DEPARTAMENTO DE

QUÍMICA AGRÍCOLA Y EDAFOLOGÍA

PLATAFORMAS ANALÍTICAS EN METABOLÓMICA Y SU APLICACIÓN PARA EL ESTUDIO DE LA RESISTENCIA-SENSIBILIDAD A HERBICIDAS

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Córdoba, septiembre de 2012

TITULO: *PLATAFORMAS ANALÍTICAS EN METABOLÓMICA Y SU APLICACIÓN PARA EL ESTUDIO DE LA RESISTENCIA-SENSIBILIDAD A HERBICIDAS.*

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TÍTULO DE LA TESIS:

PLATAFORMAS ANALÍTICAS EN METABOLÓMICA Y SU APLICACIÓN PARA EL ESTUDIO DE LA RESISTENCIA-SENSIBILIDAD A HERBICIDAS

DOCTORANDA:

DOÑA ANTONIA MARÍA ROJANO DELGADO

LOS DIRECTORES DE LA TESIS INFORMAN:

Que el presente trabajo de investigación titulado **"PLATAFORMAS ANALÍTICAS EN METABOLÓMICA Y SU APLICACIÓN PARA EL ESTUDIO DE LA RESISTENCIA-SENSIBILIDAD A HERBICIDAS"**, constituye la memoria que presenta **Doña Antonia María Rojano Delgado** para aspirar al grado de Doctor en "Biociencias y Ciencias Agroalimentarias", habiendo sido realizado en los laboratorios de los Departamentos de Química Agrícola y Edafología y de Química Analítica, ambos de la Universidad de Córdoba, bajo nuestra dirección y supervisión. Consideramos que la doctoranda cumple los requisitos legales para optar al grado de Doctor en "Biociencias y Ciencias Agroalimentarias".

A continuación se presenta una relación de los trabajos publicados o en vías de publicación a los que ha dado lugar la investigación realizada y que forma parte del cuerpo de la Tesis, o en los que la doctoranda ha aplicado los métodos que ha desarrollado para obtener datos en los que se soporten los estudios realizados por otros compañeros del grupo de investigación:

1. A.M. Rojano-Delgado, J. Ruiz-Jiménez, M.D. Luque de Castro, R. De Prado (2010). Determination of glyphosate and its metabolites in plant material by reversed-polarity capillary-electrophoresis with indirect absorptiometric detection. *Electrophoresis*, **31 (8): 1423-1430**.

2. A.M. Rojano-Delgado, F. Priego-Capote, M.D. Luque de Castro, R. De Prado (2010). Screening and confirmatory analysis of glyoxylate: A biomarker of plants resistance against herbicides. *Talanta*, **82: 1757-1762**.

3. H.E. Cruz-Hipólito, A.M. Rojano-Delgado, J.A. Domínguez-Valenzuela, A. Heredia, M.D, Luque de Castro, R. De Prado (2011). Glyphosate tolerance by *Clitoria ternantea* and *Neonotonia wightii* plants involves differential absorption and translocation of the herbicide. *Plant and Soil,* **347: 221-230**.

4. A.M. Rojano-Delgado, H.E. Cruz-Hipólito, R. De Prado, M.D. Luque de Castro, A. Rodríguez-Franco (2011). Limited uptake, translocation and enhanced metabolic degradation contribute to glyphosate tolerance in *Mucuna pruriens* var. *utilis* plants. *Phytochemistry*, **73: 34-41**.

5. L.B. de Carvalho, P.L. da Costa Aguiar-Alves, F. González-Torralva, H.E. Cruz-Hipólito, A.M. Rojano-Delgado, R. De Prado, J. Gil-Humanes, F. Barro, M.D. Luque de Castro (2012). Pool of resistance mechanisms to glyphosate in *Digitaria insularis*. *Journal* of *Agricultural and Food Chemistry*, **60: 615-622**.

6. F. González-Torralva, A. Rojano-Delgado, M.D. Luque De Castro, N. Mülleder, R. De Prado (2012). Two non-target mechanisms are involved in glyphosate resistant horseweed (*Conyza canadensis* L. Cronq.) biotypes. *Journal of Plant Physiology,* **doi:10.1016/j.physletb.2003.10.071**.

7. L. Carvalho, F. González-Torralva, H. Cruz Hipolito, A. Rojano-Delgado, J. Gil-Humanes, M.D. Luque De Castro, F. Barro, P. Alves, R. De Prado (2012). Glyphosate resistance in a *Digitaria insularis* biotype is due entirely to differential metabolism. Enviado a: *Planta*.

8. A.M. Rojano-Delgado, F. Priego-Capote, R. De Prado., M.D. Luque de Castro (2012). Ultrasound-assisted extraction with LC–TOF/MS identification and LC–UV determination of imazamox and its metabolites in plants. Enviado a: *Journal* of *Agricultural and Food Chemistry*.

9. A.M. Rojano-Delgado, F. Priego-Capote, M.D. Luque de Castro, R. De Prado (2012). Importance of imazamox metabolism in Clearfield *Triticum aestivum.* Enviado a: *Phytochemistry*.

10. A.M. Rojano-Delgado, F. Priego-Capote, R. De Prado, M.D. Luque de Castro (2012). Qualitative/quantitative strategy for determination of glufosinate and metabolites in plants. Enviado a: *Journal of chromatography A.*

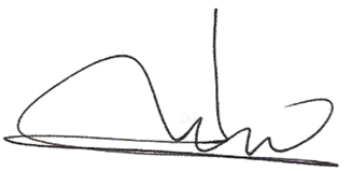
11. A.M. Rojano-Delgado, F. Priego-Capote, F. Barro, M.D. Luque de Castro, R. De Prado (2012). Liquid chromatography–diode array detection to study glufosinate metabolism in *Triticum aestivum* T-590 and importance of genetic modification on its resistance. Enviado a: *Phytochemistry.*

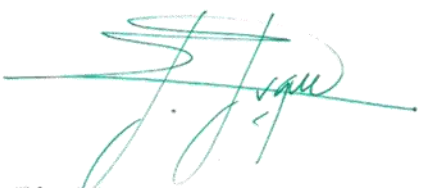
12. A.M. Rojano Delgado & M.D. Luque de Castro (2012). Capillary electrophoresis–herbicide analysis: An excellent marriage. Enviado a: *Phytochemistry Reviews*.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 28 de SEPTIEMBRE de 2012

Firma de los directores





PLATAFORMAS ANALÍTICAS EN METABOLÓMICA Y SU APLICACIÓN PARA EL ESTUDIO DE LA RESISTENCIA-SENSIBILIDAD A HERBICIDAS

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Trabajo presentado para optar al grado de

Doctora en Biociencias y Ciencias Agroalimentarias

Fdo.**: Antonia María Rojano Delgado**
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"Una experiencia nunca es un fracaso,
pues siempre viene a demostrar algo"

Thomas Alva Edison

A mi familia, a Blanca

RESUMEN

Los estudios de los fenómenos de resistencia–susceptibilidad de las hierbas a los herbicidas requieren la puesta a punto de métodos adecuados para la determinación de estos compuestos, pero también de los metabolitos a los que dan lugar las diferentes rutas en los que están implicados estos fenómenos. Para aportar nuevos métodos en los que se aprovechen las ventajas que proporcionan los avances en instrumentación analítica que han aparecido en los últimos tiempos y validar la utilidad de los métodos, se ha desarrollado la siguiente investigación:

1. Se ha puesto a punto un método para la determinación de glifosato y sus metabolitos basado en electroforesis capilar con la sensibilidad y selectividad adecuadas para la determinación de todos ellos en materia vegetal; lo que ha posibilitado su aplicación a hierbas como la *Digitaria insularis*, *Mucuna pruriens* y *Clitoria ternantea*, entre otras, sometidas a la acción del glifosato, y el estudio de los mecanismos por los que tienen lugar la tolerancia o resistencia al herbicida.

2. Los resultados obtenidos en los estudios realizados con el anterior método mostraron un metabolito como marcador de la diferencia entre plantas susceptibles y resistentes a glifosato, lo que condujo al desarrollo de un método fotométrico simple para la cuantificación de este único compuesto: el glioxilato. El método ha permitido discernir plantas resistentes de sensibles a tiempos cortos de aplicación, lo que supone un gran avance en la lucha contra la resistencia a glifosato; ya que se puede hacer una aplicación con diferente herbicida antes de 24 horas de haber aplicado glifosato.

3. Se han desarrollado métodos para la determinación de imazamox y sus metabolitos y para glufosinato y sus metabolitos a pesar de no existir patrones de los productos del metabolismo de ninguno de los herbicidas. Para ello se han usado extractos de plantas tratadas y no tratadas con el herbicida en cuestión y se han cromatografiado mediante LC utilizando como detector un DAD; se ha optimizado la separación de los picos que aparecen en presencia del herbicida y se han identificado los compuestos que los producen mediante un detector de masas de tiempo de vuelo. Una vez conocidos los tiempos de retención cromatográficos

de cada uno de los compuestos, se puede utilizar el LC–DAD para una cuantificación relativa al herbicida o a otro compuesto-patrón con estructura semejante al metabolito en cuestión. La aplicación de los métodos a biotipos diferentes de trigo resistente–susceptible (caso del imazamox) y a plantas genéticamente modificadas y no modificadas también de trigo (caso del glufosinato) han permitido la cuantificación relativa de cada metabolito en cada estudio y, por tanto, el desarrollo relativo de las diferentes vías metabólicas de los herbicidas en las diferentes plantas.

4. En todos los casos la preparación de la muestra se auxilió con ultrasonidos para acelerar la lixiviación de los compuestos de interés de las plantas en estudio, lo que redujo considerablemente el tiempo total del análisis.

Es ésta la primera vez que: (i) Se propone un método para la determinación cuantitativa de glifosato y todos sus metabolitos en plantas; (ii) se ha desarrollado un método simple, utilizable en campo, para la respuesta rápida a la acción de glifosato a través de uno de sus metabolitos; (iii) se han desarrollado métodos cromatográficos simples para la determinación de imazamox y todos sus metabolitos, y para glufosinato y todos sus metabolitos, que permiten su cuantificación relativa sencilla a pesar de no existir patrones de estos metabolitos; (iv) se han aplicado los métodos para conocer de forma inequívoca las vías de transformación de los dos herbicidas en plantas resistentes–sensibles y en plantas genéticamente modificadas y no modificadas.

ABSTRACT

The studies on the phenomena involved in the resistance–susceptibility of herbal species to herbicides require development of methods suitable for the determination of herbicides, but also for that of the metabolites resulting from the different metabolic pathways involved in these phenomena. With the aim of developing new methods which take advantage of the cutting-edge advances in analytical instrumentation and assessing their usefulness, the following research has been carried out:

1.A method for the determination of glyphosate and its metabolites has been developed, which is based on capillary electrophoresis and is endowed with the appropriate selectivity and sensitivity for determination of all them in vegetal material. The method has been applied to herbals such as *Digitaria insularis*, *Mucuna pruriens* and *Clitoria ternantea*, among others, subjected to glyphosate action, as well as to the study of the mechanisms through which either the tolerance or resistance to the herbicide takes place.

2.The results from the above studies showed a metabolite as biomarker of the difference between susceptible and resistant plants; thus leading to the development of a simple photometric method for quantitation of this single compounds: glyoxilate. The method has made possible short-time discrimination between resistant and sensitive plants before 24 h application of glyphosate.

3.Methods for the determination of imazamox and its metabolites, as well for glufosinate and its metabolites have been developed, despite the absence of available standards of these metabolic products. For the development of the methods, extracts from plants treated and nontreated with the target herbicide have been used; extracts which have been subjected to chromatography by LC using a DAD as detector; the separation among the peaks appeared in the presence of the herbicide has been optimized and the compounds which provide the peaks have been identified by a mass time-of-flight detector. Once the chromatographic retention time for each of the compounds has been known, the LC–DAD system can be used for quantitation of them relative to either the

herbicide or other standard compound with structure similar to that of the target metabolite. The application of the methods to different biotypes of resistant–susceptible wheat (subjected to imazamox) and to genetically modified and unmodified wheat (subjeted to glufosinate) has allowed relative quantitation of each metabolite in each of the studies and, therefore, to know the relative development of the different metabolic pathways of the herbicides in the different plants.

4. In all cases, sample preparation has been assisted by ultrasound to accelerate the leaching step of the target compounds from the plants under study; thus decreasing drastically the total analysis time.

This is the first time that: (i) a method is proposed for the quantitative determination of glyphosate and all its metabolites in plants; (ii) a method simple, for in field use and with short-time response to the action of glyphosate through one of its metabolites (glyoxilate) has been reported; (iii) simple chromatographic methods for the determination of imazamox and all its metabolites, and for glufosinate and all its metabolites have been developed —the methods allow relative quantitation of the metabolites despite there are not available standards for them—; (iv) the methods thus developed have been applied to know, in an unequivocal manner, the metabolic pathways of the two herbicides in resistant–sensitive plants and in genetically modified and unmodified plants.

ÍNDICE

ABREVIATURAS

ae	ácido equivalente
ai	ingrediente activo
AHAS	acetohidroxiácido sintasa
ALS	acetolactato sintasa
AMPA	ácido aminometilfosfónico
ANOVA	análisis de la varianza
ASPCR	allele-specific PCR
BGE	electrolito de fondo
CE	electroforesis capilar
Cit P_{450}	citocromo P_{450}
CTAB	bromuro de hexadeciltrimetilamonio
2,4-D	ácido 2,4-diclorofenoxiacético
DAD	detector de diodos en fila
DDA	días después de la aplicación
ddNTP	dideoxinucleótidos
DDT	días después del tratamiento
DMS	diferencia mínima significativa

DTT	ditiotreitol
ECPA	European Crop Protection Association
ED_{50}	dosis de herbicida capaz de reducir en un 50% el peso fresco de las plantas frente al control
EDTA	ácido etilendiaminotetracético
EIC	cromatograma obtenido mediante extracción de un determinado ión de cada espectro
EPSPS	enol piruvato shikimato fosfato sintasa
ESI	ionización por electroespray
FAD	flavin adenin dinucleótido
FAO	Food and Agriculture Organization of the United Nations
FMOC-Cl	cloruro de fluorenilmetoxicarbonil
FR	factor de resistencia a herbicida
G	gramo (s)
GC	cromatografía de gases
HCA	análisis jerárquico de grupos
HDT	hora (s) después del tratamiento
HILIC	cromatografía de líquidos de interacción hidrofílica

HPLC	cromatografía de líquidos de alta resolución
I_{50}	dosis de herbicida capaz de reducir en un 50% la actividad enzimática
IM	imidazolinonas
Kb	kilobase
kg	kilogramo (s)
λ	longitud de onda
LC	cromatografía de líquidos
LC–TOF/MS	cromatografía de líquidos-espectrometría de masas conanalizador de tiempo de vuelo
LD_{50}	dosis letal
LDA	análisis discriminante lineal
LLE	extracción líquido-líquido
LLME	microextracción líquido-líquido
LOD	límite de detección
LOQ	límite de cuantificación
m.a.	materia activa
MALDI	desorción/ionización de la matriz asistida por láser
MANOVA	análisis multivariante de la variaza

MEKC	cromatografía electrocinética micelar
METLIN	base de datos de metabolitos del Scripps, Centro de metabolómica y espectrometría de masas
MF	entidad molecular
MHB	ácido 4-metilfosfínico-2-hidroxi-butanóico
MIM	manejo integrado de malezas
MPB	ácido 4-hidroximetilfosfinil-butanóico
MPP	ácido 3-metilfosfínico-propanoico
MS	espectrometría de masas
nm	nanómetro (s)
nd	no detectado
No-SA	resistencia fuera del sitio de acción herbicida
PASA	amplificación por PCR específica de alelo
Pat	fosfinotricina-N-acetiltransferasa
PCR	reacción en cadena de la polimerasa
PM	peso molecular
PPO	acido 4-metilfosfínico-2-oxo-butanoico
PVPP	polivinilpolipirrolidona

RSD	desviación estándar relativa
SPE	extracción en fase sólida
SPME	microextracción en fase sólida
SU	sulfonilureas
TLC	cromatografía de capa fina
TOF	analizador de masas por tiempo de vuelo
TPP	tiamina pirofosfato
UV	ultravioleta

OBJETIVOS

El objetivo básico de la investigación realizada y que se recoge en esta Memoria fue el desarrollo de plataformas analíticas para la determinación de herbicidas y sus metabolitos y la aplicación al estudio de su comportamiento en plantas. La información así obtenida se pretendía utilizar para el estudio y elucidación de las vías a través de las cuales tienen lugar los fenómenos de sensibilidad y de resistencia de las plantas a los herbicidas en cuestión.

El objetivo concreto global fue realizar una contribución a la metabolómica vegetal que permitiera aclarar aspectos desconocidos del comportamiento planta–herbicida en los fenómenos de resistencia a la acción de este último. Este objetivo se desglosaba en dos bloques de objetivos concretos a desarrollar de forma secuencial: Uno de plataformas analíticas (A) y otro de su aplicación (B).

En el bloque A se pretendió que las plataformas desarrolladas no requirieran equipamiento más complejo de lo estrictamente necesario, de forma que su implantación en laboratorios agrícolas fuera asequible en general. Éstas fueron las premisas para la puesta a punto de (i) un método para la determinación de glifosato y sus metabolitos basada en separación individual de los analitos mediante electroforesis capilar (en adelante CE) y detección absorciométrica mediante un espectrómetro de diodos en fila (DAD). (ii) Un método fotométrico para la determinación de glioxilato en plantas tratadas con glifosato. (iii) Un método para la determinación de imazamox y sus metabolitos, y (iv) un método para glufosinato y sus metabolitos estuvieron basados en la separación mediante cromatografía líquida de alta resolución (LC) con detección fotométrica. La ausencia de patrones comerciales de casi todos los metabolitos de estos herbicidas ha hecho necesario recurrir a su identificación mediante un analizador de tipo cuadrupolo–tiempo de vuelo (Qq/TOF) previa separación mediante LC de los componentes de los extractos de plantas tratadas con los herbicidas. La asignación de los tiempos de retención cromatográficos de los compuestos identificados al método LC–DAD da lugar a la semicuantificación de los metabolitos tomando como patrón el herbicida precursor o el metabolito comercial que existe en el caso del glufosinato. Con esta última investigación se pretendió abrir una nueva vía

para la identificación de metabolitos desconocidos con la que ampliar la posibilidad de dilucidar rutas metabólicas no exploradas.

La parte B, de aplicación de las plataformas desarrolladas, ha tenido como protagonista principal el glifosato porque es el herbicida más utilizado mundialmente, pero también porque la plataforma desarrollada (CE–DAD) es suficientemente simple, rápida y segura para su aplicación masiva, tal como se requiere en metabolómica, y porque fue el primer método analítico desarrollado por la doctoranda. Los estudios realizados abarcan su aplicación al estudio diferencial frente a glifosato de *Mucuna pruriens* y *Amaranthus retroflexus*, que forma parte del cuerpo la Tesis, pero también el método lo ha aplicado la doctoranda como base de los estudios realizados por otros miembros del grupo, y cuyas publicaciones se recogen como anexos a esta Memoria. En el caso del imazamox y glufosinato, una vez identificados los compuestos formados en sus rutas metabólicas y localizados de forma inequívoca en el cromatograma obtenido mediante LC–DAD, los métodos se han aplicado al estudio de trigo (*Triticum aestivum*) con una mutación generada de forma natural y otro modificado genéticamente, respectivamente, demostrando así que esta vía de estudio de las rutas de degradación de herbicidas es más simple, barata y fiable que el uso de métodos basados en medidas de radiactividad de ^{14}C.

Por tanto, la formación de la futura doctora, que constituye el objetivo último de una tesis doctoral, ha abarcado la doble vertiente analítica y agrícola (bloque A y B, respectivamente), e incluye un estudio bibliográfico sobre herbicidas y CE que ha dado lugar a la elaboración de un artículo de revisión que constituye el Capítulo 1 de esta Memoria. Esta formación ha incluido también el programa de doctorado "Biociencias y Ciencias Agroalimentarias", en el que la doctoranda ha cursado el número correspondiente de créditos. Paralelamente ha ampliado su formación agrícola con la realización de otras actividades que se recogen como anexos, a saber:

- Anexo I: Investigación simultánea con la de la Tesis en la que ha aplicado la plataforma analítica que ha desarrollado y que constituye el Capítulo 2

de esta Memoria. Esta investigación simultánea ha dado lugar a un total de 4 artículos publicados o en fase de publicación.

- Anexo II: Comunicaciones (13) en conferencias nacionales internacionales además de las cuales muchas de ellas han formado parte de libros de agricultura aplicada y en revistas informativas

INTRODUCCIÓN

En esta introducción general de la Tesis Doctoral se pretende ofrecer una visión de los conocimientos teóricos adquiridos por la doctoranda en la materia objeto de la Tesis y que le han permitido su desarrollo. Con este propósito se han adoptado lo siguientes criterios: (i) Puesto que esta Tesis está realizada en dos áreas diferentes, Agrícola y Analítica, se expondrá una introducción que abarcará una visión general de los aspectos agrícolas relacionados con herbicidas y de los aspectos analíticos utilizados en la realización de este trabajo. (ii) Dado que la técnica de separación más novedosa de las dos empleadas —electroforesis capilar (CE) y cromatografía líquida (LC)— es la CE, se hace especial énfasis en ella y en su comparación con LC. (iii) Teniendo en cuenta que el área a la que se presenta la Tesis no es analítica se ha pretendido dar una visión de los aspectos analíticos clara y simple, evitando en la medida de lo posible los tecnicismos propios de la jerga analítica.

1. MALAS HIERBAS

1.1. Generalidades

El concepto de "mala hierba" es muy subjetivo. Las definiciones suelen ser antropocéntricas y, por tanto, subjetivas. Un ejemplo claro de esta subjetividad lo constituye el *Lolium multiflorum* ("raygrass"). Esta especie constituye un cultivo de una enorme importancia en la zona noroeste de los Estados Unidos, pero también actúa como especie infectante en los cultivos de trigo en Chile, España y Francia.

Son principalmente tres los puntos de vista con los que se aborda el concepto de "mala hierba", ya que no existen características generales que permitan catalogar de forma inequívoca a una planta como tal: El antropocéntrico, el ecológico y el mixto.

1.1.1. Punto de vista antropocéntrico

Este punto de vista antepone los intereses del hombre. Así, mala hierba es aquella que interfiere negativamente en los intereses objetivos del hombre, ya sea porque no tiene uso (o no ha sido aún descubierto), por tener características desagradables o por competir con otra planta cuya producción se persigue.

1.1.2. Punto de vista ecológico

Este criterio se basa en características comunes observadas en especies que muestran de forma repetida un comportamiento invasivo de las zonas cultivadas.

1.1.3. Punto de vista mixto

En este caso se consideran ambos criterios.

Conjugando los distintos enfoques, Pujadas y Hernández (1998) definieron mala hierba como "*aquella planta que crece siempre, o de forma predominante, en situaciones marcadamente alteradas por el hombre y que resulta no deseable por él en un lugar y un momento determinados*". Según lo anterior, cualquier planta puede comportarse como mala hierba.

El carácter invasivo es aspecto común en las malas hierbas y se debe a una competencia ventajosa frente a los cultivos comerciales, ventaja que, según Menéndez (1997) obtienen principalmente por:

1. Elevada producción de semillas.
2. Semillas de tamaño similar a las del cultivo.
3. Más de un modo de propagación o dispersión de semillas.
4. Tiempo de maduración de semillas corto.
5. Dispersión efectiva de semillas.
6. Sabor desagradable.
7. Aspecto poco llamativo.
8. Buena supervivencia en condiciones adversas
9. Crecimiento retardado de órganos vegetativos.
10. Germinación escalonada de semillas.

1.2. Importancia agronómica de los herbicidas

Las malas hierbas causan considerables pérdidas en los sistemas de producción de cultivos en todo el mundo (Tabla 1). Aunque las pérdidas son significativas en países con sistemas de producción agrícola muy desarrollados, como los existentes en Europa, Norte América y Australia, en los países menos desarrollados las pérdidas son mayores (Haigh, 2000). Estas pérdidas se deben a varias causas (Wyse, 1978; García y Fernández-Quintanilla, 1991), entre las que destacan las siguientes:

1.2.1. Reducción de los rendimientos

De un modo genérico, la disminución del rendimiento debida a la presencia de malas hierbas en un cultivo depende de factores tales como la propia especie de mala hierba, la densidad de la población y el cultivo.

1.2.2. Interferencia en la recolección

La presencia de malas hierbas retrasa y dificulta la recolección. Los problemas derivan de los atascos originados en la maquinaria debido a la presencia de materia vegetal verde y húmeda (la maduración de las malas hierbas es posterior a la del cultivo). Estos atascos provocan paradas para la limpieza de la

maquinaria y suponen un mayor desgaste de los equipos y un aumento en el número de averías.

1.2.3. Incremento de los costes de producción

El incremento se debe principalmente a los gastos originados por el control de las malas hierbas, ya sea un control mecánico (incremento del número de labores, maquinaria, carburante, etc.) o químico (herbicidas). Los costes indirectos también se ven afectados, tanto durante el tiempo de cultivo (empleo de cultivos poco rentables en la rotación, retrasos en la fecha de siembra, etc.) como en la cosecha.

1.2.4. Disminución de la calidad de los productos

La menor calidad puede expresarse como:

a) Transferencia al producto de sabores y olores desagradables procedentes de las semillas de malas hierbas o restos de éstas que confieren, además, una mayor humedad a la cosecha.

b) Presencia en el producto de sustancias tóxicas, como es el caso de las infectaciones de *Solanum nigrum* (tomatito) en el cultivo de guisante o en brásicas silvestres en los que la similitud del tamaño de las semillas de mala hierba y de cultivo hace su separación prácticamente imposible.

c) Pérdida de la calificación de semilla certificada debido a niveles superiores a los permitidos de semillas de la población infectante.

1.2.5. Reducción en el valor de la tierra

La devaluación debida a la presencia de estas semillas en un gran banco de semillas para cultivo puede ser de hasta un 50% (Menéndez, 1997).

1.2.6. Hospedaje de plagas y enfermedades

Determinadas especies de malas hierbas son huéspedes de insectos y microorganismos potencialmente dañinos, pudiendo inocular–transferir tales agentes al cultivo que infecta. En este sentido, el objetivo de la agricultura ha sido siempre producir los mayores rendimientos reduciendo la presencia de plantas indeseadas mediante la utilización de distintas técnicas.

Tabla 1. Producción real y pérdidas (billones de dólares) estimadas en 8 cultivos, por plagas y región. (Haigh, 2000).

Región	Producción real	Causa de pérdidas			
		Patógenos	Insectos	Malas hierbas	TOTAL
África	13.3	4.1	4.4	4.3	12.8
Norte América	50.5	7.1	7.5	8.4	22.9
Latino América	30.7	7.1	7.6	7.0	21.7
Asia	162.9	43.8	57.6	43.8	145.2
Europa	42.6	5.8	6.1	4.9	16.8
Oceanía	3.3	0.8	0.6	0.5	1.9

1.3. Métodos de control

La base del control de malas hierbas es encontrar la información y las herramientas necesarias para suprimirlas en cultivos, plantaciones forestales, etc. donde no son deseables. Los principales métodos utilizados para el control de malas hierbas son los siguientes:

1.3.1. Métodos físicos

Existen diversos métodos mecánicos para la eliminación de malas hierbas, entre los que se engloban todos los que desarraigan, entierran, cortan, cubren, o queman la vegetación. Consisten, entre otros, en labrar, retirar manualmente, quemar, segar, inundar, etc.

1.3.2. Métodos culturales

Las prácticas culturales usadas en el control de malas hierbas son principalmente:

a) *La prevención*: Por ejemplo, utilizando semillas de buena calidad, limpias de semillas de malas hierbas, limpiando utensilios de labranza antes de utilizarlos en sitios donde no hay malas hierbas, etc.

b) *La rotación de cultivos*: Consiste en alternar diferentes cultivos en la misma área cada año. Ciertas especies de malas hierbas están asociadas con cultivos específicos (cultivos de verano, de primavera o de invierno). Además, las poblaciones de estas malas hierbas aumentan cuando se utiliza el mismo cultivo

en la misma tierra durante varios años. Esto se debe a que las mismas condiciones ambientales o de cultivo que favorecen al cultivo están favoreciendo a las malas hierbas. Por esta razón, la rotación de cultivos ayuda a controlar las malas hierbas.

c) *Las cubiertas vegetales*: Se desarrollan dos cultivos simultáneamente, aunque uno de ellos es más importante desde el punto de vista económico. Con esto se minimiza la presencia de suelo descubierto, reduciendo la germinación de semillas de malas hierbas.

d) *La recolección*: Aunque no se considera un método de control en sí, la recolección puede promover cierto nivel de supresión de malas hierbas. Por ejemplo, es común recolectar alfalfa varias veces durante el periodo de crecimiento. El ritmo de las operaciones de recolección puede afectar a la disponibilidad de agua así como cambiar ciertas condiciones necesarias para la germinación de la mala hierba.

1.3.3. Métodos biológicos

Utilizan enemigos naturales para eliminar especies de malas hierbas, entre los que se pueden destacar:

a) *El pastoreo*: Es un método tradicional y común para el control biológico de malas hierbas. Se puede usar gran variedad de animales que se alimenten de esas las plantas: Rumiantes, pájaros, insectos, peces, etc. b) *Los micoherbicidas*: Los patógenos de plantas han sido ampliamente usados para el control de malas hierbas y tienen la ventaja de que estos organismos se obtienen en medios artificiales de una forma barata y fácil. Además, estos patógenos pueden aplicarse en el campo de la misma forma que los herbicidas. Si el organismo en un hongo recibe el nombre de micoherbicida.

c) *La alelopatía*: Es cualquier efecto dañino producido, de forma directa o indirecta, por una planta en otra a través de la producción de sustancias químicas que entran en el medio ambiente.

1.3.4. Métodos químicos

Suponen el uso de herbicidas. Aunque existen inconvenientes en su uso, presentan un gran número de ventajas que hacen que sean los métodos más empleados y eficaces. De su estudio se encarga la Malherbología, una disciplina científica que ha conseguido grandes avances en el desarrollo de herramientas y tácticas para el control de malas hierbas. La historia de la Malherbología está ampliamente relacionada con la del control químico de malas hierbas. Por ello, los principales objetivos que persigue esta ciencia son los siguientes (Hess, 1994; Sanyal et al., 2008):

a) La mejora del conocimiento del impacto económico y ecológico de las malas hierbas en diferentes cultivos.

b) El conocimiento más profundo de la biología, ecología y genética de las malas hierbas para optimizar su manejo.

c) La optimización del uso en el campo de agentes biológicos y de productos naturales.

d) El estudio de poblaciones de malas hierbas resistentes a herbicidas para mejorar su control.

e) El desarrollo de nuevas tecnologías para la aplicación de herbicidas con el fin de mejorar su actuación y minimizar su efecto en el medio ambiente. f) La puesta a punto mejores métodos para detectar residuos de herbicidas en agua, suelo y vegetación.

Para conseguir estos objetivos es necesaria la integración de, al menos, seis disciplinas diferentes (Fig. 1):

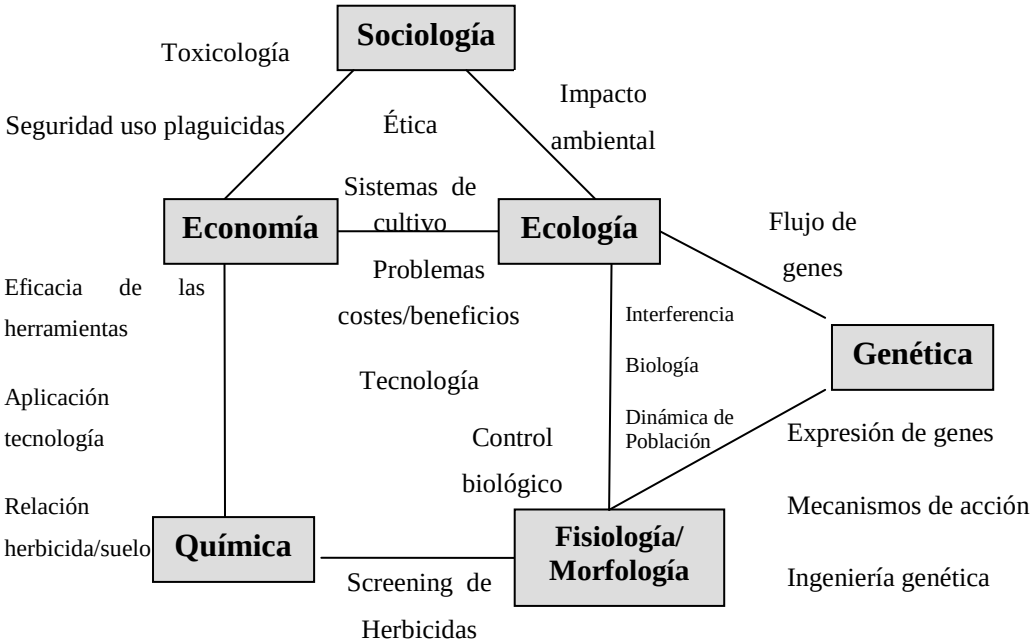

Figura 1. Interrelaciones de 6 disciplinas fundamentales en Malherbología. (Radosevich et al., 1997).

El resultado es un conjunto de información empírica que se usa para desarrollar, mejorar, modificar y, algunas veces, justificar la tecnología usada en el control de malas hierbas.

2. HERBICIDAS

Las malas hierbas, y en consecuencia la planificación de estrategias para su control, son inherentes a la actividad agrícola desde sus comienzos. Originalmente, estas técnicas implicaban la retirada manual, uso de instrumentos de labranza, de azadones primitivos (6000 a.C.), ayuda de animales (1000 a.C.), complementos mecánicos (1920 d.C.), control biológico (1930 d.C.) y control químico, iniciado con la introducción de los dos primeros herbicidas selectivos, MCPA y 2,4-D (1947) (Heap, 2011).

El herbicida se define como un producto químico que inhibe o interrumpe el crecimiento y desarrollo de malas hierbas. Está compuesto por un ingrediente activo causante del efecto sobre las plantas, surfactantes que permiten que el ingrediente activo pueda penetrar en la planta y otras sustancias llamadas excipientes que mejoran la calidad y estabilidad del herbicida.

Un herbicida posee tres nombres: el nombre químico, el común y el comercial. Por ejemplo, el herbicida comercializado con el nombre de Roundup tiene el nombre común de glifosato y el nombre químico N-fosfonometilglicina (que puede estar formulado a su vez como sal de isopropilamina, de amonio o de potasio). El nombre comercial se usa por la empresa para promocionar la venta de su marca específica y comúnmente es el nombre más conocido de un herbicida. El nombre común es el genérico dado al ingrediente activo aprobado por autoridades competentes en la materia, como la Sociedad Americana de la Ciencia de la Maleza (WSSA) y la Organización Internacional para la Estandarización (ISO). El nombre químico describe la estructura química del herbicida (Caseley, 1996).

En el mercado también existen herbicidas que son mezcla de dos o más ingredientes activos, por lo que es importante conocer los nombres comunes de los herbicidas.

Desde su implantación en los sistemas de cultivo moderno, los herbicidas han ido aumentando su importancia hasta convertirse en la principal herramienta en todos los programas de lucha contra malas hierbas de las agriculturas desarrolladas. Este hecho puede deberse a distintas razones (Coble, 1996; Menéndez, 1997; Bastida y Menéndez, 2001).

2.1. Características de los herbicidas

Los herbicidas son compuestos que se distinguen por los siguientes aspectos:

2.1.1. Son productos altamente fiables y eficaces

Con frecuencia los herbicidas ofrecen un control casi completo sobre un amplio abanico de especies indeseadas, a la vez que resultan prácticamente inocuos en los cultivos que protegen. Esta eficacia suele estar muy por encima de la presentada por otras opciones de control en la mayoría de los cultivos.

2.1.2. Son productos de fácil manejo

La mayoría de los herbicidas no requieren una maquinaria especial o prácticas culturales específicas antes o después de su aplicación.

2.1.3. Son productos económicamente rentables

Tras una buena selección y correcto uso, el coste de los herbicidas por unidad de superficie es menor que el de cualquier otro método. Esta comparación es aún más favorable cuando se incluye el tiempo requerido para aplicar las medidas de control.

2.1.4. Son productos agronómicamente versátiles

Los herbicidas ofrecen al agricultor la posibilidad de elegir cuándo y cómo controlar el problema de malas hierbas, permitiendo un margen de uso que va desde la preemergencia a la postemergencia tardía, además de diversas opciones de tratamiento en cada cultivo.

A pesar de estas características, en la actualidad existe una gran controversia sobre los efectos dañinos que producen los herbicidas. En este contexto, las iniciativas persiguen un progresivo reemplazo de herbicidas por una serie de alternativas, entre las que se encuentran.

2.2. Alternativas para la sustitución de los herbicidas

Entre las alternativas más importantes propuestas para sustitución de los herbicidas se encuentran las siguientes:

2.2.1. Uso extensivo de cultivares resistentes

En los próximos 20 años se producirá un importante incremento en el uso de cultivos transformados genéticamente. Por ejemplo, existe un buen número de cultivos transgénicos resistentes a herbicidas (Berner et al., 1997).

2.2.2. Control biológico de plagas y malas hierbas

Los bioplaguicidas son un tipo de plaguicidas que derivan de materiales naturales como son animales, plantas, bacterias o minerales. Un ejemplo de bioplaguicida es el aceite de canola, que se utiliza como tal.

2.2.3. Mejora en la formulación y en los métodos de aplicación de los productos

Las corporaciones internacionales más importantes que dominan el mercado de los plaguicidas están investigando sobre el desarrollo de productos nuevos,

menos tóxicos, menos persistentes y con un espectro menos amplio para cumplir las condiciones impuestas en los mercados más importantes de todo el mundo.

2.2.4. Uso de sistemas expertos de decisión en el control de malezas

El MIM (Manejo Integrado de Malezas, IWM en inglés) puede ser otra alternativa para reducir el uso de plaguicidas químicos. En la actualidad, después de más de 20 años desde la introducción de este concepto en EE.UU., todavía no existe una definición aceptada de MIM. Unos usuarios lo definen como parte de un amplio enfoque que conduce a una "agricultura sin química", mientras que otros lo ven como un sistema que incluye un uso más eficiente de los herbicidas. Concretando los conceptos anteriores, se puede definir MIM como un concepto que incluye la combinación de diversas medidas de control directas (medidas de tipo mecánico, químico o biológico) e indirectas (métodos culturales, siembra y fertilización) para mantener las poblaciones de malas hierbas bajo un umbral económicamente admisible (Zwerger, 1996).

En cuanto al uso de la biotecnología en la producción de cultivos, hay conceptos que preocupan en gran medida a la población; como la posible transferencia de cualidades genéticas de las plantas modificadas a las malas hierbas, creando nuevas generaciones de plantas resistentes a herbicidas y, además, las dudas sobre los efectos a largo plazo, debido al incremento en el consumo de productos modificados genéticamente, tanto para animales como para humanos. Sin embargo, en los últimos años ha adquirido gran importancia lo que se conoce como tecnología Clearfield (BASF, 2004), que es un sistema integrado de control de malas hierbas basado en el desarrollo de variedades tolerantes a las imidazolinonas mediante técnicas tradicionales de inducción de mutaciones y mejora genética convencional; por lo tanto, se trata de semillas no transgénicas. El uso de semillas de variedades imi-tolerantes más la aplicación de imazamox (única imidazolinona autorizada en la Comunidad Europea con nombre comercial PULSAR 40) proporcionan un cómodo y excelente control de las malezas (incluso las de difícil control) incrementando la calidad y el rendimiento del cultivo.

2.3. Clasificación y modo de acción de los herbicidas

Existen diversos criterios de clasificación de los herbicidas, todos ellos tienen alguna utilidad, pero puede que ninguno sea completo. Entre ellos pueden destacarse los siguientes (Tabla 2):

Tabla 2. Clasificación de los herbicidas

ÉPOCA DE APLICACIÓN	Presiembra Preemergentes Postemergentes
SELECTIVIDAD	Selectivos No selectivos
MOVILIDAD EN LA PLANTA	De contacto Sistémicos
FAMILIA QUÍMICA	25 familias químicas
MODO DE ACCIÓN	Siete modos de acción
MECANISMOS DE ACCIÓN	24 mecanismos de acción

2.3.1. Época de aplicación

Por lo general, los herbicidas "pre" se aplican después de la siembra, pero antes de que comiencen a emerger las malas hierbas y el cultivo. Este tipo de herbicidas elimina las malas hierbas en germinación o recién emergidas, lo que evita la competencia temprana con el cultivo.

Los herbicidas "post" se aplican después de la emergencia del cultivo y la maleza. La aplicación de herbicidas "post" debe realizarse sobre malas hierbas en los primeros estados de desarrollo, cuando son más susceptibles a los herbicidas y su competencia con el cultivo es mínima.

Los herbicidas de presiembra se aplican 20–30 días antes de la siembra. Realizan el control temprano de las malas hierbas.

2.3.2. Selectividad

Los herbicidas selectivos son aquellos que a ciertas dosis, formas y épocas de aplicación eliminan ciertas malezas sin dañar gravemente al cultivo; mientras los no selectivos son aquéllos que producen toxicidad en toda planta y deben utilizarse en terrenos sin cultivar o evitando el contacto con los cultivos.

2.3.3. Movilidad en la planta

Los herbicidas de contacto son aquellos que eliminan sólo las partes de la planta con las que entran en contacto en el momento de la aplicación y tienen un transporte limitado dentro de la planta. Por su parte, los herbicidas sistémicos son los que son absorbidos y transportados a toda la planta incluyendo sus raíces y otros órganos subterráneos.

2.3.4. Familia química

Esta clasificación se basa en la estructura química de los compuestos usados como herbicidas. Los herbicidas dentro de una familia química tienen propiedades químicas similares y generalmente tienen el mismo modo de acción (Retzinger y Mallory-Smith, 1997). Algunos ejemplos de las principales familias químicas de herbicidas son las triazinas, las dinitroanilinas, los fenoxiacéticos, las cloroacetamidas, las ciclohexano-dionas, las sulfonilureas y los bipiridilos (Hance y Holly, 1990).

2.3.5. Modo de acción

La clasificación más útil de los herbicidas se basa en su modo de acción (Duke y Dayan, 2001), (Schmidt, 2005), constituido por la secuencia de eventos que ocurren desde la absorción del herbicida hasta la muerte de la mala hierba. Los herbicidas con el mismo modo de acción tienen el mismo comportamiento de absorción y transporte y producen síntomas similares en las plantas tratadas (Gunsolus y Curran, 1996). Además, la clasificación de los herbicidas según su modo de acción permite predecir, en forma general, su espectro de control de maleza, época de aplicación, selectividad a cultivos y persistencia en el suelo

(Ashton y Craft, 1981). Cobb y Reade (2010), distinguen siete grandes grupos de herbicidas, que incluyen a su vez uno o más mecanismos de acción.

2.3.6. Mecanismo de acción

El mecanismo de acción de un herbicida se define como la principal reacción bioquímica o biofísica causada por el herbicida para dañar la planta tratada. El modo de acción incluye el mecanismo de acción. Este último comúnmente supone el bloqueo de algún proceso enzimático vital para la planta. Los herbicidas pueden clasificarse por su mecanismo de acción en función de los síntomas que provocan en las plantas tratadas.

La WSSA ha desarrollado un sistema de clasificación numérico mientras que el sistema de clasificación del HRAC se basa en letras. También diversos autores han propuesto clasificaciones de herbicidas según su modo y mecanismo de acción. Cada una de estas clasificaciones es diferente y no siempre coinciden los criterios.

La clasificación que se muestra en la Tabla 3 está basada en el modo de acción. Esta clasificación es la que proporciona más información sobre la interacción última entre herbicida y planta y, consecuentemente, expresa el efecto fitotóxico del herbicida.

Tabla 3. Clasificación de los herbicidas según su modo de acción. (HRAC, 2011).

Grupo	**Modo de acción**	**Familia química**
A	Inhibición de la acetil CoA carboxilasa (ACCasa)	Ariloxifenoxipropionatos, ciclohexanodionas
B	Inhibición de la acetolactato sintasa (ALS)	Imidazolinonas, sulfonilureas, triazolopirimidinas, pirimidiniltiobenzoatos
C1	Inhibición de la fotosíntesis en el fotosistema II	Triazinas, triazinonas, uracilos, piridazinona, fenil-carbonatos
C2	Inhibición de la fotosíntesis en el fotosistema II	Ureas, amida
C3	Inhibición de la fotosíntesis en el fotosistema II	Nitrilos, benzotiadiazol, fenil-piridazina

Continuación

Grupo	**Modo de acción**	**Familia química**
D	Desviación del flujo electrónico en el fotosistema I	Bipiridilos
E	Inhibición del protoporfirinógeno oxidasa (PPO)	Difeniléteres, N-fenil-ftalamidas, tiadiazoles, oxadiazol, triazolinona
F1	Decoloración: inhibición de la síntesis de carotenoides a nivel de la fitoeno desaturasa (PDS)	Piridazinona, nicotinanilida, otros
F2	Decoloración: inhibición de la 4-hidroxifenil-piruvato-dioxigenasa (4-HPPD)	Trikenona, ixosazol, pirazol
F3	Decoloración: inhibición de la síntesis de carotenoides (punto desconocido)	Triazol, isoxazolidinona, urea
G	Inhibición de la EPSP sintetasa	Glicinas
H	Inhibición de la glutamino sintetasa	Acido fosfínico
I	Inhibición del DHP (dihidropterato) sintetasa	Carbamatos
K1	Inhibición de la unión de los microtúbulos de la mitosis	Dinitroanilinas, fosforoamidatos, piridazina, ácido benzóico
K2	Inhibición de la mitosis	Carbamatos, benziléter
K3	Inhibición de la división celular	Cloroacetamidas, carbamato, acetamida, benzamida, oxiacetamida
L	Inhibición de la síntesis de la pared celular (celulosa)	Nitrilos, benzamida
M	Desacopladores (alteración de la membrana)	Dinitrofenoles
N	Inhibición de la síntesis de los lípidos (no ACCasa)	Tiocarbamatos, fosforoditioato, benzofurano, ácidos clorocarbónicos
O	Auxinas sintéticas (como la acción del ácido indolacético, AIA)	Acidos fenoxi-carboxílicos, ácido benzoico, ácido piridin-carboxílico, ácido quinolin-carboxílico, otros
P	Inhibición del AIA	Ftalamato, diflufenzopir

Continuación

Grupo	Modo de acción	Familia química
R/S/T/Z	Desconocido	Ácido arilamino propiónico, organoarsenicales, otros

2.4. Glifosato

2.4.1. Familia química

Pertenece al grupo de las glicinas, compuesto por glifosato y sulfosato (Fig. 2). Es un inhibidor de la 5-enolpiruvil-sikimato-3-fosfato sintasa (EPSPs, EC 2.5. 1.19).

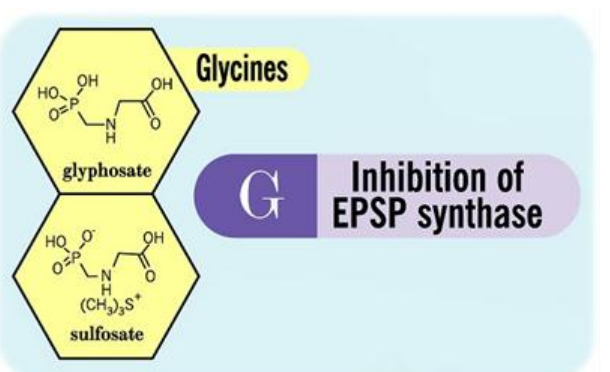

Figura 2. Familia de glicinas perteneciente al grupo G según la clasificación de la HRAC. Estructura química de las dos materias activas disponibles en el mercado mundial de herbicidas (International Survey of Herbicide Resistant Weeds, 2012).

2.4.2. Actividad de la EPSP sintasa en plantas

Esta enzima cataliza la formación de fosfoenolpiruvil-shikimato 3-fosfato (EPSP) a partir de fosfoenolpiruvato (PEP) y shikimato 3-fosfato (S3P). Los aminoácidos esenciales fenilalanina, tirosina y triptófano son productos de esta ruta metabólica, también llamada ruta del shikimato. Estos aminoácidos aromáticos son los precursores de numerosos productos secundarios en la planta, como antocianos, lignina, promotores del crecimiento (hormonas), inhibidores del crecimiento y fenoles, así como de la producción de proteínas (Franz et al.,

1997). Además, entre el 20 y el 35% del carbono fijado por la fotosíntesis se utiliza en esta ruta.

2.4.3. Acción del herbicida

El glifosato, N-fosfonometil glicina, es un herbicida no selectivo, foliar y de amplio espectro, que ha sido utilizado durante más de 25 años para controlar una amplia variedad de malas hierbas mono y dicotiledóneas (Anderson y Jonson, 1990; Atkinson, 1985; Coble, 1996). En los últimos 15 años, la expansión de cultivos modificados genéticamente para su resistencia a glifosato ha incrementado su uso (Padgette et al., 1996). Además de la alta eficacia del glifosato en el control de numerosas malas hierbas, tiene otras características favorables para el medioambiente como es su fuerte adsorción a los coloides del suelo (lo que dificulta su percolación y consiguiente contaminación de las aguas subterráneas), la similitud con los aminoácidos y baja toxicidad para mamíferos, aves y peces (Padgette et al., 1996). Estos factores, unidos a su éxito en el control de malas hierbas perennes, constituyen muchas veces la única herramienta eficaz para su control, y la rápida inactivación en suelos, que permite la siembra de cualquier cultivo casi inmediatamente después del tratamiento, y explican que el glifosato sea el herbicida más utilizado en el mundo.

Su modo de acción primario es la inhibición de la enzima 5-enolpiruvil-shikimato-3-fosfato sintasa (EPSPs), acción compleja, pero la información disponible indica que el glifosato compite con el fosfoenolpiruvato (PEP) por su sitio específico de unión a la EPSPs. La inhibición de la ruta provoca una acumulación de los intermediarios shikimato y shikimato-3-fosfato (S3P). Como consecuencia, se detiene la producción de los aminoácidos aromáticos esenciales fenilalanina, tirosina y triptófano (Fig. 3).

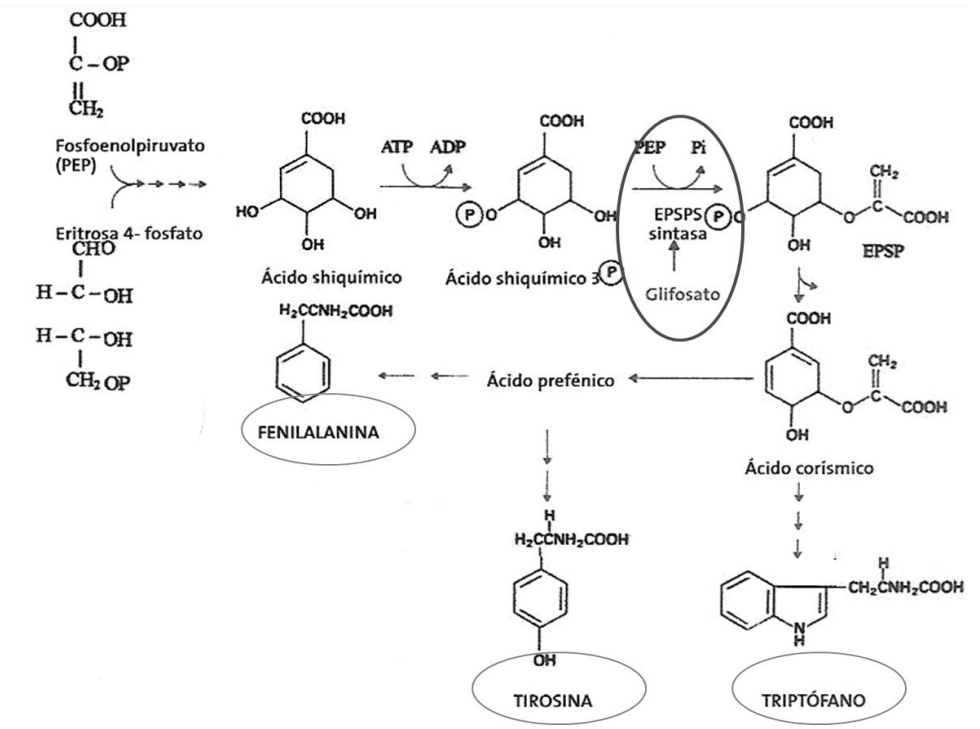

Figura 3. Inhibición de la enzima 5-enolpiruvil-shikimato-3-fosfato sintasa por el herbicida glifosato en la ruta biosintética de los aminoácidos fenilalanina, tirosina y triptófano.

