contribuir en el tratamiento de enfermedades con alta prevalencia enfocándose en análisis de efecto dirigido para la determinación de compuestos antioxidantes que puedan contribuir al tratamiento de la obesidad y enfermedades cardiovasculares; compuestos inhibidores de la α-glucosidasa para la diabetes mellitus; y compuestos inhibidores de la acetilcolinesterasa que potencialmente puedan contribuir en el tratamiento de la enfermedad de Alzheimer.

Dentro de las frutas cultivadas en Chile la chirimoya es considerada como frutal menor,

representando una importante oportunidad de generar una fruta con un alto valor comercial a partir de su biofuncionalidad. De las especies de *Annona*, la chirimoya es la que tiene menos investigaciones que puedan acreditar sus beneficiosos, sin embargo, los antecedentes respecto a similares especies demuestran una adecuada variedad de bioactividad. Además, la agroindustria de la chirimoya produce diferentes productos como pulpa de fruta envasada, helados y jugos, generando subproductos como piel y semillas que podrían tener un importante valor biológico y comercial, aportando a la revitalización de la industria mediante la extracción de compuestos con un mayor valor comercial.

2.2 Hipótesis

Conociendo los antecedentes y estableciendo la importancia del desarrollo de metodologías analíticas que contribuyan a la determinación de compuestos bioactivos útiles para el tratamiento de ECNT, este trabajo postula la siguiente hipótesis de trabajo:

"El desarrollo de una plataforma analítica de HPTLC-Bioensayo-MS de efecto dirigido permite la determinación e identificación de compuestos bioactivos *in situ* de chirimoya (*Annona cherimola* Mill.)"

2.3 Objetivos

2.3.1 Objetivo general

Establecer una plataforma analítica HPTLC-Bioensayo-MS de efecto dirigido para determinar e identificar compuestos bioactivos presentes en chirimoya (*Annona cherimola* Mill.).

2.3.2 Objetivos específicos

1. Determinar la bioactividad de las principales variedades de chirimoyas cultivadas en Chile a través de metodologías HPTLC-Bioensayo capaces de evaluar la capacidad antioxidante (ORAC) y actividad inhibitoria de las enzimas angiotensina, α-glucosidasa y acetilcolinesterasa.
2. Identificar los compuestos con mayor actividad en extractos de chirimoya a través del acoplamiento HPTLC-MS y HPTLC-NMR.
3. Establecer un proceso extractivo que permita la obtención de un extracto a base de chirimoya con alta concentración de compuestos bioactivos.

3. ESTRATEGIA ANALÍTICA

La estrategia analítica establecida para llevar a cabo los objetivos planteados en el presente trabajo puede resumirse en tres procedimientos generales. Primero se desarrolla una metodología HPTLC-bioensayo de efecto dirigido. En segundo paso la metodología fue aplicada para detectar e identificar compuestos bioactivos en chirimoya. Finalmente, el tercer paso consistió en la aplicación de un método extractivo no convencional para la obtención de un extracto rico en compuestos bioactivos. El flujo de trabajo aplicado en el presenta trabajo se esquematiza en la Figura 7.

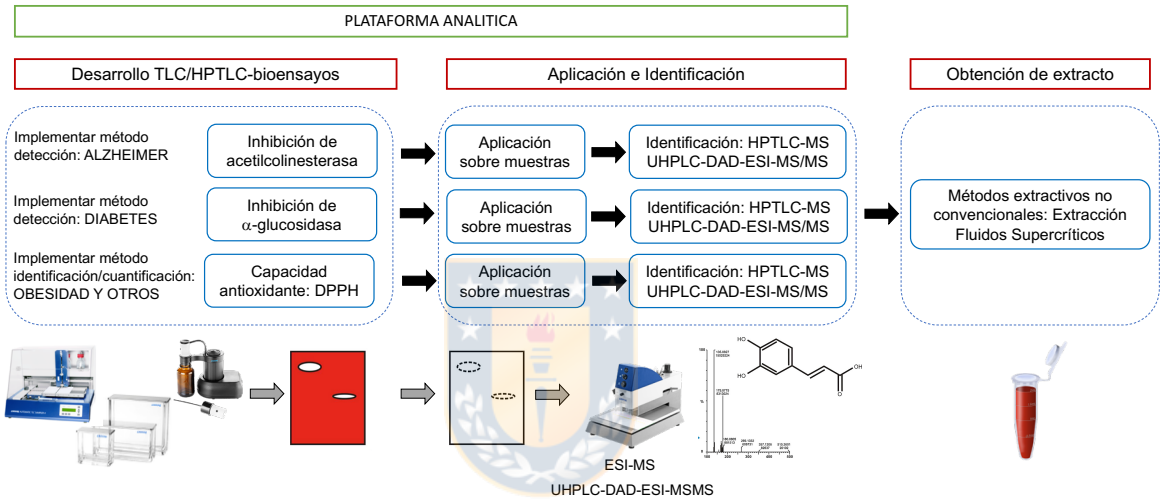

Figura 1. Esquema resumen de la estrategia analítica aplicada.

3.1 Desarrollo de una metodología HPTLC-bioensayo-MS para inhibidores de acetilcolinesterasa (AChE) y α-glucosidasa.

Para la detección de compuestos inhibidores de AChE varias condiciones de la metodología HPTLC-bioensayo fueron modificados para obtener un adecuada intensidad y definición de las bandas activas. Aplicando un diseño univariado se evaluó concentración enzimática, concentración de sustrato, concentración de revelador (sal de Fast Blue B) y tiempo de incubación. Mediante la metodología implementada se evaluaron extractos de piel, pulpa y semilla de chirimoya. La identificación se llevó a cabo en tres pasos, primero usando reactivos de derivatización para identificar posibles familias de compuestos, segundo un análisis directo TLC-MS para obtener la masa molecular, y tercero un aislamiento de los compuestos para un análisis más detallado mediante UHPLC-DAD-ESI-MS/MS.

3.2 Evaluación de una metodología HPTLC-DPPH-MS para la identificación de compuestos antioxidantes y cuantificación de la actividad antioxidante**.**

La placa HPTLC se fue inmersa en una solución de DPPH, para detectar los compuestos antioxidantes como zonas amarillo pálido. Para la cuantificación de la actividad antioxidante de las bandas activas fueron evaluados tres procedimientos: espectrofotodensitometría en modo fluorescencia, procesamiento de imagen digital y una combinación de espectrofotodensitometría en modo absorbancia/procesamiento de imagen digital. Muestras de piel y semilla fueron evaluadas, usando ácido cafeico como estándar. Una identificación preliminar se realizó mediante un análisis *in situ*, eluyendo directamente las bandas desde la placa al espectrómetro de masas usando una interfaz TLC-MS.

3.3 Extracción de compuestos bioactivos mediante fluidos supercríticos.

Previo a la optimización, el uso y tipo de co-disolvente fueron evaluados. Luego las condiciones operativas fueron optimizadas mediante un diseño experimental estudiando tres factores: temperatura, presión y porcentaje de co-disolvente. Bajo una metodología de extracción por fluidos supercríticos optimizada fueron obtenidos extractos de piel de chirimoya. La actividad de los extractos fue estudiada mediante ensayos espectrofotométricos ORAC, inhibición de AChE y α-glucosidasa. La presencia de compuestos bioactivos fue determinada y cuantificada mediante UHPLC-ESI-MS.

CAPÍTULO II: *Resultados y Discusión*

1. DETECTION AND IDENTIFICATION OF ACETYLCHOLINESTERASE INHIBITORS IN *Annona cherimola* Mill. BY EFFECT-DIRECTED ANALYSIS USING THIN-LAYER CHROMATOGRAPHY-BIOASSAY-MASS SPECTROMETRY

Detection and identification of acetylcholinesterase inhibitors in *Annona cherimola* Mill. by effect-directed analysis using thin layer chromatography-bioassay-mass spectrometry

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ABSTRACT

Introduction. Acetylcholinesterase (AChE) inhibitors are considered an important strategy in the treatment of neurological disorders such as Alzheimer's disease. A simple and fast planar chromatography-bioassay methodology has been established to detect bioactive molecules in cherimoya fruit.

Objective. Detect and identify AChE inhibitors in cherimoya by high-performance thin-layer chromatography (HPTLC)-bioassay-mass spectrometry (MS) and related techniques.

Methodology. Effect-directed analysis by planar chromatography-bioassay-mass spectrometry was applied to detect and identify AChE inhibitors in pulp, peel and cherimoya seed. Bioassay was optimized establishing the following conditions: enzymatic solution (1.0 U mL^{-1}), 1-naphtyl acetate substrate (1.5 mg mL^{-1}) and Fast Blue B salt (1.0 mg mL^{-1}). TLC/MS interface was used to directly eluted the active zones into mass spectrometer or to a micro-vial for further off-line studies.

Results. Two AChE inhibitory bands were detected in peel extracts. An analysis via HPTLC-MS and high-performance liquid chromatography (HPLC-DAD-MS/MS) allowed to characterize three potential AChE inhibitors: anonaine (*m/z* 266 $[M+H]^+$; UV λ_{max} 269.6 nm), glaucine (*m/z* 256 $[M+H]^+$; UV λ_{max} 282.9 and 300.6 nm) and xylopine (*m/z* 296 $[M+H]^+$; UV λ_{max} 278.5 nm).

Conclusions. The application of this optimized high throughput method allowed to establish the presence of three potential AChE inhibitors in cherimoya peel. For the first time AChE inhibitory capacity of these alkaloids is reported.

Keywords: alkaloids, anonaine, glaucine, HPTLC, HPLC, xylopine

1. INTRODUCTION

Worldwide, nearly 46.8 million people have Alzheimer's disease (AD) or related dementia, pathologies that generated an estimated economic impact of US$ 818 billions in 2015[1]. There has been extensively proposed that the presence of beta-amyloid (Aβ) plaques, neurofibrillary tangles and degeneration/atrophy of basal forebrain cholinergic neurons causes a reduction of cholinergic activity leading to the cognitive impairment[2]. Thus, acetylcholinesterase (AChE) inhibitors (AChEi) are considered as an important strategy in the treatment of neurological disorders such as AD or senile dementia. These drugs inhibit the action of AChE enzyme decreasing the breakdown of acetylcholine and therefore increasing cholinergic neurotransmission[3]. This kind of inhibitory molecules can be found in plant kingdom, for example the presence of relevant AChEi has been confirmed in plants such as galantamine obtained from Galanthus species, which is used to treat symptoms of mild to moderate dementia in AD. The search of bioactive compounds from plants leads also to discover of new and interesting pharmacophores, from which can be developed semi-synthetic analogues, e.g. physostigmine was isolated from Physostigma venenosum and its chemical molecular base provided a template for the development of rivastigmine improving the pharmacokinetic parameters and efficacy[4, 5]. Thus, plants and fruits have demonstrated to be a relevant source of bioactive compounds that could contribute to reduce the risk of suffering or treat some degenerative diseases[6]. *Annona cherimola* Mill. (cherimoya) is a tropical fruit with exceptional taste and strong antioxidant activity attributed to its phenolic content. These types of bioactive molecules might contribute to reduce the risk of suffering diseases associated with oxidative stress such as cancer, atherosclerosis and neurodegenerative diseases[7-9]. Additionally, alkaloids, with well-known activity in the treatment of AD[5] have also been identified in cherimoya[10]. Discovery of AChEi in nature requires suitable methods that allow a rapid bioactive molecule detection and identification. A very effective strategy is the coupling of chromatography and biological detection or specific bioassay pursuing an effect-directed analysis (EDA). This approach is particularly useful for detection of compounds with enzyme inhibitory activity[11]. In this way, for AChEi detection in plants and foods the technique of choice is high-performance thin-layer chromatography (HPTLC) coupled in situ (over the plate) with bioassay detection. Two HPTLC-bioassay methods have been reported to evaluate AChEi. The first uses acetylthiocholine iodine substrate Ellman's reagent to generate colorless (white) inhibitory bands against a yellow background;[12] and the second one uses 1-naphthyl acetate as

substrate and fast blue B salt to generate colorless (white) bands against purple background. The latter method is preferred because presents higher contrast and detection capacity[13, 14]. The objective of the present work was to detect and identify AChEi in cherimoya by HPTLC-bioassay-mass spectrometry and related techniques.

2. MATERIAL AND METHODS

2.1 Reagents and solvents

Acetylcholinesterase from Electrophorus *electricus* (electric eel) (E.C number 3.1.17), 1-naphthyl acetate, fast blue B salt, bovine serum albumin (BSA) and donepezil hydrochloride (pharmaceutical secondary standard) were purchased from Sigma (St. Louis, MO, USA). Ultrapure water (18.2 MΩ cm) was produced by means of Simplicity system from Millipore (Bedfrod, MA, USA). Dichloromethane (DCM), chloroform, methanol, ethanol, acetone, hexane, ammonia (25%), acetic acid (glacial) and hydrochloric acid (all analytical grade), tris(hydroxymethyl)aminomethane (TRIS) (>99.7%), bismuth (III) nitrate alkaline and potassium iodide were purchased from Merck (Darmstadt, Germany). Glaucine standard was a kindly donated by South American Phytochemical (SAPHYCHEM, Santiago, Chile). Chromatography was performed on 10 x 10 cm HPTLC plates from Merck, coated with 200 µm layer of silica gel 60 F_{254}. Enzymatic solution (30 U mL^{-1}) was dissolved in 50 mM Tris-HCl buffer at pH 7.8 containing 0.1% BSA to stabilize the enzyme, kept at 2ºC until its use[13].

2.2 Sample preparation

Cherimoya fruits were directly purchased from fruit store. This fruit is cultivated in the central zone of Chile, from IV to V region (29°02'27.3"S 70°36'36.0"W to 33°57'19.0"S 71°43'25.7"W). Cherimoya peel, pulp and seeds were manually separated and freeze dried for 36 hours at -55ºC using a Martin Christ (Osterode am Harz, Germany) Alpha 1-2 LD plus freeze-dryer. Dried samples were milled and stored at -18ºC until its use. Extraction procedure was carried out using a Ningbo Xinzhi Biotechnology Co. (Ningbo, China) SB5200DT ultrasonic bath. Bioactive molecules were extracted twice from 2.0 g of dried sample with 20 mL of solvent (methanol or DCM) during 30 minutes at frequency of 25 kHz and 30ºC. Thereafter, extracts were filtrated through Whatman (Clifton, NJ, USA) filter paper Nº4 and concentrated to ca. 3 mL on a Büchi (Flawil, Switzerland) rotary evaporator and

kept at -18 ºC until use. The extracts were filtered through 13 mm PVDF syringe filter (0.22 µm) just before HPTLC application.

2.3 Chromatography

Samples and standard solutions were applied with Automatic TLC Sampler 4 (ATS4, CAMAG, Muttenz, Switzerland) using the following settings: band length 6 mm, track distance 10.0 mm, dosage velocity 120 nL s^{-1} and first application *x*-axis and *y*-axis at 10.0 mm. Samples application volume ranged from 5.0 to 50 µL and donepezil standard (positive control) from 2.0 and 5.0 µL (20-50 ng/band). Chromatography was performed in 10 x 10 cm twin trough chamber (CAMAG) up to a migration distance of 60 mm using a mixture of chloroform, methanol and ethyl acetate (80:14:6 v/v/v) as mobile phase. Extracts were applied in triplicate dividing the HPLTC plate in three sections: the first section was used for bioassay, the second section for derivatization and the third for mass spectrometry analysis.

2.4 HPTLC-AChE bioassay

HPTLC-bioassay methodology was implemented following the method proposed by Mroczek[15]. Chromatography was carried out using a mobile phase that contains the enzyme substrate 1-naphthyl acetate (1.5 mg mL^{-1}). After separation, mobile phase was removed on CAMAG TLC plate heater, and enzymatic solution (1 U mL^{-1} in 50 mM TRIS-hydrochloric acid buffers at pH 7.8) was sprayed by means of TLC sprayer (Merck). Liquid excess was quickly removed using a hair dryer, but not completely in order to keep the enzyme active. Thereafter the plate was incubated at 37ºC for 10 min over a horizontal stand inside a closed plastic tank containing ca. 50 mL of ultrapure water for humidity atmosphere. Immediately after, a fast blue B salt aqueous solution (1.0 mg mL^{-1}), freshly prepared, was sprayed onto the plate to obtain a purple background, which contrasts with colorless inhibition zones (ca. 2 min). Donepezil (20-50 ng/band) was applied in parallel. Plate image was documented under illumination (reflectance) with white light by means of CAMAG Reprostar 3 documentation system. All instruments were controlled via CAMAG WinCats 1.4.7 software.

2.5 Chemical identification of AChE inhibitors

The second plate section was sprayed with Dragendorff´s reagent (Munier Macheboeuf modification) using TLC sprayer and dried at room temperature. Solution A reagent was

prepared by dissolving 0.85 g of bismuth (III) nitrate alkaline in 10 mL of acetic acid (glacial) and completed to 50 mL with distilled water; solution B was prepared with 8 g of potassium iodide in 20 mL of distilled water. Ten milliliters of solution A, 10 mL solution B, 20 mL acetic acid (glacial) and 10 mL of distilled water were mixed to produce Dragendorff's reagent. Alkaloids appear as orange bands after spraying[16].

2.6 Mass spectrometry identification of AChE inhibitors

Bands of interest were selected and marked using a soft pencil on the HPTLC plate based on retention factor (R_F) visualized under CAMAG cabinet. Inhibitory compounds were mainly identified in two steps: first, bands of interest were directly eluted and analyzed by mass spectrometry (MS); and second, bands were eluted to a micro-vial for UHPLC-DAD-ESI-MS/MS analysis. For the first step, the selected bands were eluted from plate to MS by means of CAMAG TLC-MS interface using a mixture of methanol and acetonitrile (50:50% v/v) at a flow rate of 0.2 mL min^{-1} for 60 seconds. Shimadzu (Kyoto, Japan) LCMS 8030 triple quadrupole mass spectrometer with electrospray ionization (ESI) source was operated applying the following conditions: ESI in positive mode, capillary voltage 3.0 kV, nebulizing gas (N_2) 3 L min^{-1}, drying gas (N_2) 15 L min^{-1}, DL temperature 250ºC, and block temperature 400ºC. Mass spectra were acquired in full scan mode between *m/z* values of 50 and 1000. Plate background signals were subtracted for each analysis. Data were acquired and recorded by Shimadzu LabSolution software version 5.51. In the second step, bioactive bands were eluted into a micro-vial using TLC-MS interface and concentrate under nitrogen stream. Reconstituted solution was analyzed by means of Shimadzu UHPLC-DAD-ESI-MS/MS system composed 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). Chromatography was carried out on Kromasil (Bohus, Sweden) 100-5C_{18} (250 x 4.6 mm; 5 µm) column, using a binary mobile phase composed of 0.01% v/v formic acid (A) and methanol (B), at a flow rate of 0.4 mL min^{-1}. The following gradient program was used: 0-3 min, 18-20% (B); 3-4 min, 20-30% (B); 4-6 min, 30% (B) (isocratic step); 6-10 min, 30-80% (B); 10-14 min, 80% (B) (isocratic step); 14-16 min, 80-18% (B); and 16-20 min 18-18% (B) (column conditioning). MS/MS analysis in product ion scan mode was performed using argon as collision gas and voltage of -35 V.

3. RESULTS AND DISCUSSION

3.1 HPTLC-bioassay

The method proposed by Mroczek[15] was modified to enhance the detection of AChEi. An univariate optimization was performed to qualitatively increase the contrast between inhibitory bands and plate background color. From the factors that possible affect the enzymatic reaction and detection, four factors were chosen: 1-naphthyl acetate concentration (0.5-2.5 mg mL^{-1}), enzyme concentration (1.0-2.5 U mL^{-1}), incubation time (5-30 min) and Fast Blue B salt concentration (0.5-2.5 mg mL^{-1}). Following the previous work of Mroczek[15], substrate (1.5 mg mL^{-1}) was added in the mobile phase to obtain a homogeneous contrast as well as higher detection capability. After optimization, the following conditions were established: a 1-naphthyl acetate concentration of 1.5 mg mL^{-1}, enzyme concentration of 1.0 U mL^{-1}, incubation time of 10 min and Fast Blue B salt concentration of 1.0 mg mL^{-1}. It is essential to completely remove the mobile phase after chromatographic separation, because residues of organic solvents and/or acid/base could inhibit/degrade the enzyme, which is on the most expensive item in HPTLC-bioassay. On this regard, the proposal method reduces by 3-fold the enzyme concentration reported by Mroczek[15] and 6.7-fold the one used by Marston et al[13]. The adjustment of enzyme and substrate concentration are in concordance with Yang et al[17]. Regarding incubation time, 10 min were selected because lower bands diffusion was observed, this value is in the range of those reported elsewhere[13, 15, 17]. Chromogenic reagent Fast Blue B salt was applied at 1.0 mg mL^{-1}, lower concentrations decreased purple color background, reducing the contrast with colorless inhibitory bands. Employing these optimal conditions, inhibitory bands were clearly visualized against purple background after 1 to 2 min reaction time. Compared with common assay in cuvette or microplate, this optimized method is fully compatible with organic solvents without affecting the enzymatic activity. Further, the substrate is soluble in ethanol (50 mg per mL) and it can be dissolved in most organic solvents commonly used as HPTLC mobile phases.

