
# UNIVERSIDAD DE MURCIA

## ESCUELA INTERNACIONAL DE DOCTORADO

# UPLC-QTOF-MS-Untargeted metabolomics to explain enzymatic browning of fresh-cut lettuce

# Metabolómica no dirigida por UPLC-QTOF-MS para explicar el pardeamiento enzimático en lechuga fresca cortada

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- **Garcia, C.J.,** García-Villalba, R., Gil, M.I., Tomas-Barberan, F.A., 2017. LC-MS Untargeted metabolomics to explain the signal metabolites inducing browning in fresh-cut lettuce. Journal of Agricultural and Food Chemistry. 65, 4526-4535.

- **Garcia, C.J.,** Gil, M.I., Tomas-Barberan, F.A., 2018. LC-MS untargeted metabolomics reveals early biomarkers to predict browning of fresh-cut lettuce. Postharvest Biology and Technology. 146, 9-17.

- **Garcia, C.J.,** Gil, M.I., Tomas-Barberan, F.A., 2019. Targeted metabolomics analysis and identification of biomarkers for predicting browning of fresh-cut lettuce. Journal of Agricultural and Food Chemistry. 67, 5908−5917.

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*Aprende a observar;*

*sé consciente de todo cuanto te rodea.*

*La solución siempre ha estado delante de ti.*

*A mi familia, mi guía para convertirme en lo que hoy soy.*

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# UPLC-QTOF-MS-Untargeted metabolomics to explain enzymatic browning of fresh-cut lettuce

Trabajo realizado para obtener el Título de Doctor por la Universidad de Murcia

Carlos J. García Hernández-Gil

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# GENERAL INDEX

**GENERAL INDEX**

Summary/Resumen I

List of abbreviations and symbols XIII

List of figures and tables XVIII

**CHAPTER I:** Introduction

1. LETTUCE 1

1.1. Origin 3

1.2. Horticultural types 4

1.3. Production and value 6

2. FRESH-CUT LETTUCE 9

2.1. Quality loss of fresh-cut lettuce 10

2.2. Physical and physiological responses to wounding 11

3. ENZYMATIC BROWNING 16

3.1. Polyphenol oxidase (PPO) 16

3.2. Peroxidase (POD) 18

3.3. Phenylalanine ammonia lyase (PAL) 18

4.PHENOLIC METABOLITES 19

4.1. Lettuce phenolic compounds 21

5. CONTROL OF ENZYMATIC BROWNING 22

5.1. Cold storage 23

5.2. Modified atmosphere packaging (MAP) 23

5.3. Anti-browning solutions 24

5.4. Heat shocks 26

6. NEW OMICS SCIENCES 26

6.1. Metabolomics 28

6.2. Untargeted metabolomics analysis 30

7. DATA TREATMENT 33

7.1. Data processing 33

7.2. Data normalization 34

7.2.1. Sample normalization or baselining 35

7.2.2. Scaling 35

7.2.3. Transformation 36

8. DATA ANALYSIS 37

8.1. Univariate analysis 37

8.2. Multivariate analysis 38

**CHAPTER II:** Objectives 40

**CHAPTER III:** Material and methods

1. PLANT MATERIAL 42

2. PROCESSING AND STORAGE CONDITIONS 43

3. DIGITAL IMAGE ANALYSIS 45

4. SAMPLE PREPARATION 47

5. UPLC-ESI-QTOF-MS 48

6. METABOLOMICS DATA TREATMENT 54

6.1. Data pre-processing 55

6.1.2. Data integrity test 56

6.2. Data processing 57

6.3. Data analysis and identification 57

**CHAPTER IV:** Untargeted metabolomics approach using UPLC-ESI-QTOF-MS to explore the metabolome of fresh-cut iceberg lettuce 60

**CHAPTER V:** LC-MS Untargeted metabolomics to explain the signal metabolites inducing browning in fresh-cut lettuce 73

**CHAPTER VI:** LC-MS untargeted metabolomics reveals early biomarkers to predict browning of fresh-cut lettuce 83

**CHAPTER VII:** Targeted metabolomics analysis and identification of biomarkers for predicting browning of fresh-cut lettuce 92

**CHAPTER VIII:** General discussion 102

**CHAPTER IX:** Conclusions 122

**REFERENCES** 124

# SUMMARY

**Summary**

**UPLC-QTOF-MS-Untargeted metabolomics to explain enzymatic browning of fresh-cut lettuce.**

Lettuce is the most popular vegetable grown on a commercial scale. A detailed knowledge of its physiology, technology and biochemistry is key for a correct production, processing and commercial distribution to guarantee a high quality product. The new trends of consumption and a higher concern about a healthy diet have led to an increase in the world demand for fresh-cut lettuce as ready to eat mix salads, what makes lettuce the main raw material for the fresh-cut processing industry. One of the main problems that the industry faces at when commercializing fresh-cut lettuce is the enzymatic browning, which is the main responsible for the quality loss after cutting that often leads to consumer rejection of the product.

The main objective of this Thesis was the identification of the metabolites that could explain the enzymatic browning and the selection of those that could act as biomarkers for the selection of lettuce cultivars for the industrial processing with less susceptibility to develop browning. For this purpose, an untargeted metabolomics approach with an UPLC-ESI-QTOF-MS was carried out.

To reach this objective, an essential requirement was the development of a robust methodology for the untargeted metabolomics analysis, which was the objective included in **Chapter IV**. The untargeted metabolomics analysis was able to explore the whole metabolome of lettuce cultivars with different browning susceptibilities and to detect differences in the metabolic profiles associated with browning before and after its development. The setup of this methodology included building a specific database of lettuce metabolites, which allowed the identification of specific metabolites of this plant

species during the different studies carried out in this Thesis. The first analyses allowed the correlation between enzymatic browning and the metabolic profile which included amino acids, phenolic compounds, sesquiterpene lactones, and fatty acid derivatives. The preliminary identification of these metabolites belonging to different metabolic groups suggested that a study of the role of the different metabolites in browning development was needed.

The identification of metabolites associated with browning development including phenolic compounds, lysophospholipids, and oxidixed derivatives of fatty acids (oxylipins), that also are intermediates in the jasmomic acid biosynthesis, allowed the interpretation of the biosynthetic pathway following a logical sequence of metabolic events that were initiated with the wound damage damaging the cell membranes during the cutting process, the generation and propagation of the wound signals, and the induction of the biosynthesis of phenolic compounds that are substrates of the enzymes leading to browning. The results of the kinetic study in response to the cut-induced wounding and the changes during storage on the different metabolites identified as signals, as well as the intermediate and final metabolites are included in **Chapter V**. The results showed that a higher browning susceptibility was directly associated with a higher speed in the wound response, in the biosynthesis of metabolites of the jasmonic acid pathway, which include the phenolic compounds that are substrates of the enzyme polyphenol oxidase.

These results suggested the need of understanding the impact of genetics on the changes in the metabolites associated with browning through de study of different cultivars. In **Chapter VI** thirty Romaine lettuce cultivars with different browning susceptibility harvested at three consecutive dates were studied. The results showed that there was a large variability in the browning susceptibility of the same cultivar when

harvested at different dates, and therefore, the potential impact of environmental and development factors affecting the browning susceptibility. The study allowed the selection of some metabolites that could act as biomarkers of enzymatic browning. The results included in **Chapter VI** suggested that the ratio between phenolic metabolites that are substrates of enzymatic browning (PPO substrates) and those that are not substrates (PPO inhibitors, or precursors of lignin formation), could be used to predict the level of browning development of a given cultivar. In this sense, it was established that the basal ratio of caffeoyl quinic/ferulate methyl ester (chlorogenic acid/FAME), both tentatively identified, correlated with a higher browning development after cutting and five days of storage. This ratio predicted the browning susceptibility level in more than 70% of the lettuce cultivars studied and prompted the need for its validation as a biomarker.

The results obtained on the browning susceptibility of different cultivars at different harvests dates included in **Chapter VI** also suggested that the lettuce development stage could also play a relevant role in browning as a more immature stage could led to a lower browning development. These results suggested that the basal levels of the phenolic metabolites that are substrates of the browning enzymes could be influenced by the plant development stage and this could affect the mechanism of the wound-response and finally the browning development. Therefore, in **Chapter VII**, the metabolic changes associated with lettuce browning susceptibility at two different development stages which differed in only two weeks between harvest were specifically evaluated. The metabolites associated with lettuce browning susceptibility were identified and validated in the **Chapter VII** by a targeted metabolomics approach for the whole characterization as biomarkers. The targeted analysis allowed the identification of sinapaldehyde (*m/z* 207.0663), that was tentatively identified as ferulic acid methyl ester in **Chapter VI**, and its 4-β-D-glucoside and 4-β-D-(6'-malonyl) glucoside conjugates. In

addition, the caffeoyl quinic derivatives were identified as chlorogenic acid isomers (5-*trans*-caffeoyl quinic, and 5-*cis*-caffeoyl-quinic acids). The results showed that the lettuce development stage affected directly the browning process and the related metabolites, being the browning susceptibility smaller in the samples harvested earlier, and increasing when the samples were harvested with a more advanced development stage. Results included in **Chapter VII** confirmed these biomarkers and the validation of the ratio **chlorogenic acid isomers/sinapaldehyde and conjugates** in twenty four cultivars of fresh-cut Romaine lettuce to predict browning susceptibility.

# RESUMEN

**Resumen**

**Metabolómica no dirigida por UPLC-QTOF-MS para explicar el pardeamiento enzimático en lechuga fresca cortada**

La lechuga es la hortaliza de hoja más popular cultivada a escala comercial. El conocimiento detallado de su fisiología, tecnología y bioquímica es clave para una correcta producción, procesamiento y distribución comercial para garantizar un producto de alta calidad. Las nuevas tendencias de consumo y la mayor preocupación por una dieta saludable han llevado a un aumento en la demanda de lechugas frescas preparadas y listas para comer, o en cuarta gama, lo que hace de la lechuga sea la principal materia prima para la industria de procesado en fresco. Uno de los principales problemas a los que se enfrenta la industria al comercializar la lechuga fresca cortada es el pardeamiento enzimático, el cual es el principal responsable de la pérdida de calidad después del corte el cual ocasiona a menudo el rechazo del producto por parte del consumidor.

El objetivo principal de esta Tesis Doctoral ha sido la identificación de metabolitos que pudieran explicar el pardeamiento enzimático y la selección de aquellos que pudieran actuar como biomarcadores para la selección de variedades de lechuga con menor susceptibilidad al pardeamiento para su procesado en cuarta gama. Para conseguir esta meta se establecieron los siguientes objetivos secundarios:

i) Establecer una metodología robusta mediante el análisis por metabolómica no dirigida que permita explorar el metaboloma de la lechuga.

ii) Avanzar en el conocimiento de aquellos metabolitos que se comportan como señales en el desarrollo del proceso de pardeamiento.

iii) Seleccionar en la lechuga posibles biomarcadores relacionados con el pardeamiento tras el corte teniendo en cuenta factores genéticos y la interacción con factores medioambientales.

iv) Identificar y seleccionar mediante metabolómica dirigida los metabolitos capaces de predecir el pardeamiento con el estudio de variedades con distinto grado de desarrollo y su validación.

Para alcanzar estos objetivos se ha llevado a cabo una aproximación metabolómica no dirigida utilizando un equipo UPLC-ESI-QTOF-MS en la mayoría de los ensayos, así como también el análisis por metabolómica dirigida para la identificación de metabolitos capaces de predecir el pardeamiento.

Un requisito esencial ha sido el desarrollo de una metodología robusta para el análisis metabolómico no dirigido, objetivo perseguido en el **Capítulo IV.** El análisis por metabolómica no dirigida permitió explorar el metaboloma completo de variedades con distintas susceptibilidades al pardeamiento y detectar las diferencias en el perfil de metabolitos relacionados con este proceso. La puesta a punto de esta metodología incluyó la creación de una base de datos con los metabolitos de la lechuga. Esto permitió identificar aquellos metabolitos específicos de esta especie vegetal y acometer los diferentes estudios que comprenden la presente Tesis. Los primeros análisis permitieron correlacionar el pardeamiento con un perfil de metabolitos donde se incluyeron aminoácidos, compuestos fenólicos, lactonas sesquiterpénicas, y derivados de ácidos grasos. La identificación preliminar de metabolitos pertenecientes a grupos metabólicos completamente diferentes permitió profundizar en el papel de los diferentes metabolitos en el desarrollo del pardeamiento enzimático.

La identificación de metabolitos asociados con el desarrollo del pardeamiento como los compuestos fenólicos, los lisofospolípidos y los derivados oxidados de ácidos

grasos (oxilipinas), que a su vez son intermediarios en la síntesis del ácido jasmónico, permitió la interpretación bioquímica dentro de una secuencia lógica de eventos metabólicos que se inician con el daño producido por el corte en las membranas celulares, la generación y propagación de señales, y la inducción de la síntesis de compuestos fenólicos sustratos de las enzimas que conducen al pardeamiento. Los resultados del estudio de la cinética de respuesta al daño inducido por el corte y los cambios metabólicos que se producen durante su conservación en refrigeración se han incluido en el **Capítulo V**. Se han estudiado diferentes metabolitos previamente identificados como señales del corte, así como los metabolitos intermedios y finales del proceso que conduce al pardeamiento enzimático inducido por el corte. Se identificaron diversos metabolitos pertenecientes a diferentes familias y cuyos cambios en su contenido tras el procesado y la conservación (0 h- 120 h) se estudiaron con una aproximación cinética. Estos metabolitos identificados pertenecen a las familias de los lisofosfolipidos, las oxilipinas y los fenoles. Los metabolitos del ácido hidroxijasmonico, ferulato de metilo, ácido 9-hidroperoxido-12,13-epoxi-10-octadecanoico y el coniferil alcohol glucósido se encontraron entre los que tenían mayor significación estadística para discriminar entre las variedades que se pardeaban más rápidamente de las que lo hacían más lentamente. Los resultados mostraron que la mayor susceptibilidad al pardeamiento estaba directamente relacionada con la mayor velocidad de respuesta al daño y con la biosíntesis de metabolitos de la ruta del ácido jasmónico y de aquellos metabolitos fenólicos que son sustratos de la enzima polifenol oxidasa.

Estos resultados plantearon la necesidad de conocer de qué manera la dotación genética de las variedades de lechuga afectaban a los diferentes grupos de metabolitos asociados con el pardeamiento. En el **Capítulo VI** se han incluido los resultados del estudio de 30 variedades de lechuga romana con diferente susceptibilidad al pardeamiento

y muestreadas en tres fechas de recolección consecutivas. Los resultados mostraron que un mismo cultivar presentaba diferentes niveles de pardeamiento en diferentes fechas de recolección, y por lo tanto, los factores ambientales debían jugar también un papel muy relevante en el desarrollo del pardeamiento. Este estudio permitió seleccionar una serie de metabolitos de interés que podían actuar como biomarcadores del pardeamiento enzimático. Se identificaron metabolitos que correlacionaban positivamente con el proceso de pardeamiento alcanzado tras cinco días de conservación, así como otros que correlacionaban negativamente con el pardeamiento. Los resultados de este capítulo sugirieron que un cociente entre metabolitos fenólicos basales que son sustratos de pardeamiento (sustratos de PPO) y aquellos que no lo son (inhibidores de PPO o bien precursores de síntesis de lignina), podrían predecir el desarrollo del pardeamiento en una variedad determinada. En este sentido, se pudo establecer de forma preliminar que la relación **ácido clorogénico/derivados del ácido ferúlico** (ácido clorogénico/ester metílico del ferúlico) se podrían correlacionar con el nivel de pardeamiento tras el corte. En este capítulo los metabolitos fueron identificados de forma tentativa sin proceder a la confirmación con estándares. Esta relación permitió predecir la susceptibilidad al pardeamiento de las 30 variedades de lechuga estudiadas en más del 70% de los casos y sugirió la necesidad de su validación para ser aplicada como biomarcador.

Los resultados obtenidos sobre la susceptibilidad al pardeamiento de las diferentes variedades en tres recolecciones consecutivas incluidos en el **Capítulo VI** sugirieron que a pesar de que la recolección de las lechugas se había realizado cuando todas ellas habían alcanzado la madurez comercial, las especificaciones del estado de madurez comercial que incluye a estados de desarrollo distintos, suponía la existencia de diferencias entre el estado menos maduro y el más maduro. Estas diferencias podrían explicar la variabilidad observada en el grado de susceptibilidad al pardeamiento de una misma variedad. Estos

resultados sugirieron que los niveles basales de los metabolitos fenólicos que son sustratos de las enzimas que dan lugar al pardeamiento enzimático podrían estar influenciados por el estado de desarrollo del vegetal lo que afectaría al mecanismo de respuesta al daño y finalmente al desarrollo de pardeamiento. En el **Capítulo VII,** se evaluaron específicamente los cambios metabólicos asociados al pardeamiento en dos variedades de lechuga conocidas por su diferente susceptibilidad al pardeamiento, y en dos estados de desarrollo que correspondían a fechas de recolección con dos semanas de diferencia. Los metabolitos relacionados con el pardeamiento enzimático de la lechuga se evaluaron en el **Capítulo VII** mediante un enfoque de metabolómica dirigida para identificar aquellos metabolitos propuestos en el **Capítulo VI** y validar su empleo para predecir la susceptibilidad al pardeamiento inducido por el corte en distintas variedades de lechuga. Para la confirmación de los biomarcadores, mediante los análisis de metabolómica dirigida, se adquirieron estándares comerciales de los metabolitos de interés y además se llevaron a cabo análisis de MS/MS para comprobar los patrones de fragmentación. Los análisis de metabolómica dirigida permitieron confirmar la identidad de varios biomarcadores entre los que se encontraba el sinapaldehido (207.0663 *m/z*) y sus derivados conjugados (sinapaldehido glucósido y sinapaldehido malonyl-glucósido). El sinapaldehido había sido tentativamente identificado en el **Capítulo VI** como el éster metílico del ácido ferúlico (FAME), identificación que resultó ser incorrecta. El sinapaldehido es un metabolito metilado que proviene también del ácido cafeico, como en el caso del ferulato de metilo (FAME), y que es un precursor de la lignificación. El estudio de metabolómica dirigida permitió también identificar los diferentes isómeros del ácido clorogénico ya que son sustratos directos de la enzima polifenol oxidasa y han sido correlacionados positivamente con el desarrollo del pardeamiento. Los isómeros del ácido clorogénico identificados fueron los ácidos 3-*trans*-cafeoilquinico, 4-*trans*-

cafeoilquinico, 5-*trans*-cafeoilquinico y 5-*cis*-cafeoilquinico, siendo los dos últimos los que se correlacionaban con la susceptibilidad al pardeamiento. Los resultados mostraron que el estado de desarrollo de la lechuga afectaba directamente al grado de pardeamiento enzimático siendo la susceptibilidad al pardeamiento más baja en las muestras recolectadas antes (más inmaduras o con menos estado de desarrollo), y aumentando cuando se alcanzaban un estado de desarrollo más avanzado (más maduras o mayor estado de desarrollo). Este estudio también permitió identificar otros biomarcadores y validar que el cociente **ácido clorogénico / sinapaldehído y conjugados** supone un excelente biomarcador para predecir la susceptibilidad al pardeamiento inducido por el corte en lechuga romana.

Los resultados obtenidos en esta tesis e incluidos en los **Capítulos IV**, **V**, **VI** y **VII** concluyeron que:

1. El estudio del metaboloma completo de la lechuga es extraordinariamente complejo, y de hecho el análisis llevado a cabo con un enfoque no dirigido conlleva implícitamente un enfoque dirigido hacia el tipo de metabolitos que potencialmente se pueden analizar.

2. Se ha establecido una sólida metodología para el análisis por metabolómica no dirigida aplicado a explorar el metaboloma de la lechuga.

3. Se ha creado una base de datos de metabolitos específicos de lechuga y un flujo de trabajo de metabolómica para estudiar los cambios en estos metabolitos.

4. La identificación de metabolitos en el análisis de metabolómica no dirigida en plantas representa un cuello de botella debido a la limitación de las bases de datos de metabolitos vegetales disponibles en comparación con las bases de datos de humanos y animales.

5. Los metabolitos implicados en el proceso de pardeamiento se han identificado a través de un enfoque metabólico no dirigido. Estos metabolitos pertenecen a tres grupos principales que incluyen los lisofosfolípidos, los metabolitos del grupo oxilipinas/ácido jasmónico, y los metabolitos fenólicos que permitieron explicar el proceso de pardeamiento como respuesta al estrés inducido por la herida realizada por el proceso de cortado.

6. El análisis de la cinética de aparición de los diferentes metabolitos tras el corte indicó que las variedades con un desarrollo de pardeamiento más rápido mostraron una respuesta al corte más intensa en menor tiempo dando lugar a una mayor acumulación de algunos metabolitos clave, entre los que se encontraban el fitoprostano 16F, el ácido hidroxi-jasmónico y el ácido clorogénico, que aquellos cultivares que se pardeaban más lentamente.

7. Un mayor contenido basal de metabolitos que son sustratos de la enzima PPO respecto al de otros metabolitos precursores del proceso de lignificación se asocia con una mayor susceptibilidad al pardeamiento inducido por el corte. La relación clorogénico/sinapaldehido y conjugados se puede utilizar como un biomarcador para explicar la susceptibilidad al pardeamiento de la lechuga tras el procesado en fresco.

8. La susceptibilidad al pardeamiento de la lechuga se ve afectada tanto por factores intrínsecos como la genética y el grado de desarrollo, como por factores extrínsecos, como los factores ambientales y agronómicos. Estos factores modulan el contenido basal de ácido chlorogénico y de sinapaldehido así como de sus derivados en los tejidos de lechuga.

9. La capacidad de la lechuga para acumular metabolitos fenólicos que son sustratos de la enzima PPO o precursores del proceso de lignificación es uno de los

principales determinantes de la susceptibilidad al pardeamiento (velocidad de desarrollo del mismo tras el corte). Esto se puede deber a varios factores:

i) Al contenido basal de los metabolitos acumulados antes de la recolección, que se ve afectado por el estado de desarrollo y los factores ambientales.

ii) A la velocidad e intensidad de la respuesta a la herida producida por el proceso de cortado. que puede conducir a la biosíntesis de metabolitos fenólicos que son sustratos de la enzima PPO o precursores del proceso de lignificación y reparación de la herida.

# LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| **Å** | Angstrom |
| **AA** | Ascorbic acid |
| **ACN** | Acetonitrile |
| **ANOVA** | Analysis of variance |
| **amu** | Atomic mass unit unified |
| **API** | Atmospheric pressure ionization |
| **ATP** | Adenosine triphosphate |
| **AOS** | Allene oxide synthase |
| **AOC** | Allene oxide cyclase |
| **BPC** | Base peak chromatogram |
| **°C** | Degrees Celsius |
| **CE** | Capillary electrophoresis |
| **cm** | Centimeters |
| **$CO_2$** | Carbon dioxide |
| **d** | Days |
| **Da** | Daltons |
| **DAD** | Diode array detector |
| **DIMS** | Direct infusion mass spectrometry |
| **EDTA** | Ethylene diamine tetra-acetic acid |
| **EIC** | Extract ions chromatogram |
| **ESI** | Electro spray ionization |
| **f** | Focal aperture |
| **FAME** | Ferulic acid methyl ester |
| **FAO** | Food and Agricultural Organization of the United Nations |

| | |
|---|---|
| **FDA** | Food and Drugs Administration |
| **FDR** | False discovery rate |
| **FW** | Fresh weight |
| **g** | Grams |
| **g** | Gravitational acceleration |
| **GC** | Gas chromatography |
| **h** | Hours |
| **-H** | Hydrogen |
| **Ha** | Hectare |
| **-HCOO** | Formic acid |
| **HCA** | Hierarchical clustering analysis |
| **Hg/Ha** | Hectogram/Hectare |
| **HSPs** | Heat shock protein |
| **HSB** | Hue, saturation and brightness |
| **K** | Kelvin degree |
| **kDa** | Kilo Daltons |
| **Km** | Kilometer |
| **kPA** | Kilo Pascal |
| **L** | Liters |
| **LC** | Liquid chromatography |
| **LOX** | Lipoxygenase |
| **m** | Meters |
| **$m^2$** | Square meters |
| **mDa** | Mili Daltons |

| | |
|---|---|
| **MAP** | Modified atmosphere packaging |
| **MFE** | Molecular feature extraction |
| **min** | Minutes |
| **MLR** | Multiple linear regression |
| **mm** | Millimeters |
| **MPP** | Mass profiler professional |
| **mRNA** | Messenger ribonucleic acid |
| **MS** | Mass spectrometry |
| **m/z** | Mass to charge |
| **n** | Observations |
| **NIH** | National Institute of Health |
| **NMR** | Nuclear magnetic resonance |
| **$n^2_p$** | Effect size for analysis of variance |
| **$O_2$** | Oxygen |
| **OPDA** | 12-oxo-phytodienoic acid |
| **OPLS-DA** | Orthogonal partial least square-discriminant analysis |
| **OPR** | 12-oxo-phytodienoic reductase |
| **PAL** | Phenylalanine ammonia lyase |
| **PC** | Phosphatidylcholine |
| **PCA** | Principal component analysis |
| **PE** | Phosphatidylethanolamine |
| **PLA** | Phospholipase A |
| **PLS** | Partial least square |
| **PLS-DA** | Partial least square-discriminant analysis |

| | |
|---|---|
| **psi** | Pounds-force per square inch |
| **POD** | Peroxidase |
| **ppm** | Parts per million |
| **PPO** | Polyphenol oxidase |
| **PVDF** | Polyvinylidene difluoride membrane |
| **$Q^2$** | Estimates predictive ability |
| **QC** | Quality control |
| **Q-TOF** | Quadrupole time of flight |
| **$R^2$** | Model fit of the data |
| **RGB** | Red, green and blue model color |
| **RH** | Relative humidity |
| **RNA** | Ribonucleic acid |
| **RT** | Retention time |
| **$R^2X$** | Variation in X |
| **$R^2Y$** | Variation in the response Y |
| **s** | Seconds |
| **T** | Tons |
| **UK** | United Kingdon |
| **UPLC** | Ultra high pressure liquid chromatography |
| **US** | United States |
| **USDA** | United States Department of Agriculture |
| **V** | Voltage |
| **v:v** | Volume proportion |
| **VIP** | Variable importance in the projection |

| | |
|---|---|
| **X** | Entities |
| **Y** | Categories |
| **µl** | Micro liters |
| **µm** | Micro meters |
| **∆ HUE** | Delta Hue |
| **11-HPHTrE** | 11(S)-hydroperoxy-7(Z),9(E),13(Z)-hexadecatrienoic acid |
| **13-HPOTrE** | 13(S)-hydroperoxy-9(Z),11(E) 15(Z)-octadecatrienoic acid |
| **º** | Angle degree |

# LIST OF FIGURES AND TABLES

**CHAPTER I**

**Photo 1.** *Lactuca sativa* (cultivated lettuce): romaine (A) and iceberg (B) lettuce fields in Pulpí (Almería, Spain).

**Photo 2.** *Lactuca serriola* (photo from www.euskalnet.net).

**Photo 3.** The most consumed types of lettuce: crisphead lettuce or iceberg (i) and cos lettuce or romaine (ii).

**Figure 1.** Immediate wound response from Toivonen (2010).

**Figure 2.** Secondary wound response from Toivonen (2010).

**Figure 3**. Tertiary wound response from Toivonen (2010).

**Figure 4.** Structure of a phospholipid and the sites of phospholipase activity.

**Figure 5.** Biosynthesis pathway of jasmonic acid (adapted from Weber et al., 2002).

**Figure 6.** Scheme of the two essential reactions catalyzed by polyphenol oxidase (PPO).

**Figure 7.** Phenylpropanoid pathway (Dixon and Paiva, 1995).

**Figure 8.** Scheme of omics sciences (Deida et al., 2015).

**Figure 9.** Scheme of targeted and untargeted metabolomics methodologies.

**Figure10.** Untargeted metabolomics workflow.

**Figure 11.** General scheme of pre-processing data.

**Figure 12.** General scheme of data analysis (Saccenti et al., 2014).

**Table 1.** Harvested area (hectare), production (thousand metric tons), and yield in major lettuce-producing countries and regions in 2016.

**Table 2.** Main phenolic compounds and PPO substrates identified in lettuce (Abu-Reidah et al., 2013; Mai et al., 2013; Viacava et al., 2017)

**CHAPTER III**

**Photo 1.** "El Albujon" experimental farm of Enza Zaden located in Torre Pacheco (Murcia, Spain).

**Photo 2.** The freeze-drier used (model Christ Alpha 2-4 LD Plus).

**Photo 3.** The professional blender used (Oster Professional BPST02-B).

**Photo 4.** The ultrasound equipment (Branson 5510) (left) and the centrifuge (Thermo Fisher sorvall ST 16 R) (right) used.

**Figure 1.** Excised midribs as the plant material selected in this study.

**Figure 2.** Diagram of the midrib excision and the midrib freezing process.

**Figure 3.** Digital image processing from raw image to the processed image by ImageJ.

**Figure 4.** LC system from Agilent Technologies (Waldbronn, Germany) model Agilent 1290 Infinity II multi-sampler.

**Figure 5.** Schematic diagram representing the Electrospray Ionization source (ESI).

**Figure 6.** Agilent 6550 iFunnel Accurate-Mass Quadrupole Time-of-Flight Q-TOF LC/MS system (Agilent Technologies, Waldbronn, Germany).

**Figure 7.** Schematic diagram representing the Agilent 6550 iFunnel Q-TOF LC/MS (Agilent Technologies, Waldbronn, Germany).

**Figure 8.** Schematic diagram representing the multivariate analysis types.

**CHAPTER IV**

**Figure 1.** Percent of browning obtained by image analysis of FB and SB iceberg cultivars at day 0 (d0) and day 5 (d5) of storage.

**Figure 2.** Workflow followed in the development of the current untargeted metabolomics study. FB fast-browning cultivar, SB slow-browning cultivar, d0 day 0, d5 day 5.

**Figure 3.** Unsupervised analysis of all lettuce samples [20 samples, 4 conditions/groups and 2 variables: cultivar (non-numeric variable) and storage time (numeric variable)] using pre-treated data: **a** Expanded view of the entire dendrogram obtained after hierarchical cluster analysis (HCA) to illustrate the separation between the conditions. Each row represents a metabolite with a color coding that indicates the relative intensity of each metabolite in each condition ranging from low (blue) to high intensity (red); **b** 3-D principal component analysis (PCA) score plot; **c** 2-D PCA score plot (PC1 9 PC2); **d** 2-D PCA score plot (PC1 9 PC3). FB-d0 fast browning cultivar at d0, FB-5 fast-browning cultivar at d5, SB-d0 slow-browning cultivar at d0, SB-d5 slow-browning cultivar at d5. Outlier replicate indicated by an arrow.

**Figure 4.** Volcano plot of metabolomics data obtained comparing storage time (numeric variable): d5 versus d0 in **a** fast-browning (FB) and **b** slow-browning (SB) iceberg cultivars; **c** Venn diagram showing the repartition of the features obtained as statistical differences between d0 and d5 in both cultivars.

**Figure 5.** Extracted ion chromatograms (EIC's) of the primary compounds tentatively identified after the statistical analysis between d0 and d5 of storage in FB iceberg cultivar. Compounds are numbered according to Table 1. Asterisk compounds classified as other families.

**Table 1.** Metabolites tentatively identified after the statistical analysis between 0 and 5 days of storage in FB and SB lettuce cultivars.

**CHAPTER V**

**Figure 1.** Schematic representation of the metabolomics analysis workflow.

**Figure 2.** Objective measurement of the browning process (%) in midribs of fast-browning (FB) and slow-browning (SB) Romaine lettuce cultivars. Pictures of the lettuce tissue just after cutting and after storage show the browning development.

**Figure 3.** Score plots of principal component analysis (PCA) of metabolites profiles of fast-browning (FB) and slow-browning (SB) lettuce cultivars. Cultivars by shape: FB (■) and SB (▲). Different time intervals after cutting at 7 °C by color: 0 h (red), 2 h (brown), 6 h (pink), 24 h (gray), 48 h (green), 72 h (turquoise), 96 h (dark blue), and 120 h (light blue).

**Figure 4.** Main MS/MS fragments of the lysophospholipids: (1) PI(18:3/0:0), (2) PS(18:3/0:0), (3) PS(20:5/0:0), and (4) PS(18:2/0:0).

**Figure 5.** Trends of metabolites identified: red (up-regulated); blue (down-regulated); yellow (no change); gray (not detected). See Table 1 for the identification of metabolites 1−15.

**Figure 6.** Kinetics of some selected metabolites identified as statistically significant in the fast-browning (FB) (●) and slow-browning (SB) (○) lettuce cultivars at different time intervals (2, 6, 24, 48, 72, 96, and 120 h) after cutting at 7 °C. Values are the mean ± standard deviation (n = 4).

**Figure 7.** Scheme of wound-induced metabolic changes of cut lettuce leading to browning. PA, phospholipase A; 9-LOX, 9-lipoxygenase; AOS, allene oxide synthase; POX, peroxygenase; EPH, epoxide hydrolase; PAL, phenylalanine ammonia-lyase; PPO, polyphenol oxidase.

**Table 1.** Metabolites Tentatively Identified Related to the Browning Process.

**Table 2.** MS/MS Fragmentation Pattern of Lysophospholipids.

**CHAPTER VI**

**Figure 1.** Metabolomics experiment workflow. Data management and processing steps.

**Figure 2.** Δ HUE obtained by digital image analysis as an objective measurement of browning in thirty romaine lettuce cultivars harvested at three different dates (H1, H2, and H3). See Materials and Methods for H1, H2, and H3 identification. Error bars are standard deviations (n=4 for each cultivar). Statistical differences are denoted in the graph (* $p < 0.05$ and ** $< 0.01$).

**Figure 3.** Principal component analysis (PCA) score plot for the first two components (t [1]/t [2]) of 720 samples of romaine lettuce separated by color representing storage time (green: samples at 0 d; red: samples at 5 d).

**Figure 4.** Partial least square discriminant analysis (PLS-DA) loading plot for the first two components (w*c[1]/w*c[2]) based on the full dataset; X: entities, Y: discriminant groups. Left: non-browning stage (0 d); Right: browning stage (5 d).

**Figure 5.** Multivariate model analysis of the full dataset: A) Scatter plot p(corr)/VIP and B) Column plot p(corr)/VIP. Metabolomic differences between 0 d and 5 d. Error bars are the standard deviation of the whole dataset (n=720).

**Figure 6.** Multivariate model analysis of selected cultivars with very different browning susceptibility (high and low browning potential). A) Scatter plot p(corr)/VIP; B) Column plot p(corr)/VIP. HB: Entities correlated with high browning susceptibility; LB: Entities correlated with low browning susceptibility. Error bars are the standard deviation of the whole dataset at 0 d (n=360).

**Figure 7.** Multiple linear regression model (MLR) of the full dataset. High t-vale indicates high probability to have a significant influence on the model and t-value close to 0 have non-significant on the model. Bars: t-values of variables in the model; Green bar: variables not selected; Red bar: variables selected to explain browning process; *: variables with a p-value less than 0.05, **: variables with a p-value less than 0.01.

**CHAPTER VII**

**Figure 1.** ΔHue values of LB and HB susceptibility cultivars at development stages 1 (harvested 2 weeks before commercial maturity) and 2 (harvested at commercial maturity). Error bars are standard deviations (n = 4)..

**Figure 2.** Chromatographic separation of (**A**) caffeoylquinic acid isomers and (**B**) sinapaldehyde derivatives. UPLC−QTOF−MS analyses of lettuce samples in the negative mode. (**A**) Ion extracted chromatogram at m/z 353.0878: (**1**) 3-trans-caffeoylquinic acid, (**2**) 5-trans-caffeoylquinic acid, (**3**) 4-trans-caffeoylquinic acid, and (**4**) 5-cis-caffeoylquinic acid. (**B**) Ion extracted chromatogram at m/z 207.0663: (**5**) trans-

sinapaldehyde-4- β-D-glucoside, (**6**) trans-sinapaldehyde-4-(6′-malonyl)-β-D-glucoside, and (**7**) trans-sinapaldehyde.

**Figure 3.** (**A**) Sinapaldehyde (**7**) and (**B**) ferulic acid methyl ester (**12**) UPLC−QTOF−MS fragments (m/z 207.0663) in the negative mode and at 20 eV.

**Figure 4.** UPLC−QTOF MS/MS fragments in the positive mode of (**A**) sinapaldehyde-4-β-D-glucoside and (**B**) sinapaldehyde-4-(6′-malonyl)-β-D-glucoside.

**Figure 5.** (**A**) Chlorogenic acid isomers (trans- plus cis-5-caffeoylquinic acids) and (**B**) sinapaldehyde and conjugates in the selected Romaine lettuce cultivars with LB and HB susceptibility at development stages 1 (harvested 2 weeks before commercial maturity) (light green bar) and 2 (harvested at commercial maturity) (dark green bar). Error bars are standard deviations (n = 4).

**Figure 6.** Linear regression model of romaine lettuce cultivars. N =24. Coefficients: b0 = −27.619 450 985 6, b1 = 3.879 263 702 3, and r2= 0.793 072 783 7. Ratio: Σchlorogenic acids/Σsinapaldehyde and conjugates (mg/g of fresh midribs).

**Figure 7.** Metabolic routes leading to PPO substrates (brown metabolites) or lignin biosynthesis precursors (green metabolites) with an indication of the biomarkers identified and the key enzymes.

**Table 1.** UPLC-QTOF-MS-MS Targeted Metabolomics Analysis of Phenolic Metabolites, with a Collision Energy of 20 eV.

**Table 2.** UPLC-QTOF-MS MS/MS Analysis of Metabolites in Positive Mode, with a Collision Energy of 20 eV.

**CHAPTER VIII**

**Figure 1.** Diagram of the general workflow divided into experimental design, sample extraction and analysis, data treatment, metabolite identification and biological interpretation.

**Figure 2.** General pathway of the wound-induced phenolic metabolism.

# CHAPTER: I

# INTRODUCTION

## 1. LETTUCE

Lettuce (*Lactuca sativa L.)* belongs to the largest dicotyledonous family in the plant kingdom, the *Asteraceae*, formerly known as a member of the *Compositae*. Lettuce is included in the subfamily of *Cichorioideae*, the tribe *Lactuceae* and the genus *Lactuca* (Lindqvist, 1960a). *Lactuca L.* (Compositae) includes approximately 100 species: 17 European species, 10 North American species, 33 tropical East African species, and around 40 Asian species (De Vries and Raamsdonk, 1994).

Lettuce forms a deep taproot and larger horizontal lateral roots, which can grow 60 cm or longer and denser as it gets closer to the soil for water and nutrient absorption. Lettuce leaves are spirally arranged in a dense rosette on the often shortened stem, forming a rosette of leaves. The development of the rosette continues along the vegetative life of the plant, as in leaf lettuces type, or it may form rounded heads as the crisphead and butterhead lettuce or elongated heads in case or cos lettuce. Due to the particular structure of his leaves, the lettuces is an obligate self-fertilizing species. Elongation of the style and pollen release from the anther tube occurs at the same time, therefore the germination must be very quickly.

There is a great diversity regarding color, shape, surface, margin, and texture that classify the great variability of lettuce in different types. A type is a group of cultivars that are morphologically similar, and usually, it can be subdivided into subtypes that share similar genetic and morphological characteristics.

On the other hand, a cultivar is a variety selected with the traits that the crop wants to obtain, and a variety is a taxonomic group standing below species and subspecies. Despite this, different classification systems have been suggested for lettuce (Rodenburg, 1960; Lebeda et al., 2007; Mou, 2008). However, there is no standardized classification due to the great genetic and morphological diversity between cultivars.


Photo 1. *Lactuca sativa* (cultivated lettuce): romaine (A) and iceberg (B) lettuce fields in Pulpí (Almería, Spain).

### 1.1. Origin

The species that have been involved in the origin and evolution of the lettuce have been identified. *L. serriola* has been identified as one, or the only direct ancestor as the chromosomes of *L. sativa* and *L. serriola* are morphologically similar (Feráková, 1977; Mou, 2008). The evolutionary changes suffered over time have been able to cause the changes seen in the lettuce currently consumed.

Photo 2. *Lactuca serriola* (photo from www.euskalnet.net).

In the Middle East, primitive forms of the present lettuce have been found. This provides strong support for the idea that lettuce could be originated in the Mediterranean. In Egyptian tombs, some lettuce paintings 4500 years old have been found supporting the Middle East origin of lettuce. From Egypt, it is thought that was extended to Greece,

Rome and the whole Mediterranean basin (Lindqvist, 1960b). In the rest of Europe, it started to be cultivated later. In the northwest of Europe, it was where the first indications of cultivation of lettuce were found in a plantation of Schöffer (1485) (de Vries, 1997).

Regarding the beginning of lettuce cultivation in America, Christopher Columbus brought lettuce to America in one of his first trips (Ryder, 1997). In the following years, different types of lettuce were introduced with different types of leaves. At the beginning of the 20th century, the batavia lettuce (crisphead) began to predominate because it could maintain its quality for 10-12 days for shipment and marketing. The crosses between different varieties of batavia gave rise to the iceberg lettuce, which soon gained popularity in America and Europe.

### 1.2. Horticultural types

There are six main types of lettuce based on size, head formation, texture, leaf shape and stem type (Mou, 2008). These are: *i*) crisphead lettuce or iceberg lettuce (var. *capitata* L. nidus jaggeri Helm), *ii*) romaine or cos lettuce (var. *longifolia* Lam., var. *romana* Hort. in Bailey), *iii*) butterhead (var. *capitata* L. nidus *tenerrima* Helm), *iv*) leaf or cutting lettuce (var. *acephala* Alef., syn. var. *secalina* Alef., syn. var. *crispa* L.), *v*) stem or stalk (*Asparagus*) lettuce (var. *angustana* Irish ex Bremer, syn. var. *asparagina* Bailey, syn. L. *angustana* Hort. In Vilm.), and *vi*) Latin lettuce (no scientific name) (Ryder, 1999). The most consumed lettuce types over the world are the crisphead or iceberg and romaine or cos.

i) Crisphead or iceberg lettuce

ii) Cos or romaine lettuce

Photo 3. The most consumed types of lettuce: crisphead lettuce or iceberg (i) and cos lettuce or romaine (ii).

i) *Crisphead lettuce*. This type of lettuce is also known as "iceberg" or head lettuce. Crisphead lettuce is characterized by having a firm spherical shape. The development of this type of lettuce in the field begins by increasing the length of the leaves and the width gradually more than the length alone as the maturation progresses. After about 12 leaves, the head begins to change to a cup shape and overlap each other to form a head structure.

The younger leaves that keep growing inside the head make it bigger and firmer. The overripe head, with too many leaves superimposed inside it, can break up causing a bitter taste, which translates into one of the problems that the cultivation of this type of head-shaped lettuce has. When the plant grows correctly without breaking the leaves inside, the leaves have a rough, brittle and soft taste. In this case, it reaches a weight in slightly less mature state and forms a very important subtype within the crisphead type called Batavia.

ii) *Cos lettuce or romaine*. Cos lettuce has been traditionally grown in the Mediterranean basin and the Kos island in the eastern Mediterranean near Turkey is the one that gives the name. This type of lettuce forms elongated leaves, firm, thick, moderately crunchy and with great veins crossing through the middle. This type of lettuce has completely closed or slightly open heads arranged vertically in the field.

At commercial maturity, they can reach weights between 750-1000 g, where the outermost leaves have a strong green color while the inner leaves have a slightly yellowish color. The leaves are elongated, the texture is pretty robust and the rosette usually have a vertical stature.

These two types of lettuces cover a large part of the consumer's demand due to the different range of flavors from sourer and crispier in the case of iceberg, to sweeter flavors in the case of romaine.

### 1.3. Production and value

Lettuce is one of the major leaf crops in the world, widely grown and popularly consumed worldwide. It is grown in most temperature and subtropical areas in the world.

Table 1**.** Major lettuce-producing countries/ regions, harvested area (hectares), yield (hectogram/hectare) and production (thousand metric tons).

| Country/Region | Harvested area (ha) | Yield (hg/ha) | Production (Tons) |
|---|---|---|---|
| Australia | 8,679 | 161,194 | 139,900 |
| Austria | 1,669 | 291,887 | 48,716 |
| Bangladesh | 7,233 | 55,436 | 40,097 |
| Belgium | 1,294 | 406,244 | 52,568 |

| | | | |
|---|---|---|---|
| Canada | 3,967 | 261,510 | 103,741 |
| Chile | 6,237 | 133,598 | 83,320 |
| Egypt | 4,443 | 228,196 | 101,392 |
| France | 8,765 | 260,557 | 228,378 |
| Germany | 14,278 | 233,250 | 333,034 |
| Greece | 5,916 | 117,565 | 69,547 |
| Guatemala | 2,865 | 305,052 | 87,412 |
| India | 171,618 | 63,456 | 1,089,025 |
| Iran | 17,293 | 305,626 | 528,533 |
| Israel | 2,169 | 125,849 | 27,291 |
| Italy | 34,343 | 214,297 | 735,967 |
| Japan | 21,804 | 268,763 | 586,000 |
| Jordan | 1,771 | 398,408 | 70,558 |
| Mexico | 20,066 | 219,195 | 439,831 |
| Netherland | 3,524 | 335,414 | 118,200 |
| New Zealand | 1,333 | 260,018 | 34,661 |
| Niger | 8,688 | 200,769 | 174,434 |
| Peru | 6,741 | 109,121 | 73,559 |
| Portugal | 2,181 | 238,368 | 51,988 |
| South Africa | 2,194 | 158,301 | 34,736 |
| Spain | 34,862 | 266,788 | 930,081 |
| Switzerland | 3,293 | 200,538 | 66,037 |
| Turkey | 22,431 | 213,295 | 478,442 |
| United kingdon | 4,813 | 221,382 | 106,551 |
| United States of America | 113,980 | 357,390 | 4,073,530 |

| | | | |
|---|---|---|---|
| Venezuela | 2,559 | 201,192 | 51,485 |
| Africa | 27,238 | 176,648 | 481,149 |
| Asia | 891,040 | 203,230 | 18,108,568 |
| East Europe | 2,942 | 210,721 | 62,001 |
| Western Europe | 32,836 | 258,057 | 847,360 |
| N & C America | 169,053 | 299,719 | 5,066,829 |
| South America | 24,208 | 133,341 | 322,786 |
| World | 1,223,407 | 218,893 | 26,779,564 |

Source: Food and Agricultural Organization of the United Nations (FAO, 2016).