2.5. Imazamox

2.5.1. Familia química

Desde su aparición en los años 80 (Shaner et al., 1984), las imidazolinonas han tenido un gran éxito en la agricultura moderna, no sólo por su alta eficacia en cultivos de cereales y leguminosas, sino también por su baja toxicidad en mamíferos y su bajo impacto ambiental (Aichele y Penner, 2005; FAO, 2012), así como por la amplia selectividad de cultivos y gran número de formulaciones indicadas tanto en pre- como en post-emergencia (Shaner y O'Connor, 1991). En 1998 apareció una nueva imidazolinona conocida con el nombre de imazamox (Brady et al., 1998; European comission, 2002). Este herbicida presenta gran efectividad en campo debido a que es fácilmente absorbido por las hojas y por las raíces; además, presenta translocación y bajo metabolismo. Las imidazolinonas tienen en su estructura un núcleo 4-isopropil-4-metil-5-oxo-2-imidazolin-2-il

unido en la posición 2 a un anillo aromático (usualmente heterocíclico), según se muestra en la Fig. 4. La estructura del imazamox está representada en la Fig.5. Actúan principalmente como inhibidores de la acetolactato sintasa (ALS).

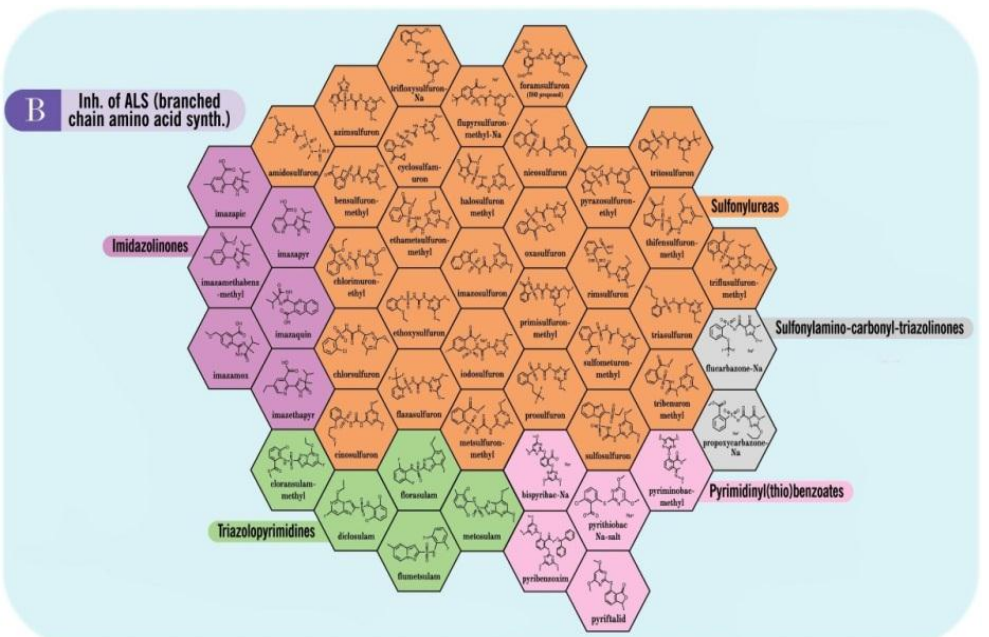

Figura 4. Herbicidas inhibidores de la ALS: sulfonilureas, imidazolinonas, triazolopirimidinas, pirimidiniltiobenzoatos,etc.

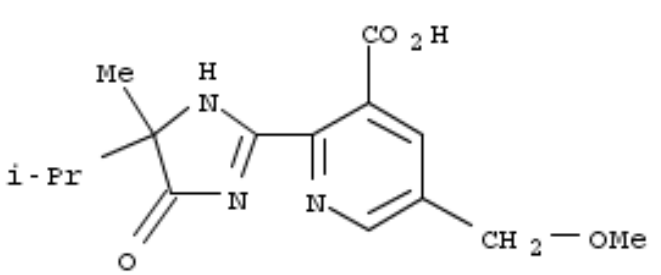

Figura 5. Estructura del imazamox.

2.5.2. Actividad ALS en plantas

La acetolactato sintasa (ALS, acetohidroxiácido sintasa, AHAS, E.C. 2.2.1.6) cataliza el primer paso común en la biosíntesis de los aminoácidos de cadena ramificada valina, leucina e isoleucina en plantas y microorganismos (Saari y Mauvais, 1996). El estudio de esta ruta de biosíntesis en plantas es importante debido, entre otras razones, a que al ser 3 de los 10 aminoácidos esenciales, representa un aspecto crítico de gran valor nutricional. Además, estos aminoácidos son precursores de metabolitos secundarios, como glucosinolatos y glicósidos.

2.5.3. Acción del herbicida

Su efectividad en campo es debida a una combinación de varios factores, entre los que se encuentran su fácil translocación, bajo metabolismo y persistencia en el suelo. En el caso concreto del imazamox, este es fácilmente absorbido por las hojas y también por las raíces. Son herbicidas que controlan un amplio espectro de malas hierbas y se usan de forma generalizada en cultivos de cereales, leguminosas y en plantaciones forestales.

Su modo de acción primario es la inhibición de la enzima acetolactato sintasa. En una revisión realizada por Powles y Yu en 2010, se muestra cómo la enzima ALS es inhibida por el herbicida. Esta enzima está formada por dos subunidades, una catalítica y otra reguladora. La segunda estimula la actividad de la subunidad catalítica y confiere sensibilidad a la inhibición por retroalimentación de aminoácidos de cadena ramificada. Este trabajo ha revelado que el sitio catalítico de la ALS se encuentra en un canal profundo y, por tanto, los herbicidas inhibidores de la ALS no se unen dentro del sitio catalítico, sino a través de un dominio de unión a los herbicidas, que se extiende hasta la entrada del canal bloqueando el paso del sustrato al sitio catalítico (Fig. 6). A través de este dominio, 18 residuos de aminoácidos se implican en la unión con el herbicida.

Los herbicidas inhibidores de la ALS estructuralmente diferentes se orientan también de manera diferente en el dominio de unión al herbicida, con superposición parcial (Fig. 6). Por lo tanto, una sustitución de aminoácidos

dentro del dominio de unión puede conferir resistencia a algunos, pero no a otros herbicidas.

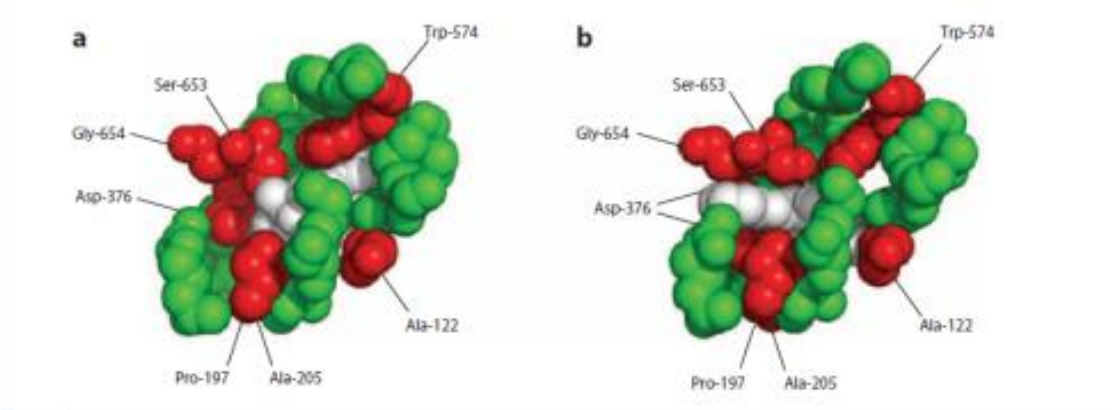

Figura 6. Modelo de simulación de la estructura de ALS de *Arabidopsis* con el herbicida clorsulfurón (a) y el herbicida imazaquin (b). Los herbicidas son de color blanco, las sustituciones de los residuos que han desarrollado resistencia son de color rojo. Hay que tener en cuenta que el herbicida clorsulfurón se une más y tiene más contacto con el sitio catalítico de lo que lo hace el herbicida imizaquin. Se observa en las imágenes que las moléculas de los herbicidas en la parte inferior izquierda son las que están en la entrada del canal, y las que están en la parte superior derecha se encuentran dentro del canal, que conduce al sitio catalítico. (Powles y Yu, 2010).

Como consecuencia de la inhibición de la enzima ALS se detiene la producción de aminoácidos de cadena ramificada tales como la valina, leucina e isoleucina (Fig. 7), deteniendo el crecimiento y produciendo posteriomente la muerte en plantas susceptibles poco tiempo después de la aplicación (en 4–6 semanas después de la aplicación).

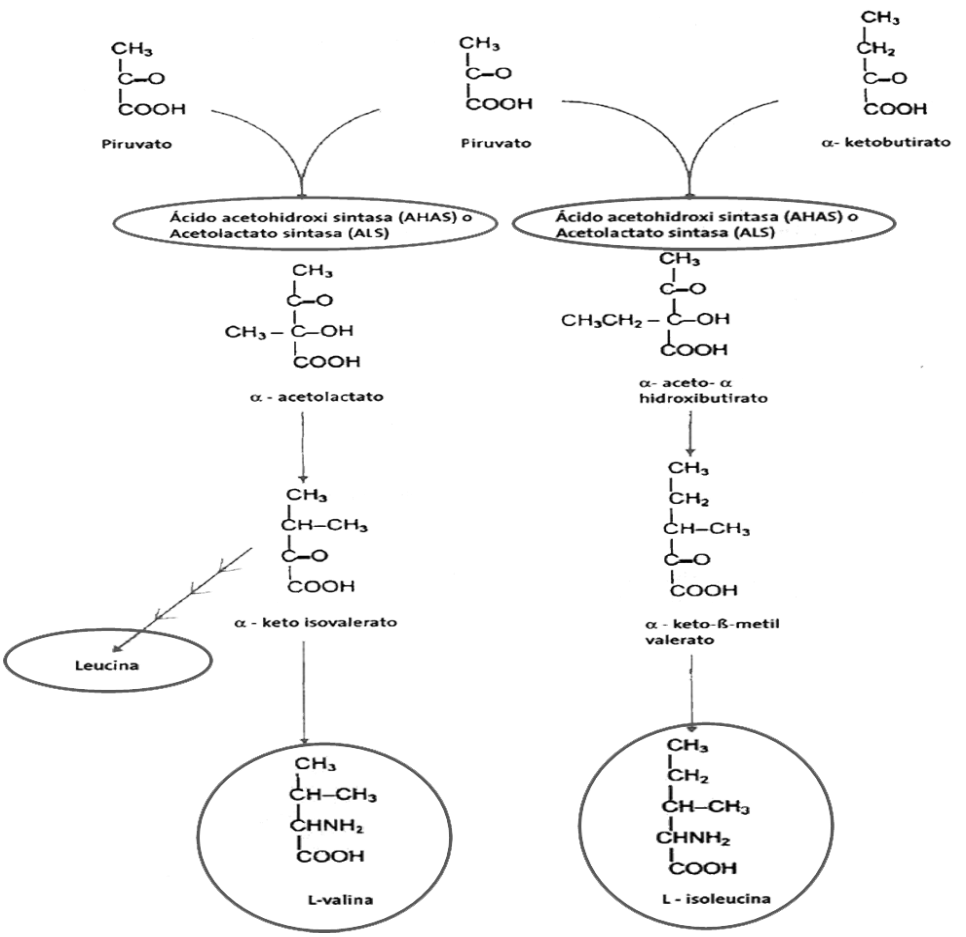

Figura 7. Ruta biosintética de los aminoácidos de cadena ramificada valina, leucina e isoleucina.

2.6. Glufosinato

2.6.1. Familia química

El glufosinato es un herbicida que se descubrió en el año 1972 en unos laboratorios de Alemania cuando se estaba aislando un aminoácido de una especie de bacteria del suelo llamada *Streptomyces viridochromogenes* (Bayer et al., 1972), demostrándose en poco tiempo que este aminoácido presentaba actividad herbicida. El glufosinato pertenece a la familia química de los derivados de los ácidos fosfónicos. Este herbicida inhibe la actividad de la enzima glutamina sintetasa (GS) (Fig. 8), es no selectivo y de origen natural. No es absorbido por tallos lignificados, lo que permite su uso en frutales y vides. La

estructura del glufosinato es el tripéptido fosfinotricina (PPT) unido a 2 moléculas de alanina. El glufosinato de amonio es una mezcla de D y L fosfinotricina. Su nombre químico es (2-amino-4-(hidroximetilfosfinil) butanoato amónico.

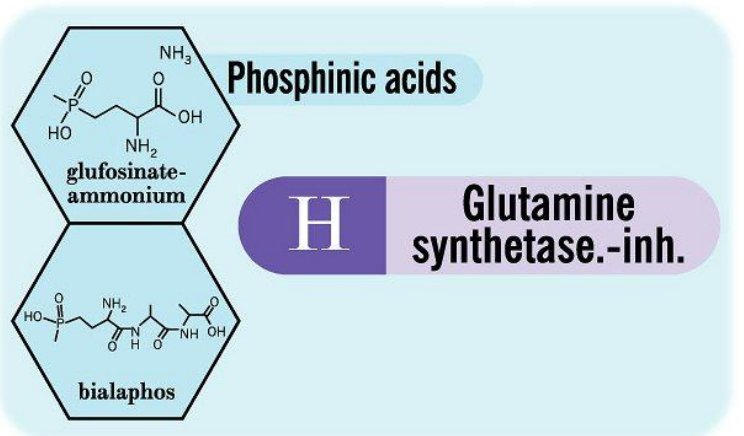

Figura 8. Herbicidas inhibidores de la GS, entre los que se encuentra el glufosinato y el bialafos.

2.6.2. Actividad GS en plantas

La glutamina sintetasa (GC, EC 6.3.1.2) cataliza la síntesis de L-glutamina a partir de L-glutamato ATP y NH_3 (Fig. 9). La GS utiliza el NH_3 producido por la reducción del nitrato, la degradación de aminoácidos y la fosforilación, y cataliza la condensación dependiente de ATP del glutamato que carga un amonio dando glutamina. La hidrólisis del ATP direcciona el primer paso de un mecanismo consistente en dos etapas. El ATP fosforila el glutamato para formar ADP y el intermediario acetil fosfato para dar un γ glutamil fosfato que reaccionará con el ión amonio, formando glutamina y un fosfato inorgánico.

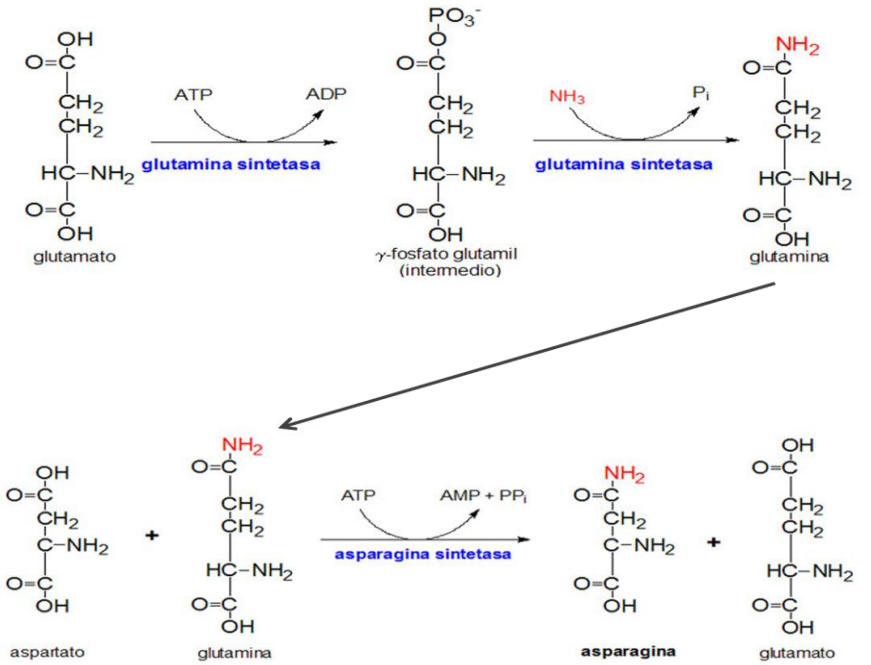

Figura 9. Reacción de catálisis de la GS y su efecto en la biosíntesis de la asparagina.

2.6.3. Acción del herbicida

El glufosinato es un herbicida no selectivo postemergente. Desde el punto de vista biológico el glufosinato de amonio es un análogo natural del acido glutámico. Este análogo se usa para una gran variedad de malezas de grandes hojas y para pastos con árboles frutales u orquídeas, viñedos, plantaciones de caucho y palma, árboles ornamentales y tierras no cultivadas, además de en cultivos de vegetales antes de la germinación.

El principal mecanismo de acción del glufosinato es mediante inhibición de la GS. El herbicida se une al ATP formando un complejo glutamina sintetasa-glufosinato fosfato con el cual el NH_3 no puede reaccionar, quedando la enzima inhibida irreversiblemente provocando una disminución en los niveles de acido aspártico y asparagina, entre otros fenómenos. El flujo de electrones en la fotosíntesis se ve inhibido por la disminución de los grupos amino que provee el acido glutámico los cuales los utiliza el glioxilato en el peroxisoma. El glioxilato se acumula reduciendo la fijación del CO_2 en el ciclo de Calvin, inhibiendo la fase luminosa de la fotosíntesis. En presencia de luz, la inhibición del flujo de electrones produce clorofila en estado triplete (estado inestable altamente

energético) lo que conlleva la peroxidación de lípidos y el daño irreversible de las membranas biológicas. Además de acumularse el glutamato también lo hace el amonio, ya que es una ruta de fijación de nitrógeno en plantas, inhibiéndose la fosforilación en la fotosíntesis (Tachibana et al., 1986; Sauer et al., 1987; Hoerlein 1994).

2.7. Resistencia de las malas hierbas a los herbicidas

2.7.1. Concepto de resistencia–tolerancia

Aunque existe un número elevado de herbicidas que resultan activos frente a cualquier tipo de planta, no cabe duda de que, hoy día, los más importantes son los herbicidas capaces de controlar un amplio espectro de malas hierbas sin afectar a los cultivos a los que se aplican. Tal acción selectiva supone que determinadas especies de plantas cultivadas, y también de malas hierbas, son capaces de vivir y crecer a las dosis recomendadas de aplicación agrícola del herbicida, aunque puedan ser controladas a dosis mayores. Este tipo de respuesta se conoce generalmente como tolerancia natural y ha sido definida por el HRAC como la *"habilidad/aptitud heredable de una especie vegetal a sobrevivir y reproducirse después de un tratamiento, pudiendo considerarse como una característica a nivel de especie"*. El término tolerancia se usa no sólo para referirse a variaciones entre especies sino también en relación con la variabilidad dentro de una misma especie (Lebaron y Gressel, 1982).

Como consecuencia de la presión selectiva impuesta por la aplicación continuada de herbicidas, es posible el desarrollo de biotipos que dejan de ser controlados por un determinado producto al que originalmente eran susceptibles. Tal respuesta se conoce generalmente como resistencia, siendo una característica adquirida por una población (biotipo) de una especie que carecía de ella (HRAC).

En este contexto, la resistencia es un proceso evolutivo en el que una población cambia de ser susceptible a ser resistente. Las plantas individuales no pasan de ser susceptibles a ser resistentes, sino que es la proporción de individuos originalmente resistentes dentro de la población la que se incrementa a lo largo del tiempo.

El término resistencia suele modificarse con diversos adjetivos que hacen alusión a la posible pluralidad existente tanto en los mecanismos de resistencia que posee un individuo como en los herbicidas a los que es resistente. Surgen así los conceptos de resistencia cruzada y resistencia múltiple. Dependiendo de los autores consultados, estas definiciones se asocian a mecanismos de resistencia (Jutsum y Graham, 1995), o a familias de herbicidas (Rubin, 1991), Tabla 4.

Tabla 4. Clasificación y definiciones de resistencia según distintos autores.

Clasificación	**Según mecanismo de resistencia**	**Según familia de herbicidas**
Resistencia cruzada	Aquélla por la que un individuo es resistente a dos o más herbicidas debido a un solo mecanismo de resistencia.	Aquélla por la que una población es resistente a dos o más herbicidas que actúan en el mismo sitio primario de acción.
Resistencia múltiple	Aquélla por la que un individuo posee más de un mecanismo de resistencia para uno o varios herbicidas.	Aquélla por la que una población es resistente a dos o más herbicidas que actúan en distintos sitios de acción.

El término resistencia cruzada se usa a menudo para describir casos en los cuales una población de malezas es resistente a dos o más herbicidas (de la misma o diferente clase química) debido a la presencia de un mecanismo de resistencia único.

El término de resistencia múltiple se refiere a aquellas plantas resistentes a dos o más grupos diferentes de herbicidas con dos o más mecanismos de resistencia. La resistencia cruzada negativa se refiere a aquellos casos en los que un biotipo resistente a un herbicida exhibe un aumento en la sensibilidad a otros herbicidas con distinto modo de acción o de degradación (De Prado et al., 1992). Por último, los cultivos resistentes a herbicidas son cultivos que poseen genes que les confieren resistencia a un cierto herbicida al que habían sido previamente sensibles.

De ambos tipos de resistencia, la agronómicamente más compleja y de difícil solución es la múltiple, ya que se atribuye a diferentes mecanismos que

operan a nivel de absorción, retención, translocación y metabolismo (Retzinger y Mallory-Smith, 1997).

En este caso tolerancia y resistencia son expresiones que denotan diferencias en intensidad de un mismo fenómeno, considerándose la resistencia como un caso extremo y menos frecuente de tolerancia (Holt y LeBaron, 1990) o considerando la tolerancia como un mecanismo poligénico y la resistencia como uno monogénico (Gressel, 1985).

2.7.2. Mecanismos de resistencia

Existen dos formas en que la resistencia se puede manifestar en una población de malas hierbas. En primer lugar, pueden estar presentes un gen o un grupo de genes que proporcionen la resistencia debido a mutaciones aleatorias. En este caso, el herbicida mata a la mayoría de las plantas susceptibles, pero los individuos resistentes sobreviven y se reproducen. La proporción de individuos resistentes en la población se incrementa gradualmente hasta el punto en el que se produce una disminución en la efectividad del herbicida (entre un 10 y un 20% de plantas sobreviven a la aplicación). Es importante tener en cuenta que la proporción de genes resistentes en toda la población puede haberse incrementado durante años antes de que se advierta un problema de control en campo. El grado de resistencia depende de la relación entre individuos resistentes y susceptibles en una misma población (Moss, 2002).

En segundo lugar, la selección puede actuar sobre la variación cuantitativa mediante un proceso menos conocido adquiriendo un incremento gradual y progresivo en la resistencia a lo largo de varias generaciones. Estas variaciones cuantitativas pueden ser causadas por un cierto número de poligenes, cada uno de los cuales produce un efecto mínimo, pero tiene la posibilidad de generar un nuevo rasgo en el fenotipo. El término de variación cuantitativa implica que existe un continuo de respuestas al herbicida dentro de la población, las cuales van desde susceptible, parcialmente resistente hasta altamente resistente. Esta gradación ocurre debido a un incremento progresivo en el nivel de resistencia en toda la población y no a un incremento en la proporción de individuos resistentes (Moss, 2002).

Dependiendo del tipo de mecanismo de resistencia detectado la mala hierba presentará un patrón específico en su resistencia a herbicidas, que podrá variar desde un alto grado de resistencia a determinados compuestos de una misma familia química, a una moderada resistencia a un amplio espectro de herbicidas. Asimismo, el conocimiento de estos mecanismos permite prever la posible respuesta de la población resistente al conjunto de métodos (químicos, mecánicos, culturales) seleccionados para su control, su efectividad a corto y largo plazo y la posible aparición de nuevos problemas. La aplicación anual de plaguicidas es de millones de toneladas por año; sin embargo, se ha estimado que sólo un pequeño porcentaje de estos productos alcanzan el organismo objetivo, el resto se deposita en el suelo y en organismos no objetivo y se mueve hacia la atmósfera y el agua (Rodríguez-Castellanos y Sánchez-Hernández, 2007).

Existen al menos cinco mecanismos generales, no necesariamente excluyentes, que pueden explicar la resistencia a herbicidas (Sherman et al., 1996). En la Fig. 10 se muestran cada uno de estos cinco mecanismos, así como las tres fases del proceso de metabolización de las moléculas.

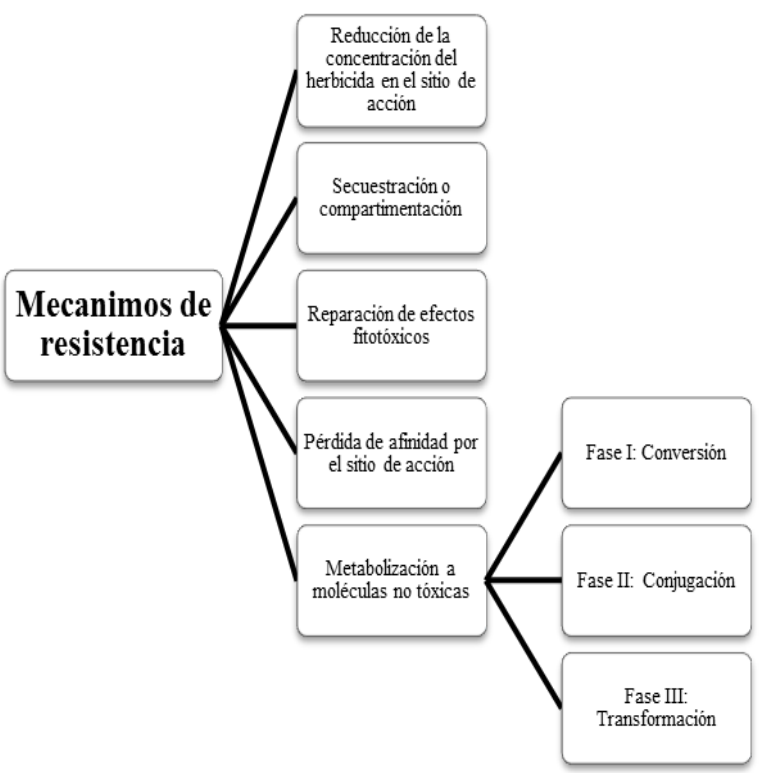

Figura 10. Diagrama de los mecanismos de resistencia de plantas a herbicidas.

a) Reducción de la concentración de herbicida en el sitio de acción. Una condición necesaria para lograr la efectividad de un herbicida es que una concentración suficiente alcance su sitio de acción para que el efecto sea letal. La falta de movimiento de un herbicida reduce la concentración de éste en el sitio de acción, lo que le permite mantenerse funcional. Las bajas concentraciones pueden deberse a la reducción en la penetración, absorción, translocación o a la existencia de fenómenos de secuestración en orgánulos celulares más o menos translocables. La falta de absorción, penetración o translocación de herbicidas es básicamente un mecanismo de tolerancia existente en numerosos cultivos y en algunas malas hierbas (Hess, 1985) (De Prado et al., 2001) (Michitte et al., 2004) (Ruiz-Santaella et al., 2004) (Cruz-Hipolito et al., 2009).

b) Secuestración o compartimentación son mecanismos de resistencia o tolerancia poco conocidos debido a que los indicios en los que los apoyan son circunstanciales en su mayoría (Coupland, 1991;Owen y Pallutt, 1991; Ge et al., 2010). Los pocos casos encontrados en la bibliografía justifican la resistencia, tanto en líneas de cultivos celulares como en plantas enteras, como un incremento en la capacidad de secuestrar el herbicida o los metabolitos potencialmente fitotóxicos en la vacuola celular. Los procesos subyacentes a estos mecanismos de secuestración son todavía desconocidos**.**

c) Reparación de efectos fitotóxicos. Algunos herbicidas ariloxifenoxi-propanoatos (inhibidores de la enzima acetil coenzima A carboxilasa), como diclofop-metil y haloxifop, despolarizan el potencial de la membrana plasmática en células parenquimáticas de *Avena sativa*, *Triticum aestivium*, *Lolium rigidum*, etc. La capacidad despolarizadora del diclofop-metil se atribuye al flujo específico de protones que este compuesto produce hacia el interior de la célula (Shimabukuro y Hoffer, 1997). Recientemente se han identificado biotipos de malas hierbas cuyo mecanismo de resistencia al diclofop-metil parece ser debido a la capacidad de recobrar el potencial de membrana una vez que se ha retirado el herbicida causante de la despolarización (De Prado et al., 1999).

d) Pérdida de afinidad por el sitio de acción. Los herbicidas resultan letales para las plantas debido a su actividad sobre un sitio de acción primario, generalmente

una proteína de especial relevancia biológica. Este sitio primario suele ser específico y la acción del herbicida sobre él suele conducir al desarrollo de efectos secundarios de naturaleza mucho más general, que normalmente producen la muerte de la planta (Corbett et al., 1994). Una o varias mutaciones en la secuencia aminoacídica del sitio primario de acción pueden resultar en una pérdida de afinidad del herbicida por ese sitio, imposibilitando la unión efectiva de ambos e impidiendo así la continuidad del proceso vital mediado por dicho sitio (Devine y Shimabukuro, 1994; Grownwald, 1994; Saari et al., 1994). Este tipo de mecanismo es el que exhiben la mayoría de los biotipos resistentes descritos hasta el momento. Se caracteriza por conferir un alto grado de resistencia al herbicida empleado e incluso a otras moléculas pertenecientes a la misma familia química (Fig. 11).

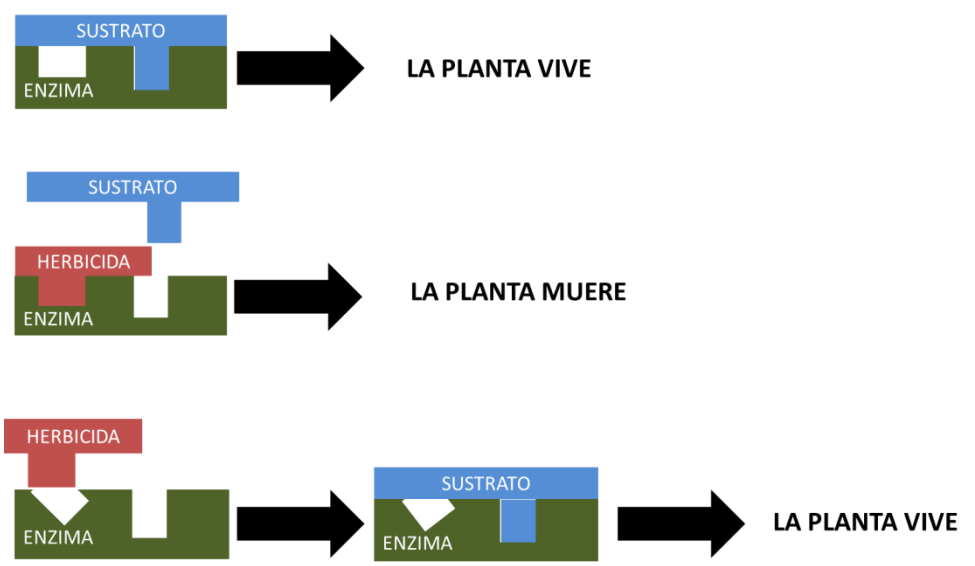

Figura 11. Representación esquemática de la inhibición de la acción de un herbicida debida a pérdida de afinidad por el sitio de acción. En el primer caso nada impide que el sustrato llegue al sitio de unión, por lo que la planta vive; en el segundo el herbicida que está unido a la enzima impide que el sustrato llegue al sitio de unión provocando la muerte de la planta. Por último, el herbicida no puede ocupar el sitio de acción puesto que ya no es afín a él y, por tanto, la planta vive.

e) Metabolización a especies no tóxicas (destoxificación). Los procesos de destoxificación metabólica se caracterizan por ser procesos biológicos en los que las moléculas tóxicas son metabolizadas a compuestos inocuos o menos

tóxicos. En los procesos de destoxificación metabólica los biotipos resistentes son capaces de degradar el herbicida antes de que éste cause daños irreversibles. La velocidad de degradación enzimática puede variar debido a factores endógenos y exógenos como el estadio de crecimiento de la planta o las condiciones climáticas, entre otros.

Los procesos de destoxificación metabólica de herbicidas en tejidos vegetales pueden dividirse en tres fases (Tabla 5) (Shimabukuro, 1985; Hatzios, 1991):

Tabla 5. Resumen de las tres fases del metabolismo de plaguicidas (adaptado de (Shimabukuro, 1985) (De Prado et al., 2004).

Características	**Propiedades iniciales**	**Fase I**	**Fase II**	**Fase III**
Reacciones	Compuesto inicial	Oxidación, hidrólisis, reducción	Conjugación	Conjugación secundaria o incorporación a biopolímeros
Solubilidad	Lipofílico	Ambifílico	Hidrofílico	Hidrofílico o insoluble
Fototoxicidad	Tóxico	Modificada o menos tóxico	Muy reducida o no tóxico	No tóxico
Movilidad	Selectiva	Modificada o reducida	Limitada o inmóvil	Inmóvil

Fase I (conversión). Algunos herbicidas pueden ser conjugados directamente, sin embargo, otros no poseen sustituyentes disponibles en sus moléculas (grupos amino, hidroxilo, sulfhidrilo, etc.) que puedan reaccionar para formar conjugados con constituyentes celulares. Estos herbicidas deberán ser convertidos en compuestos más solubles en agua y menos tóxicos mediante algunas de las siguientes reacciones:

–*Hidrólisis:* Estas reacciones están catalizadas por enzimas hidrolíticas (esterasas, fosfatasas o amidasas, dependiendo del sustrato). En las transformaciones hidrolíticas se rompen los enlaces de un sustrato por adición a cada producto de H u OH proveniente del H_2O. Hay muchas enzimas hidrolíticas

capaces de metabolizar una gran variedad de sustratos, particularmente aquellos que contienen grupos funcionales amida, carbamato o éster. Estas enzimas pueden estar compartimen-talizadas o ser extracelulares, y las reacciones pueden ocurrir tanto en condiciones aerobias como anaerobias. La hidrólisis de enlaces éster de herbicidas ha sido ampliamente estudiada y analizada en plantas y microrganismos (Incledon y Hall, 1997; Hoagland y Zablotowicz, 1998). La hidrólisis del enlace éster se realiza principalmente por esterasas y en menor medida por lipasas y proteasas. Con respecto a la hidrólisis de enlaces amida, el propanil es el herbicida más estudiado.

–*Reducciones:* La metabolización reductora de los herbicidas es un proceso que raramente se da en plantas, pudiéndose destacar únicamente la desaminación reductora de las s-triazinonas (metamitrona y metribu-zina), en cultivos tolerantes y malas hierbas resistentes (Fedtke, 1983).

–*Oxidaciones, oxigenaciones e hidroxilaciones*: Todas ellas se pueden incluir en un grupo denominado transformaciones oxidativas. La oxigenación es el primer paso más frecuente en la biotransformación de herbicidas, siendo las hidroxilaciones las más frecuentemente observadas en plantas. La destoxificación por hidroxilación y la posterior formación de un conjugado glicósido son especialmente importantes como mecanismos de selectividad y resistencia a herbicidas en monocoti-ledóneas. Muchas de estas reacciones están mediadas por enzimas oxidativas (p.e. citocromo P_{450}) que son las enzimas más importantes en la primera fase del metabolismo de un herbicida (Barrett, 2000). Los agroquímicos pueden influir en los sistemas citocromo P_{450} actuando como efectores, modificando o regulando así el metabolismo de los herbicidas en una planta. Además de las enzimas citocromo P_{450}, las plantas producen otras enzimas oxidativas (peroxidasas, polifenoloxidasas, lacasas y tirosinasas) que catalizan la polimerización de varias anilinas y fenoles (Dec y Bollag, 2001). Las peroxidasas que median el metabolismo de herbicidas que funcionan de manera similar a las P_{450} intervienen en descarboxilaciones, oxidaciones sulfúricas, N-desmetilaciones, hidroxila-ciones del anillo y oxidaciones aromáticas del grupo metilo (Lamoureux y Frear, 1979). En plantas, a menudo las enzimas peroxidasas

funcionan en la tercera fase del metabolismo (p.e. formación de residuos ligados).

Fase II (conjugación). Los conjugados suelen ser los metabolitos finales en los procesos de destoxificación de herbicidas. La naturaleza de estos conjugados suele ser muy diversa, con azúcares, aminoácidos, péptidos y lignina como grupos orgánicos y enlaces de tipo éster, éter, tioéter, amida o glicosídico.

–*Conjugación con glutatión*: Constituye un mecanismo de destoxificación de gran importancia en muchos tejidos vegetales. Se trata de una sustitución nucleofílica en la que el anión glutatión GS- sirve de nucleófilo, actuando los grupos cloro, *p*-nitrofenol o alquil-sulfóxido como posibles grupos a sustituir en la molécula de herbicida (Lamoureux y Frear, 1979). La conjugación con el tripéptido glutatión está catalizada por una familia de enzimas denominadas glutatión-S-transferasas más o menos específicas que se encuentran de manera constitutiva o inducible en muchos tejidos vegetales (Devine et al., 1993).

–*Conjugación con aminoácidos*: De forma general la hidrólisis de glutatión en este tipo de conjugados suele producir un conjugado de cisteína que puede ser posteriormente malonizado. Sin embargo, en el caso del herbicida clorfemprop sólo se ha descrito en trigo el conjugado de cisteína, y no el de glutatión (Pont y Collet, 1980), siendo posible que la cisteína pueda actuar como nucleófilo en una reacción de conjugación similar a la descrita en el glutatión.

–*Conjugación con azúcares*: Los conjugados glicósidos más frecuentes en plantas son los ß-D-glucopiranósidos junto con los N-glicósidos, O-glicósidos y ésteres de glucosa. Este tipo de reacciones están catalizadas por glucosil-transferasas que utilizan UDP-glucosa como donante de glucosa (Mansager et al, 1983). De todas estas reacciones, la formación de O-glucósidos es la más común. Esta formación sigue normalmente a la introducción de grupos hidroxilo en la molécula de herbicida por monooxigenación e implica la conjugación del herbicida o de sus metabolitos con un azúcar, aminoácido o glutatión, incrementando su solubilidad en agua y reduciendo la toxicidad del compuesto.

Generalmente, los metabolitos formados en la fase II tienen poca o nula fitotoxicidad y pueden ser almacenados en orgánulos celulares.

Fase III (deposición). La ruta metabólica seguida por un herbicida afecta en gran manera el uso final de los metabolitos terminales y conjugados. Los conjugados glicósidos se depositan en la vacuola donde quedan almacenados, mientras que los conjugados de origen aminoacídico se excretan a la pared celular donde se integran en el componente de lignina de aquélla, formando un residuo insoluble (Pillmoor y Caseley, 1984). Es decir, esta fase implica la transformación de los metabolitos de la fase anterior en conjugados secundarios con nula toxicidad (Hatzios, 1991). Cabe mencionar que estos procesos de deposición no son completamente irreversibles, la reentrada de las agliconas herbicidas o sus productos de conversión en el pool de herbicida activo intracelular es muy lenta (Devine et al., 1993).

Esta división no constituye una regla general, dado que alguna de las fases puede no estar presente en los procesos de destoxificación. La molécula de herbicida puede ser un proherbicida inactivo que debe ser enzimáticamente convertido en un compuesto activo. A veces, ciertos procesos de conjugación son de carácter reversible, por lo que sólo afectan de manera parcial a la cantidad de herbicida libre intracelular.

La resistencia a herbicidas por destoxificación es un proceso muy frecuente. Sin embargo, este mecanismo suele estar asociado a fenómenos de resistencia cruzada, lo que implica que un mismo individuo tiene la capacidad de metabolizar moléculas muy diferentes pertenecientes a diversas familias químicas. Esta moderada resistencia a un amplio espectro de productos hace extremadamente difícil el control de estos biotipos de malas hierbas con métodos químicos únicamente.

3. MÉTODOS DE ANÁLISIS

3.1. Justificación del uso de métodos analíticos

El uso masivo de herbicidas ha conducido a la situación considerada en las secciones anteriores que conlleva la necesidad del estudio de sus vías

metabólicas, de la naturaleza de los metabolitos y del impacto que los productos resultantes de los procesos metabólicos pueden tener en los vegetales de consumo animal o humano y en el ambiente.

Existen, por tanto, dos áreas en las que es cada vez más necesario estudiar el efecto de los herbicidas: La ambiental, que abarca los aspectos nocivos sobre los seres vivos —y que requiere conocer las concentraciones a las que pueden encontrarse en los diferentes ecosistemas— y la agrícola, en la que el estudio se orienta principalmente a las vías de degradación de los herbicidas y su interacción con la hierba en cuestión para dar lugar a los biotipos sensibles y resistentes (cualidades deseables o no), como en el caso de la *Mucuna pruriens* (Rojano et al., 2011) o *Canavalia ensiformis* (Cruz-Hopólito et al., 2011), entre otras muchas.

En el área ambiental preocupan los residuos de herbicidas y de sus productos de degradación que se encuentra en aguas, suelos, aire, e incluso en animales y en especies vegetales destinados a la alimentación, que en seres humanos pueden provocar alteraciones de diversa índole y gravedad en función de la toxicidad que presentan algunos de ellos. Investigación en este sentido realizaron Andrè C. et al. en 2005 en la que buscaban una relación entre los metales y la asociación herbicida–gamma-synnucleína con posible influencia en enfermedades neurodegenerativas como el Parkinson. Asimismo, existen estudios sobre la interacción de los componentes del suelo con herbicidas tipo atrazina (Lima et al., 2010), del efecto de los ácidos húmicos sobre la adsorción del herbicida paraquat en goethita (Brigante et al., 2010), o de la interacción de metales con las sulfonilureas (Guo et al., 2009), por citar algunos.

En muchos países desarrollados existe legislación sobre los niveles máximos de residuos (MRL) de herbicidas y algunos de sus metabolitos en alimentos, tanto los de consumo humano como los de consumo animal o piensos (Reglamento (UE) Nº 396/2005), así como en los cultivos. Sin embargo, la necesidad de determinar estos compuestos a baja concentración ha hecho imprescindible el desarrollo de nuevos métodos que permiten su identificación y cuantificación de forma rápida, eficaz y en cualquier tipo de matriz. El carácter polar de los herbicidas y la necesidad de separarlos de matrices complejas ha

conducido a que la mayoría de los métodos desarrollados estén basados en el uso de la electroforesis capilar (CE) o de la cromatografía líquida de alta resolución (HPLC o LC) como técnica de separación. Más escasamente se ha utilizado la cromatografía de gases (GC).

3.2. Tipos de muestras/analitos

La mayoría de los métodos desarrollados para la determinación de herbicidas se han aplicado a muestras de agua (Dinelli et al., 1996), una minoría a otras muestras líquidas más complejas como zumos, aceites, etc., y menos aún son los aplicados a muestras sólidas como vegetales, alimentos, etc.

Es conocido que las muestras de agua fortificadas con el herbicida en cuestión requieren poca o nula etapa de limpieza, que se complica cuando se trata de aguas naturales de ríos, lagos, o subterráneas, que presentan cantidades variables de interferentes en función de los cuales varía el número de etapas de preparación y su complejidad.

La importancia de los análisis de herbicidas en aguas desde el punto de vista agronómico se debe a la existencia de una legislación medioambiental que establece el control de los niveles máximos permitidos de estos compuestos en aguas de ríos y de lagos y en aguas subterráneas con el fin de evitar posibles intoxicaciones o destrucción de la fauna de esas zonas. No obstante, la mayor dificultad desde el punto de vista analítico la presentan las muestras sólidas como frutas, semillas, hojas de tabaco, suelo y vegetales, ya que todas ellas requieren una etapa de disolución (digestión) o de extracción más o menos selectiva de los compuestos en estudio mediante lixiviación previa a las etapas de preconcentración, limpieza, separación individual y detección. Por otra parte, en los últimos años se ha incrementado el desarrollo de métodos para la determinación de herbicidas en muestras clínicas, promovido por el número de intoxicaciones, accidentales o no (West et al., 1997; Wu and Tsai, 1998), en las que ha estado implicado de forma mayoritaria el glifosato, uno de los herbicidas más utilizado (Franz et al., 1997).

Respecto a los analitos para los que se han propuesto métodos, cabe destacar una minoría de artículos en los que se determinan conjuntamente los

herbicidas y sus productos de degradación o metabolitos (Aga et al., 1999; Amelin et al., 2012; Arribas et al., 2011; Berger et al., 1998; Chang and Liao, 2002; Chicharro et al., 2004; De Rosssi et al., 2005; Desiderio et al.,1997 a; Garrison et al., 1996; Godwin et al., 2003; Hsieh and Huang, 1996; Hsu and Whang, 2009; Ishiwata et al., 2007; Ishiwata et al., 2004; Iwamuro et al., 2010; Kawai et al., 2011; Komarova and Kartsova, 2003; Komarova and Kartsova, 2002; Lara et al., 2008; Loos and Niessner, 1999; Rojano et al., 2010; Schmitt et al., 1996; See et al., 2010 a; See et al., 2010 b; Tomita et al., 1991; Tříska and Vrchotová, 2002; Tsunoi et al., 2000; Yi et al., 2007; Zerbinati et al., 2000; Zerbinati et al., 1998). La importancia de estos métodos radica en la necesidad de conocer su presencia y concentración para establecer su toxicidad para las plantas, animales e incluso el ser humano, además de para conocer el modo de actuación del herbicida en plantas y suelos. La revisión realizada recientemente por Aliferis and Chrysayi-Tokousbalides (2011) ha puesto de manifiesto la importancia de la metabolómica en el estudio de herbicidas, aspecto al que se ha intentado contribuir con las plataformas desarrolladas en esta Tesis y su posterior aplicación. Es de destacar que muchos de los metabolitos de los herbicidas son estereoisómeros (Marina and Crego, 1997), lo que supone un problema adicional, tal como se pone de manifiesto en las investigaciones realizadas para la optimización de la separación de estos isómeros (André and Guillaume, 2003).

Considerando el uso de la CE en esta área, el trabajo de Hernández-Borges et al. (2004) muestra que la mayoría de los métodos puestos a punto para la determinación de herbicidas se ha aplicado a muestras acuosas naturales (Amelin et al., 2012; Arribas et al., 2011; Aturki et al., 2001; Baggiani et al., 2001; Barroso et al., 1999; Carabias-Martínez at al., 2002; Carabias-Martínez et al., 2000; Chang and Liao, 2002; Chicharro et al., 2005; Chicharro et al., 2003; Chicharro et al., 2004; Desiderio and Fanali, 1992; Frías et al., 2004; Godwin et al., 2003; Hernández-Borges et al., 2005 b; Hickes and Watrous, 1999; Horčičiak et al., 2012; Hsieh and Huang, 1996; Hsu and Whang, 2009; Islam et al., 2012; Komarova and Kartsova, 2003; Komarova and Kartsova, 2002; Kubilius and Bushway, 1998; Núñez et al., 2002; Penmetsa et al., 1996; Pérez-Ruíz et al., 1996; Qin et al., 2002; Rogers et al., 2000; Safarpour et al., 2004; See et al., 2010

a; Tříska and Vrchotová, 2002; Xu et al., 2005; You et al., 2003; Yu et al., 2005; Zhang et al., 2002; Zhou et al., 2010), o incluso fortificadas con los analitos en cuestión (Asami and Imura, 2006; De Rosssi et al., 2005; Desiderio et al.,1997 a; Galceran et al., 1997; Kawai et al., 2011; Loos and Niessner, 1999; Machett et al., 1996; Núñez et al., 2002; Núñez et al., 2001; See et al., 2010 a; See et al., 2010 b; Springer and Lista, 2010; Tříska and Vrchotová, 2002; Zerbinati et al., 2000; Zerbinati et al., 1998). Pocos han sido los métodos desarrollados que se han utilizado para muestras más complejas, como suelo (Aga et al., 1999; Berger et al., 1998; Fu et al., 2009; Garrison et al., 1996; Hernández-Borges et al., 2005 a; Islam et al., 2012; Iwamuro et al., 2010; Liu et al., 2006 a; Orejuela and Silva, 2005; Pérez-Ruíz et al., 1996; Yi et al., 2007), vegetales (Aliferis and Chrysayi-Tokousbalides, 2011; Godwin et al., 2003; Iwamuro et al., 2010; Pérez-Ruíz et al., 1996; Rojano et al., 2010), semillas (Liu et al., 2006 a), alimentos (Carabias-Martínez et al., 2007; Chicharro et al., 2008; Hsu and Whang, 2009; Quesada-Molina et al., 2010; Rodriguez-Gonzalo et al., 2009; Wang et al., 2011), zumos (Khrolenko et al., 2002), aceites (Amelin et al., 2012), leche de soja (Hernández-Borget et al., 2005 c), orina (Lanaro et al., 2011; Lara et al., 2008; Pérez-Ruíz et al., 1996; West et al., 1997; Wu and Tsai, 1998) o sangre (Ishiwata et al., 2007; Ishiwata et al., 2004; Lanaro et al., 2011; Pérez-Ruíz et al., 1996; Tomita et al., 1991; West et al., 1997).

Cuando se considera el binomio CE–muestras complejas conteniendo herbicidas hay que citar las investigaciones de Aga et al. (1999) sobre herbicidas de acetanilida y sus metabolitos en suelos, las de Amelin et al. (2012) sobre glifosato y su metabolito más conocido (AMPA) en aguas superficiales y en aceite vegetal; así como las de Rojano et al., (2010) también sobre glifosato y AMP, además de otros metabolitos del mismo herbicida no determinados conjuntamente en plantas con anterioridad.

Cabe también destacar los métodos desarrollados mediante CE para la determinación de mezclas de herbicidas, bien de la misma familia química (Carabias-Martínez at al., 2002; Carabias-Martínez et al., 2000; Chicharro et al., 2008; Chicharro et al., 2005; Desiderio et al.,1997 a; Desiderio and Fanali, 1992; Galceran et al., 1997; Hernández-Borges et al., 2005a; Hernández-

Borges et al., 2005 c; Hernández-Borges et al., 2005 b; Hickes and Watrous, 1999; Hsieh and Huang, 1996; Ishiwata et al., 2007; Islam et al., 2012; Komarova and Kartsova, 2003; Komarova and Kartsova, 2002; Machett et al., 1996;Núñez et al., 2002; Núñez et al., 2001; Orejuela and Silva, 2005; Pérez-Ruíz et al., 1996; Qin et al., 2002; Quesada-Molina et al., 2010; Quesada-Molina et al., 2007; Rodriguez-Gonzalo et al., 2009; Springer and Lista, 2010; Tsunoi et al., 2000; Wang et al., 2011; Wu and Tsai, 1998, Xu et al., 2005; Zerbinati et al., 2000; Zerbinati et al., 1998; Zhang et al., 2002; Zhou et al., 2010) o de diferentes familias (Aturki et al., 2001; Baggiani et al., 2001; Barroso et al., 1999; Carabias-Martínez et al., 2007; Chang and Liao, 2002; Chicharro et al., 2003; De Rosssi et al., 2005; Frías et al., 2004; Fu et al., 2009; Iwamuro et al., 2010; Kawai et al., 2011; Penmetsa et al., 1996; See et al., 2010 b). Estos métodos resultan de especial interés debido al diferente comportamiento electroforético que presentan las diferentes familias de herbicidas en función de su polaridad, tal y como se describe en el trabajo de Karcher y Rassi (2000) en el que se hizo necesario el uso de medios micelares para la adecuada separación.

Similar es la situación en el caso de los métodos basados en LC, en los que predomina la aplicación a muestras acuosas frente a muestras más complejas. Ishiwata et al. (2007) puso de manifiesto esa tendencia en el estudio de muestras acuosas. Sin embargo, comparada con la CE se puede hablar de mayor aplicación de la LC a muestras complejas como suelo, alimentos, zumos, entre otros (Soler et al., 2008; Yang y Ghosh appl; Hammel et al., appl).