3.2 Evaluation of AChE inhibitory activity of cherimoya extracts

Applying the procedure described in Sample Preparation section, extracts of pulp, seed, and cherimoya peel were obtained with water, methanol, ethanol, acetone, DCM, chloroform, and hexane. A screening of AChEi was performed for each extract using the optimized HPTLC-bioassay. From all extracts, only methanolic and DCM peel extracts showed clearly

two inhibitory bands, initially named as band 1 and band 2 with R_F=0.32 and R_F=0.62 respectively (Figure 1a and 1b). Therefore, peel was chosen for the identification of new AChEi, which were localized by R_F comparison under fluorescence at 366 nm (Figure 1c), and UV at 254 nm (Figure 1d). A first structural approximation was carried out spraying several detection reagents (ninhydrin, ferric chloride and anisaldehyde solutions) on plates developed in parallel to HPTLC-bioassay. In concordance with other reports that ascribed to alkaloids the AChE inhibitory activity[5], a positive reaction for alkaloids using Dragendorff's reagent was observed (data not shown). Once the chemical group was identified, three different extraction methodologies were assayed to increase the extraction yield, i.e. liquid-liquid extraction (LLE), solid phase extraction (SPE) and ultrasound-assisted extraction (UAE). Briefly, 4.0 g of freeze-dried peel were mixed with 80 mL of methanol or DCM in a Boeco (Staufen, Germany) OS-20 orbital shaker for 2 h at 180 rpm. Thereafter extracts were filtered and divided into two parts. One part was used for extraction of alkaloids by LLE using 1% HCl and chloroform, while the second part was evaporated to dryness and then dissolved with 10 mL of 1% HCl: methanol solution (1:1 v/v). The later was used for SPE using Waters (Milford, MA, USA) Oasis MCX cartridge (6 cc) following the method reported by Chen et al[18]. Afterward, LLE and SPE extracts were dried and dissolved in 2 mL of methanol. Both extraction procedures were submitted to HPTLC-bioassay observing a high extraction efficiency only for AChEi present band 1. The low extraction observed for compounds from band 2 could be explained due to the use of an acid solution for LLE and SPE methodologies. This behavior could be explained by the lower polarity nature compared with compound(s) from band 1 and consequently more soluble in organic solvents. Therefore, an ultrasound-assisted extraction was assayed using only organic solvents. For this extraction, 2.0 g of sample was placed in 50 mL centrifuge tube and extracted twice with 20 mL methanol or DCM in an ultrasonic bath for 30 min each. Both extracts (40 mL each) were filtered and concentrated in rotary evaporator up to 3 mL approximately. HPTLC-bioassay showed a higher inhibitory activity (higher contrast) in comparison with the first two methodologies. Both solvents showed an efficient extraction, but methanolic extract had comparatively fewer interfering compounds compared with DCM extract, which was rich in oils causing detection problems. In view of these results, ultrasound-assisted extraction with methanol was chosen as an appropriate alternative to extract of AChEi from cherimoya peel.

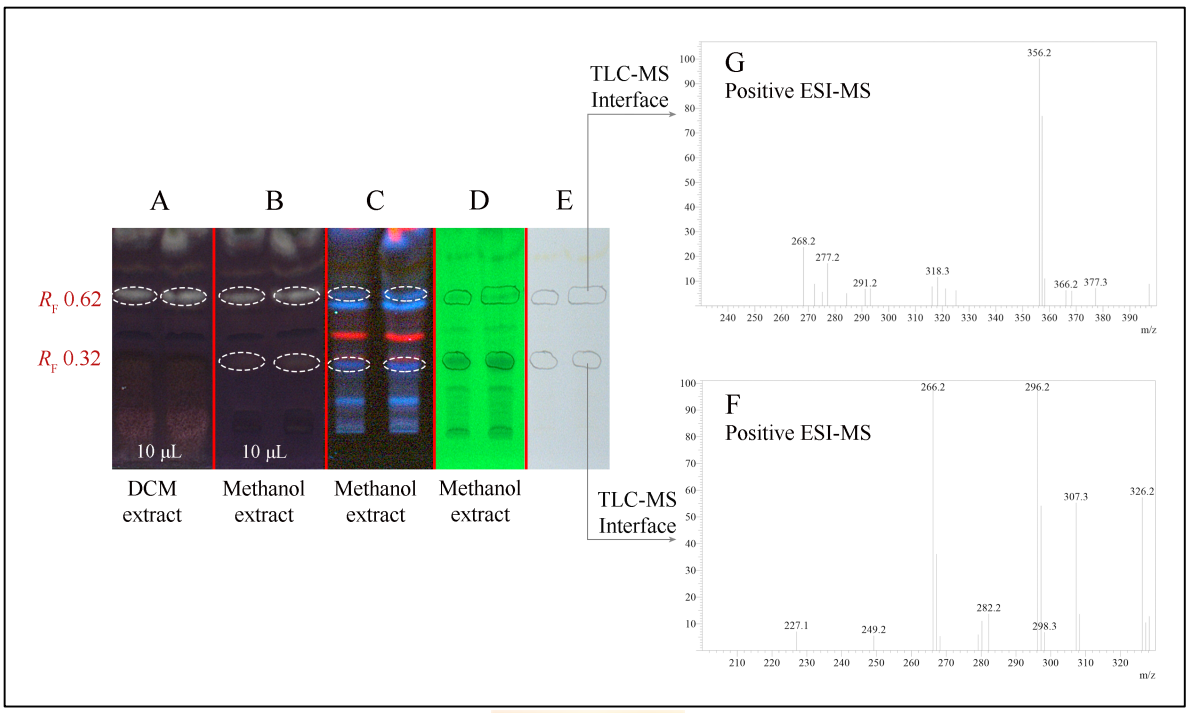

FIGURE 1. HPTLC chromatograms of methanolic and DCM extracts from cherimoya peel on silica gel 60 F_{254} plates using a mobile phase composed of chloroform-methanol-ethyl acetate (80:14:6 v/v/v). HPTLC-AChE bioassay photo-documented under white light of DCM (A) and methanolic extract (B); photo-documentation at 366 nm-fluorescence (C) and 254 nm-UV (D); selected bands marked with soft pencil for elution to MS via TLC/MS interface (E) and ESI-MS spectra of selected bands (F and G).

3.3 Identification of AChE inhibitors in cherimoya peel

HPTLC system can be easily coupled to several analytical tools for a fast compound identification. In this regard, TLC-MS interface is an important tool for unknown compounds identification because allows a direct band (compound) elution from HPTLC plate to MS interface (ESI or APCI) or the elution to micro-vial for a complementary analysis by other analytical techniques such as UPLC-ESI-qTOF and NMR[19]. Recently, it was reported a direct connection of TLC-MS interface with HPLC column for two dimensional separation coupled to diode array detection (DAD) and ESI-TOF MS[20]. In this report AChEi from cherimoya peel present in methanol extract were identified by direct analysis by means of TLC-MS interface (Figure 1e). Molecular mass data were obtained for each active zone: band 1 showed two probable compounds with *m/z* values of 266 and 296 $[M+H]^+$, named compound 1 and compound 2 respectively (Figure 1f); and band 2 the most probable m/z

values was 356 $[M+H]^+$, named as compound 3 (Figure 1g). According to some reports of alkaloids isolated from Annona species, these inhibitory compounds could correspond to the following aporphine alkaloids: compound 1 anonaine (*m/z* 266 $[M+H]^+$), compound 2 norlaureline or xylopine (both with *m/z* 296 $[M+H]^+$), and band 2 glaucine (*m/z* values: 356 $[M+H]^+$)[21-23]. Figure 2 shows the chemical structures of proposed compounds and similarity between norlaureline and xylopine, which just differ in the position of methoxy group. To obtain more structural information, bioactive compounds were isolated applying two methods. The first one consisted in eluting to a micro-vial more than 300 active bands selected by R_F using the conditions described in Chromatography section. The eluates were evaporated to dryness and dissolved in 1.5 mL of methanol (ca. 4-fold concentrate).

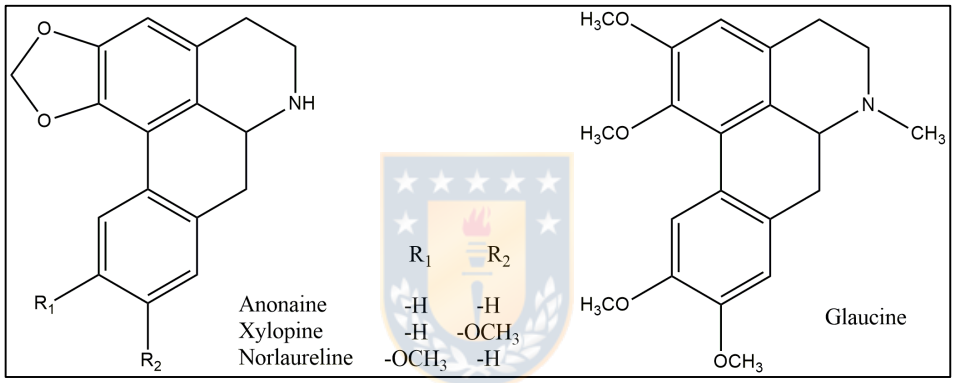

FIGURE 2. Chemical structures of AChEi found in cherimoya peel.

The second alternative was to apply the sample on HPTLC plates (10x10 cm) in lines of 8 cm using ATS4 system to scrape out de interesting band, thereafter the active compounds were extracted with methanol: chloroform mixture (1:1 v/v), evaporated to dryness and dissolved in 1.5 mL of methanol (ca. 4-fold concentrate). Even when both isolation/elution methods were suitable for liquid chromatography analysis, TLC-MS interface method showed higher selectivity. Preliminary assignment of AChEi based on the HPTLC-MS analysis was confirmed by UHPLC-DAD-ESI-MS/MS. Compounds were characterized by comparison of their mass spectra (fragment pattern) with those found in literature. Compound 1 showed the following fragment pattern: *m/z* 249 $[M+H-17]^+$, 219 $[M+H-47]^+$, 201 $[M+H-65]^+$, 191 $[M+H-75]^+$ and 178 $[M+H-88]^+$, which are consistent with anonaine (Figure 3a)[18, 24]. According to registered fragment patterns, *m/z* 279 $[M+H-17]^+$, 249 $[M+H-47]^+$, 234 $[M+H-62]^+$, 221 $[M+H-75]^+$ 206 $[M+H-90]^+$ and 178 $[M+H-118]^+$, the potential candidate for compound 2 was xylopine (Figure 3b).[23, 25] These fragment patterns were less

concordant with reported fragment patterns for norlaureline.[26] In band 2 only compound 3 was detected and it was identified as glaucine, the fragment patterns: 294 $[M+H-62]^+$, 279 $[M+H-77]^+$, 251 $[M+H-105]^+$, and 236 $[M+H-120]^+$, are in agreement with previous reports[27] (Figure 3c) and with glaucine standard spectrum (data not shown). Additional structural information was obtained from UHPLC chromatogram and UV spectrum (Figure 4). Compound 1 UV λ_{max} were 235, 270 and 308 nm, which are consistent with reported values for anonaine[28, 29]. Compound 2 showed UV λ_{max} at 238, 278 and 320 nm were in concordance with UV spectrum of norlaureline[26] and xylopine[23] (Figure 4a). Minimal variations in UV λ_{max} values could be due to the acid mobile phase. According to reported UV λ_{max}, compounds 3 with 282 and 301 nm matched with glaucine spectrum[30], further, compound 3 was compared with glaucine standard showing the same UV spectrum (Figure 4b). Due to the lower quantity of isolate compounds it was not possible to carry out other structural elucidation techniques such as HRMS or NMR. As reported elsewhere, isolation of these kind of compounds has shown low yields which limit a detailed structural study. Villar et al obtained 28 mg of anonaine from 15 kg of *Annona cherimola* leaves (1 mg anonaine per 536 g leaves),[31] a slight yield improvement was achieved by Chen et al with 20 mg of anonaine and 8 mg of xylopine from 4.0 kg of *Annona cherimola* stems (1 mg anonaine per 200 g stems)[23], and highest yield was reported by Hu et al reaching 14.6 mg of anonaine from 2.0 kg of *Nelumbo nucifera* Gertn. leaves (1 mg anonaine per 137 g leaves) using high-speed counter-current chromatography (HSCCC)[32]. Similar yield was reported by da Silva et al obtaining 7.1 mg of glaucine/norglaucine mixture from ca. 1 kg of *Unonopsis duckei* leaves (1 mg glaucine/norglaucine per 139 g leaves)[27]. Additionally, the isolation from peel is more complicated since only 10 g of freeze-dried peel is obtained from each cherimoya, therefore is necessary to establish an optimal extraction/isolation system/conditions. Overall, the proposed HPTLC-bioassay-MS method allowed the detection of AChE inhibitory activity of two aporphine alkaloids, anonaine and glaucine, and a potential candidate, xylopine. To the best of our knowledge this activity is reported for the first time. Although anonaine and xylopine were identified as a mixture, both would have the inhibitory action considering the aporphine alkaloids structure-activity relationship. The substituent at C-1 and C-2 position, and N atom could have a direct relation with activity, which may form an H-bond with AChE active site[33]. In this case, the substituent in both molecules are the same (Figure 2). Besides AChE inhibitory activity, these alkaloids have shown several interesting activities, e.g. anonaine presents antiplasmodial, antibacterial,

antifungal, antioxidant, anticancer, antidepression and vasorelaxant activity,[34] and glaucine has demonstrated anti-inflammatory, analgesic, antipyretic,[35] bronchodilator and antitussive effects[36]. Contrarily, no bioactivity has been ascribed so far to xylopine.

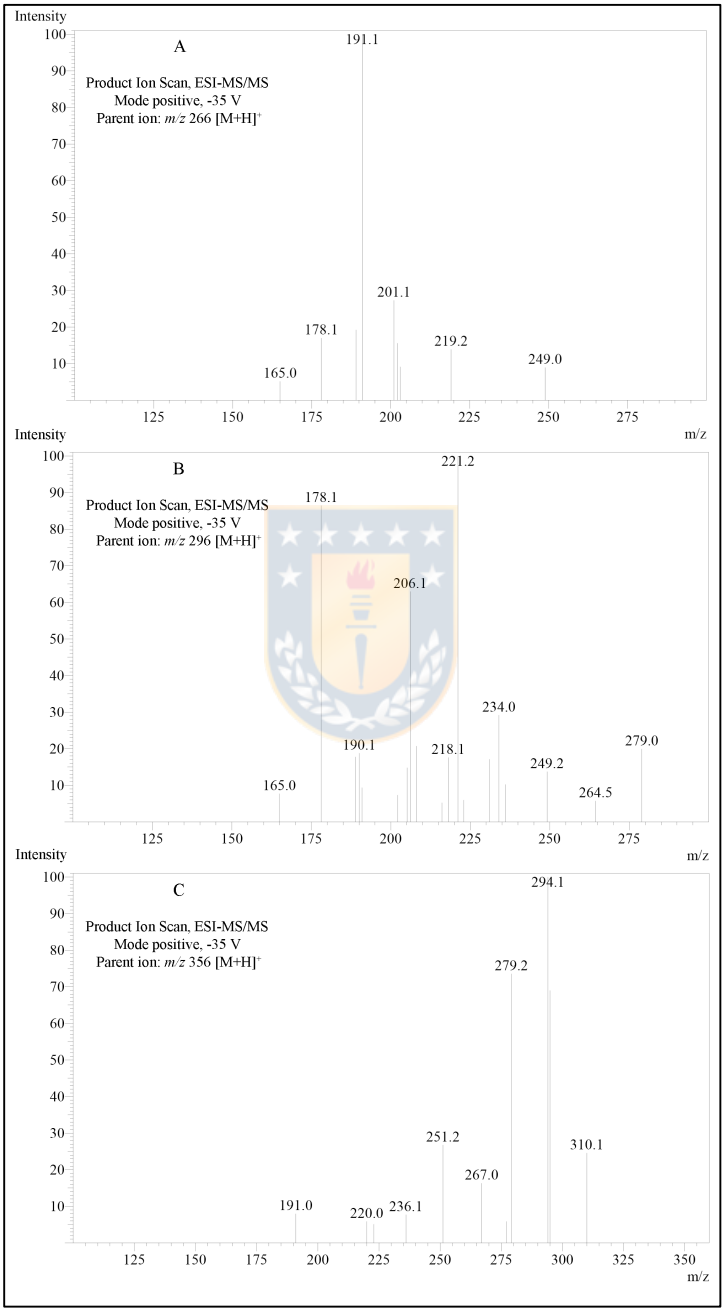

FIGURE 3. ESI-MS/MS spectra of AChEi found in cherimoya peel. Product ions of anonaine *m/z* 266 $[M-H]^+$ (A); xylopine *m/z* 296 $[M-H]^+$ (B); and glaucine *m/z* 356 $[M-H]^+$ (C).

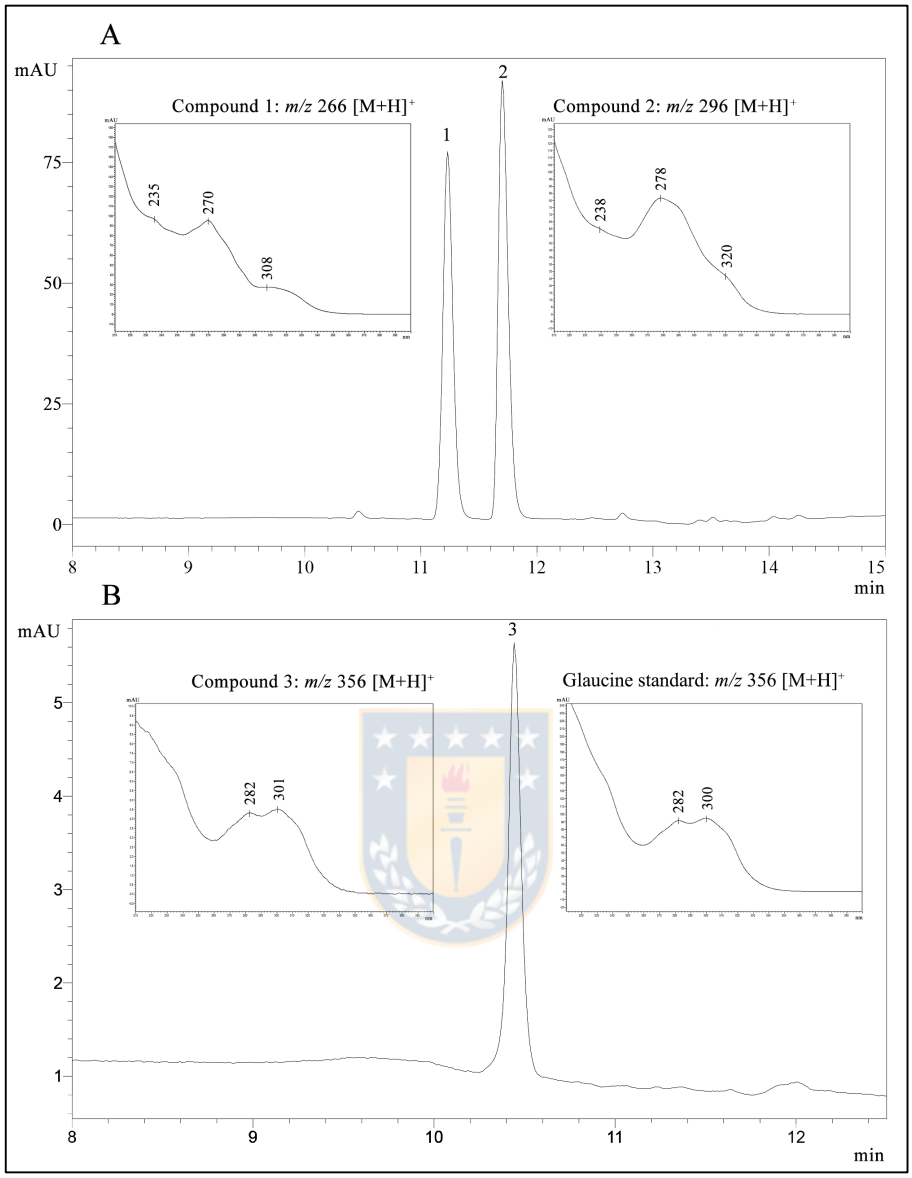

FIGURE 4. UHPLC/DAD chromatograms and UV spectra of inhibitory compounds extracted from HPTLC plate: anonaine and xylopine (A) and glaucine (B).

Overall, this work showed for the first time the evaluation of bioactive compounds in cherimoya fruit cultivated in Chile. HPTLC-bioassay conditions for AChEi detection were improved reducing the enzyme concentration and incubation time, which resulted in a fast and cost-efficient methodology. Combination of HPTLC-bioassay and parallel screening with new analytical tools like TLC-MS interface increase method versatility and applicability. Bioactive compounds were identified directly from HPTLC plate or selectively eluted into micro-vial to confirm their identities applying complementary analytical techniques.

Application of this direct-effect methodology allowed to establish the presence of two potential AChEi in cherimoya peel: anonaine and glaucine, and a possible candidate, xylopine. Although the presence of these alkaloids has been reported in *Annona* species, the present work shows for the first time the AChE inhibitory activity of these alkaloids.