Lettuce crops represent the eighth most consumed vegetable, producing 27 million tons per year worldwide. The largest producer of lettuce in the world is Asia, which produces mainly stem lettuce (*L. sativa* L. var. *angustana*) not commonly consumed in the US or Western Europe (Mou, 2008). The US and Western Europe contribute with 22% and 13% of the total lettuce production worldwide, respectively (Mou, 2008). In the US, lettuce ranks as the 3rd most consumed vegetable (USDA, 2016). Australia, Japan, and Israel are also important lettuce producers. In the UK, around 75% of the production is iceberg lettuce, 15% is butterhead, and 10% is romaine. Netherlands and Belgium are very important producers, but the majority is produced in the greenhouse. The production in France is primarily butterhead and Batavia lettuce for home consumption and iceberg for export. In Germany, about 70% of the production is butterhead lettuce and 30% iceberg and most of the production is in only three areas, Baden-Wurttemberg, Rhineland-Westphalia, and the Palatinate. Italy is the largest producer of vegetables in Western Europe and an important lettuce producer, in which more than half is butterhead, and about one-third is romaine, and the rest belong to iceberg and leaf lettuces. In Spain,

lettuce is produced for both home consumption and export to other European countries, particularly iceberg lettuce. The Region of Murcia accounts for 70.2% of all the lettuce exported by Spain, reaching a production about one-third of the crop, followed by Andalucía, Valencia, and Catalonia. Lettuce in all its varieties, is the product with the greatest contribution to Murcia's vegetable exports. In the 2017-2018 campaign, exports increased by 7.9% compared to the previous season, reaching 576,598 tons worth 463.8 million Euro (2.4% more) (Proexport, 2018).

Although lettuce is a vegetable commonly consumed, it has never been considered as one of the nutritious vegetables due to its high-water content. However, the composition in nutrients is similar to that of other vegetables considered nutritious. The nutritional contribution of lettuce to the human diet is mainly a combination of vitamins, minerals as well as fiber and a large amount of water. The nutritional value depends on the lettuce type. Romaine and leaf lettuces have higher vitamin and mineral contents than the iceberg type. The outer green leaves have higher nutrient content than the inner leaves because the synthesis of many nutrients that are light dependent. In the case of iceberg lettuce, as is an enclosure head structure, the leaves are protected from the direct radiation a this causes a lower nutritional value (Mou and Ryder, 2004).

## 2. FRESH-CUT LETTUCE

The term fresh-cut also known as minimally processed, lightly processed, partially processed, precut, pre-prepared or cut prepared, identifies fresh products that have been cut into small serving-size portions ready to eat (Cantwell, 1992; Gil and Kader, 2008). Consumption of fresh-cut products has increased during the last years, and new products are continually being developed. Fresh-cut lettuce was the first commodity requested by

the fast food service, and it later arrived at the retail store. It may represent more than 85% of the total production of fresh-cut produce (Cook, 2004).

### 2.2. Quality loss of fresh-cut lettuce

Fresh-cut processing increases perishability rather than making the products more stable (Rolle and Chism, 1987). This type of processing differs from traditional processing because the tissue remains fresh after cutting and during storage. The behavior of the tissue after cutting is that of a plant tissue that has been wounded or exposed to stress conditions (Brecht, 1995). The operations implicated in the processing of fresh-cut lettuce such as cutting, washing, drying, and packaging, cause tissue damage, increasing the respiration rate and the rapid quality deterioration and shelf-life reduction (Bolin and Huxsoll, 1991; Varoquaux and Wiley, 1994; Martinez-Sanchez et al., 2011). The shelf-life of fresh-cut lettuce after storage is limited due to microbial growth, texture loss and off-odors although the main problem is the enzymatic browning that occurs on the cut surface area (López-Gálvez et al., 1996; Toivonen and Brummell, 2008; Martinez-Sanchez et al., 2011). Enzymatic browning is a discoloration that causes a great visual impact, decreasing the commercial quality, the organoleptic acceptance and the nutritional value. To extend the post-cutting life of fresh-cut lettuce requires an understanding of the physical and physiological processes in response to wounding. Effective postharvest treatments such as heat-shock treatments have been developed but the basic strategies for minimizing physical damage, maintaining optimum ranges of temperature and relative humidity, and avoiding microbial contamination remain the most relevant ones (Tomás-Barberán et al., 1997; Peiser et al., 1998; Saltveit, 2003; Altunkaya and Gökmen, 2008).

### 2.3. Physical and physiological responses to wounding

The preparation of fresh-cut lettuce entails physical wounding of the tissue. The unavoidable physical injury causes first an immediate response to the cells that are cut following by a subsequent physical and physiological response of the adjacent cells.

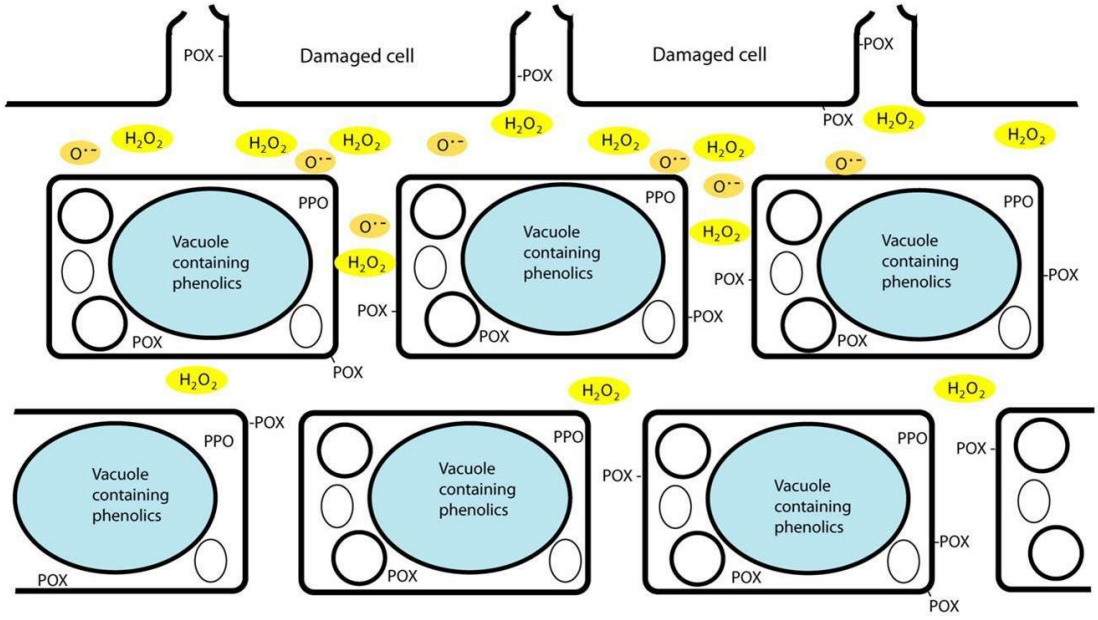



Figure 1. Immediate wound response from Toivonen (2010).

The immediate response to the physical injury is the propagation of the wound signal, depolarization of the membranes and the decompartmentalization (Toivonen, 2010). As represented in Figure 1, a plant cell contains many compounds that are kept in separate compartments by semipermeable membranes. The cell membrane that surrounds the living cytoplasm of the cell establishes a boundary between it and its external environment. The vacuole, separates the cytoplasm with its many enzymes, from the stored organic acids and phenolic compounds.

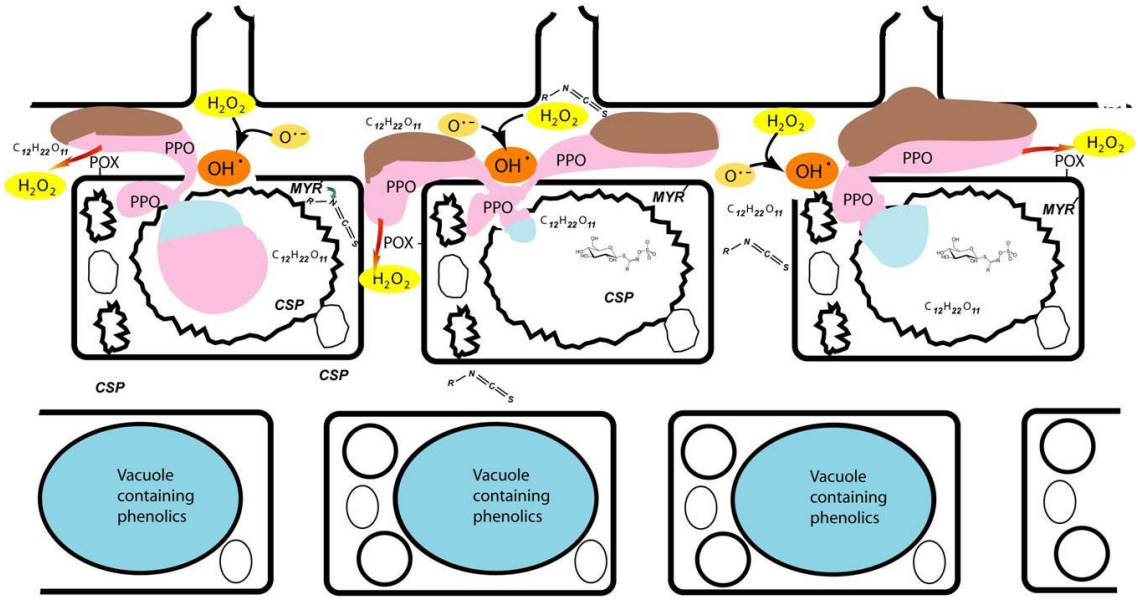



Figure 2. Secondary wound response from Toivonen (2010).

Wounding not only physically damages the membranes in the injured cells but also disrupts membrane function in adjacent cells, so that incompatible compounds mix and uncontrolled reactions happen (Figure 2). For example, phenolic compounds from the vacuole mix with enzymes (e.g., polyphenol oxidase, PPO) in the cytoplasm to produce brown compounds that can discolor the tissue (Saltveit, 2003; Toivonen, 2010).

Wound-induced changes include moisture loss, elevated respiration, production of ethylene, and the activation of the phenylpropanoid metabolism that result in the accumulation of phenolic compounds and subsequent tissue browning (Saltveit, 1997). The interaction of these constituents with enzymes such as ascorbate oxidase, polyphenol oxidase, cytochrome oxidase, and peroxidase, could also promote the degradation of the nutrient content (Figure 3). In fresh-cut lettuce, these responses are usually detrimental to the overall quality of the product. For example, wound-stimulated phenylpropanoid metabolism promotes the synthesis and accumulation of phenolic compounds that promote browning of fresh-cut lettuce.

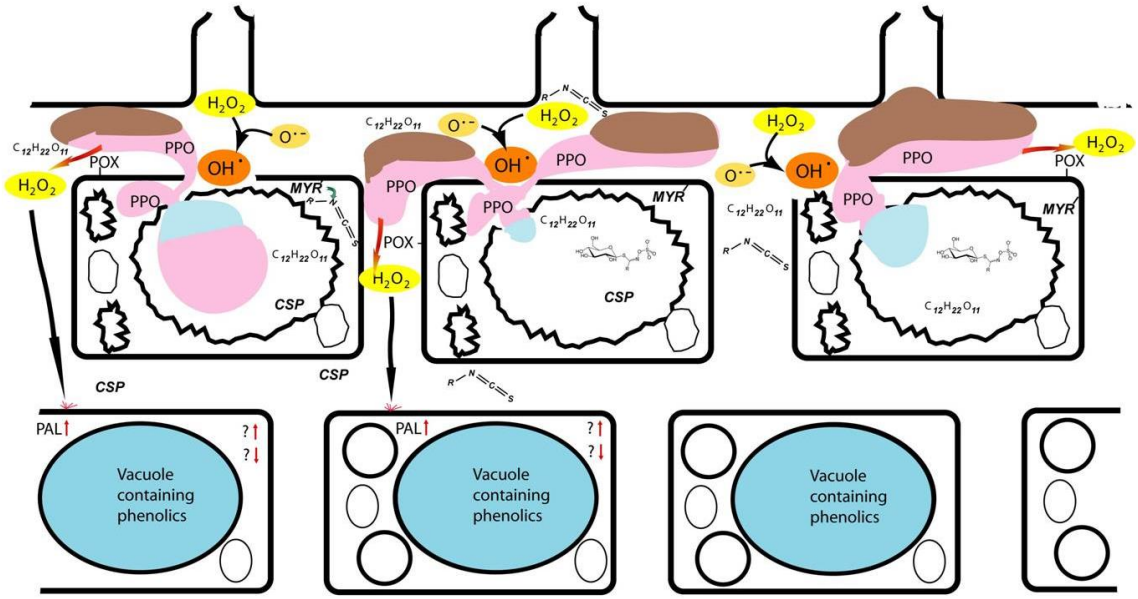



Figure 3. Tertiary wound response from Toivonen (2010).

The abiotic stresses such as wounding not only cause physical damage, but also cause a physiological damage that triggers a cascade of signals that entails a plant response. This response has been associated with the defense role of the plants (Kachroo and Kachroo, 2009). Different enzymes, phospholipids and oxylipins are involved in the defense response and the final octadecanoic-derived compounds are considered the signaling molecules (Schaller, 2001).

The phospholipase A (PLA) is the first enzyme involve in the membrane damage. The phospholipase A hydrolyze phospholipids to produce lysophospholipids and free fatty acids. PLA 1 and PLA 2 release a fatty acid from the sn-1 and sn-2 position (Figure 4).

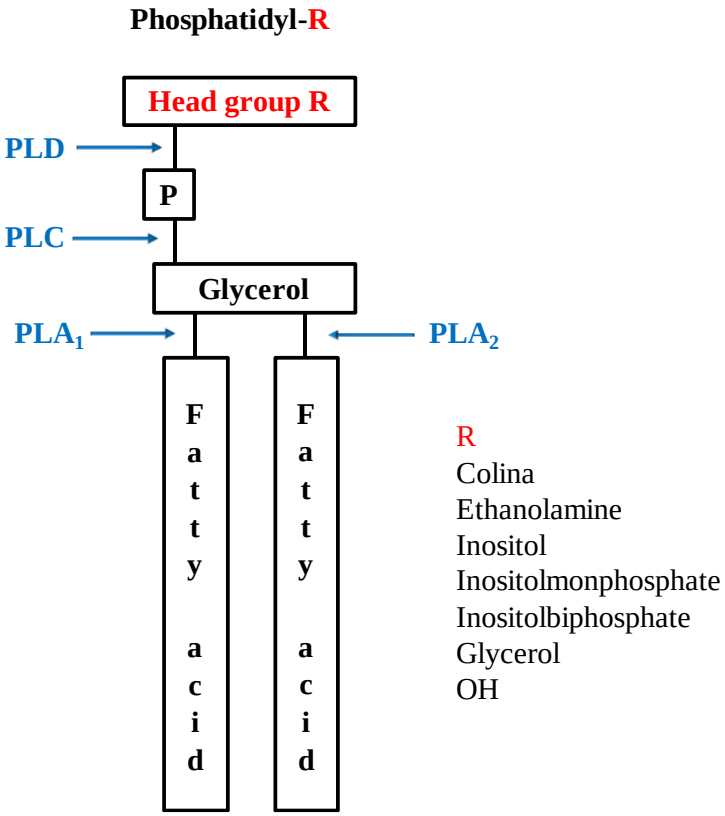



Figure 4. Structure of a phospholipid and the sites of phospholipase activity.

Wounding causes a release of α-linolenic acid (octadecatrienoic acids (18:3)) in the chloroplast membrane by the action of phospholipase A (PLA), which is incorporated in the octadecanoid pathway even though there is a hexadecanoid pathway in parallel (hexadecatrienoic acids (16:3)) (Weber., 2002). The enzyme lipoxygenase (LOX) catalyzes the oxygenation of fatty acids to their corresponding hydroperoxy derivatives, 11(S)-hydroperoxy-7(Z),9(E),13(Z)-hexadecatrienoic acid (11-HPHTrE) and 13(S)-hydroperoxy-9(Z),11(E) 15(Z)-octadecatrienoic acid (13-HPOTrE). The enzymes allene oxide synthase (AOS) and the allene oxide cyclase (AOC) jointly are implicated in the biosynthesis of jasmonic acid by dehydration of the hydroperoxy and cyclization. The 12-oxo-phytodienoic acid (OPDA) as the jasmonic acid precursor, leaves the chloroplast and the jasmonic acid is produced in the peroxisome by β-oxidation. The increase of jasmonic

acid such as a signaling molecule by wounding has been described in the induction of phenylpropanoid pathway (Saltveit et al., 2005).

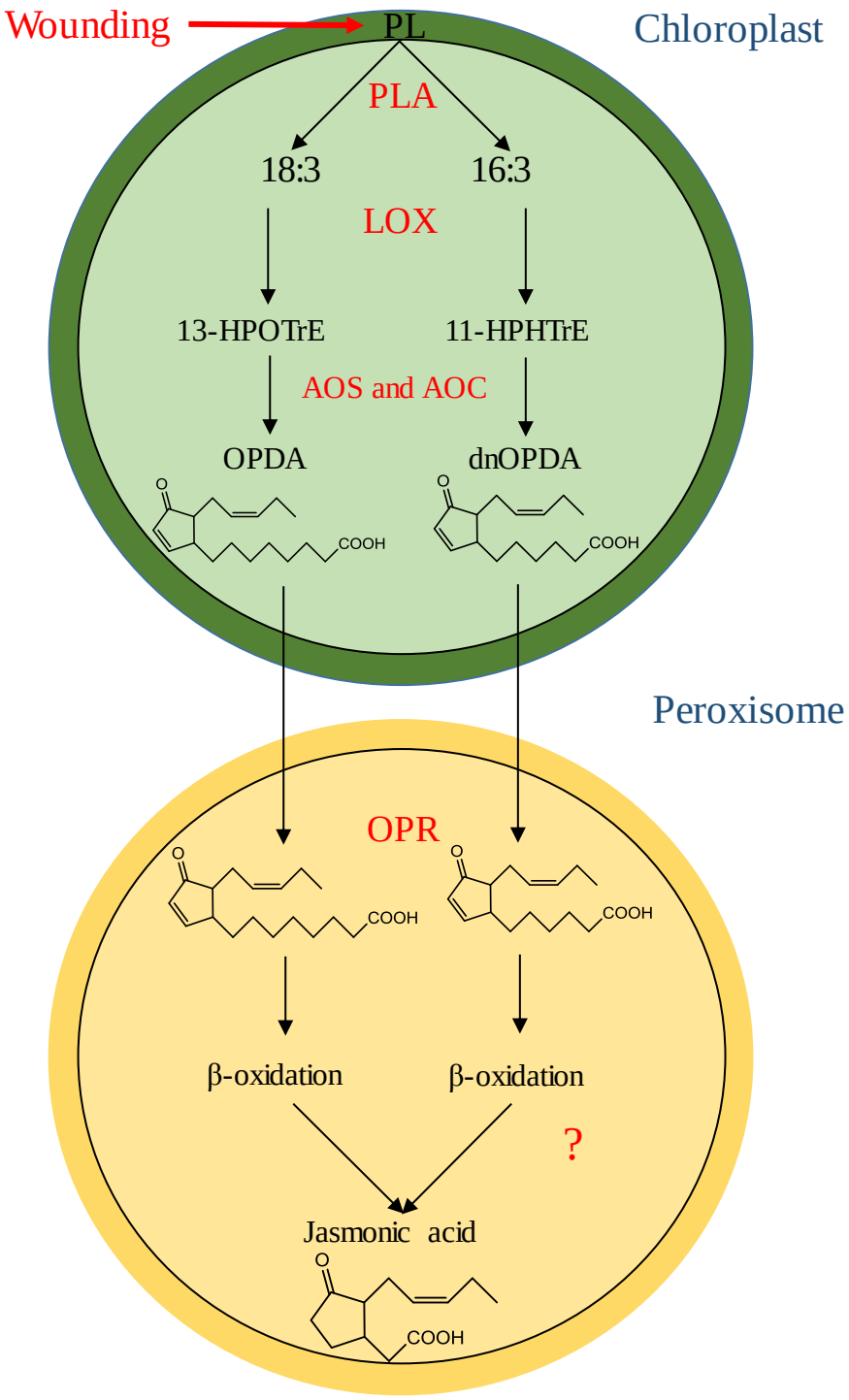



Figure 5. Biosynthesis pathway of jasmonic acid (adapted from Weber et al., 2002).

## 3. ENZYMATIC BROWNING

Enzymatic browning causes harmful effects to the quality maintenance of the fresh-cut fruit and vegetable industry, although it is beneficial for the development of color and flavor in some food items such as tea, coffee, and cocoa. A variety of fruits and vegetables, such as apple, pear, banana, lettuce and potato, are especially susceptible to enzymatic browning after processing and storage. Browning not only has a negative effect on visual appearance but also may impair other sensory properties including taste, odor, and texture, as well as nutritional value. In lettuce, enzymatic browning is the most important biochemical disorder that leads to significant quality loss after cutting, and it is promoted during storage, causing product rejection. Enzymatic browning is a general color reaction occurring on the cut edges of lettuce that involves the interaction of oxygen, phenolic compounds and oxidative enzymes. Enzymatic browning does not occur in intact cells as the phenolic compounds located in the vacuoles are separated from the oxidative enzymes located in the cytoplasm. However, once the tissue is damaged by cutting, the phenolic compounds and the oxidative enzymes come into contact and result in rapid browning reaction. The extension of enzymatic browning depends on the activity of oxidative enzymes such as polyphenol oxidase (PPO), peroxidase (POD) as well as phenylalanine ammonia lyase (PAL). There is evidence that increases of PPO, POD and PAL activities cause an increase in browning (Cantos et al., 2001). The understanding of the enzymatic browning and its control from harvest to consumption is important for minimizing losses and maintaining the economic profitability of fresh-cut lettuce.

### 3.1. Polyphenol oxidase (PPO)

Polyphenol oxidase (PPO) (1,2-benzenediol: oxygen oxidoreductase; EC 1.10.3.1) is also known as catechol oxidase, catecholase, diphenol oxidase, o-

diphenolase, phenolase, tyrosinase, and cresolase. It contains copper in the active site, which is essential for enzyme activity (Taranto el al., 2017). Browning is normally initiated by the enzymatic oxidation of monophenols into o-diphenols and o-diphenols into quinones, which suffer further non-enzymatic polymerization leading to the formation of pigments (Toivonen and Brummell, 2008). Thus, PPO catalyzes two essential reactions: *i*) hydroxylation of the phenolic substrate at the *o*-position, contiguous to an existent hydroxyl group (monophenol oxidase activity or cresolase activity); and *ii*) oxidation of diphenol to *o*-benzoquinones (diphenol oxidase activity or catecholase). Both reactions use molecular oxygen as a co-substrate. To date, all the known PPOs have the property to transform *o*-dihydroxyphenols to *o*-benzoquinones, but not all PPOs can hydroxylate monophenols (Figure 6). Both reactions consume oxygen and the overall stoichiometry is 1 mol of oxygen for 1 mol of monophenol, giving 1 mol of *o*-quinone and 1 mol of water.

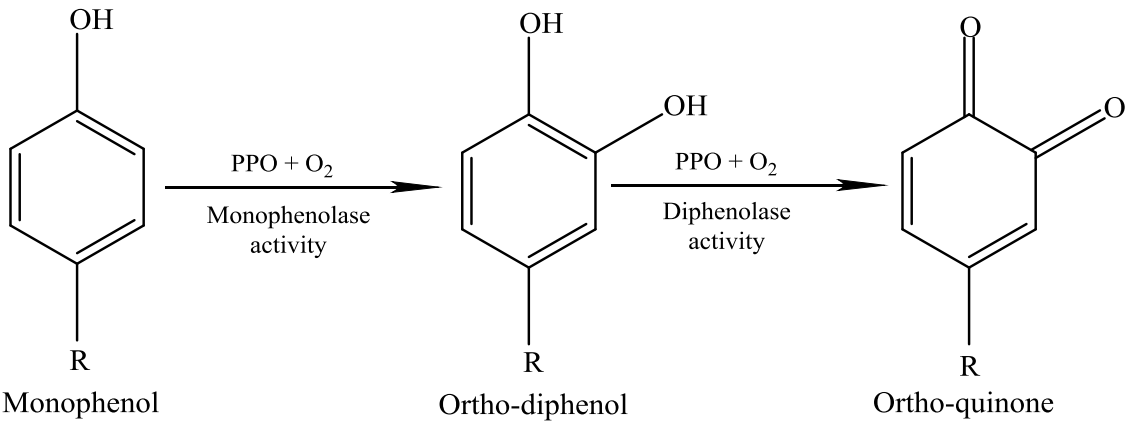



Figure 6. Scheme of the two essential reactions catalyzed by polyphenol oxidase (PPO).