3.3. Alternativas para la optimización de las etapas de un método analítico

Se puede definir la optimización de un método analítico como la búsqueda de las mejores condiciones para conseguir la mayor eficacia posible en la etapa de preparación de la muestra y los valores más altos de sensibilidad y selectividad en la determinación. Para ello se deben estudiar con la adecuada profundidad las diferentes variables de las que depende la consecución de esas condiciones óptimas.

Existen dos formas genéricas de llevar a cabo la optimización de una etapa del proceso analítico: Los métodos univariantes y los multivariantes. En los primeros se fija un valor base de cada una de las variables y se cambia el de una de ellas en un rango hasta conseguir el valor máximo de la señal que se utiliza para medir su efecto. Conseguido este valor óptimo para esa variable, se fija su valor en ese óptimo y se estudia otra de las variables de la misma forma hasta que todas ellas se han optimizado. Estos métodos presentan el inconveniente de que son muy laboriosos (requieren un número grande de experimentos) y si las variables están interrelacionadas nunca se conseguirá el verdadero valor óptimo al mantener una de ellas fija mientras se varía otra.

Actualmente se utilizan más frecuentemente los métodos multivariantes, en los que mediante fórmulas matemáticas se varían simultáneamente los valores de todas las variables que se incluyen en el estudio. Es usual realizar un estudio preliminar de cribado para acotar el rango de los valores de las variables objeto del estudio. El diseño y aplicación de un estudio multivariante proporciona una superficie de respuesta en la que se visualiza la variación del sistema en estudio para cada valor conjunto que se da a las variables.

Tantos los estudios de optimización como los de tratamiento posterior de los datos obtenidos al aplicar el método a una serie de muestras en estudio utilizan como herramienta básica la quimiometría, una ciencia metrológica a caballo entre la química y las matemáticas, que aplica conocimientos matemáticos, especialmente estadísticos, a procesos químicos.

La quimiometría es una disciplina estadística y matemática que permite diseñar o seleccionar procedimientos experimentales óptimos y conseguir la máxima información química relevante de los sistemas químicos mediante el análisis de los datos generados experimentalmente (Massart et al., 1997). Para ello, la quimiometría hace uso de algoritmos matemáticos y de los datos experimentales que le han permitido convertirse en una herramienta imprescindible en química analítica, sobre todo en aquellas técnicas instrumentales que generan una gran cantidad de datos difícilmente procesables sin el uso de herramientas de este tipo.

Existe un gran número de métodos quimiométricos que se utilizan con una amplia variedad de propósitos como, por ejemplo, la optimización de una etapa del proceso analítico con el fin de operar en las mejores condiciones, análisis cualitativo mediante métodos de clasificación o cuantitativos mediante calibración multivariante.

3.3.1. Optimización del proceso analítico: Diseños experimentales

El término "diseño experimental" se utiliza tanto para describir el grupo de experimentos que se realizan con el fin de desarrollar un modelo, bien de calibración o ANOVA, como para la optimización de procesos. En el caso de optimización mediante ANOVA, el diseño experimental se emplea para determinar de una forma eficaz los valores óptimos de las condiciones experimentales que afectan de forma significativa o influyen en el proceso en cuestión. En este contexto, las condiciones experimentales que se pretende optimizar reciben el nombre de factores, que se pueden definir como las variables que se cambian de manera controlada para estudiar su posible efecto en las características de interés del proceso en estudio. Los diferentes valores que se dan a los factores se llaman niveles. La definición de diseño experimental se refiere a la optimización de varios factores, ya que normalmente se estudian todas las posibles variables que se cree pueden afectar al sistema; por lo que el diseño experimental es una aproximación multivariante. Las caracterísi-cas de tipo cuantitativo del proceso que se pretende optimizar se llaman respuestas, mientras que el modelo que relaciona la respuesta con el efecto de los factores se llama función respuesta o superficie de respuesta.

a) Objetivo del diseño experimental. El objetivo del diseño experimental es optimizar de forma eficaz procesos para que se desarrollen de la manera deseada, lo que significa poner de manifiesto el efecto de los factores y/o modelar la relación existente entre ellos y la respuesta con el mínimo número de experimentos. En muchos casos se combinan ambos objetivos de modo que se empieza determinando qué factores afectan a la respuesta y en qué medida (significativamente o no). El siguiente paso consiste en obtener un modelo que describa de forma cuantitativa estos efectos. Finalmente, se define el conjunto

óptimo de los valores que deben tener los factores para conseguir el mejor desarrollo del proceso. Es importante seleccionar de forma apropiada el dominio experimental o, lo que es lo mismo, los niveles extremos a los que se van a estudiar los factores.

El diseño experimental se aplica en muchas áreas de la química y ciencias relacionadas.

b) Estrategias de optimización. Existen dos estrategias de optimización multivariante: Secuencial y simultánea. La primera consiste en llevar a cabo sólo unos pocos experimentos que establecen las condiciones del siguiente experimento, mientras que en la estrategia simultánea se planean todos los experimentos que se van a realizar y, una vez obtenidos los resultados, se evalúan.

Los diseños simultáneos, conocidos también como diseños factoriales, se usan frecuentemente para investigar superficies de respuesta multifactoriales (Deming y Morgan, 1993). Los factores se varían simultáneamente de un modo sistemático para detectar cuáles son significativos y estimar la magnitud de su efecto. Una de las mayores ventajas de los diseños factoriales es que pueden usarse para revelar la existencia de interacciones entre factores si están presentes en un sistema.

Los diseños factoriales se definen mediante el número de factores implicados en el diseño o el número de niveles asignados a cada factor. El diseño factorial más directo y fácil de entender comprende medidas de la respuesta para todas las combinaciones posibles de factores a todos los niveles que les han sido asignados. Así, la definición de un diseño factorial da el número de combinaciones de factores posibles. En general, si P es el número de factores que va a investigarse y k es el número de niveles de cada factor, se generan k^P combinaciones de factores por cada diseño factorial completo. El modelo más frecuentemente ajustado a los datos obtenidos a partir de un diseño factorial a dos niveles 2^2 es:

$$y_{1i} = b_o + b_1 x_{1i} + b_2 x_{2i} + b_{12} x_{1i}\, x_{2i} + r_{1i}$$

En este caso, el número de parámetros a determinar coincide con el número de combinaciones de factores. Por tanto, el número de grados de libertad es cero y no es posible evaluar la falta de ajuste del modelo. Este problema se soluciona eliminando en el modelo aquellos efectos que no parecen ser significativos y permitiendo que aparezcan en los residuales a la variación adscrita a esos efectos. Además, si no se realizan réplicas, no se dispone de grados de libertad para estimar la incertidumbre de las medidas experimentales, lo que se supera normalmente realizando réplicas en el centro del diseño.

Uno de los problemas de los diseños factoriales completos se presenta cuando el número de factores a estudiar es moderado o alto, con lo que el número de experimentos a realizar es muy grande. En estos casos es necesario recurrir a los diseños factoriales fraccionados (Bosé y Carter, 1954), que sirven para llevar a cabo el estudio de un número grande de factores realizando un número moderado de experimentos. En tales casos, sólo se realiza una fracción de los experimentos, que normalmente es 1/2, 1/4 ó 1/8 del total. Al realizar menos experimentos se pierde parte de la información de algunas o incluso todas las interacciones.

En ciertos casos el estudio de las interacciones no interesa, es decir, sólo se pretende identificar qué factores son significativos. En estos casos, los diseños que se emplean son los diseños factoriales fraccionados saturados o el de Placket-Burman (Brereton, 1990), que sirven también para determinar la influencia colectiva de un número elevado de factores en la varianza de los resultados de un proceso sin entrar a distinguir qué factores afectan más, por lo que se han usado en estudios de robustez (Plaket y Burman, 1946; Van der Heyden et al., 1995).

Los diseños factoriales fraccionados y el de Placket-Burman ofrecen una visión general aproximada y son adecuados para construir modelos lineales acerca de la influencia de las distintas variables en el sistema, mientras que cuando se quiere describir una función respuesta en el óptimo se deben usar diseños de superficie de respuesta que consideren al menos tres niveles para cada factor.

c) Variables a optimizar. Como ejemplo de variables a optimizar en una etapa del proceso analítico se consideran los factores o variables que influyen en la separación en CE y las razones que hacen necesaria su optimización: La composición, concentración y pH del electrolito; el voltaje aplicado entre los dos electrodos; la longitud y el diámetro del capilar; la temperatura y la presencia de disolventes orgánicos.

c.1. La *composición, concentración y pH del electrolito* establecen, para cada tipo de analitos, el signo de su carga o su ausencia y, por tanto, el electrodo hacia el que migrará cada uno de ellos; de ahí que la selección del electrolito sea clave para una separación óptima.

c.2. El *voltaje aplicado* permite la manipulación del tiempo de migración teniendo en cuenta que un aumento del voltaje conlleva una disminución de este tiempo y, como consecuencia, un empeoramiento en la separación. Además, voltajes altos pueden dar lugar a intensidades de corriente elevadas, provocando gradientes de temperatura que originan fenómenos de convección, impidiendo una buena resolución.

c.3. La *longitud y el diámetro del capilar* son claves en esta técnica. La primera influye decisivamente en el grado de separación; mientras que cuanto más pequeño es el diámetro mayor superficie de intercambio de calor (producido por efecto Joule) existe en el sistema. El efecto Joule se incrementa con el aumento del voltaje.

c.4. La *temperatura* influye decisivamente en el flujo electroosmótico ya que su aumento disminuye la viscosidad de la disolución del electrolito. Un incremento de temperatura de 1 ^{o}C (por ejemplo, de 20 a 21 ^{o}C), reduce la viscosidad del agua un 2.4%. Por otra parte, el incremento de temperatura disminuye la constante dieléctrica y, por tanto, produce una reducción en el flujo electroosmótico. En el ejemplo anterior, en el que el electrolito es acuoso, la disminución de la constante dieléctrica es del 0.5%, por lo que el efecto neto del aumento de temperatura es un aumento del flujo electroosmótico.

c.5. Los *disolventes orgánicos* aumentan o disminuyen el flujo electroosmótico dependiendo de su naturaleza y su porcentaje, ya que afectan a la viscosidad, a la constante dieléctrica y al potencial Zeta. Por ejemplo, al añadir metanol al agua hasta un porcentaje del 50% aumenta la viscosidad de la disolución, que disminuye cuando el porcentaje de metanol es superior al 50%. Por el contrario, en el caso del acetonitrilo en agua, la viscosidad de la mezcla disminuye progresivamente desde el 0 al 100% de acetonitrilo.

d) Estudio de la precisión del método. Desde un punto de vista estadístico, la precisión se define como la medida de la dispersión de los resultados obtenidos al aplicar repetida e independientemente el mismo método analítico a la misma muestra, entre sí y con su media, lo que se materializa en los errores aleatorios o indeterminados debidos al azar.

La quimiometría es el soporte estadístico para el estudio de la precisión, ya que los parámetros que la caracterizan son la desviación estándar, la varianza, etc. Estas connotaciones cuantitativas de la precisión se complementan con aspectos cualitativos que describen la manera en la que se han obtenido los resultados (experimentación desarrollada, instrumentos, reactivos, patrones, tiempos, etc.), ya que cuanto más diferentes sean las condiciones experimentales mayor será la dispersión de los resultados y menor la precisión. De estas connotaciones cualitativas nacen dos conceptos que matizan la precisión: La repetitividad y la reproducibilidad. La repetitividad es, según la ISO (ISO 1993; ISO 1994), "la dispersión de resultados de ensayos mutuamente independientes, utilizando el mismo método aplicado a alícuotas de la misma muestra, en el mismo laboratorio, por el mismo operador, usando el mismo equipamiento en un intervalo de tiempo corto". Por otro lado, la reproducibilidad la define la ISO (ISO 1993; ISO 1994) como "la dispersión de resultados de ensayos mutuamente independientes utilizando el mismo método aplicado a alícuotas de la misma muestra en diferentes condiciones: Distintos operadores, diferente equipamiento y/o diferentes laboratorios".

En resumen, la repetitividad es la precisión obtenida en las mejores circunstancias posibles y la reproducibilidad es la precisión obtenida en las circunstancias más adversas. Sin embargo, hay situaciones intermedias de modo

que un laboratorio no puede determinar la reproducibilidad como tal, porque tiene que recurrir a otros laboratorios, pero la ISO considera que pueden establecerse unas condiciones intermedias de precisión, definiendo lo que se denomina como repetitividad y reproducibilidad "dentro del laboratorio". Si el que opera es el mismo analista, otros autores prefieren el nombre variabilidad en un mismo día (inter-day variability) o entre días (intra-days variability) para definir la repetitividad y reproducibilidad, respectivamente. En la investigación desarrollada por la autora de esta Tesis se decidió utilizar los términos establecidos por la ISO, aunque no se rechazan los relativos a la variabilidad en un día o entre días.

La metodología seleccionada para el estudio de la precisión consiste en realizar una serie de experimentos con duplicados cada día durante un determinado número de días (Massart et al., 1997). De la tabla ANOVA que se genera con los resultados se obtienen los cuadrados de la media de los residuales, que se denominan cuadrados de la media "en el día". Esto representa s_r^2 en condiciones de repetitividad (variabilidad en el día), siendo s_r la repetitividad. Para determinar la varianza debida al efecto "entre días" ($s^2_{"entre\ días"}$) se usa la siguiente ecuación:

$$s^2_{"entre\ días"} = (ms_{"entre\ días"} - ms_{"en\ el\ día"})/n_j$$

donde *ms* es la media de los cuadrados y n_j es 2.

La reproducibilidad "en el laboratorio", s_{WR}, se calcula mediante,

$$s^2_{WR} = s_r^2 + s^2_{"entre\ días"}$$

El número de réplicas y días varía según las referencias. Por ejemplo, la Société Française des Sciences et Techniques Pharmaceutiques (Commision SFSTP, 1992) establece 6 réplicas y 3 días dependiendo del tipo de precisión intermedia que se necesite evaluar. El National Committee for Clinical Standards recomienda 20 días con determinaciones en duplicado. En la investigación recogida en esta Memoria se han calculado la repetitividad y la reproducibilidad "dentro del laboratorio" mediante la realización de 2 medidas durante 7 días.

3.4. Etapas de la preparación de la muestra

La preparación de la muestra consta, o puede constar, de una serie de etapas, unas imprescindibles y otras que dependen del estado físico de la muestra y de su naturaleza, del tipo de análisis a realizar y del equipamiento analítico a utilizar. Previa a ella ha de realizarse la toma de muestra.

La primera etapa del proceso analítico es la toma de muestra, de la cual depende una de las características analíticas supremas: La representatividad. La dificultad de esta etapa es función de la heterogeneidad del sistema en estudio y de la información que se posea sobre esta característica. La bibliografía está plagada de ejemplos en los que una toma de muestra no representativa ha conducido a unos datos analíticos erróneos y a una interpretación del sistema en estudio más o menos alejado de la realidad, dependiendo de la magnitud del error cometido en la toma de muestra, aun cuando las restantes etapas del proceso analítico se hayan desarrollado correctamente.

A continuación se comentan las diferentes etapas de las que puede constar la preparación de la muestra, que es la parte del proceso analítico más laboriosa, en la que el analista está más implicado y a la que tradicionalmente se le ha prestado menor atención (Luque de Castro y Luque-García, 2002).

3.4.1. Muestras sólidas: Etapa de digestión o lixiviación

Existen pocos casos en los que se puede hacer el análisis directo de la muestra sólida. Las medidas directas en muestra sólidas presentan como principal inconveniente la ausencia de patrones comerciales sólidos de los analitos a determinar en una matriz similar a la de la muestra (Luque de Castro y Luque-García, 2002). Por tanto, la situación más usual es la de análisis por vía húmeda. Las muestras sólidas requieren diferentes tratamientos para llevar a disolución los analitos problema, tratamientos que dependen del binomio matriz–analito.

En la investigación realizada en esta Tesis las muestras han sido siempre sólidas: Plantas de diferentes características y sometidas a diferentes tratamientos. Una etapa fundamental en la preparación de este tipo de muestras previa a la digestión o a la lixiviación es la homogeneización, que puede realizarse mediante: (i) Pulverización de la planta fresca, con la que se favorece

la posterior extracción de los analitos. Puede llevarse a cabo en presencia de nitrógeno líquido para evitar la degradación de ciertos analitos, tal como ocurre en el estudio del glifosato y sus metabolitos en plantas (Rojano et al., 2010). (ii) Pulverización de la planta seca, tras la eliminación de la humedad mediante estufa, o por liofilización. Especial cuidado requiere el control de la temperatura de secado, que no debe afectar a la estabilidad de los compuestos en estudio.

La siguiente etapa —la disolución— puede ser completa mediante digestión más o menos suave dependiendo de la matriz, o parcial mediante lixiviación. Se prefiere esta segunda opción siempre que se asegure la completa disolución de los analitos problema, si se pretende cuantificarlos, ya que la presencia de interferentes se reduce con respecto a la que existe en la muestra totalmente disuelta. La selección del lixiviante adecuado, es decir, suficientemente selectivo, requiere experiencia. La etapa de lixiviación puede ser larga, por lo que se suele acelerar mediante energías auxiliares tales como las microondas (Delgado-Torre et al., 2012; García-Ayudo et al., 1998) ultrasonidos(Delgado-Torre et al., 2012; Priego-Capote y Luque de Castro, 2007) o presión+temperatura altas en la llamada extracción/lixiviación con líquidos sobrecalentados (Delgado-Torre et al., 2012; González-Rodriguez et al., 2004).

3.4.2. Etapa de limpieza y/o preconcentración

Cuando se parte de muestras líquidas —y en el caso de las sólidas una vez realizada la etapa de digestión o lixiviación— generalmente se requiere la separación de especies interferentes y/o la preconcentración de los analitos. Las diferentes técnicas o modalidades de ellas mediante las que se pueden conseguir estos objetivos son las siguientes:

a) Extracción 1íquido–líquido (LLE). En una LLE la disolución de la muestra se pone en contacto con un líquido inmiscible por el que tienen afinidad los analitos. Se produce una transferencia de masa que se favorece con el aumento de la superficie de contacto entre ambas fases. Una vez establecido el equilibrio de transferencia, la relación de concentración de los compuestos entre las dos fases inmiscibles viene dada por el llamado coeficiente de partición. Un problema tradicional en la LLE es la formación de emulsiones que en el caso de las disoluciones que contienen herbicidas comerciales se incrementa ya que incluyen

surfactantes en la formulación. Una tendencia actual para acelerar el proceso de transferencia es precisamente el aumento de la superficie de contacto entre ambas fases mediante la formación de una fina emulsión con auxilio de ultrasonidos (Chicharro et al., 2008; Chicharro et al., 2003; Eash and Bushway, 2000; Font et al., 2008).

b) Microextracción líquido–líquido (LLME). En esta versión miniaturizada de la LLE se utilizan sólo unos pocos microlitros de disolventes en lugar de los hasta cientos de mililitros característicos de la LLE convencional. Existen diferentes variantes en las que puede implementarse esta modalidad miniaturizada, que resulta más barata y más fácil de automatizar que la convencional y que se adapta a las actuales tendencias de la "química analítica verde". En los últimos años, la LLME se ha combinado con LC y CE, además del acoplamiento generalmente usado con GC, y se ha aplicado a diversas matrices, biológicas, ambientales, así como a alimentos. La versión más fácilmente automatizable y que requiere una mínima implicación del usuario es la LLME en fibra hueca desechable (Rasmussen and Pedersen-Bjergard, 2004).

c) Extracción en fase sólida (SPE) o extracción líquido–sólido. La SPE es, en sus diferentes versiones, la técnica de limpieza y/o preconcentración más utilizada en la actualidad. Consiste en poner la muestra líquida (el digerido o el lixiviado, si se ha partido de una muestra sólida) en contacto con un material sólido (llamado sorbente y generalmente empaquetado en un cilindro constituyendo la llamada columna o cartucho de SPE) que retiene de forma selectiva compuestos con unas determinadas características químicas. El resto de la fase líquida sale por el extremo inferior de la columna, que se lava con un líquido capaz de eliminar los restos no retenidos. El paso subsiguiente a través de la columna de un disolvente puro o mezcla de disolventes de características adecuadas separa los compuestos retenidos en un volumen tanto más pequeño cuanta más afinidad tengan las especies retenidas por el eluyente, dando lugar al llamado eluato. Además de las columnas o cartuchos de SPE, el sorbente puede utilizarse en los llamados discos, que permiten mayores flujos y la automatización de esta etapa. Mientras la modalidad manual se utiliza para el

caso de análisis esporádicos o poco frecuentes, la modalidad en línea con la etapa de separación individual (por LC, CE o GC) es cada vez más frecuente y prácticamente la única utilizada en análisis sistemático.

Los sorbentes utilizados en la SPE de herbicidas han sido de muy diferentes características a tenor de las de las especies a retener. Así, se han usado cartuchos de estireno-divinil benceno para el caso de mezclas de sulfunilureas e imidazolinonas(Aturki et al., 2001). Ishiwata et al. (2007) utilizó óxido de titanio para eliminar interferentes como ferrofitinas, hemoglobina y otros en la determinación de glifosato, glufosinato, y de dos de sus metabolitos principales (AMPA y MPP) en muestras de sangre. También se ha utilizado alúmina con recubrimiento de nanopartículas de óxido de hierro para la limpieza y preconcentración de glifosato y AMPA en agua y fruta (Hsu and Whang, 2009) y se ha comparado la sílice con grafito poroso en el caso de herbicidas catiónicos en aguas, demostrándose que el segundo proporciona una mayor limpieza y un mejor factor de preconcentración (Carneiro et al., 2000). En los casos en los que no es posible mejorar ambos aspectos (limpieza y preconcentración) se adopta una solución de compromiso o se mejora uno en detrimento del otro, según convenga. Un ejemplo de este último caso se recoge en el trabajo realizado por Baggiani et al. (2001) en el que se utiliza un polímero molecularmente impreso como material sorbente para la eliminación de fenoxiácidos clorados de muestras acuosas de herbicidas comerciales como el fenoprop y el diclorprop, entre otros. En comparación con C18 en fase reversa, el polímero sorbente presenta menor recuperación, pero mayor limpieza.

En general la SPE se viene utilizando frecuentemente en las últimas décadas para la preconcentración de herbicidas, tal como en el método propuesto por García-Barroso et al. (1999) para la preconcentración de cinco fenilureas en agua de bebida y muestras ambientales, con el que se consiguen límites de cuantificación del orden de 0.05 ppm para estos herbicidas.

Vidal et al. (2012) han publicado una revisión en la que discuten algunas de sus recientes contribuciones y proporcionan una visión general del estado actual de los líquidos iónicos modificados como eluyentes en SPE.

d) Microextracción en fase sólida (SPME). Es una microtécnica que se utiliza para la extracción y separación de compuestos orgánicos, como la mayoría de los herbicidas, e inorgánicos (Pawlyszin, 1997). Es rápida, económica y su aspecto más interesante desde el punto del ahorro de disolventes orgánicos (y por tanto soportando las directrices de la química verde) es que no se utilizan disolventes, es decir, las especies retenidas en el sorbente no se eluyen con un disolvente, sino que se desorben mediante un cambio de temperatura. Se basa en la retención de los componentes de interés en una fibra de sílice fundida (puede estar recubierta con un material sorbente) que forma parte de una jeringa especial y que se expone directamente a la muestra líquida o gaseosa (espacio de cabeza, en este último caso). En la fibra se combinan el muestreo y la preconcentración en una única etapa y en la optimización de esta etapa se consideran variables tales como el tiempo de exposición de la muestra a la fibra, la temperatura, el pH, la fuerza iónica y el tiempo de desorción, entre las más importantes.

La SPME se ha aplicado a la separación de una amplia gama de herbicidas como glifosato y su metabolito AMPA (Hsu and Whang, 2009), triazinas (Zambonin and Palmisano, 2000; Ji et al., 2008), en diferentes matrices como muestras acuosas (Hsu and Whang, 2009), fruta (Ji et al., 2008) y suelo (Zambonin and Palmisano, 2000) con diferentes técnicas como son CE (Hsu and Whang, 2009), LC (Ji et al., 2008) y GC (Zambonin and Palmisano, 2000). Es de destacar que la técnica de alta capacidad de resolución que más frecuentemente se ha acoplado a la SPME ha sido la LC.

e) Microextracción en barra agitada (stirring bar microextraction o SBME). Es una modalidad de la microextracción en fase sólida en la que un pequeño cilindro magnético recubierto de un sorbente apropiado se introduce en la disolución de la muestra y se somete a la acción de un agitador magnético (Magi et al., 2012).

3.4.3. Derivatización

La derivatización puede utilizarse con muy diversos propósitos que dependen del momento del proceso analítico en el que se realiza:

a) Para favorecer la disolución o la lixiviación de la muestra: El reactivo derivatizante forma parte de la disolución de digestión o del lixiviante. En estos

casos el proceso se facilita, según la ley de Le Chatelier, al ir formándose el producto de la reacción.

b) Para mejorar la etapa de limpieza y/o preconcentración: La formación de un producto más fácilmente retenible en un sorbente o que se eluya en un menor volumen de eluyente cuando se trata de SPE; que se extraiga con más facilidad o con mayor rendimiento a la fase aceptora (generalmente por un cambio de polaridad del producto respecto al analito o reactante) en el caso de LLE. En estos casos la reacción de derivatización tiene lugar antes o durante la etapa de preconcentración y/o limpieza (derivatización *in situ*, esta última).

c) Para facilitar o hacer posible la separación individual de los analitos. En este caso, y dependiendo del objetivo perseguido, la derivatización tiene que ser previa (como es el caso de la GC, en el que el propósito suele ser conseguir una mayor volatilidad de los analitos (Seiber, 1991) o una menor termolabilidad), o puede realizarse in situ si el reactivo forma parte de la fase móvil (LC) o del electrolito base (CE).

d) Para hacer posible, más sensible o más selectiva la detección cuando el analito en cuestión no proporciona respuesta en el detector, el factor de respuesta es bajo o la señal es común con la de especies interferentes, respectivamente.

Según lo anterior, se tendrán que utilizar diferentes reactivos dependiendo del fin perseguido y del tipo de analito. Como ejemplo, entre los reactivos más utilizados para conseguir un producto fluorescente (o para aumentar la fluorescencia en el caso de que el analito posea fluorescencia intrínseca, pero débil) están el FMOC-Cl (9- fluorenilmetil-cloroformato) (Bernal et al.,2005; Chang and Liao, 2002), el OPA (*o*-ftaldehído) (Lutchtefeld, 1985), el 6-aminoquinolil-N-hidroxisuccinimidil carbamato (AQC) (Bosch et al., 2008), 7-aminonaphthalene-1,3-disulfonic acid (Mechref and Rassi, 1996) fenilisotiocianato (Amelin et al., 2012), cloruro de *p*-toluensulfonil (Tomita et al., 1991) destacando el FMOC-Cl por su baja toxicidad y su rápida cinética de formación de productos fluorescentes con compuestos que poseen grupos amino.

3.5. Etapa de separación individual de los analitos

Varias son las técnicas analíticas que se han utilizado para la separación individual de herbicidas entre sí, de sus productos de degradación y de otros componentes de la muestra. La EC se ha utilizado con frecuencia (Chicharro et al., 2005; Chicharro et al., 2004; Dinelli et al., 1996, Hickes and Watrous, 1999; Komarova and Kartsova, 2003; Kubilius and Bushway, 1998; Lanaro et al., 2011; Safarpour et al., 2004; Santilio et al., 2009; Tomita et al., 1991), la LC (Berger et al., 1998; Brigante et al., 2010; Hickes and Watrous, 1999), mientras que la GC lo ha hecho en menor proporción (Cserháti et Forgàcs, 1998; Dinelli et al., 1994), debido, sobre todo, a su inadecuación para compuestos no volátiles, termolábiles o muy polares, como es el caso de la mayoría de herbicidas; por lo que, para su uso, es necesaria en la mayoría de los casos una reacción de derivatización que convierta a los analitos en productos menos polares y/o más volátiles. Se aumenta de esta forma el número de etapas y, por tanto, el tiempo requerido para la preparación de la muestra. LC y EC sí permiten la separación de estos compuestos sin necesidad, en la mayoría de los casos, de una etapa de derivatización. En la revisión realizada por Berrada et al. en 2003 se muestra la aplicabilidad de cada una de estas técnicas para la separación de herbicidas como las fenilureas y las sulfonilureas y de insecticidas como las benzoilureas; o la realizada por Fangshi y Juan en 2005 sólo para fenilureas.

Se comenta a continuación la CE por ser la menos conocida de las dos técnicas que se han utilizado en la investigación recogida en esta Memoria para la separación individual de los herbicidas y sus metabolitos.

3.5.1. La electroforesis capilar

El fundamento de la CE es la diferente velocidad con la que se desplazan los analitos en función de su relación carga–tamaño a lo largo de un capilar en cuyos extremos existen dos electrodos entre los que se establece una adecuada diferencia de potencial.

a) Tipos de CE. Las modalidades de CE que se han utilizado con más profusión para la separación individual de herbicidas son las siguientes:

a.1. La *electroforesis capilar de zona (CZE)* es una de las modalidades más utilizada debido, probablemente, a su simplicidad y elevado poder de separación. Basada, por supuesto, en la separación de los analitos según la relación carga/tamaño, en esta modalidad la composición del tampón es constante en todo el capilar. El potencial aplicado hace que los diferentes componentes iónicos de la mezcla migren cada uno según su movilidad y se separen en zonas que pueden estar completamente resueltas o parcialmente solapadas. Entre las zonas completamente resueltas el capilar está ocupado por el tampón. Su principal inconveniente es que no permite separar los compuestos neutros. Los tampones a utilizar en esta modalidad electroforética son muy variados (e.g. formiato–ácido fórmico, fosfato–ácido fosfórico, borato–ácido bórico y acetato–ácido acético), siendo los más usados el tampón fosfato en el intervalo de pH 2 – 7 (Barroso et al., 1999; Chicharro et al., 2004; Lanaro et al., 2011; Pérez-Ruíz et al., 1996) y el borato en el entorno de pH 9 (Qin et al., 2002; Quesada-Molina et al., 2007; Springer and Lista, 2010; Zhang et al., 2002).

a.2. El *enfoque isoeléctrico* se utiliza especialmente para la separación de proteínas aprovechando la diferencia de sus puntos isoeléctricos, por lo que, en relación con los herbicidas, se ha utilizado para comprobar el efecto de los segundos sobre las primeras. En esta modalidad se establece un gradiente de pH estable en una disolución o en un gel.

a.3. En la *isotacoforesis* (electroforesis a velocidad uniforme) la muestra se introduce entre un tampón frontal y otro terminal, de manera que sus componentes se separan en zonas entre ambos tampones que se mueven a la misma velocidad. En esta modalidad las bandas están en contacto y se requieren usualmente desarrollos independientes para la separación de iones positivos y negativos.

a.4. La *cromatografía electrocinética capilar (MEKC)* requiere el uso de un tensoactivo a una concentración en la que se formen micelas (por encima de la concentración micelar crítica, CMC) capaces de alojar compuestos no polares en su interior.

De las modalidades CE las más utilizadas para estudios de herbicidas han sido la CZE y la MEKC. Matchett et al. (1996) hizo una comparación entre ambas técnicas para la separación de 7 sulfonilureas en agua con posterior detección mediante espectrometría de masas (MS), llegando a la conclusión de que la eficacia de cada una de ellas para la separación de herbicidas viene dada por las características del tampón de desarrollo utilizado.

b) Modos de inyección de la muestra en CE. Son dos las formas más comunes de introducción de la muestra en CE: Inyección electrocinética, e inyección hidrostática.

b.1. La inyección hidrodinámica de la muestra se basa en el establecimiento de una caída de presión a lo largo del capilar, lo que puede conseguirse mediante tres alternativas experimentales:

–*Inyección a presión*: Mediante un gas auxiliar se establece una presión sobre la muestra en la que está sumergido el capilar, lo que requiere un sistema cerrado.

–*Inyección por gravedad*: Se crea una presión hidrostática por gravedad mediante el llenado del capilar hasta una altura dada en un tiempo definido.

–*Inyección por vacío*: Se aplica vacío por el extremo del capilar, lo que induce la succión de la muestra por el extremo en contacto con la misma.

b.2. En la inyección electrocinética la muestra se desplaza al interior del capilar mediante el movimiento electroforético o electroendosmótico producido por la generación de un campo eléctrico.

En todos los casos los volúmenes de muestra inyectada son muy pequeños, del orden de 2 a 20 pL. Estos modos de inyección sirven en ocasiones para aumentar la sensibilidad por preconcentración, tal y como ocurre con la inyección hidrodinámica (Aturki et al., 2001) o electrocinética (Carabias-Martínez et al., 2000), o para la limpieza de la muestra (Rojano et al., 2010).

3.6. Técnicas de detección

Los detectores utilizados en CE y en LC están basados en una amplia variedad de técnicas, tanto ópticas espectrométricas —espectrometría de absorción molecular

(EAM) en la zona ultravioleta y visible, espectrofluorimetría molecular (EFM)— como en electroquímicas (amperometría, potenciometría, conductimetría). Especial mención merece la espectrometría de masas.

3.6.1. Técnicas ópticas

Existen dos modos de detección en las técnicas ópticas: Directa e indirecta.

La detección directa se utiliza cuando los analitos (o sus productos de reacción) son activos en el detector mientras que la fase móvil o el tampón de desarrollo (en LC y en CE, respectivamente) no lo son o lo son débilmente. El paso por la célula de medida del detector de la fase móvil o el tampón de desarrollo originará una señal constante que corresponderá al cero o blanco instrumental; mientras que el paso del analito o su producto de reacción producirá un aumento de la señal (pico positivo).

La detección indirecta es la vía que se adopta cuando los analitos no son activos en el detector. En este caso se utiliza un tampón de desarrollo o una fase móvil que sí es activo en el detector en cuestión. El blanco se establece en un valor alto de señal del detector y al paso del analito por la célula de medida se producirá una disminución brusca de la señal (pico negativo). De esta forma se evita la derivatización de los compuestos que no son activos en el detector. Esta es la forma de detección utilizada en una de las plataformas analíticas propuestas por la doctoranda para la determinación de glifosato y sus metabolitos mediante CE–EAM (Rojano et al., 2010), en la que se usa un tampón formado por fosfato, CTAB (hexadeciltrimetilamonio), acetonitrilo y ftalato potásico, siendo este último el compuesto que tiene el grupo cromóforo, que responde en el DAD (detector de fotodiodos en fila). También la fluorescencia con detección indirecta se ha utilizado para la determinación de herbicidas (Chang and Liao, 2002).

a) La *espectrometría de absorción molecular en la zona UV-Visible* (especialmente con el uso de DAD) es la más utilizada debido al precio de los instrumentos basados en ella, a la estabilidad, la robustez y los valores aceptables de sensibilidad y rango dinámico de trabajo. La escasa selectividad de esta forma de detección es un problema fácilmente superable con una buena y reproducible separación individual de los analitos. Existe en la bibliografía un

amplio número de métodos para herbicidas en los que se hace uso de esta forma de detección. En la investigación desarrollada por la doctoranda se ha utilizado este tipo de detección, tanto en CE como en LC. El uso del DAD permite la medida simultánea de la absorción molecular a diferentes longitudes de onda, lo que aumenta la sensibilidad del método en cuestión y especialmente la selectividad ya que reduce la necesidad de separación individual cuando los espectros de absorción de los analitos lo hace posible (longitudes de onda de absorción no comunes).

b) La *espectrofluorimetría molecular* aventaja a la EAM en sensibilidad y selectividad. La ausencia de grupos fluoróforos en muchos herbicidas hace necesaria una etapa de derivatización con la que se introduce este grupo en la molécula. La variante más interesante de la técnica es aquella en la que la excitación se realiza mediante un láser (fluorescencia inducida por láser o LIF), ya que permite aumentar la sensibilidad en varios órdenes de magnitud. Una forma de obviar la etapa de derivatización en CE cuando los analitos no son fluorescentes es la determinación indirecta (utilizar un tampón que sí es fluorescente), tal como en el método propuesto por Chang and Liao (2002) para la determinación de glifosato, AMPA, glufosinato y MPP mediante fluorescencia indirecta, utilizando fluoresceína como tampón fluoróforo y un láser de ion Ar para inducir la fluorescencia. Este tipo de medida puede, en ocasiones, evitar etapas de limpieza, generalmente laboriosas (Orejuela and Silva, 2005).

c) La *detección electroquímica*, poco frecuente en métodos para la determinación de herbicidas, ha estado basada en el uso de la conductimetría, la amperometría o la potenciometría, y más que para análisis sistemático se ha utilizado en investigación, poniéndose de manifiesto que pueden proporcionar límites de detección uno o dos órdenes de magnitud más bajos que la EAM. Si bien lo usual es utilizar estas técnicas para compuestos electroactivos, también se ha utilizado para los no electroactivos, previa derivatización. La combinación de conductimetría y EAM en CE ha permitido conseguir una excelente sensibilidad y selectividad en análisis de herbicidas (Chicharro et al., 2008; Chicharro et al., 2005; Chicharro et al., 2003).

d) La *espectrometría de masas* es actualmente la técnica de detección más sensible (a excepción de la LIF) y selectiva que existe. Dependiendo del tipo de instrumento en el que se materialice la técnica, la identificación absoluta o relativa es otra de las ventajas de la MS. De las diferentes interfaces que hacen posible la conexión de un sistema dinámico líquido (tal como un LC o CE) con un detector de masas, la ionización por electrospray (ESI) es una de las más utilizadas. Esta interfaz se considera la más adecuada para el acoplamiento CE–MS, sobre todo porque permite que las moléculas pasen directamente al estado gaseoso. El primer acoplamiento CE–MS se realizó hace más de 20 años y proporcionó una excelente herramienta para una amplia variedad de aplicaciones, entre las cuales se encontraba el análisis de alimentos, con especial énfasis en la seguridad y calidad alimentarias. La idoneidad del acoplamiento CE–MS para la determinación de herbicidas y plaguicidas fue puesta de manifiesto por Lee et al. (1989) con la separación de sulfonilureas. En 2005 (Huck et al., 2005) se publicó una revisión de la aplicación del acoplamiento al análisis de compuestos fenólicos y sus derivados, en la que se consideraban especialmente las diferentes interfaces CE–MS usadas hasta la fecha.

3.7. Uso de estándares internos

Los estándares internos se emplean en aquellos métodos instrumentales, bien sea de emisión o absorción atómica, que permiten la determinación multielemental. Un patrón interno es una sustancia que se añade a todas las muestras, blancos y patrones de calibrado en una cantidad fija. También puede ser un componente mayoritario de las muestras que se agrega a los patrones en concentración lo suficientemente elevada como para considerar que es la misma en todas las muestras. En este caso, el calibrado es una representación gráfica del cociente entre la señal del analito y la señal del patrón interno en función de la concentración de analito en los patrones. Este cociente también se utiliza para determinar la concentración del analito en las muestras a partir de una curva de calibrado. Si se elige y se usa adecuadamente un patrón interno, se pueden compensar algunos errores aleatorios o sistemáticos, como derivas, ruido de parpadeo, algunas interferencias no espectrales producto de la matriz, etc. ya que

el cociente de las señales del analito y del patrón interno es independiente de las fluctuaciones y de algunos efectos de la matriz. Cuando el patrón interno es el componente mayoritario de las muestras y de los patrones también puede suceder que se compensen los errores producidos en la preparación de las muestra. La mayor dificultad para aplicar el método del patrón interno es encontrar la sustancia adecuada que sirva para compensar estos efectos, así como para incorporarla a las muestras y a los patrones de forma reproducible y barata. El patrón interno deberá dar una señal similar a la del analito en la mayoría de los casos. De manera adicional, las interferencias no espectrales deben afectan al estándar interno de igual manera que al analito. Por esta razón la elección del estándar interno es clave para la aplicación rigurosa del método. Los criterios de elección del estándar interno son variables dependiendo de la técnica analítica a emplear en la determinación. En el caso de los detectores de masas es común el uso de isótopos estables como estándares internos (Häubl et al., 2005). Los elementos utilizados como estándares internos deben cumplir una serie de requisitos como no estar presentes en las muestras, no interferir espectralmente con la masa del analito, tener un potencial de ionización similar al del analito y utilizarlo para un grupo de analitos dentro de un rango de masas similar.

Algunos de los patrones internos más comunes son: ^{9}Be, ^{45}Sc, ^{59}Co, ^{74}Ge, ^{89}Y, ^{103}Rh, ^{115}In, ^{169}Tm, ^{175}Lu, ^{187}Re y ^{232}Th.

3.8. Comparación de los métodos de determinación de herbicidas basados en CE y en LC

Debido a su mayor antigüedad y a la variedad de fases estacionarias y móviles existentes, los métodos propuestos para el análisis de herbicidas basados en LC superan en número a los desarrollados mediante CE. No obstante, existe en CE una gran variedad de tampones y aditivos, la mayoría de ellos no tóxicos y con un consumo extremadamente bajo, aspecto este último también asequible actualmente en las modalidades micro- y nano de LC. En términos de instrumentación la CE es más simple, ya que estos equipos no requieren inyector, bomba o mezclador de disolventes. En CZE se utilizan capilares de gran longitud y con secciones pequeñas, lo que significa que la resistencia eléctrica a lo largo

del capilar es grande. De esta forma se favorece la disipación de calor (que causa el calentamiento de la disolución por efecto Joule). Como consecuencia, el ensanchamiento de banda debido a la mezcla por convección térmica no es significativo; de modo que las anchuras de los picos en electroforesis capilar se aproximan a los valores teóricos propios de la difusión longitudinal y se encuentran así eficacias de varios cientos de miles de platos. También hay que destacar que en EC se usan capilares de sílice que son mucho más económicos que las columnas de LC. El mismo capilar puede utilizarse para separaciones y análisis de compuestos polares, no-polares, quirales o biomoléculas grandes, tales como proteínas; se trabaja a temperatura ambiente y a baja presión sin necesidad de bombeo del líquido en el capilar. Comparando con las columnas cromatográficas en LC, los capilares son relativamente baratos, fáciles de utilizar y de larga duración, si bien se reemplazan con cierta frecuencia a causa de la obturación. En LC con elución isocrática los tiempos de retención son más largos y los picos más bajos, debido a la dispersión que experimentan los solutos en la columna. Si se comparan las eficiencias de la separación, N, es de cientos de miles de platos hasta millones de platos teóricos en la CE frente a los aproximadamente 20.000 en LC. La razón es la creación de un flujo plano en CE frente al flujo laminar de la LC. El volumen de nuestra es de microlitros en CE y del orden de los microlitros en LC (ahora bastante igualados en las modalidades micro y nano de la LC). El consumo de reactivos en un día de trabajo es de 500–1000 mL de fase móvil para LC, mientras que es sólo de pocos mL de tampón en el caso de la CE.

En LC al igual que en CE existen diferentes modos, con un cierto paralelismo entre los modos de ambas técnicas. Por ejemplo, la cromatografía de intercambio iónico es similar a la electroforesis capilar en zona (ECZ) o electroforesis capilar en zona libre y al isoelectroenfoque capilar (IEEC), ya que las separaciones se basan en las diferencias de carga y tamaño entre los analitos; la cromatografía en fase reversa se asemeja a la MEKC; la cromatografía de exclusión por tamaños y la electroforesis capilar en gel separan atendiendo al tamaño molecular de las sustancias a separar, y las interacciones por afinidad usadas en cromatografía se aprovechan también en CE.

Otros aspectos que asemejan o diferencian la LC de la CE son los siguientes:

Los sistemas de LC también están automatizados al igual que en CE.

La separación de macromoléculas en LC es menos eficiente que en CE, ya que los biopolímeros son resistentes a las transferencias de masa, y por tanto, se separan más eficientemente mediante técnicas "non-partitioning". Las moléculas pequeñas sí se ajustan bien a las capacidades de la cromatografía debido a su buen coeficiente de difusión y sus características en lo que a transporte de materia se refiere.

En LC las fuerzas de fricción entre el sólido y el líquido dan lugar a perfiles de flujo laminares o parabólicos, existiendo un gradiente de velocidad importante que da lugar a un perfil de velocidades que es mayor en el centro y menor en las proximidades de las paredes. En CE, el flujo final tiene un perfil prácticamente plano, por lo que todos los solutos presentan la misma velocidad y presentan picos estrechos.

Los tiempos de retención en LC no tiene correlación con los tiempos de migración en CE, el orden de elución no es el mismo.

La LC consume mayor cantidad de fase móvil que la CE en tampón de desarrollo.

La longitud del paso de luz en la celda de detección en LC es mayor que en CE (milímetros frente a micrómetros); lo que hace que la sensibilidad en LC sea mayor que en CE.

En LC la separación de los analitos se realiza en columna y la detección tiene lugar una vez que se han eluido. El detector está localizado después de la columna separativa y la detección se lleva a cabo fuera de ella. La velocidadd a la cual viajan los analitos a través del detector es independiente de la columna y de la separación. Ésta sólo depende del flujo de la bomba de alta presión. Si el flujo es constante, entonces todos los analitos se moverán a través del detector a una velocidad constante. Por el contrario en CE la detección es en columna. Como la separación se basa en que los analitos migran a diferente velocidad, éstos pasarán a través del detector a diferente velocidad: Por lo tanto, mientras los cromatogramas pueden integrarse, los electroferogramas no.

Las comparaciones de ambas técnicas por autores diferentes han conducido a veces a resultados contradictorios. Así, mientras en un artículo de Dinelli et al. (1994) se compara la CE, la LC y el inmunoensayo con detección UV–Vis y se enfatiza la facilidad de manejo de la CE, la velocidad de la LC y la selectividad y sensibilidad del inmunoensayo, Safarpour et al. (2004) ponen de manifiesto la escasa preparación de la muestra requerida en CE frente a la necesaria en LC cuando se trabaja con muestras vegetales. Menne et al. (1999) destaca que aunque en ambas se da la separación de todos los compuestos, en LC se desarrolla en la mitad de tiempo que en CE; mientras que en la comparación entre NACE y LC con detección UV–Vis en el estudio de herbicidas en aguas realizado por Carabias-Martínez et al. (2007) se consiguen límites de detección más bajos con NACE. Por tanto, cada una de las técnicas presenta sus ventajas y limitaciones características, que en muchos casos son consecuencia de las características concretas de los equipos utilizados.

En ocasiones se ha utilizado una de las técnicas para la confirmación de los resultados obtenidos con la otra, como en el caso de la determinación de metsulfuron-metil en suelos (Zhu et al., 2007), en la que un método basado en LC sirve como confirmatorio de otro basado en CE.

Dado que las modalidades de micro y nano LC son muy recientes no existen en la bibliografía datos de su comparación con la CE para herbicidas.

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HERRAMIENTAS AGRONÓMICAS Y ANALÍTICAS UTILIZADAS

En este apartado se comentan algunos de los dispositivos e instrumentos más importantes utilizados en el desarrollo de la parte experimental de la investigación recogida en esta Memoria.

1. Sistemas para la preparación de muestras

Dispositivos de ultrasonidos

Para la etapa de lixiviación de los analitos de las plantas problema se utilizaron un baño de ultrasonidos equipado con una unidad de control de temperatura, así como una sonda ultrasónica. Existen dos dispositivos básicos en el laboratorio analítico para aplicar ultrasonidos: Los baños y las sondas.

Aunque los baños se utilizan con mayor frecuencia debido a su menor precio y omnipresencia en el laboratorio, las sondas tienen las ventajas de concentrar la radiación de ultrasonidos en una zona concreta, aumentando así su acción, y de no sufrir el fenómeno de fatiga con el tiempo, proporcionando, por tanto, una mayor reproducibilidad de los resultados, particularmente en tratamientos largos.

La sonda de ultrasonidos utilizada fue una Branson 450 digital, que permite la selección de la amplitud de la radiación así como el modo irradiación, continuo o discontinuo. Esta sonda se utilizó para, simultáneamente, formar una emulsión y acelerar el proceso de extracción. Para ello la sonda se introdujo directamente en un recipiente que contenía la muestra sólida y los extractantes.

Dispositivo de microondas

Se utilizó un digestor comercial Microdigest 301 fabricado por Prolabo, basado en microondas focalizadas, para la extracción de los compuestos objetivo de las plantas.

Centrífuga

La centrifugación de los extractos se realizó con una centrífuga Coulter Avanti J-25, dotada con un sistema de control de temperatura.

Baños de agua termostatizados

Para la incubación de muestras en reacciones enzimáticas o no se utilizaron baños de agua con sistema de recirculación que permite homogeneizar en todo momento la temperatura del baño.

Cámara de pulverización de herbicidas

Consiste en una cámara cerrada con un mecanismo mecánico de desplazamiento del sistema de pulverización cuya velocidad y altura pueden controlarse por el operador mediante un panel de control. Además de ser compatible con los diferentes tipos de boquilla existentes en el mercado (salida en abanico, en cono, etc.), la cámara está dotada de un sistema de limpieza para evitar la contaminación entre muestras.

Oxidador biológico

Se trata de un sistema de combustión de muestras sólidas que permite transformarlas en muestras líquidas, evitando reacciones indeseadas y problemas de solubilidad. Muy útil en estudios de radiactividad, en los que es necesario que la manipulación de la muestra sea mímina. Además, el modelo utilizado (A307 Sample Oxidizer) aumenta la sensibilidad en el contador de centelleo puesto que evita solapamiento de espectros, reacciones de quimioluminiscencia y amortiguación de señales debido a los reactivos utilizados y al sistema de llama abierto/cerrado, con el que consigue una combustión del 100%. Este sistema permite recuperar el carbono marcado en un 99%.

2. Instrumentos

Electroforesis capilar

Se ha utilizado la electroforesis capilar en su modalidad de zona, con electroinyección (es decir, con aplicación de un voltaje capaz de introducir los componentes cargados de la muestra a través del capilar, evitando así la entrada de compuestos neutros). El equipo (3-D Capillary Electrophoresis Agilent G1600A Instrument) equipado con una unidad Peltier de termostatación, está acoplado a un espectrofotómetro de diodos en fila, DAD (190-600 nm), que permite adquirir al mismo tiempo los espectros de cada uno de los analitos en un

mismo análisis, para la identificación a través de su espectro y por comparación con los espectros de los patrones. Debido a la falta de grupos cromóforos en los analitos se utilizó detección indirecta, originándose picos negativos.

Espectrofotómetro

El espectrofotómetro utilizado es un sistema autónomo completo que incorpora: la CPU, la interfaz gráfica de usuario, el monitor, el teclado, el ratón, sistema de control de temperatura, bomba de intercambio gaseoso y puertos E/S.El rango de longitud de onda va desde 190 a 1100 nm. El equipo cuenta con programas para estudios a longitud de onda fija, barrido de longitud de onda, corrección de la dispersión, determinación cinética y la manipulación de los espectros obtenidos. Este equipo permite hacer análisis cuantitativo de la proteína total, estudiar el mecanismo de actuación de la enzima (determinación de la Km, Vmax, Kcat, Ki), actividad enzimática, análisis cuantitativo individual de varios componentes, análisis espectral simultáneo de varios componentes y validación del rendimiento.

Contador de centelleo líquido

Para la detección de la actividad radiactiva. Las muestras se introducen en viales especiales de centelleo. La detección está basada en el recuento de los centelleos que produce una muestra que contiene agentes radiactivos al ser excitada (fluorescencia). Puede trabajar con volúmes de muestras de 20, 7 o 3 mL, con una resolución de hasta 0.002 Kev. El tiempo entre cada muestras es de aproximadamente 5 segundos. Las unidades de medida son DPM, CPM y Kbq (para isótopos). Además, puede programarse y corrige el "quenching" o atenuación. Como patrones sólo pueden utilizarse ^{14}C y ^{3}H.