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Declarations of interest: none

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2. AN IMPROVED METHOD FOR A FAST SCREENING OF α-GLUCOSIDASE INHIBITORS IN CHERIMOYA FRUIT (*Annona cherimola* Mill.) APPLYING EFFECT-DIRECTED ANALYSIS VIA HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY-BIOASSAY-MASS SPECTROMETRY

An improved method for a fast screening of α-glucosidase inhibitors in cherimoya fruit (*Annona cherimola* Mill.) applying effect-directed analysis via high performance thin-layer chromatography-bioassay-mass spectrometry

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Abstract

α-glucosidase inhibitors (AGIs) are very attractive bioactive compounds due to their therapeutic profile that includes beneficial effects over glycemic control in type 2 diabetes mellitus and viral infections. Its detection and identification in plants and fruits has gained growing attention, and certainly requires efficient screening methodologies. The objective of the present work was to develop a fast methodology by effect-directed analysis-HPTLC-bioassay-mass spectrometry for to identify AGIs in cherimoya fruit (*Annona cherimola* Mill.). Both, HPTLC and bioassay conditions were optimized accomplishing 50% and 90% reduction on enzyme concentration and incubation time, respectively. Additionally, inhibitory band contrast was also enhanced by enzyme substrate impregnation on HPTLC plate. Optimized detection conditions were established as follow: 5.0 U mL^{-1} of enzyme solution, 1.0 mg mL^{-1} of 2-naphthyl-α-D-glucopyranoside substrate, 1.0 mg mL^{-1} of Fast Blue B salt solution and 10 min as incubation time. Applying this methodology, coupled to HPTLC-MS and UHPLC-DAD-MS/MS, three AGIs were for the first time detected and identified in cherimoya peel and seeds. Compounds were tentatively assigned as phenolamides (phenylethyl cinnamides): N-*trans*-feruloyl tyramine (*m/z* 314 $[M+H]^+$; UV λ_{max} 293 and 316 nm), N-*trans*-*p*-coumaroyl tyramine (*m/z* 284 $[M+H]^+$; UV λ_{max} 296 nm) and N-*trans*-feruloyl phenethylamine (*m/z* 298 $[M+H]^+$; UV λ_{max} 288 nm). To the best of our knowledge, the presence of latter compound is reported for the first time in cherimoya.

Keywords: HPLC-bioassay, α-glucosidase inhibitors, cherimoya, mass spectrometry, phenolamides.

1. Introduction

Cherimoya (*Annona cherimola* Mill) is a tropical fruit that has become an important crop in Spain. This native fruit from Peru and Ecuador has been related with several beneficial effects, e.g. antioxidant, anti-neoplastic activities, and treatment of skin and digestive diseases. These biological activities have been mostly ascribed to polyphenol compounds [1, 2]. However, compounds responsible of the bioactivity or specific functionality assays have not been reported. Only a few articles have described hypoglycemic and anti-diabetic effect of ethanolic extracts of *Annona squamosa* leaves, a well branched tree that belongs to the same family than cherimoya (Annonaceae) [3]. Diabetes is currently a serious threat to public health and one of the most important chronic non-communicable diseases (NCDs). Population with diabetes has nearly quadrupled since 1980, from 108 to 422 million, with a global prevalence of 9%, being responsible of 1.6 millions death per year [4]. Glycosidases enzymes play a fundamental role in carbohydrate metabolism, they participate in several fundamental functions from food digestion to defense against microbial infection. Thus, search and development of glycosidase inhibitors is extremely attractive considering their therapeutic potential in the treatment of different diseases like lysosomal storage, diabetes, cancer, and bacterial and viral infections (including influenza and HIV) [5, 6]. Particularly, α-glucosidase inhibitors (AGIs) are important therapeutic agents that have been investigated as antidiabetic agents because improve glycemic control in Type 2 diabetes mellitus [6]. AGIs delay carbohydrates breakdown during digestion, slowing down its absorption, which result in a decrease of postprandial blood glucose levels, favoring glycemic control in diabetic patients [7]. Acarbose is one of the main AGIs with a demonstrated efficacy, similar to metformin [8, 9]. The most relevant AGIs have been discovered from natural sources, e.g. acarbose was obtained from fermentation broth of *Actynoplanes* strain, and miglitol and emiglitate semisynthetic derivatives were obtained from mulberry tree (*Morus spp.*) [6]. Other kind of compounds with inhibitory activity have been also isolated, including terpenes, alkaloids, quinones, flavonoids, phenols, phenylpropanoids and sterides. Hence, the potential of plants as a source of AGIs becomes rational. Promising biological activity of AGIs and their wide distribution in nature offers the opportunity to find novel compounds as possible therapeutic agents [10]. In this scenario, the hyphenation of high performance thin-layer chromatography (HPTLC) and effect direct analysis (EDA) is an excellent alternative in both domains, autography coupled with chemical analysis (e.g. DPPH) and bioautography coupled with biological system or biological evaluation (α-and β-glucosidase inhibition). This

approach coupled to different detection system such as UV, Vis, FLD, MS, and NMR, allows a rapid and unequivocal identification of bioactive compounds [11]. In this regard, two important HPTLC bioassay methods have been reported to detect AGIs. The first alternative involves the use of *p*-nitrophenyl-α-D-glucopyranoside substrate, with which colorless inhibitory bands (AGIs presence) are observed against a faint yellow background. The second alternative uses 2-naphthyl-α-D-glucopyranoside as substrate and Fast Blue B salt as derivatization reagent generating colorless inhibitory bands on a purple background [7]. The latter method is preferred because presents higher contrast and therefore better detection capacity. The objective of the present work was to develop a fast methodology to detect and identify AGIs in cherimoya fruit (*Annona cherimola* Mill.) applying effect-directed analysis via HPTLC-bioassay-mass spectrometry.

2. Materials and methods

2.1 Reagents, chemical and solvents

α-glucosidase from *Saccharomyces cerevisiae* (E.C. number 3.2.1.20), Fast Blue B salt, anhydrous magnesium sulfate (≥97.0%), ammonium formate (≥99.0%) and caffeic acid (≥98%) were purchased from Sigma (St. Louis, MO, USA). 2-naphthyl-α-D-glucopyranoside (substrate) was obtained from Glycosynth (Warrington, Cheshire, UK). Ultrapure water (18 MΩ cm) was produced using a Simplicity system from Millipore (Bedfrod, MA, USA). LC-grade acetonitrile and methanol, dichloromethane (DCM), chloroform, methanol, propanol, ethyl acetate, ammonia (25%), acetic acid (glacial) and hydrochloric acid, di-potassium hydrogen phosphate (≥99.0%), potassium dihydrogen phosphate (≥99.5%), potassium hydroxide hydrate (≥ 99.9%), sodium chloride, bismuth (III) nitrate and potassium iodide were purchased from Merck (Darmstadt, Germany). Chromatography was performed on 10 x 10 cm HPTLC plates from Merck, coated with a 200 µm layer of silica gel 60 F_{254}. Enzymatic stock solution was prepared at a concentration of 250 U mL^{-1} in 100 mM phosphate buffer, pH 7.4, divided in 100 µL aliquots and kept at -18 ºC until use.

2.2 Sample preparation

Cherimoya fruits cultivated in Chilean central zone (29°02'27.3"S 70°36'36.0"W to 33°57'19.0"S 71°43'25.7"W approximately) were purchased directly from fruit store and immediately frozen. Peel, pulp and seeds were manually separated and then freeze-dried for 36 hours at -55ºC using an Alpha 1-2 LDplus freeze-dryer from Martin Christ (Osterode

am Harz, Germany). Dried samples were milled and stored at -18ºC until its use. One gram of dried sample was vortex-mixed for 1 min with 10 mL of 1.0% NH_4OH solution into 50 mL centrifuge tube using a Thermolyne vortex (Dubuque, USA). Then, 5 mL of acetonitrile were added and the mixture was submitted to sonication for 30 min into a Ningbo Xinzhi Biotechnology Co. Ltd. SB5200DT ultrasonic bath (Ningbo, China) using a frequency of 25 kHz at 30ºC. Suspension was centrifuged for 10 min at 3992 x *g* and 20ºC using a Hettich (Tuttlingen, Germany) Universal 32R centrifuge, thereafter, filtered into 50 mL centrifuge tube and completed with acetonitrile to ca. 15 mL. Following our previous work [12], bioactive compounds were transferred to organic phase by salting-out extraction adding 4 g of $MgSO_4$ and 2 g of NaCl, vortex mixed for 90 seconds and centrifuged for 10 min at 3992 x *g* and 20ºC. Organic phase was then separated, evaporated to dryness under nitrogen stream and re-dissolved in 1.0 mL of methanol. Prior to chromatography, extracts were filtered through a 13 mm PVDF syringe filter (0.22 µm pore size).

2.3 Chromatography

Samples and standard solutions were applied by means of CAMAG (Muttenz, Switzerland) Automatic TLC Sampler 4 (ATS4), using the following settings: band length 6 mm, track distance 10.0 mm, dosage velocity 120 nL s^{-1} and first application x-axis and y-axis at 10.0 mm. Samples application volume ranged from 5.0 to 25 µL and caffeic acid standard (positive control) from 1.0 to 10.0 µL (50-500 ng/band). Chromatography was performed in 10 x 10 cm twin trough chamber (CAMAG) up to a migration distance of 70 mm for peel extracts using a mobile phase composed of chloroform, propanol and ethyl acetate (84:8:8 v/v/v) and up to 55 mm for seed extracts using a mixture of chloroform and methanol (90:10 v/v). Both extracts were applied in triplicate dividing the HPLTC plate in three sections: the first section was used for bioassay, the second section for derivatization and the third for mass spectrometry analysis.

2.4 EDA-HPTLC-α-glucosidase bioassay

HPTLC-bioassay methodology was developed following the method proposed by Simões-Pires, Hmicha, Marston and Hostettmann [7] with slight modifications. Briefly, HPTLC plates were development with a methanolic solution containing 1.0 mg mL^{-1} of 2-naphthyl-α-D-glucopyranoside (enzyme substrate) in a twin trough chamber up to a migration distance of 80 mm and dried at 60ºC for 20 min on TLC plate heater (CAMAG). After chromatography,

mobile phase was removed at 60ºC for 10 min on TLC plate heater, and then the enzymatic solution (5.0 U mL^{-1} in 100 mM phosphate buffer, pH 7.4) was sprayed on the plate using TLC sprayer (Merck). Liquid excess was quickly eliminated using a hair dryer, but not completely in order to keep the enzyme active. Plate incubation was carried out at 37ºC in a closed vessel for 10 min over a horizontal stand inside a closed plastic tank containing ca. 50 mL of ultrapure water for humidity atmosphere. Enzyme product (2-naphthol) was detected by atomization with freshly prepared Fast Blue B salt aqueous solution (1.0 mg mL^{-1}) to obtain a purple background, which contrasts with colorless inhibition bands (2 min reaction). Caffeic acid (0.25 µg per band) was applied in parallel. Plate image was documented under illumination (reflectance) with white light using CAMAG Reprostar 3 documentation system. All instruments were controlled via WinCats 1.4.7 software from CAMAG.

2.5 Chemical identification of AGIs

Second plate section was sprayed with Dragendorff's reagent (Munier Macheboeuf modification) using a TLC sprayer and dried at room temperature. Reagent was prepared according to [13]. Briefly, solution A was prepared dissolving 0.85 g of bismuth (III) nitrate alkaline in 50 mL of 20% acetic acid (glacial), and solution B was prepared with 8 g of potassium iodide in 20 mL of distilled water. Ten milliliters of solution A, 10 mL of solution B, 20 mL of acetic acid (glacial) and 10 mL of distilled water were mixed to produce Dragendorff's reagent. Alkaloids appear as orange bands after spraying.

2.6 Mass spectrometry identification of AGIs

Inhibitory bands were selected and marked using a soft pencil on the HPTLC plate based on retention factor (R_F) visualized under CAMAG cabinet (254 and 366 nm). All AGIs were tentatively identified in two complementary steps: i) bands of interest were directly eluted to and analyzed by mass spectrometry (MS); ii) bands were eluted to a micro-vial for UHPLC-DAD-ESI-MS/MS analysis. For the first step, selected bands were eluted from plate to MS by means of CAMAG TLC-MS interface assembled with oval elution head (4.0 x 2.0 mm) using a mixture of methanol and acetonitrile (50:50% v/v) 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 (ESI) source operated with the following conditions: ESI in positive mode, capillary voltage 3.0 kV, nebulizing gas (N_2) 3 L

min^{-1}, drying gas (N_2) 15 L min^{-1}, DL temperature 250ºC, and block temperature 400ºC. Mass spectra were acquired in full scan mode between *m/z* values of 50 and 1000. Plate background signals were subtracted for each analysis. Data were acquired and recorded by Shimadzu LabSolution software version 5.51. For second step, bands of interest with AGIs (colorless bands) were eluted from HPTLC plate into a micro-vial using TLC-MS interface, evaporated to dryness with N_2 and dissolved in 1.0 mL of methanol. Filtered solution (0.22 µm pore size) were analyzed by means of Shimadzu UHPLC-DAD-ESI-MS/MS system composed 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. Chromatography was carried out on Phenomenex (Torrence, USA) Kinetex 5µ XB-C18 100A (150 x 4.6 mm; 5 µm) column connected to a C_{18} guard column (4.6 ID), both set at 30ºC, using a binary mobile phase composed of acetonitrile (A) and 10 mM ammonium formate, pH 3.5 (B) at a flow rate of 0.5 mL min^{-1}. The following gradient program was used: 0-5 min, 50-70% (A); 5-8 min, 70% (A) (isocratic step); 8-12 min, 70-50% (A); and 12-15 min 50-50% (A) (column conditioning). MS/MS analysis was done using argon as collision gas and collision cell voltages from -20 and -40 V.

3. Results and discussion

3.1 Development of HPTLC-bioassay method

HPTLC-bioassay commonly requires a large enzyme amount compared with microplate methods. Additionally, enzyme-substrate ratio must be optimized in order to achieve an adequate detection capability (contrast) on HPTLC plate. In this scenario, a univariate optimization was performed considering the conditions established by Simões-Pires, Hmicha, Marston and Hostettmann [7] and the possible parameters that can affect the contrast of inhibitory bands. Accordingly, the following factors were chosen: type (100 mM of acetate or phosphate buffer) and buffer pH (6.9 or 7.4) used for enzyme-substrate reaction on the plate; α-glucosidase concentration (2.0 to 10.0 U mL^{-1}); 2-naphthyl-α-D-glucopyranoside concentration (0.75 to 1.50 mg mL^{-1}); incubation time (5-60 min); incubation temperature (25 and 37ºC); and Fast Blue B salt concentration (0.5-2.5 mg mL^{-1}). First, the effect of buffer and pH over enzymatic reaction was studied; a higher intensity of purple background was observed when enzyme was dissolved in phosphate buffer. This contrast was not affected by buffer pH (6.9 or pH 7.4), chosen the latter for further analysis. The

inclusion of 2-naphthyl-α-D-glucopyranoside in mobile phase was discarded due to its low solubility in organic solvents like chloroform and ethyl acetate. A second alternative was applied, which was to impregnate on the plate the enzyme substrate at a concentration of 1.0 mg mL^{-1} (5 mL). With this substrate amount it was possible to reduce the enzyme concentration from the original 10 U mL^{-1} to 5 U mL^{-1} without affecting the contrast between purple background color and colorless inhibition bands. This 50% reduction on enzyme concentration is very important considering that this reagent is one the most expensive item in HPTLC-bioassay. To the best of our knowledge, this is the lowest enzyme concentration reported. Regarding incubation time, after factors optimization, only 10 min were required to achieve adequate product yields (intense purple background). This incubation time is 6-fold lower than the one reported in the original method (60 min) [7]. Incubation time reduction is quite important because extensive time under humidity atmosphere causes band diffusion on the plate losing resolution, which negatively affect bioactive compound identification. Incubation temperatures assayed did not affect product yields, both ones showed adequate contrast between colorless inhibitory bands and purple background, therefore, 25 or 37ºC can be used without observable changes. Enzyme product (2-naphthol) react with Fast Blue B salt in a diazotization reaction. The minimum chromogenic reagent concentration required to achieve a suitable purple background color was 1.0 mg mL^{-1}. This concentration is consistent with the original method (1.25 mg mL^{-1}) and other works that report detection via diazotization, such as acetylcholinesterase inhibitors detection [14]. To the best of our knowledge, for the first time is presented an approach with substrate inclusion via plate impregnation, with which a homogeneous color and contrast was accomplished on the plate, improving detection capability. The latter was confirmed by the low detection limit achieved for caffeic acid (50 ng per band). Traditional methods such as cuvette or microplate assays are not compatible with organic solvents commonly used for extraction; thus, middle and nonpolar compounds are not studied or considered due to the requirement of aqueous solubility [15]. Instead, proposed HPTLC-bioassay method is compatible with several type of extraction solvent like water, methanol, chloroform, ethyl acetate, etc. without affecting enzymatic activity, allowing a rapid *in situ* detection of AGIs.

3.2 Evaluation of α-glucosidase inhibitory activity of cherimoya extracts

Interference of sugars, oils and derivates was avoided performing an extraction with aqueous solvent and a subsequent salting out step. Initially, basic and acid extractions were

assayed using 10 mL of 1% HCl or 1% NH_4OH solutions following the procedure described in sample preparation section. Although with both solvents clear extracts were obtained, using 1% NH_4OH was observed a superior inhibitory activity (higher contrast) in HPTLC-bioassay. Basic extracts of peel and seed showed an adequate chromatographic separation, and after HPTLC-bioassay, it was possible to visualize two inhibitory bands in peel extracts (R_F=0.76 and R_F=0.66, AGI-P1 and AGI-P2, respectively) and two in seed extracts (R_F=0.56 and R_F=0.44, AGI-S1 and AGI-S2, respectively, Fig. 1).

3.3 Identification of α-glucosidase inhibitor compounds in cherimoya

Currently, several modern analytical tools have been coupled to HPTLC system to facilitate compounds identification directly from the plate. In this regard, TLC-MS interface is a versatile instrument that has become an essential tool because allows a direct band (compound) elution from HPTLC plate to MS ionization source [16, 17] or the elution to micro-vial for a complementary analysis by other analytical techniques such as UHPLC-ESI-qTOF and NMR [18]. As described in chromatography section, seed and peel extracts were applied in triplicate dividing the HPLTC plate in three sections: the first section was used for bioassay, the second section for derivatization and the third for mass spectrometry analysis. A first structural approximation was carried out by spraying several detection reagents on second plate section, which was studied in parallel to HPTLC-bioassay. Inhibitory bands from seed and peel extracts were slightly positive for alkaloids reaction using Dragendorff's reagent (data not shown). Although alkaloids are important constituents in *Annona* genus, its activity as α-glucosidase inhibitors has scarcely reported in comparison with other secondary metabolites such as flavonoids and terpenes [10]. Dragendorff's reagent can also react with non-nitrogenous oxygenated compounds [19] or with secondary amines (at high concentration), which could produce weak colored bands [20]. Thus, detected compounds can also be studied among iminosugars (or polyhydroxylated alkaloids) and amide alkaloids groups, like nojirimycin or *N-p*-coumaroyl-*N'*-feruloyl putrescine respectively, which are potent AGIs [21]. MS analysis was performed on third plate section, tracks scanning via TLC-MS interface allowed the acquisition of individual mass spectra of four inhibitory bands. In seed extract, AGI-S1 showed a protonated molecule $[M+H]^+$ at *m/z* value of 314 and AGI-S2 at *m/z* value of 284. In the case of peel extract, the most probable *m/z* values were 298 and 314 for AGI-P1 and AGI-P2, respectively (Fig. 1).

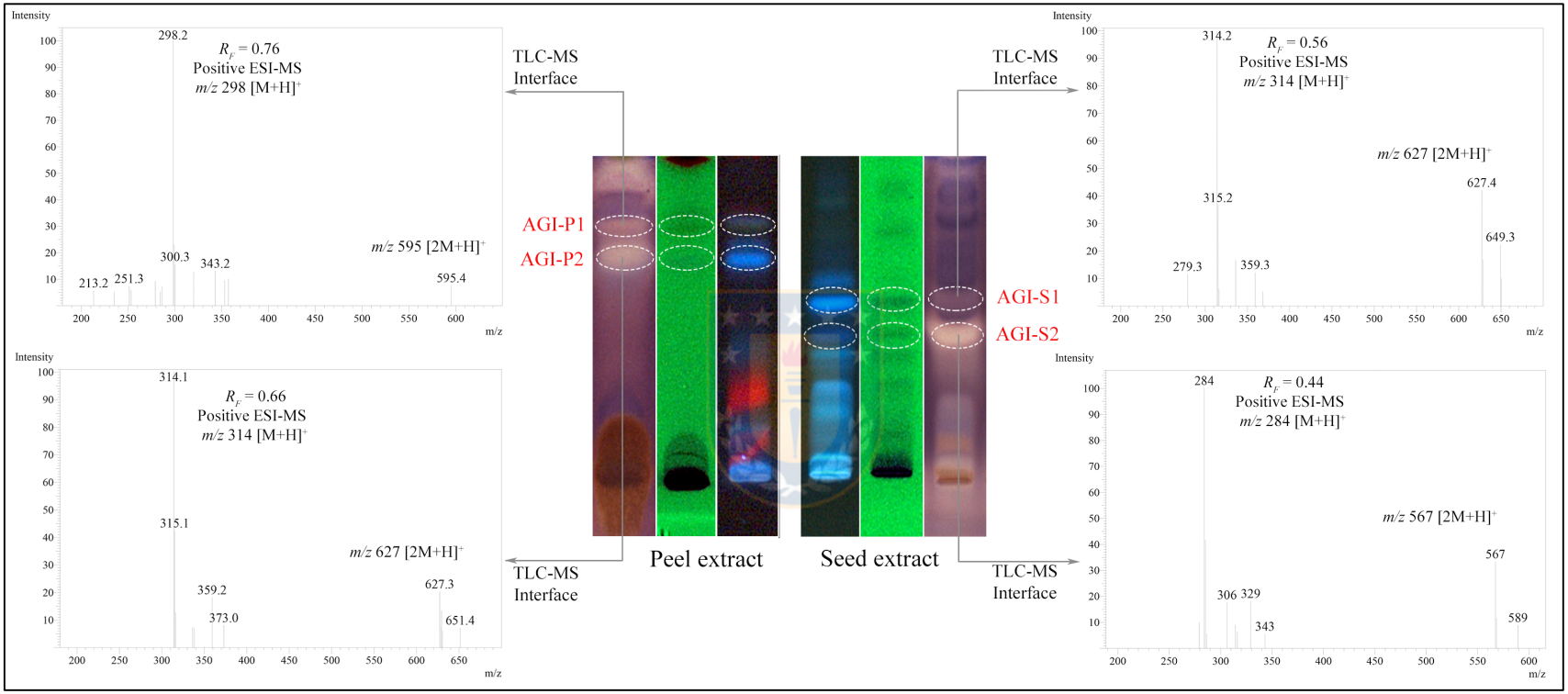

Fig. 1. HPTLC chromatograms of peel and cherimoya seed extracts on silica gel F_{254} plates. Detection of AGIs via HPTLC-bioassay photo-documented under white light, 254 nm-UV and 366 nm-fluorescence and HPTLC-ESI-MS mass spectra of selected bands.