Diphenol oxidases have received considerably more attention than monophenol oxidases due to the high catalytic rate and the association with the formation of quinones, which lead to the production of brown pigment. Some types of PPO differ only in a few

subunits, and these are usually different in the chemical and kinetic properties. The differences in the subunits may be responsible for the different catalytic activities of mono- and di-phenolic substrates (Gandhi et al. 2018).

### 3.2. Peroxidase (POD)

Peroxidases (POD, EC 1.11.1.7) are a group of enzymes which general first function is to oxidize hydrogen donors. They are very specific for hydrogen peroxide but can accept a large range of hydrogen donors. In browning, the peroxidase oxidizes a single electron of the phenolic compounds in the presence of hydrogen peroxide. Peroxidases are glycoproteins with a hematin compound as a cofactor, and their molecular weights range between 30 and 55 kDa. The optimum pH for the activity of the enzyme fluctuates between 4 and 7 depending on the source of the enzyme and the hydrogen donor substrate. The formation of hydrogen peroxide in the oxidation of some phenolic compounds catalyzed by PPO can produce a synergistic function between PPO and POD, suggesting the involvement of the POD enzyme in the browning mechanism. The involvement of peroxidase in tissue browning of the fresh-cut products has been confirmed (Subramanian et al., 1999; Hisaminato et al., 2001; Cantos et al., 2002).

### 3.3. Phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) is the first enzyme in the phenylpropanoid pathway related to browning as this pathway is the principal source of phenolic PPO substrates (Mai and Glomb., 2013). Wound stress of plant tissues triggers an increase in the levels of phenolic compounds, particularly phenylpropanoids. This wound-induced phenolic biosynthesis is mediated through an increase in PAL activity. The increase in PAL activity occurs immediately after lettuce is cut and has been found

to be closely related to browning development (Hyodo et al., 1979; Lopez-Galvez et al., 1996b). Some authors have observed an apparent relationship between PAL activity and enzymatic browning of fresh-cut lettuce during storage (Hisaminato et al., 2001). These authors found the possibility of preventing browning by inhibiting PAL activity.

## 4. PHENOLIC METABOLITES

The main substrates of lettuce browning are phenolic compounds that are formed throughout the phenylpropanoid pathway (Figure 7). The phenylpropanoids are a miscellaneous group of compounds, derived from a carbon skeleton of phenylalanine, that are implicated in plant defense, conferred structural support and survival sustenance (Vogt et al., 2010). The compounds belonging to this pathway are known as secondary metabolites because this pathway has no apparent implication in any of the basal cell functions such as photosynthesis, respiration or protein and nucleic acid synthesis processes which are the role the primary metabolites. As a result, it is possible to think that secondary metabolism is not strictly necessary for the immediate survival of the organism. However, the importance of secondary metabolites in the interaction of plants with biotic and abiotic stresses is crucial (Fraser and Chapple, 2011). Specifically, the phenylpropanoid pathway is essential for plants due to for example, the production of the hydroxycinnamoyl alcohols, also known as monolignols or the building blocks of lignin that confers structural support, vascular integrity and pathogen resistance as well as other small molecules such as flavonoids, coumarins, hydroxycinnamic acid conjugates and lignans.

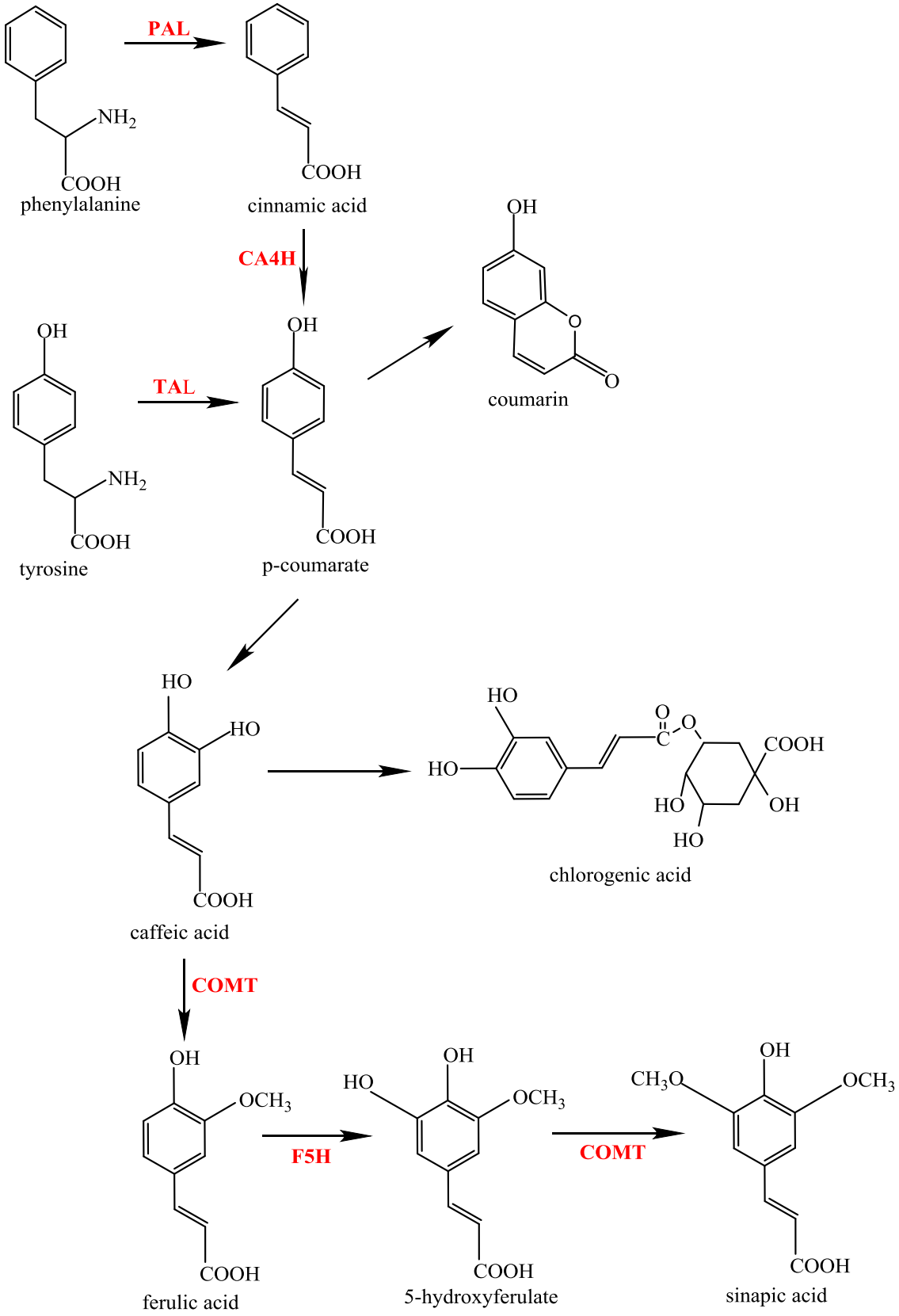



Figure 7. Phenylpropanoid pathway (Dixon and Paiva, 1995).

### 4.1. Lettuce phenolic compounds

The phenolic compounds in lettuce are a large group of compounds previously studied (Llorach et al., 2008; Abu reidah et al., 2013). The phenolic compounds present in lettuce are mostly hydroxybenzoic acids, hydroxycinnamic acids and flavonoids, specifically anthocyanins only present in the red types. The type and content of phenolic compound depend on the type of lettuce for example, iceberg, butterhead, romaine, red leaf and green leaf contain different amounts of phenolic compounds (Tomas-Barberan and Espin, 2001). The total phenolic content also changes depending on the growing season (Bunning et al., 2010) being red leaf and green leaf the types of lettuce with higher content followed by butterhead, romaine and iceberg as the last one.

Caffeic acid derivatives are the phenylpropanoid compounds widely extended in all lettuce types. Caffeic acid derivatives are plant polyphenol oxidase substrates and therefore substrates of browning. The caffeic acid derivative occurring in lettuce which is the most relevant compound in browning development is caffeoylquinic acid also called chlorogenic acid. Caffeic acid derivatives such as *O*-caffeoyltartaric acid (caftaric acid), di-O-caffeoyl- tartaric acid (chicoric acid), 5-O-caffeoylquinic acid (chlorogenic acid) and 3,5-di-O-caffeoylquinic acid (isochlorogenic acid) are constitutively present in lettuce and can be accumulated after wounding (Tomas-Barberan et al., 1997). These compounds are oxidized by PPO to quinones, which spontaneously polymerize to brown pigments which are responsible for tissue browning (Ke and Saltveit, 1986, 1989).

Table 2. Main phenolic compounds and PPO substrates identified in lettuce (Abu-Reidah et al., 2013; Mai et al., 2013; Viacava et al., 2017)

| **Phenolic compound family** | | | |
|---|---|---|---|
| ***Class*** | ***Type*** | | |
| Hydroxybenzoic acids | Protocatechuic acid | | |
| Hydroxyphenylacetic acids | Trihydroxyphenyl acetyl derivatives | | |
| Hydroxycynnamic acids | Caffeic acid derivatives<br>Coniferylalcohol derivatives | | |
| Flavonoids | Anthocyanins, flavones | | |
| **PPO substrates** | | | |
| ***Caffeic acid derivatives*** | | | |
| **Compound** | **Common name** | **Formula** | **Mass** |
| 5-Caffeoylquinic acid | Chlorogenic acid | $C_{16}H_{18}O_9$ | 354.0951 |
| Dicaffeoylquinic acid | Isochlorogenic acid | $C_{25}H_{24}O_{12}$ | 516.1268 |
| Caffeoyltartaric acid | Caftaric acid | $C_{13}H_{12}O_9$ | 312.0481 |
| Dicaffeoyltartaric acid | Chicoric acid | $C_{22}H_{18}O_{12}$ | 474.0798 |

## 5. CONTROL OF ENZYMATIC BROWNING

As mentioned before, minimal processing of fresh-cut lettuce entails physical wounding of the tissue that damages cell membranes, mixing PPO located in the cytoplasm and the existing phenolic compounds from the vacuole to produce brown compounds (Saltveit et al., 2003). Thus, the most relevant elements that can control browning are the activity of PPO and the content of phenolic compounds present, pH, temperature and the availability of oxygen in the tissue. All these factors and the understanding of their interaction are needed for the control of enzymatic browning. To

date, different mechanisms and techniques have been studied to control enzymatic browning of fresh-cut vegetables. Usually, browning control methods attempt to eliminate one or more essential components (such as oxygen, enzyme or substrate) from the browning reaction (Queiroz et al., 2008). Accordingly, some strategies have been established to prevent browning and help to maintain the initial color. The most relevant ways to control browning are cold storage and modified atmosphere packaging (MAP) with low $O_2$ levels. However, other strategies such as immersion in anti-browning solutions and heat shock have been also described as effective treatments.

### 5.1. Cold storage

The quality of the fresh-cut lettuce depends mainly on the initial quality of the raw material. The raw material should be of the highest quality as possible and must be stored at temperature < 2 °C when they are received from the field as whole heads. Before and after processing, lettuce heads should be stored at a minimum temperature of 1 °C and a maximum of 3 °C to guarantee the quality of the fresh-cut produce avoiding the freezing potential of the product during handling, distribution and storage (Barth et al., 2016).

### 5.2. Modified atmosphere packaging (MAP)

In recent years, the extraordinary growth of fresh-cut products has been possible due to the innovation and development of MAP technology. In MAP, there is a balance between oxygen ($O_2$) and carbon dioxide ($CO_2$) that it is achieved through the control of $O_2$ and $CO_2$ transmission rate through the packaging film and the respiration rate of the produce. Several studies have shown the control of lettuce browning under low $O_2$ and moderate $CO_2$ concentrations (Luna et al., 2016). Some authors previously observed that despite enzymatic browning can be inhibited under low $O_2$ and moderate $CO_2$, there is

not a simple relationship with total ascorbic acid content and PPO activity in iceberg (Heimdal et al.,1995) and romaine lettuce (Hamza et al., 1996). In previous studies with "Lollo rosso" a reduction of the wound-induced increase of phenylpropanoids was observed after MAP storage (Gil et al., 1998).

The control of browning in lettuce pieces can be achieved under low $O_2$ conditions (0.3-0.5 kPa) (Smyth et al., 1998; Martinez-Sánchez et al., 2011). However, it is very difficult to understand the changes caused by the modified atmosphere and the complex interaction between phenylpropanoids and oxidation reactions. This is because fresh-cut lettuce is a non-homogenous material with different parts of the plant tissues, photosynthetic and vascular, which entails a great difficulty to establish correlations between enzymes and substrates. Previous studies have shown that the use of MAP decreases wound-induced PAL activity and therefore the biosynthesis of substrates of PPO reactions such as caffeic acid. MAP also inhibits the activation of the conversion of latent PPO to the active form as well as under MAP the content of ascorbic acid is preserved (Luna et al., 2016).

### 5.3. Anti-browning solutions

Reducing compounds, such as ascorbic acid (AA) and its derivatives, cysteine, and glutathione, are the most effective additives to control browning. Some salts of ascorbic acid such as calcium ascorbate, are commercially used in many fresh-cut products that have a high browning development. Reducing compounds can play an important role in controlling browning either irreversibly by reacting with quinones to form colorless products or reversibly by reducing *o*-quinones to colorless diphenols.

Ascorbic acid and its derivatives have been reported to be effective in different concentration ranges, from 0.4-5%. They are generally recognized as safe (GRAS)

antioxidants for their use as browning-preventing compounds. This effect is due to the reduction of *o*-quinones produced by the action of PPO to original phenolic substrates (Gorny et al., 2002; Abbott et al., 2004; Gonzalez-Aguilar et al., 2004).

Sulfites have also shown to be effective in controlling browning. However the use of sulfites on fresh-cut products was banned by the FDA due to their effects on the allergic population as they can cause asthmatic crises.

Other compounds such as L-cysteine and glutathione which several thiol-residues can reduce *o*-quinones to their corresponding phenolic substrate. Cysteine, glutathione and ascorbic acid were compared to evaluate the effect of thiol-containing compounds and the inhibition of PPO (Eissa et al., 2006). These authors observed that cysteine and glutathione showed a significantly higher effect than ascorbic acid. However, they cannot be used in Europe.

Acidulant solutions can modify the pH and change the ionizable groups of the protein structure of the PPO, POD, and PAL and therefore their activity. These ionizable groups must be in the right ionic form to maintain the conformation of the active site, bind substrates or catalyze the browning reaction. Changes in the ionization of PPO are generally reversible, but it can also occur an irreversible inactivation under drastic pH conditions. PPO can become inactive by adjusting the pH below that necessary for its optimal activity with acidulants such as citric acid, malic acid and phosphoric acid. Citric acid is one of the most widely used acidulants in the food industry. It is usually used in combination with other anti-browning agents such as ascorbic acid (Luo et al., 2011; Grimm et al., 2012).

Chelating agents such as sorbic acid, polycarboxylic acids (citric, malic, tartaric, oxalic and succinic acids), polyphosphates (ATP and pyrophosphates), macromolecules (porphyrins, proteins) and ethylene diamine tetra-acetic acid (EDTA), which prevent the

enzymatic activity by binding to metals in the metal-enzyme complex, are widely used in fresh-cut although for their approval it is convenient to check the additive regulations in each country (Du et al., 2012).

### 5.4. Heat shocks

Enzymatic browning can be controlled by mild heat shock treatments, thus it has been reported to be one of the most successful methods to prevent browning of fresh-cut lettuce (Saltveit, 1998; Loaiza-Velarde et al., 2003). The application of mild heat treatment of 45-60 °C for 90 sec inhibits browning through the formation of heat shock proteins (HSPs) (Loaiza-Velarde et al., 2003). The exposure of plant tissue to mild temperatures is accompanied by the synthesis of HSPs in detriment of other proteins such as synthesis of the wound-induced PAL (Murata et al., 2004). This response is ubiquitous to plants and protects the plant from other stresses instead of the synthesis of enzymes involved in phenolic metabolism that are induced by the wounding. This is the mechanism by which the heat shock treatment could decrease browning of fresh-cut lettuce.

## 6. NEW OMICS SCIENCES

The new technological advances of recent years have favored the way in which we observe biological systems. The new omics sciences emerged as a powerful way to study biological systems to understand the cellular functioning of organisms and their biological alterations (Rochfort, 2005; Ryan and Robards, 2006). The omics sciences cascade includes genomics (the study of gene alteration), transcriptomics (the study of the gene expression through the changes in the RNA transcription), proteomics (the study of changes in the translated proteins) and metabolomics (the study of the change in metabolites). Changes of the expression in the cascade of the omic sciences always

correspond to a certain phenotype (Sales et al., 2014; Hasin et al., 2017). Metabolomics is the link between the gene expression and phenotyping which is one of the keys of understanding biological systems.

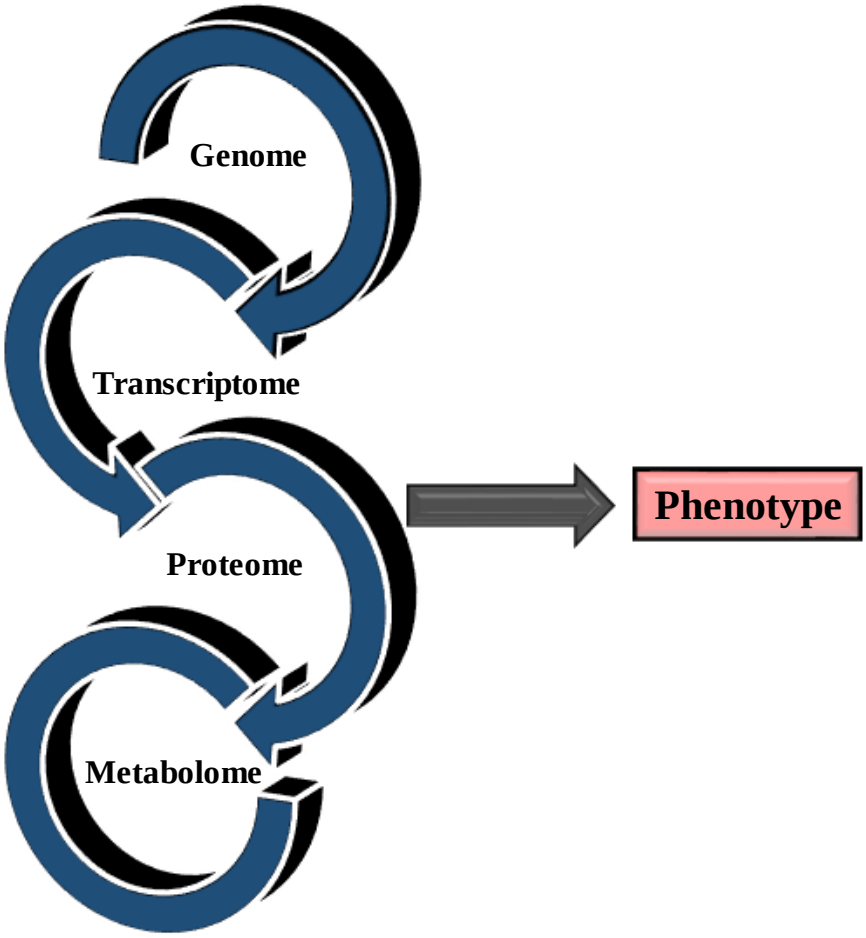



Figure 8. Scheme of omics sciences (Deidda et al., 2015).

Through the advances in gene and nucleotide sequencing, it has been possible the sequencing of a large number of genomes such as *Arabidopsis thaliana* (The Arabidopsis Initiative, 2000) and humans (Venter et al., 2001). The advances in the new technologies for mRNA profiling (Kehoe et al., 1999; Lorenzi et al.,2018) allow a comprehensive analysis of the transcriptome (Sumner et al., 2003; Fan et al., 2018). Finally, advances in

mass spectrometry have allowed analyzing proteins and metabolites with a higher degree of precision. The sum of the progression in all these fields has allowed the understanding of the complex biological systems (Ideker et al., 2001).

### 6.1. Metabolomics

Metabolites are the intermediate or final products of biological metabolism that include the complete set of metabolites of low molecular weight (less than 1500 Da) that are constitutive of a biological system or are formed when the system is altered. The whole set of metabolites in a system is known as the metabolome. The study derived from the identification of these metabolites in biological samples, and consequently their metabolome, is known as metabolomics (Oliver et al., 1998; Fiehn, 2002; Dettmer and Hammock, 2004). It is possible to know the metabolic status of a given biological system through metabolomics. All biological samples have a set of metabolites that belong to the same biochemical pathway. This is known as the metabolic profile and the set of metabolites that appear after a controlled biological process is known as metabolic fingerprinting (Nicholson et al., 1999; Kell et al., 2005; Nielsen et al., 2010).

Nowadays, it is difficult to cover all the metabolism due to the great chemical variability of the metabolites. Metabolomics uses all the technologies trying to explain the maximum possible percentage of the metabolome. There are different platforms capable of detecting and mapping the quantitative changes of the metabolites and the total composition of the metabolome. These include nuclear magnetic resonance (NMR), gas chromatography (GC-MS) (Beale et al., 2018; Prinsloo et al., 2018) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Cordeiro et al., 2018; Cui et al., 2018).

Nuclear magnetic resonance (NMR) is a robust technique that needs little or no manipulation of the sample so that the intact samples can be analyzed (Jarak et al., 2018; Lima et al., 2018). A great advantage of this technique is that it requires very little sample quantity and that it is a non-destructive method, despite having to add deuterated solvents. On the other hand, it has low sensitivity and selectivity as disadvantages.

Mass spectrometry (MS) is the most used technique in metabolomics because it provides sensitivity, selectivity and speed. In metabolomics analysis, MS can be used by direct infusion (DIMS) but often leads to problems in the analysis due to difficulty in the ionization of the sample. These problems caused by direct infusion, especially in matrices of complex samples, can be solved through the coupling of continuous separative techniques to the analysis of mass spectrometry. These separative techniques include gas chromatography (GS), liquid chromatography (LC), and capillary electrophoresis (CE). The coupling of chromatographic separation techniques also provides parameters such as the retention time that helps the subsequent identification of the metabolites.

Gas chromatography (GC) is one of the most used chromatographic techniques for the analysis of the volatile metabolome (Hill and Roessner, 2013). This analysis is characterized by being robust and repeatable. Sometimes this technique can cause problems because some volatile compounds can be lost in the cases where it is necessary to apply derivatization of the samples and also often limits the number of samples since derivatization can be a tedious procedure. The availability of spectra libraries (based on retention time and fragmentation pattern) for the metabolite identification that can be used in gas chromatography is one of the great advantages of this separative technique that gives credibility to the identification.

Liquid chromatography (LC) is the most used technique in metabolomics studies due to its high robustness, selectivity and sensitivity. It is a complete technique in what

refers to the analysis of different kinds of compounds, the available mobile and stationary phases and the multiple separation options.

Regarding the identification of metabolites, there are two approaches: *i*) targeted metabolomics as the quantitative or qualitative analysis of one or more previously selected metabolites; *ii*) untargeted or non-targeted metabolomics as the qualitative analysis of the largest number of metabolites of all classes.