Generador de imagen de fósforo

Este sistema de tipo ciclón permite obtener imágenes de las muestras de alta resolución sin necesidad de realizar autorradiografías que se afectan mucho por la temperatura, la luz y por la presencia de impurezas en los reactivos. Este equipo es compacto, rápido y fácil de usar. Proporciona una alta sensibilidad y

precisión en todas las imágenes radiométricas. No necesita reactivos reveladores puesto que basta con las pantallas o láminas de fósforo para detectar la actividad de las muestras. El diseño de exploración helicoidal permite captar la señal de manera más eficiente para una mayor sensibilidad y un amplio rango dinámico lineal. Las pantallas de fósforo se analizan mediante un láser, creándose una imagen latente de alta resolución cuya intensidad permite la cuantificación. La imagen se visualiza en la pantalla para el análisis con el software OptiQuant ™ y puede imprimirse, exportarse y archivarse para usos posteriores.

Equipos cromatográficos

Se ha utilizado la cromatografía de líquidos de alta presión en su modalidad de fase reversa, es decir, una fase móvil polar y una fase estacionaria apolar (C18 en todos los métodos desarrollados, excepto para los metabolitos del glufosinato, que se utilizó una fase estacionaria de interacción hidrófila —HILIC— diseñada para la separación de compuestos polares o iónicos, que tienen poca o ninguna retención en las columnas de fase reversa. Durante el desarrollo de esta Tesis se han utilizado distintos modelos de cromatógrafos de líquidos de diferentes firmas, entre ellos los modelos Agilent series 1200 y Beckman Coulter Gold, todos ellos acoplados a detectores tales como los de masas triple cuadrupolo Agilent, modelos 6410 y 6460, ambos con ionizadores por electrospray (ESI), el segundo mejorado respecto al primero por introducción de la tecnología Jet Stream, que incrementa la sensibilidad gracias a un gradiente térmico focalizado de N2 (ESI Jet Stream).. Completan la gama de los detectores utilizados un detector de masas cuadrupolo-tiempo de vuelo, modelo 6540; un detector fotométrico ultravioleta-visible de diodos en fila (DAD), modelo HP 1100 y, por último, un detector ultravioleta PDA, Varian ProStar 330.

Cada uno de los instrumentos empleados se detalla en los capítulos en los que se ha utilizado.

3. Técnicas quimiométricas

La metodología del diseño de experimentos se ha utilizado en la optimizacion de los métodos analíticos.

La precisión de los métodos propuestos se ha estudiado como reproducibilidad dentro del laboratorio y repetibilidad mediante series de experimentos por triplicado usando análisis de varianza (ANOVA) a diferentes niveles de concentración de los analitos.

El tratamiento de datos se ha realizado con distintos programas informáticos: Statgraphics, Sigma Plot y Statistix, The Unscrambler and Mass Profiler Professional y METLIN necesarios para realizar estudios univariantes mediante regresión lineal simple y regresión logística simple, así como estudios de tratamientos multivariantes e identificativos.

PARTE EXPERIMENTAL

Parte A:

DESARROLLO DE PLATAFORMAS ANALÍTICAS PARA EL ESTUDIO DEL METABOLISMO DE HERBICIDAS

Las plataformas que se han desarrollado y que se recogen en esta Parte A de la Memoria constituyen la base para obtener la información necesaria para profundizar en el estudio del metabolismo de los herbicidas en cuestión. La optimización de las plataformas, la caracterización y la validación mediante aplicación a muestras concretas han dado lugar las publicaciones que constituyen los capítulos de esta parte de la Memoria.

El Capítulo 1 recoge una revisión bibliográfica sobre el uso de la electroforesis capilar (CE) para el estudio de herbicidas, que se ha enviado a la revista Phytochemistry para su publicación y que es el fruto de la experiencia adquirida por la doctoranda en este campo.

El Capítulo 2 está constituido por un método basado en CE para la identificación y cuantificación de todos los metabolitos del glifosato en plantas, que no habían sido determinados de forma simultánea hasta el momento. El estudio se realizó con muestras reales cuya resistencia a este herbicida era conocida, pero cuyo metabolismo no estaba totalmente dilucidado. Los resultados obtenidos con este estudio dieron pie a la investigación que se recoge en el siguiente capítulo.

La investigación que se recoge en el Capítulo 3 se orientó al desarrollo de un método rápido y fiable para la determinación de glioxilato, ya que este compuesto intermedio en el metabolismo del glifosato presenta problemas para su determinación en plantas. Por ello se optimizó un método ya existente que se había utilizado en muestras acuosas, pero no en vegetales. Se consiguió así un método espectrofotométrico simple, rápido y eficaz que puede utilizarse como método de rutina en laboratorio y en campo.

En el Capítulo 4 se recoge un método simple basado en cromatografía de líquidos con detección por absorción UV (LC–UV) para la determinación simultánea de imazamox y sus dos metabolitos (el compuesto hidroxilado y el conjugado glucosídico). Debido a la ausencia de estándares comerciales, la presencia de metabolitos del imazamox fue confirmada por LC–TOF/MS, siendo cuantificadas de forma relativa respecto al imazamox. Este estudio se validó con muestras de trigo tratadas con 200 gramos de ingrediente activo de imazamox por hectárea (200 g de ia Ha^{-1}).

El método para el estudio del metabolismo del glufosinato recogido en el Capítulo 5 está basado en LC–V previa derivatización de algunos analitos con cloruro de fluorenilmetoxicarbonilo (FMOC-Cl) para facilitar la separación. La identificación de metabolitos no disponibles comercialmente se realizó mediante LC–TOF/MS, con el que también se confirmaron todos los analitos. Este estudio se realizó utilizando plantas de trigo tratadas con glufosinato a una dosis normal de aplicación (300 g de ia Ha^{-1}).

Un aspecto a destacar de los estudios experimentales que conforman esta parte de la Memoria es la mejora realizada en la preparación de la muestra, en la que la etapa de lixiviación se ha acelerado de forma drástica con auxilio de ultrasonidos.

Capítulo 1

Capillary electrophoresis–herbicide analysis: An excellent marriage

Capillary electrophoresis–herbicide analysis: An excellent marriage

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Capillary electrophoresis–herbicide analysis: An excellent marriage

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Abstract This article does not intend for an exhaustive review on the existing literature on capillary electrophoresis (CE)–herbicides, but only to provide a general overview on the potential of this binomial. To this end, the features of the methods for herbicides based on CE, the types of samples and analytes to which the methods have been applied, the sample preparation steps required, mainly for cleanup and preconcentration, but also derivatization steps, and the types of detection applied are critically discussed and CE compared with liquid chromatography when appropriate. The role of MS detection in present and future analytical research in this field (both in identification and quantitation) are commented. The foreseeable and desirable trends in the analysis of herbicides are also outlined in the light of the present trends in metabolomics as a way of knowing the pathways, the intermediate and final degradation products which can influence the crops and, in general, the food chain of humans and other animals.

Keywords Herbicides • Capillary electrophoresis • Sample preparation • Detectors

Abbreviations

AMPA aminomethyl phosphonic acid
BGE background electrolyte
CE capillary electrophoresis
ESI electrospray ionization
FESI Field-Enhanced Sample Injection
GC gas chromatography
HPLC high-performance liquid chromatography
LC liquid chromatography
LLE liquid-liquid extraction
MCPA 2-methyl-4-chlorophenoxyacetic acid

MS mass spectrometry
MEKC micellar electrokinetic chromatography
SPE solid-phase extraction
SPME solid-phase microextraction
TOF time of flight
2,4-D 2,4-dichlorofenoxiacetic acid

1. Introduction

Herbicides are chemicals mainly of polar character used to kill or inhibit the growth of plants, especially weeds. Since the introduction of the first growth regulator herbicides (MCPA and 2,4-D) in 1945, the use of such compounds has increased in such as manner that far more than two hundred of herbicides of different chemical nature exist at present worldwide (HRAC, 2012). These products have revolutionized the agricultural sector in just 60 years by increasing production. However, despite their beneficial effects, the abuse in the use of herbicides is causing major agricultural and environmental problems.

Agricultural problems come from the increase in the number of resistant weeds (Heap 2011), resulting in uncontrol of them and a competition with the crop, which results in economic losses. This raises the need for understanding the increased resistance, which can be due to detoxification by metabolism of the given herbicide in the plant, mechanisms inherent to the mutation itself, herbicide uptake by increasing the waxy coating on leaves, herbicide translocation, etc. (Powles and Yu, 2010). Therefore, studies dealing with degradation pathways of herbicides and interaction with the target plant to yield sensitive and resistant biotypes, as is the case of Mucuna pruriens (Rojano et al., 2011) or Canavalia ensiformis (Cruz-Hopólito et al., 2011) among many others, are agricultural cutting-edge aspects.

Environmental problems result from herbicide residues and their degradation products found in water, soil, air, and even in animals and food vegetables, sometimes interacting with humans and causing somatic alterations or diseases. Andrè. et al. (2005) conducted a study looking for a relationship between the association of metals and the herbicide-gamma-synuclein as a

possible factor in neurodegenerative diseases like Parkinson's. Other studies deal with interaction of atrazine herbicides and soil (Lima et al., 2010), the effect of humic acids on adsorption of the herbicide paraquat in goethite (Brigante et al., 2010), or the interaction of metals with sulfonylureas (Guo et al., 2009), just to name some of them. Nevertheless, more research is necessary on the harmful aspects of herbicides on living beings, on the concentrations that both herbicides and their metabolites can be found in the environment, and on the interaction of herbicides and metabolites with other environmental pollutants which can produce new compounds of unknown characteristics.

Rules to control the maximum allowed residue levels (MRL) of herbicides and certain metabolites in human food, animal feed and crops have been established in many developed countries (Regulation (EU) No. 396/2005). The need for identifying these compounds at trace levels has pushed the development of new methods for fast and effective identification and quantitation of them in a number of matrices.

2. Capillary electrophoresis (CE) as a separation technique for herbicides

The separation of herbicides between them, from their degradation products and other matrix components makes most times necessary to use a high-resolution separation technique such as liquid chromatography (LC) (Berger et al., 1998; Brigante et al., 2010, Cserháti et Forgàcs, 1998; Fangshi and Juan, 2005; Hickes and Watrous, 1999; 129, 197, Zhu et al., 2007), gas chromatography (GC) (Cserháti et Forgàcs, 1998; Dinelli et al., 1994), or CE (Arribas et al., 2011; Barroso et al., 1999; Berger et al., 1998; Chicharro et al., 2005; Chicharro et al., 2004; Cserháti et Forgàcs, 1998; Dinelli et al., 1996; Eash and Bushway, 2000; Fangshi and Juan, 2005; Font et al., 2008; Hickes and Watrous, 1999; Komarova and Kartsova, 2003; Kubilius and Bushway, 1998; Machett et al., 1996; Safarpour et al., 2004; Santilio et al., 2009; Tomita et al., 1991; Wu and Tsai, 1998; Zhang et al., 2002; Zhu et al., 2007). Among these separation techniques it is noteworthy that GC is not a suitable technique for non-volatile, thermolabile or polar compounds, as is the case for most herbicides. When GC is used, a derivatization reaction to approach the features of the product to those required in

GC is mandatory, thus increasing the number of steps and time for sample preparation. Both LC and CE do permit the determination of these compounds without, in most cases, an additional derivatization step, providing a suitable separation efficiency and, in general, appropriate analytical features of the given method. Berrada et al. discussed in their review in 2003 the applicability of LC and CE in determining phenylurea herbicides as sulfunilureas and benzoylureas; or in the review by Fangshi and Juan (2005), only for phenylureas, where it was shown that the suitability of using one or the other technique mainly depends on the physicochemical characteristics of the analytes to be determined. Comparison of different techniques (micellar electrokinetic chromatography, CE, LC and GC) for separation of phenoxyacetic acids and dichlorprop was made by Cserhati et al. (1998). The results showed a better performance of LC and CE with shorter development time and less user involvement.

CE is a very suitable technique for separation of polar compounds — characteristic of most herbicides— (Zhang et al., 2002), as the basis for separation is charge-to-mass ratio. Other well recognized characteristics of CE are fast and effective separations after simple or no-sample preparation. Rapidity is a desirable characteristic of the technique to be used in dealing with biological samples (e.g. vegetable, clinical) owing to the presence of low-stability analytes and/or enzymes that degrade them. The review from Boyce (2007) emphasizes the applicability of CE to determine additives and organic contaminants in food as well as to overcome problems from complex matrices. Other remarkable aspect of CE is low consumption of organic solvents, sample, and reagents as compared to LC. Also, the simple silica capillaries of CE are cheaper than LC columns, and of easier use. CE has also demonstrated to be the best alternative for separation of chiral compounds of environmental interest (Aga et al., 1999; Polcaro et al., 1999), as in the case with L-glufosinate (Marina and Crego, 1997) or enantiomers of imazaquin (Yi et al., 2007), diclofop and imazamethabenz (Penmetsa et al., 1996).

However, the CE has as a major drawback the lower sensitivity as compared with LC: a consequence of the smaller volume at the detection point. This problem is more significant for UV-visible molecular absorption detection

and may be minimized by operational modes such as indirect UV–Vis (Galcerán et al., 1997; Rojano et al., 2010 a), or by using fluorescence detectors, either with a conventional excitation source (Asami and Imura, 2006), laser induced fluorescence (Mechref and Rassi, 1996; Orejuela and Silva, 2005; Penmetsa et al., 1996; Rogers et al., 2000), indirect fluorescence (Chang and Liao, 2002), electrochemical detection —either amperometry (Islam et al., 2012), conductimetry (Xu et al., 2007), or potentiometry (Mostafa, 2010)— and even using more sensitive and selective but more expensive mass spectrometry (MS) detection (Font et al., 2008; Godwin et al., 2003; Hernández-Borges et al., 2005 c; Huck et al., 2005; Iwamuro et al., 2010; Kawai et al., 2011; Machett et al., 1996;Núñez et al., 2002; Safarpour et al., 2004). CE also permits sensitivity and selectivity to be increased by preconcentration in the capillary (See et al., 2010 b) and by avoiding interferents to enter the capillary, respectively (Arribas et al., 2011).

The present review is intended both to give an overview on the scope of CE for the determination of herbicides and show the necessity for going further in the study of these compounds through metabolomics in the light of the scarce publications dealing with the herbicides–metabolomics binomial. These publications demonstrate that metabolomics helps to a better understanding of the pathways through which the sensibility/resistance of the plant to a given herbicide takes place. A glance through the overall analytical process shows the most remarkable steps for sample preparation developed prior to insertion of the treated sample into the capillary, which depend on the matrix–analytes binomial.

3. Samples and analytes

The work of Hernández-Borges et al. (2004) shows that most of the studies in the field of CE and herbicides were applied to liquid samples (Amelin et al., 2012;, Arribas et al., 2011; Aturki et al., 2001; Baggiani et al., 2001; Barroso et al., 1999; Carabias-Martínez at al., 2002; Carabias-Martínez et al., 2000; Chang and Liao, 2002; Chicharro et al., 2005; Chicharro et al., 2003; Chicharro et al., 2004; Desiderio and Fanali, 1992; Frías et al., 2004;Godwin et al., 2003; Hernández-Borges et al., 2005 b; Hickes and Watrous, 1999; Horčičiak et al.,

2012; Hsieh and Huang, 1996; Hsu and Whang, 2009; Islam et al., 2012; Komarova and Kartsova, 2003; Komarova and Kartsova, 2002; Kubilius and Bushway, 1998; Núñez et al., 2002; Penmetsa et al., 1996; Pérez-Ruíz et al., 1996; Qin et al., 2002; Quesada-Molina et al., 2010; Rogers et al., 2000; Safarpour et al., 2004; See et al., 2010 a; Tříska and Vrchotová, 2002; Xu et al., 2005; You et al., 2003; Yu et al., 2005; Zhang et al., 2002; Zhou et al., 2010), many of them being water spiked with the target herbicides (Asami and Imura, 2006; De Rosssi et al., 2005; Desiderio et al.,1997 a; Galceran et al., 1997; Kawai et al., 2011; Loos and Niessner, 1999; Machett et al., 1996; Núñez et al., 2002; Núñez et al., 2001; See et al., 2010 a; See et al., 2010 b; Springer and Lista, 2010; Tříska and Vrchotová, 2002; Zerbinati et al., 2000; Zerbinati et al., 1998). Few methods deal with complex solid samples such as soils (Aga et al., 1999; Berger et al., 1998; Fu et al., 2009; Garrison et al., 1996; Hernández-Borges et al., 2005a; Islam et al., 2012; Iwamuro et al., 2010; Liu et al., 2006 a; Orejuela and Silva, 2005; Pérez-Ruíz et al., 1996; Yi et al., 2007; Zhu et al., 2007), vegetables (Aliferis and Chrysayi-Tokousbalides, 2011;Godwin et al., 2003; Pérez-Ruíz et al., 1996; Rojano et al., 2010 a), seeds (Liu et al., 2006 a), food (Carabias-Martínez et al., 2007; Chicharro et al., 2008; Hsu and Whang, 2009; Quesada-Molina et al., 2010; Rodriguez-Gonzalo et al., 2009; Whang et al., 2011), or complex liquids like juice (Khrolenko et al., 2002), oils (Amelin et al., 2012), soy milk (Hernández-Borges et al., 2005 c), urine (Lara et al., 2008; Pérez-Ruíz et al., 1996; West et al., 1997; Wu and Tsai, 1998) or blood (Ishiwata et al., 2007; Ishiwata et al., 2004 a; Pérez-Ruíz et al., 1996; Tomita et al., 1991; West et al., 1997). It can be said that about 60% of the published methods have been applied to aqueous samples and only a 40% to solid or complex liquid samples.

The importance of the analysis of herbicides in aqueous samples in the agronomic field is due to the existence of environmental legislation that controls the maximum allowed levels of herbicides in rivers, lakes and underground waters, to avoid poisoning or destruction of wildlife in those areas.

The analysis of herbicides in solid samples such as fruits, seeds, leaves snuff, soil, plants, etc. by CE makes mandatory total or partial dissolution of the

target solid (digestion or leaching, respectively) before introduction into the capillary. In recent years the number of publications dealing with herbicides and clinical samples, mainly related to accidental or provocated poisoning, has increased (West et al., 1997; Wu and Tsai, 1998).

Concerning to the analyte to be determined, it is noteworthy that a minority of the published methods deals with both herbicides and their metabolites (Aga et al., 1999; Amelin et al., 2012; Arribas et al., 2011; Berger et al., 1998; Chang and Liao, 2002; Chicharro et al., 2004; De Rosssi et al., 2005; Desiderio et al.,1997 a; Garrison et al., 1996; Godwin et al., 2003; ; Hsieh and Huang, 1996; Hsu and Whang, 2009; Ishiwata et al., 2007; Ishiwata et al., 2004; Iwamuro et al., 2010; Kawai et al., 2011; Komarova and Kartsova, 2003; Komarova and Kartsova, 2002; Kubilius and Bushway, 1998; Lara et al., 2008; Loos and Niessner, 1999; Malik et al., 2009; Rojano et al., 2010 a; Schmitt et al., 1996; See et al., 2010 a; See et al., 2010 b; Tomita et al., 1991; Tříska and Vrchotová, 2002; Tsunoi et al., 2000; Yi et al., 2007; Zerbinati et al., 2000; Zerbinati et al., 1998), despite determination of metabolites is of great interest both because they are, in most cases, toxic to plants, animals and even humans; and because they inform on the mode of action of the herbicide in plant and soil. Among these works, those deserving special mention are that from Aga et al. (1999) in which acetanilide herbicides and their metabolites are determined in soil; that from Amelin et al. (2012) who determined glyphosate and its metabolite AMPA in vegetable oil; and the contribution by Rojano-Delgado et al. (2010) who determined glyphosate and AMPA with other metabolites (viz. glyoxylate, formaldehyde and sarcosine) from glyphosate not determined jointly in plants so far.

The review by Aliferis and Chrysayi-Tokousbalides, (2011) illustrates the importance of metabolomics in the study of herbicides. However, it should be noted that most of the metabolites are usually stereoisomers (Marina and Crego, 1997); that means an additional problem as evidenced by a study conducted by André in 2003 (André and Guillaume, 2003) on the optimization of stereoisomers.

Other studies deal with mixtures of herbicides, either from the same chemical family (Carabias-Martínez at al., 2002; Carabias-Martínez et al., 2000; Chicharro et al., 2008;Chicharro et al., 2005; Desiderio et al.,1997 a; Desiderio and Fanali, 1992; Galceran et al., 1997; Hernández-Borges et al., 2005a; Hernández-Borges et al., 2005 c; Hernández-Borges et al., 2005 b; Hickes and Watrous, 1999; Hsieh and Huang, 1996; Islam et al., 2012; Komarova and Kartsova, 2003; Komarova and Kartsova, 2002; Machett et al., 1996; Núñez et al., 2002; Núñez et al., 2001; Orejuela and Silva, 2005;Pérez-Ruíz et al., 1996; Qin et al., 2002; Quesada-Molina et al., 2010; Quesada-Molina et al., 2007; Rodriguez-Gonzalo et al., 2009; Springer and Lista, 2010; Tsunoi et al., 2000; Whang et al., 2011; Wu and Tsai, 1998; Xu et al., 2005; Zerbinati et al., 2000; Zerbinati et al., 1998; Zhang et al., 2002; Zhou et al., 2010; Zhu et al., 2007) or from different chemical families (Aturki et al., 2001; Baggiani et al., 2001; Barroso et al., 1999; Carabias-Martínez et al., 2007; Chang and Liao, 2002; Chicharro et al., 2003; De Rosssi et al., 2005; Frías et al., 2004; Fu et al., 2009; Iwamuro et al., 2010; Kawai et al., 2011; Penmetsa et al., 1996; See et al., 2010 b). Interestingly, efficient separation of analytes when several herbicide families with different electrophoretic behavior were involved required, most times, the use of micellar media, as described by Karcher and Rassi (2000).

4. Sample preparation

A preliminary step of sample preparation is sampling, which must provide a representative sample, for which important factors such as homogeneity of the population, state of aggregation, stability of the analytes, or environmental factors can always be taken into account. In dealing with vegetables, homogenization by spraying, which in turn serves to improve extraction, or liquid nitrogen to prevent some analytes from degradation, as in the study of glyphosate and its metabolites in plants (Rojano et al., 2010 a) should be applied. Also spray drying has been used to obtain a solid easy to be homogeneized (Schwegman, 2009).

The aggregation state, the characteristics of the analytes and matrix, and the type of detector condition the steps in sample preparation. Solid state of the

sample makes mandatory a step (either digestion or leaching) to put the analytes in solution. Auxiliary energies such as ultrasound (Priego-Capote and Luque de Castro, 2007 a; Rodríguez-Gonzalo et al., 2009), microwaves (Delgado-Torre et al., 2012) or temperature+pressure (Luque de Castro and Luque-García, 2002; González-Rodríguez et al., 2004) are presently used to accelerate and improve leaching or digestion. Leaching is preferred if complete extraction of the target analytes is ensured as the leachate is always less complex than the digested.

In dealing with liquid samples (or solid samples after leaching or digestion) the most simple sample preparation is dilution, which is used to prevent blockage of the capillary or to lead the concentration of the target analytes within the linear range of the detector, if too concentrated. It is used for not complex liquid samples (e.g. relatively clean water samples spiked with the analytes (You et al., 2003, Penmetsa et al., 1996). Nevertheless, as the small dimensions of the capillary dramatically decreases the sensitivity of the CE methods based on any type of detection, preconcentration is most times mandatory as sample preparation in CE, which can also involve cleanup. The most common techniques for achieving these goals are as follows:

Solid phase extraction (SPE)

SPE is the technique more widely used both for preconcentration and cleanup of herbicides. It is based on putting the liquid sample into contact with a sorbent in a minicolumn or cartridge through which the sample matrix circulates and the target analytes and compounds with similar chemical characteristics are retained. After washing the minicolumn, if required, a small volume of a solvent (the eluent) with high affinity to the analytes elutes them from the column.

The nature of the sorbent material can be very varied. Cartridges of styrene-divinyl benzene (Aturki et al., 2001) have been compared to those of silica porous carbon graphite for the determination of cationic herbicides in water demonstrating that the latter material provides higher recovery (Carneiro et al., 2000); while Ishiwata et al. (2007) used titania to remove interferents such as ferrofitins and hemoglobin prior to the determination of glyphosate, glufosinate, AMPA and MPP in blood samples. Alumina has also been used with nanoparticles coated iron oxide for SPE prior to determination of glyphosate and

AMPA in water and fruit (Hsu and Whang, 2009). Materials such as molecularly imprinted polymers have been used by Baggiani et al. (2001) for retention of phenoxy herbicides, thus avoiding the interference from chlorine in aqueous samples. Comparison of the polymer sorbent with reverse phase C18 showed a lower recovery but higher cleanup provided by the former.

Vidal et al. (2012) has published a review that highlights some of their recent progress in CE, LC and GC for the development of methods for herbicides determination and provides an overview on the current state of modified ionic liquids used in sample preparation steps by both SPE and LLE.

Highlight deserves the work from Hernández-Borges et al. (2005c) which shows the great potential for herbicides studies of the combined use of SPE–FESI(Field-Enhanced Sample Injection) –CE–UV to improve sensitivity, thus allowing to obtain LODs similar to those provided by other analytical techniques like GC or HPLC.

Solid phase microextraction (SPME), the micromode of SPE implemented by Pawliszyn (1997), is based on the enrichment of the components of interest in a fused silica fiber coated or not with sorbents and exposed to the sample, with subsequent desorption by raising the temperature, without the help of an eluant. Therefore, cleanup/concentration and sampling are combined in the fiber in a single step with "green" desorption.

Families of herbicides such as triazines (Zambonin and Palmisano, 2000), and herbicides plus their metabolites, as is the case with glyphosate and AMPA (Hsu and Whang, 2009) have been subjected to SPME, mainly for cleanup as the small dimensions of the fiber does not permit high concentration factors.

Stirring bar microextraction (SBME)

It is a form of liquid Phase Microextraction based upon the sorption of the investigated analytes from the liquid matrix onto a thick film of polydimethylsiloxane (PDMS) coated on a glass-coated magnetic stir bar. Recovery of the analytes can be performed either by thermal desorption (TD) followed by gas chromatography coupled to mass spectrometry detection (GC–MS) or liquid desorption (LD) which is compatible with LC–MS analysis.

TD technique is a ‘‘one-shot’’ process and once the sample is desorbed there is no chance for method development and possible re-analysis (Magi et al., 2012). This limitation can be overcome by SBSE–LC technique which is cheaper and easier to use.

Liquid–liquid extraction (LLE)

In LLE the liquid sample is put into contact with an immiscible phase to which the analytes or their derivatization products have a high affinity. The partition coefficient of the analytes between the two phases and the ratio between the volumes of the two immiscible phases, together with the presence of species with chemical characteristics similar to those of the target analytes determine the efficiency of both preconcentration and cleanup. The main problem with this step is formation of emulsions, increased in the case of commercial herbicides, as they are formulated with surfactants. LLE has been implemented prior to CE for preconcentration of five sulfonylurea herbicides using chloroform as extractant and providing a preconcentration factor of 20 for a volume ratio of 10 (Zhou et al., 2003).

Micromodes of LLE (LLME) have also been implemented prior to CE taking advantage of the small volume required for injection into the capillary and the additional advantage of inserting the mode within “green analytical methods”. As an example, Lambropoulou and Albanis (2007) implemented an LLME method in which only few microliters of solvents were used instead of up to several hundreds of milliliters in LLE. In recent years, LLME has been combined with liquid chromatography (LC) and capillary electrophoresis (CE), in addition to the generally used coupling to GC, which has been applied to various matrices —biological, environmental and food matrices. The need for automation in LLME combined with the decreased volumes of organic solventes has led to the recent development of LLME based on disposable hollow fiber (Rasmussen and Pedersen-Bjergard, 2004). Asensio-Ramos et al.(2011) highlights the advantages of this extraction over other conventional methods in food analysis. Vidal et al. (2012) have published a review which highlights some of their recent research on LC, GC and CE and provides an overview on the

current state of modified ILs used in both SPE and LLE for sample preparation in herbicides analysis.

5. Derivatization

The purpose for which derivatization is implemented depends on the analytical step where it is included, which can be:

(i) To favor sample dissolution or leaching: the derivatizing reagent is a part of the digestion solution or of the leachant. In these cases, the process is facilitated by formation of the derivatization product, according the Le Chatelier law.

(ii) To improve the cleanup and/or preconcentration step: formation of a product which is easily retained on a sorbent or eluted in a lower eluant volume in dealing with SPE; which is easily extracted or with higher efficiency to the acceptor phase (usually by a polarity change of the product as compared with the analyte or reactant) in the case of LLE. In these cases derivatization reaction takes place before or during preconcentration and/or cleanup (in situ derivatization, in the latter case).

(iii) To facilitate or make possible individual separation of the analytes. In this case, and depending of the pursued objective, derivatization must be previous (as is the case of GC, in which the purpose is most times to achieve a higher volatility of the products as compared with the target analytes) (Seiber, 1991), or a decreased thermolability, or in situ derivatization if the reagent is in the mobile phase (LC) or in the background electrolyte (BGE) in the case of CE.

(iv) To make detection possible, more sensitive or more selective when the target analyte does not provide response at the detector, the response factor is low, or the signal is common with those of the interferents, respectively.

In CE, derivatizations are usually performed off-line, prior to introduction of the sample into the capillary (Mechref and Rassi, 1996; Chang and Liao,

2002), but also in-capillary column has been performed (Priego-Capote and Luque de Castro, 2006).

6. Individual separation of the target analytes or their reaction products

The most used analytical techniques for individual separation of herbicides between them or from other sample components has been LC (Berger et al., 1998; Brigante et al., 2010, Cserháti et Forgàcs, 1998; Fangshi and Juan, 2005; Hickes and Watrous, 1999; Zhu et al., 2007), which is the oldest; then, EC (Barroso et al., 1999; Berger et al., 1998; Chicharro et al., 2005; Chicharro et al., 2004; Cserháti et Forgàcs, 1998; Dinelli et al., 1996; Eash and Bushway, 2000; Fangshi and Juan, 2005; Font et al., 2008Hickes and Watrous, 1999; Komarova and Kartsova, 2003; Kubilius and Bushway, 1998; Machett et al., 1996; Safarpour et al., 2004; Santilio et al., 2009; Tomita et al., 1991; Wu and Tsai, 1998; Zhang et al., 2002; Zhu et al., 2007). LC and CE both allow separation of herbicides and their metabolites without derivatization, in most cases. The review by Berrada et al., (2003) shows the applicatibility of each of these techniques for separation of herbicides such as phenylureas, sulfonylureas and insecticides such as benzoylureas; or that by Fangshi and Juan, in 2005, only on phenylureas.

7. Capillary electrophoresis

The different displacement rate of the target analytes as a function of their charge-to-mass ratio in the presence of an electrical field created in between the ends of a capillary in which two electrodes are located is the basis of individual separation in CE.

The CE modes more used so far for the separation of herbicides are as follows:

(i) Capillary zone electrophoresis (CZE) is among the most used CE modes because its simplicity and high separation power based on the load/size ratio. In this CE mode the composition of the BGE is constant throughout the separation zone. The applied potential causes the different ionic components of the mixture migrate each according

to their own mobility and separate areas that can be completely resolved or partially overlapped. Among areas completely resolved there are gaps occupied by the BGE. The main drawback of CZE is its inability to separate neutral compounds. A wide variety of BGEs such as formiate, phosphate, borate, acetate–acetic acid and phosphate have been used for separation of herbicides, the most common being phosphate within the pH range 2–7 (Barroso et al., 1999; Chicharro et al., 2004; Pérez-Ruíz et al., 1996), and borate at pH close to 9 (Qin et al., 2002; Quesada-Molina et al., 2007; Springer and Lista, 2010; Zhang et al., 2002).

(ii) Isoelectric focusing (IF) is used to separate proteins as a function of their isoelectric points. It is based on establishment of a stable pH gradient created by ampholytes which migrate until they reach their isoelectric points. This mode has been used to study the effect of herbicides on given proteins (Kang et al., 2003).

(iii) Isotacophoresis is a type of electrophoresis at a constant development rate. Because the sample is introduced between front and terminal buffers, its components are separated between the two buffer zones moving at the same rate. This means that the travel time in the capillary under isotacophoretic conditions is independent of speed (Fernández-Gutiérrez et al., 2005).

(iv) Micellar electrokinetic capillary chromatography (MEKC) requires a surfactant at a concentration above its critical micelle concentration (CMC); therefore, the micelles accommodate non-polar compounds in its interior to favor separation.

The most used electroforetic modes are CZE and MEKC. Matchett (1996) made a comparison between both techniques for the determination of 7 sulfonylureas in water. After detection by MS in both cases, the author concluded that the efficiency of each mode was given by the characteristics of the developing BGE used.

Sample injection in CE can be done in three different ways: (i) electrokinetic injection, also known as electromigration; (ii) hydrostatic injection,

also called suction-injection; and (iii) direct injection by pressure. In all three cases a sample volume of the order of 2 to 20 pL is injected.

The advantages and disavantages of each type of injection are discussed somewhere (Hernández-Borges et al., 2005 a). In general, increased sensitivity by preconcentration is achieved by hydrodynamic injection (Aturki et al., 2001) or electrokinetic (Carabias-Martínez et al., 2000); while cleaner analytical samples are inserted into the capillary by electrokinetic injection (Rojano et al., 2010 a).

8. Optimization in capillary electrophoresis

In capillary electrophoresis optimization search for greater efficiency in the separation and increased sensitivity. To this end the most influential factors are as follows:

(i) The composition, concentration and pH of the BGE are the key variables in CE as depending on the pH the analytes are charged positively, negatively, or they are neutral, so that their migration is strongly influenced by the pH, which in turn depends on the selected BGE.

(ii) The applied voltage, which must be carefully selected as its decrease increases the migration time and the analysis time as a result; nevertheless, an increased voltage involves a poorer separation. In addition, high electric fields can give rise to an electric current high enough to created a temperature gradient that can result in convection, preventing full resolution.

(iii) The capillary length and diameter of the capillary are the key for appropriate both separation and elimination of heat created by Joule effect, respectively, caused by application of high voltage.

(iv) The temperature is also a very influential factor as its increase also increases the electroosmotic flow by a decrease of the viscosity of the BGE solution. In the case of water, a temperature rise of 1 °C (20 to 21 ° C) reduces 2.4% the viscosity of water; nevertheless, this rise in temperature decreases the dielectric constant 0.5%, which decreases electroosmotic flow, so the overall effect of increased the temperature is also an increase of electroosmotic flow.

(v) The presence of organic solvents in the BGE affect its viscosity, dielectric constant, and Zeta potential. Addition of methanol to water increases the viscosity of the solution up to a percentage of methanol of 50% (v/v), but the viscosity decreases when the percentage in methanol is above 50% (v/v). Different is the behavior of acetonitrile that added to water gradually reduces the viscosity of the mixture by increasing its percentage from 0 to 100.

Optimization of influential variables has been traditionally carried out by a univariate method; that is, by changing one by one the variables while keeping constant the others. Presently, interrelated variables, as is the case with those in CE methods, are optimized by a multivariate design which allows simultaneous variation of all variables, thus obtaining more realistic optimum values (Deming and Morgan, 1993).

9. Types of detection and detectors in CE

There are two modes of measurement used in absorption and emission molecular spectrometry: direct and indirect detection depending on the active or inactive behavior, respectively, of the target analytes with respect to the given technique.

Direct detection is used when the analytes, separated in the CE, are active at the detector; in this case a BGE that is not active or minimally active at the detector must be selected. In this way, passage of the BGE through the measurement cell will produce a zero or close to zero baseline (instrumental blank), while passage of the given analyte will produce a risen signal (positive peak). Núñez et al. (2002) determined quaternary ammonium herbicides in drinking water using as BGE CTAB (cetyltrimethylammonium bromide) (0.8 mM, at pH 4, and with 5% methanol), which is not functional at the monitoring wavelength. Detection limits, based on a signal-to-noise ratio of 3, were lower than 0.3 μg/L.

Indirect detection is the choice when the target analytes are no active at the detector. In this case a very active development BGE (that is, with high molar absorptivity or high quantic yielding for absorption or luminescent techniques, respectively) must be used. In this way the baseline or blank signal is established

at a very high value of the instrumental signal. A negative peak appears when the non-active analytes pass through the flow cell. This is the case with the CE–UV absorption method for the determination of glyphosate and 4 of its metabolites (Rojano et al.,2010), using a buffer consisting of phosphate, acetonitrile, potassium phthalate, and CTAB as the absorbent compound. Also direct and indirect fluorescence measurements of herbicides have been reported after CE separation (Orejuela and Silva, 2005 and Chang and Liao, 2002, respectively).

A wide variety of measurement cells has been developed for CE, the characteristics of which closely depended on the technique on which detection was based. Therefore, absorptiometry cells —sometimes consisting of a zone of the capillary transparent to the monitoring wavelength—, fluorescence cells of different shape, electrochemical cells for amperometry, potentiometry or conductimetry measurements have been designed and liquid interfaces as those used in LC–MS have been adapted to CE–MS to use mass detectors.

(i) The UV–Visible molecular absorptiometry detector is quite robust and reasonably sensitive; it is also stable under different conditions of separation and has good linear dynamic range. A number of applications support the usefulness of this detector, particularly in the diode array version, which allows scanning in the whole area of the spectrum. Corbera et al. (2005) highlight the need for preconcentration of diluted analytes when this detector is used, which has even been used after chiral separation of herbicides using cyclodextrins as selectors.

(ii) Molecular fluorimetry, specially when excitation is induced by laser (laser-induced fluorescence, LIF), provides a more selective and sensitive detection, but its use is restricted to molecules with intrinsic fluorescent properties or acquired after derivatization. Most herbicides and their metabolites are not fluorescent, therefore derivatization to a fluorescent product or indirect fluorescence is required with this type of detector. Chang and Liao(2002) have determined glyphosate, AMPA, glufosinate and MPP by indirect fluorescence using fluorescein as fluorophore BGE and an Ar-ion laser to induce fluorescence and establish the high-fluorescence baseline. The selectivity of this technique as compared to molecular absorption can prevent from cleanup steps (Orejuela and

Silva, 2005). The most common commercial lasers used for excitation and their nominal wavelengths are that of He-Cd (325 nm), Ar-ion (488 nm), and He-Ne (633 nm).

(iii) The electrochemical techniques more commonly coupled to CE have been conductometry, amperometry or potentiometry. Despite electrochemical detection can provide detection limits one or two orders of magnitude lower than UV–Vis absorptiometry, this type of detection has not been widely used in routine analysis of herbicides. The research on herbicides using electrochemical techniques is scarce. They are used for electrochemically active compounds, so these techniques requires derivatization for not electroactive analytes. The combination of UV and electrochemical detection was used Chicharro et al. (Chicharro et al., 2008; Chicharro et al., 2005; Chicharro et al., 2003) after CE separation, thus providing high sensitive analyses of herbicides.

(iv) Mass spectrometry is at present the most sensitive, selective and versatile detection technique. The design of interfaces to introduce a liquid into the mass spectrometer, first designed for LC, has also been successfully used in CE (Smith et al., 1990). Electrospray ionization (ESI) is considered the most appropriate interface for coupling MS to CE, which has been, therefore, that used for the study of different herbicides with excellent sensitivity (Hernández-Borges et al., 2005 c). The potential of the CE–MS coupling for the determination of herbicides was first reported by Lee et al. (1989) in dealing with the separation of sulfonilureas. Nevertheless, one of the areas of herbicide analysis in which the characteristics of the MS are specially required is for food safety and food quality applications, as demonstrated by Font et al. (2008) in the determination of organic food contaminants and residues. Huck et al. (2005) reviewed the use of capillary electrophoresis (CE) coupled to mass spectrometry (MS) for analysis of phenolic compounds and their derivatives in herbices mixtures, in which attention was paid to the different interfaces used. Ion-trap, time-of-flight or Orbitrap modes of MS, of great potential for identification, are the key for knowing the intermediate and final metabolites in unknown metabolic pathways which can provide light on the normal and special behavior of herbicides.

10. Foreseeable–desirable trends in research on herbicides analysis

In the authors' opinion, the most desirable trends in research on herbicides analysis should be focused on:

(i) The design of fast methods for herbicides implemented in small equipment for in-field studies, thus providing an almost in real time response to (and possible remediation of) an undesirable situation caused for a bad use/bad response of given herbicides (Rojano et al., 2010 b).

(ii) The development of fast, low price, and green methods for the analysis of herbicides, as those implemented by CE, which allow determining as many metabolites as possible with the aim of detecting and quantifying them to know the real level of toxicity caused by the presence of the herbicide used as a function of its dose of application, the time since application and the climatological conditions (Rojano et al., 2010 a; Rojano et al., 2011). A low consumption of cheap reagents will make possible to perform as many analyses as necessary for a strict control of the crops, which can be achieved by CE without the need for micro or nano devices which are easily obstructed and difficult to manipulate (Priego-Capote and Luque de Castro, 2007 b). The use of micro- or nano-LC equipment is much more expensive than CE.

(iii) The report of more sensitive methods, mainly based on MS detection, for determination of herbicides and their metabolites in the food chain at concentrations at low as possible to avoid potential damage to humans and other animals (Ishiwata et al., 2007).

(v)The use of equipment based on MS or NMR for identification of primary and secondary metabolites of herbicides to establish in an unequivocal manner their degradation pathways and the toxicity of the degradation products for establishment of the allowed MRL.

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Capítulo 2

Determination of glyphosate and its metabolites in plant material by reversed-polarity CE with indirect absorptiometric detection

Determination of glyphosate and its metabolites in plant material by reversed-polarity CE with indirect absorptiometric detection

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A simple CE method for simultaneous determination of glyphosate and its metabolites (i.e. aminomethylphosphonic acid, glyoxylate, sarcosine and formaldehyde) in plants is reported here. A BGE of pH 7.5, 10% ACN, 7.5mM phthalate, containing 0.75mM hexadecyltrimethylammonium bromide as an electro-osmotic flow modifier, an applied voltage of –20 kV and absorptiometric monitoring at 220 nm were the optimal chemical and instrumental parameters. The method, with development time 20 min, shows linear calibrations within the range 5–500 μg/mL (for all target analytes) with correlation coefficients between 0.999 and 0.998. It has been validated by application to samples of *Lolium spp*. The electroinjection mode hinders most interferents to enter the capillary, thus providing a clean electropherogram and making unnecessary long samplepreparation steps.

Keywords:
Aminomethylphosphonic acid / Formaldehyde / Glyoxylate / Glyphosate /Sarcosine

1. Introduction

Glyphosate (GLY) [N-(phosphonomethyl)glycine] is a nonselective post-emergence herbicide for control of long grasses and broad-leafed weeds. After application, it is absorbed by the leaves and translocated through the plant tissue, where it inhibits the action of the enzyme 5-enolpyruvate-shikimate-3-phosphate synthase (EPSP synthase) in the shikimate pathway, which produces aromatic amino acids such as phenylalanine for protein synthesis and other secondary plant products [1]. GLY was first commer-cialized by Monsanto in 1974 under the name of Roundrup and due to its effectiveness and relatively low mammal

toxicity became one of the most extensively used herbicides worldwide. In recent years, plants resistant to GLY have appeared in the farms, causing millionaire loss (http://www.weedscience.com. Accessed: May 2009) [2, 3].

The present accepted mechanism for metabolic degradation of GLY could explain the plants resistance to this pesticide, but the lack of methods for simultaneous determination of GLY and all its metabolites —*viz*. aminomethylphosphonic acid (AMPA), glyoxylate (GLYO), sarcosine (SAR) and formaldehyde (FA)— at residual levels in complex matrices hinders confirmation of this mechanism [4, 5].

The characteristics of GLY and its metabolites —relatively high solubility in water, insolubility in organic solvents, high polarity and low volatility make mandatory derivatization prior to GC analysis to decrease their polarity and enhance volatility [6, 7]. On the other hand, the use of HPLC or CE with absorption or luminescence detectors has been limited by the lack of fluorophore or chromophore groups in the analytes. Therefore, either pre- or postcolumn derivatization is mandatory for the determination of the analytes by absorption or fluorescence detection [8, 9]. To avoid this tedious and usually time-consuming step, indirect UV-vis absorption detection [10], indirect LIF detection [11], electrospray ionization MS [12, 13], flame ionization detection [14], electrochemi-luminescence [15] and electrospray condensation-nucleation light-scatter-ing detection [16] have been used for detection of these analytes. Indirect UV–visible absorption, widely used with CE, has also been the approach selected for detection in the research here presented because of its universality. The main problem of indirect detection is most times the lack of sensitivity; meanwhile other detection modes require more expensive and complicated equipment.

There are no methods in the literature for the simultaneous determi-nation of GLY and all its metabolites; so, a such a method would be very useful taking into account the two known metabolic pathways of this herbicide. Measurement in a single step of all compounds involved in GLY metabolism would provide a reliable way to study the metabolic pathways of GLY in plants, and thus, to know the resistance of a given plant to this herbicide. The aim of the research here presented was the development of a simple, fast and low-cost method to monitor

GLY and its metabolites for a comprehensive study of its behaviour in plants resistant to this herbicide.

2. Materials and methods

2.1. Equipment and apparatus

Application of GLY to plants was made inside a closed spray chamber furnished with a flat jet-spray tip Teejet 80.02 (Wheaton, USA). A thermostated centrifuge Beckman Coulter Avanti J-25 (Fullerton, USA), furnished with a rotor No. 20; a magnetic stirrer from Bunsen (Spain), a Selecta (Barcelona, Spain) ultrasonic bath, furnished with temperature control —generator power 50 W—; a porcelain mortar, furnished with a pestle from Pobel (Madrid, Spain), nylon filters 45 µm pore-size × 13mm id from Millipore (Carrigtwohill, Ireland); and round bottom sterile polystyrene tubes (Deltalab, Spain) were used for sample preparation. A 3-D Capillary Electrophoresis Agilent G1600A Instrument, equipped with a DAD (range 190–600 nm); capillary tubing of 88.5 cm (effective length 80 cm) × 50 µm id × 375 µm od (Análisis Vínicos, Ciudad Real, Spain), and thermostated by a Peltier unit, was used to separate and quantify the analytes. The instrumental setup was controlled and the data acquired and processed by the Agilent ChemStation software.

2.2. Reagents

Acetone, sodium hydroxide, ACN, FA, hydrochloric acid and potassium phthalate from Panreac (Barcelona, Spain), CTAB from Fluka (Buchs, Switzerland), SAR and GLYO from Sigma (St. Louis, USA), AMPA from Supelco (Bellefonte, USA), and GLY from Riedel-Haën (Seellze, Germany) were used. The commercial GLY used for plant treatment was 36% Roundup from Monsanto (St. Louis, USA). The stock standard solutions were prepared by dissolving 0.1–g each analyte in 100–mL water. The BGE was 10mM potassium phthalate, 0.5mM CTAB and 10% ACN (pH 7.5); 18mΩ deionized water from a Millipore Milli-Q water purification system was used throughly. The stock standards can be stored for six months at –20 ºC without degradation. The standard working solutions were daily prepared by dilution of the appropriate

volume of the stock solutions in BGE. Liquid nitrogen (Praxair, Spain) was used for sample preparation.

2.3. Samples

Seeds from five different weed biotypes (*Lolium multiflorum*), collected in different zones, were germinated in Petri dishes using a damp filter paper as substrate. The seedlings were transplanted to plastic pots —three plants per pot— filled with 1:2 v/v peat:clay as substrate. When plants averaged six leaves, commercial GLY was applied to 90% of plants, the rest being used as blank. Spraying, at 200 kP, was done inside the closed spray chamber calibrated at 0.5m height above the target surface, with a relative volume of 200 L/ha. After 0 and 96 h since GLY application, all plants —treated and blank— were cut, frozen by liquid nitrogen and stored at –40 ºC until use.

2.4. Proposed method

2.4.1. Sample preparation procedure

The procedure is divided into two steps: extraction of the target analytes from the solid matrix and preconcentration–cleanup of the extracts.

Before extraction, the frozen samples were washed three times with 20 mL water to remove traces of GLY and soil in the leaf surface. In total, 1.5 g sample was placed in the porcelain mortar and flash-frozen using 20 mL liquid nitrogen. Then, the sample was grinded to a fine powder using the porcelain pestle for 5 min, and the powder transferred to a plastic beaker and extracted three times with 8 mL 1:1 water–acetone, each time involving magnetic stirring for 10 min, ultrasonication for 5 min, and centrifugation at 4 ºC and 10 000 rpm for 15 min.

In the preconcentration–cleanup step, the supernatants obtained with the three 8 mL portions of extractant were pooled and evaporated to dryness under nitrogen flow. The extract was reconstituted with 2 mL BGE and filtered through a nylon filter before CE analysis.

2.4.2. Individual separation – detection by CE – UV absorption

The filtered extract was injected for 5 s at –10 kV into the BGE (10mM potassium phthalate, 0.5mM CTAB and 10% ACN at pH 7.5). The analysis voltage was –20 kV, and the monitoring wavelength 220 nm for all analytes. In order to maintain the capillary under optimal working conditions, its surface was regenerated after each run by sequential washing with water (2 min), 0.1M sodium hydroxide (2 min), 1 min waiting and BGE (10 min). In addition, the capillary was activated every-day by sequential washing with water (1 min), 0.1M sodium hydroxide (10 min), 5 min waiting and water (1 min).

3. Results and discussion

The optimization sequence consisted of two steps: the first, focused on the best separation–determination of the compounds using CE–DAD, and, the second, on the extraction and cleanup–preconcentration of the target compounds. Optimization of the electrophoretic separation was carried out with both standards and extracts obtained from blank samples, not sprayed with GLY. The extracts were spiked with the target analytes to take into account the presence of possible co-extracted interferents. In preliminary experiments, the optimal wavelength for analytes monitoring, the capillary length and the best modality between hydrodynamic and electrokinetic injection were established.

3.1. Optimization of individual separation–detection by CE–UV absorption

The influence of the main variables involved in the electrophoretic separation was studied to obtain the best separation of the analytes in the shortest time. The variables studied were capillary temperature, analysis voltage, injection voltage, injection time and those related with BGE (i.e. pH, hydrogen phthalate concentration, CTAB concentration and organic modifier percentage). The response variables used for optimization of this step were the peak half-width, the peak height and the resolution expressed as (tm2–tm1)/ tm1, where tm is the migration time. The first variable should be the narrowest possible and the highest for the two others in order to achieve the best resolution in the shortest time with maximum sensitivity.

Potassium hydrogen phthalate had the dual role of fixing the pH, and acting as a chromophore. The BGE pH was that high enough to ensure all the analytes to be charged (pH 7.5 sufficed this requirement), and a 10 mM potassium hydrogen phthalate solution provided the best sensitivity; meanwile 0.5 mM CTAB in the BGE was enough to fulfil the objective of inverting the electroosmotic flow. 10% ACN was used as organic BGE modifier for better resolution between peaks. The capillary temperature was also exhaustively studied. Despite the best resolution was obtained at 30 ºC, degradation of the target analytes hinders its use; therefore, 20 ºC was selected as the temperature, which provided the best resolution without degradation. A voltage of –20 kV provided the best resolution between analytes and sample matrix in the shortest time.

The injection mode is a key variable to reduce interferents from the sample matrix. It is well known by CE users that the electroinjection mode reduces these interferences as charged compounds are preferentially introduced into the capillary. Therefore, this injection mode was selected for subsequent experiments. Two injection variables (*i.e*. voltage and time) were optimized to obtain the best sensitivity and peak resolution with minimum interferences, for which their values were fixed at –10 kV and 5 s, respectively. Higher injection voltage and longer injection time yielded increased peak-width and interferences from the matrix both detrimental to the electropherogram.

After the optimization step, a study of the injection repeatability and reproducibility —in terms of areas and retention times— with standard and extract solutions yielded standard deviations lower than 6%; therefore, the use of an internal standard was considered unnecessary. Under the optimum working conditions, a standard solution containing all analytes at 400 µg/mL, a blank extract and a blank extract spiked with the analytes at the same concentration as the standard solution are shown in Fig. 1.