According to some reports about compounds isolated from *Annona* species and considering the possible presence of nitrogenous groups, the potential candidates were N-*trans*-feruloyl tyramine (or moupinamide) (or isomer N-*cis*-feruloyl tyramine) assigned to AGI-S1 and AGI-P2 (both with *m/z* value of 314) and N-*trans*-p-coumaroyl tyramine for AGI-S2 (*m/z* value of 284) [22-24]. For AGI-P1 (*m/z* value of 298) two possible compounds could be assigned, stepharine (proaporphine alkaloid) [22, 24] or N-*trans*-feruloyl phenethylamine [25]. Thus, except for stepharine, the possible AGIs could be classified as amides [22, 23], hydroxycinnamic acid amides [26], phenolamides [27] or phenylethyl cinnamides [28]. Structure of the proposed phenolamides are shown in Fig. 2.

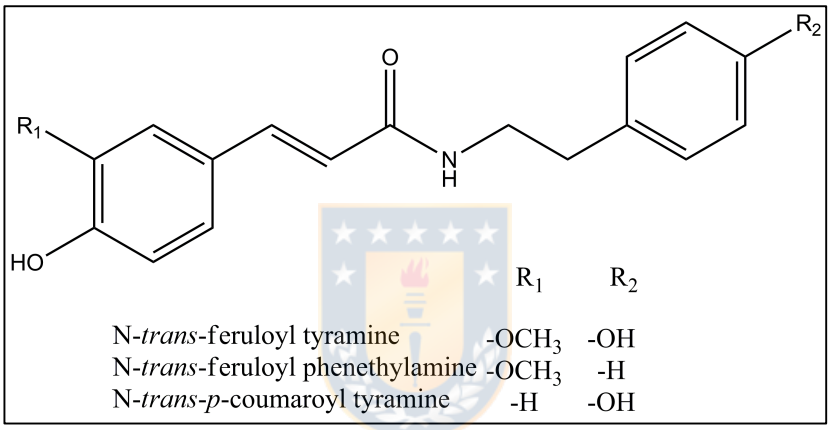

Fig. 2. Chemical structure of proposed phenolamides as AGIs.

To obtain more structural information AGIs were analyzed by UHPLC-DAD-ESI-MS/MS. For this purpose, 400 µL of extracts were applied on HPTLC plate in lines of 8 cm using ATS4. After chromatography, each AGIs were isolated from HPTLC plate, extracted twice with methanol: chloroform mixture (1:1 v/v), dried under gently nitrogen stream and dissolved in 1.0 mL of methanol. MS/MS fragmentation patterns of each AGIs compound were compared against database and literature data. Thus, it was possible to observe the same fragmentation patterns for AGI-S1 and AGI-P2 showing *m/z* values of 177.1 $[M+H-137]^+$, 145.1 $[M+H-169]^+$, 121.1 $[M+H-193]^+$, 117.1$[M+H-197]^+$ and 103.1$[M+H-211]^+$, which were consistent with *N-trans*-feruloyl tyramine (Fig. 3a) [24, 26]. Further, two peaks were observed in each AGI-S1 and AGI-P2 UHPLC chromatograms (Fig. 3b), which share the same fragment pattern, and therefore, could be tentatively identified as *cis* and *trans* isomers. This conversion might be triggered when bioactive band was positioned under UV light. According to Hwang, Kim, Jang, Oh, Lim, Lee and Rho [29], *cis* isomer eluted first

under similar chromatographic conditions, thus, both isomers were tentatively assigned (Fig. 3b). Additional identification points were obtained from UV spectrum; first peak (t_R=6.61 min) showed λ_{max} at 276 nm and second one (t_R=7.38 min) at 293 and 316 nm (Fig. 3b), which were in agreement with values reported for *N-cis*-feruloyl tyramine and *N-trans*-feruloyl tyramine, respectively [30]. Following the same approach, *m/z* values for AGI-P1 were 177.0 $[M+H-121]^+$, 145.1 $[M+H-153]^+$, 117.1 $[M+H-181]^+$ and 105.1 [M+H-193] (Fig. 4a), with UV λ_{max} at 288 nm (Fig. 4b), these results are consistent with N-*trans*-feruloyl phenethylamine [26]. Thus, two phenolamides were tentatively identified, N-*trans*-feruloyl tyramine and N-*trans*-feruloyl phenethylamine, which showed similar fragment patterns corresponding to hydroxycinnamoyl moieties derived from ferulic acid.

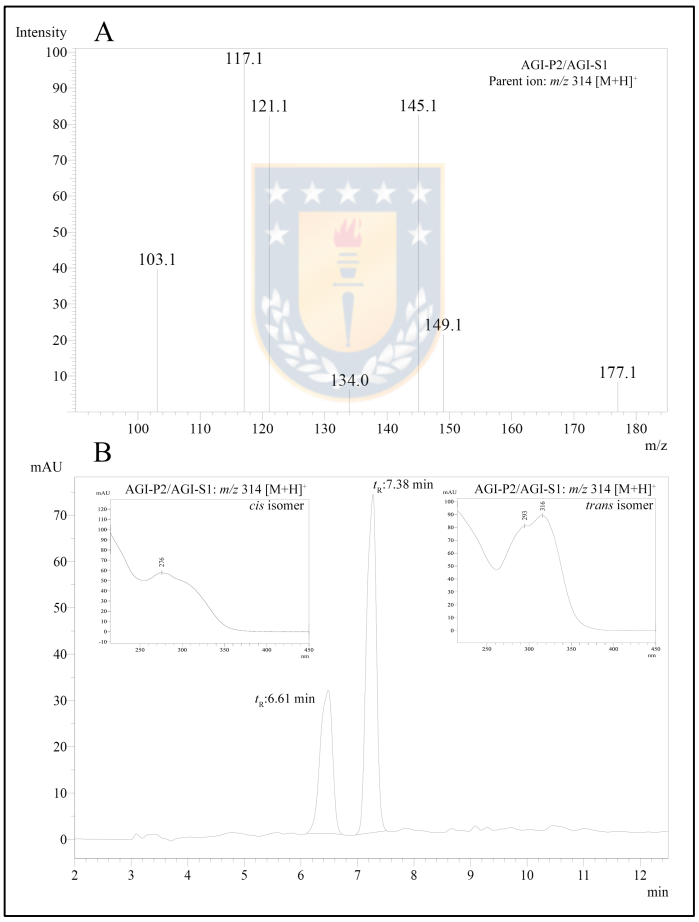

Fig. 3. UHPLC-DAD-ESI-MS/MS analysis of AGIs: N-*trans* feruloyl tyramine (AGI-P2 and AGI-S1), found in cherimoya peel and seeds. ESI-MS/MS spectrum in positive mode at -40V of parent ion *m/z* 314 $[M-H]^+$ (A); and UHPLC/DAD chromatogram and UV spectra of AGI N-*trans* and *cis*-feruloyl tyramine (B).

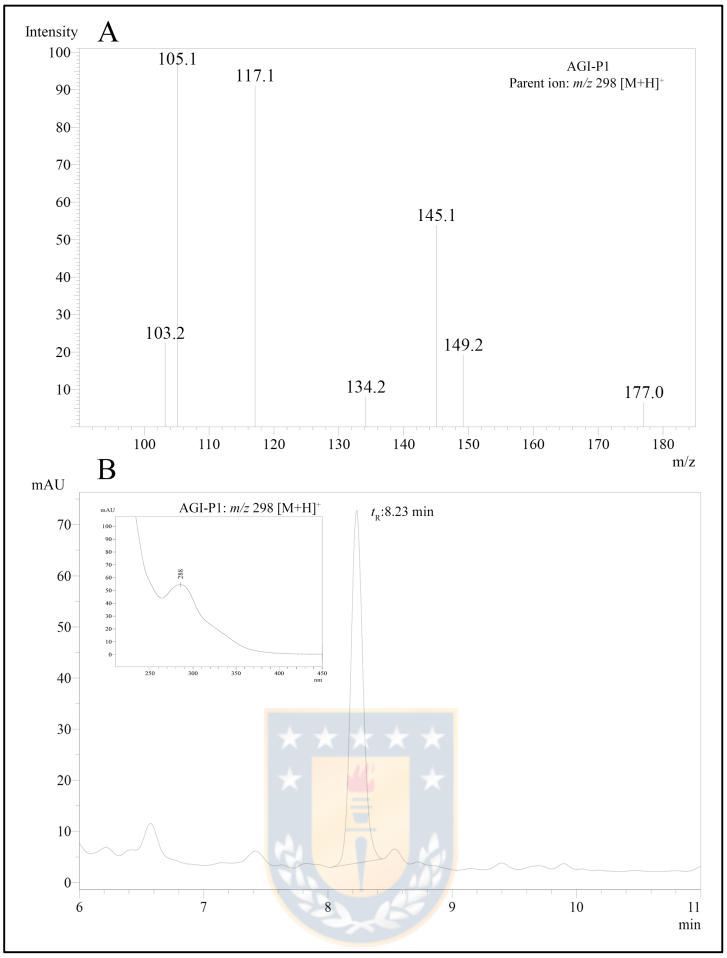

Fig. 4. UHPLC-DAD-ESI-MS/MS analysis of AGI: N-*trans*-feruloyl phenethylamine (AGI-P1) found in cherimoya peel. ESI-MS/MS spectra in positive mode at -40V of parent ion *m/z* 298 $[M-H]^+$ (A); and UHPLC/DAD chromatogram and UV spectrum of N-*trans*-feruloyl phenethylamine (B).

Second bioactive compound found in seed extract, AGI-S2, was tentatively identified as N-*trans*-*p*-coumaroyl tyramine. Its fragmentation pattern showed *m/z* values of 147.1 $[M+H-137]^+$, 121.1 $[M+H-163]^+$, 119.1 $[M+H-165]^+$ and 103.1 $[M+H-181]^+$ that are in agreement with previous reports (Fig. 5a) [22, 31]. Chromatographic analysis showed also the presence of two peaks (Fig. 5b) with the same fragmentation patterns, probably due to the conversion of *trans* isomer (t_R=6.85 min) to *cis* isomer (t_R=6.08 min), following the same behavior of first amide when exposed to UV light. Identification of N-*trans*-*p*-coumaroyl tyramine was complemented by comparison of UV spectrum that showed a λ_{max} at 296 nm, which was in agree with early reports [32], while N-*cis*-*p*-coumaroyl tyramine showed λ_{max} of 276 nm (Fig. 5b). *N*-*trans*-feruloyl tyramine and N-*trans*-*p*-coumaroyl tyramine and their *cis* isomers have

been reported in stems of *Annona cherimola* [23] and other species of the Annonaceae family [33]. Its capacity of α-glucosidase inhibition has been studied before, being classified as non-competitive inhibitors [28].

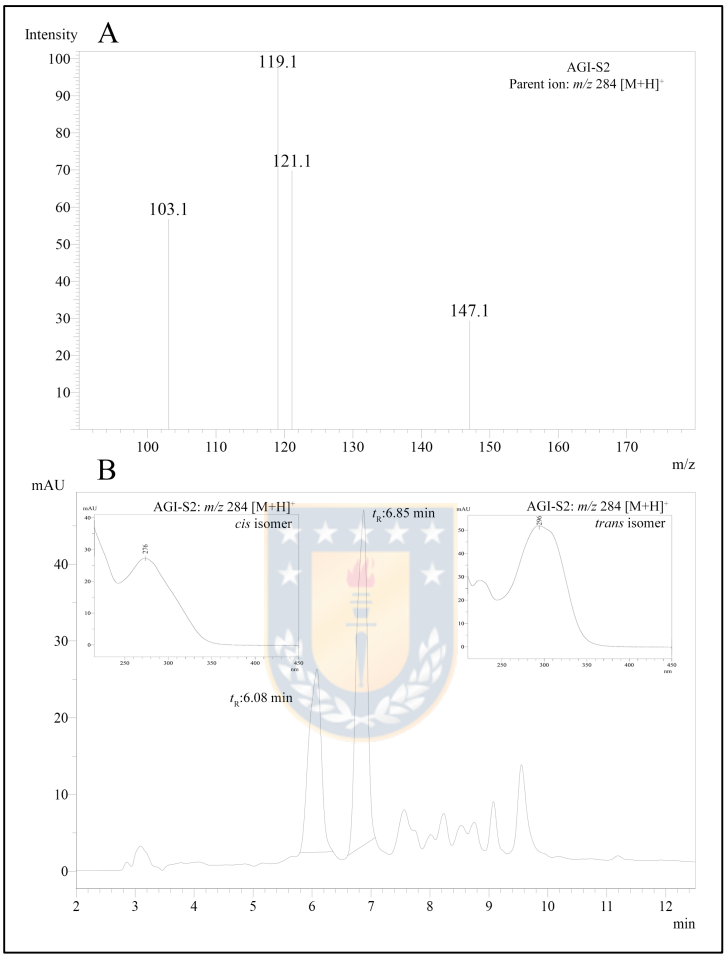

Fig. 5. UHPLC-DAD-ESI-MS/MS analysis of AGI: N-*trans*-*p*-coumaroyl tyramine (AGI-S2) found in cherimoya seeds. ESI-MS/MS spectra in positive mode at -40V of parent ion *m/z* 284 $[M-H]^+$ (A); and UHPLC/DAD chromatogram and UV spectra of AGI N-*trans* and *cis*-*p*-coumaroyl tyramine (B).

Among phenolamides family, N-*trans*-*p*-coumaroyl tyramine and *N*-*trans*-feruloyl tyramine present a stronger inhibition with IC_{50} values 37.9 and 18.7-fold [30] and 117.4 and 1.4-fold higher than acarbose and 1-deoxynojirimycin [32], respectively. Structure-activity relationship studies suggested that methylation of hydroxycinnamic hydroxyl group reduces α-glucosidase inhibition capacity. Further, Zhang, Tu, Yuan, Wang, Xie and Fu [30] and Liu, Luo and Kong [28], reported that *cis* isomers have lower inhibitory activity. To the best of our knowledge, for the first time is reported the presence of N-*trans*-feruloyl phenethylamine

in cherimoya and its inhibitory capacity over α-glucosidase. In view of this results, future work is planned to isolate and evaluate its inhibitory capacity as well as the type of inhibition. Besides AGIs activity, several relevant bioactivities have been also ascribed to phenolamides, e.g. antiviral, antibacterial, antifungal, insecticidal [27], and excellent anti-inflammatory properties [26]. In particular, *N-trans*-feruloyl tyramine has shown very interesting bioactivities like antitumoral, antimycobacterial, and cyclooxygenase inhibition [27], and important capacity of decreasing hypertriglyceridemia, insulin resistance and blood pressure [34].

4. Conclusions

To the best of our knowledge for the first time the presence of AGIs in cherimoya fruit (*Annona cherimola* Mill.) applying a rapid effect-directed analysis via a HPTLC-bioassay-mass spectrometry is reported. This approach clearly demonstrated its versatility to perform a rapid detection of α-glucosidase inhibitors in complex matrices. HPTLC-bioassay conditions were considerably improved through reduction of enzyme concentration (50% lower), incubation time (90% lower) and substrate inclusion (impregnation), resulting in a fast and cost-efficient methodology capable of detecting bioactive molecules with potential health benefits in less than 45 min. Application of this high throughput method permitted the detection and identification of three AGI compounds: N-*trans*-*p*-coumaroyl tyramine, N-*trans*-feruloyl tyramine and N-*trans*-feruloyl phenethylamine. To the best of our knowledge, the presence of latter compound is reported for the first time in cherimoya.

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Conflict of Interest: Oscar Galarce-Bustos, Jessy Pavón-Pérez , Karem Henríquez- Aedo and Mario Aranda declare that they have no conflict of interest.

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3. EVALUATION OF THREE ANALYTICAL METHODOLOGIES FOR AN *IN SITU* QUANTIFICATION OF THE ANTIOXIDANT CAPACITY OF CHERIMOYA EXTRACTS USING A HPTLC- DPPH ASSAY

Evaluation of three analytical methodologies for *in situ* quantification of the antioxidant capacity of cherimoya extracts using a HPTLC-DPPH assay

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ABSTRACT

Antioxidant compounds are constituents of plant and have proven to contribute to the prevention of physiological diseases that involve oxidative stress, such as Alzheimer and cardiovascular diseases. HPTLC-DPPH assay is a simple and fast method for the *in situ* detection of antioxidant compounds, however a quantitative procedure has not been clearly established. From the bioactive bands, visualized in the HPTLC plates as pale yellow bands on a purple background, was carried out a digital image processing, densitometric evaluation, and both combination. These three procedure allowed to quantify the antioxidant capacity, but the digital image processing using ImageJ® software was adequate and economic (free access) for routine analyses. Additionally, the HPTLC-DPPH-MS coupling allowed preliminarily to identify the antioxidant compounds from peel and seed cherimoya extracts, establishing the initial conditions of a full and powerful tool to detect, identify and quantify from vegetable extracts. Four compounds were tentatively identified in peel extract, highlighting *N-trans*-feruloyl tyramine with a strong antioxidant activity (7.47±0.80 μg EAC/g sample). Similarly, in the seed extract, three possible acetogenins were identified and quantified with values ranged from 0.99±0.07 to 1.35±0.07 μg EAC/g sample.

1. Introduction

Currently, the antioxidants derived from plants are considered a family of compounds of major interest among the research. This interest is justified by their ability to reduce and neutralize reactive oxygen species (ROS), which are involved in the pathophysiology of several diseases (Amorati & Valgimigli, 2018). Sufficient evidence has shown that free radicals mediated damage involved several human diseases, such as cancer, cardiovascular disease, neuronal disease, and aging (Gu, Wu, & Wang, 2009). Also, antioxidants play a crucial role in maintaining life and in preserving the quality of food. For these reasons, there is an increasing interest to search for the antioxidant molecules present in vegetables and fruits (Gu et al., 2009; Yang et al., 2018).

Antioxidants are a heterogeneous molecular family and a quick detection is difficult due to the complex compositions of vegetable extracts. In this scenario, several assays have used for screening and quantification antioxidant activity, which included DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging capacity assay (Gu et al., 2009). In this assay, the purple chromogen radical is reduced by the presence of antioxidant compounds to the pale yellow hydrazine, involving an electron transfer reaction-based (Karadag, Ozcelik, & Saner, 2009). DPPH assay has been widely used to evaluate the antioxidant activity by monitoring the absorbance at 515-528 nm using cuvette or microplate. However, this assay measures free radical scavenging ability of the whole extract, without individualizing the most potent free radical scavengers. Thus, the use of techniques separation based, prior to the biodetection, have been as an important strategy to detect and quantify of new active compounds. (Cieśla, Waksmundzka-Hajnos, Wojtunik, & Hajnos, 2015). The combination of the on-line HPLC–DPPH assay has been reported, which is a laborious technique and complex equipment is required (Wang et al., 2019; Zhang et al., 2013). Regarding, high-performance thin-layer-chromatography (HPTLC) is a simple technique that combined with biological or chemical detection as DPPH assay allows to assess the activity of individual compounds found in complex samples. So this approach, also named autography, allows to separate the compounds and evaporate the organic solvent of the mobile phase prior to the reaction with DPPH (Cieśla et al., 2015), avoiding the incompatibility between the sample solvent and the solubility of DPPH (Karadag et al., 2009). HPTLC-DPPH assay has become a powerful analytical tool, which has been mainly used for the detection and guide-isolation of antioxidant compounds from vegetable extract (Bhadane & Patil, 2017; Gu et al., 2009; Kassim, Lim, Ismail, & Awang, 2019). Additionally, the identification of active bands has been enhanced by the coupling of HPTLC with a mass spectrometer (MS), allowing the

transfer of active band directly to MS using a TLC-MS interface (Azadniya & Morlock, 2018; Krüger, Hüsken, Fornasari, Scainelli, & Morlock, 2017). As well this autography has been associated with image processing to compare quantitatively free radical scavenging potential of the analyzed compounds (Cieśla et al., 2015). The image of selected chromatograms can be mainly acquired by a flat-bed scanner or digital camera. This last one is considered easily accessible and inexpensive in-house image capturing tools that can be used in high-resolution plate imaging. Then, the image taken can be could be processed with several software packages such as SorbfilTLC®, ImageJ® y Just TLC® (Ibrahim, Khairy, Zaatout, Hammoda, & Metwally, 2018; Ristivojević, Trifković, Vovk, & Milojković-Opsenica, 2017). Initially, the image processing involves some stages including a correction of the image by baseline removal, denoising, target peak alignment and normalization. Regardless of the procedure of image processing, the resultant must closely resemble the original image (Ristivojević et al., 2017). Thus, nowadays, the greater accessibility to image capturing devices and image processing software has caused an increasing scientific interest for the use of HPTLC-DPPH assays coupled with image analysis as quantitation method of the antioxidant activity in natural extracts (Ibrahim et al., 2018). In that context, the aim of the present work was to establish the methodological principles for a simple and fast analysis by HPTLC-DPPH assay to detect, *in situ* identify and quantify of antioxidant compounds, applying a densitometric evaluation and image processing of the thin-layer chromatogram. This methodology will is part of an analytical platform for the identification of bioactive compounds in cherimoya.