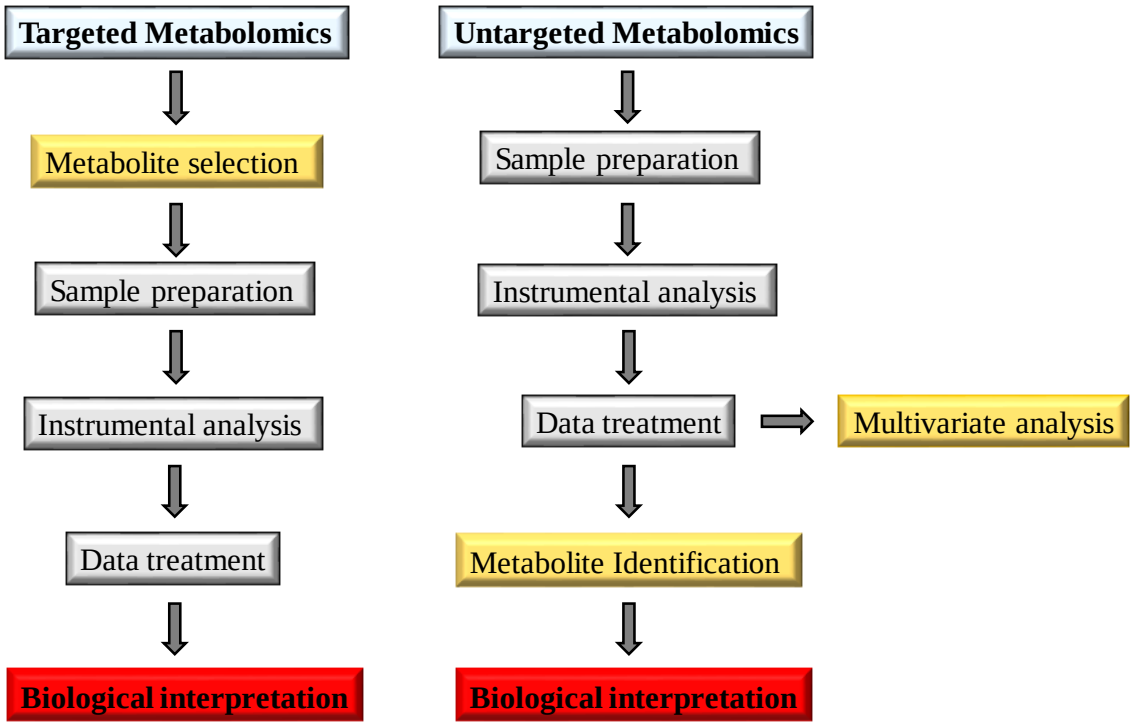



Figure 9. Scheme of targeted and untargeted metabolomics methodologies.

### 6.2. Untargeted metabolomics analysis

Untargeted metabolomics analysis is characterized by the simultaneous measurement of a large number of metabolites in biological samples from which, a priori, their composition is unknown (Alonso et al., 2015). The complexity of the untargeted study versus targeted metabolomics is of a greater extent because the changes must be

detected without applying any filter than the chemical selectivity of the chromatographic method, therefore, detecting a wide range of spectra. LC-MS applied to the non-targeted metabolomics approach has led to a revolution in the study of metabolites. Thus, LC-MS has been applied to the study of all types of metabolites including metabolites relevant to biomedical studies (Cui et al., 2013; Shahfiza et al., 2017), food science (Farneti et al., 2014), nutritional studies (Oresic, 2008) and microbiology (Luzzatto-Knaan et al., 2015).

The goal of untargeted metabolomics is to measure as many metabolites as possible, including the unknowns, and generate a metabolic fingerprint characteristic of each biological sample. The most common way of working in an untargeted metabolomics is through a comparative analysis (Dudzik et al., 2017). Thus, the fingerprints of different study cases are overlaid to select those signals that can be statistically different.

Data generated in the analysis of non-targeted metabolomics are abundant and complex. Therefore the use of appropriate data processing tools has to be applied to avoid errors and maintain the nature of the biological systems studied. The link between non-targeted metabolomics and bioinformatics is a fact. Thus, the combination of data preprocessing tools, statistical analysis, and multivariate analysis is essential for the correct use of this powerful tool. The correct handling of the amount of data generated by this technique is essential to obtain reliable results as well as the establishment of a thorough and complete workflow is a critical point. All untargeted metabolomics analyses are composed by the first stage of data acquisition, a data pre-processing, subsequent data processing, identification of metabolites and after the biological interpretation (Figure 10).

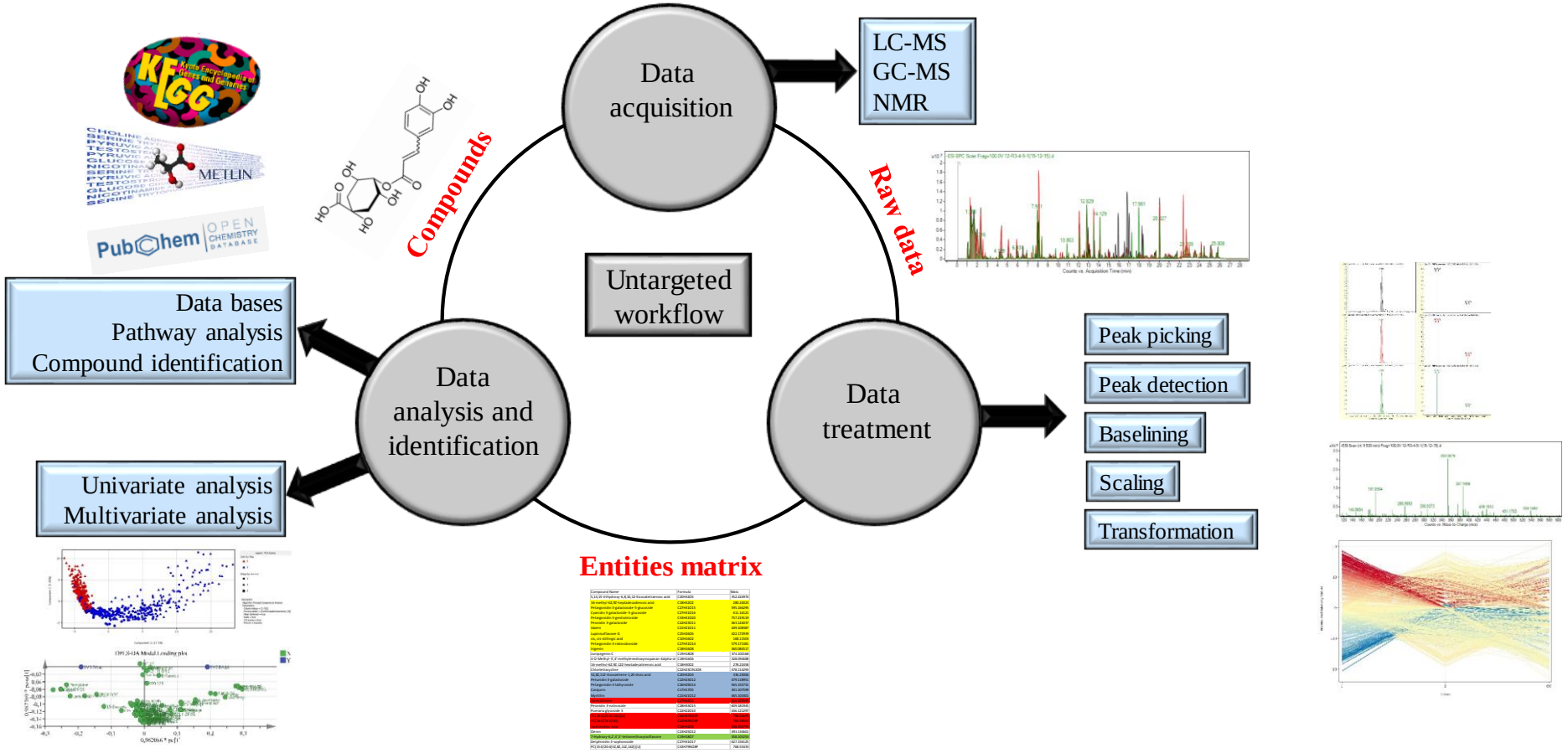



Figure10. Untargeted metabolomics workflow.

## 7. DATA TREATMENT

The metabolomics platforms provide an immense amount of data that in the case of LC-MS include chromatographic and mass spectrometry data. The untargeted metabolomics methods use this combined information to extract the maximum information about the global metabolome.

From the peak and ions extraction at the beginning of the analysis to the identification and biological interpretation, there is a long list of important operations that the detected ion must pass satisfactorily in order to be a relevant candidate. The purpose of data treatment is to transform the raw data obtained into a data matrix processed and ready for analysis (Jonsson et al., 2005).

There are different strategies to carry out the data treatment according to the software used. These strategies are marked by the algorithms and by the criticisms of each software applied and can determine in a very precise way the final results.

### 7.1. Data processing

The data preprocessing consists of several stages that will depend on the software used although it can be grouped in different steps: peak detecting, peak picking and alignment. These steps are critical as they determine completely the subsequent analysis and the results. Therefore, the knowledge and application of the correct parameters in this first phase are crucial (Nielsen et al., 2010; Tian et al., 2017).

In the logical sequence of the pre-processing, the first action on the raw data is the peak picking. The peak picking is used to extract and filter the peaks detected in the experimental chromatogram. The minimal operations applied in the peak picking are usually a restriction for retention time and *m/z* and also a peak height filter. The alignment is a critical step, especially when comparing between a large and complex data set. The

alignment of species provides the same peak depending on a tolerance parameter in the retention time and the tolerance of *m/z*.

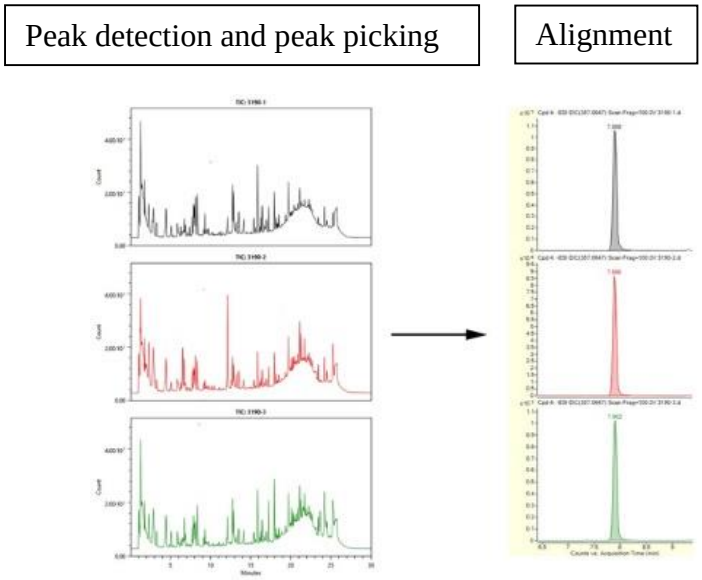



Figure 11. General scheme of pre-processing data.

### 7.2. Data normalization

The set of operations that finally gives an entity value to the information extracted from the data processing is included within the general concept of data normalization. At the beginning of the data treatment (data processing), the computer tools allow to extract all the information detected and evaluate their relevance across the full data set although the data normalization converts the information into an entity matrix (Sugimoto et al., 2012). Applying a normalization is necessary to give the status of entity to the extracted and aligned peaks and extracted ions. The normalization process includes the sample normalization or baselining, scaling and the transformation.

### 7.2.1. Sample normalization or baselining

The baselining is the firt step in the data normalization and aims to normalize the way in which entities are treated according to their intensity value. When data are heteroscedastic, the effect of the normalization method is not always enough to standardize how relevant the entities are to each other and if it is necessary to apply more normalization operations.

### 7.2.2. Scaling

The scaling methods are different data normalization approaches that divide each variable by a scaling factor that is different from the variable. The objective is to adjust the intensity differences of the different metabolites by converting the data into differences of intensity relative to a scale factor. Applying a correct scale is essential since scaling often results in the overvaluation of small values that increase with the presence of the measurement error that is usually large for measurement of small values (Van der Berg et al., 2006).

There are two subclasses of scaling. In the first class, the measure of data dispersion (such as the standard deviation) is applied as a scale factor. The second class of scaling uses a measure of size such as the average.

*Data dispersion scaling*

The scaling methods by data dispersion are: *i*) Auto scaling, *ii*) Pareto scaling, *iii*) Range scaling and *iv*) Vast scaling (Keun et al., 2003; Smilde et al., 2005).

i) *Auto-scaling*. Auto-scaling applies that the standard deviation of all the entities is equal to 1. Therefore the comparison is based on the correlations and not on the

covariance. Auto-scaling considers all entities of equal importance, and this can cause an increase in measurement errors.

ii) *Pareto scaling*. This applies the square root of standard deviation instead of standard deviation. After Pareto scaling, the data present a greater decrease in large fold changes than in small fold changes. Therefore, this scaling downplays the big changes compared to the data without scaling. This scaling method keeps intact the original data more than auto-scaling (Eriksson et al.,1999). The Pareto scaling is one of the best data dispersion scaling methods for the data obtained through LC-MS.

iii) *Range scaling*. This scaling method is very similar to the auto scaling method. Range scaling, as in auto-scaling, considers all entities of equal importance and can give relevance to measurement errors.

iv) *Vast scaling*. Aims to the entities that show small fluctuations.

*Scaling based on size measure*

This subclass of scaling methods aims to level scaling. This scaling method is focused on the relative response and can also present an increase in measurement error such as auto-scaling and range scaling.

### 7.2.3. Transformation

Data transformation is the last step of the data treatment. It is a nonlinear conversion applied to correct the heteroscedasticity and reduce the large values of the dataset relatively more than the small values, one step more in the scaling. Data transformation converts multiplicative relations into additive relations and makes skewed distributions more symmetric (Van der Berg et al., 2006).

The transformation widely used is the log transformation. This can remove the heteroscedasticity if the standard deviation is constant, but as a consequence the log

transformation emphasize the large values of deviation which present the entities with low intensity. On the other hand, it is incompatible with the zero value that occurs when the logarithm approaches infinity as the value to be transformed approaches zero (Kvalheim et al., 1994; Yang et al., 2015).

## 8. DATA ANALYSIS

Data analysis represents the last stage of the untargeted metabolomics analysis. It is dependent on the previous steps, and it determines the precision and success of the analysis. It is necessary to convert correctly the signals obtained in the data acquisition into a matrix of identities in order to carry out the analysis of the data. Data analysis includes the statistical analysis, grouped in the univariate and multivariate analyses, and the identification.

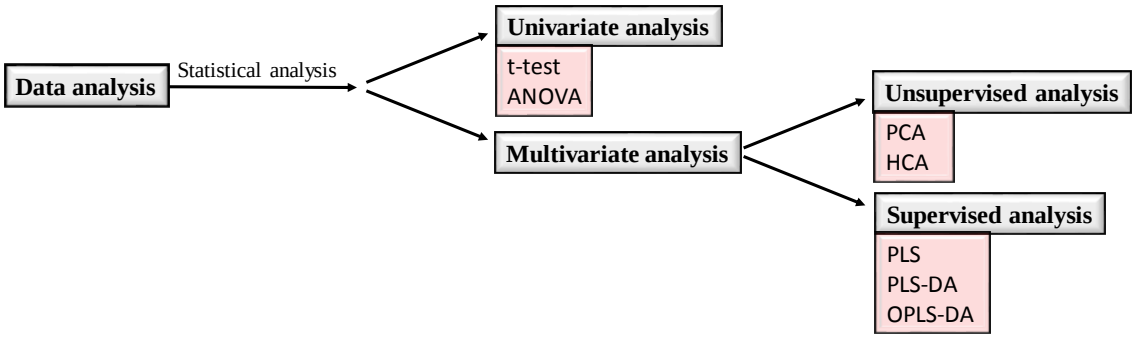



Figure 12. General scheme of data analysis (Saccenti et al., 2014).

### 8.1. Univariate analysis

It is possible to apply statistical tools to the data matrix to extract the most relevant information. Statistics offers univariate analysis solutions including different variants depending on the study groups and the number of those variables (groups). In univariate analyses, the studied variables (entities or metabolites) are evaluated separately, and their

relationships are discarded. Some of these univariate statistical tests include fold change analysis, t-test, volcano plot and one-way analysis of variance (ANOVA).

### 8.2. Multivariate analysis

The multivariate analysis techniques allow analyzing more than two variables at the same time. The objective of the multivariate analysis is the analysis of the variance, data trends, classification or discrimination of the groups of entities or metabolites responsible for differentiating the samples evaluated by analyzing a previously obtained data matrix. It differs mainly from the univariate analysis in that the study variables are evaluated at the same time and the relationships between them are examined.

The multivariate analysis allows us the use of unsupervised and supervised methods of analysis. The criteria to select a technique is the type of data (nominal or ordinal) and the reason for the analysis (explore the trend of the data, reduce the dimensionality and classify).

*Unsupervised methods*

The supervised methods do not treat any variable as a dependent. The unsupervised method studies the relationship between the variables or the variable and the cases trends. Principal component analysis (PCA) and hierarchical clustering analysis (HCA) and self-organizing maps (SOMs) are the most used and important ones (Barker and Rayens, 2003; Alonso et al., 2015). Principal component analysis (PCA) is based on the linear transformation of metabolic characteristics into a set of linearly uncorrelated variables known as principal components. This decomposition method maximizes the variance explained by the first component, while the other components explain the variance reductions. Moreover, the PCA minimizes the covariance between the components, which are independent of each other. The PCA method generates a set of

loading vectors and scoring vectors. The loading vectors represent by their coefficients the individual contribution of each variable to the main components and the scoring vectors represent the projection of each sample in the new representation base. On the other hand, hierarchical clustering analysis (HCA) is a powerful grouping and visualization tool that provides a classification method at the sample and function levels according to a predetermined distance measure. In the case of self-organizing maps (SOMs), they are used in studies of metabolomics to identify phenotypes and metabolic characteristics and give more importance to the metabolites of interest as a function of their similarity.

*Supervised methods*

The supervised methods treat one or more variables as dependent variables that are going to be explained or predicted by others. Some supervised methods are Multiple regression analysis, partial least square (PLS), partial least square discriminant analysis (PLS-DA), and orthogonal partial least square discriminant analysis (OPLS-DA) (Bilesjo et al., 2006; Brereton and Lloyd, 2017). Partial least square (PLS) is used either as a regression analysis or as a binary classifier (PLS-DA) to explain the covariance between the variable of interest and the metabolomics data. PLS does not maximize the variance of the set of data explained unlike PCA. The variables (entities or features) of a PLS loading plot represent how much of an entity o feature contributes to the discrimination of the different sample groups. One of PLS models drawbacks is that some of the entities that are not related to the study variety can influence the results. The orthogonal PLS (OPLS and OPLS-DA) can solve this problem because factorize the data variance into two components: the first component which is correlated with the study variable and the second which is orthogonal and uncorrelated.

# CHAPTER: II

# OBJECTIVES

## Objectives

Lettuce is one of the most important vegetable crops in the world and it has a great economic value for the fresh-cut industry. The knowledge of lettuce physiology, biology, technology, and biochemistry is essential for the correct production, industrial processing, and commercial distribution to assure a high-quality product.

Enzymatic browning is the most important discoloration disorder that decreases the quality after cutting and storage and causes consumer rejection. Even though this process has been studied extensively during the last 20 years, the detailed knowledge of the browning mechanism, the implicated metabolites and the factors involved in the development still need to be studied with the novel omics technologies to understand better the browning process for prevention.

An untargeted metabolomics approach has been carried out in this Thesis to study the metabolites implicated in the browning process and identify the early biomarkers that can predict browning after cutting.

The main objectives of this Thesis are:

1. To establish a robust untargeted metabolomics methodology to explore the metabolome of lettuce by the creation of a specific lettuce metabolites database and a metabolomics workflow.

2. To identify the signal metabolites that can trigger enzymatic browning development of fresh-cut lettuce by LC-MS untargeted metabolomics.

3. To select early biomarkers to predict browning of fresh-cut lettuce by LC-MS untargeted metabolomics: Impact of internal and external factors such as genetics (cultivars), and environmental conditions.

4. To identify early biomarkers of the browning process by LC-MS targeted metabolomics: Impact of lettuce development and validation for the selection of lettuce cultivars less susceptible to browning after cutting and storage.

# CHAPTER: III

# MATERIAL AND METHODS

## 1.PLANT MATERIAL

The plant material used in this Thesis was provided by Enza-Zaden ESPAÑA, S.L. (Experimental farm "El Albujon," Torre Pacheco, Murcia, Spain). All lettuce types and cultivars used were grown in the Campo de Cartagena area (37.44ºN, 0.59ºE) (Torre Pacheco, Murcia, Spain). Commercial agricultural practices were used for growing and differences in planting time, and harvest date was programmed as needed.


Photo 1. "El Albujon" experimental farm of Enza Zaden located in Torre Pacheco (Murcia, Spain).

Iceberg and romaine lettuce types were selected as the most relevant lettuce types from primary production and processing interest. Two cultivars of iceberg lettuce, one of low browning potential and another of high browning potential previously characterized

by our research group were selected to explore the metabolome of lettuce (Chapter IV). In the case of romaine lettuce, two cultivars with very different browning susceptibility were chosen to evaluate the kinetics of browning development (Chapter V) and a set of 30 different cultivars harvested at three different times were compared to evaluate the impact of internal and external factors such as genetics (cultivars), and environmental conditions affecting browning (Chapter VI). Two of them with different browning susceptibility were examined to understand browning as affected by the maturity stage (Chapter VII).

## 2. PROCESSING AND STORAGE CONDITIONS

The midrib tissue was selected as the plant material studied. The reason was that in this vascular tissue, the enzymatic browning occurs earlier and faster than in the photosynthetic tissue. Another reason for this selection was the low basal content of phenolic substrates of the enzymatic browning just after harvest, and also as this type of tissue has a much simpler and consistent metabolic composition than the photosynthetic tissue which is more complex and variable in composition making more difficult the identification of the metabolites associated to the browning process.

Lettuces were harvested the day before processing and stored for 24 h at 7 °C and 80% relative humidity. Ten heads per cultivar were processed. First, the external leaves were discarded and then from the middle part of the head, four leaves for iceberg and six leaves for romaine were selected. Then the midribs were separated and cut into pieces of 1.5 cm length. All the processing was carried out in less than 20 min in a cold room at 4 ºC to reduce as much as possible the induction of the wound response and thus the changes in the initial composition of the midribs. Each replicate was comprised of 30 g of midribs.

Midribs were packaged in plastic bags (120 mm × 200 mm) without sealing to maintain an air atmosphere and high humidity, avoiding water loss. There were five replicates per sampling time, just after cutting (day 0) and after five days of storage at 7 ºC.

For the objective evaluation of browning, image analysis was carried out. The procedure for the sample processing was the same in all the trials with some minor modifications. These modifications were mainly related to the image analysis as both lettuce types differed morphologically in midrib size and shape.