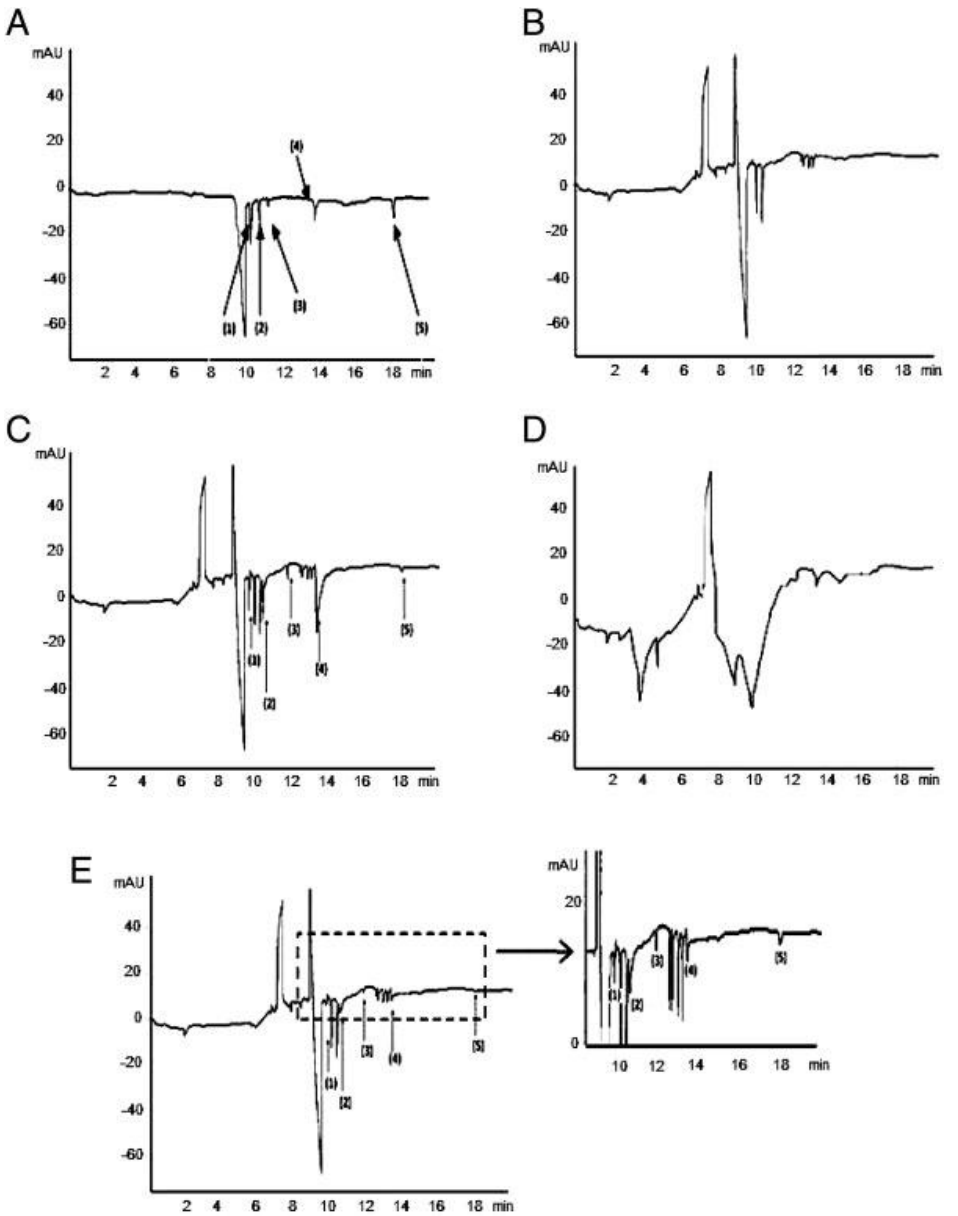

Figure 1. Electropherograms of standards (A), blank sample (B), blank sample spiked with the target analytes (C), blank sample spiked with the target analytes reconstituted in water (no cleanup procedure) (D); and natural sample treated with GLY and cut 96 h after application (E). (1) GLY, (2) AMPA, (3) GLYO, (4) SAR, (5) FA.

This figure shows that baseline separation of the target analytes, without interferences from the matrix, is achieved in less than 18 min. GLY and AMPA can be baseline isolated in less than 11 min. This time is similar to that found in the literature for the determination GLY and AMPA in plant materials by HPLC

or CE [17, 18]. In addition, the proposed method is also able to determine, glyoxilate, SAR and FA. It is worth emphasizing that individual separation and determination methods proposed so far using HPLC [20–23], CG [24–26] or CE [15, 27–29] allow identification and quantitation of only GLY and AMPA (most of them in water), but not those of other metabolites such as the GLYO, SAR or FA. The method here reported allows the determination of the pesticide and compounds resulting from the two known metabolic pathways in a single electrophoretic step. The proposed method also avoids both time-consuming derivatization [6, 8, 9] – which can degrade the analytes – and the use of more complex detection systems such as MS detectors [12].

3.2. Sample-preparation: Optimization of the extraction–preconcen-tration–cleanup procedure

3.2.1. Extraction procedure

The optimum separation–detection conditions were used to monitor optimization of the extraction–preconcentration–cleanup procedure, aimed at achieving maximum removal of the target compounds from the matrix in the shortest time. Obviously, real samples were used to optimize sample preparation, divided into two steps: extraction (more properly, leaching) of the analytes and preconcentration– cleanup of the extract.

The variables optimized in the extraction step were: extractant, extraction time – both conventional and with ultrasonic assistance – and number of extractions for complete removal. Table 1 lists these variables, the ranges studied and their optimum values as well as the extractants assayed (water–methanol and water–acetone mixtures).

Table 1. Optimization of the extraction step

Variable	Tested range	Optimum value (efficiency)
Water–methanol (%)	0–100	—
Water–acetone (%)	0.1–100	50
Convential extraction (min)	1–30	10
Ultrasonic extraction (min)	1–30	5
Conventional+ultrasonic extraction (min)	0–30+0–30	10–5
Number of extraction cycles	1–10	3

The response variable used for optimization of this step was the peak area of the target compounds. This variable should be as higher possible in order to achieve maximum extraction efficiency in the shortest time without degradation.

Water–acetone mixtures provided better extraction efficiencies than water–methanol mixtures. This fact can be explained because acetone has a higher power for protein denaturation than methanol. The best extraction efficiencies were achieved for 1:1 ratio; which was selected for subsequent experiments.

Kinetics study of the conventional stirring method and that assisted by ultrasound were aimed at knowing both if ultrasonic irradiation shortened the extraction time and/or if this type of energy degraded the target analytes.

Extractions by conventional stirring, by ultrasound assistance and with sequential use of both types of extraction were compared using the peak areas as response variable. As can be seen in Fig. 2, the first two procedures did not provide the maxima extraction efficiency, which was achieved by sequential application of conventional and ultrasonic extraction. The best extraction efficiencies for extraction by each independent procedure were obtained after 10 and 5 min of conventional stirring and ultrasonic assistance, respectively; far from the extraction efficiency obtained by sequential application of both

procedures. Furthermore, in the case of ultrasonic extraction, the highest extraction efficiency was achieved after 5 min and decreased for times longer than 12 min. These results —short irradiation time to obtain the maxima extraction efficiency with minimum degradation of the target analyte— prevent from direct insertion of an ultrasonic probe into the extraction system or in a water-bath surrounding the extraction device. The fact that sequential application of 10 min stirring and 5 min ultrasonic irradiation provided maximum extraction efficiency of the target compounds can be explained because conventional stirring fragments the vegetal material (it can be visually observed) but it is not enough energetic to extract completely the analytes from inside the cells. Ultrasound breaks the cells, thus facilitating analytes transfer to the extractant, but degrades the analytes for irradiation times longer than 12 min.

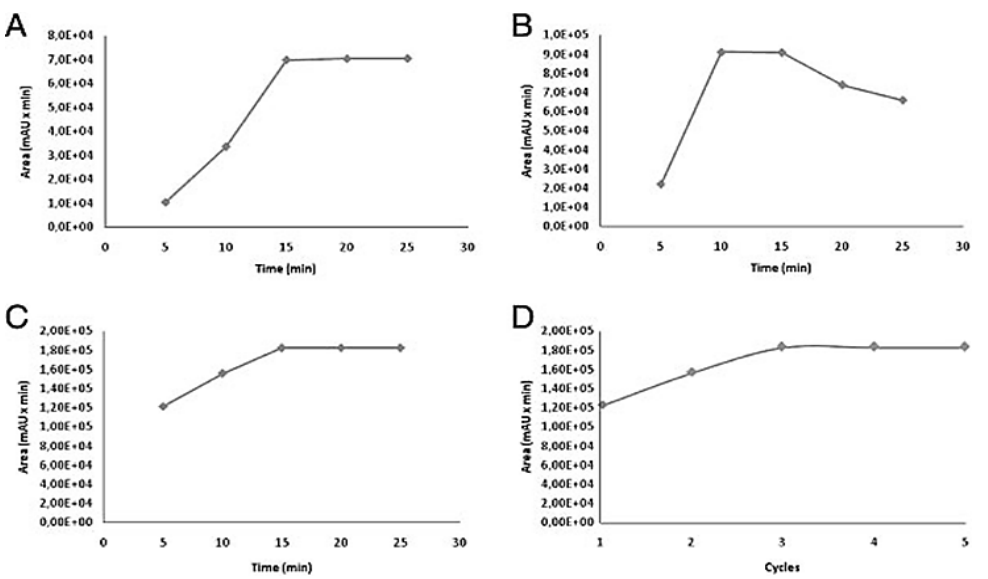

Figure 2. Extraction kinetics for the target analytes using: (A) conventional extraction, (B) ultrasound-assisted extraction, and (C) sequential conventional plus ultrasound assisted extraction. (D) Extraction efficiency as a function of the number of extraction cycles.

The number of extractions necessary to achieve the maximum extraction efficiency was also tested. As can be seen in Fig. 2D, after three extraction cycles the amount of extracted analytes does not increase; thus, three extraction cycles was selected for subsequent experiments.

According to the results obtained in this study, the extraction procedures reported in the literature based on either conventional stirring or long-time ultrasound-assistance for the extraction of only GLY and AMPA from plant material [17, 18] do not achieve complete extraction or degrade the analytes, respectively. The use of sequential traditional stirring and short periods of ultrasound irradiation improves extraction efficiency and avoids degradation of the target compounds.

3.2.2.Preconcentration–cleanup procedure

The preconcentration step consisted of extractant evaporation to dryness using a nitrogen stream. Despite increased temperature accelerated this step, 25 ºC was adopted to avoid analytes degradation.

Extract cleanup was aimed at separation of analytes from proteins, polysaccharides and other compounds by exploiting the solubility of the latter in the extractant and insolubility in a low-ionic-strength solution containing CTAB. BGE solutions with different concentrations of CTAB (0.1–1 mM) were tested. The best results (complete dissolution of the target analytes with minimum presence of bigger molecules) were obtained with the CTAB concentration used for electrophoretic separation of the analytes (0.5 mM); therefore, BGE as such was also used for selective reconstitution of the dried extract. Filtration was required for removal of the solid residue from the solution containing the target analytes. To ensure no retention of the analytes in the filter, a recovery study was developed, and no retention was observed; therefore, this step was included in the method.

Two experiments were carried out to check the cleanup effect of extract reconstitution with BGE: (i) comparison of the electropherogram obtained from a standard solution containing the analytes at 400 µg/mL with that from a blank extract spiked with the analytes at the same concentration. Figures 1A and C show the similarity of both; therefore, standard solutions can be used in the calibration step. (ii) Comparison of the electropherograms provided by two aliquots of an extract (one of them subjected to the overall sample-preparation procedure and the other differing in the reconstitution step, using water instead of BGE solution) can be made from Figs. 1C and D. This comparison clearly shows

that the use of BGE is the key to obtain a clean electropherogram of the target analytes.

3.3. Characterization of the method

Calibration plots were run for all analytes using the peak area, at the monitoring wavelength, as a function of the standard concentration of each compound. The calibration equations, regression coefficients and linear dynamic ranges are listed in Table 2.

Table 2. Features of the method

Compound	Calibration equation[a]	R^2	Linear range	LOD[b]	LOQ[b]
GLY	Y=0.495+0.326X	0.999	LOQ–500	0.1	0.3
AMPA	Y=–0.543+0.297X	0.999	LOQ–500	0.1	0.3
GLYO	Y=0.641+0.244X	0.998	LOQ–500	0.2	0.7
SAR	Y=–0.323+0.181X	0.999	LOQ–500	0.1	0.3
FA	Y=0.113+0.203X	0.998	LOQ–500	0.1	0.3

[a]Y expressed as absorbance units x 10^{-3}; X as µg/ml; [b] expressed as µg/ml

The LOD is expressed as the concentration of the given analyte which provides a signal 3σ above the mean blank signal —obtained after analysis of the extract from a plant, which was not treated with GLY; where σ is the standard deviation of the blank signal. The LODs are between 0.1 and 0.2 µg/mL for all analytes. The LOQs expressed as the concentration of analyte, which gives a signal 10σ the mean blank signal, are within 0.3 and 0.7 µg/mL for all analytes (see Table 2). These LODs and LOQs are similar to those obtained by LIF detection [11] and one order of magnitude lower than those provided by UV detection [19]. In comparison with MS detection, the LODs and LOQs are more than one order of magnitude higher [12].

3.4. Precision assessment

Within laboratory reproducibility and repeatability were evaluated for the proposed method in a single experimental setup with duplicates by experiments carried out with natural samples —a pool of plants from five weed biotypes cut

96 h after GLY application. Two measurements of these samples per day were carried out on 7 days. Equation (1) was used to determine the between-day variance:

$$s_{between} = (MS_{between} - MSwithin)/n_j \quad (1)$$

where MS is the mean square (residual sum of squares rated by the freedom degrees) and nj is the number of replicates per day. The within-laboratory reproducibility, s^2_{WR}, was calculated by Eq. (2)

$$s^2_{WR} = s^2_r + s^2_{between} \quad (2)$$

where s^2_r is the residual mean squares within-days and $s^2_{between}$ is the variance due to the between-day effect.

The results obtained are listed in Table 3.

Table 3. Average concentration, repeatability and within-laboratory reproducibility expressed as relative standard deviation (RSD) for natural samples cut 96 h after GLY application.

Parameter	GLY	AMPA	GLYO	SAR	FA
Average[a]	23.6	1.0	2.7	1.3	1.3
s_b[b]	3.7	7.2	2.6	5.6	5.0
s_{wr}[b]	8.9	9.3	5.0	8.1	6.1

[a] expressed as µg/g. [b] expressed as %

The repeatability, expressed as RSD, ranged between 2.6 and 7.2% for the target analytes; the within-day laboratory reproducibility, also expressed as RSD, ranged between 5.0–9.3%.

3.5. Application of the method

In order to assess the applicability of the proposed method, it was used to determine the analytes in samples from five weed biotypes treated with GLY, which were cut 0 and 96 h after application. The accuracy of the method and potential matrix effects were established by analyzing samples spiked at two concentration levels, 5 and 400 µg/g, after sample grinding. In order to favor interaction between the matrix sample and the analytes, the samples were aged in the dark at –40 ºC for 1 week.

Table 4. Analysis of glyphosate and its metabolites in natural samples by the proposed method (errors, in parenthesis, are expressed

Sample	Added	GLY		AMPA		GLYO		SAR		FA	
		Found[a]	Recovery[b]	Found[a]	Recovery[b]	Found[a]	Recovery[b]	Found[a]	Recovery[b]	Found[a]	Recovery[b]
Lolium 1 (0 h)	—	—	—	—	—	—	—	—	—	—	—
	L1	4.9	97.1	4.6	92.5	4.5	91.6	4.6	93.1	4.9	99.4
	L2	405.5	101.3	391.3	97.8	389.2	97.3	413.3	103.3	418.7	104.6
Lolium 1 (96 h)	—	20.7 (1.4)	—	0.8 (1.0)	—	2.6 (2.4)	—	1.1 (1.2)	—	1.3 (0.9)	—
	L1	25.7	99.8	5.3	90.4	6.9	90.8	5.6	91.3	6.5	103.3
	L2	427.5	101.6	392.1	97.8	401.4	99.6	391.1	97.5	419.2	104.4
Lolium 2 (0 h)	—	—	—	—	—	—	—	—	—	—	—
	L1	4.7	94.7	4.7	93.2	4.7	95.6	4.7	94.2	4.6	92.1
	L2	403.0	100.7	384.0	95.9	397.4	99.3	402.8	100.7	377.9	94.4
Lolium 2 (96 h)	—	26.8 (1.3)	—	1.2 (2.7)	—	1.0 (0.9)	—	1.1 (4.5)	—	1.4 (1.2)	—
	L1	30.9	97.1	5.8	93.6	5.6	92.4	5.8	95.9	6.0	94.4
	L2	430.6	100.8	393.6	98.1	412.7	102.9	381.4	95.0	434.0	108.1
Lolium 3 (0 h)	—	—	—	—	—	—	—	—	—	—	—
	L1	4.4	88.6	4.8	96.5	4.5	91.2	4.7	94.2	4.5	90.3
	L2	375.0	93.7	387.0	96.7	386.1	96.5	379.1	94.7	378.3	94.5
Lolium 3 (96 h)	—	28.6 (2.0)	—	2.8 (6.9)	—	1.5 (7.1)	—	1.4 (2.8)	—	1.2 (1.5)	—
	L1	29.4	87.2	7.7	99.0	6.0	92.4	6.1	95.6	5.1	81.4
	L2	402.9	93.9	402.2	99.8	385.9	96.1	373.0	92.9	379.5	94.5
Lolium 4 (0 h)	—	—	—	—	—	—	—	—	—	—	—
	L1	4.6	92.8	4.8	95.6	4.8	97.3	4.9	98.0	4.4	89.0
	L2	366.8	91.7	389.9	97.4	374.4	93.6	359.6	89.9	354.6	88.6
Lolium 4 (96 h)	—	19.8 (2.4)	—	1.1 (0.9)	—	3.5 (4.3)	—	1.2 (5.8)	—	1.3 (5.1)	—
	L1	24.0	96.4	6.3	102.0	8.1	94.9	6.0	96.7	5.8	90.8
	L2	385.4	91.7	402.0	100.2	357.2	88.5	380.3	94.7	356.1	88.7
Lolium 5 (0 h)	—	—	—	—	—	—	—	—	—	—	—
	L1	4.4	87.9	4.8	95.3	4.6	92.3	5.2	104.2	4.8	97.6
	L2	393.9	98.4	394.3	98.58	390.0	97.5	410.1	102.5	400.5	100.1
Lolium 5 (96 h)	—	20.7 (2.0)	—	0.9 (1.7)	—	0.9 (4.2)	—	1.4 (1.1)	—	1.2 (0.8)	—
	L1	22.7	88.2	5.8	99.4	5.7	96.2	6.0	94.4	6.3	100.8
	L2	415.2	98.6	410.4	102.3	382.5	95.4	393.0	97.9	412.6	102.8

Table 4 shows that recoveries ranging between 81.4 and 108.1% were obtained. The paired *t* test revealed the absence of significant differences between the concentrations added and those found (α 0.05). Figure 1E shows a typical electropherogram from a natural sample.

4. Concluding remarks

The proposed method constitutes a valuable analytical tool for determination of GLY and its metabolites in plants by a fast technique as CE and simplified sample preparation by avoiding derivatization.

The method has been applied to the determination of the target analytes in plants, where the high amounts of proteins, amino acids and polysaccharides usually difficult the analysis. The use of CTAB as cleaning reagent seems to be a good option to avoid interferents in CE.

The good analytical characteristics of the method concerning repeatability, reproducibility and LOQs allow its use to monitor the target analytes in plants at trace levels.

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The authors have declared no conflict of interest.

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Capítulo 3

Screening and confirmatory analysis of glyoxylate: A biomarker of plants resistance against herbicides

Screening and confirmatory analysis of glyoxylate: A biomarker of plants resistance against herbicides

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Screening and confirmatory analysis of glyoxylate: A biomarker of plants resistance against herbicides

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ABSTRACT

The evidence that glyoxylate is a biomarker of tolerance or susceptibility to the action of herbicides belonging to the glycine family makes necessary to develop simple methods for the determination of this metabolite. Glyoxylate level allows both to know the presence/absence of members of the glycine family in plants and plant response to these herbicides. With this aim, a colorimetric-screening method has been developed for determination of glyoxylate based on formation of a phenylhydrazone, then oxidised to red coloured 1,5-diphenylformazan. Simultaneous optimization of ultrasound-assisted extraction of glyoxylate from plants and derivatiza-tion by a multivariate design has allowed the determination of the target analyte in fresh plants without interferences from pheophytines and compounds with carbonyl groups. Limits of detection and quantification are 0.05 μg ml^{-1} and 0.17 μg ml^{-1}, respectively, with precision, expressed as relative standard deviation, of 3.3% for repeatability and 5.6% for the within-day laboratory reproducibility. Only 50 mg of plant is necessary for determination of glyoxylate within 32 min. Confirmatory analysis by capillary electrophoresis–diode array detection in samples of *Lolium spp*. subjected to treatment with glyphosate shows that the relative error of the proposed method is always lower than 7%.

Keywords:

Biomarker, Capillary electrophoresis, Colorimetric screening method, Glyoxylate, Glyphosate resistance

1. Introduction

Glyoxylate, a metabolite endowed with aldehyde and carboxylate groups, is involved in different metabolic pathways in bacteria, plants and animals [1–5].

There are two main sources of glyoxylate formation in plants. One comes from photorespiration, through the activity of glycolate oxidase; the other from the glyoxylate cycle that bypasses the steps in the citric acid cycle where carbon is lost as CO2 [6]. Several enzymes (mainly dehydrogenases and transaminases) are involved in the synthesis and catabolism of glyoxylate [7–12]. These biocatalysts can be inhibited, or even mutated, by the action of external agents such as herbicides or by internal agents such as feedback, in both cases with lethal effects on the regulation of glyoxylate levels [13–15]. For this reason, glyoxylate could be considered a biomarker to detect these abnormal enzyme behaviours and, therefore, the development of methods for screening and confirmatory analysis of this metabolite are of interest.

Glyoxylate has been determined in different matrices, from biological fluids such as urine or blood [16–19] to plant extracts [20,22]. Among the different methods, one alternative is that reported by Kramer et al. [21] for glyoxylate determination in aqueous solutions. This method is based on the formation of glyoxylate phenylhydrazone and subsequent oxidation, in acid medium, to 1,5-diphenylformazan, an intensely coloured red compound with maximum absorption at 520 nm. The main limitation of this approach is the lack of selectivity since any molecule with carbonyl groups could interfere the determination of glyoxylate. The method was improved by Bräutigam et al. for simultaneous determination of glyoxylate and ammonium in tobacco plants by inclusion of liquid–liquid extraction with chloroform [22]. The toxic character of chloroform as extractant together with a long extraction time and number of steps are the main drawbacks of this method. Glyoxylate has also been determined using separation techniques prior to detection such as GC [16], LC [17,18] or CE [20,23]. These methods require more sophisticated, no portable instrumentation and, usually, they do not provide a fast response; aspects which are critical for in-field methods. An approach is here proposed for screening and confirmatory analysis of glyoxylate in extracts from different parts of the leaf treated with glyphosate, a representative of the glycine herbicide family. The screening method is a modification of the colorimetric test reported by Kramer et al. [21]. The main variables influencing the derivatization reaction, as well as those

involved in the extraction of the target analyte from plants, have been optimized using multivariate approaches to minimize or avoid the influence of interferences for more accurate and simple determination of glyoxylate and for quantitative extraction from the samples, respectively. The approach has been completed with a confirmatory method based on capillary-electrophoresis separation of glyoxylate from plant extracts and diode-array detection/quantification.

2. Materials and methods

2.1. Reagents

Potassium ferricyanide, methanol and hydrochloric acid from Panreac (Barcelona, Spain) and glyoxylate sodium salt and phenylhydrazine from Sigma (St. Louis, USA) were used in the screening method. Stock-standard solutions were prepared by dissolving 0.1 g glyoxylate in 100 ml deionized water (18 mΩ) from a Millipore Milli-Q water purification system. Acetone, sodium hydroxide and acetonitrile from Panreac, and hexadecyltrimethylammonium bromide (CTAB) from Fluka (Buchs, Switzerland) were also used. Glyphosate (36% concentration) was obtained from Monsanto (St. Louis, USA) as a commercial herbicide for plants treatment.

2.2. Samples

Seeds from different *Lolium spp*. biotypes were collected in different geographical zones of Spain for germination in Petri dishes using a damp filter paper as substrate. The seedlings were transplanted to plastic pot s—three plants in each pot— filled with 1:2 (v/v) peat:clay as substrate. When plants averaged six leaves, commercial glyphosate was applied to 90% of the plants while the remainder 10% was used as control. Application was performed by spraying at 200 kPa in a closed chamber calibrated at 0.5 m height above the target surface, with a relative volume of 200 l ha^{-1}. All plants —treated with herbicide and control— were cut after 0, 24, 48, 72 and 96 h since application and stored at −40 ºC until use. The sample thus prepared was used for screening analysis. For confirmatory analysis, the plant was placed in a porcelain mortar, flash-frozen

using 20 ml liquid nitrogen, grinded to a fine powder using the porcelain pestle for 5min and stored at −40 ºC until use.

2.3. Instruments and apparatus

Herbicide was applied to plants located in a closed spray chamber furnished with a flat jet-spray tip Teejet 80.02 (Wheaton, USA). Eppendorf vials (Eppendorf GA, Germany) were used in the extraction and derivatization steps. A thermostated centrifuge Beckman Coulter Avanti J-25 (Fullerton, USA) furnished with a rotor no. 20 and an ultrasonic bath from Selecta (Barcelona, Spain) —generator power 50 W— equipped with a temperature control unit were used in the extraction step. Nylon filters 45µm pore-size and 13mm i.d. from Millipore (Carrigtwohill, Ireland) were used to remove solid particles. A thermostated water-bath from Selecta (Barcelona, Spain) was used for acceleration of extraction and derivatization steps.

A 3D Capillary Electrophoresis Agilent G1600A Instrument, equipped with a diode array detector (DAD, range 190–600 nm); capillary tubing of 88.5cm (effective length 80 cm) × 50 µm i.d.×375 µm o.d. (Tecnokroma, Spain), and thermostated by a Peltier unit, was used for confirmatory analysis of glyoxylate. The instrumental setup was controlled by the Agilent ChemStation software, which also enabled data acquisition and processing. A 8453 Agilent diode-array spectrophotometer (190–1100 nm) was used to obtain absorption spectra.

2.4. Screening method

50 mg of the target plant and 1 ml water are put into contact in an Eppendorf vial, which is subject to ultrasound for 10 min at 20 ºC. Two 450 µl aliquots of the extract are used: each aliquot is mixed with 50 µl of a freshly prepared 1% (w/v) solution of phenylhydrazine in 100 mM HCl, and kept at 60 ºC for 10 min; then acidified by adding 250 µl concentrated HCl. 100 µl of an1.6% (w/v) $K_3Fe(CN)_6$ in water solution is added to one of the aliquots (aliquot A), and 100 µl distilled water to aliquot B and the absorbance of both aliquots is monitored at 520 nm after reaction development for 12 min. The difference in absorbance between

aliquots A and B corresponds to glyoxylate extracted from the given plant. The scheme of the overall process is in Fig. 1.

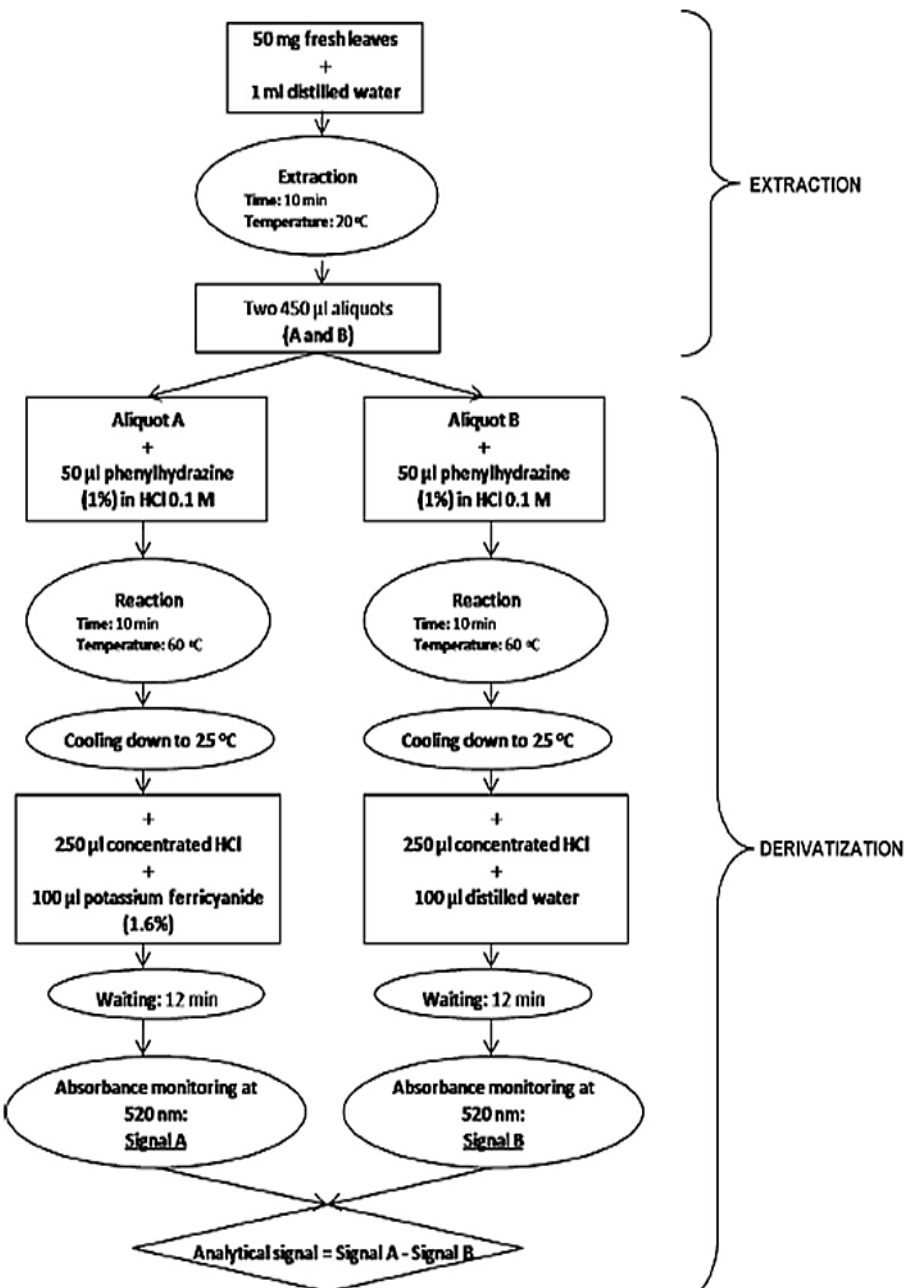

Fig. 1. Scheme of the proposed screening method.

2.5. Confirmatory method

The method used for confirmation of screening is that proposed by the authors for determination of glyphosate, glyoxylate, sarcosine, aminomethylphosphonic acid and formaldehyde, based on ultrasound-assisted extraction and separation-quantification by capillary electrophoresis-diode array detection [23]. In this method, 1.5 g sample is transferred to a plastic beaker andextracted three times with 8 ml 1:1 water–acetone, each time involving magnetic stirring for 10 min, ultrasonication for 5 min, and centrifugation at 4 ºC and 12,096 × g for 15 min. The supernatants obtained with the three 8 ml portions extractant are pooled and evaporated to dryness under nitrogen flow. The extract is reconstituted with 2 ml background electrolyte (BGE) (10 mM potassium phthalate, 0.5 mM CTAB and 10% acetonitrile at pH 7.5) and filtered through a nylon filter before CE analysis. The filtered extract is injected for 5 s at −10 kV into the BGE. The analysis voltage is −20 kV, and the monitoring wavelength selected for indirect detection of all analytes 220 nm. To maintain the capillary under optimal working conditions, its surface was regenerated after each run by sequential washing with water (2 min), 0.1 M sodium hydroxide (2 min), 1 min waiting, and BGE (10 min). In addition, the capillary was activated everyday by sequential washing with water (1 min), 0.1 M sodium hydroxide (10 min), 5 min waiting, and water (1 min).

3. Results and discussion

3.1. Optimization of the method for screening of glyoxylate

The method reported by Kramer *et al*. for the determination of glyoxylate was applied only to analysis of water [21]. Use of this method after extraction of the target analyte from plants requires the joint optimization of the extraction and derivatization steps to assure the highest efficiency of glyoxylate isolation with minimum interferences from species with carbonyl groups, because of the scant selectivity of the derivatization reaction. Extraction of glyoxylate from plants is influenced by temperature, process time, pH of the extraction medium (HCl concentration), and applied auxiliary energy. Meanwhile, the derivatization step

is influenced by incubation temperature and reaction time, in addition to reagents concentration.

Preliminary experiments on extraction in the presence and absence of ultrasound energy showed that this energy accelerates sample leaching, which is complete after 10 min, causes degradation for longer irradiation times, and modifies the presence of interferents in the extract. The time required for a similar extraction efficiency using magnetic stirring is 40 min.

After checking both that the concentrations of reagents proposed by Kramer *et al*. are in a high excess with respect to that of glyoxylate in the extract from plants and none of the reagents absorb at the monitoring wavelength, these concentrations were adopted for the screening method. Thus, the optimization study was centered on extraction temperature, extraction time, hydrochloric acid concentration in the extractant, temperature of the first step of the derivatization reaction development (to avoid reaction of other species with carbonyl groups present in the extract), and derivatization reaction time (because of the instability of the reaction product). These influential variables were optimized by a multivariate approach using as response variable the difference in absorbance between the two aliquots (A and B) used for each optimization point: one in which the 1,5-difenilformazan is formed after derivatization and the other, corresponding to the background signal (contribution from other species absorbing at 520 nm), without addition of the redox solution which gives place to the coloured compound from the target analyte. Therefore, the 1,5-difenilformazan formation could be maximized (both by favouring analyte extraction and formation of the coloured reaction product); while the background signal (contributing pheophytins formed in acid medium by conversion of extracted chlorophylls) could be minimized.

The use of a multivariate experimental design enabled drastic reduction of the number of experiments to be carried out. First, a factorial design involving 19 experiments and 3 central points provided information about the most influential variables as well as their possible interactions, so that the main effects and interactions were statistically evaluated. Fig. 2 shows the variables found to be significant for extraction and derivatization of glyoxylate, which were the

concentration of HCl in the extractant and the time for development of the first step of the derivatization reaction; that is, after addition of phenylhydrazine.

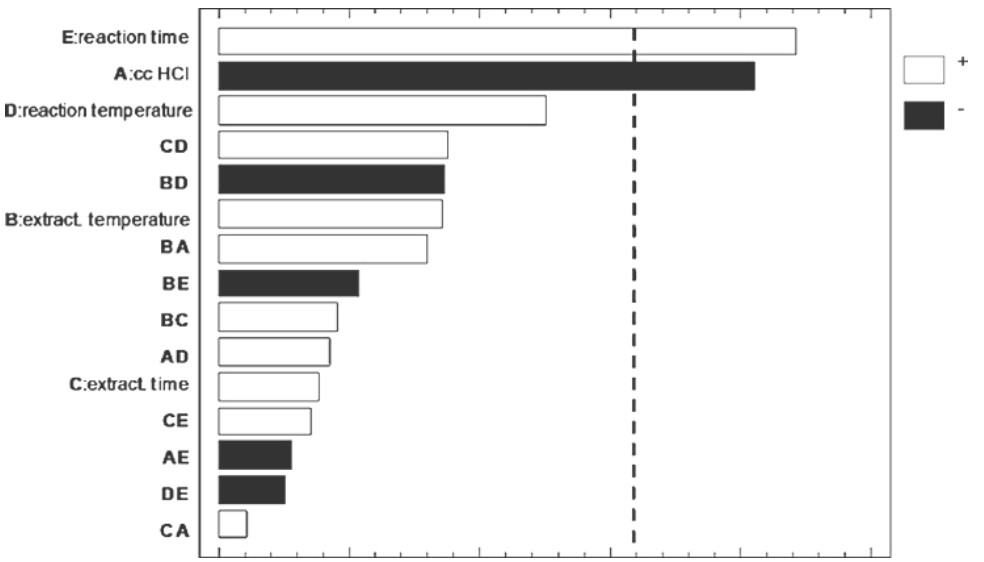

Fig. 2. Significance level of each variable studied in the screening experimental design for extraction and derivatization variables. Only two variables are significant: reaction time (positive effect) and HCl concentration (negative effect).

The acid concentration showed a negative effect, while the effect of the reaction time was positive. Therefore, the variable response increased by lowering acid concentration and increasing the reaction time. Nonsignificant factors were fixed at their optimum values, which were extraction temperature 20 ºC, extraction time 10 min, and temperature for formation of the phenylhydrazone (after addition of the phenylhydrazine solution) 60 ºC. The two significant variables were subsequently studied by a surface-response design in which they were in the range 0–0.1M for hydrochloric acid and 10–20 min for reaction time. The estimated response surface in Fig. 3 shows a better behaviour without acid addition and 20 min incubation time.

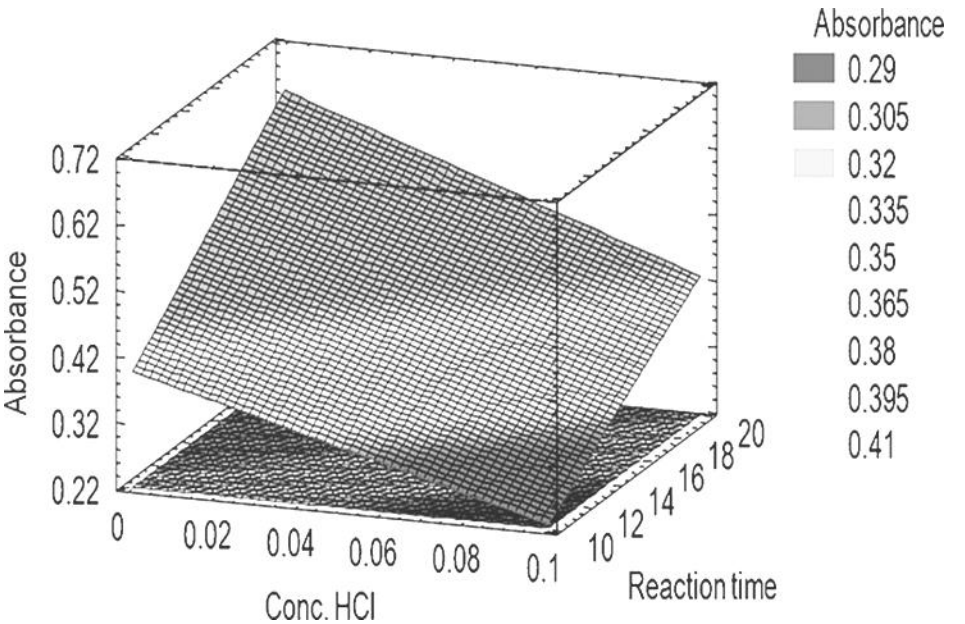

Fig. 3. Contours of the estimated response surface in the second experimental design. The optimum values (maximum absorbance) are represented by the less coloured area of the figure, indicating the values of the variables to which they must work to obtain the maximum signal.

The optimum development of extraction in the absence of HCl in the extract is in contradiction with usual favourable effect of acids in extraction media for isolation of intracellular metabolites. This behaviour can be explained because chlorophylls have scant solubility in water and, in addition, their undesirable conversion to pheophytins (with absorption at the monitoring wavelength of the target reaction product) is favoured in an acid medium. Therefore, the interference of pheophytins is avoided at a close-to-neutral pH and ambient temperature in order to avoid chlorophylls extraction. In this way, the signal from aliquot B is drastically decreased and, thus, the difference between signals from aliquots A and B increased.

Optimization was completed with a kinetics study of the last step of the derivatization reaction because the monitored product showed instability.

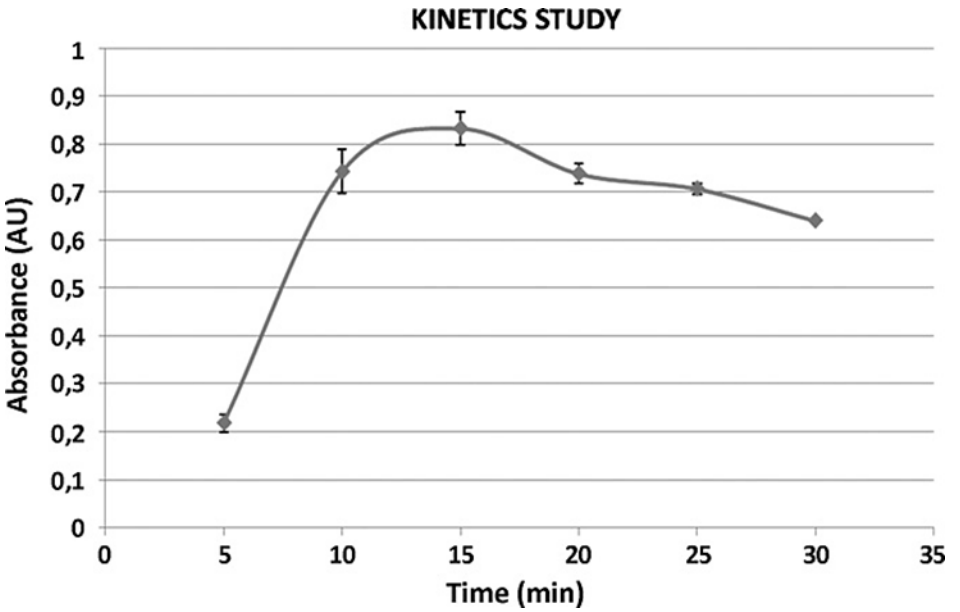

Fig. 4.. Kinetics study of the redox reaction. Degradation of the formazan complex starts 12 min after mixing with the oxidant (potassium ferricyanide).

As Fig. 4 shows, 12 min, from mixture of the reactants to monitoring, is the time necessary to obtain an almost plateau resulting from the equilibrium between formation of the reaction product and its degradation. After this period, degradation predominates over formation and the absorbance of the analytical signal decreases.

3.2. Validation of the proposed approach

Calibration plots were run for glyoxylate using the absorbance data as a function of the standard concentration of glyoxylate. The calibration equation, regression coefficient and linear dynamic range are listed in Table 1.

Table 1. Features of the two methods for a pool of natural samples cut 96 h after glyphosate application

Method	Calibration equation[a]	R^2	Linear range	LOD[b]	LOQ[b]	Average[c]	S_b[d]	S_{wr}[d]
Screening	Y=0.0678+0.0886X	0.997	LOQ-600	0.05	0.5	38.2	3.3	5.6
CE	Y=0.641+0.244X	0.998	LOQ-500	0.2	0.7	2.7	2.6	5.0

[a]*Y* expressed as absorbance units×10^{-3}; *X* as µg ml^{-1}.[b]Expressed as µg ml^{-1}.[c]Expressed as µg g^{-1}.[d]Expressed as %.

The limit of detection (LOD), expressed as the concentration of analyte providing a signal 3σ above the mean blank signal, where σ is the standard deviation of the blank signal, was 0.05 μg ml^{-1}. The blank signal was obtained from plants which were not treated with glyphosate. The limit of quantification, LOQ, expressed as the concentration of analyte which gives a signal 10σ the mean blank signal, was 0.17 μg ml^{-1} (see Table 1).

Within laboratory reproducibility and repeatability were evaluated for the proposed method in a single experimental setup with triplicates by experiments carried out with natural samples—a pool of plants from four weed biotypes of *Lolium spp*. cut 96 h after glyphosate application. Three measurements of these samples per day were carried out on 3 days. Eq. (1) was used to determine the between-day variance:

$$s^2_{between} = (MS_{between} - MS_{within}) / n_j \quad (1)$$

where MS is the mean square (residual sum of squares rated by the freedom degrees) and nj is the number of replicates per day. The within-laboratory reproducibility, s^2_{WR}, was calculated by Eq. (2).

$$s^2_{WR} = s^2_r + s^2_{between} \quad (2)$$

where s^2_r is the residual mean squares within-days and $s^2_{between}$ is the variance due to the between-day effect.

The results obtained are listed in Table 1. The repeatability, expressed as relative standard deviation (RSD), is 3.3% for glyoxylate in this pool of plants; the within-day laboratory reproducibility, also expressed as RSD, is 5.6%.

3.3. Study of potential interferences in the analysis of glyoxylate

After eliminating interferences such as those caused by chlorophylls and their degradation products by optimization of the extraction step, other potential interferences of the derivatization reaction were studied.

The derivatization reaction between phenylhydrazine and glyoxylate and subsequent oxidation with formation of the coloured compound is common to other compounds with carbonyl groups such as aldehydes and ketones, but in this case, with yellow colour. Therefore, compounds usually present in plants extracts such as sugars (glucose, fructose) or derivatives (ascorbic acid) could be potential

interferents in the determination of glyoxylate. An interference study was carried out spiking glucose and fructose at concentrations ranging from 0.1 to 1000 mg ml^{-1} to a 5 mg ml^{-1} glyoxylate standard solution. The average measurement resulted in a glyoxylate concentration of 5.026 ± 0.139mg ml^{-1}, which involved a relative standard deviation below 3%. This enabled to onclude that there is no interference at the monitoring wavelength.

3.4. Influence of sample humidity

The humidity content of the samples is other relevant factor which deserved to be studied because of a drying step (to homogenize humidity between different plants). Heating could favour the conversion of chlorophylls into pheophytins, the latter being soluble in the extractantmedium.The influence of sample humidity was tested by experiments involving portions of Lolium spp., which were cut and independently dried at temperatures of 30, 60 and 70 ºC for 12 h. Fig. 5 illustrates the absorbance at 520nm of aliquots A and B of extracts (that is, with and without reaction with ferricyanide).

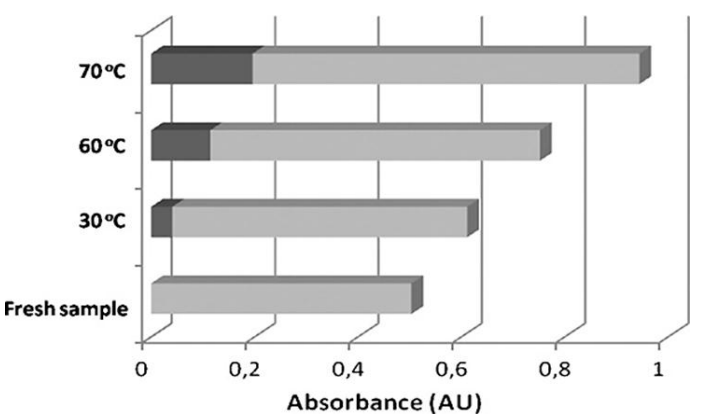

Fig. 5. Changes in absorbance at 520 nm of the extracts from fresh samples and samples dried at 30, 60 and 70 ºC prior to (dark zone) and after (clear zone) reaction with potassium ferricyanide. The extracts from dried samples are coloured due to the presence of pheophytins. The absorbance of these compounds is summed up to that from the product of the derivatization reaction.

As can be seen, the absorbance in aliquots B (caused by the presence of pheophytins in the extract) is drastically influenced by the drying temperature that influences the formation of pheophytins, which is nil in fresh material. This

is an additional key favourable aspect of the method, which allows the plant to be subject to extraction without any prior treatment.

3.5. Sampling study for glyoxylate determination

To ensure the homogeneity of the samples when taken from different parts of the leaf, a study of glyoxylate distribution on the leaf was performed.

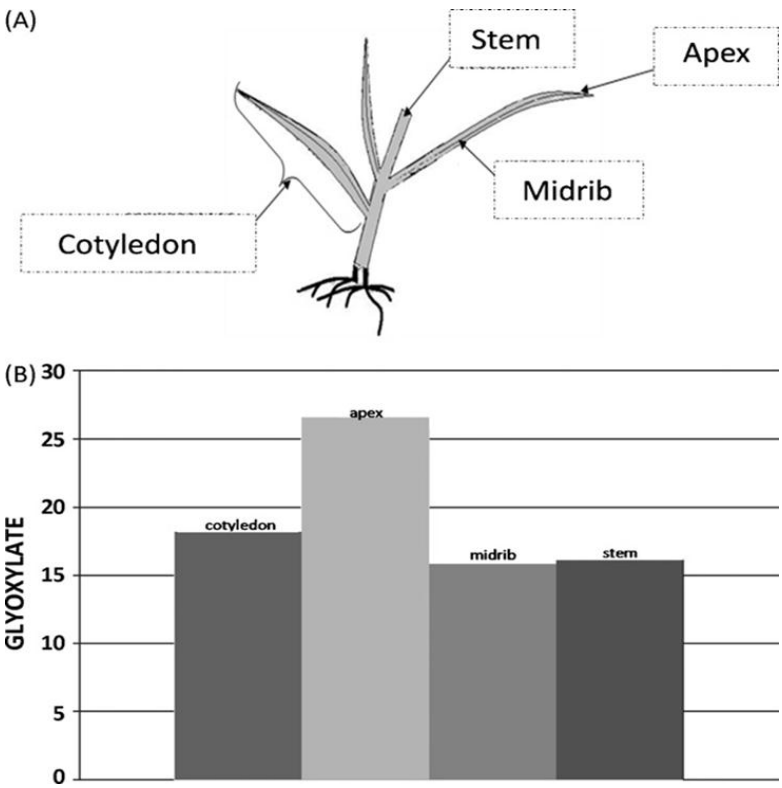

Fig. 6. (A) Scheme of Lolium spp. signaling the different parts selected for determination of glyoxylate. (B) Accumulation of glyoxylate in the different parts of *Lolium spp*. As can be seen, the accumulation of glyoxylate is higher in the apex, while it is lower in midrib and stem, both with similar accumulation.

Fig. 6A shows the different sampling areas of leaves selected for this study: cotyledon, apex, midrib and stem; while Fig. 6B shows the glyoxylate concentration as a function of the sampled area. As can be seen, the concentration of glyoxylate was significantly higher in the apex area, behaviour ascribed to the fact that the apex is the part of the plant more exposed to light, with a higher photorespiration activity.

3.6. Confirmatory analysis

The confirmatory analysis of the proposed screening approach is based on CE, which allows determination of glyoxylate in plants together with glyphosate aminomethylphosphonic acid, sarcosine and formaldehyde, previously proposed by the authors [23]. Both screening and confirmatory methods were applied to the same samples following the procedures under "Experimental". Table 2 shows the results provided by both methods, while Table 3 summarizes the differences between the two methods based on the equation of relative error (in which the data provided by the CE method are considered as the true value of the concentration of glyoxylate in the samples).

Table 2. Analysis by the screening and CE methods of glyoxylate in samples cut 0, 24, 48, 72 and 96 hours after glyphosate application (*n*=9 replicates)

Samples	**Concentration of glyoxylate[a]**					**Method**
	0 h	**24 h**	**48 h**	**72 h**	**96h**	
Lolium 1	13.794	27.256	34.479	55.909	51.545	**Screening**
	14.738	29.236	34.173	56.752	52.432	**CE**
Lolium 2	25.962	59.453	42.169	53.411	58.761	**Screening**
	26.745	60.2949	43.053	54.063	59.648	**CE**
Lolium 3	51.944	23.561	38.189	46.165	27.256	**Screening**
	52.727	24.506	39.172	47.029	28.148	**CE**
Lolium 4	27.211	94.096	105.059	130.77	103.922	**Screening**
	28.096	94.981	105.904	132.046	104.931	**CE**

[a]Expressed as µg g^{-1}.

Table 3. Difference between the screening and the CE methods based on the equation of relative error (data from the CE method are considered as true value)

Sample	**Relative error (%)**				
	0 h	**24 h**	**48 h**	**72 h**	**96 h**
Lolium 1	6.399	6.772	0.896	1.485	1.691
Lolium 2	2.929	1.396	2.0529	1.207	1.488
Lolium 3	1.486	3.855	2.510	1.837	3.171
Lolium 4	3.152	0.932	0.798	0.966	0.961

4. Conclusions

The screening method here proposed allows a fast determination of glyoxylate in plants treated with herbicides using simple instrumentation. In addition to its simplicity and rapidity, the method is endowed with appropriate reproducibility and sensitivity, so it can be used to monitor changes in the level of glyoxylate (or other members of the glycine family) in plants treated with herbicides, thus relating variations in the level of this metabolite with the application of herbicides.

The in-depth optimization study has allowed elimination of interferences and avoidance of pretreatment steps, thus making possible the development of the method in 32 min, which in turn makes possible its simultaneous application to a high number of samples in a short time, as desirable for a screening method.