2. Materials and methods

2.1 Reagents, chemical and solvents

2,2-Diphenyl-1-picrylhydrazyl free radical, caffeic acid (≥ 98.0%) and anhydrous magnesium sulfate $MgSO_4$, ≥ 97.0%) were purchased from Sigma (St. Louis, MO, USA). Ultrapure water (18 MW cm) was produced using a Simplicity system from Millipore (Bedfrod, MA, USA). Acetonitrile and methanol, both HPLC grade, chloroform, methanol, ethanol, ammonia (25%), formic acid (FA, 98–100%), hydrochloric acid and sodium chloride (NaCl) were purchased from Merck (Darmstadt, Germany). Chromatography was performed on 20 x 10 cm HPTLC plates from Merck, coated with a 200 µm layer of silica gel 60 F_{254}.

2.2 Sample preparation

Cherimoya peel, pulp and seeds were manually separated and freeze-dried for 36 hours at

-55ºC using a Martin Christ (Osterode am Harz, Germany) Alpha 1-2 LD plus freeze-dryer. Dried samples were milled and stored at -18ºC until its use. Bioactive molecules were extracted from 1.0 g of dried sample was vortex-mixed with 10 mL of 1.0% NH_4OH solution into 50 mL centrifuge tube using a Thermolyne vortex (Dubuque, USA). Then, 5 mL of acetonitrile were added and the mixture was submitted to sonication for 30 min into a Ningbo Xinzhi Biotechnology Co. Ltd. SB5200DT ultrasonic bath (Ningbo, China) using a frequency of 40 kHz at 30ºC. Suspension was centrifuged for 12 min at 3992 x *g* and 20ºC using a Hettich (Tuttlingen, Germany) Universal 32R centrifuge, thereafter, filtered into 50 mL centrifuge tube and completed with acetonitrile to ca. 15 mL. Extraction and clean procedure was performed following our previous work (Galarce-Bustos, Novoa, Pavon-Perez, Henriquez-Aedo, & Aranda, 2018), bioactive compounds were transferred to organic phase by salting-out extraction adding 4 g of $MgSO_4$ and 2 g of NaCl, vortex-mixed for 90 seconds and centrifuged for 10 min at 3992 x *g* and 20ºC. The organic phase was then separated, evaporated to dryness under a nitrogen stream and re-dissolved in 1.5 mL of methanol. Prior to chromatography, extracts were filtered through a 13 mm PVDF syringe filter (0.22 µm pore size).

2.3 Chromatography

Samples and standard solutions were applied with Automatic TLC Sampler 4 (ATS4, CAMAG, Muttenz, Switzerland) using the following settings: band length 6 mm, track distance 10 mm, dosage velocity 120 nL/s and first application x-axis and y-axis at 10 mm. Calibration curve was constructed applying a volume ranged from 1.0 to 8.0 µL (2.0 to 10 ng/band) of caffeic acid solution in methanol. Plates were developed with mixture chloroform, methanol and formic acid (78:10:12 v/v/v) in 20 cm x 10 cm twin trough chamber (CAMAG) up to a migration distance of 80 mm.

2.4 HPTLC-DPPH assay

After development the plates were dried for 30 min at 60ºC on TLC Plate Heater III (CAMAG), to evaporate the solvent used. Subsequently, the derivatization was carried out following the method proposed by Azadniya and Morlock (2018) with modifications. Briefly, HPTLC plate was immersed for 3 s in a 0.1% (w/v) DPPH solution in methanol, by using Chromatogram Immersion Device III (CAMAG) with 3 cm s^{-1} immersion speed. After 30 min of drying in the dark at room temperature, the plates were photographed under illumination (reflectance) with white light using Reprostar 3 (CAMAG) documentation system equipped

with a digital CCD camera with a resolution of 82 µm on the plate. Finally, the plate was scanned at 516 nm using TLC Scanner 3 under software control of WinCats v. 1.4.6 (CAMAG). Two densitograms were obtained using a tungsten-halogen lamp, first by in the fluorescence mode (inverse scan)to obtain directly positive peaks for the inhibiting zones (absorption measurement would lead to negative peaks on a high background); and second in absorbance scan (normal scan) mode obtaining negative peaks.

2.5 Digital image processing

The image processing was applied for quantification of the antioxidant activity on two images: first, a photo made with the use of TLC Reprostar 3 (digital camera), and second an image obtained from the densitogram in normal scan mode. Both files were obtained in TIFF format. The first image was obtained with a resolution of 500 dpi and pre-treatment was done using Photoshop CC v. 2017.1.1 software (Adobe System Incorporated) obtaining a photo in 8-bit. Then, image processing was carried out according to the procedure described by Ibrahim et al. (2018) using ImageJ v. 1.52a software (Wayne Rasband, National Institutes of Health, USA). The required track was outlined by the 'Rectangle' selection tool. The tracks widths should be adjusted to preserve uniform of the bands thicknesses. Line profile plots were generated by using 'Analyze' drop menu then 'Gels' and after 'Plot lanes' option. Subsequently, a baseline was drawn for the desired peak using the 'Straight line' selection tool and to define a closed area for each peak, the 'Wand' was used. Finally, the peak area was obtained by 'Measure' option in 'Analyze' drop menu. The second image also obtained with 500 dpi of resolution and each negative peak was manually selected using 'Polygon Selections'. Then the peak area was measured with 'Measure' option.

2.6 Mass spectrometry identification of antioxidant compound

Inhibitory bands were selected and marked using a soft pencil on the HPTLC plate based on retention factor (R_F) visualized under CAMAG cabinet (254 and 366 nm). Then, bands of interest were directly eluted and analyzed by mass spectrometry (MS). Then, the selected bands were eluted from plate to MS by means of CAMAG TLC-MS interface using a mixture of methanol and acetonitrile (50:50% v/v) at a flow rate of 0.2 mL min^{-1} for 60 seconds. Shimadzu (Kyoto, Japan) LCMS 8030 triple quadrupole mass spectrometer with electrospray ionization (ESI) source was operated applying the following conditions: ESI in positive mode, capillary voltage 3.0 kV, nebulizing gas (N_2) 3 L min^{-1} drying gas (N_2) 15 L

min^{-1}, DL temperature 250ºC, and block temperature 400ºC. Mass spectra were acquired in full scan mode between *m/z* values of 50 and 1000. Plate background signals were subtracted for each analysis. Data were acquired and recorded by Shimadzu LabSolution software version 5.51.

3. Results and discussion

3.1 Evaluation methods of the antioxidant capacity in HPTLC-DPPH assay

DPPH is a deep purple colored, stable free radical that is reduced in antioxidants presence, which appear as yellow spots against a purple background on the HPTLC plate. This reaction can be evaluated at a wavelength between 515 and 528 nm to a stable color (Marston, 2011). Some possible experimental factors that can affect the HPTLC-DPPH assay are the influence of adsorbent type, DPPH concentration, solvent of used for DPPH dissolution and time between immersion and documentation. Most of the factors were studied by Cieśla, Kryszeń, Stochmal, Oleszek, and Waksmundzka-Hajnos (2012), and according to the results, silica gel plate could enhance the antioxidant activity of the compounds, which improves detection. Similarly, caffeic acid was considered a calibration standard adequate for to quantify due to its antioxidant activity outstanding, which was enhanced when methanol was used as solvent for DPPH dissolution. The measurement of antioxidant capacity was stable for the standard from 20 min after immersion, therefore, the measurement was carried out at 30 min. Commonly the concentration of the DPPH solution used corresponds to 0.2% w/v (Azadniya & Morlock, 2018; Krüger et al., 2017; Pozharitskaya, Ivanova, Shikov, & Makarov, 2008; Wojtanowski & Mroczek, 2018), however, the use of 0.1 % w/v (Gu et al., 2009; Olech, Komsta, Nowak, Cieśla, & Waksmundzka-Hajnos, 2012) and 0.02% w/v (Krüger, Bergin, & Morlock, 2018) have been reported. In the present work, the concentration used was 0.1% w/v, considering that it provides an adequate contrast for the evaluation of antioxidant capacity.

In order to evaluate the quantification methodologies, the caffeic acid standard was applied in 5 levels (2.0 to 10.0 ng/band) and peel and seed extract were studied applying 5 µL on the plate. After the development of the plate, an image of the plate was captured at a wavelength of 366 nm (Figure 1a), which allowed to select the bands with activity. After 30 min of the immersion of the plate in the DPPH solution, an image was captured under white light, with upper and lower illumination to reduce the shadows on the plate. Thereby, HPTLC-DPPH assay allowed, by visual inspection, to establish the presence of pale yellow bands, corresponding to antioxidant compounds present in the extracts of cherimoya (Figure 1b).

To quantify the antioxidant activity, the image of the chromatogram was saved as a digital image (TIFF format) with a resolution of 500 dpi for its analysis. According to some reports, image processing has been used to quantify antioxidant activity *in situ* by HPTLC-DPPH (Cieśla et al., 2012; Ibrahim et al., 2018; Olech et al., 2012). Image processing has been used mainly ImageJ® and Sorbfil TLC® software packages, both are efficient, reliable and user-friendly (Ibrahim et al., 2018; Kowalska et al., 2013; Olech et al., 2012). ImageJ® program was selected based on its accessibility (free access from https://imagej.nih.gov/ij/) and simple operation.

Previously to the quantification, a pre-treatment of the image was carried out with the Adobe Phosotshop software, to increase the contrast of active bands, reduce the noise (median filter of five pixels), invert the colors and convert to grayscale. Then, the enhanced image was opened with the ImageJ software and the baseline drift caused by inhomogeneous illumination was removed. The pretreated image obtained was able to be analyzed for peak-based quantification (Figure 1c). Subsequently, each track, standard and sample, was selected and each band with antioxidant activity was transformed to a chromatographic peak as shown in Figure 2a. From these new chromatograms, the area values were obtained according to the procedure described in the methodology section and adjusted to a linear regression model with a coefficient of determination (R^2) equal to 0.997 as indicated in Table 1.

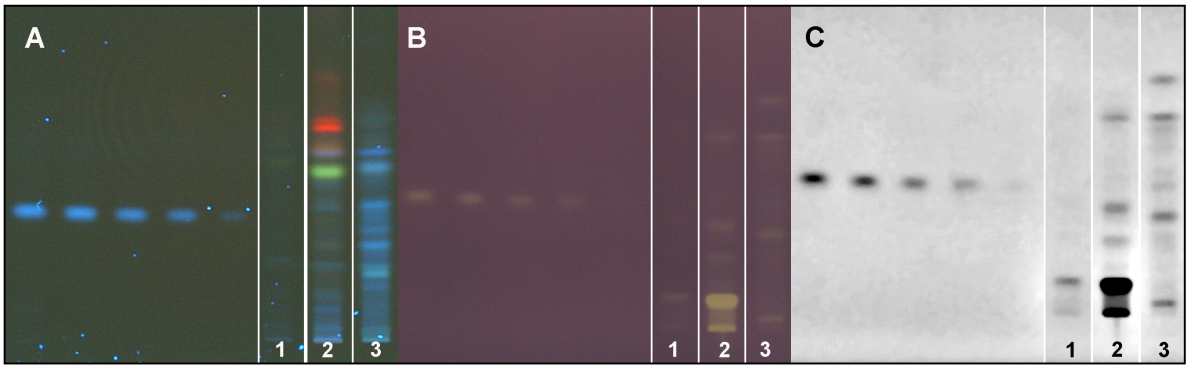

Figure 1. Chromatograms of the calibration curve, diluted peel extract (1), peel extract (2) and seed extract (3); under UV light at 360 nm before to the derivatization (A), DPPH assay (B) and after of image processing (C).

In addition to image processing, in planar chromatography the quantitative evaluation has commonly been carried out by scanning densitometry of the sample tracks (Fichou & Morlock, 2018), thus the derivatized plate can be evaluated by densitometry at a wavelength

of 516 nm. A first option, inverse scan (Azadniya & Morlock, 2018), measures the absence of fluorescence from the background plate caused by the presence of DPPH, which increases in the active band where the reaction was neutralized for the antioxidant compound presence. Thereupon in this active zone, a fluorescence emission of the HPTLC plate (fluorescence indicator, F_{254}) was produced generating a positive peak. A second option, normal scan (Pozharitskaya et al., 2008), measures the absorbance generated by DPPH (violet color) that decreases in the band with antioxidant activity (pale yellow color) and generates a negative peak.

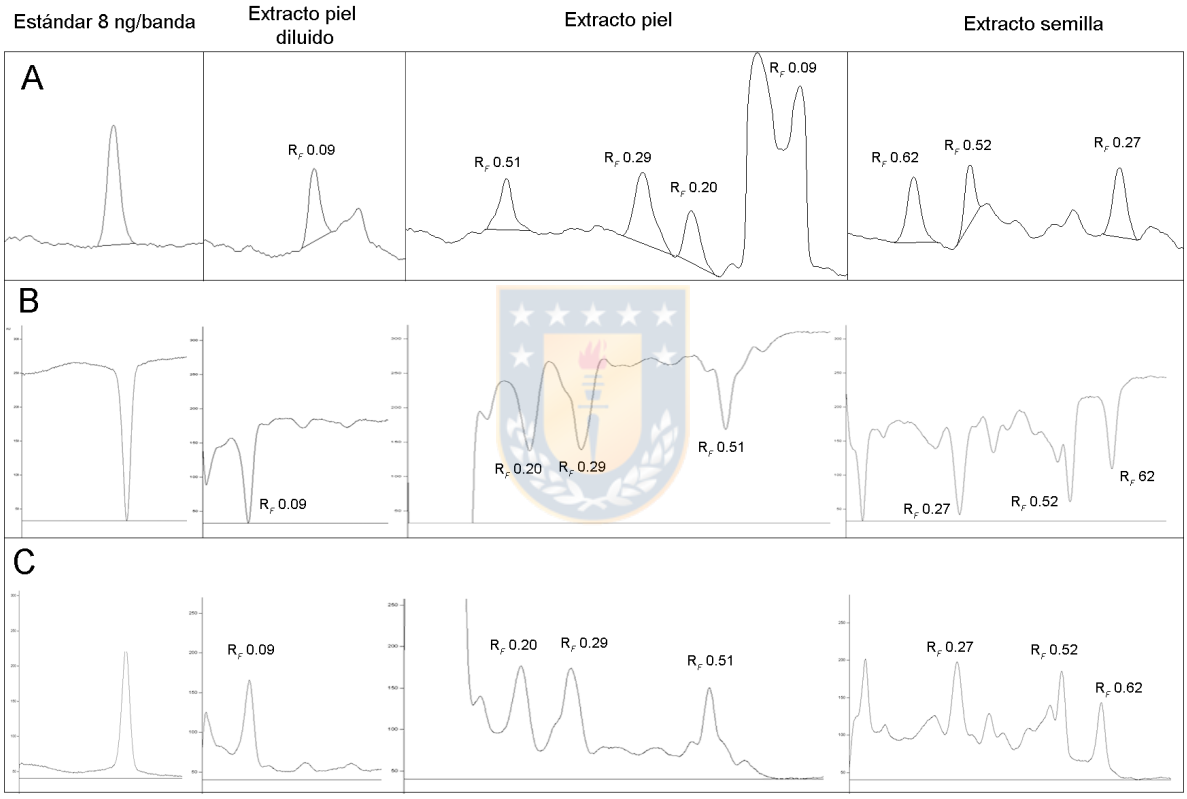

Figure 2. The results obtained from HPTLC-DPPH assay to quantify the antioxidant activity using image processing with ImageJ program (A), absorbance mode densitometry (*normal scan*) + image processing (B), and fluorescence mode densitometry (*inverse scan*) (C).

For each densitometric methodology, inverse scan and normal scan mode, a chromatogram was obtained for each track. Fluorescence evaluation was easy and fast due to the advantages of the software used, WinCats, which allows the integration of the area of the positive peaks obtained the area from each band with activity (Figure 2c). However, absorbance mode was laborious because the WinCats software does not allow the integration of negative peaks. Then, the negative peak area was obtained by the image

processing of a chromatogram generated by the software (Figure 2b), becoming a procedure that combines densitometry and image processing. Each negative peak was delineated manually with the ImageJ software and then the area was obtained. The values obtained in inverse scan and normal scan were adjusted to linear regression, as described in Table 1 with R^2 greater than 0.99.

Table 1. Calibration curves of the quantification methods applied for HPTLC-DPPH assay.

	Regression equation $y = ax \pm SD + b \pm SD$	R^2
Fluorescence mode densitometry	$y = 2261.4x \pm 69.6 - 1529.9 \pm 443.2$	0.994
Image processing	$y = 651.2x \pm 15.6 - 738.0 \pm 113.3$	0.997
Absorbance mode densitometry + image processing	$y = 290.3x \pm 10.9 - 360.5 \pm 67.1$	0.991

The antioxidant compounds present in the peel and seed extracts were detected (Figure 1a), and quantified by the three methodologies evaluated (Figure 2). In the peel extract four compounds were detected (1, 2, 3 and 4) and three in the seed extract (5, 6 and 7). The results showed that the antioxidant capacity of each compound was similar among the three methodologies evaluated, as shown in Table 2. By Kruskal-Wallis test (α = 0.05) no statistically significant differences were established ($p \geq 0.05$) between the values of the samples quantified by densitometry (inverse scan) and image processing. Similarly, no significant differences were found between the two procedures that include image processing, except for the values determined for compound 7 ($p < 0.05$). The main differences were observed between the procedure of densitometry (inverse scan) and densitometry (normal scan) plus image processing, with statistically significant differences in the results of compound 4, 5 and 6. According to the results, Compound 1 showed the highest antioxidant capacity and could be measured after diluting 10 times the peel extract, while the other compounds showed ranged from 0.88 ± 0.02 to 1.94 ± 0.08 µg EAC/g sample. These results cannot be compared against other reports that have determined the antioxidant capacity peel and seed extracts by IC_{50} values (Albuquerque et al., 2016; Loizzo et al., 2012), which correspond to a neutralization kinetic of the DPPH radical.

Hence, the three methodologies allowed the quantification of the antioxidant activity. The main advantage of the use of densitometry was the simplicity and quicklness of the analysis without requiring of a qualified personnel, however it has a high cost due to the equipment used (densitometric scanner and software). Also, a fundamental requirement is that the

compounds should not emit fluorescence between 510 and 520 nm. Similarly in the quantification by the combination of densitometry plus image processing, the compounds should not absorb between 510 and 520 nm, making screening difficult with the presence of anthocyanins. In addition, each chromatographic peak must be manually delineated to obtain the area. Overall, these characteristics do not contribute to the development of a routine and easily accessible methodology. On the other hand, image processing quantification using ImageJ® presented several features, proving to be a suitable methodology and easy access for routine analysis. For this quantification only is an adequate lighting system and a camera to capture images. However, an operator with basic knowledge in image analysis is required to correct the baseline drift and not lose information during processing. Additionally, image processing quantification can also be extended for any photo of TLC or HPTLC plate, even using a domestic digital camera.

Table 2. Scavenging activity against DPPH radical (μg CAE*/g sample) of peel and seed cherimoya extract..

	R_F	Fluorescence mode densitometry (*inverse scan*)	Image processing	Absorbance mode densitometry (*normal scan*) + image processing
		Peel extract		
Compound 1	0.09	6.99 ± 0.52	7.47 ± 0.80	8.07 ± 0.54
Compound 2	0.20	1.25 ± 0.06	1.25 ± 0.01	1.37 ± 0.06
Compound 3	0.29	1.69 ± 0.09	1.94 ± 0.08	1.75 ± 0.09
Compound 4	0.51	1.45 ± 0.08	1.18 ± 0.01	1.05 ± 0.04
		Seed extract		
Compound 5	0.27	1.54 ± 0.12	1.35 ± 0.07	1.23 ± 0.04
Compound 6	0.52	1.08 ± 0.03	0.99 ± 0.07	0.88 ± 0.02
Compound 7	0.62	1.31 ± 0.09	1.33 ± 0.06	1.02 ± 0.02

* CAE: Caffeic acid equivalents

3.2 Mass spectrometry identification of antioxidant compounds

For identification of the bioactive compounds, a plate was developed in parallel to HPTLC-DPPH assay, then the antioxidant bands were selected were directly eluted from HPTLC plate to ESI-MS interface. Plate background signals were subtracted for each analysis, in order to decrease the contaminants, solvents effect and plate components. MS analysis was carried out in scan mode for each band with antioxidant activity, and the *m/z* values obtained are shown in Table 3. These results allowed an approximation for the identification of the antioxidant compounds, however, to increase the identification certainty additional steps are

required such as the use of commercial standards or isolating sufficient amount (milligrams) for analysis by nuclear magnetic resonance (NMR). Occasionally, when compounds are found in large amounts in the sample, it is possible to isolate them by preparative TLC (Azadniya & Morlock, 2018). In this regard, preliminary assignment of antioxidants compound was based on the HPTLC-MS analysis, so the compounds were characterized by comparison of their mass spectra (fragment pattern) with those found in the literature.