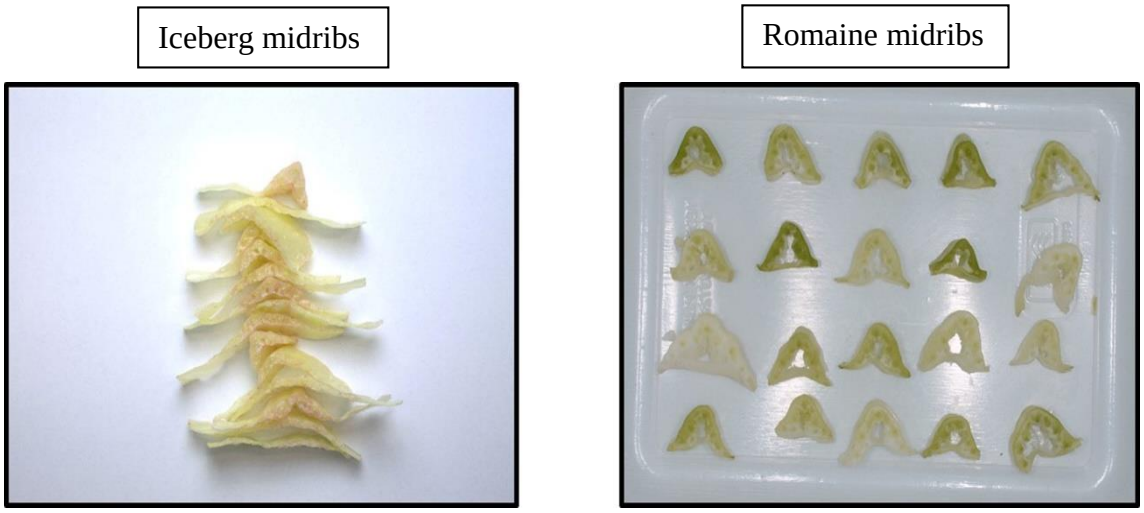



Figure 1. Excised midribs as the plant material selected in this study.

For the analysis of metabolites, the plant material was frozen in liquid nitrogen immediately after cutting (day 0) and also after storage (day 5). Samples were then freeze-dried and finally milled to a fine powder before extraction.

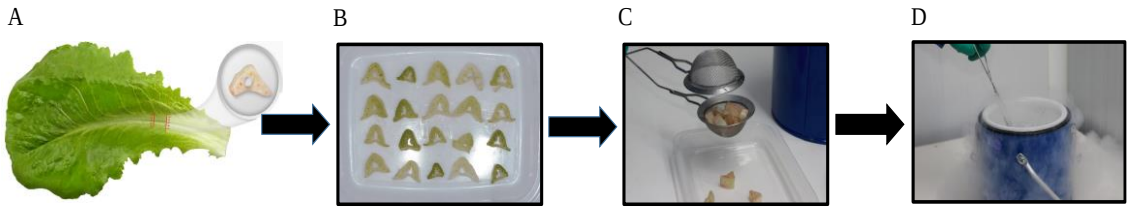



Figure 2. Diagram of the midrib excision and the midrib freezing process.

## 3. DIGITAL IMAGE ANALYSIS

The assessment of browning has been generally done by the evaluation of the visual appearance as a subjective measurement with a large influence of the operator. An objective measurement of browning level was needed to enable the assessment without the problems of the subjective evaluation. Image analysis was chosen as an easy and fast procedure without sample manipulation to estimate the browning level.

The image analysis methodology includes the use of a digital camera, a prepared photography scene that isolates the background of the product image and the image analysis software for processing.

*Digital camera*

A digital camera model Nikon D7100 was used for the image capture and Nikon camera lens Advanced Multi-CAM 3500DX and 35mm focal length. The camera was set to synchronize flash Speed 1/125 s and the focal aperture (f) 22.

*Photography scene*

The camera was positioned 27 cm above the base and the light was provided by Metz Mecablitz flash with a color temperature of 5600 K. All measurements were made

in a dark room with a temperature of 20 ºC. For each image, 20 pieces of midribs were positioned with the cut surface looking up to capture the image on a plate.

*Digital image software*

The digital images were analyzed using the software ImageJ version 1.48v (Image NIH, National Institute of Health, Bethesda, USA). ImageJ is a public domain digital image processing program programmed in Java developed at the National Institute of Health. ImageJ was designed with an open architecture that provides extensibility via Java plugins and writable macros (macroinstructions). Image analysis processes the image by selecting the whole midrib tissue by color contrast as the study area. Images were analyzed using the HSB color model, and the values of hue, saturation, and brightness of the surface area were measured. To identify the brown pixels a range of 20–35 hue, 40–195 saturation, and 0–225 brightness was applied.

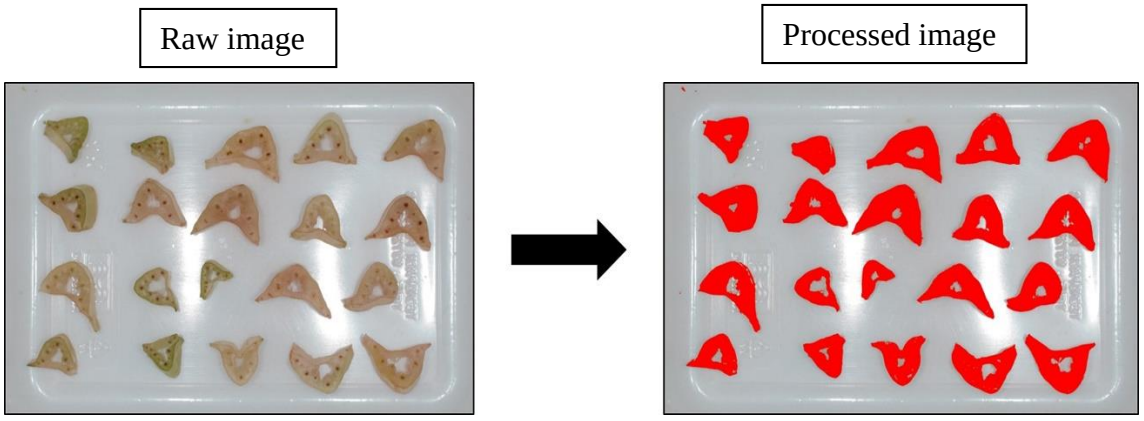



Figure 3. Digital image processing from raw image to the processed image by ImageJ.

## 4. SAMPLE PREPARATION

All samples were frozen in liquid nitrogen and then stored at -80 ºC for at least 48 h before the preparation of fine powder for the analysis. Once the samples were completely frozen, they were freeze-dried for at least 72 h in a freeze-drier (model Christ Alpha 2-4 LD Plus).

Photo 2. The freeze-drier used (model Christ Alpha 2-4 LD Plus).

The dried samples were milled to a fine powder using a blender (Oster Professional BPST02-B) with independent jars (Butlers 324Y91, Bormioli).

Photo 3**.** The professional blender used (Oster Professional BPST02-B).

Freeze-dried samples (0.3 g) were mixed with 10 mL of methanol/water (80:20; v:v). The extraction mixture was sonicated at 30 °C for 30 min, centrifuged at 5000 g for 15 min and directly filtered through a 0.22 μm PVDF filter before the analysis by UPLC-ESI-QTOF.


Photo 4. The ultrasound equipment (Branson 5510) (left) and the centrifuge (Thermo Fisher sorvall ST 16 R) (right) used.

## 5. ULTRA HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO ELECTROSPRAY IONISATION AND QUADRUPOLE TIME-OF-FLIGHT MASS SPECTROMETRY (UPLC-ESI-QTOF-MS)

In this Thesis, all the analyses were done by using a UPLC-ESI-QTOF-MS as the most powerful analytical tool for characterizing metabolites in lettuce extracts. The complete untargeted metabolomics analysis was developed by a metabolomics platform provided by Agilent Technologies. The analyses were carried out using an Agilent 1290

Infinity II multi-sampler LC system coupled to the 6550 iFunnel Accurate-Mass QTOF (Agilent Technologies, Waldbronn, Germany) with an electrospray interface (Jet Stream Technology).

*Agilent 1290 Infinity II multi-sampler LC system*

The chromatographic method was set to separate as many metabolites as possible to describe the complete lettuce metabolome. The chromatographic column used was a reversed phase Poroshell 120 EC-C18 column (3 x 100 mm, 2.7 μm) (Agilent Technologies, Waldbronn, Germany). The Poroshell 120 EC-C18 bonded phase is made of a dense monolayer of dimethyl-n-octadecyl silane stationary phase chemically bonding to the porous shell of the Poroshell 120 silica support. The EC-C18 is characterized by a pore size of 120 Å, temperature working limit of 60 ºC, pH range of 2.0-8.0 and surface area of 130 $m^2$/g. The column was operating at 30 ºC with a flow rate of 0.4 mL/min. The chromatographic method was set to a large range of polarity to separate as many metabolites as possible.

The mobile phases used were acidified water (0.1 % formic acid) (phase A) and acidified ACN (0.1 % formic acid) (phase B). The mobile phases were acidified to improve the resolution and ionization as well as avoid peak "tailing".

Compounds were separated using the following gradient conditions: 0–10 min, 1–18 % phase-B; 10–16 min, 18–38 % phase-B; 16–22 min, 38–95 % phase-B. Then, the column was re-equilibrated for 5 min with the initial conditions before a new analysis.

Figure 4. LC system from Agilent Technologies (Waldbronn, Germany) model Agilent 1290 Infinity II multi-sampler.

*6550 iFunnel Accurate-Mass QTOF*

The Agilent 6550 Q-TOF LC/MS system operates with the Jet Stream electrospray ionization (ESI) which is an atmospheric pressure ionization (API) working mode. The electrospray ionization source (ESI) has a chemical base for the generation of ions of the metabolites in solution before the analyte reaches the mass spectrometer. The eluent is nebulized into a spray chamber in the presence of a strong electrostatic field and heated drying gas at atmospheric pressure. The electrostatic field occurs between the capillary and the nebulizer which is at a high voltage. The spray flows with a 90º angle to the capillary. This design reduces the background noise of the drops, increases sensitivity, and keeps the capillary clean for a longer period.

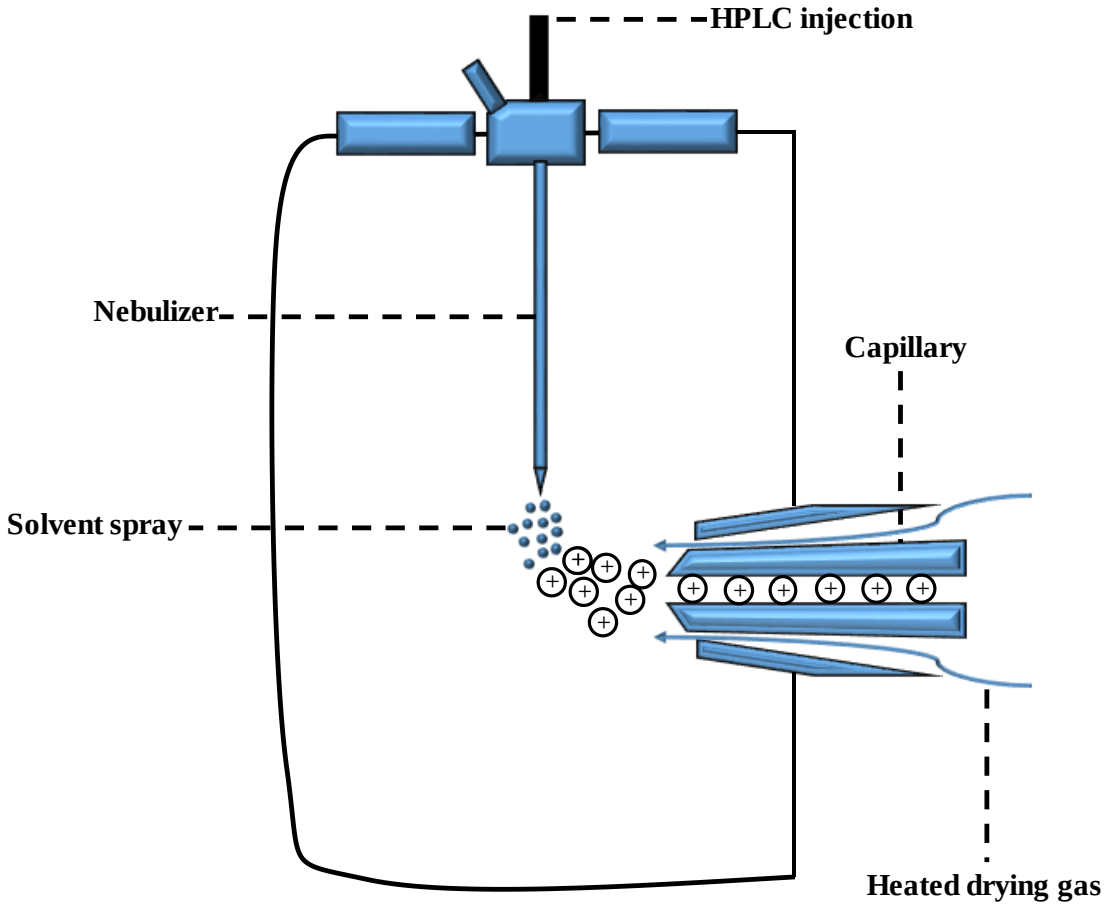



Figure 5. Schematic diagram representing the Electrospray Ionization source (ESI).

The electrospray ionization involves four steps including the formation of the ions, nebulization, desolvation, and ion evaporation. The formation of the analyte ions occurs before the nebulization if the analyte and solvents are correct, giving a high concentration of ions and improving the sensitivity of ESI, or they can also be formed by the strong electrical charge on the surface of spray droplets caused by nebulization, desolvation and ion evaporation. The optimal conditions for the electrospray interface were set up as 280 ºC gas temperature, 9 L/min drying gas, 35 psi nebulizer pressure, 400 ºC sheath gas temperature, and 12 L/min sheath gas flow.

The Agilent 6550 Series iFunnel Q-TOF LC/MS is a liquid chromatograph Q-TOF mass spectrometer that performs MS/MS using a quadrupole, a hexapole (collision cell) and a time-of-flight unit to produce spectra. The quadrupole selects precursor ions that are fragmented in the collision cell into product ions. After that, the product ions are pushed at an angle perpendicular to the original trajectory, to the detector.

Figure 6. Agilent 6550 iFunnel Accurate-Mass Quadrupole Time-of-Flight Q-TOF LC/MS system (Agilent Technologies, Waldbronn, Germany).

The iFunnel technology of the Agilent 6550 iFunnel Q-TOF equipment provides mainly two innovative enhancements. The first innovation is the hexabore capillary which increases up to ten times the entry of ion-rich gas from the ionization source and also transmits to high ions volume into the ion optic system. Ion Funnel technology is the next enhancement. This technology eliminates the gas and neutral noise but retains the ions and can transmit these ions efficiently at the highest possible pressure. The iFunnel has

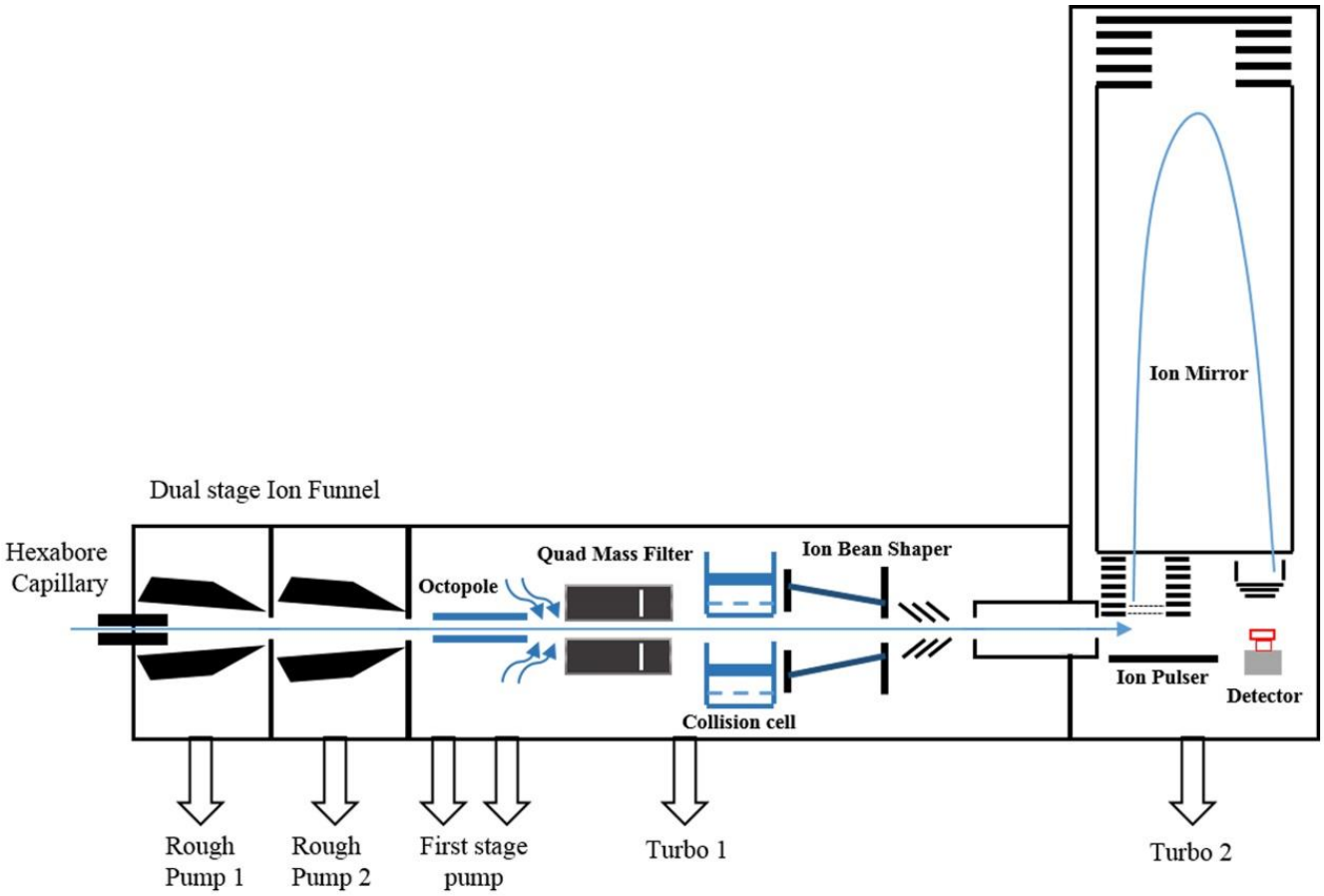



Figure 7. Schematic diagram representing the Agilent 6550 iFunnel Q-TOF LC/MS (Agilent Technologies, Waldbronn, Germany).

two pressure filters, the first between 7 and 14 Torr and the second at a lower pressure between 1 to 3 Torr.

The MS system was operated in the negative ion mode with the mass range set at *m/z* 100–1100 in the full scan resolution mode due to the nature of the untargeted metabolomics expected. The Fragmentor voltage was established at 100 V, and the acquisition rate was set at 1.5 spectra/s. The targeted MS/MS mode was also applied to add confidence for the identification of the compounds due to the difficulties in looking for patterns when there are no matchings with metabolites in databases. The MS/MS product ion was collected at an *m/z* range of 50-800 using a retention time of a minute and an isolation window of 4.0 amu. A collision energy range of 15-40 was applied to elucidate the molecule fragments.

The equipment was calibrated before the analysis of each batch of samples with the standard mix of reference masses provided by Agilent Technologies to guarantee the correct mass pressure throughout the analysis. Continuous internal calibration was also performed during the analyses by looking at the signals *m/z* 112.9855 and *m/z* 1033.9881 in negative polarity.

## 6. METABOLOMICS DATA TREATMENT

The data acquisition system Agilent 6550 iFunnel Q-TOF LC/MS stored the data in a ".d" data file. The raw data included the chromatogram data from the LC system and the recorded data from the mass detector. The system Agilent 6550 iFunnel Q-TOF LC/MS can acquire the chromatographic data and the mass spectral data in centroid and profile format. The profile format is the entire set of data points from the memory system. The advantage of data profiling is the easy detection and extraction of the signals as true

peaks differentiated from the noise of the instrument. The Centroid format summarizes the data points into a dimensionless line, and the advantage of centroid data is that the file size is significantly smaller and easier to handle in informatics terms. The data was stored in both formats for safety reasons for the data treatment.

### 6.1. Data pre-processing

The data pre-processing was performed with the software provided by Agilent technologies. The Mass Profinder version B.08.00 was the software used for pre-processing data including the peak picking and peak alignment steps. The Mass Profinder contains different molecular features extraction (MFE) algorithms to carry out the steps for pre-processing. The Mass Profinder was used to apply the molecular and recursive feature extraction algorithms to peak picking and alignment. An untargeted method to extract as many entities as possible to explore the complete metabolome was developed based on several parameters of the extractions algorithms applied.

*Extraction parameters*

The extraction parameters included a retention time restriction of 1.0-25.0 minutes, an *m/z* restriction of 100.0000-1100.000, a peak filter for the spectrum of the 1000 account profile format, and a negative ion allowance of -H and -HCOO.

*Compound binning and alignment*

The alignment parameters were a tolerance of retention time of 0.30 min and a tolerance mass of ±20.00 ppm and 2.00 mDa.

*Algorithms filters*

Absolute height filter of >= 20000 counts and a score of 70.00% were applied. The minimal match filter applied retains all the entities that appear in at least one sample.

*Find by ions (tolerance and scoring)*

The extraction ions tolerance for chromatogram extraction was ±35.0, and the limit extraction range was 1.50 minutes. The method established a score for the mass of 100.00%, for isotope abundance of 60.00% and for isotope spacing score of 50.00%.

*Peak integration and filtering*

The integration algorithm used was the“ agile 2” which is an algorithm created by Agilent Technologies. The peak filter was set to filter on peak height with absolute height >= 20000 counts. The method used the centroid chromatogram format data for the extraction.

*Spectrum extraction*

The peak spectrum includes an average scan > 10% of peak height, and the TOF spectra exclude data if above 20% of saturation. The method used the centroid mass spectral format data for the extraction.

### 6.1.2. Data integrity test

In untargeted metabolomic analysis, it is often necessary to check the data integrity to ensure a correct structure of the data matrix (i.e., labels and numerical values) and filters if necessary.

*Missing value estimation*

The missing values can cause a problem in the subsequent analysis of the data. There are different methods to remove them. The default method removes the entites with >50% of missing values which is usually configured according to the needs. It is important to consider if the pre-processing software used detect the missing values such as the empty value or zero to apply the missing value estimation correctly.

If it is required,there are different methods to estimate de remaining missing values such as the replace by small value close to the detection limit or by a columm with a mean or median close to the entity value but in this Thesis this was not applied.

*Data filtering*

The objective of the data filtering is to detect and remove the variables that probably are not useful to data modeling. The data filtering is widely used in untargeted metabolomics datasets which are composed by a large number of variables and it is possible that many of them are from baseline noise. The data filtering type is applied depending on the entities number and the variable characteristic.

### 6.2. Data processing

After pre-processing data, it was possible to extract the data in a text file entity, before the final step of processing, to process them in external processing software, or to extract the data in a specific Agilent format file named ".cef " to further process data in Mass Profiler Professional (MPP) 14.9.1 provided by Agilent technologies or other softwares or platform such as Metaboanalyst, SIMCA or R.

Data processing includes the baselining (normalization), scaling and transformation steps. Regardless of the software used for data processing, a baselining to median, logarithmic transformation and pareto scaling were always applied.

### 6.3. Data analysis and identification

Once the data processing was finished, the raw data was transformed into a matrix of entities for the analysis. Data analysis included the univariate and multivariate analyses that were both performed using different software and different tools depending on the goal.

Mass Profiler Professional (MPP), and Metaboanalyst 4.0 web platforms were used to execute univariate analyses such as t-test, correlation analyses, fold change, clustering, volcano plot, and ANOVA depending on the experiment grouping based on *p*-value computation.

Mass Profiler Professional (MPP), The Unscrambler X 10.4 (CAMO software), and SIMCA 14.1 (MKS Umetrics) software were used to apply the modeling of multivariate analysis. Unsupervised and supervised multivariate analyses were applied to analyze the behavior of all the variables and their relationship. The unsupervised models contribute to the overview of the data including the quality control as it allows the detection of outliers and it also shows the trend of the data. The supervised methods used are discriminant models that inform about the biomarkers candidates.