Application of this method to obtain information of the mode of action of herbicides in different plants (both resistant and susceptible to herbicides) is our present research, to demonstrate the usefulness of the method in agronomic studies.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Capítulo 4

Ultrasound-assisted extraction with LC–TOF/MS identification and LC–UV determination of imazamox and its metabolites in plants

Ultrasound-assisted extraction with LC–TOF/MS identification and LC–UV determination of imazamox and its metabolites in plants

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Ultrasound-assisted extraction with LC–TOF/MS identification and LC–UV determination of imazamox and its metabolites in plants

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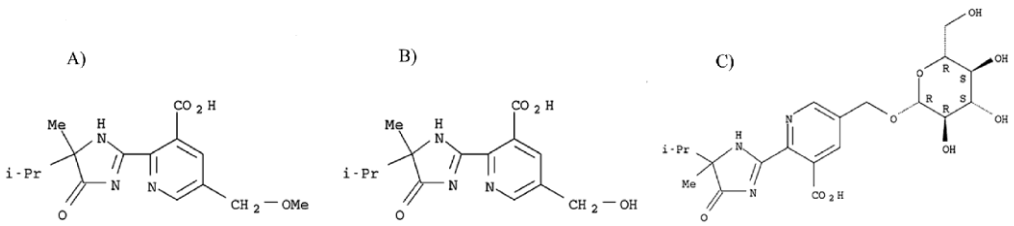
ABSTRACT: A simple method based on liquid chromatography–UV absorption detection (LC–UV) for simultaneous determination of imazamox and its metabolites (*i.e.* hydroxy and glucose conjugated metabolites) in plants is reported here. Sample preparation was carried out by ultrasound-assisted extraction with subsequent filtration of the extracts and cleanup/concentration prior to chromatographic separation and detection. The linear dynamic range of the calibration curve was within 0.27–600 μg mL^{-1} with correlation coefficient of 0.998 and precision —studied at two concentration levels, 0.1 and 2 μg mL^{-1}— of 2.9 and 5.0% for repeatability, and 4.7 and 6.9% for reproducibility, respectively. Identification and confirmatory analysis of the presence of imazamox and metabolites in extracts from treated plants was carried out by LC–TOF/MS in high resolution mode for precursor ion. The method was validated by analyzing wheat samples treated with 200 g of active ingredient Ha^{-1} of imazamox.

KEYWORDS: *imazamox, metabolites, ultrasound-assisted extraction, LC–TOF/MS, high resolution mass spectrometry, LC–UV separation–determination*

INTRODUCTION

Imazamox [3-pyridinecarboxylic acid, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-(methoxymethyl)-] is one of the herbicides with imidazolinone structure, selective for many legominous and cereals crops, which was developed by BASF (American Cyanamid Company, Princeton, NJ, USA) in the 80's of the past century. This compound is relatively polar because of the presence of carboxyl and amino groups in its structure (Supplementary Fig. 1), which are susceptible to pH changes able to confer charge to the imazamox molecule. This herbicide is used at doses equal or lower

than 200 g active ingredient Ha^{-1} owing to its classification as poorly degradable substance by photolysis respect to other imidazolinone compounds.[1–3]



S- Figure 1. Chemical structures of imazamox (A) and its two metabolites: hydroxy (B) and glucoside (C) metabolites.

There are few methods reported in the literature for determination of imazamox in environmental or food samples[4–15] as compared with those existing for other herbicides such as glyphosate, glufosinate, etc. The study by Ohba *et al.*[6] showed that the metabolism of imazamox occurs through a single pathway involving the gradual formation of hydroxy and glucose metabolites. Other authors have explained the presence of metabolites by the appearance of radiactivity in extracts of plants treated with ^{14}C-imazamox[12,13] as the target metabolites are not commercially. The absence of the standards has delayed research on degradation of this herbicide, which has been restricted to studies involving either radiactivity-based methods[12,13] or required the synthesis of metabolites by the authors.[6] Working on radiactivity-based methods, Harir *et al.* have ventured the presence of metabolites on the amount of measured radioactivity, which is not a reliable information as radiactivity can originate from a non-metabolic degradation occurred outside the plant by photolysis.[4] The main problems in dealing with metabolites synthesis are costs (both reagents and time) and purity achieved. The search for fast and effective procedures for identification of metabolites without standards is a challenge in the agronomical field due to the need for knowing the behavior of a plant against a given herbicide. In the case of resistant weeds the finding of a such procedure would constitute a useful tool to raise new attack strategies.

Capillary electrophoresis (CE) in its different modes has been the most widely used separation technique for determination of imidazolinones.[16] Thus, Ohba *et al.*[6] described a method using reverse micellar electrokinetic chromatography

(MEKC) with UV detection which allowed determination of imazamox and its hydroxy and glucoside metabolites (synthesized standards). The capability of CE can be improved by coupling to a mass spectrometry (MS) detector, which has been the subject of several studies dealing with trace analysis of herbicides including imazamox.[5–7, 14, 17–19] Apart from CE–MS, separation of imazamox from mixtures with other herbicides and pesticides has involved gas chromatograpy (GC)[15] and, particularly, liquid chromatography (LC),[4, 8–13] with MS detection in all instances. Despite the potential of MS for identification of degradation products from herbicides,[4] no MS-based studies have been proposed to confirm the presence of imazamox metabolites in plants.

The present research was aimed at the development of a method for the simultaneous determination of imazamox and its metabolites in plants based on ultrasound-accelerated extraction, individual separation by HILIC and UV detection. With this aim and taking into account the absence of commercial standards of the target metabolites, previous identification of them in extracts from plants treated with the herbicide by high-resolution MS was planned, together with final quantitation of the identified metabolites relative to their precursor.

MATERIALS AND METHODS

Reagents. Chromatographic grade methanol, acetic acid, and acetonitrile were acquired from Panreac (Barcelona, Spain). LC–MS grade acetonitrile and acetic acid (Scharlab, Barcelona, Spain), and deionized water (18 MΩ·cm) from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA) were used for preparation of chromatographic mobile phases in LC–MS analyses. imazamox herbicide standard was provided by Sigma (St. Louis, USA). The stock standard solutions were prepared by dissolving 0.1 g of imazamox in 100 mL of 90:10 (v/v) methanol–water. The commercial imazamox product used for plant treatment was PULSAR 40 (4% w/v of imazamox) from BASF (Princenton, NJ, USA).

Samples. Seeds from two biotypes (resistant and susceptible varieties) of *Triticum aestivum* with different resistance to imazamox were collected in Chile for germination in Petri dishes using a damp filter paper as substrate. The ED_{50} values (measurement of active ingredient per Ha with capability to cause 50% growth inhibition of plants as compared to the untreated control) were 289.55 and 25.15 g of active ingredient Ha^{-1} for the resistant and susceptible biotypes, respectively. The seedlings were transplanted to plastic pots (three plants in each pot), filled with 1:2 (v/v) peat–clay as substrate. When plants averaged five–six leaves, commercial imazamox was applied to 90% of the plants while the remainder 10% was used as control. Application was performed by spraying at 300 kPa in a closed chamber calibrated at 0.5 m height above the target surface, with a relative volume of 200 L Ha^{-1} and with one dose of 5 L Ha^{-1} PULSAR 40 (200 g of active ingredient Ha^{-1}). Plants —both treated with herbicide and controls— were cut 0, 3, 6, 24 h and 8 days after herbicide application, and stored at −40 ºC until use.

Instruments and apparatus. A flat jet spray tip Teejet 80.02 (Wheaton, USA) was used to apply the herbicide to plants inside a closed spray chamber. A Microdigest 301 digestor of maximum power 200 W (Prolabo, Paris, France) furnished with a microprocessor programmer (Prolabo) to control the microwave unit, and a Branson 450 digital sonifier (20 kHz, 450W) with tunable amplitude and duty cycle, equipped with a cylindrical titanium alloy probe (12.7 mm in diameter) were used for microwave- and ultrasound-assisted extractions, respectively. Test tubes (10 mL) from Pyrex (London, England) were used for extraction. Nylon filters 45 μm pore-size and 13 mm i.d. from Millipore (Billerica, Massachusetts,USA) were used to remove solid particles. Centrifugation of the extracts was carried out by a Mixtasel (Selecta, Barcelona, Spain) centrifuge.

A 15 Gold HPLC System from Beckman Coulter (Fullerton, USA) equipped with a 26 System Gold Diode Array detector (DAD, wavelength range 190–600 nm) and a HILIC column (20 cm × 4.6 cm, 3 μm particle size) from Teknokroma (Barcelona, Spain) was used for analysis. The instrumental setup

was controlled by the Karat 3.0.7 software, which also enabled data acquisition and processing.

A series 1200 LC system (Agilent Technologies, Palo Alto, CA) coupled to an Agilent UHD TOF accurate mass spectrometer (Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source was used for identification and confirmatory analysis. Nitrogen was provided by a high purity generator from CLAN Tecnológica (Sevilla, Spain) and used as the source gas, while ultra pure nitrogen (99.999%) from Carburos Metálicos (Sevilla, Spain) was used as collision gas. MassHunter Workstation software version 3.01 (Agilent Technologies) was used to control data acquisition and support qualitative analysis including extraction of molecular features, generation of molecular formula.

Sample pre-treatment. Before extraction, the frozen samples were washed three times with 20 mL water to remove traces of imazamox on the leaf surface. Each sample was placed in a porcelain mortar and flash-frozen using 20 mL liquid nitrogen, and grinded to a fine homogeneous powder using a porcelain pestle for 5 min.

Conventional extraction method. The method described by Pester *et al.*[12] was used as reference, for which 0.5 g of the sample powder was mixed with 15 mL of a 95:5 (v/v) methanol–water mixture, then shaken for 1 h, followed by 15 min centrifugation at 2.900 × g.

Microwave-assisted extraction method. 0.5 g of sample was placed into the extraction vessel with 10 mL 90:10 (v/v) methanol–water. The vessel was positioned at the suited zone for irradiation with focused microwaves. The auxiliary energy was applied at 70 W irradiation power for 4 min (4 × 1 min cycles), after which the solid residue was removed by centrifugation (15 min at 2.900 × g).

Proposed ultrasound-assisted extraction method. 0.5 g of sample powder was placed into the extraction vessel with 10 mL 90:10 (v/v) methanol–water. The ultrasonic probe was immersed into the extraction mixture for sonication at

70 W irradiation power for 10 min with a duty cycle of 70% (0.7 s/s irradiation). After that, the extract was isolated by centrifugation (15 min at 2.900 × g).

Clean-up and preconcentration. 6 mL of the supernatant (extract) was taken and evaporated to dryness under a nitrogen stream. The solid residue was reconstituted by 0.5 mL of the extractant (90:10 methanol–water) and filtered through a nylon filter syringe before chromatographic analysis.

Chromatographic analysis. The same chromatographic method was used for identification of imazamox metabolites and for determination of imazamox and its metabolites in extracts from plants by LC–UV. 50 µL of the reconstituted phase was injected into the LC with a 1% (v/v) acetic acid in water as mobile phase A, and pure methanol as mobile phase B. The elution program started with 5% mobile phase B and followed the linear gradient: step 1, 5 to 20% methanol in 10 min; step 2, 20 to 80% methanol in 10 min; step 3, 80% to 100% methanol in 5 min; step 4, 100 to 5% methanol in 10 min. The constant flow rate and column temperature were 1.0 mL min^{-1} and 40 $^{\circ}C$, respectively. Chromatographic grade and LC–MS grade solvents were used for LC–UV and LC–MS analysis, respectively.

Identification of imazamox metabolites by LC–TOF/MS. Identification of imazamox metabolites was carried out by LC–TOF/MS in high resolution mode. The samples were analyzed both with positive and negative ionization modes. The operating conditions for the mass spectrometer were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L min^{-1}; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3000 and 4000 V for negative and positive ionization modes; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V. Data acquisition (2.5 Hz) in both the centroid and profile modes was governed via the Agilent MassHunter Workstation software. The mass range for detection was set at *m/z* 60–1100. Reference mass correction on each full scan was performed with a continuous infusion of Agilent TOF biopolymer mixture containing purine (*m/z* 121.0508) and hexamethoxyphosphazyne (*m/z* 322.0481). The analyses were carried out with a resolution of 20.000 to examine the isotopic

profiles for imazamox and its metabolites. The analytes were identified by accurate mass detection and formula elucidation based on isotopic distribution.

Once the samples were analyzed by LC−TOF/MS, the molecular features were extracted from the raw data files prior to formula generation. Ions with identical elution profiles and related *m/z* values (representing different adducts, ions generated after specific neutral losses or isotopic forms from the monoisotopic ions) were extracted as molecular features (MFs) in a matrix characterized by retention time (RT) and accurate mass and containing intensity in apex of chromatographic peaks as data. The isotope model corresponded to common organic molecules with peak spacing tolerance of *m/z* 0.0025 ± 7.0 ppm. The MF extraction algorithm limited extraction to ions exceeding 5000 counts with charge state limited to a maximum of two. The allowed negative ions were deprotonated species and formate adducts, while allowed positive ions were protonated species and sodium adducts. Dehydratation, glucosidation and phosphate neutral losses were also allowed. Candidate formula generation was supported on C, H, O, N, S and P elements by setting a cut-off value of mass accuracty at 2 ppm.

Determination of imazamox and metabolites by LC–UV. After identification and confirmatory analysis by LC–TOF/MS, imazamox and metabolites were determined by LC–UV analysis at the absorption wavelength of 240 nm. Chromatographic peaks in LC–UV were assigned according to retention times using as reference the imazamox peak detected by spiking extracts with the commercial standard. Quantification of imazamox metabolites was based on the calibration model prepared for imazamox and expressing the results as µg of the analyte equivalent to imazamox g^{-1} of plant.

RESULTS AND DISCUSSION

Optimization of the chromatographic separation of imazamox and metabolites. The first aim of this research was to confirm the presence of imazamox metabolites in real samples treated with the herbicide following common agronomical practices. For this purpose, samples extracted by the

conventional protocol described under Experimental were analyzed. The resistant biotype was selected for this study. Chromatographic profiles obtained with LC–UV under different conditions were preliminary compared to detect peaks that could be associated to tentative imazamox metabolites. Figure 1 shows the main results obtained in this study by analysis of extracts from: (1) non-treated plant as an absolute blank of the sample matrix; (2) treated sample at time 0 after herbicide application when imazamox has not been metabolized; (3) treated sample at time 0 after herbicide application which was spiked with imazamox standard (50 μg mL^{-1}) to identify imazamox chromatographic signal; (4) treated plant collected 6 h after herbicide application to detect imazamox and the new chromatographic peaks that could be associated to tentative metabolites at short-term by comparison with the chromatogram illustrated in Fig. 1.B; (5) treated plant collected 8 days after herbicide application to check the metabolic effect at long-term. This strategy was designed due to the lack of commercial metabolites and the need for identifying them as naturally generated in the plants. As can be seen, Fig. 1.C shows that imazamox, detected in those samples after herbicide application, was eluted at *ca.* 19 min. Apart from that, the chromatogram obtained by analysis of the sample collected 6 h after imazamox application did not show a significantly different profile from that corresponding to the sample collected just after herbicide application (0 h). Short-term metabolism of imazamox was poorly detected since small chromatographic signals appeared in the elution range 15–17 min. On the other hand, Fig. 1.E reveals significant differences by detection of new chromatographic peaks in this elution window, which could be tentatively ascribed to imazamox metabolites. Both signals would be associated to metabolites more polar than imazamox according to the gradient program used; this behavior is quite logical considering that imazamox metabolism should lead to more polar compounds. The two chromatographic peaks appeared at *ca.* 16 and 17 min. It is also worth mentioning that the imazamox chromatographic signal showed a low intensity, indicative of almost complete metabolism of the herbicide in the resistant biotype.

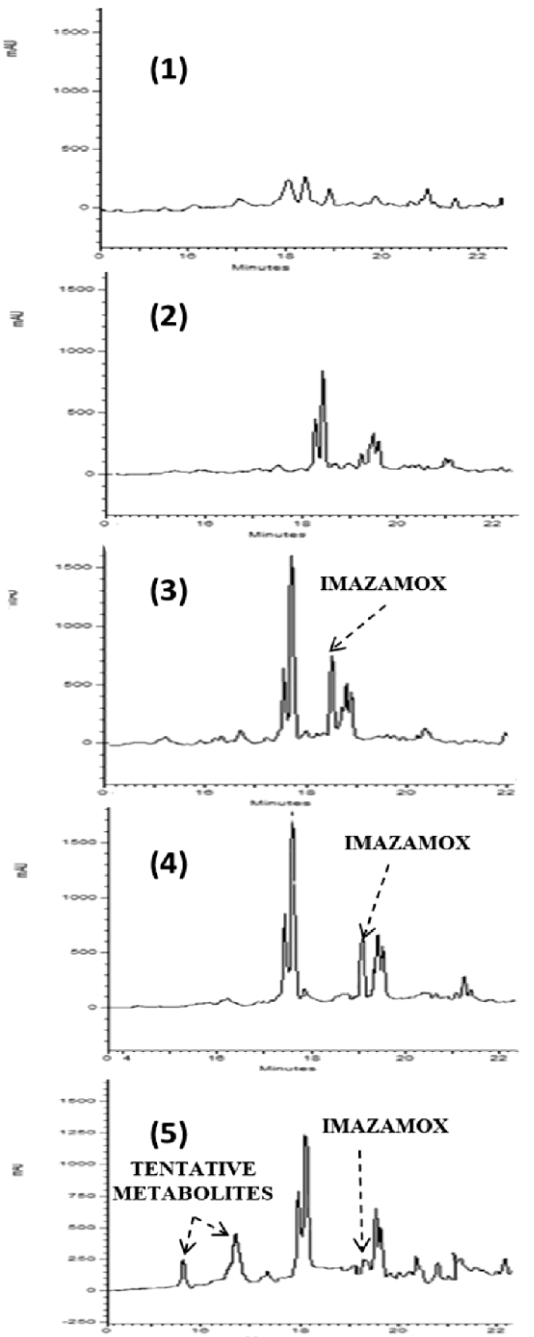


Figure 1. Chromatograms of extracts from resistant wheat plants (*Triticum aestivum*) subjected or not to imazamox treatment obtained by the conventional extraction method: (1) non-treated plant as an absolute blank; (2) treated sample at time 0 since the herbicide application; (3) treated sample at time 0 since the herbicide application spiked with imazamox standard (50 μgmL); (4) treated plant at time 6 h since the herbicide application; (5) treated plant at time 8 days since the herbicide application.

After location of chromatographic peaks which could be tentatively associated to imazamox metabolites, the influence of the main variables involved in the chromatographic separation was studied to obtain the highest resolution in the shortest time. The variables studied were column length, temperature, % acetic acid, and programmed gradient. The response variables were the peak area and resolution expressed as the peak width at half-height. An increase of temperature up to 40 ºC shortened the chromatogram time without peaks overlapping effects. However, the chromatographic resolution at temperatures above 40 ºC was quite poor owing to peak broadening effects. Therefore, 40 ºC was adopted as temperature in the column compartment. Taking into account the structure of imazamox and its main metabolites, a slight acidic pH, within 4.6–4.7 (by adding 1% acetic acid), favored sensitivity by thickening chromatographic peaks (pKa 2.3). pH values below 4.0 affected negatively the stability of the chromatographic column and degraded the metabolites. The selection of the column length was based on retention times (t_r). A 15 cm column provided partial separation with significant overlapping; while that of 25 cm length yielded very long t_r and wide peaks; therefore an intermediate 20 cm column was selected. The gradient study resulted in the optimum program described under section 2.9, which involves 4 steps and provides a high resolution, particularly in the first part of the chromatogram, where elution of imazamox metabolites was foreseeable.

A study of the injection repeatability and reproducibility —in terms of peak area and t_r— with imazamox standard and extract solutions yielded standard deviations below 7%. These results make unnecessary the use of an internal standard.

After optimization of the chromatographic process the extracts were analyzed by LC–TOF/MS to confirm the identity and presence of imazamox metabolites in resistant plants treated with the herbicide, if present. These tests were carried out with the LC–TOF/MS analyzer in order to take benefits from high resolution mass spectrometry in full scan. The imazamox peak was associated to the ion *m/z* 306.1454 in positive ionization mode, which was identified as the $[M+H]^+$ precursor. Figure 2 shows the extracted ion

chromatogram (EIC) for this experimental *m/z* value. Concerning the signals corresponding to two tentative metabolites, ions at *m/z* 454.1817 and 292.1295 were detected at retention times 16 and 17 min, respectively. Theoretical formulae for these precursor ions and their isotopic distributions revealed the presence of glucoside and monohydroxy imazamox derivatives. Errors of experimental *m/z* values as compared to theoretical ones were below 2 ppm, which supported assignations of formulas. Table 1 lists parameters such as retention time, formula, experimental and theoretical masses, and identification error.

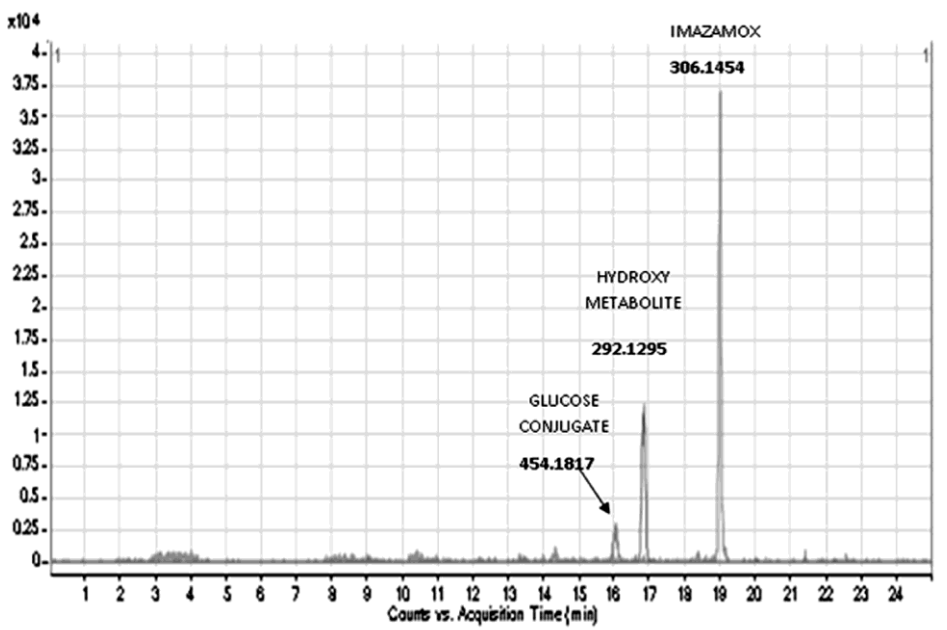
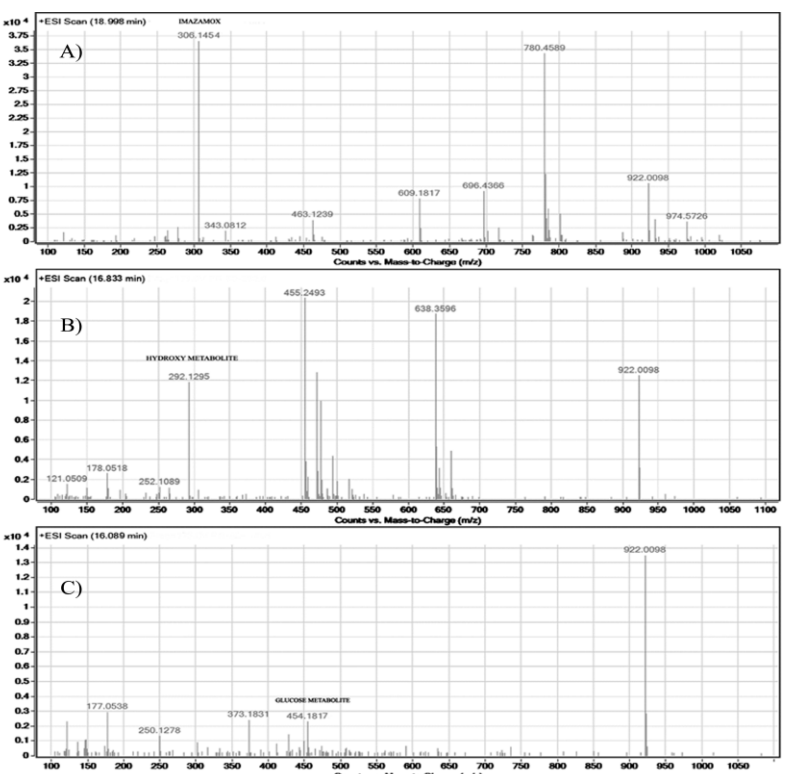


Figure 2. Extracted ion chromatograms for imazamox and its hydroxy and glucoside metabolites obtained by LC–TOF/MS analysis of wheat resistant biotype (treated with 200 g of active ingredient Ha^{-1}) collected 8 days after herbicide treatment. The chromatograms confirm the presence of imazamox and allow the identification of hydroxy and glucoside metabolites.

Table 1. Accurate mass measurements for the identification of imazamox and its metabolites in *Triticum* plants obtained by LC–TOF/MS analyses.

COMPOUND	FORMULA	RETENTION TIME (min)	ION	*m/z* ACTUAL	*m/z* EXPERIMENTAL	ERROR (μg mL^{-1})
Imazamox	C_{15} H_{19} N_3 O_4	18.998	$[M+H]^+$	306.1448	306.1454	1.9599
Hydroxy metabolite	C_{14} H_{17} N_3 O_4	16.833	[M+H]+	292.1292	292.1295	1.0269
Glucose metabolite	C_{20} H_{27} N_3 O_9	16.089	[M+H]+	454.1820	454.1817	0.6605

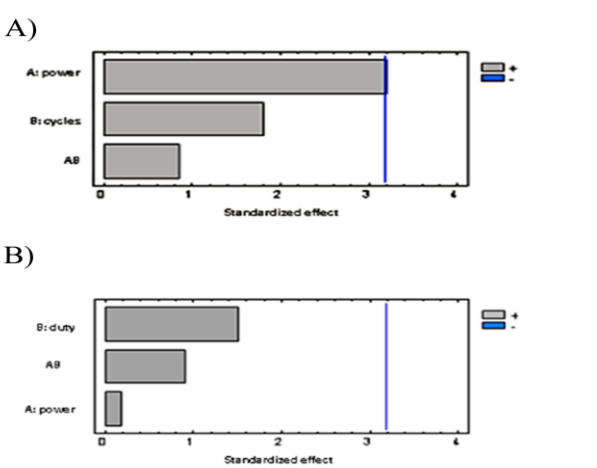
On the other hand, EICs for ions associated to both imazamox metabolites are also illustrated in Fig. 2, while the mass spectrum in full scan for each chromatographic signal is shown in Supplementary Fig. 2).



S- Figure 2. Mass spectrum in full scan mode of the chromatographic signals associated to: imazamox (A), hydroxy derivative of imazamox (B), and glucose derivative of imazamox (C) obtained by LC–TOF/MS analysis of an extract from resistant wheat plants (treated with 200 g of active ingredient cut at 8 days since herbicide application).

Sample preparation: optimization of accelerated extractions. Two types of auxiliary energy were assayed to accelerate the extraction (leaching) step: ultrasound and microwaves. In both cases, the influential variables were optimized by a multivariate approach that enables drastic reduction of the number of experiments to be carried out. The response variable used in all instances for optimization was the peak area of imazamox and those of the putative metabolites. First, a factorial design involving 7 experiments including 3 center points provided information about the most influential variables and their possible interactions, so that the main effects and interactions were statistically evaluated. The optimum separation–detection conditions were used to monitor optimization of extraction aimed at achieving maximum removal of imazamox and potential metabolites from the matrix in a time as short as possible.

Microwave-assisted extraction. The characteristic variables of this type of energy are irradiation power and number of cycles (cycle duration selected: 1 min — selected to avoid boiling of the liquid phase). The results, expressed in the Pareto chart (in Supplementary Fig. 3.A) shows the non-significance of the number of cycles and the significance of irradiation power. The results of this study are shown in Table 2. The significant effect of microwave irradiation power promoted a univariate study within the range 10–90 W at 4 cycles. The best behavior corresponded to 70 W.



S- Figure 3. Significance level of each variable studied in the screening experimental design for microwave-assisted extraction (A) and for ultrasound-assisted extraction (B).

Table 2. Optimization of the extraction step

Auxiliary energy	**Variable**	**Tested range**	**Optimum value (efficiency)**
Ultrasound	Power (W)	10–70	70
	Duty cycle (s/s)	0.1–0.7	0.7
Microwaves	Power (W)	10–90	70
	Number of cycles	1–5	4

Since the stability of imazamox and its metabolites under microwave radiation is not well-known, a kinetics study was performed with different number of cycles. As Fig. 3 shows, 4 cycles were necessary to obtain the maximum extraction efficiency, but a sharp decrease owing to imazamox degradation occurs after the cycle 4, making very critical the application time of this energy.

Ultrasound-assisted extraction. The variables with potential influence on ultrasonic extraction are irradiation amplitude and duty cycle applied for a total time of 3 min in all instances for a comparative basis. The results, expressed in the Pareto chart in Suppplementary Fig. 3B, show the non-significant effect of ultrasonic irradiation variables on the leaching efficiency of imazamox. As can be seen, positive effects were observed for both variables and, for this reason, 70 W and 0.7 s/s duty cycle were adopted for further experiments. Table 2 shows the results of this study.

Optimization of ultrasound-assisted extraction was completed by a kinetics study of the irradiation time to maximize the extraction efficiency. Figure 3 shows that an extration time of 10 min was required to obtain the maximum extraction efficiency for imazamox, which was constant in the range 10–13 min. Longer times allowed detecting that the concentration of the herbicide decreased, possibly owing to degradation. Nevertheless, this degradation effect was less patent than when the process is assisted by microwaves, in which the extraction efficiency decreased dramatically after extraction for 5 min. Attending to operative reasons, the extraction protocol assisted by ultrasonic energy was adopted as the preferred sample preparation for

isolation of imazamox and metabolites. It is worth mentioning that this protocol reduced the extraction time to 10 min as compared to the convenional protocol described under Experimental. The kinetics study of the conventional method proved that the extraction efficiency after 30 min extraction was half of that obtained under ultrasonic irradiation (see Fig. 3).

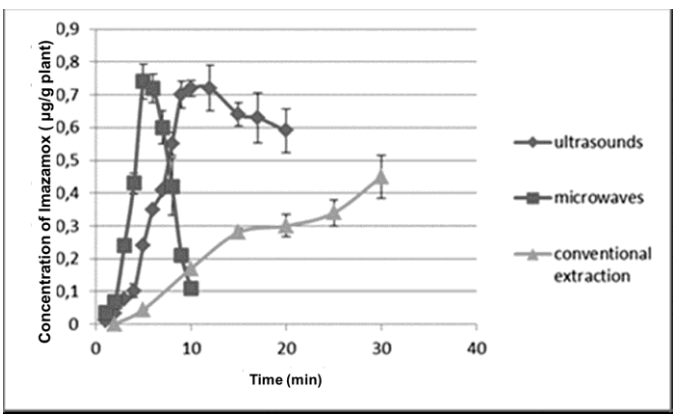


Figure 3. Extraction kinetics for isolation of imazamox using conventional extraction, ultrasound-assisted extraction, and microwave-assisted extraction.

Characterization of the LC–UV method for determination of imazamox. The calibration curve for imazamox was plotted using the peak area as analytical signal, at the monitoring wavelength of 240 nm, as a function of the standard concentration of this compound spiked at different concentrations in an extract of non-treated plant. The calibration equation for imazamox, regression coefficient and linear dynamic range are listed in Supplementary Table 1.

S-Table 1. Features of the method for determination of imazamox by LC–UV.

Method	**Calibration equation** [a)]	**R^2**	**Linear range**	**LOD**[b)]	**LOQ**[b)]
LC–UV	Y=1.967 + 93.003*X	0.998	LOQ–600	0. 081	0.270

a) Y expressed as absorbance units 10^{-3}; X as µg mL^{-1}.

b) Expressed as µg mL^{-1}.

The limit of detection (LOD) was expressed as the concentration of the given analyte which provides a signal 3σ above the mean blank signal —obtained by using the extract from a plant non treated with imazamox—, where σ is the standard deviation of the blank signal. With this expression, the LOD was 0.081 mg L^{-1}. On the other hand, the limit of quantitation (LOQ), expressed as the concentration of analyte which gives a signal 10σ above the mean blank signal, was 0.270 mg L^{-1} (see Table 2). These values of LOD and LOQ are similar to those obtained by previous studies based on CE–MS[7]. This calibration model was adopted for relative quantitation of imazamox metabolites.

Within laboratory reproducibility and repeatability were evaluated for the proposed method in a single experimental setup carried out with extracts from plants —a pool of plants from two biotypes cut 24 h after application of a standard of imazamox at 0.1 μg mL^{-1} and 2 μg mL^{-1}. Three measurements of the extract per day were carried out on 7 days. Equation (1) was used to determine the between-day variance:

$$s_{between}^2 = (ms_{between} - ms_{within})/n_j \quad (1)$$

where ms is the mean square (residual sum of squares rated by the freedom degrees) and nj is the number of replicates per day. The within-laboratory reproducibility, s_{wR}^2, was calculated by Eq. (2):

$$s_{wR}^2 = s_r^2 + s_{between}^2$$

where s_r^2 is the residual mean squares within-days and $s_{between}^2$ is the variance due to the between-day effect. The results obtained are listed in Supplementary Table 2. The repeatability, expressed as RSD, ranged between 2.9 and 5.0% for the imazamox in the different concentrations and biotypes; the within-day laboratory reproducibility, also expressed as RSD, ranged between 4.7–6.9% for different concentrations and biotypes.

S-Table 2. Average concentration, repeatability and within-laboratory reproducibility expressed as RSD for wheat samples (resistant and susceptible biotypes) cut 24 h after imazamox application. The extracts were spiked with imazamox standard at 0.1 and 0.2 μg mL^{-1}.

Biotype	**Added (μg mL^{-1})**	**Average (%)**	**S_b (%)**	**s_{wr} (%)**
R	0.1	99.3 ± 4.6	4.5	5.3
	0.2	104.6 ± 2.8	5.0	6.9
S	0.1	101.1 ± 3.2	2.9	5.8
	0.2	102.4 ± 3.7	4.1	4.7

R: resistant biotype; S: susceptible biotype

Application of the method. In order to assess the applicability of the proposed method, it was used to determine imazamox and its metabolites in plants of resistant and suscetible biotypes treated with the herbicide. Plants from both biotypes were cut 0, 3, 6, 24 h and 8 days after application of the herbicide following agronomical practices. The samples were analyzed with the proposed method involving ultrasound-assisted extraction and LC–UV determination using the calibration curve for imazamox. The concentrations were expressed as μg of imazamox g^{-1} of plant and as μg of the analyte equivalent to imazamox g^{-1} of plant for the precursor and metabolites, respectively. Table 3 lists the concentration of imazamox and metabolites found in the two biotypes at different collection times. As expected, the resistant biotype presented clear evidences of imazamox metabolism in contrast to the susceptible biotype, which only allowed detection of the glucoside metabolite at trace levels. Concerning the resistant biotype, the monohydroxy metabolite was detected at higher concentration than the glucoside metabolite. This suggests that the formation of the monohydroxy derivative is a transition step to the conjugated glucoside, the final product of imazamox metabolism. This would explain why the glucoside derivative was also detected in the susceptible biotype even when the plant is fully affected and without survival probabilities. Thus, once the monohydroxy derivative is formed, the –OH group would react with the anomeric carbon of the glucose unit to form the glucose conjugated by condensation.

In relation to the results obtained by analysis of plant biotypes, it is worth mentioning that the method allows discrimination of them by quantitation of imazamox and relative quantitation of the two metabolites, mainly formed in imazamox resistant plants.

Table 3. Analysis of imazamox and its metabolites in wheat samples (treated with 200 g of active ingredient Ha^{-1}) by the proposed LC–UV method (values expressed as μg/g ± standard error of the mean, n=7 replicates)

Biotype	*Time*	*imazamox*	*Hydroxy metabolite*	*Glucose conjugated metabolite*
R	**0 h**	–	–	–
	3 h	0.34 ± 0.03	–	–
	6 h	0.72 ± 0.08	–	–
	24 h	5.11 ± 0.12	0.98 ± 0.04	0.30 ± 0.02
	8 days	3.68 ± 0.05	46.72 ± 0.23	19.34 ± 0.09
S	**0 h**	–	–	–
	3 h	0.56 ± 0.08	–	–
	6 h	2.29 ± 0.05	–	–
	24 h	9.89 ± 0.13	–	–
	8 days	68.14 ± 0.09	0.29 ± 0.04	–

R: resistant biotype; S: susceptible biotype.

Finally, the accuracy of the method was established by analyzing real samples (resistant wheat treated with 200 g of herbicide Ha^{-1} and collected at 0, 3, 6 and 24 h and 8 days after application) spiked with 0.1 and 0.2 μg mL^{-1} of imazamox using the optimized method. As Supplementary Table 3 shows, recovery factors were from 98.7 to 111.4% for both concentration levels. The paired *t* test revealed the absence of significant differences between the concentrations added and those found (α= 0.05). It is worth mentioning that higher errors were detected at shorter sampling times, 0 and 3 h after application of the herbicide, probably due to a poorer homogeneization of herbicide distribution.

S-Table 3. Accuracy of the proposed LC–UV method obtained by analysis of wheat samples (resistant biotype) treated with imazamox (200 g of active ingredient Ha^{-1}). The extracts were spiked with imazamox at two concentration levels (recovery values ± standard errors of the mean, n=9 replicates).

Sampling time	Added ($\mu g\ mL^{-1}$)	Recovery (%)
0 h	**0.1**	110.4±8.3
3 h		104.9±1.1
6 h		99.2±2.1
24 h		99.5±2.6
8 days		98.7±3.6
0 h	**0.2**	111.4±7.4
3 h		109.2±3.3
6 h		102.8±3.6
24 h		100.1±5.2
8 days		99.9±0.9

The proposed strategy constitutes a valuable tool for determination of imazamox and its non commercial metabolites by LC–UV analysis after ultrasound-assisted extraction. The method allows relative quantitation of imazamox metabolites after confirmatory analysis by LC–TOF/MS of their presence in resistant plants treated with the herbicide. The sample preparation step has been expedited by ultrasonic energy by comparison to conventional extraction based on maceration. The overall method has shown its excellent performance without influence of potential interferences associated to the plant matrix. The analytical characteristics of the method make it recommend in studies to evaluate metabolism of imazamox in plants.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Capítulo 5

Qualitative/quantitative strategy for determination of glufosinate and metabolites in plants

Qualitative/quantitative strategy for determination of glufosinate and metabolites in plants

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Qualitative/quantitative strategy for determination of glufosinate and metabolites in plants

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ABSTRACT

A simple method for simultaneous determination of glufosinate and its metabolites in plants based on liquid chromatography–UV absorption detection (LC–UV) after derivatization with fluorenylmethoxycarbonyl chloride (FMOC-Cl) of some analytes to facilitate separation is reported here. Non-available standard metabolites were identified by LC–TOF/MS, which also confirmed all the target analytes. Then, glufosinate and its metabolites for which standards are available (N-acetyl-glufosinate and 3-methylphosphinico-propanoic acid or MPP) were quantified; while the others were relatively quantified taking as standard that more similar to them. Ultrasound-assisted extraction was used for sample preparation (70 W power and duty cycle of 0.7 s/s for 10 min) with subsequent evaporation of the extractant, reconstitution and filtration as cleanup/concentration step prior to derivatization, chromatographic separation and detection at 270 nm for underivatized compounds and 340 nm for derivatized compounds. The chromatographic analysis was completed in 40 min using a hydrophilic interaction liquid chromatography column (HILIC). The analytical characteristics of the method were linear dynamic range of the calibration curves within 0.047–700 μg/mL with correlation coefficient of 0.999 for glufosinate; 0.077–700 μg/mL with correlation coefficient of 0.998 for N-acetyl-glufosinate; and 0.116–600 μg/mL with correlation coefficient of 0.998 MPP. The precision for the determination of glufosinate—studied at two concentration levels, 0.1 and 5 μg/mL— was of 2.7 and 6.0% for repeatability, and 4.7 and 7.2% for within-laboratory reproducibility, respectively. Identification and confirmatory analysis of the presence of glufosinate and metabolites in extracts from treated plants was carried out by LC–TOF/MS in high resolution mode for the precursor ion. The method was validated by analyzing wheat (*Triticum aestivum*) samples (resistant and susceptible biotypes) treated with glufosinate following conventional agronomical practices (300 g of active ingredient/Ha).

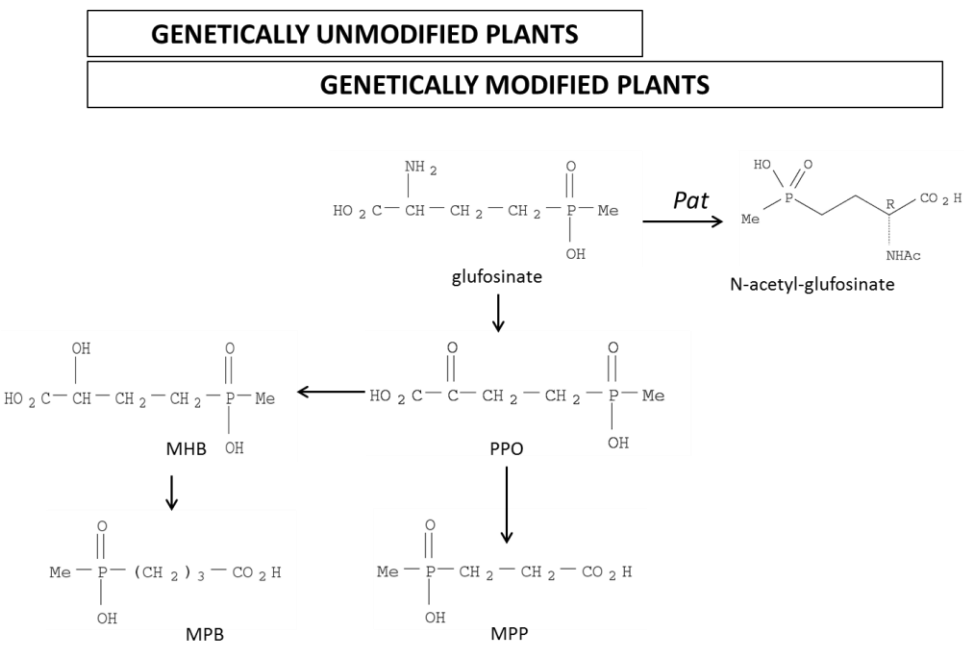
Keywords: Glufosinate, Metabolites, Ultrasound-assisted extraction, FMOC-Cl derivatization, LC–TOF/MS, High resolution mass spectrometry, LC–UV separation–determination

1. Introduction

Glufosinate (phosphinothricin) is an herbicide discovered in 1972 in a German laboratory when an amino acid isolated from the soil bacterium *Streptomyces viridochromogenes* [1] showed herbicide activity. Glufosinate inhibits the activity of the enzyme glutamine synthetase (GS) by competitive fixation at the glutamate (substrate) binding site, which inhibits the synthesis of L-glutamine and causes a decrease in the levels of aspartic acid, asparagine and other amino acids [1–3]. In addition to the accumulation of glutamate, ammonium concentration is also increased, thus inhibiting the phosphorylation pathway in photosynthesis as the formation of L-glutamine in plants is a nitrogen fixation pathway [4–6].

In particular cases GS inhibition does not occur owing to a mutation that causes loss of affinity for the binding site. This is the case with the mutation of the plastidic GS2 gene of *Lolium* spp., in which an aspartic acid residue is substituted by an asparagine residue at position 171 [7]. The combination of other mechanisms such as absorption, translocation and metabolism can also reduce the activity of glufosinate by GS inhibition. In the case of glufosinate metabolism the herbicide is converted into non toxic products. This metabolism can be innate in the plant or induced artificially as it is the case of genetically modified varieties. For this particular herbicide, the introduced gene (*pat*) expresses the synthesis of the enzyme Phosphinotricine-N-acetyltransferase (Pat) composed of 183 amino acids [8,9] which catalyzes the acetylation of glufosinate in the N-terminal site to generate N-acetyl-glufosinate without herbicidal activity. Studies on glufosinate metabolism in unmodified plants [10–12] have shown that this herbicide is rapidly transformed into PPO (4-methylphosphinico-2-oxo-butanoic acid), which led to the formation of MPP (3-methylphosphinico-propanoic acid) [11,12] and MHB (4-methylphosphinico-2-hydroxy-butanoic

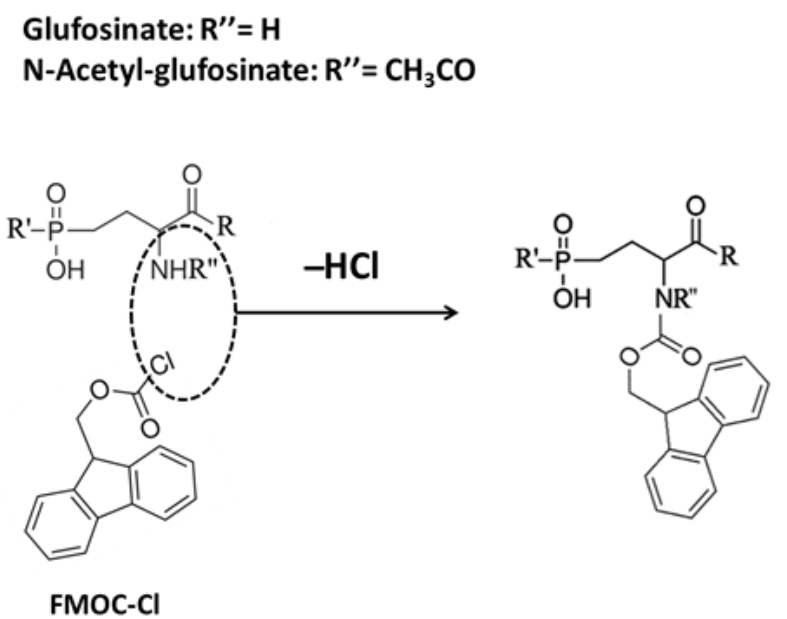
acid) [12]. Incubation of plant material with ^{14}C-glufosinate has allowed identification of MPB (butanoic acid, 4-(hydroxymethylphosphinyl), which is a metabolite of MHB [13]. All these metabolites are nontoxic to plants.The two glufosinate degradation pathways both in modified and unmodified plants are shown in Supplementary Fig. 1.



S-Figure 1. Metabolism pathways of glufosinate for genetically modified and unmodified plants.

Glufosinate has been the single subject of different studies involving aqueous samples [14,15], but also human fluids [16,17] or plants [18]. Mixtures of glufosinate with other herbicides have been also determined in water samples [19–24] and in more complex matrices such as fruits and soils [25], or human fluids [26]. Optimization of the extraction and cleaning procedure for each target sample has been mandatory [27]. Concerning determination of the analytes, this has been frequently carried out after separation by liquid chromatography (LC) [15,16,17,22,23], gas chromatography (GC) [18,24,25] or capillary electrophoresis (CE) [19,20,21,26] coupled to different detection techniques. In cases involving metabolism studies, the preferred methods for analysis of glufosinate and metabolites in plants are based on LC separation and

radioactivity detection [12,13,28,29], which require simple sample preparation protocols (usually precipitation of polysaccharides and protein/peptides and subsequent filtration or centrifugation), thus avoiding or minimizing potential losses of the target analytes (particularly low concentrated metabolites) [13]. Nevertheless, radiactivity-based methods present limitations such as the high cost of reagents, loss of radioactivity by quenching, difficulties for wastes management and inability for identification of chemical metabolite structures. Apart from radioactivity-based methods, photometry with a diode array detector (DAD) [14,17,20], fluorescence detection [15,16,19,21] and mass spectrometry (MS) [18, 22–25] have also been used but only for glufosinate determination. For fluorescence detection a derivatization step has been required, most times using fluorenylmethoxycarbonyl chloride (FMOC–Cl) as fluorogenic reagent [15,16,24] which reacts with the amino group as shows Supplementary Fig. 2.



S-Figure 2. Derivatization by FMOC reagent of compounds containing an amino group.

The aim of the present research was the development of a dual strategy for analysis of glufosinate and metabolites in plants. Taking into account the absence of available standards of most glufosinate metabolites, identification and confirmatory analysis of their presence in extracts from plants treated with the herbicide was planned with LC–TOF/MS in high resolution mode. As a second

part of the strategy, a quantitative method for determination of glufosinate and metabolites was developed by accelerated extraction from plants, LC individual separation and photometric detection. The method should be fast enough to be implemented in routine analysis; therefore, it should require reliable analytical equipment. This dual qualitative/quantitative strategy was planned to replace radioactivity methods as a key tool for analysis of glufosinate and metabolites in plants.

2. **Experimental**

2.1. Chemicals and reagents

Preparation of the chromatographic mobile phases for LC–DAD analyses required chromatographic grade acetone, formic acid and acetonitrile, which were acquired from Panreac (Barcelona, Spain). LC–MS grade acetonitrile and formic acid from Scharlab (Barcelona, Spain), and deionized water (18 MΩ·cm) provided by a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA) were used for LC–MS analyses in high resolution mode. Sodium hydroxide (purity 99.9%) and FMOC-Cl (purity 99.0%) were used for derivatization. Standards of glufosinate and the MPP metabolite were purchased in Sigma (St. Louis, USA). N-Acetyl-glufosinate was quantitatively synthesized by using a mixture of 5 mM glufosinate solution (pH 8) and an excess of acetic anhydride from Sigma–Aldrich (St. Louis, USA), under vigorous agitation (by magnetic stirrer) at room temperature for 1 h [12] with conversion higher than 99%. Stock standards solutions were prepared by dissolving 0.1 g of glufosinate, N-acetyl-glufosinate or MPP in 100 mL of 10 mM KH_2PO_4 in 90:10 (v/v) water–acetonitrile. The commercial herbicide used for plant treatment was Finale® (15% w/v of glufosinate) from Bayer (Frankfurt, Germany).

2.2. Samples

Seeds from two biotypes (resistant and susceptible varieties) of *Triticum aestivum* with different resistance to glufosinate were used for this study. These seeds were provided by the Higher Council for Scientific Research (CSIC) and germinated in Petri dishes using a damp filter paper as substrate. The

measurement of active ingredient per Ha with capability to cause 50% growth inhibition of plants as compared to the untreated control (ED_{50}) were 478.59 and 32.65 g of active ingredient per Ha (g ai/Ha) for the resistant and susceptible biotypes, respectively. The seedlings were transplanted to plastic pots (three plants in each pot), filled with 1:2 (v/v) peat–clay as substrate. Once the plants had an average of five or six leaves, commercial glufosinate was applied to 90% of the plants while the resting 10% was used as control. Application was performed by spraying at 200 kPa in a closed chamber calibrated at 0.5 m height above the target surface, with a relative volume of 300 L/Ha and with one dose of 2 L/Ha Finale® (300 g ai/Ha). Plants —both treated with herbicide and controls— were cut at 0, 3, 6, 24 and 48 h after the herbicide application, and stored at −40 ºC until use.

2.3. Instruments and apparatus

The herbicide was applied to plants by using a flat jet spray tip Teejet 80.02 (Wheaton, USA) inside a closed spray chamber. A Microdigest 301 digestor of maximum power 200 W (Prolabo, Paris, France) furnished with a microprocessor programmer (Prolabo) to control the microwave unit was used for microwave-assisted extractions. Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (20 KHz, 450 W) with tunable amplitude and duty cycle, which was equipped with a cylindrical titanium alloy probe (12.70 mm in diameter). A porcelain mortar furnished with a pestle from Pobel (Madrid, Spain) and test tubes (10 mL) from Pyrex (London, England) were used for extraction. A magnetic stirrer from Bunsen (Spain) and a water bath furnished with temperature controller from Selecta (Barcelona, Spain) were used for derivatization. Nylon filters 45 µm pore-size and 13 mm i.d. from Millipore (Billerica, Massachusetts, USA) were used to remove solid particles from the extracts. Centrifugation of the extracts was carried out by a Mixtasel centrifuge (Selecta, Barcelona, Spain).