The compounds present in peel extract could correspond to nitrogen compounds such as alkaloids or phenolamides. In concordance with Wang, Suh, Zheng, Wang, and Ho (2017), the Compound 1 was identified as *N-trans*-feruloyl tyramine. Also previous reports this compound has been determined as an inhibitor of α-glucosidase present in the peel cherimoya. The band called Compound 2, possibly presents two compounds with different *m/z* values, the *m/z* 330.2 $[M+H]^+$ was possibly identified as *N-trans*-feruloyl dopamine (Wang et al., 2017), while the *m/z* value 286.2 $[M+H]^+$ it could not be identified. Similarly the Compound 4 was not identified. The potential candidates for Compounds 3 with *m/z* 342.2 $[M+H]^+$, were magnoflorin or isochoridine, both alkaloids reported in *Annona* species (Avula et al., 2018). Both class compounds, alkaloids and phenolamides, have demonstrated an outstanding antioxidant activity. The amidic derivatives of the hydroxycinnamic acids (phenolamides) are interesting bioactive compounds with a potent antioxidant activity and a greater physiological stability than other molecules (Rajan et al., 2002; Wang et al., 2018).

In the same way, some aporphine alkaloids, common in Annona species, have showed potent antioxidant activity due to the presence of hydroxyl groups, such as boldine (O'Brien, Carrasco-Pozo, & Speisky, 2006). The *m/z* values founded in seed extract can linked to the presence of acetogenins, which have been widely reported in seed of *Annona* species (Jamkhande, Ajgunde, & Jadge, 2017). These compounds have proved a scavenger DPPH radical capacity strong, even similar to ascorbic acid, commonly used as standard in antioxidant activity measure (Lima, Pimenta, & Boaventura, 2010). According to some reports, acetogenins forming adducts with sodium $[M+Na]^+$ (Avula et al., 2018), also the three compounds of the seed extracts, the presence of *m/z* values was found $[M+H+Na+14]^+$ or $[M+ H+Na+28]^+$, which may correspond to nitrogen contamination or formation of adducts with methyl groups. Thus, Compounds 7 and 8 were identified as acetogenins, but their individualization was not possible with the obtained information.

Although in this work the identification certainty of the antioxidant compounds was incomplete, the application of this streamlined workflow proved to be important analytical

tools, which resulted in a fast and cost-efficient methodology that allows obtaining valuable information for the identification of compounds from plant extracts

Table 3. Molecular mass (as *m/z* values) founded in each band with antioxidant activity and proposed compounds

	R_F	*m/z* value	Preliminary identification
		Peel extract	
Compound 1	0.09	314.2 $[M+H]^+$	*N-trans*-feruloyl tyramine
Compound 2	0.20	286.2 $[M+H]^+$ / 330.2 $[M+H]^+$	Unidentified / N-*trans*-feruloyl dopamine
Compound 3	0.29	342.2 $[M+H]^+$; 383.2 $[M+CH_3CN]^+$	Magnoflorine o Isocoridine
Compound 4	0.51	339.1 $[M+H]^+$; 377.1 $[M+K]^+$	Unidentified
		Seed extract	
Compound 5	0.27	651.5 $[M+Na]^+$; 679.5 $[M+Na+C_2H_4]^+$	Unidentified
Compound 7	0.52	619.5 $[M+Na]^+$; 633.5 $[M+Na+CH_2]^+$	Squadiolin C, Glaucafilin o Anonacine
Compound 8	0.62	617.4 $[M+Na]^+$; 645.4 $[M+Na+C_2H_4]^+$	Squamicin B

4. Conclusions

Some studies have reported by independent methodologies the DPPH scavenging activity of peel and seed cherimoya extracts, and the compounds responsible for this activity. However, the present work presented for the first time a methodology that allowed the identification and quantification of antioxidant compounds. In this regard, this work evaluated and determined the most appropriate methodology for quantification, contributing to establish the methodological principles to develop a simple, affordable and optimal methodology for *in situ* determination of compounds with biological activity in cherimoya extracts. Therefore, this proof of concept will allow the quantification of other activities such as α-glucosidase, acetylcholinesterase or another bioassay. In addition, the association of HPTLC-DPPH-MS allowed beginning to elucidate the structure and identify compounds with antioxidant activity, developing a complete tool in the analysis of bioactive compounds in plant extracts.

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4. SUPERCRITICAL FLUID EXTRACTS FROM CHERIMOYA BY-PRODUCTS AS A PROMISING SOURCE OF BIOACTIVE ALKALOIDS

Supercritical fluid extracts from cherimoya by-products as a promising source of bioactive alkaloids

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Formato manuscrito enviado a: Industrial Crops and Products

ABSTRACT

A huge concern of the agro-industrial sector is reducing the environmental impact of by-products obtained from pruning activities (leaves, branches and bark) and food industry (peel and seeds). Cherimoya by-products are valuable natural sources of bioactive compounds. In order to obtain extracts rich in alkaloids from cherimoya peel, the conditions of supercritical fluid extraction were optimized by central composite design. Prior to extraction, a simple heat drying was adequate for the raw material pre-treatment. Results showed a high alkaloid extraction yield using 100 bar of pressure, 75 ºC and 15% methanol as co-solvent. These conditions were applied to peel of three cherimoyas varieties as well as in cherimoya leaves. The evaluation of the bioactivity showed a high antioxidant capacity (5304.2 ± 73.6 to 21705.2 ± 1069.3 μmol TE/100 g), which correlated with the presence of alkaloids (r = 0.954). Both peel and leaf extracts showed an interesting acetylcholinesterase (IC_{50} = 87.69 ± 3.42 − 515.02 ± 29.25 μg mL^{-1}) and α-glucosidase inhibitory capacity (IC_{50} = 1097.76 ± 121.12 − 3206.88 ± 97.06 μg mL^{-1}). The inhibition of both enzymes was higher in leaf extracts, showing a high correlation of the total alkaloids with the acetylcholinesterase inhibition (r = 0.990). Additionally, the presence of some bioactive alkaloids was studied by UHPLC-ESI-MS analysis, highlighting the concentration of N-*trans*-feruloyl phenethylamine in peel extracts and anonaine in leaf extracts. This work provides novel information about the bioactivity of cherimoya by-products and their potential as a source of bioactive compounds.

Keywords: cherimoya, by-products, supercritical fluid extraction, alkaloids, experimental design, bioactivity.

1. Introduction

Annona cherimola Mill. (Annonaceae), commonly known as cherimoya, is a tropical and subtropical fruit native to the Andean valleys, cultivated in Peru and Ecuador, but its cultivation has been extent to other countries such as Chile, Peru, Bolivia, United States, Mexico, Spain and Portugal (Jamkhande et al. 2017). Nowadays, the coast of Granada-Malaga (Spain), the so-called 'Costa Tropical', is the main producer of cherimoya worldwide. This region enjoys a favorable mild climate where the two most important varieties are 'Fino de Jete' and 'Campas' (García-Salas et al. 2015). In the northern zone of Chile it is also cultivated, but to a much lesser extent, with the most important varieties being 'Concha Lisa' and 'Bronceada' (Manríquez et al. 2014). The cherimoya fruit is highly appreciated by consumers. The pulp stands out for its exceptional taste, smooth texture and high organoleptic qualities. It is usually consumed '*in natura'* or used for the agro-industry for the production of juices, smoothies, desserts and ice creams. However, agricultural crop residues and other by-products generate by cherimoya processing industry, such as the peel, seeds and leaves, have also a great importance. They have been used in folk medicine as antimicrobials and insecticides, and as an effective treatment for digestive disorders and skin diseases, though their potential has not been extensively studied (Loizzo et al. 2012).

In recent years, the production of fruits worldwide has increasing and consequently agro-industrial by-products and waste too, which poor management could cause serious environmental problems. However, by-products and waste from processed fruits are enrich-source of bioactive compounds (Silvan and Martinez-Rodriguez 2019). In addition, the valorization of agricultural by-products contributes to the recovery of agro-industrial waste, with major economic and environmental effects. Nowadays, tropical fruits and its by-products have attracted increasing attention from researchers and industry, as they are a great source of phytochemicals useful for the manufacture of different products in the fields of cosmetics, pharmacy, nutraceutics and food. In this scenario, identifying and quantifying phytochemicals in agricultural by-products is of the utmost importance to substantiate their potential health benefits and the added value of food by-products (Carriço et al. 2018).

Regarding cherimoya, several bioactive compounds such as phenolic compounds, phenylpropanoids (Díaz-de-Cerio et al. 2018; Loizzo et al. 2012), fatty acids, sterols (García-Salas et al. 2016), acetogenins (Barreca et al. 2011) and alkaloids (Lúcio et al. 2015) have been identified in leaves, roots, fruit and seeds, making the assessment of their by-products interesting. Among the alkaloids from *Annonaceae*, the predominant group are the aporphine alkaloids, which also provide antioxidant, antipyretic, anti-inflammatory (Debnath

et al. 2018) and acetylcholinesterase inhibitory activity (Yang et al. 2012).

An important stage to recover bioactive compounds from food by-products is the extraction process. Currently, the use of green extraction methods is essential to preserving the environmental and sustainable chemical production. Thus, supercritical fluid extraction (SFE) is an interesting alternative to extract bioactive compounds that provide attractive features, overcoming many of the limitations of conventional extraction methods, including the use of a green solvent as it is carbon dioxide (CO_2), faster and more selective processes, and the possibility to carried out the extraction at mild temperatures and in an inert ambient which avoid the degradation of sensitive compounds (Pavlić et al. 2018). SFE has been widely used to recovery bioactive compounds from numerous agro-industrial and food by-products (Wijngaard et al. 2012). Novel non-polar compounds, like lycopene, have been extracted from tomato pomace (Perretti et al. 2013). But also, by adding a co-solvent such as methanol, ethanol or other organic solvents, an efficient extraction of moderately to highly polar compounds, mainly phenolics and anthocyanins, has been achieved (Fernández-Ponce et al. 2012; Otero-Pareja et al. 2015). The use of SFE, with or without co-solvents, to extract alkaloids has been also reported; different classes of alkaloids such as colchicine (Bayrak et al. 2019), aporphine (del Valle et al. 2005), isoquinoline and indole (Brandão et al. 2017; Gañán et al. 2016) have been extracted from plants.

In cherimoya agro-industrial production, leaves, peel and seeds could be considered as the main by-products, and the extraction of bioactive compounds from them using SFE has scarcely been studied. Only the extraction of acetogenins using SFE has been reported, but from other *Annona* species (Yang et al. 2009). This background information serves to demonstrate the feasibility of obtaining bioactive compounds like alkaloids from cherimoya by-products by SFE. In addition, to the best of our knowledge, previous studies on alkaloid extraction from cherimoya by-products using SFE have not been reported in the literature. Therefore, the aim of the present study was to obtain an extract with a high content of bioactive alkaloids from cherimoya peel and leaves by using an optimized SFE process. The extracts were evaluated considering the total alkaloid extraction yield, inhibitory acetylcholinesterase and α-glucosidase capacity and antioxidant activity. Additionally, some varieties of cherimoya leaves were evaluated using the optimized method.

2. Material and methods

2.1 Materials and reagents

Carbon dioxide (99.99%) was provided by Abello-Linde S.A. (Barcelona, Spain).

Acetylcholinesterase from *Electrophorus electricus* (electric eel) (E.C number 3.1.17), α-glucosidase from *Saccharomyces cerevisiae* (E.C number 3.2.1.20), donepezil hydrochloride (pharmaceutical secondary standard), *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG, ≥99.0%), acetylthiocholine iodide (ATCI; ≥98.0%), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, ≥98.0%), pyrogallol red (PGR), 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH; 97%), (±)-6-Hydroxy-2,5,7,8-tetramethylchromano-2-carboxylic acid (Trolox, 97%), Folin & Ciocalteu's phenol reagent 2 N, bromocresol green (95%), gallic acid (97.5–102.5%) and boldine were obtained from Sigma-Aldrich (St. Louis. MO, USA). Glaucine standard was a kind donation from South American Phytochemical (SAPHYCHEM) (Santiago, Chile). Potassium dihydrogen phosphate (≥99.5%), di-potassium hydrogen phosphate (≥99.0%), potassium hydroxide hydrate (≥99.9%), tris(hydroxymethyl)aminomethane (TRIS; >99.7%), sodium dihydrogen phosphate anhydrous (≥99.9%), disodium hydrogen phosphate (≥99.0%) and hydrochloric acid (all analytical grade) were purchased from Merck (Darmstadt, Germany). Acarbose active pharmaceutical ingredient was a kind donation from a Chilean pharmacy. The organic solvents ethanol, methanol and chloroform, all HPLC gradient grade, and ammonia (30% as NH_3) were provided by Panreac (Barcelona, Spain). The water used in all experiments was double-distilled milliQ grade.

2.2 Samples and pre-treatment

Two varieties of cherimoya fruit grown in Spain and one variety grown in Chile were studied. 'Fino de Jete' and 'Campas', Spanish varieties, were provided by the research group at the Institute for Mediterranean and Subtropical Horticulture IHSM 'La Mayora', Malaga, Spain (36°45'29.4"N 4°02'37.2"W) and harvested in December 2017. The Chilean variety was purchased directly from a fruit store in September 2017. This fruit is cultivated in the central zone of Chile, between the IV and V regions (29°02'27.3"S 70°36'36.0"W to 33°57'19.0"S 71°43'25.7"W).

Cherimoya peel was manually separated and two drying methodologies were evaluated: freeze drying and heat drying. For the first procedure, a freeze dryer was required to dry the sample for 48 h. The second procedure was carried out in a laboratory oven at 45ºC for 24 h. Then 5 g of each sample was milled and extracted for 60 min in an ultrasonic bath with 150 mL of chloroform. Afterward, the samples were filtered prior to the measurements. Additionally, three varieties of cherimoya leaves - 'Fino de Jete', 'Campas' and 'Alboran' - were analyzed. These samples were also provided by the Institute for Mediterranean and

Subtropical Horticulture IHSM 'La Mayora'. The leaves were dried at room temperature for 48 h. All dried samples were ground and kept frozen in absence of light.

2.3 Supercritical fluid extraction procedure

The extraction tests were carried out in a model SF100 high-pressure apparatus (Thar Technology, Pittsburg, PA, USA). A schematic diagram of the equipment is shown in **Fig. 1**. This set-up included an extraction vessel (100 mL capacity) with a thermostatic jacket to control the extraction temperature, two pumps with a maximum flow rate of 50 g min^{-1} (one for carbon dioxide and the other for co-solvent), a back pressure valve regulator to control the system pressure, and a cyclonic separator to allow periodic discharge of the extracted material during the extraction process. For every test, the extraction vessel was loaded with approximately 15 g of sample. Prior to extraction, the milled samples were impregnated with a solution of 2% ammonia in methanol and dried again at 45ºC for 24 h, enabling the extraction of alkaloids in the form of free bases. Then, the extraction procedure was as follows: the sample was put into a filter paper cartridge and loaded into the extraction vessel, which was fitted with metal filters at the bottom and top, and was pre-heated to 75ºC. Then, the high-pressure pumps began to flow CO_2 and co-solvent (methanol/ethanol) at a constant pressure of 100 bar. Tests were carried out at a flow of 20 g min^{-1} of CO_2 and 3.5 g min^{-1} of co-solvent during 3 h. Finally, extracts were collected in amber glass bottles and stored in darkness at -20ºC prior to the assays.

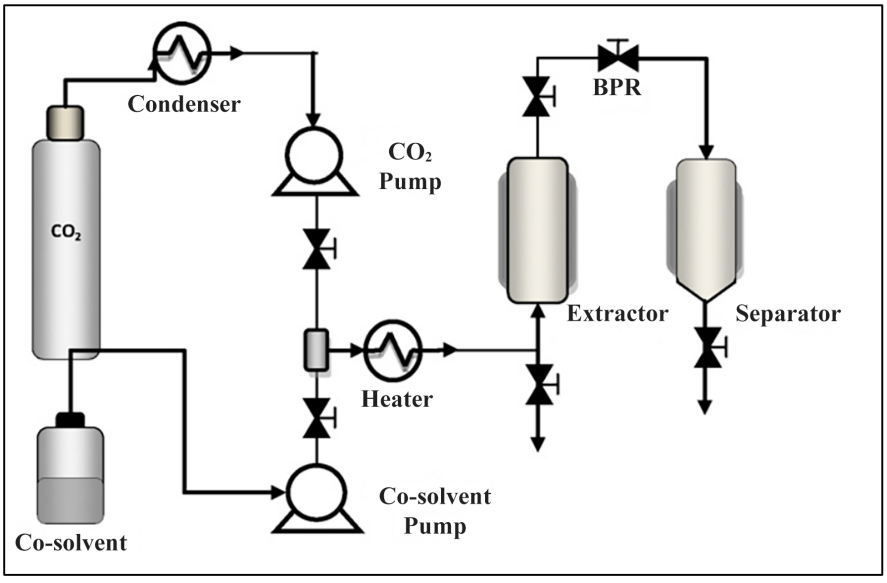

Fig. 1. Schematic diagram of the supercritical extraction system.

The efficiency of the extraction process was evaluated in terms of global yield and total alkaloid content. The global yield for all extraction conditions tested was calculated as the ratio between the dry mass of extract and that of the dry raw material.

2.4 Total alkaloids content

The total alkaloid (TA) content was determined according to the previous work of Li et al. (2015). For the alkaloid standard, a stock solution of boldine (1 mg mL^{-1}) was prepared in chloroform and diluted at six levels (2 to 24 μg mL^{-1}) to make the calibration curve. For the sample, an adequate volume was evaporated to dryness and re-dissolved in chloroform. Then, 3 mL of phosphate buffer (pH 4.7) and 3 mL of 0.43 mM bromocresol green solution were mixed in a separatory funnel, then 5 mL of standard or sample was added and shaken for 2 min. After 30 min, the organic phase was collected in a flask and the extraction was continued one more. All fractions were collected and the volume completed up to 10 mL with chloroform. For the blank, the same procedure was repeated using 5 mL chloroform instead of the standard solution. Similarly, the background sample was measured using 6 mL of phosphate buffer (pH 4.7) and 5 mL of sample. The absorbance of the extract was measured at 410 nm. Each procedure was performed in triplicate (n=3) and each extract was measured three times. The results were expressed as micrograms of boldine equivalent per gram of dried sample (μg BE g^{-1}).

2.5 Experimental design

Prior to the application of experimental design, two co-solvents were evaluated to extract alkaloids from cherimoya peel: methanol and ethanol. The initial extractions were done using the operating conditions proposed by Gañán et al. (2016) for an efficient extraction of alkaloids. The CO_2 flow rate was 22 g min^{-1} including 10% of co-solvent at pressure of 120 bar and 55ºC for 3 h.

Once the co-solvent was selected, a response surface methodology (RSM) was employed to optimize the alkaloid extraction process. The experimental design was generated with the Statgraphics Centurion XVI version 16.1.18 software (Rockville, MD, USA). Central composite design (CCD) was used to evaluate the impact of the factors and to maximize the response. The factors and experimental range were set on the basis of a review of the literature and by conducting preliminary experiments on extraction. The factors chosen for extraction experiments were pressure (100 to 400 bar) (X_1), temperature (35 to 75ºC) (X_2) and co-solvent percentage (5 to 15%) (X_3). The dependent response chosen to evaluate the

efficiency extraction was TA (*Y*). Seventeen experiments were performed according to face-centered CCD including three central points. Each experiment was evaluated in triplicate (*n*=3) to minimize the effects of uncontrolled factors.

2.6 Determination of antioxidant activity

Antioxidant capacity of extracts was determined by a microplate-based oxygen radical absorbance capacity (ORAC) assay using PGR as the probe, as reported by Ortiz et al. (2012), with slight modifications. The assay was performed using an Infinite 200 Pro microplate reader (Tecan, Gröedig, Austria) with temperature control. The reaction was carried out in a 96-well microplate with a final volume of 250 μL. The mixture for the assay was as follows: 50 μL of blank, sample or Trolox, 150 μL of 75 mM sodium phosphate buffer (pH 7.4) and 25 μL of PGR (5 μM final concentration). The mixture was pre-incubated for 30 min at 37°C, before rapidly adding the AAPH solution (25 μL; 10 mM, final concentration), previously incubated at 37ºC. The microplate was shaken prior to each reading. All the reagents were dissolved in sodium phosphate buffer and the sample or Trolox in methanol. The microplate was immediately placed in the reader, automatically shaken, and the absorbance (A) of the PGR consumption was read and recorded at 540 nm every 60 s for 180 min. A calibration curve was taken in triplicate (*n*=3) each time with the standard Trolox (50 to 500 μM), the blank was PGR and AAPH using methanol instead of the antioxidant solution (A_0). The reaction mixtures of the samples were also prepared in triplicate (*n*=3), and the background sample was measured using 50 μL of sample and 200 mL sodium phosphate buffer, which was subtracted in each measurement.

To determine antioxidant capacity, values of the relative absorbance (A/A_0) were plotted as a function of the incubation time. Then, the integration of the area under the curve was performed up to a time that A/A_0 reached a value of 0.2. These areas were employed to obtain ORAC values, which were expressed as μmol Trolox equivalents (TE)/100 g of sample.