The different types of multivariate models were built based on the observation (N), the variables (K), the entities (X) and the categories (Y).

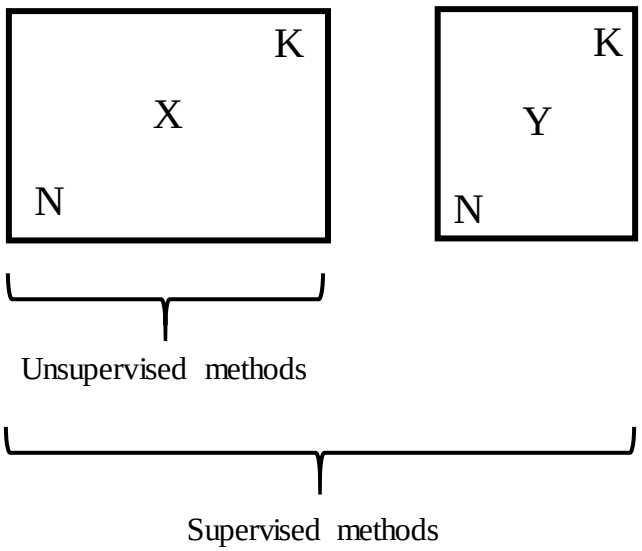
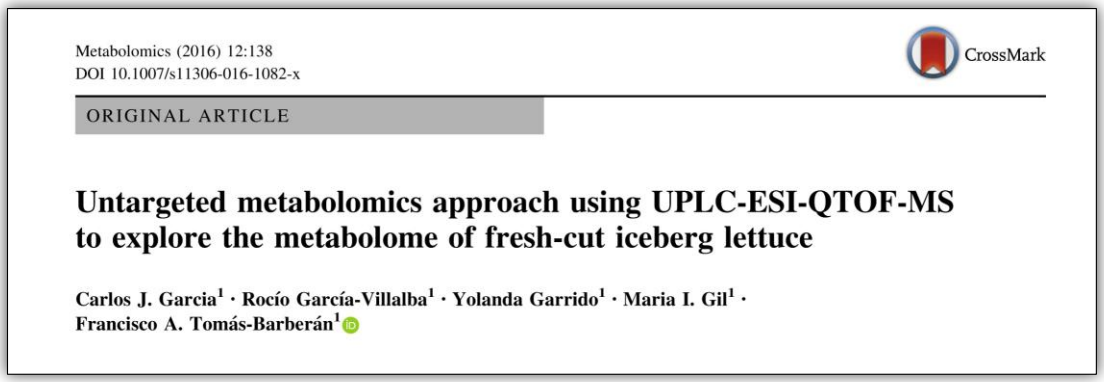



Figure 8. Schematic diagram representing the multivariate analysis types.

Principal component analysis (PCA) and multiple linear regression (MLR) were the unsupervised methods used. The principal component analysis (PCA) models were build based on the full entities matrix list including the quality control (Qc) observation. PLS-DA and OPLS-DA were the supervised discriminant methods used.

# RESULTS

# CHAPTER IV



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ORIGINAL ARTICLE

# Untargeted metabolomics approach using UPLC-ESI-QTOF-MS to explore the metabolome of fresh-cut iceberg lettuce

Carlos J. Garcia[1] · Rocío García-Villalba[1] · Yolanda Garrido[1] · Maria I. Gil[1] · Francisco A. Tomás-Barberán[1]



## Abstract

*Introduction* The shelf-life of fresh-cut lettuce after storage is limited by several factors that affect its quality and lead to consumer rejection. Different metabolic events occur after cutting as an abiotic stress response.

*Objectives* This study aims to explore the metabolome of iceberg lettuce and to understand the changes related to storage time and genetics applying an untargeted metabolomics approach.

*Methods* Two cultivars with different browning susceptibility, fast-browning (FB) and slow-browning (SB), were analyzed by UPLC-ESI-QTOF-MS just after cutting (d0) and after five days of storage (d5). Extraction, metabolic profiling, and data-pretreatment procedures were optimized to obtain a robust and reliable data set.

*Results* Preliminary principal component analysis and hierarchical cluster analysis of the full dataset [around 8551 extracted, aligned and filtered molecular features (MFs)] showed a clear separation between the different samples (FB-d0, FB-d5, SB-d0, and SB-d5), highlighting a clear storage time-dependent effect. After statistical analysis applying

Student’s t test, 536 MFs were detected as significantly different between d0 and d5 of storage in FB and 633 in SB. Some of them (221) were common to both cultivars. Out of these significant compounds, 22 were tentatively identified by matching their molecular formulae with those previously reported in the literature. Five families of metabolites were detected: amino acids, phenolic compounds, sesquiterpene lactones, fatty acids, and lysophospholipids. All compounds showed a clear trend to decrease at d5 except phenolic compounds that increased after storage.

*Conclusion* The untargeted metabolomics analysis is a powerful tool for characterizing the changes on lettuce metabolome associated with cultivar and especially with storage time. Some families of compounds affected by storage time were reported to be closely related to quality loss.

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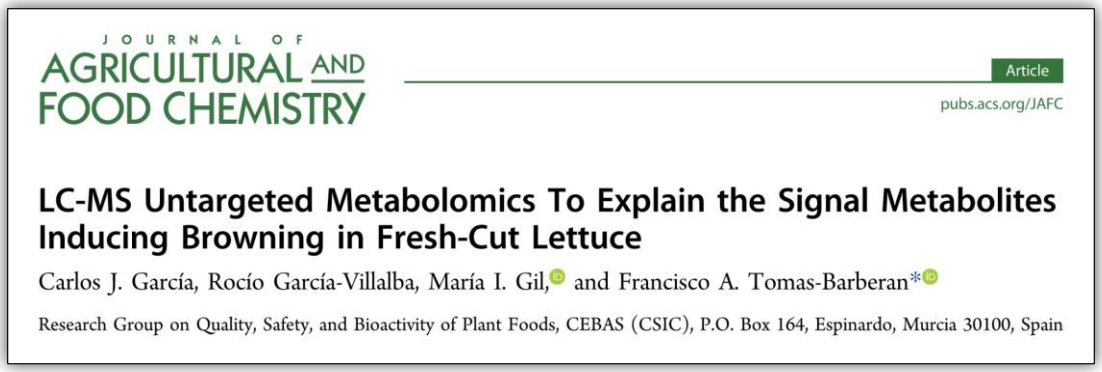
DOI:10.1007/s11306-016-1082-x

# CHAPTER V






## Abstract

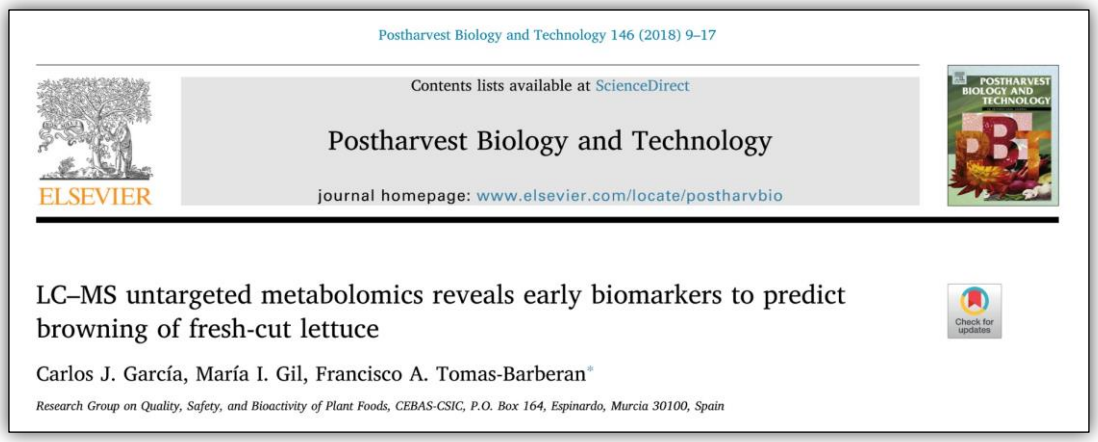
Enzymatic browning is one of the main causes of quality loss in lettuce as a prepared and ready-to-eat cut salad. An untargeted metabolomics approach using UPLC-ESI-QTOF MS was performed to explain the wound response of lettuce after cutting and to identify the metabolites responsible of browning. Two cultivars of Romaine lettuce with different browning susceptibilities were studied at short time intervals after cutting. From the total 5975 entities obtained from the raw data after alignment, filtration reduced the number of features to 2959, and the statistical analysis found that only 1132 entities were significantly different. Principal component analysis (PCA) clearly showed that these samples grouped according to cultivar and time after cutting. From those, only 15 metabolites belonging to lysophospholipids, oxylipin/jasmonate metabolites, and phenolic compounds were able to explain the browning process. These selected metabolites showed different trends after cutting; some decreased rapidly, others increased but decreased thereafter, whereas others increased during the whole period of storage. In general, the fast-browning cultivar showed a faster wound response and a higher raw intensity of some key metabolites than the slow-browning one. Just after

cutting, the fast-browning cultivar contained 11 of the 15 browning-associated metabolites, whereas the slow-browning cultivar only had 5 of them. These metabolites could be used as biomarkers in breeding programs for the selection of lettuce cultivars with lower browning potential for fresh-cut applications.

**URL direction**

DOI: 10.1021/acs.jafc.7b01667

# CHAPTER VI






**Abstratc**

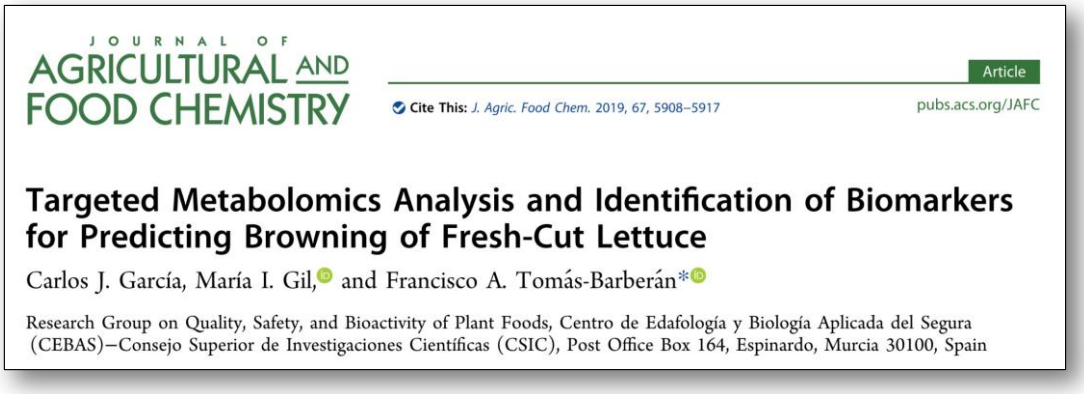
A complete untargeted metabolomics study was developed to identify biomarkers related to the browning of fresh-cut lettuce which is the main cause of quality loss. For this purpose, UPLC-MS-QTOF analysis was optimized to explore the metabolome of 30 selected cultivars of romaine lettuce with different browning susceptibility harvested at three different harvest dates. Different multivariate analyses and statistics software, such as Agilent Mass Profiler Professional (MPP), SIMCA and The Unscrambler, were used for the selection of entities correlated with browning induced after cutting and storage. A group of metabolites that were identified through the analysis of different databases and comparison with authentic standards when available, highly correlated with browning measured by image analysis measuring Hue angle difference between day 0 and day 5 of storage at 7 °C. A Multiple Linear Regression (MLR) model combined entities matrix and browning. At day 0 the metabolites that correlated positively ($P \leq 0.01$) with browning development at day 5 were caffeoylquinic acid and 3-hydroxy-tetradecadienoic acid while ferulic acid methyl ester and 2-O-p-hydroxyphenyl-6 O-galloyl glucose correlated

negatively (P≤0.01). This study also confirmed the involvement of different types of metabolites (phenolic compounds, lipids and, terpenes) in the development of browning. A ratio ferulic acid methyl ester/caffeoylquinic acid at time 0 was able to predict browning after 5 days of storage in 70% of the cases.

**URL dirección**

https://doi.org/10.1016/j.postharvbio.2018.07.011

# CHAPTER VII






## Abstract

The metabolism of phenolic compounds is a key factor in the development of wound-induced enzymatic browning of fresh-cut lettuce. In the present study, the lettuce midribs discriminant metabolites, selected in a previous untargeted metabolomics study, were thoroughly identified. Our results showed that their basal contents correlated with browning developed after 5 days of storage. 5-trans-Chlorogenic acid and 5-cis-chlorogenic acid were positively correlated with browning, while sinapaldehyde and its 4-β-D-glucoside and 4-(6′-malonyl)-β-D glucoside conjugates were negatively correlated. Using targeted metabolomics, the metabolites were analyzed in lettuce heads with different degrees of development and different browning susceptibility and these biomarkers were confirmed. Despite the large variability in the browning process of lettuce, the chlorogenic acids/sinapaldehyde derivatives ratio showed a linear correlation ($r2 = 0.79$) with the fresh-cut lettuce browning developed in 24 Romaine lettuce cultivars, validating the relevance of these biomarkers. These results show that the analysis of the basal content of these metabolites could be used in lettuce breeding programs to select cultivars that are more appropriate for the fresh-cut industry.

**URL dirección**

DOI: 10.1021/acs.jafc.9b01539

# CHAPTER: VIII

# DISCUSSION

## General discussion

The general objective of this Thesis was to get insight into the metabolic events leading to wound-induced lettuce enzymatic browning by using an HPLC-MS untargeted metabolomics approach, and to identify metabolic biomarkers that could be used for the identification of cultivars with low browning susceptibility for the fresh-cut industry.

The Thesis was divided into four specific objectives to reach this general objective.

1. To establish a robust untargeted metabolomics methodology to explore the metabolome of lettuce.

2. To identify the signal metabolites that trigger enzymatic browning of fresh-cut lettuce.

3. To evaluate the impact of internal and external factors such as genetics (cultivars), and environmental conditions that affect browning and select early biomarkers to predict browning.

4. To identify and confirm by LC-MS targeted metabolomics the metabolites related to lettuce browning and validate them as early biomarkers of the browning process that can be used for the selection of lettuce cultivars with less susceptibility to browning after cutting and storage.

**Standardized methodology for the metabolomics study of lettuce**

The untargeted metabolomics methodology to study plant metabolism has been less advanced than that used for metabolomics in animals and humans (van Treuren et al., 2018). The available plant metabolite databases are less comprehensive than the animal-based ones because there are many unknown molecules when characterizing the filtered features. Therefore, the first task of this Thesis was to establish a robust methodology for the metabolomic analysis of lettuce. To reach this objective, a lettuce metabolite database was built, and this included all the metabolites previously identified in our laboratory and those found in the literature regarding lettuce composition. The selected workflow (**Chapter IV**) included the extraction of the metabolic features, the metabolic profiling, and a data pre-treatment. The procedures were optimized and a robust data-set was prepared. This methodology was slightly modified in the different metabolomics studies carried out in the present Thesis (**Chapters IV-VII**).

The general workflow was divided into different steps:

A) Experimental design.

B) Sample extraction and analysis.

C) Data treatment:

$C_1$. Raw data pre-processing and processing.

$C_2$. Data analysis to identify discriminant metabolites.

D) Metabolite identification.

E) Evaluation of the biological significance of the results.

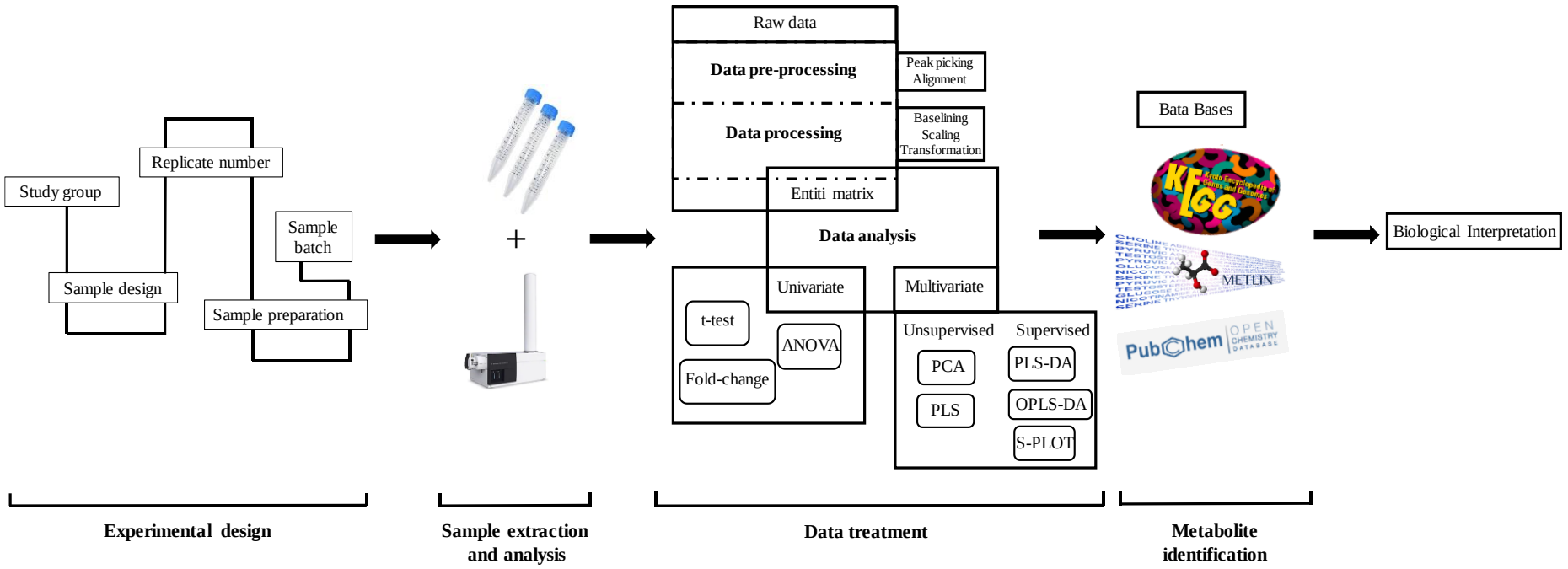



Figure 1. Diagram of the general workflow divided into experimental design, sample extraction and analysis, data treatment, metabolite identification and biological interpretation.

*A) Experimental design*

This was an essential part of the workflow as it determined the outcome of the study. It is not easy to find reports in the literature indicating the methodology for a correct experimental design to carry out a successful untargeted metabolomics study. After the studies carried out in this Thesis, it was evident that the best results were obtained when two well-differentiated groups of samples were analyzed and compared. This is the case of two cultivars or groups of cultivars with different phenotype, the same cultivar at two different development stages, or the same cultivar just after cutting and after storage. These groups of samples showed significant metabolic differences and a robust metabolome differentiation that enabled the further identification of key metabolites and suggested their biological significance. The untargeted metabolomics approach can lead to the discovery of new metabolites involved in a specific physiological, biological or technological processes and can lead to a groundbreaking advance in knowledge.

*B) Sample extraction and analysis*

Although the methodology used for plant tissue handling, extraction and further UPLC-Q-TOF MS analysis has been well defined and similar methods have been used in the different chapters (see Materials and Methods section), this methodology was previously optimized as a general protocol needed to be completed before starting the untargeted metabolomics studies. There is no general methodology for the extraction and analysis of metabolites that can be applied for the study of all the metabolites present in a plant tissue. There are large differences in metabolite solubility (polar *versus* non-polar volatile metabolites), chromatographic behavior and ionization in the MS detector, that make impossible to analyze the whole plant metabolome in a single analysis. Therefore, metabolomics analyses are always incomplete and therefore, the selection of the

extraction and further analytical methodology has to be appropriate for the type of metabolites that are relevant for the study. The detector can also be programmed to detect metabolites in the positive or negative ionization modes and this also leads to significant differences in the number and type of features detected. In the present Thesis, the metabolomics study was focused on those metabolites that could be responsible for the enzymatic browning process and this was limited by the analytical method available for the metabolites analysis, UPLC-QTOF. For these reasons, the extraction was completed with methanol, the chromatographic separation was carried out on a reversed-phase UPLC column using water and acetonitrile as mobile phase and the MS detection was set in the negative mode as the most robust and reproducible results were observed under these conditions. The positive mode, however, can give complementary information regarding the metabolite fragmentation and therefore can be useful for identification purposes with a targeted metabolomics approach.

*C) Data treatment*

Data processing is one of the most important steps in the untargeted metabolomics workflow. The data treatment strategies and the criteria that are applied for the data treatment can substantially change the results and therefore, the correct use of the data processing tool is essential.

The data treatment concept involves the development of the most efficient method to convert the raw data obtained by the analytical equipment into a manageable data matrix for further analysis. In the present Thesis, the data treatment has progressed from **Chapter IV** to **Chapter VII**, improving the setting up, testing and refining data treatment methodology that allows the extraction of the most useful and relevant results.

One of the most important drawbacks of the data treatment after using untargeted metabolomics techniques is the complexity of the standardization, as most of the data acquisition equipment manufacturers allow only the use of their own data treatment software for this purpose. This represents a problem in developing a robust and reproducible methodology. Usually, the metabolomics methodologies for data processing are summarized in a single step, but in this Thesis, a division of the data treatment is proposed in order to control better the criteria of data processing to get a more reproducible and robust methodology. Therefore, we suggest to separate data pre-processing (peak detection, peak picking and alignment) from the data processing (baselining, scaling and transformation) to enable using a software which does not belong to the manufacturer of the analytical equipment for the processing treatments. Thus, the data pre-processing was suggested to be completed by the software of the manufacturer to get a better performance while the data processing can be processed by using open software to provide more flexibility and data sharing.

*C1. Raw data pre-processing and processing*

The data pre-processing is the stage that determines what raw data from the acquisition system will be extracted to be normalized, transformed and scaled up during the further processing that leads to the final data matrix. Therefore, this stage is vital as it acts as a kind of raw data filtering. In this study, Agilent Technologies pre-processing software was used to ensure the extraction with the parameters recommended for this purpose. The parameters used from the peak picking (extraction parameters, compound filters, etc.) for the alignment were optimized along **Chapters IV, V, VI** and **VII**, comparing the pre-processing results to get the most representative version of the metabolome.

The data processing operations ended up giving entity to the raw signals pre-processed previously. This process requires prior knowledge of the acquisition systems and the experimental design to give the correct relevance of some signals with respect to the others (van der Berg et al., 2006). In this Thesis, different software has been used to carry out the processing operations such as Mass Profiler Professional, Agilent technologies, Metaboanalyst website platform; The Unscrambler X, CAMO; SIMCA, UMETRICS. As a conclusion of this study, the use of pareto scaling for LC-MS data and also a log transformation to create a representative data matrix was suggested. This ensures a data treatment methodology to deal with the adequate transfer from the raw data to a manageable data matrix to be further analyzed.

*$C_2$. Data analysis to identify discriminant metabolites*

The management of the data analysis allows obtaining a clearer discrimination of the most significant entities and therefore, a closer approximation to the candidate biomarkers for later identification.

Univariate and multivariate models have been tested in the different chapters. The experimental design is a critical point that limits the type of model that can be built to evaluate the variables studied. The groups included in the experimental design as well as the number and type of the variables determinate the models and the functions. In this Thesis, the experimental designs were stratified into two case studies to make the most of the multivariate models of discrimination (OPLS-DA) and the tools of discrimination (S-plot) to find candidates for biomarkers.

D) *Metabolite identification*

The identification of the metabolites is the final step that converts the raw data into a biological context. Therefore, the final identification of metabolites is essential to

understand the biological significance of the metabolomics studies and after that, the metabolites should be rigorously validated.