A 15 Gold LC System from Beckman Coulter (Fullerton, USA) equipped with a 26 Gold DAD System (wavelength range 190–600 nm) was used for individual separation and UV detection. The instrumental setup was controlled by the Karat 3.0.7 software, which also enabled data acquisition and processing.

Chromatographic separation was carried out, using a Luna HILIC column (200 × 4.6 mm, 3 µm particle size) from Phenomenex Inc. (Torrance, California, USA), furnished with HILIC 2 × 4 mm (5 µm particle size) guard cartridges.

A 1200 LC system (Agilent Technologies, Palo Alto, CA) coupled to an Agilent UHD TOF accurate mass spectrometer (Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source was used for identification and confirmatory analysis. Nitrogen was provided by a high purity generator from CLAN Tecnológica (Sevilla, Spain) and used as the source gas, while ultra pure nitrogen (99.999%) from Carburos Metálicos (Sevilla, Spain) was used as collision gas. MassHunter Workstation software version 4.0.1 (Agilent Technologies) was used to control data acquisition and support qualitative analysis including extraction of molecular features, generation of molecular formula.

2.4. Sample pre-treatment

Frozen samples were washed three times with 20 mL water before extraction to remove glufosinate traces on the leaves surface. Each sample was placed in a porcelain mortar and flash-frozen using 20 mL liquid nitrogen, and grinded to a fine homogeneous powder using a porcelain pestle for 5 min.

2.5. Conventional extraction method

The method described by Druart *et al.* [27] for extraction of glufosinate from soils was used as reference, for which 1 g of the sample powder was mixed with 10 mL of a 90:10 (v/v) water–acetonitrile mixture, then shaken for 1 h, equilibrated for 1.5 h and shaken again for 1 h, followed by 15 min centrifugation at 2.900 × g.

2.6. Microwave-assisted extraction method

One gram of sample was placed into the extraction vessel with 10 mL 90:10 (v/v) water–acetonitrile and positioned at the suited zone for irradiation with focused microwaves. The auxiliary energy was applied at 90 W irradiation power for 5 min (5 × 1 min cycles), after which the solid residue was removed by centrifugation (15 min at 2.900 × g).

2.7. Proposed ultrasound-assisted extraction method

One gram of sample powder was placed into the extraction vessel with 10 mL 90:10 (v/v) water–acetonitrile. The ultrasonic probe was immersed into the extraction mixture for sonication at 280 W irradiation power for 10 min with a duty cycle of 70% (0.7 s/s irradiation). After that, the extract was isolated by centrifugation (15 min at 2.900 × g).

2.8. Clean-up and preconcentration

The method proposed by Jansen *et al*. [13] to remove polysaccharides and protein/peptides was used. Thus, 5 mL of the extract was 1:1 (v/v) mixed with acetone with subsequent centrifugation (4.000 × g). The liquid phase was evaporated to dryness under a nitrogen stream. The solid residue was reconstituted in 1 mL of 90:10 (v/v) water–acetonitrile and filtered through a nylon filter syringe before chromatographic analysis.

2.9. Derivatization

The method proposed by Druart *et al*. [27] was followed for derivatization of glufosinate and its acetylated metabolite to obtain products allowing a better chromatographic separation between them and from other glufosinate metabolites. Briefly, 250 µL of 5 mM FMOC-Cl and 250 µL of water were added to 3 mL of the reconstituted phase and subjected to agitation by magnetic stirrer for 1 h at 30 ºC. Supplementary Fig. 2 shows the selectivity of the derivatization process for glufosinate and N-acetyl-glufosinate since only they present an amino group in their chemical structures.

2.10. Chromatographic analysis

The chromatographic method was carried out by injecting 50 µL of the reconstituted sample and using 0.1% (v/v) formic acid in water and acetonitrile as mobile phases A and B, respectively. The elution program started with a linear gradient from 10% mobile phase B to 45% in 32 min (step 1); 45% mobile phase B to 70% in 3 min (step 2); 70% to 10% acetonitrile in 5 min for equilibration (step 3). The constant flow rate and column temperature were 1.0 mL/min and 30 $^{\circ}$C, respectively.

2.11. Identification of glufosinate metabolites by LC–TOF/MS

Glufosinate metabolites were identified by LC–TOF/MS analysis in high resolution mode. The samples were analyzed both with positive and negative ionization modes. The operating conditions for the mass spectrometer were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3000 and 4000 V for negative and positive ionization modes; skimmer, 65 V; octopole radio frequency voltage, 750 V; focusing voltage, 90 V. Data acquisition (2.5 Hz; mass range 60–1100 *m/z*) in both the centroid and profile modes was governed via the Agilent Mass Hunter Workstation software. The instrument provided typical resolution 15000 FWHM (Full Width at Half Maximum) at *m/z* 112.9856 and 30000 FWHM at *m/z* 1033.9881. To assure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses with the use of signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3H tetrafluoropropoxy) phosphazine or HP-921] in positive ion mode. In negative ion mode, ions with *m/z* 119.0362 (proton abstracted purine) and *m/z* 1033.988109 adduct of HP-921 were used.

The analytes were identified by accurate mass detection and formula elucidation based on isotopic distribution. For this purpose, once the samples were analyzed by LC–TOF/MS molecular features (MFs, *m/z* value and retention time pairs) were extracted from raw data files prior to formula generation. Ions with identical elution profiles and related *m/z* values (representing different adducts, ions generated after specific neutral losses or isotopic forms from the monoisotopic ions) were associated to the same MF to avoid redundant information. The isotopic model corresponded to common organic molecules with peak spacing tolerance of *m/z* 0.0025 ± 6.0 ppm. The MF extraction algorithm limited extraction to ions exceeding 5000 counts with charge state limited to a maximum of two. The allowed negative ions were deprotonated species and formate adducts, while allowed positive ions were protonated species and sodium adducts. Dehydratation, glucosidation and phosphate neutral losses

were also allowed. Candidate formula generation was supported on C, H, O, N, S and P elements by setting a cut-off value of mass accuracty at 6 ppm.

2.12. Determination of glufosinate and metabolites by LC–DAD

Glufosinate and metabolites were determined by LC–DAD analysis at two absorption wavelength (270 nm for underivatized compounds and 340 nm for derivatized compounds). Chromatographic peaks in LC–DAD were assigned according to their retention times taking as reference the peaks of glufosinate and N-acetyl-glufosinate derivatives (d-glufosinate and d-N-acetyl-glufosinate since now) and that of MPP, which were located by spiking extracts from plants treated with the herbicide with known amounts of the commercial and synthesized standards. Quantification of non-available commercially glufosinate metabolites was based on the calibration model built for the available compound with structure more similar to them (MPP) and the results expressed as µg of the analyte equivalent to MPP/g of fresh plant.

3. **Results and discussion**

3.1. Optimization of the chromatographic separation of glufosinate and metabolites

The first test to be carried out was the confirmation of the presence of glufosinate metabolites in plants treated with the herbicide following common agronomical practices. For this qualitative study, samples extracted by the reference protocol described under Experimental [27] were analyzed. The resistant biotype was selected for this study as it is intended to detect the presence of glufosinate metabolites. Preliminary studies using the extracts as such revealed low chromatographic resolution for glufosinate and N-acetyl-glufosinate as compared to the resting metabolites. For this reason, a derivatization step with FMOC-Cl was included in the protocol for separation of derivatized glufosinate and N-acetyl-glufosinate from the non-derivatized metabolites. The derivatization step clearly improved separation as Fig. 1 shows. This figure summarizes the main results obtained by LC–DAD analysis of extracts from: (1) non-treated plants as absolute blank of the sample matrix; (2) non-treated plants spiked with standards

of glufosinate, N-acetyl-glufosinate and MPP (50 µg/mL each) for potential identification of the chromatographic signals of the non-derivatized compounds; (3) non-treated plants spiked with glufosinate, N-acetyl-glufosinate and MPP standards (50 µg/mL each) and subjected to the derivatization reaction; (4) treated plants cut 48 hour after herbicide application (300 g ai/Ha) and subjected to derivatization to correlate those chromatographic signals corresponding to glufosinate, N-acetyl-glufosinate and MPP as well as those new chromatographic peaks that could be associated to tentative metabolites at short-term. This strategy was designed due to the lack of available metabolites and the need for identifying them as naturally generated in the plants.

Comparison of Fig. 1.2 and 1.3 revealed a clear and logical displacement of the chromatographic peaks associated to the two derivatized compounds (glufosinate and N-acetyl-glufosinate) taking as reference the non-derivatized compounds due to the change of polarity. This led to an improvement of chromatographic resolution for both analytes, but also for MPP, the metabolite unable for derivatization since they were eluted in regions without mutual interferences. Figure 1.3 shows that the two derivatized analytes and MPP, detected in extracts after herbicide and metabolites addition, were eluted at *ca.* 9.046, 5.299 and 2.624 min, for d-glufosinate, d-N-acetyl-glufosinate and MPP, respectively. Figure 1.4 confirms the presence of the three compounds in treated plants, but also the presence of other chromatographic peaks in the elution window 3.0–4.0 min and 8.5–9.0 min, which could be tentatively associated to other glufosinate metabolites. The appearance of these potential metabolites in extracts of treated plants after glufosinate application led to take them into account in the optimization of the chromatographic process.

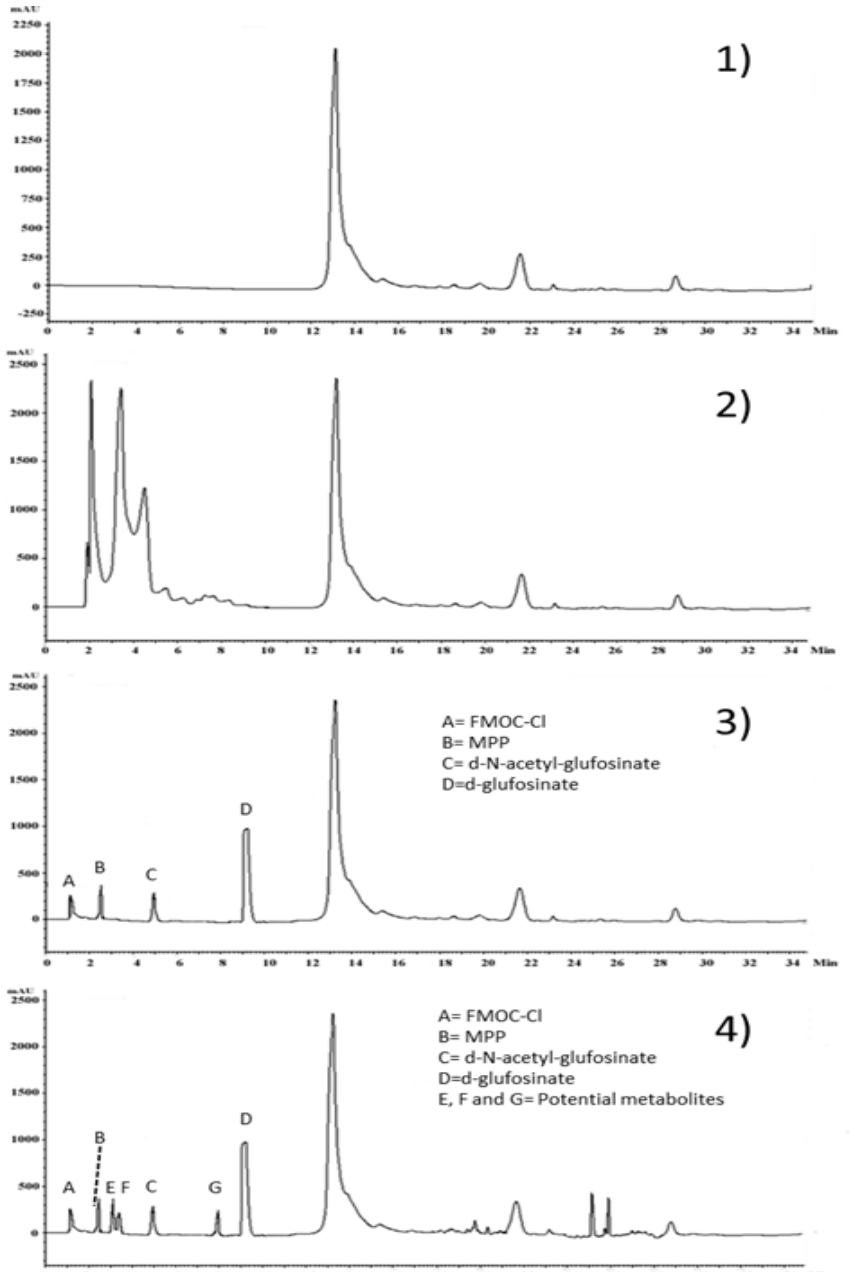


Figure 1. LC–DAD chromatograms obtained by analysis of extracts from resistant wheat plants (*Triticum aestivum*) subjected or not to glufosinate treatment obtained by the conventional extraction method: **(1)** non-treated plant as an absolute blank of the sample matrix; **(2)** non-treated plant spiked with glufosinate, N-acetyl-glufosinate and MPP standards (50 µg/mL each); **(3)** non-treated plant spiked with glufosinate, N-acetyl-glufosinate and MPP standards (50 µg/mL each) and derivatized; **(4)** treated plant collected 48 h after herbicide application (300 g ai/Ha) and derivatized.

The variables studied in this process were column length, temperature, % formic acid, and gradient program. The response variables were the peak area and resolution expressed as the peak width at half-height of the chromatographic signals associated to glufosinate and metabolites. An increase of temperature up to 30 ºC shortened the chromatogram time but led to partial overlapping of peaks. On the other hand, the chromatographic resolution at temperatures above 30 ºC was quite poor owing to peak broadening effects. Therefore, 30 ºC in the column compartment was adopted as compromise solution. Taking into account the structure of glufosinate, N-acetyl-glufosinate and most part of other metabolites, an acidic pH (within 2.0–2.8, obtained by adding 0.1% formic acid) favored sensitivity by thickening the chromatographic peaks. The selection of the column length was based on retention times (t_r). A 15-cm column provided partial separation with significant overlapping and fast elution of the metabolites; while that of 25-cm length yielded very long t_r and wide peaks; therefore, an intermediate 20-cm length was selected. The gradient study resulted in the optimum program described under section 2.10, which involves 3 steps and provides an optimum resolution, particularly in the first part of the chromatogram where tentative glufosinate metabolites with high polar character were eluted. Finally, a study of the injection repeatability and reproducibility —in terms of peak area and t_r— carried out with glufosinate standards and extract solutions provided standard deviation values always below 7%.

After optimization of the chromatographic process the extracts were analyzed by LC–TOF/MS to confirm the identity and presence of glufosinate, N-acetyl-glufosinate and MPP in resistant plants treated with the herbicide, if present, but also to identify those other potential metabolites. These tests were carried out with the LC–TOF/MS analyzer in order to take benefits from high resolution mass spectrometry in full scan. Chromatographic peaks from glufosinate, N-acetyl-glufosinate and MPP were associated to the precursor ions *m/z* 402.1124 *m/z* 420.1210 and *m/z* 151.0157, respectively, in negative ionization mode, which were identified as the $[M–H]^-$ precursors. The extracted ion chromatograms (EIC) for these three experimental *m/z* values are shown in Fig. 2. Concerning the signals corresponding to the three tentative metabolites,

ions at *m/z* 165.0317, 181.0271 and 179.0116 were detected at retention times 3.137, 3.661 and 8.680 min, respectively. Theoretical formulas for these precursor ions and their isotopic distributions were associated to $C_5H_{11}O_4P$, $C_5H_{11}O_5P$ and $C_5H_9O_5P$, respectively. Attending to these candidate formulas, they were associated to MPB, MHB and PPO glufosinate metabolites.

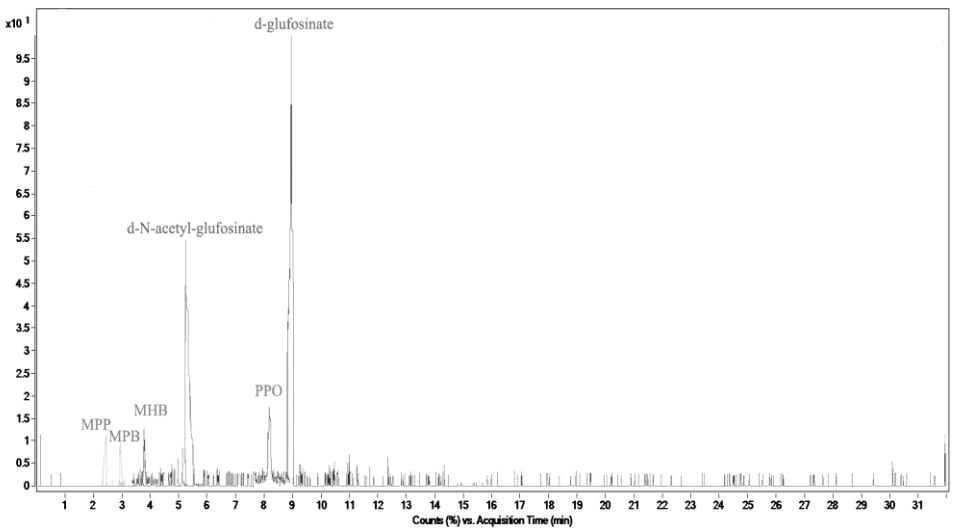


Figure 2. Extracted ion chromatograms for glufosinate and metabolites obtained by LC–TOF/MS analysis of extracts from wheat resistant biotype (treated with 300 g ai/Ha) collected 48 h after herbicide treatment. The chromatograms confirm the presence of glufosinate, N-acetyl-glufosinate, MPP and allow identification of PPO, MHB and MPB.

Accuracy errors estimated by comparing experimental and theoretical *m/z* values were below 6 ppm, which supported assignations of formulas. Table 1 lists parameters such as retention time, formulae, experimental and theoretical masses, and identification error. EICs for ions associated to glufosinate metabolites are also illustrated in Fig. 2.

Table 1. High resolution mass spectrometry for identification and confirmatory analsysis of glufosinate and metabolites in *Triticum aestivum* resistant plants treated with the herbicide

Compound	Theoretical formulae	Retention time (min)	Detected ion	Theoretical ion (*m/z*)	Experimental ion (*m/z*)	Accuracy error (ppm)
glufosinate	$C_{20}\,H_{22}\,N\,O_6\,P$	9.046	$[M–H]^-$	402.1112	402.1124	2.9842
N-acetyl-glufosinate	$C_{22}\,H_{24}\,N\,O_7\,P$	5.299	$[M–H]^-$	420.1218	420.1210	1.9042
MPP	$C_4\,H_9\,O_4\,P$	2.624	$[M–H]^-$	151.0166	151.0157	5.9596
PPO	$C_5\,H_9\,O_5\,P$	8.680	$[M–H]^-$	179.0115	179.0116	0.5586
MHB	$C_5\,H_{11}\,O_5\,P$	3.661	$[M–H]^-$	181.0271	181.0271	0
MPB	$C_5\,H_{11}\,O_4\,P$	3.137	$[M–H]^-$	165.0322	165.0317	3.0297

3.2. Sample preparation: optimization of accelerated extractions

Two types of auxiliary energy (ultrasound and microwaves) were assayed to accelerate the extraction step. The influential variables in both cases were optimized by multivariate approaches that enabled drastic reduction of the number of experiments to be carried out. The response variable used in all instances for optimization was the peak area of glufosinate, N-acetyl-glufosinate, MPP and those of the tentative metabolites. The strategy followed in both cases was the same. First, a factorial design involving 7 experiments including 3 center points provided information about the most influential variables and their possible interactions, so the main effects and interactions were statistically evaluated. The optimum separation–detection conditions were used to monitor optimization of extraction aimed at achieving maximum removal of glufosinate and metabolites from the matrix in a time as short as possible without degradation.

3.2.1. Microwave-assisted extraction

The characteristic variables of this type of energy are irradiation power and number of cycles (cycle duration selected: 1 min —selected to avoid boiling of the liquid extractant phase). The results showed the non-significance of the number of cycles and the significance of irradiation power. The results of this study are listed in Table 2. The significant effect of microwave irradiation power promoted a univariate study within the range 10–90 W at 5 cycles. The best behavior corresponded to 90 W.

Table 2. Optimization of the extraction step

Auxiliary energy	Variable	Tested range	Optimum value
Ultrasound	Power (W)	10–70	70
	Duty cycle (s/s)	0.1–0.7	0.7
Microwaves	Power (W)	10–90	90
	Number of cycles	1–5	5

Since the stability of glufosinate and its metabolites under microwave radiation is not well-known, a kinetics study was performed with different number of extraction cycles. As Fig. 3 shows, 5 cycles were required to achieve the maximum extraction efficiency, but a sharp decrease owing to glufosinate degradation occurs after cycle 5. Therefore, the time for application of microwaves was critical.

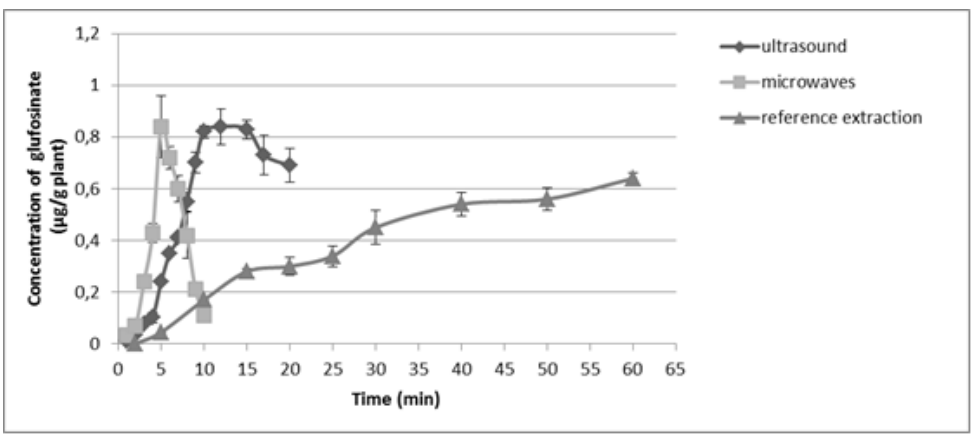


Figure 3. Extraction kinetics for isolation of glufosinate using reference extraction, ultrasound-assisted extraction, and microwave-assisted extraction.

3.2.2. Ultrasound-assisted extraction

The variables with potential influence on ultrasonic extraction are irradiation amplitude and duty cycle applied for a total time of 5 min in all instances for a comparative basis with microwave extraction although the extraction mechanisms are different. The results showed the non-significant effect of ultrasonic irradiation variables on the leaching efficiency of glufosinate and metabolites. As can be seen, positive effects were observed for both variables and, for this reason, 70 W and 0.7 s/s duty cycle were adopted for further experiments. Table 2 lists the results of this study.

Optimization of ultrasound-assisted extraction was completed by a kinetics study of the irradiation time to maximize the extraction efficiency. Figure 3 shows that an extration time of 10 min was required to obtain the maximum extraction efficiency for glufosinate, which was kept constant within the range 10–13 min. Longer times allowed detecting that the concentration of

the herbicide decreased significantly ($p<0.0001$), possibly owing to degradation. Nevertheless, the degradation was lower than when the process was assisted by microwaves, in which the extraction efficiency decreased dramatically after extraction for 5 min. According to operative reasons, the extraction protocol assisted by ultrasonic energy was adopted as the preferred sample preparation for isolation of glufosinate and metabolites. It is worth mentioning that this protocol reduced the extraction time to 10 min as compared to the reference protocol described by Druart *et al.* [27]. The kinetics study of this reference method proved that the extraction efficiency after 1 h extraction was around 85% of that obtained under ultrasonic irradiation (see Fig. 3).

3.3. Characterization of the LC–DAD method for determination of glufosinate, N-acetyl-glufosinate and MPP

The calibration models for glufosinate, N-acetyl-glufosinate and MPP were built using extracts from non-treated plants spiked with known concentrations of the three compounds. Calibration curves were plotted using the peak area as analytical signal at the monitoring wavelengths (270 nm for underivatized compounds and 340 nm for derivatized compounds) as a function of the standard concentration of these compounds.The resulting calibration equation for glufosinate, N-acetyl-glufosinate and MPP as well as the regression coefficient and the linear dynamic range are listed in Supplementary Table 1.

S-Table 1. Features of the method for determination of glufosinate, N-acetyl-glufosinate and MPP by LC–DAD.

Compound	**Calibration equation**[a)]	**R^2**	**Linear range**	**LOD**[b)]	**LOQ**[b)]
Glufosinate	Y=0.935 + 75.084*X	0.999	LOQ–700	0. 014	0.047
N-Acetyl-glufosinate	Y=1.367 + 59.011*X	0.998	LOQ–700	0.025	0.077
MPP	Y=0.350 + 10.071*X	0.998	LOQ–600	0. 035	0.116

a) *Y* expressed as absorbance units 10^{-3}; *X* as µg/mL.

b) Expressed as µg/mL.

The limit of detection (LOD) in all cases was expressed as the concentration of the given analyte which provides a signal 3σ above the mean blank signal —obtained by repetitive analysis of an extract from a non-treated plant with glufosinate—, where σ is the standard deviation of the blank signal. With this expression, the LODs were 0.014, 0.025 and 0.035 mg/L for glufosinate, N-acetyl-glufosinate and MPP, respectively. On the other hand, the limit of quantitation (LOQ), expressed as the concentration of analyte which gives a signal 10σ above the mean blank signal, were 0.047, 0.077 and 0.116 mg/L for glufosinate, N-acetyl-glufosinate and MPP, respectively. The calibration model of MPP was adopted for relative quantitation of PPO, MHB and MPB metabolites because of their similar chemical structure.

Within laboratory reproducibility and repeatability were evaluated for the proposed method in a single experimental setup carried out with extracts from plants —a pool of plants from the two biotypes cut 48 h after spiking of a standard of glufosinate at 0.1 µg/mL and 5 µg/mL. Three measurements of the extract per day were carried out on 10 days. Equation (1) was used to determine the between-day variance:

$$s_{between}^2 = (ms_{between} - ms_{within})/n_j \quad (1)$$

where ms is the mean square (residual sum of squares rated by the freedom degrees) and nj is the number of replicates per day. The within-laboratory reproducibility, s_{wR}^2, was calculated by Eq. (2):

$$s_{wR}^2 = s_r^2 + s_{between}^2$$

where s_r^2 is the residual mean squares within-days and $s_{between}^2$ is the variance due to the between-day effect. The results obtained are listed in Supplementary Table 2. The repeatability, expressed as RSD, ranged between 2.7 and 6.0% for glufosinate in the different biotypes and at both concentrations levels; the within laboratory reproducibility, also expressed as RSD, ranged between 4.7–7.2% for different applied concentrations of glufosinate and biotypes.

S-Table 2. Average concentration, repeatability and within-laboratory reproducibility expressed as RSD for extracts from wheat plants (resistant and susceptible biotypes) cut 48 h after glufosinate application (300 g ai/Ha). The extracts were spiked with glufosinate standard at 0.1 and 5 µg/mL.

Biotype	Added (µg/mL)	Average (%)	S_b (%)	s_{wr} (%)
R	0.1	99.8 ± 3.1	2.7	6.3
	5	101.0 ± 4.1	3.3	4.8
S	0.1	100.3 ± 5.7	6.0	7.2
	5	100.2 ± 2.9	3.1	4.7

R: resistant biotype; S: susceptible biotype.

3.4. Application of the method

In order to assess the applicability of the proposed method, this was applied to determine glufosinate and metabolites in resistant and susceptible biotypes of *Triticum aestivum* plants treated with the herbicide. Plants from both biotypes were cut 0, 3, 6, 24 and 48 h after application of the herbicide following agronomical practices (300 g ai/Ha). The samples were analyzed with the proposed method involving ultrasound-assisted extraction, derivatization with FMOC and LC–DAD determination. The relative concentrations of PPO, MHB and MPB were expressed as µg of the target metabolite equivalent to MPP/g of plant. Table 3 lists the concentration of glufosinate and metabolites found in the two biotypes at different collection times.

As expected, the resistant biotype presented clear evidences of glufosinate metabolism in contrast to the susceptible biotype, in which only the PPO and MHB metabolites were detected. Concerning the resistant biotype, the N-acetyl-glufosinate metabolite was detected at higher concentration than MPP and PPO metabolites. This suggests that the formation of the N-acetyl-glufosinate is the crucial pathway in the metabolism of glufosinate as compared to that leading to formation of MPP and MHB, which is part of natural metabolism in plants. This would explain why the biotype genetically modified with the *pat* gen (biotype R) possesses a high resistance to glufosinate as opposite to the other biotype, which has only natural metabolism.

Table 3. Analysis of glufosinate and metabolites in extracts from wheat plants (treated with 300 g ai/Ha) by the proposed LC–DAD method (values expressed as µg/g ± standard error of the mean, n=10 replicates)

Biotype	*Time*	*Glufosinate*	*N-Acetyl-glufosinate*	*PPO*[a]	*MPP*	*MHB*[a]	*MPB*[a]
R	**0 h**	–	–	–	–	–	–
	3 h	0.69 ± 0.03	–	–	–	–	–
	6 h	1.23 ± 0.04	–	–	–	–	–
	24 h	1.76 ± 0.02	0.30 ± 0.02	0.78 ± 0.03	0.42 ± 0.05	–	–
	48 h	1.86 ± 0.03	7.48 ± 0.08	0.84 ± 0.02	0.79 ± 0.07	0.19 ± 0.03	0.08 ± 0.02
S	**0 h**	–	–	–	–	–	–
	3 h	1.12 ± 0.07	–	–	–	–	–
	6 h	2.73 ± 0.06	–	–	–	–	–
	24 h	5.09 ± 0.07	–	1.52 ± 0.05	–	–	–
	48 h	9.47± 0.04	–	2.45 ± 0.02	–	3.86 ± 0.07	–

R: resistant biotype; S: susceptible biotype.
[a]equivalent to MPP.

In relation to the results obtained by analysis of plant biotypes, it is worth mentioning that the method allows discrimination of them by quantitation of glufosinate, N-acetyl-glufosinate, MPP and relative quantitation of the three metabolites, mainly formed in glufosinate resistant plants.

Finally, the accuracy of the method was established by using extracts from treated plants (resistant wheat treated with 300 g of herbicide/Ha and collected at 0, 3, 6, 24 and 48 h after application), which were spiked with 0.1 and 0.5 µg/mL of glufosinate, N-acetyl-glufosinate and MPP; then, the solutions thus prepared were subjected to the overall optimized method. Supplementary Table 3 shows that the recovery factors were from 97.9 to 104.1 % for both concentration levels. The paired *t* test revealed the absence of significant differences between added and found concentrations (α= 0.05). Poorer homogenization of herbicide distribution at shorter sampling times, 0 and 3 h after application of the herbicide resulted in higher errors in the determination.

S-Table 3. Accuracy of the proposed LC–DAD method obtained by analysis of wheat samples (resistant biotype) treated with glufosinate (300 g ai/Ha). The extracts were spiked with glufosinate at two concentrations (recovery values ± standard errors of the mean, n=10 replicates).

Sampling time	Added (µg/mL)	Recovery (%)
0 h	**0.1**	100.6 ± 5.9
3 h		99.5±6.2
6 h		97.9±5.0
24 h		99.9±2.8
48 h		102.9±3.3
0 h	**0.5**	101.4±8.5
3 h		104.1±6.3
6 h		100.1±2.3
24 h		100.0±2.7
48 h		99.9±5.1

4. Conclusions

The proposed strategy constitutes a valuable tool for determination of glufosinate and metabolites by LC–DAD analysis with FMOC-Cl derivatization of glufosinate and N-acetyl-glufosinate to improve chromatographic resolution. Ultrasound-assisted extraction was optimized for isolation of all target compounds. The method allows relative quantitation of non-available glufosinate metabolites after confirmatory analysis of their presence in resistant plants treated with the herbicide by LC–TOF/MS. This method would also allow a relative quantification of the acetylated product expressed as glufosinate equivalence thus avoiding its synthesis, because it is clearly identified and endowed with a structure similar to glufosinate.

The sample preparation step has been both accelerated and enhanced by ultrasonic energy in comparison to conventional extraction based on maceration. The overall method showed excellent performance without influence of potential interferents associated to the plant matrix, avoiding the use of tedious cleaning

steps. The analytical characteristics of the method enable its recommendation in studies for evaluation of glufosinate metabolism in plants without the need for using ^{14}C-based methods.

Conflict of interest

The authors declare no conflict of interest.

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Parte B.

APLICACIONES DE LAS PLATAFORMAS ANALITICAS DESARROLLADAS

Las plataformas analíticas desarrolladas y recogidas en la Parte A de la Memoria se aplicaron para los diferentes estudios que constituyen esta Parte B.

Son de destacar las aplicaciones del método basado en electroforesis capilar, que han permitido corroborar la existencia de metabolismo en plantas y el efecto producido en ellas. Entre estos estudios se engloban los recogidos en el Capítulo 6 y los trabajos 1, 2, 3 y 4 que forman parte del Anexo 1, en los cuales la doctoranda ha participado aplicando el método a las muestras en cuestión.

Las aplicaciones del glioxilato no se han recogido en la Memoria debido a que están en fase experimental.

En el Capítulo 7 se presenta la aplicación del método desarrollado en el Capítulo 4 sobre plantas de trigo que con diferente resistencia a imazamox. Este método ha permitido dilucidar el mecanismo de la metabolización del herbicida y su importancia en la resistencia, puestas de manifiesto por las diferencias cuantitativas encontradas en los niveles de imazamox y sus metabolitos en ambos biotipos.

El Capítulo 8 lo conforma el estudio metabolómico de glufosinato en plantas de trigo modificadas genéticamente y en otras no modificadas, mediante el que se comprobó la existencia de metabolismo en ambos tipos. En este caso el diferente comportamiento respecto a los niveles de resistencia que mostraban fue debido a las rutas de metabolización, diferentes en abos casos.

Capítulo 6

Limited uptake, translocation and enhanced metabolic degradation contribute to glyphosate tolerance in Mucuna pruriens var. utilis plants

Limited uptake, translocation and enhanced metabolic degradation contribute to glyphosate tolerance in *Mucuna pruriens* var. *utilis* plants

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Limited uptake, translocation and enhanced metabolic degradation contribute to glyphosate tolerance in *Mucuna pruriens* var. *utilis* plants

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ABSTRACT

Velvet bean (*Mucuna pruriens*, *Fabaceae*) plants exhibits an innate, very high resistance (*i.e.* tolerance) to glyphosate similar to that of plants which have acquired resistance to this herbicide as a trait. We analyzed the uptake of [^{14}C]-glyphosate by leaves and its translocation to meristematic tissues, and used scanning electron micrographs to further analyze the cuticle and 3D capillary electrophoresis to investigate a putative metabolism capable of degrading the herbicide. Velvet bean exhibited limited uptake of glyphosate and impaired translocation of the compound to meristematic tissues. Also, for the first time in a higher plant, two concurrent pathways capable of degrading glyphosate to AMPA, Pi, glyoxylate, sarcosine and formaldehyde as end products were identified. Based on the results, the innate tolerance of velvet bean to glyphosate is possibly a result of the combined action of the previous three traits, namely: limited uptake, impaired translocation and enhanced degradation.

***Keywords**:*

Mucuna pruriens, *Amaranthus retroflexus*, Glyphosate, Tolerance, Resistance, Metabolism, Degradation, Translocation, Capillary electrophoresis

1. Introduction

Velvet bean (*Mucuna spp*.) is a vigorous annual legume with many valuable agronomic attributes. As a ground cover, it reduces erosion, fixes nitrogen (Harrison *et al.*, 2004), suppresses weeds (Baijkya *et al.*, 2005), reduces populations of some plant-parasitic nematodes (Vargas-Ayala and Rodríguez-Kábana, 2001), assimilates and sequesters leftover nutrients, and increases crop yields (Abdul-Baki *et al.*, 2005).

Since its inception in 1974, glyphosate has grown in use to the point of currently dominating herbicide usage worldwide (Powles and Preston, 2006). Its widespread use has imposed massive selection pressure on treated weeds, many of which [*e.g.*, *Eleusine indica* (L.) Gaertn. (Baerson *et al.*, 2002), *Amaranthus palmeri* (Culpepper et al., 2006), *Conyza canadensis* (L.) Cronquist (Van Gessel, 2001), *Lolium species*, and miscellaneous others (Powles *et al.*, 1998; Powles and Preston, 2006; Owen and Powles, 2010)] have evolved resistance to this pesticide. In addition to plants acquiring resistance to glyphosate as a new trait, some naturally occurring plants possess an innate, high resistance (tolerance) to this herbicide (Cruz-Hipólito *et al.*, 2009).

Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3- phosphate synthase (EPSPS, EC 2.5.1.19), which catalyzes the conversion of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate in the shikimate pathway (Geiger and Fuchs, 2002). Glyphosate is a competitive inhibitor of PEP, occupies its binding sites and forms part of the intermediate state of the ternary enzyme–substrate complex (Schönbrunn *et al.*, 2001). The inhibition of EPSP synthase results in shikimate accumulation and prevents the biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan, and usually leads treated plants to die 1 or 2 weeks after treatment.

Acquired resistance to glyphosate has been ascribed to the following factors: (1) the presence of a reduced concentration of glyphosate in meristematic tissues as a consequence of impaired or limited uptake and/or translocation of the herbicide (Feng *et al.*, 2004; Wakelin *et al.*, 2004); (2) mutation of the DNA sequence coding leading to an altered, more resistant form of EPSPS (Powles and Yu, 2010); (3) sequestration of the herbicide in cell compartments such as vacuoles (Ge *et al.*, 2010); and (4) overexpression of EPSPS through gene amplification (Gaines *et al.*, 2010). To our knowledge, none of the glyphosate-resistant plants studied to date has been shown to exhibit a metabolic pathway capable of either modifying or degrading the herbicide to an extent rendering it harmless to the plant. The former two mechanisms are the most frequent in plants which have acquired resistance as a new trait. Impaired glyphosate uptake or/and

translocation generally increases plant resistance by a factor of 8–12 relative to sensitive populations. Such is the case, for example, with Italian ryegrass (*Lolium multiflorum Lam*.), rigid ryegrass (*Lolium rigidum Gaudin*) and horseweed [*C. canadensis* (L.) Cronq.] (Lorraine-Colwill *et al.*, 2002; Wakelin *et al.*, 2004; Feng *et al.*, 2004; Koger and Reddy, 2005; Dinelli *et al.*, 2006; Michitte *et al.*, 2007; Preston and Wakelin, 2008). By contrast, the presence of an altered form of EPSPS generally confers lower resistance to glyphosate (usually only 2–4 times higher than in sensitive plants); such a modest efficiency has been deemed ineffective for plants to develop resistance in the field (Dinelli *et al.*, 2006; Sammons *et al.*, 2007). Usually, altered EPSPS forms replace the equivalent *Lolium* Pro106 by Ser, Thr or Ala (Baerson *et al.*, 2002; Ng *et al.*, 2003; Wakelin and Preston, 2006; Perez-Jones *et al.*, 2007; Yu *et al.*, 2007; Simarmata and Penner, 2008). Both traits are inherited as single and dominant genes (Preston *et al.*, 2009).

Little is known about the fate of glyphosate in plants. The presence of putative glyphosate oxidoreductase (GOX) activity was first observed in soybean cell cultures yielding (aminomethyl) phosphonic acid (AMPA) as an end product (Komoba *et al.*, 1992). Since then, it is generally assumed that plants either cannot degrade or metabolize glyphosate, or metabolize at too low a rate for plant resistance or tolerance to develop (Feng *et al.*, 2004).

In this work, *Mucuna pruriens*, a plant with a high innate tolerance to glyphosate, was characterized. We found the plant to exhibit impaired uptake of glyphosate, translocation of the herbicide to meristematic tissues to be very slow and the species to efficiently degrade the herbicide via two different, concurrent pathways. In our opinion, the joint presence of these three traits can explain the high level of innate glyphosate tolerance in this plant.

2. Results and discussion

2.1. Dose–response assays

Glyphosate application reduced shoot biomass production in both *Amaranthus retroflexus* (Amaranthaceae) and *M. pruriens* (Fig. 1). Based on the results of the whole-plant dose–response bioassay, *M. pruriens* was more than 7 times as

resistant to glyphosate as was *A. retroflexus* (Table 1). Consequently, *A. retroflexus* was fully sensitive to glyphosate, whereas *M. pruriens* was highly tolerant to the herbicide.

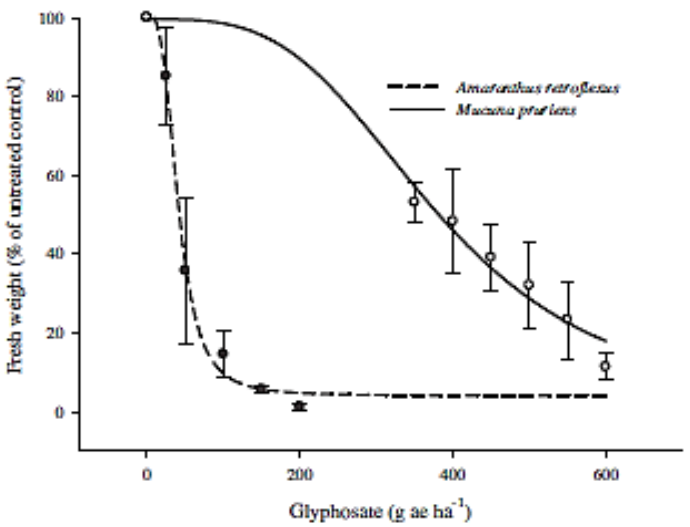

Fig. 1. Shoot biomass in A. retroflexus and M. pruriens plants treated with glyphosate at a variable rate.

Table 1. Parameters of the Log-Logistic equation used to calculate the herbicide dose required for 50% Reduction of the Fresh Weight (ED50) of *Amaranthus retroflexus* and *Mucuna pruriens.*

Plant	*c*	*d*	*b*	ED_{50} (g ae ha^{-1})	*P*[b]	RF[c]
A. retroflexus	4.09 ± 2.14	100 ± 4.61	2.91 ± 0.83	54.26 ± 6,82	<0.001	
M. pruriens	2.18 ± 0.14	94.73 ± 5.38	2.53 ± 0.28	403.78 ± 3.25	<0.001	7.44

Equation Y = c + {(d-c)/[1 + (x /g)b]}, where Y is the percentage of plant injury, x (independent variable) is the herbicide rate, c and d are the lower and upper symptotes, respectively, b is the slope of the line, and ED50 is the effective dose required for 50% plant injury. Data were pooled and fitted to nonlinear regression model. Data are means of four replicates. b Probability level of significance of the nonlinear model c RF, resistance factor = ED50 of tolerant/ED50 of susceptible biotype.

2.2. Whole-plant shikimic acid assays

The fact that EPSPS inhibition by glyphosate was accompanied by accumulation of shikimic acid in treated plants led us to perform the experiment depicted in Fig. 2.

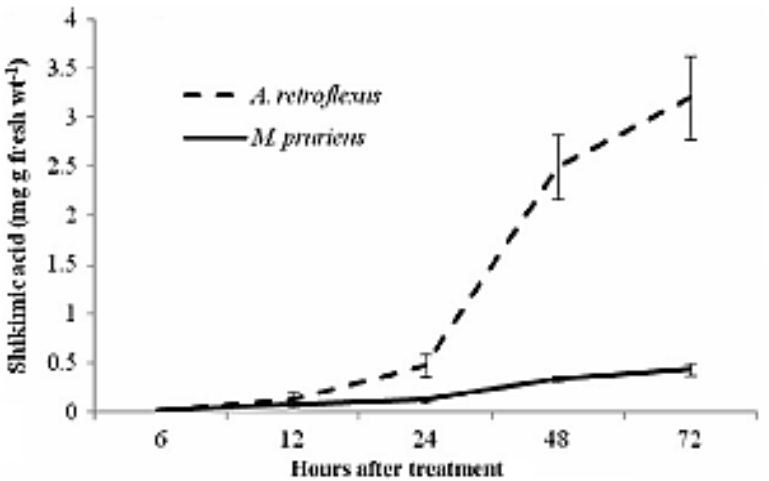

Fig. 2. Shikimic acid accumulation after treatment with 500 g ae ha^{-1} glyphosate. *A. retroflexus* accumulated more shikimic acid than did *M. pruriens*.

As can be seen, *A. retroflexus* accumulated substantially higher levels of shikimic acid than *M. pruriens*. Similar results were previously obtained by Perez-Jones *et al.* (2005) on glyphosate-resistant and susceptible Italian ryegrass biotypes isolated in Oregon.

The high tolerance (RF = 7.44) and little shikimic acid accumulation observed are consistent with those of plants which have acquired resistance or possess a high innate tolerance to glyphosate by effect of limited uptake and/or translocation of the pesticide; both reduce accumulation of the herbicide in meristematic tissue, which requires the synthesis of aromatic amino acids at this stage (Cruz-Hipólito *et al.*, 2009, 2011; Hutchinson *et al.*, 2010).

2.3. [^{14}C]-glyphosate uptake and translocation assays, and SEM analysis

There were marked differences in glyphosate uptake between the glyphosate-susceptible *A. retroflexus* and the tolerant *M. pruriens*. Fig. 3 shows the kinetics of [^{14}C]-glyphosate uptake by leaves of both species following application of the

herbicide. *Amaranthus retroflexus* plants accumulated ca. 94% of the total amount of glyphosate applied within 24 HAT. By contrast, *M. pruriens* plants accumulated a much lower fraction of applied [*14*C]-glyphosate throughout the experiment. Glyphosate accumulation in *Mucuna* plants 24 h after treatment (HAT) was less than 40% and increased slowly to less than 50% 72 HAT. Clearly, *M. pruriens* exhibited limited uptake of glyphosate compared with *A. retroflexus*.

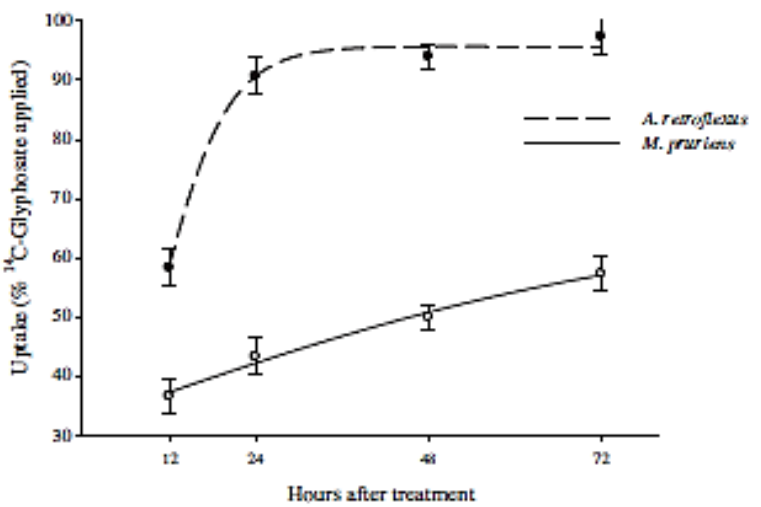

Fig. 3. [^{14}C]-glyphosate uptake by leaves of both plant species after glyphosate application. *A. retroflexus* accumulated *ca.* 94% of total glyphosate applied 24 HAT; by contrast, *M. pruriens* never accumulated more than 40% 24 HAT.

The limited glyphosate uptake by *M. pruriens* can be ascribed partly to the differences in leaf outer surfaces between the two species revealed by the scanning electron micrographs. As can be seen in Fig. 4, *M. pruriens* cuticle contained massive amounts of epicuticular wax forming a nonuniform 3D cover.

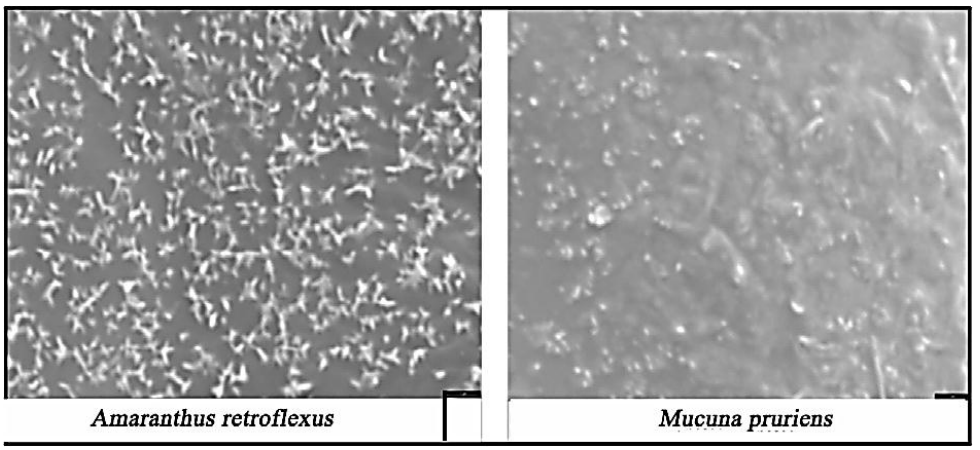

Fig. 4. Scanning electron micrographs exposing differences in leaf outer surfaces between *M. pruriens* and *A. retroflexus* (scale: bar 10 μm). The *M. pruriens* cuticle exhibited a massive presence of epicuticular waxes forming a nonuniform 3D cover. On the other hand, the *A. retroflexus* cuticle formed a more uniform covered surface

In addition, wax coverage differed significantly between the two species (in *M. pruriens* and in *A. retroflexus*). The presence of wax on *M. pruriens* leaves may have prevented direct, extensive contact of glyphosate-containing drops with leaf surfaces, thus limiting glyphosate penetration. On the other hand, *A. retroflexus* cuticle exhibited a more uniformly covered leaf surface with less wax density and areas lacking wax; this disrupted the regular structure and facilitated contact with herbicide-containing drops. Although a deeper study including measurements of cuticle width and chemical composition would be required to confirm whether the cuticle was truly responsible for the limited glyphosate uptake observed, such association is clearly apparent from the scanning electron micrographs and was previously reported in other plants (De Prado *et al.*, 2005; Shepherd and Wynne, 2006).

There were also differences in the amount of [^{14}C]-glyphosate translocated from treated leaves to the rest of the plant in both species. This is clearly visible in the phosphor images of Fig. 5.

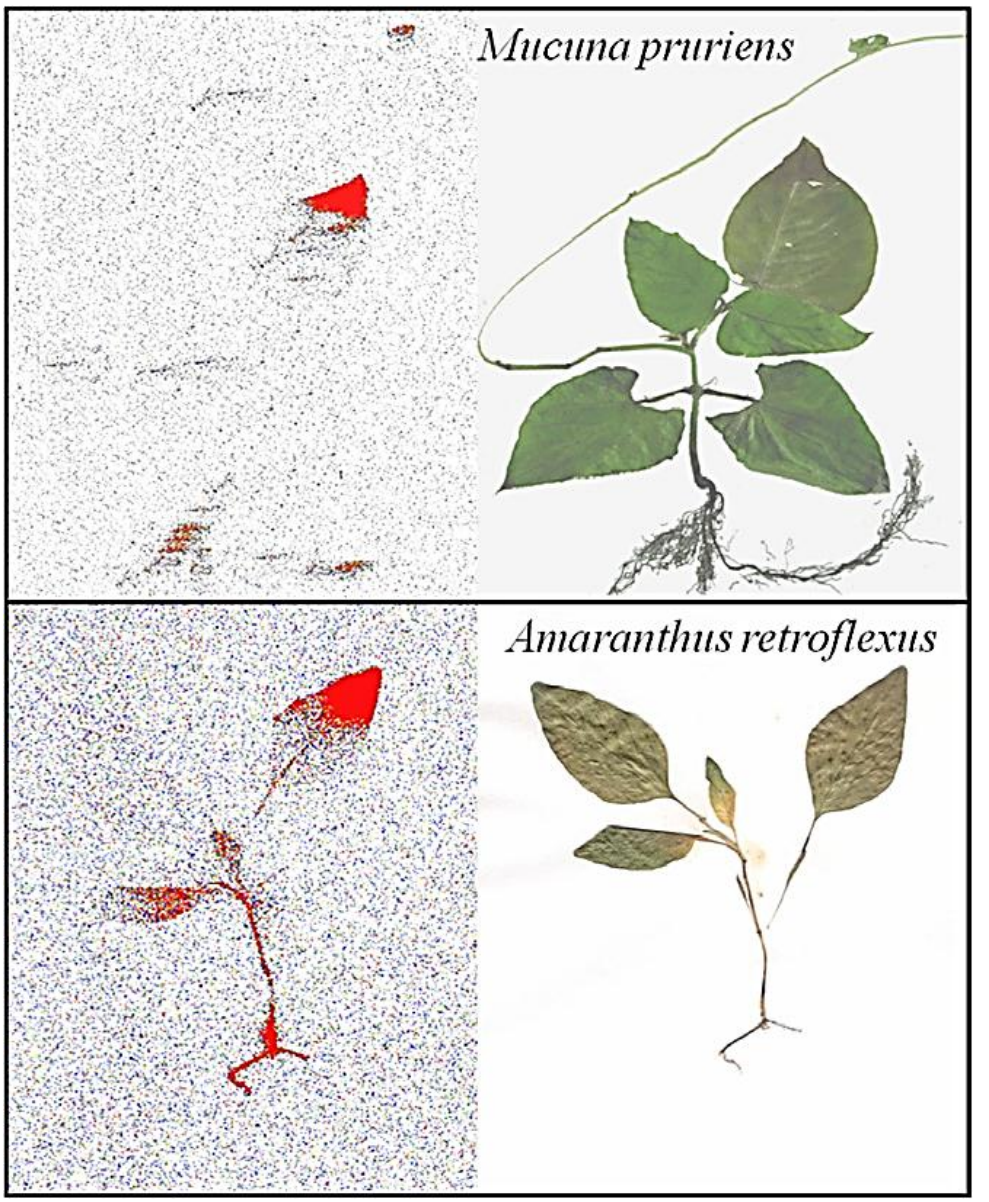

Fig. 5. Phosphor images of *M. pruriens* and *A. retroflexus* 96 HAT. *M. pruriens* plants retained more labeled glyphosate in treated leaves, and translocated less herbicide to the rest of the plant, than did *A. retroflexus* plants.