2.7 Determination of total phenolic content

The total phenolic content was determined by the Folin-Ciocalteu assay based on the work of Speisky et al. (2012). Briefly, the assay was performed in a 96-well plate mixing 50 μL of standard or sample, 200 μL of 0.2 N Folin-Ciocalteu aqueous solution and 50 μL of 5.0% (w/v) sodium carbonate. The mixture was incubated at 37ºC for 30 min and absorbance was measured using a microplate reader at 740 nm. A calibration curve was constructed with

gallic acid (20 to 400 μg mL^{-1}), using methanol as the blank. The sample background was measured using 200 μL of water instead of the Folin-Ciocalteu solution, which was subtracted in each measurement of the sample. All standards and samples were analyzed in triplicate (*n*=3) and the results were expressed as micrograms of gallic acid equivalents per gram of sample (μg GAE g^{-1}).

2.8 Acetylcholinesterase (AChE) inhibition assay

For the enzymatic assays, the samples were dissolved in 10% DMSO in buffer, ensuring the dissolution of weakly soluble compounds in the aqueous assay mixture. To determine AChE inhibition, the assay was carried out based on Ellman's method, following the method proposed by Di Giovanni et al. (2008) with some modifications. Briefly, in a 96-well microplate 150 μL of 0.25 mM DTNB solution (in 50 mM TRIS-HCl buffer pH 7.8), 25 μL of buffer and 30 μL of a 0.37 U mL^{-1} enzyme solution in buffer and 25 μL of sample or positive control (donepezil) were mixed. Then, the absorbance was registered at 405 nm using a microplate reader. After that, the enzymatic reaction was initiated by adding 20 μL of 8.75 mM ATCI solution in water and immediately shaken for 2 s. Then, the absorbance was monitored at 405 nm for 180 s at 25ºC. Each assay was done in triplicate (*n*=3) and the percentage inhibition of AChE was calculated by comparing the reaction of the samples to the blank sample (10% DMSO in buffer). Results were expressed as the sample concentration required to inhibit 50% of the enzyme activity (IC_{50}).

2.9 α-glucosidase inhibition assay

The α-glucosidase inhibitory activity was determined as described previously by Mojica et al. (2015), with some modifications. The enzyme reaction was performed using *p*-NPG as the substrate, which was cleaved by α-glucosidase to release *p*-nitrophenol, a chromogenic agent that can be measured spectrophotometrically. In brief, 25 μL of enzyme solution (1 U mL^{-1} of α-glucosidase solution in 100 mM phosphate buffer, pH 6.9), 50 μL of sample solution with varying concentrations and 125 μL of buffer were mixed in a 96-well microplate and incubated at 37ºC for 5 min prior to initiation of the reaction by adding the 50 μL of 5 mM *p*-NPG solution in buffer. After 10 min of incubation, the absorbance was registered at 405 nm by a microplate reader. All assays were measured in triplicate (*n*=3) and acarbose was used as the positive control. The α-glucosidase activity was calculated and expressed using a relationship similar to that used for AChE inhibition.

2.10 UHPLC-ESI-MS analysis

The presence of some alkaloids with enzymatic activity was determined directly from the extracts. Mass spectrometry analyses were carried out using a Shimadzu (Kyoto, Japan) UHPLC-ESI-MS system composed of Nexera X2 UHPLC coupled online with a LCMS 8030 triple quadrupole as the electrospray ionization (ESI) interface. Data were acquired and recorded using the Shimadzu LabSolutions software version 5.54. Chromatography was performed on a Phenomenex (Torrence, USA) Kinetex XB-C_{18} 100A (150 × 4.6 mm; 5 µm) column connected to a C_{18} guard column (4.6 id), both set at 30°C, using a mobile phase composed of acetonitrile (A) and 10 mM ammonium formate pH 3.5 (B). The following gradient program was used at a flow rate of 0.5 mL min^{-1}: 0−5 min, 50−70% (B); 5−8 min, 70% (B) (isocratic step); 8−12 min, 70-50% (B); and 12−15 min 50−50% (B) (column conditioning). To quantify the bioactive alkaloids, the mass spectrometer was operated in Selected Ion Monitoring (SIM) mode under the following conditions: ESI in negative mode with a voltage of 4.5 kV; nebulizer gas (N_2) 3 L min^{-1}, desolvation gas (N_2) 15 L min^{-1}; desolvation line temperature 250°C and heat block temperature 400°C. The concentrations of anonaine, xylopine and N-*trans*-feruloyl phenethylamine (also classified as alkaloid) were quantified as micrograms of boldine equivalent per gram of sample (µg BE g^{-1}) due to the absence of commercial standard; whereas glaucine has a standard and the concentration was calculated from its calibration curve. Prior to chromatography, all solutions were filtered through a 13 mm PVDF syringe filter (0.22 µm pore size).

3. Results and discussion

3.1. Evaluation of sample pre-treatment

Cherimoya is a fruit extremely susceptible to browning caused mainly by polyphenol oxidase enzyme activity, and this activity can reduce stability of some components (Olmedo et al. 2018). A simple pre-treatment is, therefore, a fundamental step to keep the active compounds and prevent microbial and enzymatic degradation. In this scenario, the drying of the peel was evaluated by freeze drying and heat drying. The global extraction yields of extraction obtained with both drying procedures were similar with value of 84.56 ± 2.54 and 91.19 ± 1.02 mg extract g^{-1} for freezer and heater dried respectively (**Fig. 2a**). The total alkaloid (TA) content in the lyophilized samples showed a value of 178.33 ± 14.54 µg BE g^{-1}, whereas the value of the sample dried in the oven was 202.93 ± 12.25 µg BE g^{-1}. According to the results, no significance differences were observed for TA between the two procedures

(P = 0.089). These results are important because they make it possible to the industry to choose a simple, rapid and cheap drying method, such as oven drying.

3.2 Optimization of SFE conditions

3.2.1 Co-solvent evaluation

Most alkaloids are polar compounds; therefore, the extraction with pure CO_2 presents very low yields (Liu et al. 2005). Also, they exist mainly in the form of salts in plants, which decreases the solubility in acid non-polar solvents like CO_2. For these reasons, two strategies were considered to increase the alkaloid extraction: the first was to alkalize the sample to extract the alkaloids in the form of free bases, and the second was to increase the polarity and diffusional properties by adding a co-solvent or CO_2 modifier, such as isopropanol, ethanol and methanol. In addition, the choice of the co-solvent type is limited to a suitable solvent considering safety, toxicity and environmental aspects (Gañán et al. 2016; Xiao et al. 2010). On this basis, in the present work two co-solvents (methanol and ethanol) were evaluated to extract alkaloids from cherimoya peel.

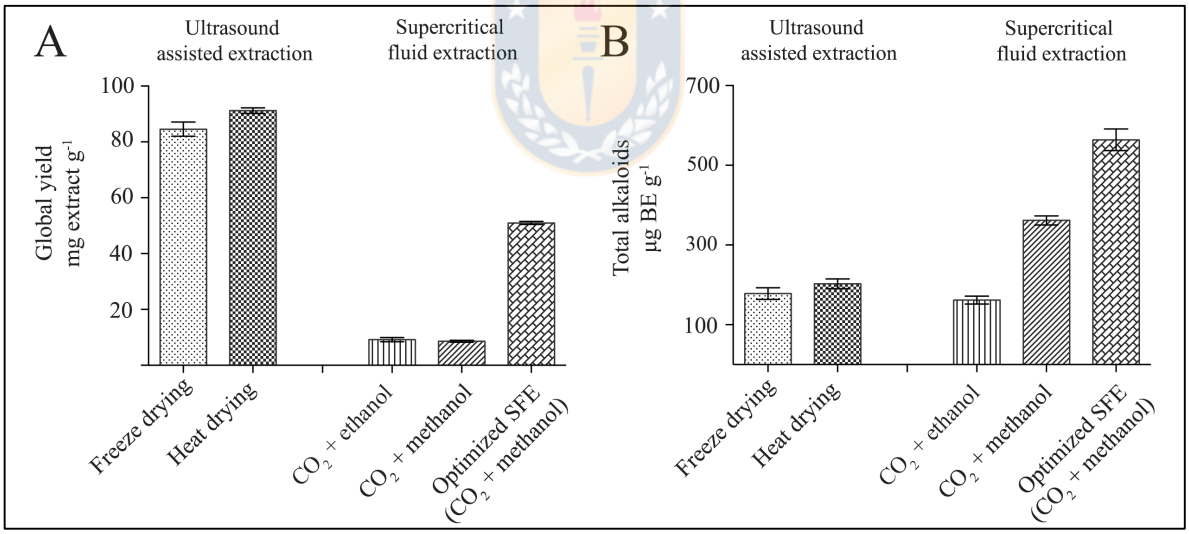

Fig. 2. Total alkaloid content and global yield of cherimoya peel extracts obtained by different extraction conditions. (A) Comparison between freeze drying and heat drying using ultrasound assisted extraction. (B) Comparison between different extraction condition using heat drying and SFE.

The results are plotted in **Fig. 2b**. The extraction using the CO_2/ethanol mixture showed a higher global yield than the CO_2/methanol mixture. However, CO_2/methanol extraction showed greater efficiency and extraction selectivity due to the amount of alkaloids extracted

per unit mass was close to twice the amount extracted with ethanol. These results can be explained by the increase in the solubility when a more polar solvent as methanol was added and the enhancement of diffusional properties because of the swelling of the sample by the liquid solvent. Consistent with these results, other authors have also concluded that the use of methanol as co-solvent enhances the extraction yield of alkaloids as compared to other modifiers like water or ethanol (Choi et al. 1999; Liu et al. 2010). The preliminary experiments were useful for the selection of the type of co-solvent; moreover, the extraction efficiency obtained with SFE was higher than that observed with ultrasound-assisted extraction using chloroform (**Fig. 2b**). Accordingly, the operating conditions for the SFE from cherimoya peel should be optimized in order to maximize the extraction of bioactive alkaloids.

3.2.2 Experimental design

The extraction of active compounds by supercritical fluids depends on several factors that could potentially affect the efficiency of the process. In this way, CCD is an effective statistical tool that allows to study the influence of various factors simultaneously and optimizing the extraction yield with a reduced number of experiments. This design is based on RSM and has been applied extensively to SFE process to determine the optimal conditions of different experimental parameters (Ameer et al. 2017). The main factors studied are temperature, pressure, CO_2 flow rate, time extraction and modifier concentration, and commonly three or four factors being studied simultaneously (Sharif et al. 2014). Since possible experimental factors that can affect the extraction - pressure, temperature and co-solvent percentage - were optimized by applying a face-centered CCD. All the experiments were conducted with a constant CO_2 flow rate (20 g min^{-1}) and time extraction (3 h). The results of the experimental runs are described in **Table 1**. Experimental data from TA fitted a second-grade model, which was validated through an ANOVA ($P < 0.05$) showing a determination coefficient (R^2) of 91.4%. The equation for total alkaloids (Y) was the following:

$$Y = -179.21 - 0.85\,X_1 + 5.16\,X_2 + 25.66\,X_3 + 0.0040\,X_1^2 - 0.028\,X_1X_2 - 0.012\,X_1X_3 + 0.040\,X_2^2 + 0.086\,X_2X_3 - 0.52\,X_3^2$$

Thus, 100 bar of pressure, 75ºC of temperature and 15% methanol were found as the optimal conditions for a maximum response. The results showed that the three factors had a significant impact ($P < 0.05$) on extraction efficiency, as illustrated in the Pareto chart (**Fig.**

3a), which is sorted from most to least significant. The positive effect of methanol concentration on alkaloid extraction yield could be explained by the increase in the polarity of the extraction mixture, which increases the solubility of alkaloids. The interaction between methanol concentration and other factors was not significant. Generally, the addition of organic solvents (methanol, ethanol and isopropanol) increases the extraction yields due to the polar nature of alkaloids, while higher yields could be attained when the polarity of the fluid matched the polarity of the analytes (Ruan et al. 2016). In SFE, the solubility of the compounds also depends on the solute vapor pressure and the solvation power of the fluid, with the latter in turn depending on the fluid density. In this way, the increase in temperature can cause two antagonistic effects: decreased density and thus a lowered solubility, and increased solute vapor pressure, which increases compounds solubility (Brandão et al. 2017). According to the results, the predominant effect was the solute vapor pressure, achieving a high extraction when the temperature increases. In the same way, the effect of vapor pressure on the extraction pressure was predominant. Pressure had a negative effect on the extraction yield although raising pressure causes an increase in fluid density, because raising pressure could cause a decrease in solute vapor pressure (Liu et al. 2010). Considering the factors studied, a response surface plot (**Fig. 3b**) with a co-solvent percentage constant can provide a better understanding of the behavior of the extraction process.

Table 1. Experimental runs for a central composite design with the corresponding responses.

Run	Factors			Response
	Pressure (bar) (X_1)	Temperature (ºC) (X_2)	Co-solvent (%) (X_3)	Total alkaloids[a] (μg BE g^{-1}) (Y)
1	100	75	5	331.26 ± 13.61
2	100	35	5	9.60 ± 0.38
3	400	35	5	44.17 ± 4.03
4	400	55	10	113.62 ± 5.59
5	100	35	15	193.53 ± 5.88
6	400	35	15	206.50 ± 5.09
7	250	55	15	121.85 ± 5.87
8	100	55	10	253.50 ± 11.55
9	250	55	10	121.51 ± 7.49
10	250	55	10	153.92 ± 10.81
11	400	75	5	50.07 ± 3.98
12	400	75	15	232.11 ± 14.62
13	250	75	10	98.28 ± 8.16
14	250	35	10	119.02 ± 3.67
15	250	55	5	37.51 ± 3.11
16	100	75	15	564.01 ± 27.13
17	250	55	10	100.77 ± 6.56

[a] Each sample were analyzed in triplicate, results are expressed as mean ± standard deviation
μg BE g^{-1}: μg boldine equivalent per gram of sample

Then, the statistical model made it possible to maximize the extraction to obtain alkaloid-rich extracts by SFE. The comparison of the optimal conditions against other reports becomes difficult, taking into account that the extraction efficiency will depend on the operating parameters, as well as on the matrix and the analyte type. In addition, the concentration of alkaloids varies among plant species as well as according to the part of the plant used, *e.g.* leaves, roots or fruit. Accordingly, some works have shown different operating conditions for an efficient alkaloid extraction Liu et al. (2010) achieves high yields of evodiamine and rutacarpine from fruit of *Evodia rutaecarpa* at 62ºC, 280 bar and approx. 7.5% methanol. Also using methanol as co-solvent, but different operating conditions, 40ºC and 200 bar, Carrara et al. (2017) extracted pyrrolidine alkaloids from leaves. Xiao et al. (2010) obtained also from leaves highest yield of nuciferine (isoquinoline alkaloid) when the extraction was carried out at 70°C under 300 bar, with diethylamine/water/methanol mixture as the co-solvent. Gañán et al. (2016) obtained a high extraction efficiency of alkaloids from the aerial part using ethanol/diethylamine mixture as co-solvent at 120 bar pressure and 55ºC. The variating conditions of the described reports demonstrate the difficulty of comparing the results among different works and the necessity of optimizing the factors and to study the behavior of the extraction process to maximize the concentration of the analyte.

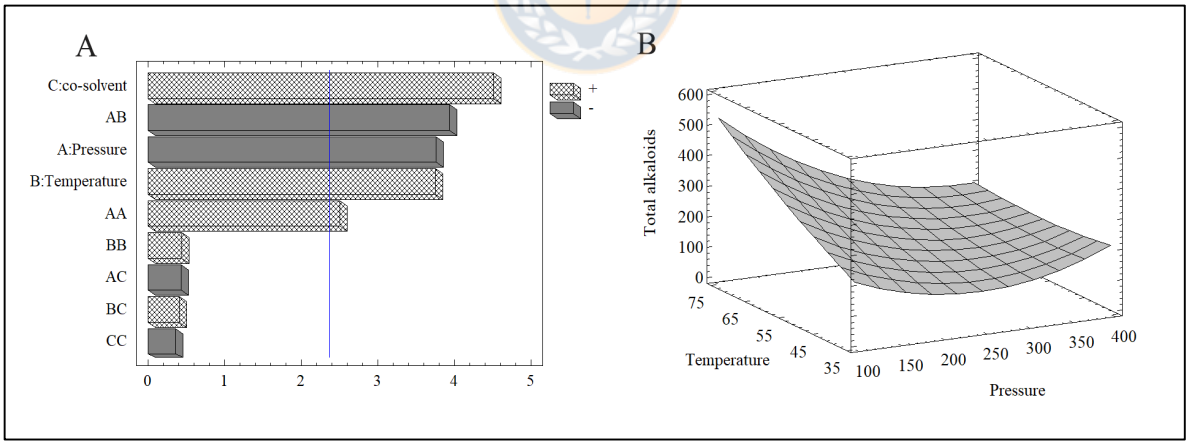

Fig. 3. (A) Standardized Pareto chart for the total alkaloids content showing the effects of factors on the response. (B) Response surface plot for the response optimization showing the effect of temperature and pressure with a co-solvent percentage constant at 15%.

3.3 Chemical and functional characterization of extracts

3.3.1 Alkaloids content

The optimized operating conditions were applied to extract alkaloids from peel samples of the two most important cherimoya varieties of Spain, 'Fino de Jete' and 'Campas', and 'Chilean variety'. It is noteworthy that in Chile, cherimoya fruit is marketed without distinction of its variety, and is commonly acquired in fruit stores as a mixture of the main varieties, 'Concha Lisa' and 'Bronceada', thereupon for the purpose of the present work were named as 'Chilean variety'. Moreover, considering that leaves also could be considered by-products of the cherimoya agro-industrial, a preliminary evaluation of alkaloid content of three Spain varieties of cherimoya leaves ('Fino de Jete', 'Campas' and 'Alboran') was carried out using the same operating conditions. The alkaloid content was determined as TA and the results are shown in **Table 2**. The highest alkaloid content was obtained in the peel of the 'Chilean variety' cherimoya with a value of 1172.60 ± 27.61 μg BE g^{-1}. The peel of Spanish varieties showed lower values, with significant differences between them (P = 0.0268). The total alkaloid content in the peel of cherimoya or another species of *Annona* has not been previously reported. As far as the leaves is concerned, the mean alkaloid content (4126.06 ± 634.03 μg BE g^{-1}) was four times higher than that determined in the peel (998.64 ± 158.47 μg BE g^{-1}). According to the results, 'Fino de Jete' leaves contain a larger amount of alkaloids than the leaves of 'Campas' variety (P = 0.011), whereas the 'Alboran' variety showed the lowest content. Thus, the cherimoya leaves initially showed a greater potential as source of bioactive alkaloids. These results are consistent with the overall distribution of alkaloids in the plant organs for *Annona* species, with the leaves being the main source of alkaloids with 42% of the alkaloids of the plant (Egydio-Brandão et al. 2017).

3.3.2 Antioxidant Capacity

The main alkaloids of the *Annona* species are aporphine alkaloids, like boldine, glaucine and other related alkaloids, which have demonstrated a potent antioxidant activity (O'Brien et al. 2006). The relationship antioxidant activity and total alkaloids has been few studied, even when a simple measurement is useful to preliminarily evaluate the qualities of extracted alkaloids. In the present work, the possible relation between total alkaloid content and antioxidant activity in cherimoya was investigated for the first time.

ORAC is a method based on hydrogen atom transfer used to evaluate the antioxidant capacity of fruits and vegetables, which could reflect the antioxidant preventive action in biological systems (Karadag et al. 2009). Then, the obtained extracts were measured and

the ORAC values were described in **Table 2**. According to the results, the 'Chilean variety' showed the greatest ORAC value (16602.33 ± 588.96 μmol TE/100 g), which is consistent with the value reported in fresh cherimoya by Speisky et al. (2012) (graphically between 16000 and 17000 μmol TE/100 g approx.). Among the Spanish varieties, the 'Fino de Jete' peel extract has a greater antioxidant capacity than 'Campas' (P = 0.0045). The cherimoya leaves presented a high antioxidant capacity with a mean value of 16421.26 ± 4734.86 μmol TE/100 g, which was 1.7-fold higher than the peel extracts (9532.93 ± 6152.01 μmol TE/100 g). The high ORAC value determined for 'Fino de Jete' leaf extract (21705.20 ± 1069.26 μmol TE/100 g) was similar to those reported for some fruits recognized for their high antioxidant capacity, such as blueberries (Speisky et al. 2012).

The correlation between ORAC data and their corresponding TA content was studied. A high correlation was observed between antioxidant capacity, expressed as ORAC, and the presence of alkaloids, with a r value of 0.954. The correlation increased when the results were studied according to the studied by-products, with a r value of 0.998 and 0.969 for peel and leaves, respectively. These results could be linked to the presence of a phenolic hydroxyl group in the structure of the alkaloids with the ability to stabilize peroxyl radicals (Tian et al. 2018). Also, the presence of co-extracted compounds in the extracts, such as phenolic compounds, should be considered since high amounts have been reported in peel (García-Salas et al. 2015) and leaves (Díaz-de-Cerio et al. 2018). Then, the TP content was determined (**Table 2**), and the correlation with the antioxidant capacity was studied. The results showed a high correlation between TP and the ORAC values of the leaf extracts with a r value of 0.965, whereas a very low correlation was observed in the peel extracts with a r value of 0.691. The established correlation for the leaf extracts could be the sum of the alkaloid effects and phenolic compounds on the antioxidant capacity. In this case, the Folin-Ciocalteu assay should have shown the total reducing capacity of the extracts, but it could not differentiate the type of compound responsible for the activity: alkaloids or phenols with a hydroxyl group. Moreover, the low correlation between TP and the ORAC values in peel extract indicates that the alkaloid content is possibly responsible for the antioxidant activity. These results showed the importance of alkaloids present in the extracts to the antioxidant activity, and it is important to highlight the potentiality of agro-industrial cherimoya by-products as a rich source of antioxidant compounds.