Once the discriminant entities were detected after multivariate analysis, the next step was the identification of the corresponding metabolites. The first approach was to launch the dataset, including the discriminant entities, against the different available databases, and also against the specific lettuce metabolomics database built in-house. The validation of this identification type is considered level 2, which leads to the putatively annotation (identification) of metabolites (e.g. without chemical reference standards), based upon physicochemical properties and/or spectral similarity with public/commercial spectral libraries (Sumner et al., 2007).

After this process, some potential metabolites were suggested. The general trend is to find out that many of the entities selected corresponded to unknown metabolites (not found in the databases) or to metabolites that have not been or cannot be found in plants.

In a second and fundamental step, additional experiments are needed to confirm the suggested metabolites. The confirmation can be done by comparison with an authentic standard, in which the retention time, exact mass and fragments are used for metabolite confirmation. The validation of this identification type is considered level 1 (Summer et al., 2007). The identification level 1 has to be validated with a commercial authentic standard. This was done in the present Thesis with phenolic metabolites such as chlorogenic acid, caffeic acid, ferulic acid methyl ester, sinapaldehyde, some flavonoids and sesquiterpene lactones. In many cases, the authentic standards were not available or were very expensive, and therefore surrogate standards were used (Clifford and Madala, 2017). This approach uses extracts of well-characterized plant materials for the identification of metabolites. This is the case of using green coffee bean extracts for the

identification of a whole set of caffeoyl and di-caffeoyl quinic derivatives in lettuce extracts after wounding as previously described (Tomás-Barberán et al., 1997). Phenolic metabolites such as 3-caffeoyl-quinic (neochlorogenic acid), 4-caffeoyl-quinic acid (kryptochlorogenic acid), 5-caffeoyl-quinic acid (chlorogenic acid), *p*-coumaroyl-quinic acid, feruloyl-quinic acid 3,4-di-caffeoyl-quinic acid, 3,5-di-caffeoyl-quinic acid (isochlorogenic acid), and 4,5-di-caffeoyl-quinic acid, were therefore annotated in lettuce in the present study.

Another approach to characterize or identify metabolites is to complete it with additional analytical methods such as the study of the MS-MS exact mass fragments obtained in the UPLC-QTOF analysis. This approach was used in the present Thesis for the identification of lysophospholipid metabolites of lettuce included in **Chapter V**. The fragments obtained were also compared with those generated in previous studies with similar metabolites.

*E) The biological significance of the results*

The final outcome of the study was the proposition of the biological implications of the identified metabolites. To achieve this successfully, a deep knowledge of the plant metabolism, particularly the metabolic changes related to the biological process of browning development was needed. In the present Thesis, a deep understanding of the biochemical processes associated to the wound-induced enzymatic browning, and the phenolic metabolism induction by abiotic stress were essential. A validation of the results found, including a targeted metabolomics approach with quantification of specific metabolites was also crucial to confirm the relevance of the findings and the potential use of the selected metabolites as biomarkers of lettuce browning.

**Wound-signal and metabolic changes induced by wounding that lead to enzymatic browning**

The metabolomics study carried out could be also used to complete the different metabolic features that have a role in the wound-induced phenolic metabolism leading to the enzymatic browning in lettuce. This approach could also be applied to other plant species. Lettuce is an excellent model to study metabolite changes as the basal content of phenolic substrates of the browning enzymes is very low and the biosynthesis of these metabolites is induced after cutting (wounding), which allows the sequential study of the biochemical events that lead to enzymatic browning.

A general pathway of the wound-induced phenolic metabolism which includes the metabolite changes found in the different Chapters is summarized in Figure 2. This pathway can be divided into different steps:

1) The release of the wound-signals,
2) The oxidative metabolism of the released lipids,
3) The synthesis of jasmonic acid and related metabolites,
4) The induction of phenylpropanoid biosynthesis,
5) The synthesis of substrates of PPO or other related phenolic metabolites.
6) The synthesis of precursors of lignin formation.

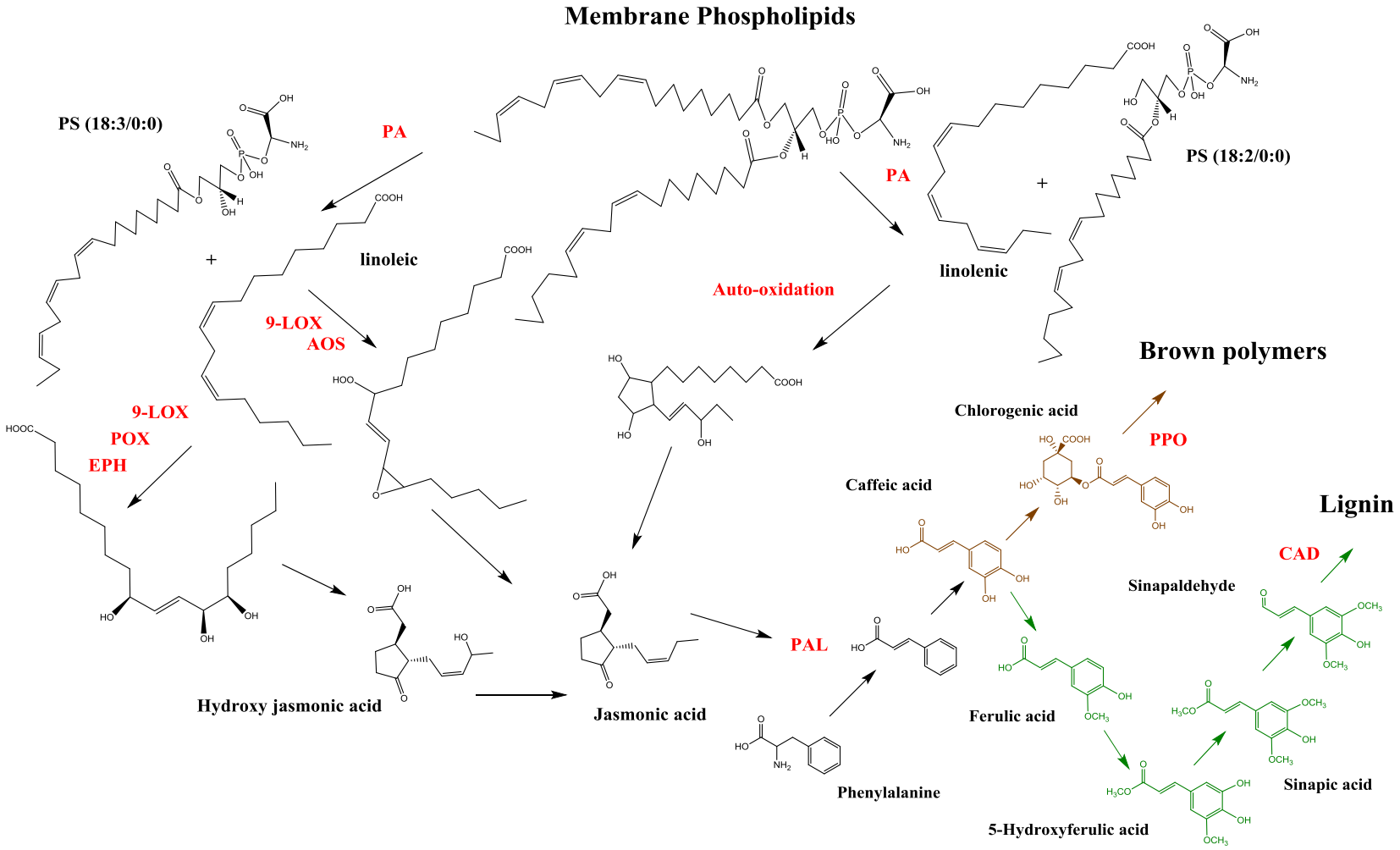



Figure 2. General pathway of the wound-induced phenolic metabolism

Wounding leads to the breakdown of membranes, and release of phospholipids, that by the action of phospholipases, particularly phospholipase A, release polyunsaturated fatty acids (linoleic and linolenic acids) and the remaining lysophospholipid residue.

Due to analytical methodology restrictions, the released fatty acids were not detected by UPLC-Q-TOF as they are non-polar metabolites that must be analyzed by GC-MS. The oxidized metabolites of the released fatty acids (oxylipins), however, were easily detected in the UPLC-QTOF system as well as the corresponding lysophospholipids that were further characterized by the analysis of the MS-MS fragments and comparisons with MS-MS data of similar metabolites reported in the microalga *Nitzschia closterium* (Yan et al., 2010) shown in **Chapter V**.

The released fatty acids were then enzymatically oxidized by 9-lipooxygenase (9-LOX) and allene-oxide synthase (AOS) to give 9-hydroxy-12,13-epoxy, 10-octadecenoic acid in the case of linoleic acid. These fatty acids could also be oxidized by peroxygenase and epoxide hydrolase to produce 9S, 12S, 13S-trihydroxy-10-Z-octadecenoic acid. These are oxylipin intermediates in the jasmonic acid biosynthesis pathway and the metabolite 11-hydroxy-jasmonic acid was also detected. Jasmonic acid, however, was not identified under the present analytical conditions as it should be detected by using GC-MS method.

Other polyunsaturated fatty acids such as linolenic acid, could also suffer auto-oxidation without the concourse of oxidative enzymes, leading to 16-F-phytoprostane, another precursor of jasmonic acid as shown in Figure 2.

Jasmonic acid and related metabolites are known to activate the phenylpropanoid biosynthesis through the induction of the enzyme PAL which is the first regulatory

enzyme in the biosynthesis of hydroxycinnamic metabolites. These include metabolites that are substrates of the enzyme PPO such as caffeic acid derivatives (caffeoyl quinic and di-caffeoyl quinic derivatives) and other phenolic metabolites derived from the same pathway that are substrates for the biosynthesis of lignin (coniferin and sinapaldehyde).

The kinetic study included in **Chapter V** about the occurrence of the different metabolites after wounding and storage for 5 days under the conditions described in the Materials and Methods, shows that some metabolites such as lysophospholipids and 9S, 12S, 13S-trihydroxy-10-Z-octadecenoic acid were immediately detected after cutting, and they decreased during the first hours after wounding, which confirms their role in the early steps of the process as they were converted to other metabolites. Other oxylipin metabolites such as 9-hydroperoxy-12,13-epoxy-10 octadecenoic acid increased during the first 24 hours after cutting to decrease steadily after the next days of storage while the jasmonate metabolite 11-hydroxy-jasmonic acid increased to reach a maximum after 48 hours to decrease slightly during the rest of the storage period.

The hydroxycinnamic acid metabolites increased steadily over the storage period as can be seen for chlorogenic acid (5-caffeoyl quinic acid), while this increase was not observed for other caffeic acid derivatives such as caffeoyl tartaric acid which remained rather stable during the storage period. Coniferyl alcohol glucoside and related metabolites that are substrates for the synthesis of lignins, also increased through the storage period as it was observed for the phenolic PPO substrates. All these metabolic events reflect and confirm the response of plant metabolism to repair wounding through the lignification and the browning process.

Other metabolites were detected that correlated negatively with the browning process, and these include sesquiterpene lactones that were negatively correlated with

browning, as well as benzoic acid derivatives and flavonoids, that although were phenolic metabolites, they are not substrates of the enzyme PPO (**Chapters IV, V, VI**, and **VII**).

### Effect of intrinsic and extrinsic factors on lettuce enzymatic browning

Browning discoloration of the lettuce cut edge depends on both intrinsic factors such as genetics and development stage and extrinsic factors such as environmental and agronomical practices. These factors can promote the susceptibility of the plant tissues, inducing the biosynthesis of enzymatic browning substrates. They can also reduce the susceptibility through the biosynthesis of enzyme inhibitors or by redirecting the phenolic metabolism to the biosynthesis of metabolites that are not substrates of PPO.

The studies carried out showed that genetics (cultivar-depended factor) was significantly relevant for both lettuce types: cultivars of Iceberg lettuce covered in **Chapter IV** and Romaine lettuce in **Chapter V**. The results illustrated the fast and slow development of the enzymatic browning discoloration in different cultivars.

These results were confirmed in the study carried out in **Chapter VI** in which 30 different cultivars of Romaine lettuce with different browning susceptibility were studied. The same lettuce cultivars were cultivated and harvested in three consecutive periods, each time in the same experimental field and harvested at commercial maturity stage. The results showed that the harvest date had a relevant effect on browning development, and this was even more significant than the differences between cultivars. It was suggested that these results could be explained by the influence of extrinsic factors such as light intensity, UV radiation, maximum and minimum temperature, and irrigation/rain, affected largely the incidence of browning development. These environmental factors have been described to affect phenolic metabolism, as it can divert the carbon flow to

phenolic metabolism leading to metabolites that are not substrates of the browning enzymes, as is the case of anthocyanins and flavonols, therefore decreasing the caffeic acid derivatives biosynthesis. Some environmental factors could also lead to the biosynthesis of di-hydroxycinnamates that are the main substrates of PPO. It has been shown that higher UV irradiation and lower temperatures favor the synthesis of anthocyanins and flavonols. The results included in **Chapter V**I also suggested that although all samples were harvested at commercial maturity, there was a wide range between the less developed and the more developed stages that were all included as commercial maturity stage. This fact could be responsible for some of the differences in the development stage (intrinsic factor) that could affect the susceptibility to browning development. Specifically, this hypothesis was evaluated in detail in **Chapter VII**.

Considering this approach, in **Chapter VII** two cultivars with significant different browning susceptibility were selected and harvested at two different development stages with only a two-week difference in harvest (development stage 1 and 2). A targeted approach was used to identify and confirm the metabolites previously pointed out as related to browning development. This targeted analysis allowed the detection of chlorogenic acid isomers and their full characterization. This targeted analysis also allowed the complete identification of sinapaldehyde (*m/z* 207.0663), that was tentatively identified as ferulic acid methyl ester (FAME) in **Chapter VI**, and its conjugates sinapaldehyde 4-β-D-glucoside and 4-β-D-(6'-malonyl)-glucoside. The chlorogenic acid isomers correlated with browning development, and sinapaldehyde and conjugates correlated negatively with browning development, were identified and confirmed with authentic standards by chromatographic comparisons and MS/MS analyses. Lettuce harvested at the early development stage 1 clearly showed less susceptibility to browning development after cutting and significant differences in the basal metabolic composition

detected immediately after cutting. The samples at development stage 1 had a lower ratio chlorogenic acid isomers/sinapaldehyde and conjugates than those harvested at the more advanced development stage 2. Lettuce samples classified as the least browning susceptible ones also presented a lower ratio chlorogenic acid isomers/sinapaldehyde and conjugates than the most susceptible ones to browning. These results suggested than the metabolites associated with browning development increased their basal content with the development stage and that this increase was higher in the cultivars most susceptible to browning. On the other hand, the metabolites associated with browning prevention (negatively correlated with browning) were found at higher amounts when samples were harvested earlier at a less developed stage and also in cultivars that were less susceptible to browning. As a result, the enhancement of phenolic metabolism can lead to the synthesis of PPO substrates such as caffeic acid derivatives and also to the synthesis of other phenolic metabolites that are not PPO substrates such as flavonoids, benzoic acids or precursors of lignin biosynthesis as it is the case of sinapaldehyde and its conjugates. The new ratio chlorogenic acid isomers/sinapaldehyde and conjugates was validated and confirmed in 24 Romaine lettuce cultivars.

The oxylipins 9-hydroperoxy-12,13-epoxy-10-octadecenoic acid and 11,12,13-trihydroxy-9-octadecenoic acid were also identified. These metabolites were previously identified (Chapters **V** and **VI**) as potential wound signal intermediates and associated with an increase in phenolic metabolism. These oxylipins were more relevant in samples harvested at the early development stage, and they decreased with plant development. A higher presence of these metabolites does not mean always a higher browning development as the increase in phenolic metabolism can also lead to an increase in the carbon flow leading to the biosynthesis of higher amounts of precursors of lignin biosynthesis rather than PPO substrates. Therefore, further research is needed to

understand the role of these oxylipin metabolites and the related enzymes in lettuce phenolic metabolism and in the development of enzymatic browning.

**Search for biomarkers of lettuce browning susceptibility**

One of the main objectives of the present Thesis was the identification, through an untargeted metabolomics approach, of biomarkers that could be used for the selection of cultivars with less susceptibility to cut edge browning as raw material for the fresh-cut industry. This is a relevant objective involving advances in the scientific knowledge of the browning process, in plants and particularly in lettuce as well as the technological advances that could be used to maintain the quality of the fresh-cut lettuce.

In **Chapter IV**, 15 metabolites were identified as potential biomarkers for the browning process development after 5 days of storage. The Fast Browning (FB) cultivar showed a faster and more intense response to biosynthesize of metabolites that correlate with the browning process than that observed in the Slow Browning (SB) cultivar. The selected metabolites included lysophospholipids (4 metabolites), oxylipin/jasmonate metabolites (5 metabolites) and phenolic metabolites (6 metabolites) (**Chapter V**). Just after cutting, the FB cultivar had 11 out of the 15 biomarkers, while the SB cultivar had only 5 of them. In this chapter, it was suggested that these metabolites could be used as biomarkers for the selection of cultivars with less browning susceptibility after cutting in lettuce breeding programs.

In **Chapter VI**, the study was focused on the detection of early biomarkers (measured just after cutting) that could be correlated with the browning development of the cut lettuce after 5 days of storage. In this case, 30 different cultivars of Romaine

lettuce with different browning susceptibilities were examined. Some metabolites correlated positively with browning, and these included caffeoyl quinic acid (chlorogenic acid), *p*-coumaroyl quinic acid, 3-hydroxy-tetradecenoic acid, 9-F1-Phytoprostane, and TriHOME. On the other hand, other metabolites detected just after cutting correlated negatively with the browning developed after 5 days, and these included ferulic acid methyl ester (FAME), quercetin acetyl-glucoside, and 2-*p*-hydroxybenzoyl-6-*O*-galloyl glucose.

The basal occurrence of metabolites related to enzymatic browning at time 0 (immediately after cutting), can be considered as a relevant information for the predisposition to a fast development of browning. Thus, plants that have higher basal levels of PPO substrates such as chlorogenic acid and *p*-coumaroyl quinic acid or intermediate metabolites in the oxylipin-jasmonic acid pathway that leads to an increase in phenylpropanoids biosynthesis, are often more prone to develop browning faster. On the contrary, lettuce cultivars that have higher basal levels of phenolic metabolites that are not substrates of PPO as is the case of metabolites that are precursors of lignin biosynthesis as is the case of sinapaldehyde, are less susceptible to develop browning after cutting and storage (or do this process at a slower rate).

This suggests that the basal balance of phenolic metabolism in lettuce plays a key role in the final susceptibility to develop wound-induced browning.

The ratio chlorogenic acid/FAME was suggested to be a useful tool to predict browning in a large percentage of the cultivars of Romaine lettuce tested (higher than 70%). This metabolite ratio was successfully validated as a potential biomarker in **Chapter VII**. It was confirmed by targeted and MS/MS analyses that sinapaldehyde and its conjugates were in fact the metabolites that were previously identified as FAME. The

ratio chlorogenic acid isomers/sinapaldehyde conjugates was confirmed to show a significant correlation with browning development in samples at different development stages. The study showed that this ratio was much higher in lettuce samples harvested at a higher development stage which developed more browning than in the lettuce samples harvested at an earlier development stage which showed less browning after cutting, therefore validating the potential application of this biomarker.

**PPO substrates and lignin precursors ratio to explain browning**

According to **Chapters IV**, **V**, **VI**, **VII**, phenolic metabolites which are **i) substrates of PPO** or **ii) precursors of lignin biosynthesis** were found in all lettuce samples evaluated in this Thesis. These compounds were identified both in Iceberg lettuce (**Chapter IV**) and in Romaine lettuce (Chapters **V, VI, VII**). These metabolites belong to the phenylpropanoids pathway and increased as a response to the metabolic signals released after wounding (**Chapter V**).

**i) The metabolites which are substrates of PPO** include chlorogenic acid isomers and they were associated with browning development in all the Chapters. After the induction of phenylpropanoid biosynthesis by wounding, which it is known to follow the activation of the enzyme PAL, the biosynthesis of di-hydroxycinnamates (caffeic acid derivatives), that are the main substrates of PPO, increases as well as does browning development. The chlorogenic acid derivatives were detected in higher basal contents in the cultivars most susceptible to browning.

**ii) The precursors of lignin biosynthesis** were also induced by wounding and from caffeic acid monolignol derivatives were formed. These metabolites were detected as coniferyl alcohol glucoside (coniferoside) (**Chapter IV and V**), 5-

hydroxyconiferaldehyde (**Chapter V**), and ferulic acid metyl ester (FAME) (**Chapter VI**). This last metabolite was later fully identified as sinapaldehyde (Chapter **VII**) and its β–D-glucoside and its-4-β-D-(6'malonyl) glucoside were also detected and identified (**Chapter VII**). All these metabolites are precursors of lignin biosynthesis and were identified in higher amounts in the cultivars less susceptible to browning.

The basal content of these phenolic metabolites in lettuce plants can be affected by intrinsic (genetic and developmental) or extrinsic factors (environmental and agronomic) (**Chapter VI**). The ratio of these metabolites will determine the susceptibility to browning after cutting and storage.

# **CHAPTER IX:**
# CONCLUSIONS

**General conclusions of untargeted metabolomics methodology**

1. The exploration of the complete metabolome of any plant material is extremely complex, and therefore the untargeted analysis is, in fact, paradoxically targeted to the type of metabolites that are considered potentially responsible through the analytical tools selected and the data processing methods.

2. A robust untargeted metabolomics methodology has been established to explore the metabolome of lettuce.

3. A specific lettuce metabolites database and a metabolomics workflow has been generated to study lettuce metabolites.

4. The identification of plant metabolites by untargeted metabolomics analysis represents a bottle-neck due to the limitation of available plant metabolite databases compared to those of human and animal databases.

5. An adequate data treatment methodology is necessary to reach, from the large amount of data generated by untargeted metabolomics, metabolite identification and its biological interpretation.

6. The targeted strategy for metabolite identification can be a further complementary analysis and a simpler tool for validation and confirmation.

**Specific conclusions for the untargeted metabolomics analysis to understand lettuce enzymatic browning**

7. The metabolites implicated in the browning process have been identified through an untargeted metabolomics approach. These metabolites belong to three main groups including lysophospholipids, oxylipin/jasmonate metabolites, and phenolic compounds that were able to explain the browning process. They were biosynthetically connected to each other through the wound-response signals.

8. The kinetic analysis of the relevant metabolites indicated that the fast-browning cultivars showed a faster wound-response and a higher content of some key metabolites, including 16F phytoprostane, hydroxy-jasmonic acid, and chlorogenic acid, than the slow-browning cultivars.

9. A higher basal occurrence of metabolites that are PPO substrates with respect to those that are precursors of lignin biosynthesis is associated with a higher susceptibility to wound-induced browning. The chlorogenic isomers / sinapaldehyde and conjugates ratio can be used as a biomarker to explain the browning susceptibility of lettuce to fresh-cut processing.

10. Lettuce browning susceptibility is affected by both intrinsic factors, such as genetics and development stage, and extrinsic factors such as environmental conditions and agronomical practices. These factors modulate the basal occurrence of chlorogenic acid isomers and sinapaldehyde and conjugates in lettuce.

11. The ability of the lettuce to accumulate phenolic metabolites that are PPO substrates or lignin formation precursors is one of the main determinants of wound-induced browning susceptibility. This metabolic ability can be affected by two factors:

i) The basal content of the metabolites that are present before harvest which is affected by the developmental stage and environmental factors.

ii) The response to wounding that can lead to the biosynthesis of phenolic metabolites that are either PPO substrates or precursors of lignin formation.

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