3.3.3 AChE inhibitory activity

In order to search and evaluate the presence of AChE inhibitors, as a possible strategy for the treatment of the Alzheimer's disease, the extracts obtained have been evaluated using Ellman's *in vitro* enzymatic method. In this regard, it is well-known that organic solvents can affect enzymatic activity, so keeping the concentration of the organic solvent in the assay mixture as small as possible is recommended. Moreover, the final concentration of organic solvent must prevent the precipitation of weakly soluble compounds in the aqueous assay mixture (Bisswanger 2014). Hence, prior to assays, the methanolic extract obtained by SFE was dried, and a solution composed by 10% (v/v) DMSO in TRIS-HCl buffer was adequate to re-dissolve the sample, with a DMSO final concentration of 1% (v/v). This percentage was within the range used in other works (0.5 to 1.6% v/v DMSO final) (Abbas-Mohammadi et al. 2018; Di Giovanni et al. 2008; Dong et al. 2015). In this study, donepezil was used as the positive control, which had an IC_{50} value of 0.039 ± 0.001 μM (0.015 ± 0.001 μg mL^{-1}) in agreement with the data in the literature (Martinez et al. 2000).

The extracts obtained from the cherimoya by-products showed an interesting inhibitory activity on the AChE, proving the presence of bioactive compounds. The results of the assay are shown in **Table 2** and revealed that leaves extracts have greater activity than peel extracts. The 'Fino de Jete' leaf extract exhibited a better activity with an IC_{50} value of 87.69 ± 3.42 μg mL^{-1}, whereas the least activity was found in the 'Chilean variety' peel extract with an IC_{50} value of 515.02 ± 29.25 μg mL^{-1} despite its high alkaloid content.

According to the results of the leaf extracts, the alkaloid content showed a high correlation with the inhibitory activity (r = 0.990), i.e., when the alkaloid content increased, the potency of the extract also increased. Then, AChE inhibition of the leaf extracts could be associated with the presence of alkaloids due to previous reports indicating these metabolites as the main AChE inhibitors, such as physostigmine and galantamine (Mukherjee et al. 2007). By contrast, a high alkaloid content produces a decrease in the activity of the peel extracts with a high correlation (r = 0.97); a behavior which could be explained by the presence of co-extract compounds that interact with the alkaloids, which do not allow the alkaloid-enzyme interaction. This however, requires an in-depth investigation due to the scant information of the constituents in the cherimoya peel.

3.3.4 α-glucosidase inhibitory activity

α-glucosidase inhibitors (AGIs) are currently attractive bioactive compounds owing to their therapeutic potential in the treatment of type II diabetes mellitus, cancer, and bacterial and

viral infections (including influenza and HIV) (Gloster and Davies 2010). The main *in vitro* assay used for the evaluation of natural extracts is based on the measurement of *p*-nitrophenol (yellow), which is measured at 405 nm. A solution of 10% v/v DMSO in phosphate buffer (2% v/v DMSO final concentration) was necessary to dissolve the sample and avoid the incompatibility of the organic solvent with the enzyme.

A positive control was analyzed using acarbose as the control inhibitor; its effectiveness was observed when an IC_{50} value of 245.86 ± 25.80 μg mL^{-1} (0.38 ± 0.04 mM) was obtained, which was consistent with previous data reports (Zhang et al. 2016). In general, the effect of cherimoya extracts on α-glucosidase inhibition showed a lower inhibition, with IC_{50} values ranging between 1097.76 ± 121.12 μg mL^{-1} and 3206.88 ± 97.06 μg mL^{-1}, which are described in **Table 2**. In this scenario, the extracts of cherimoya leaves were most active on the enzyme in comparison with peel extracts, because it showed the lowest IC_{50} value, 1798.12 ± 85.28 μg mL^{-1}, with this activity being 7.3-fold lower than acarbose. The most active inhibitor was 'Fino de Jete' leaf extract with an IC_{50} of 1097.76 ± 121.12 μg mL^{-1}, followed by 'Campas' leaf extract with an IC_{50} of 1281.29 ± 155.27 μg mL^{-1}. The capacity of the extract of 'Chilean variety' peel to inhibit α-glucosidase was also noted, with an IC_{50} value of 1550.50 ± 100.85 μg mL^{-1}.

Table 2. Evaluation of alkaloids content, total phenols and in vitro bioactivity[a] of extracts obtained by an optimized SFE process of different varieties of peel and leaf cherimoya.

Sample	Total alkaloids (μg BE g^{-1})	Total phenols (μg GAE g^{-1})	ORAC (μmol TE/100 g)	IC_{50} (μg mL^{-1})	
				AChE inhibitory	α-glucosidase inhibitory
Leaf variety					
'Alboran'	3496.49 ± 280.68	341.31 ± 15.36	12563.22 ± 237.26	136.76 ± 7.15	1798.12 ± 85.28
'Fino de Jete'	4764.45 ± 226.52	480.05 ± 6.61	21705.20 ± 1069.26	87.69 ± 3.42	1097.76 ± 121.12
'Campas	4117.24 ± 322.95	411.13 ± 9.82	14995.35 ± 75.28	106.80 ± 7.74	1281.29 ± 155.27
Peel variety					
'Fino de Jete'	960.81 ± 12.33	128.55 ± 4.51	6602.25 ± 160.95	287.67 ± 30.45	3206.88 ± 97.06
'Campas'	862.51 ± 18.89	270.71 ± 6.90	5394.20 ± 73.61	244.59 ± 19.01	2877.72 ± 49.55
'Chilean'	1172.60 ± 27.61	472.29 ± 2.80	16602.33 ± 588.96	515.02 ± 29.25	1550.50 ± 100.85

[a] Each sample were analyzed in triplicate, results are presented as mean ± standard deviation

By a simple correlation study, it was established that the alkaloid content in the extracts was not correlated with the inhibitory activity. Alkaloids have been reported as AGIs, although they are not the main chemical class; some studies report that phenolic compounds, peptides and terpenoids have an outstanding inhibitory activity (Di Stefano et al. 2018). Thus, the co-extracted phenolic compounds, measured as PT, are correlated with α-

glucosidase inhibitory activity, with a r = 0.93, then the increase in PT decreases the IC_{50} values. The correlation increases when the sample types are considered separately, with r values of 0.97 and 0.96 for peel and leaf extract, respectively. These results could be explained by the presence of hydroxycinnamic acid amides (also classified as alkaloids, amides or phenolamides) (Borges de Melo et al. 2006), like *N-trans*-feruloyltyramine and *N-p*-coumaroyltyramine, which are potent AGIs and have been isolated from *Annona* species (Avula et al. 2018; Chang et al. 2000). The hydroxycinnamic acid amides are formed by a phenolic fraction bound to an amine. Then, the phenolic fraction was able to react more strongly with Folin-Ciocalteu reagent (TP) than the amine group with the bromocresol green reagent (TA). The presence of AGI compounds in by-product extracts and potential bioactivity have been demonstrated by these analyses. However, it is also important to isolate and identify these compounds to assess their bioactivity.

3.3.5 Preliminary quantification of bioactive alkaloids

In previous studies conducted by our research group, some alkaloids with enzymatic activity have been identified from cherimoya peel. The aporphine alkaloids with possible AChE inhibitory activity are the following: glaucine, anonaine and xylopine (Galarce-Bustos et al. 2019). Also, one phenolamide, N-*trans*-feruloyl phenethylamine, was identified as a potential AGI. Therefore, the presence of the compounds that possess bioactivity was studied in the peel and leaf extracts. A simple quantitative analysis was carried out using Shimadzu UHPLC-ESI-MS with SIM mode, as shown in **Fig. 4**. This quantification aided a preliminary evaluation of the content of bioactive compounds.

As shown in **Table 3**, the leaf extracts showed a higher concentration of AChE inhibitory alkaloids than the peel extracts. These results agreed with the greater inhibitory activity established in the leaf extracts. Individually, anonaine was the main alkaloid found in leaf extracts. The mean value of the anonaine content was 15.49 ± 3.25 μg BE mL^{-1}, which was 16-fold higher than the value determined in peel extracts (0.95 ± 0.37 μg BE g^{-1}). The 'Fino de Jete' variety leaves presented the highest anonaine content with a concentration of 18.83 ± 0.24 μg BE mL^{-1}. Also, a larger content of xylopine, with a mean value of 3.14 ± 1.76 μg BE mL^{-1}, was found in leaf extracts than in the peel extracts (1.43 ± 0.66 μg BE mL^{-1}), and the highest concentration was determined in 'Campas' leaves (4.41 ± 0.11 μg mL^{-1}). With respect to glaucine, the peel extracts exhibited a content 4.5-fold higher than the leaf extracts, showing mean values of 3.01 ± 2.05 μg mL^{-1} and 0.67 ± 0.39 μg mL^{-1} respectively. According to these results, the main contribution of

glaucine was given by 'Chilean variety' peel with a concentration of 5.35 ± 0.08 μg mL^{-1}. The presence of these aporphine alkaloids add value to the by-products studied because they have also shown other bioactivities. In particular, anonaine presents antiplasmodial, antibacterial, antifungal, antioxidant, anticancer, antidepression and vasorelaxant activity (Li et al. 2013), and glaucine has demonstrated anti-inflammatory, analgesic and antipyretic activity (Pinto et al. 1998).

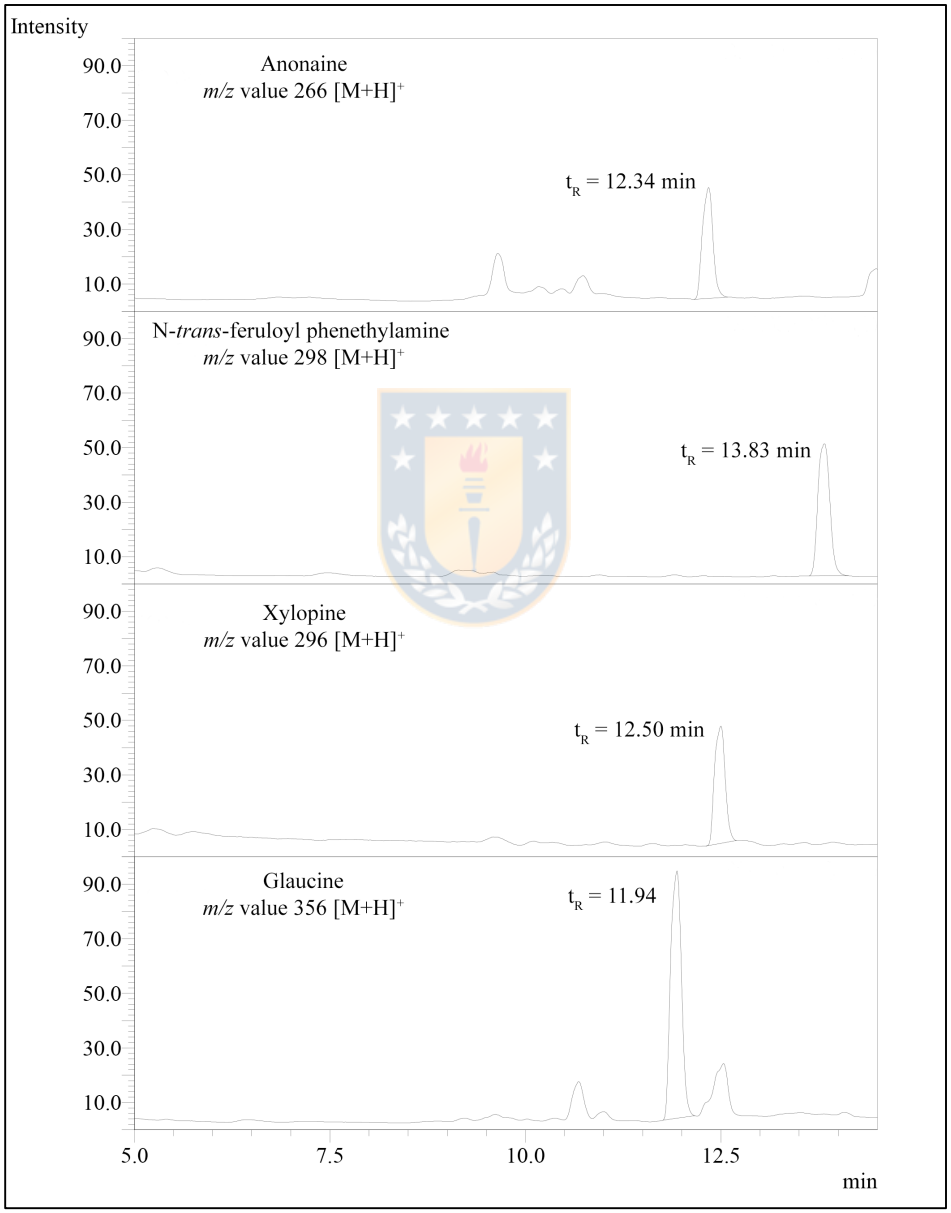

Fig. 4. Representative UHPLC-ESI-MS chromatogram with selected-ion monitoring (SIM) from the quantitative determination of alkaloids in peel extract.

Regarding the AGI N-*trans*-feruloyl phenethylamine, the concentration was the second highest, with a mean value of 8.71 ± 5.86 μg BE mL^{-1} in peel extracts, whereas its presence in leaf extracts was only detected. With these results, the inhibitory activity of the extracts cannot only be attributed to this alkaloid, and the compounds responsible for this activity should be investigated. However, individually N-*trans*-feruloyl phenethylamine has shown a strong inhibition of α-glucosidase, with an IC_{50} value 18.7-fold of acarbose (Zhang et al. 2016). In addition, phenolamides have been described as interesting compounds with several bioactivities, such as antiviral, antibacterial, antifungal (Bassard et al. 2010), hypotensive, anti-inflammatory and antidiabetic activity (Zhang et al. 2016).

The individual content of the alkaloids studied should not be correlated with the antioxidant and enzymatic activity of the extracts, because there may be synergistic and antagonistic effects of their constituents on the total activity of a crude extract. Therefore, this research only included the quantification of some alkaloids; however, other alkaloids and non-alkaloid compounds could exert an effect on the bioactivities.

Table 3. Alkaloids content in leaves and peel cherimoya extracts obtained by SFE and quantification by UHPLC-DAD-ESI-MS with SIM mode[a].

Sample	Anonaine* (μg BE g^{-1})	Xylopine* (μg BE g^{-1})	Glaucine** (μg g^{-1})	N-*trans*-feruloyl phenethylamine* (μg BE g^{-1})
Leaf variety				
'Alboran'	15.32 ± 1.16	3.89 ± 0.40	1.01 ± 0.02	Detected
'Fino de Jete'	18.83 ± 0.24	1.13 ± 0.03	0.25 ± 0.01	Detected
'Campas	12.33 ± 0.17	4.41 ± 0.11	0.75 ± 0.05	Detected
mean	15.49 ± 3.25	3.14 ± 1.76	0.67 ± 0.39	--
Peel variety				
'Fino de Jete'	0.99 ± 0.04	2.05 ± 0.07	1.50 ± 0.04	15.10 ± 1.10
'Campas'	1.30 ± 0.03	0.73 ± 0.01	2.19 ± 0.01	7.17 ± 0.12
'Chilean'	0.56 ± 0.03	1.52 ± 0.07	5.35 ± 0.08	3.77 ± 0.05
mean	0.95 ± 0.37	1.43 ± 0.66	3.01 ± 2.05	8.71 ± 5.86

[a] Each sample were analyzed in triplicate, results are presented as mean ± standard deviation
* Alkaloids content expressed as μg boldine equivalent per gram of sample (μg BE g^{-1})
** Alkaloids content expressed as μg glaucine per gram of sample (μg g^{-1})

4. Conclusions

This work reports for the first time the use of supercritical fluids to extract bioactive compounds from cherimoya peel and leaves. The experimental design, CCD, made it possible to know the influence of the three factors on extraction yield, establishing that the solute vapor pressure was the predominant effect. The optimal conditions of the SFE process increased the extraction efficiency and alkaloid-rich extracts were obtained using low pressures and high temperature. From the *in vitro* bioactivity assays, it was found that

peel and leaf extracts have significant antioxidant potential and an interesting inhibitory effect on α-glucosidase and AChE, linked with a high content on interesting bioactive alkaloids with potential pharmaceutical applications such as anonaine, glaucine, xylopine and N-*trans*-feruloyl phenethylamine. In this way, the data obtained in the present work contribute to the added value to these agro-industrial by-products as sources of interesting bioactive compounds and provide valuable information to current knowledge of their bioactivity. Additionally, with this information, several studies can be conducted, with opportunities to isolate valuable compounds, thereby providing a positive economic and environmental impact.

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CAPÍTULO IV: *Conclusiones finales*

CONCLUSIONES

A partir de los resultados obtenidos y de la discusión realizada en el presente trabajo de tesis doctoral, se pueden obtener las siguientes conclusiones:

1. Una metodología HPTLC-bioensayo fue desarrollada para detectar compuestos inhibidores de acetilcolinesterasa (AChE). Las condiciones del ensayo fueron mejoradas en relación a concentración enzimática y tiempo de incubación, resultando en una metodología eficiente respecto a tiempo y costo. Extractos de piel, semilla y pulpa de chirimoya fueron evaluados mediante esta metodología. La aplicación de un análisis *in situ* de las bandas activas, mediante TLC-MS y otras técnicas complementarias (UHPLC-DAD-ESI-MS/MS), permitió la identificación en piel de dos alcaloides, anonaina y glaucina, como potenciales inhibidores, y xilopina como un posible candidato. De esta manera este trabajo mostró por primera vez el efecto inhibitorio sobre AChE de estos alcaloides.
2. Compuestos inhibidores de α-glucosidasa fueron detectados mediante la implementación metodología HPTLC-bioensayo mejorada. La metodología permitió la detección de tres compuestos inhibidores en piel y semilla de chirimoya. La aplicación de un análisis de las bandas activas directamente desde la placa mediante TLC-MS, y TLC semi-preparativa, permitió identificar los componentes activos como: N-*trans*-feruloil tiramine, N-*trans*-feruloil fenetilamina y N-*trans*-*p*-coumaroil tiramina. Estas fenolamidas (o feniletil cinamidas) presentan varias actividades potencialmente terapéuticas y podrían contribuir en la búsqueda de nuevos agentes terapéuticos. Además, el presente trabajo reportó por primera vez la presencia de N-*trans*-feruloil fenetilamina en chirimoya.
3. La metodología HPTLC para compuestos inhibidores de enzima convertidora de angiotensina (ECA) no fue desarrollada. La enzima resultó inestable sobre una placa HPTLC. Bajo diferentes condiciones, de humedad y pH, la enzima no mostró actividad y su alto costo (1.0 U, U$ 850 sin IVA) no permitió realizar un mayor número de experimentos. Una metodología de *screening* para compuestos bioactivos de alto costo tiene bajas probabilidades de ser aplicada dentro de una plataforma analítica.
4. La actividad antioxidante fue evaluada mediante un ensayo HPTLC-DPPH, debido que el ensayo DPPH es ampliamente utilizado y requerir de poco equipamiento especializado, traduciéndose en una metodología fácilmente aplicable y repetible por

otros laboratorios, en comparación con ORAC. De esta manera, la actividad antioxidante de los compuestos presentes en extracto de semilla y piel de chirimoya fue cuantificada a partir de un ensayo de efecto dirigido HPTLC-DPPH mediante procesamiento de imagen digital de un cromatograma en placa usando el software ImageJ. El ensayo HPTLC-DPPH-MS permitió además identificar preliminarmente dos grupos de compuestos, fenolamidas y acetogeninas, como destacados compuestos antioxidantes. La metodología resultó confiable y fácil de implementar como análisis de rutina, pudiendo ser escalada al uso de una simple cámara fotográfica.

5. Mediante una metodología extractiva no convencional por fluidos supercríticos se obtuvo un extracto de piel de chirimoya con una destacada actividad inhibitoria de AChE, α-glucosidasa y antioxidante. El proceso fue optimizado mediante un diseño central compuesto (DCC) y se obtuvo un rendimiento extractivo óptimo usando un 15% de metanol como co-solvente, 100 bar de presión y 75ºC de temperatura. La presencia de compuestos bioactivos en los extractos fue establecida mediante análisis UHPLC-ESI-MS. Adicionalmente la extracción de compuestos bioactivos desde hojas de diferentes variedades de chirimoya fue realizada con la metodología propuesta, resultando en una alta presencia de alcaloides inhibidores de AChE.
6. El desarrollo de la plataforma analítica fue dirigido hacia ensayos económicos, reproducibles y repetibles, alcanzando en parte una universalidad analítica. De esta manera, las metodologías propuestas permitieron la determinación e identificación *in situ* de compuestos bioactivos en chirimoya usando HPTLC-UV/Vis-bioensayo-MS y seguido de UHPLC-MS/MS. El análisis de bioactividad, mediante ensayos de efecto dirigido y caracterización de los componentes activos logró ser integrado en una placa cromatográfica, y el flujo de trabajo resultó en una opción simple en comparación con los métodos tradicionales, principalmente por ser rápido, de fácil uso y amigable con el medio ambiente por su bajo consumo de solventes orgánicos. Es así como, sí se considera una adecuada disponibilidad de equipos y fase móvil, la aplicación de la plataforma analítica desarrollada permite la detección y una importante aproximación de la identidad de los compuestos bioactivos en menos de un día de trabajo.