Also, as can be seen in Table 2, *A. retroflexus* plants consistently retained less labeled glyphosate in treated leaves, and translocated more herbicide to the root and the rest of the plant, than did *M. pruriens* plants a few hours after application.

Table 2. Translocation of ^{14}C-glyphosate in *A. retroflexus* and *M. pruriens*

		[^{14}C]glyphosate (% absorbed)		
HAT	**SPECIES**	**TREATED LEAF**	**ROOT**	**REST OF PLANT**
24 h	***A. retroflexus***	81±2	9.3±0.5	9.5±0.5
	M. pruriens	94.2±4.6	4.2±0.34	1.7±0.1
48 h	***A. retroflexus***	62±2.5	15.3±0.51	22.4±0.55
	M. pruriens	88.1±1.85	2.4±0.34	9.6±0.1
72 h	***A. retroflexus***	42.5±1.6	28.7±0.7	29.2±0.98
	M. pruriens	71±1	17.9±0.1	21.8±0.3

The low absorption and translocation of glyphosate found in *Mucuna* plants might by themselves account for the high innate tolerance of this plant. Similar results were recently obtained in naturally tolerant *Canavalia sp*. plants, which exhibited comparable resistance (Cruz-Hipólito *et al.*, 2009).

2.3. Glyphosate metabolism

Fig. 6 summarizes available knowledge about glyphosate degradation pathways in living organisms. Glyphosate degradation to glyoxylate and AMPA in some plants and bacteria present in soil by a glyphosate oxidoreductase (GOX) gene has been reported by some authors (Liu *et al.*, 1991; Komoba *et al.*, 1992; Al-Rajab and Schiavon, 2010). Also, C–P lyase activity can degrade glyphosate to sarcosine and inorganic phosphate, which eventually form formaldehyde, glycine and CO2 in a reaction catalyzed by sarcosine oxidase (Liu *et al.*, 1991). After the first evidence of the presence of putative GOX activity in plants in aseptic soybean cell cultures was reported, a number of studies on monocotyledonous and dicotyledonous plants have revealed that a metabolism capable of degrading glyphosate to AMPA is either missing or so weak that it can hardly account for plant resistance or tolerance (Feng *et al.*, 2004; Lorraine-Colwill *et al.*, 2002). Neither the presence of C–P lyase nor glyphosate-dependent production of sarcosine or formaldehyde has so far been observed in plants.

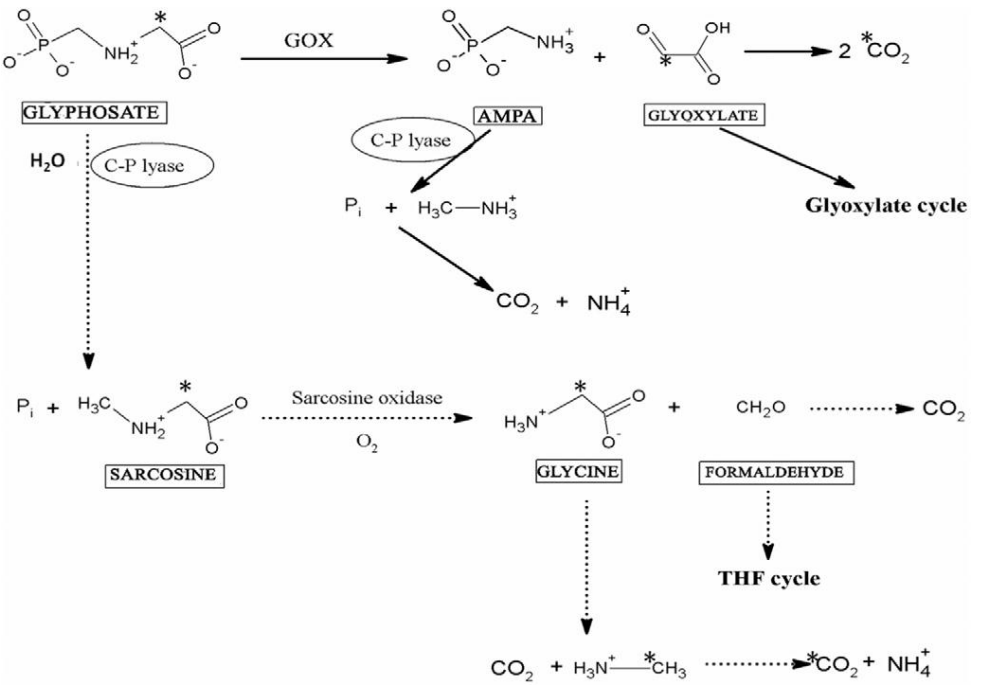

Fig. 6. Potential degradation pathway for glyphosate (⟶ primary pathway in plants and soils, ⋯▸ primary pathway in bacteria).

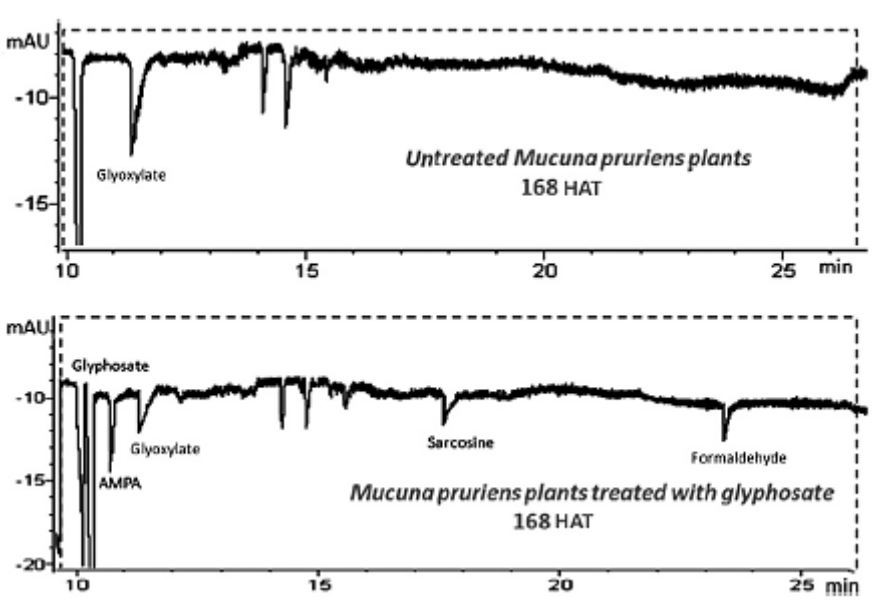

Fig. 7. Electropherograms for extracts of glyphosate-treated and untreated *M. pruriens* plants. Only the extracts from glyphosate-treated plants contained AMPA, sarcosine and formaldehyde.

Fig. 7 shows the electropherograms for extracts from glyphosate- treated *M. pruriens* plants and untreated specimens used as controls.

As can be seen, glyphosate, AMPA, sarcosine and formaldehyde were only present in the extracts from the glyphosate-treated plants. This clearly indicates that *Mucuna* plants can metabolize glyphosate and also that the two degrading pathways depicted in Fig. 6 may coexist in it. To our knowledge, this is the first time a plant has been found to degrade glyphosate to sarcosine and formaldehyde. By contrast, only glyphosate and very low traces of AMPA, but neither sarcosine nor formaldehyde, were detected in a similar experiment with glyphosate-treated *A. retroflexus* plants (results not shown).

As expected, glyoxylate was present in both treated and untreated *M. pruriens* plants (Fig. 7), since this compound is formed both by Rubisco oxygenase reaction in photorespiration and by cleavage of isocitrate in a reaction catalyzed by isocitrate lyase in the glyoxylate cycle. In our opinion, most of the glyoxylate found in our experiments could came from these sources rather than from glyphosate degradation. This is supported on the level of accumulation of glyoxylate seen in *Mucuna* untreated plants (45.36 ± 2.81 μg g^{-1} fw), which is very similar to that found in treated plants (53.99 ± 4.18 μg g^{-1} fw) at 168 HAT (Fig. 7). In fact, it is hard to ascertain how much of the glyoxylate accumulated in treated plants come from glyphosate degradation because this compound is readily being formed and recycled at the same time by the healthy *Mucuna* plants. AMPA accumulation, however, can only come from the added glyphosate.

Fig. 8A and B shows the time course appearances of these metabolites in greater detail.

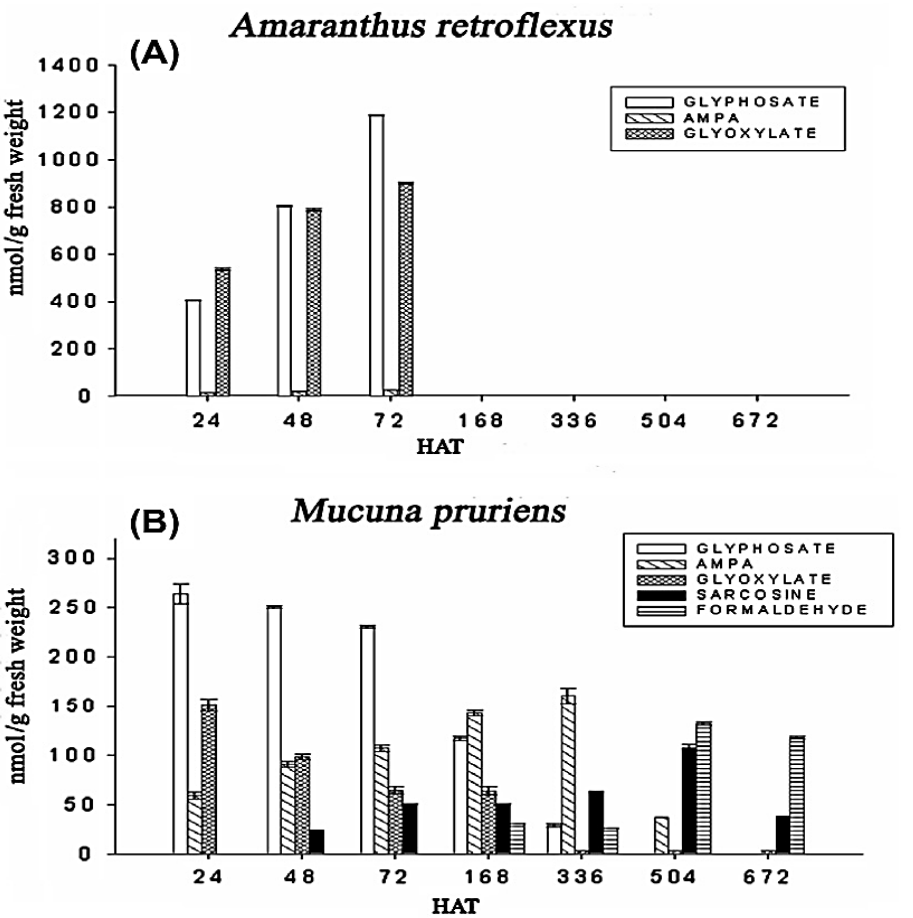

Fig. 8. Variation of the concentrations of glyphosate metabolites (nmol/g fresh weight) in (A) *A. retroflexus* and (B*) M. pruriens* plants at different times after treatment with 500 g ae ha^{-1} glyphosate.

No data for *A. retroflexus* 72 HAT are included because the plants were strongly affected by the herbicide and exhibited chlorosis symptoms by then. On the other hand, *Mucuna* plants appeared fully healthy throughout the experiment. The glyphosate and glyoxylate concentrations in *A. retroflexus* treated plants increased with time, whereas the AMPA concentrations never exceeded trace levels. Therefore, this species has no active pathway capable of degrading glyphosate, which is consistent with previous results for other plants (Yuan *et al.*, 2007; Yu *et al.*, 2009). Glyphosate must have rapidly and effectively inhibited EPSPS activity in these plants, thus suppressing the synthesis of aromatic amino acids and some secondary metabolites, protein turnover and, eventually,

accumulation of glyoxylate, which could not be reincorporated into metabolic pathways. These processes invariably lead to cell death.

Mucuna plants exhibited a rather different behavior. Thus, the maximum level of intracellular glyphosate was reached soon after application of the herbicide (24 h). Then, the herbicide concentration decreased with time, concomitantly with an increase in AMPA (detected 24 HAT) and then in sarcosine (detected 48 HAT). No glyphosate was detected after 504 HAT. Formaldehyde only appeared 168 HAT, when the plants contained high enough concentrations of sarcosine. These results suggest that a putative GOX enzyme could be either constitutively or very rapidly induced in *Mucuna* plants, which is responsible for the appearance of AMPA from glyphosate, and also that the C–P lyase and sarcosine oxidase activities needed for sarcosine and formaldehyde production must be induced or activated at a later stage. Further testing, however, is required to confirm these assumptions.

Some authors have reported additive effects of concurrent glyphosate resistance mechanisms in the same plant (Powles and Preston, 2006; Gaines *et al.*, 2010). Thus, impaired glyphosate translocation together with the presence of an altered form of EPSPS in the same plant were found to increase its glyphosate resistance as compared with populations possessing a single resistance mechanism (Yu *et al.*, 2007). Similarly, the joint presence of a GOX transgenic gene and an altered EPSPS form has made plants with a high enough tolerance to glyphosate commercially attractive —a trait which was not acquired when either of these two genes was separately present in the same plant (Dill, 2005). In fact, this idea has been exploited to raise commercial transgenic glyphosate tolerant plants harboring a bacterial GOX gene (Zhou *et al.*, 1994).

In our case, a new combination of three resistance traits acting together may have led to the high innate tolerance of *Mucuna* plants. The observed impaired uptake and translocation may have played a major role here as it has in other plants. However, the presence of both glyphosate-degrading pathways in *Mucuna* may reduce the amount of intracellular glyphosate that can reach the target enzyme, EPSPS, thus adding further tolerance to the herbicide. Although a similar level of tolerance in *Clitoria* plants was recently ascribed to impaired

uptake and translocation of glyphosate (Cruz-Hipólito *et al.*, 2011), further testing is required to ascertain whether this latter plant can degrade the herbicide.

3. Experimental

3.1. Plants and growing conditions

For comparative purposes, we used *M. pruriens*, a glyphosate tolerant plant, and *A. retroflexus*, a glyphosate-susceptible biotype. Seeds of both species were collected in 2009 in Martínez de la Torre, Veracruz, Mexico (97º 04'W, 20º 04'N), then germinated in pots (three plants per pot) containing peat and sandy loam potting mixture (1:2, v/v) in a growth chamber at 28/18ºC (day/night) with a 16 h photoperiod under 850 µmol m^{-2} s^{-1} photosynthetic photonflux density and 80% relative humidity. Special care was exercised to keep the soil water-saturated throughout.

3.2. Dose–response assays

Treatments with glyphosate (Roundup®, Monsanto) were applied to the third pair of true leaves in both plants by using a laboratory track sprayer furnished with a Tee Jet 80.02.E.VS flat-fan nozzle and delivering a spray volume of 200 l ha^{-1} at 200 kPa. Glyphosate was applied at a rate of 0, 12, 25, 50, 100 or 200 g acid equivalent (ae) ha^{-1} to *A. retroflexus* and 0, 350, 400, 450, 500, 550 or 600 g ae ha^{-1} to *M. pruriens*. Above-ground fresh weight per pot was determined 504 h (21 days) after spraying and data were expressed as percentages of the figures for the corresponding untreated plant used as control. The herbicide rates needed to inhibit plant growth of each species by 50% with respect to the untreated control (ED_{50}) were determined according to Menéndez *et al. (*2006). The tolerance/-susceptibility ratio was computed as ED_{50} (*M. pruriens*)/ED_{50} (*A. retroflexus*) in terms fresh weight since watering of the soil was strictly controlled throughout. Experimental treatments were replicated four times and each test was conducted three times. Data were pooled and fitted to a nonlinear log–logistic regression model, $Y = c + \{(d - c)/[1 + (x/g)b]\}$, where Y is the fresh above-ground weight expressed as a percentage of the untreated control; c and d are coefficients corresponding to the lower and upper asymptotes; b is the slope of the curve; g is

the herbicide rate at the point of inflection halfway between the upper and lower asymptotes: and x (independent variable) is the herbicide rate. Regression analysis was conducted by using Sigma Plot 10.0 statistical software (Cruz-Hipólito *et al.*, 2009).

3.3. Whole-plant shikimic acid assays

Plants of *M. pruriens* and A. retroflexus were sprayed as described above with commercially formulated glyphosate at 500 g ae ha^{-1}. Plants of both species (at the same stage as before) were harvested 6, 12, 24, 48, 72, and 96 h after treatment (HAT). Several leaf tissue samples each containing 0.1 g of fresh weight were homogenized and frozen in liquid nitrogen. Shikimic acid accumulation was determined optically on a Beckman DU-640 spectrophotometer according to Perez-Jones *et al. (*2005). The standard curve was obtained by using untreated plants and a known concentration of shikimic acid. The experiment was repeated three times, using 10 replications per harvest time per species.

3.4. [^{14}C]-glyphosate uptake and translocation assays

Glyphosate [glycine-2-^{14}C] (specific activity 273.8 MBq $mmol^{-1}$, American Radiolabel Chemicals, Saint Louis, MI) was mixed with commercially formulated glyphosate to prepare emulsions with a specific activity of 1.85 kBq μl^{-1} (both absorption and translocation studies) and a glyphosate concentration of 3.6 g active ingredient per liter (ai l^{-1}), corresponding to 720 g of ai ha^{-1} at 200 l ha^{-1}. The labeled herbicide was applied to the adaxial surface of the second leaf of each plant in four 0.5 µl droplets by means of a PB 600 TA micro applicator from Hamilton Co. (Switzerland). A total of 3.7 kBq was applied to each plant. Plants of both species were harvested at the three pairs of true leaves in batches of three replications 12, 24, 48, and 96 HAT, and split into treated leaf, upper leaf, root, and rest of shoot. Unabsorbed [^{14}C]-glyphosate was removed from the leaf surface by rinsing the treated area with 3 ml of 80% (v/v) methanol. The rinses from each batch were pooled and analyzed by liquid scintillation spectrometry (LSS) on an LS 6500 TA spectrometer from Beckman Instruments, Inc. (Fullerton, CA). Plant tissue was dried at 55 ºC for 72 h and combusted in a

Tri Carb model 307 oxidizer from Packard Instrument Co. (Connecticut, NE). Evolved $^{14}CO_2$ was trapped and counted in 10 ml of a 3:7 (v/v) mixture of Carbo-Sorb E and Permafluor E+(Perkin–Elmer, Packard Bioscience BV, Groningen, The Netherlands). Radioactivity was quantified by LSS and the proportion of herbicide absorbed was expressed as [kBq in combusted tissue/(kBq in combusted tissue + kBq in leaf rinses)] × 100 (De Prado *et al.*, 2005). The experiment was repeated three times. Treated plants of both species were removed from pots at the same times for the translocation tests. Roots were rinsed and whole plants oven-dried at 50 ºC for 96 h, pressed against a 25 × 12.5 cm phosphor storage film for 6 h and scanned for radiolabel dispersion on a Cyclone (Perkin–Elmer, Packard Bioscience BV). Means and their standard errors were computed for all plant parts, and means were tested for group differences and compared via analysis of variance (ANOVA) and Tukey's HSD posthoc test.

3.5. Scanning electron micrographs

Small pieces (0.5 × 0.5 cm) of fresh *M. pruriens* and *A. retroflexus* leaves were cut off with a sharp razor blade and fixed in 2% glutaraldehyde (v/v) in 0.2 M pH 7 phosphate buffer at 4 ºC overnight. Samples were thoroughly rinsed in fresh phosphate buffer and then dehydrated through an ethanol solution series [20%, 40%, 60%, 80% and 100% (v/v)] for increasing times from 15 to 90 min. Plant pieces were placed on a metal holder using a double-faced adhesive and coated with a 0.05 lm thin film of gold. A JEOL JSM-840 scanning electron microscope operated at 10–20 kV was used to examine samples. Wax coverage was digitally captured, and color differences between covered and uncovered areas were enhanced by using photo-processing software (Adobe Photoshop). Wax coverage (expressed as μm^{-2}) was quantified with the image processing software ImageJ 1.31 v.

3.6. Glyphosate metabolism

Plant growth stage and herbicide treatment were similar to those described under Whole-Plant Shikimic Acid Assays. Treated and untreated plants were harvested 0, 72, 96, 168, 336, 504 and 672 HAT. Leaf tissues were washed with distilled

water, flash-frozen in liquid nitrogen and stored at –40 ºC until use, following the protocol of Rojano-Delgado *et al.* (2010). Glyphosate and its metabolites (*viz.* AMPA, glyoxylate, sarcosine and formaldehyde) were determined on a 3D Capillary Electrophoresis Agilent G1600A instrument equipped with a diode array detector (DAD, wavelength range 190–600 nm). An aqueous solution containing 10 mM potassium phthalate, 0.5 mM hexadecyltrimethylammonium bromide (CTAB) and 10% acetonitrile at pH 7.5 was used as background electrolyte. Calibration equations were obtained from data for untreated plants and standards of known concentration of glyphosate and its metabolites supplied by Sigma–Aldrich (St. Louis, MI). Quantitation was based on electropherogram peak areas. The experiment was repeated four times per species, with five replicates per harvest time.

Conflict of interest

The authors have declared that no conflict of interest exists.

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Capítulo 7

Importance of imazamox metabolism in Clearfield Triticum aestivum

Importance of imazamox metabolism in Clearfield Triticum aestivum

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Enviado a:

Phytochemistry

Importance of imazamox metabolism in Clearfield Triticum aestivum

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Abstract

The biochemical resistance/susceptibility to imazamox in two cultivars — resistant (R) and susceptible (S)— of Clearfield wheat (*Triticum aestivum*) population was studied. This wheat is the bread variety Pandora from Chile, where these crops are of great importance. Experiments in a controlled growth chamber showed that the R cultivar presented a high resistance to imazamox with an ED_{50} value of 289.66 g active ingredient per hectare (g ai ha^{-1}) *versus* 25.15 g ai ha^{-1} for the S cultivar. The activity of acetolactate synthase (ALS) in leaves extract from both cultivars was investigated to evaluate the effect of imazamox by comparison to other imidazoline herbicides. The R cultivar showed the highest resistance to imazamox, but variable cross-resistance was observed to all tested imidazolinone herbicides with a resistance order: imazamox ≈ imazethabenz-methyl > imazapyr > imazaquin > imazethapyr). Metabolism studies showed a higher and fast penetration of imazamox in cultivar S than in the Pandora R wheat. LC–TOF/MS analysis of imazamox metabolism at 8 days after herbicide treatment (200 g ai ha^{-1}) revealed a 67% conversion of the herbicide into the hydroxy metabolite and 27.7% into the glucoside metabolite in the R cultivar, while in the S cultivar only traces of the hydroxy metabolite were detected. These results suggest that metabolism of imazamox in the R cultivar is a key mechanism of resistance that explains such high levels of herbicide tolerated by the plant, together with a second mechanism due to the loss of imazamox affinity for its target site.

Keywords: *Triticum aestivum* var. Pandora, Clearfield crops, imazamox, resistance, susceptibility, metabolism, degradation, LC–DAD.

1. Introduction

Imidazolinones have gained a great success in modern agriculture since they were discovered in the 80's of the last century (Shaner et al., 1984). This is due to their high efficiency in cereal and legume crops, their low mammalian toxicity and low environmental impact (Aichele and Penner, 2005; FAO, 2012), a wide crop selectivity and large number of formulations suitable for pre- and post-emergence (Shaner and O'Connor, 1991). Imazamox [3-pyridinecarboxylic acid, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-(methoxymethyl)-] is a imidazolinone appeared in 1998 (Brady et al., 1998; www.ec.europa.eu). This herbicide is endowed with great effectiveness as it is easily absorbed by leaves and roots; also, it presents translocation and low metabolism —nevertheless, absorption, translocation and metabolism may vary according to the plant species and the surfactants added (Ball et al., 1999; Pester et al., 2001).

The primary mode of imazamox action is the inhibition of the enzyme acetolactate synthase (ALS, former E.C. 4.1.3.18 and changed into E.C. 2.2.1.6 since 2002), which catalyzes the first common step in the biosynthesis of branched chain amino acids as valine, leucine and isoleucine, in plants and microorganisms (Saari y Mauvais, 1996). Powles and Yu described in 2010 the mechanism of ALS inhibition by the herbicide. This enzyme is formed by two subunits: one with regulatory activity and the other with catalytic activity. The former stimulates the activity of the catalytic subunit and confers sensitivity to feedback inhibition of branched chain amino acids. The catalytic site of ALS is located within a deep channel and, therefore, the ALS-inhibitor herbicides do not bind to the catalytic site: rather, they bind by a binding domain to herbicides which extends from the channel entry and block the passage of the substrate to the catalytic site. With this domain, 18 amino acid residues are involved in binding with the herbicide. ALS inhibitor herbicides structurally different are oriented differently in herbicide binding domain, with partial overlap (Duggleby et al., 2008; McCourt et al., 2006 a and b). Therefore, a mutation which causes an amino acid substitution inside the binding domain may confer resistance to some herbicides, but not to others (Christoffers et al.,2006; Kolkman et al., 2004;

Plaza et al., 2003) or even to several herbicides with the same mode of action by cross-resistance (Baumgartner et al., 1999; Bozic et al., 2012; Han et al., 2012; Kaloumenos et al., 2012). Five mutations of the ALS gene have been described in the amino acid positions Ala122, Pro197, Ala205, Trp574 and Ser653 (Tan et al., 2005). The mutation at Trp574 is cross-resistant to all families of ALS-inhibiting herbicides. The codon Pro197 mutation is more tolerant to sulfonylureas. Mutations at codons Ala122, Ala205, and Ser653 confer resistance to imidazolinones, the Ala122 conferring higher levels of resistance to imidazolinones than mutation Ser653 (Li et al., 2008). Recently, a new mutation at the position Ser-Asn627 has shown to be responsible for resistance in *Tritordeum* (Rodríguez-Suárez et al., 2009). As the mutated imidazolinone-resistant ALS genes are semi-dominant, higher levels of resistance can be a consequence of two or more resistant genes in a given genotype (Newhouse et al., 1992; Pozniak and Hucl 2004a).

The need to manage weeds resistant to herbicides, widely used in wheat, corn, rice, etc., led to the search for new cultivars with mutations that could endow them with resistance/tolerance to imidazolinones (Bond et al., 2005; Clemmer et al. 2004). This search emerged in the 90's of the past century, being the first variety used a winter wheat called "Fidel" (Anderson et al., 2004) with a mutation that conferred resistance to imazethapyr (Newhouse et al., 1992; Ball et al., 1999). The last induced resistance in wheat was to imazamox (Seefeldt et al., 1998), but it was not until the year 2000 when these crops began to be marketed with the name of Clearfield® (BASF Corporation). They were then considered as an integrated weed control (Colquhoun et al., 2003) based on the development of varieties tolerant to imidazolinones using traditional induction mutations and conventional breeding. They are non-genetically modified seeds the success of which has been widely assessed (Pfeninng et al., 2008; Tan et al., 2005).

Several studies have been developed on the plants resistance/tolerance to imazamox, namely: on enzymatic activity (Rainbolt et al., 2005), absorption/translocation (Bond & Walker, 2011; Bukun et al., 2012; Pester et al., 2001, Vassios et al., 2011), and metabolism (Bukun et al., 2012; Ohba et al., 1997; Pester et al., 2001, Vassios et al., 2011), among the most salient.

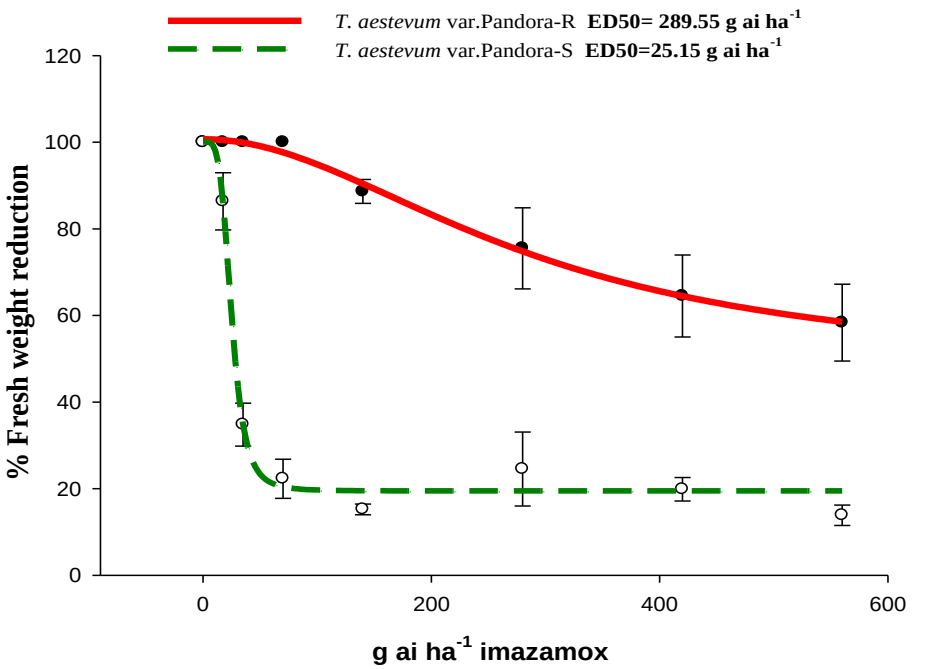
Metabolism studies have frequently been developed using ^{14}C-imazamox to determine some metabolites based on radiactivity measurements; nevertheless, the measured signal can also be due to degradation products of non-metabolic herbicide pathway (Harir et al., 2007). More convincing are the results of the work by Ohba et al. (1997) who identified two metabolites of the imazamox in adzuki bean (*Vigna angularis*) using standards of both metabolites synthesized by the American Cyanamid Company (Ohba et al., 1997).

The wheat market has great relevance in the Chilean agricultural economy both because of the cultivated extension and the growing number of farms devoted to this crop. Pandora wheat variety (*Triticum aestivum*) resistant to imazamox was used in Chile as an effective way to control a large number of weeds tolerant and/or resistant to herbicides. The aim of our research was to study the metabolism of imazamox in two cultivars of *Triticum aestivum* var. Pandora (Mellado et al., 2007) with different degrees of resistance to imazamox. The absence of commercial standards of imazamox metabolites promoted the developmend of a method for determination of the precursor and metabolites based on liquid chromatography–absorptiometry detection by a diode array detector (LC–DAD) prior identification of the latter by LC–time-of-flight mass spectrometry (LC–TOF/MS) in high resolution mode. Thus, the difference between the metabolism of both cultivars can be checked, verifying whether the metabolism is in fact a key mechanism of resistance in Clearfield crops.

2. Results and Discussion

2.1. Dose−Response Assays

The application of imazamox on Pandora plants reduced shoot biomass production in both R and S cultivars (Supplementary Fig. 1).



S-Fig. 1. Shoot biomass in the R and S cultivars of *Triticum aestivum* var. Pandora plants treated with imazamox at different doses.

The ED_{50} (herbicide concentration required for a 50% reduction of plants fresh weight) for R cultivar was 289.55 grams of active ingredient per hectare (g ai ha^{-1}) versus 25.15 g ai ha^{-1} for the S cultivar (Table 1). These results, obtained from the whole plant dose–response bioassay, showed that the R cultivar was 11.5 times more resistant to imazamox than cultivar S; therefore, the cultivar R proved to be highly resistant to imazamox.

Table 1. Parameters of the Log–Logistic equation used to calculate the imazamox doses required for a 50% reduction of fresh weight (ED_{50}) in both cultivars of *Triticum aestivum* var. Pandora.

	c	***d***	***b***	**ED_{50}[a]**	***P*[b]**	**RF[c]**
Cultivar S	3.71 ± 0.90	100 ± 2.67	2.34 ± 0.46	25.15 ± 4.82	<0.001	
Cultivar R	1.33 ± 0.28	95.40 ± 4.21	2.09 ± 0.30	289.55 ± 2.27	<0.001	11.51

Equation $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where Y is the percent plant injury; x (independent variable) the herbicide rate; c and d are the lower and upper asymptotes, respectively; b is the slope of the curve; and ED_{50} is the effective dose required for 50% plant injury. Data were pooled and fitted to a nonlinear regression model. All values were calculated by mean average of four replicates

[a]Expressed as g ae ha^{-1}

[b]Probability level of significance of the nonlinear model

[c]RF, resistance factor = ED_{50} of tolerant/ED_{50} of susceptible cultivar.

2.2. ALS Activity Assays

The specific *in vitro* activity of the ALS enzyme obtained from shoot Pandora tissue was similar in R and S cultivars (279.3 ±11.5 and 284.0 ± 27.9 nmol of acetoin mg^{-1} of protein h^{-1}, respectively).

The I_{50} values (herbicide concentration required for 50% reduction of the ALS activiy) for the different herbicides are shown in Table 2. In all cases the R cultivar tolerated higher concentrations of herbicide than the S cultivar. The order of the resistance factors estimated with the ALS *in vitro* assay to differents imidazolinones herbicides (IMI) was, imazamox ≈ imazethabenz-methyl > imazapyr > imazaquin > imazethapyr (Table 2).

Table 2. Parameters of the equation used to calculate the herbicide concentration required for 50% reduction of the ALS activiy (I_{50}) of resistant (R) and susceptible (S) cultivars of *Triticum aestivum* var. Pandora.

Herbicides (µM)	A	c	D	b	I50 (µM)	Pseudo R	P	RF
Imazamox	**S**	0.964	100.242	0.387	1.469	0.978	<0.0001	
	R	13.802	100.785	1.023	378.002	0.998	<0.0001	257.319
Imazethapyr	**S**	0.120	99.873	0.976	0.989	0.975	<0.0001	
	R	3.361	100.045	0.459	6.514	0.982	<0.0001	6.586
Imazaquin	**S**	0.286	100.228	2.834	1.347	0.980	<0.0001	
	R	6.279	100.146	0.907	12.464	0.989	<0.0001	9.253
Imazethabenz-methyl	**S**	4.745	101.005	2.830	1.281	0.977	<0.0001	
	R	9.285	97.269	3.531	317.120	0.998	<0.0001	247.56
Imazapyr	**S**	8.442	99.998	1.048	3.842	0.995	<0.0001	
	R	0.779	100.697	0.656	315.056	0.994	<0.0001	82.003

[a]Equation Y = c + {(d - c)/[1 + (x/g)b]}, where Y is the percentage of plant injury, x (independent variable) is the herbicide concentration, c and d are the lower and upper asymptotes, b is the slope of the line, and I_{50} is the effective dose required for 50% reduction in ALS activity. Data were pooled and fitted to a nonlinear regression model.
b Approximate coefficient of determination of nonlinear models with a defined intercept calculated as pseudo r^2 = 1 - (sums of squares of the regression/corrected total sums of squares). [c]Probability level of significance of the nonlinear model. d RF = resistant factor = I_{50} of resistant /I_{50} of susceptible cultivar.

These resistance factors found showed cross-resistance to imidazolinones, especially with regards to imazamox and imazethabenz-methyl. This behavior suggests as resistance mechanism an alteration of the ALS enzyme produced by a mutation that causes the loss of affinity for the binding site (Hans et al., 2012) in the R cultivar. This mechanism was discussed in different varieties of wheat and *Tritordeum*, where the most important mutations were Ala122 and Ser627 (Li et al., 2007; Rodríguez-Suárez et al., 2009). Both mutations can occur simultaneously, thus conferring higher levels of resistance (Newhouse et al., 1992; Pozniak and Hucl 2004a), as is the case of the R cultivar.

2.3. Imazamox Metabolism

Two imazamox metabolites, hydroxy and glucoside derivatives, were accurately identified in extracts from R cultivars by LC–TOF/MS, as shown Table 3.

Table 3. Total concentration of imazamox and its metabolites in extracts from R and S cultivars of wheat at different times and application doses, (metabolites expressed as imazamox µg/g fresh weight, average values obtained by LC–DAD and LC–TOF/MS ± standard deviation).

Time	Cultivar	Dose (g a.i. ha^{-1})	Imazamox	Hydroxy metabolite	Glucose conjugated metabolite
0 h	R	50	–	–	–
		100	–	–	–
		200	–	–	–
	S	50	–	–	–
		100	–	–	–
		200	–	–	–
3 h	R	50	–	–	–
		100	0.37 ± 0.06 C	–	–
		200	0.34 ± 0.03 C	–	–
	S	50	0.23 ± 0.06 DE	–	–
		100	0.42 ± 0.02 B	–	–
		200	0.56 ± 0.08 A	–	–
6 h	R	50	0.79 ± 0.08 DE	–	–
		100	0.70 ± 0.06 E	–	–
		200	0.72 ± 0.08 DE	–	–
	S	50	0.60 ± 0.02 EF	–	–
		100	0.99 ± 0.05 D	–	–
		200	2.29 ± 0.05 A	–	–
24 h	R	50	1.25 ± 0.04 F	–	–
		100	2.79 ± 0.06 E	0.35 ± 0.02 F	–
		200	5.11 ± 0.12 CD	0.98 ± 0.04 F	0.30 ± 0.02 F
	S	50	2.83 ± 0.06 E	–	–
		100	6.02 ± 0.07 C	–	–
		200	9.89 ± 0.13 A	–	–
8 d	R	50	0.82 ± 0.07 F	5.31 ± 0.09 F	–
		100	1.17 ± 0.05 F	21.61 ± 0.16 E	10.15 ± 0.03F
		200	3.68 ± 0.05 F	46.72 ± 0.23 C	19.34 ± 0.09 E
	S	50	15.74 ± 0.03EF	–	–
		100	51.23 ± 0.11 B	–	–
		200	68.14 ± 0.09 A	0.29 ± 0.04 F	–

[a] Means within a file (the same hours) followed by the same letter were not significantly different at the 5% level as per Tukey's test. Mean values ± standard errors of the means.

Figure 1 shows the extracted ion chromatograms (EICs) overlapped by monitoring the precursor ions at *m/z* 454.1820, 292.1292 and 306.1448 corresponding to $[M+H]^+$ adducts with theoretical formula $C_{20}H_{27}N_3O_9$, $C_{14}H_{17}N_3O_4$ and $C_{15}H_{19}N_3O_4$, respectively.

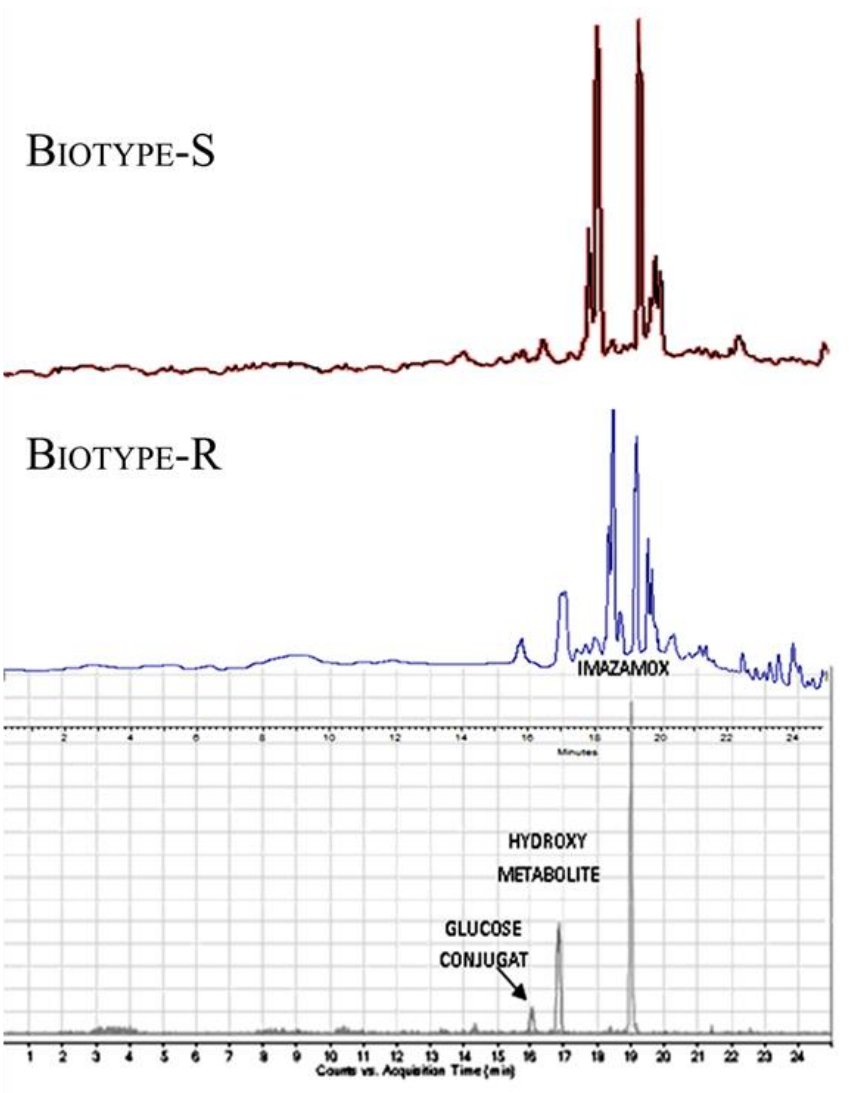
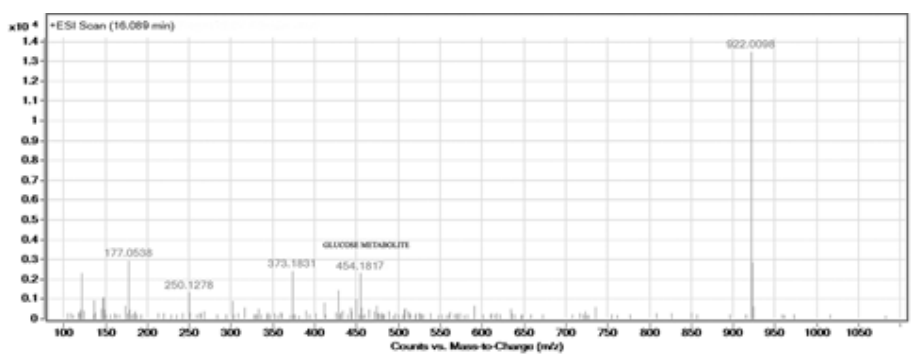
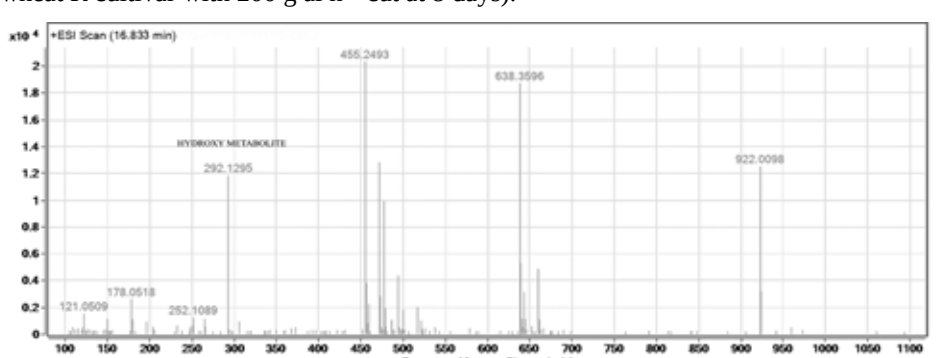


Fig. 1. Chromatograms obtained by LC–DAD analysis of extracts from S and R cultivars of *Triticum aestivum* var. Pandora plants 8 days after treatment with 200 g ai ha^{-1} imazamox. Extracted ion chromatograms (EICs) for $[M+H]^+$ adducts of imazamox and metabolites obtained by LC─TOF/MS analysis of extract from R cultivar plants after herbicide treatment.

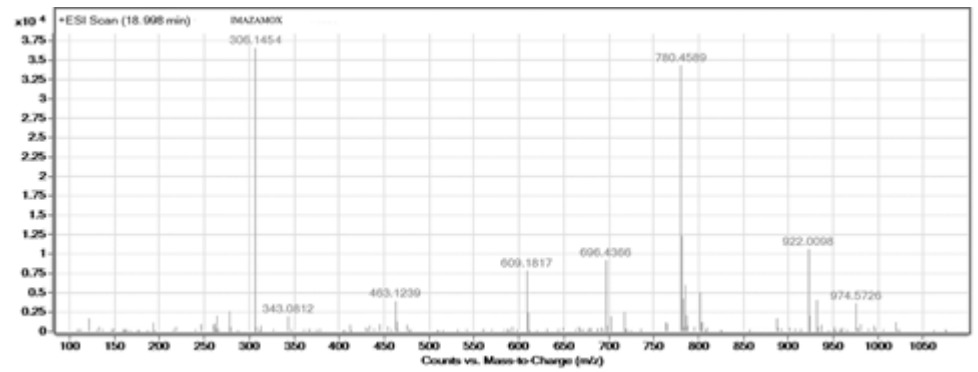
The first peak was identified as 3-pyridinecarboxylic acid, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-[(β-D-glucopyranosyloxy)methyl]- (glucose conjugated metabolite) associated to the ion [M+H]+ with *m/z* 454.1817 (Supplementary Figure 2), the second eluted compound was identified as 3-pyridinecarboxylic acid, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-(hydroxymethyl)- (hydroxylated metabolite) associated to the ion [M+H]+ with *m/z* 292.1295 (Supplementary Figure 3) and the third compound was the 3-pyridinecarboxylic acid, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-(methoxymethyl)- (imazamox) associated to the ion [M+H]+ with *m/z* 306.1454 (Supplementary Figure 4), the structures of which appears in Figure 2.



S-Figure 2. Mass spectrum in full scan for the chromatographic peak of the imazamox glucoside metabolite obtained by LC–TOF/MS analysis of extract from a treated sample (wheat R cultivar with 200 g ai h^{-1} cut at 8 days).



S-Figure 3. Mass spectrum in full scan for the chromatographic peak of the imazamox hydroxy metabolite obtained by LC–TOF/MS analysis of extract from a treated sample (wheat R cultivar with 200 g ai h^{-1} cut at 8 days).



S-Figure 4. Mass spectrum in full scan for the chromatographic peak of imazamox obtained by LC–TOF/MS analysis of extract from a treated sample (wheat R cultivar with 200 g ai h^{-1} cut at 8 days).

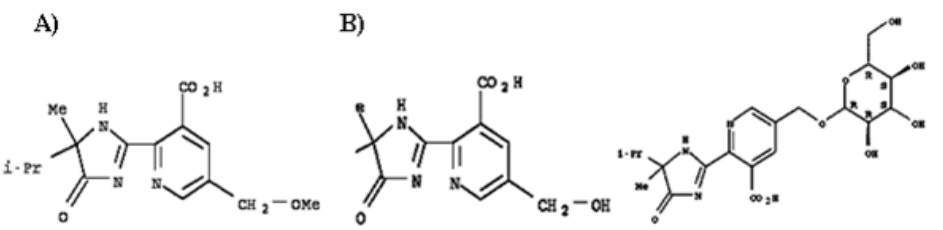
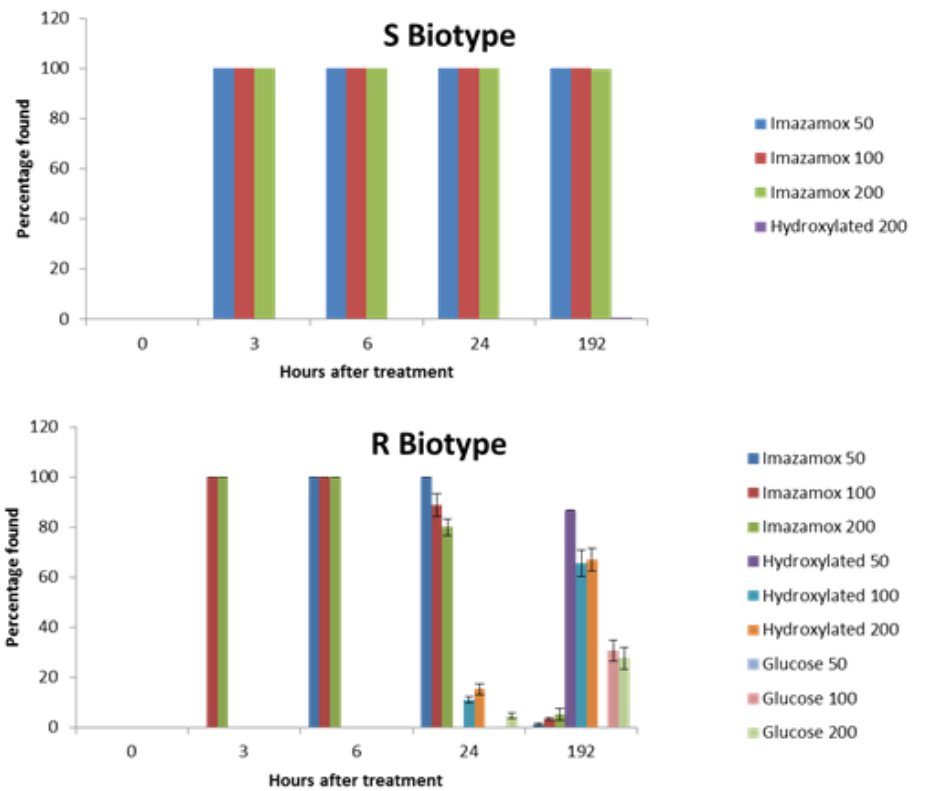


Fig. 2. Chemical structures of imazamox (A) and its two metabolites: hydroxy (B) and glucoside metabolites (C).

Once the compounds and their retention times were known (being for the glucose conjugated metabolite 16.024±0.28 min, for the hydroxylated metabolite 16.830±0.12 min and for imazamox 19.06±0.10 min) the results obtained by LC–DAD showed a clear difference between cultivars with respect to the exposure time and different doses used (Table 3). The first difference was in the amount of imazamox contained in both cultivars. At 24 h after treatment at 100 g ai of herbicide the R cultivar contained 2.16 times less amount of imazamox than the S cultivar; therefore, lower absorption would be a mechanism of resistance in the R cultivar. Comparison of the amount of metabolites in both cultivars showed that the R cultivar contained higher amount of metabolites (in time and dose) than the S cultivar. The latter contained an insignificant amount of a single metabolite at 8 days after treatment with a high dose of 200 g ai ha^{-1}. Considering that all metabolites come from imazamox and taking the sum of all them as 100% (imazamox+hydroxylated matabolite+glucose conjugated metabolite=100%) the

percentage of metabolized imazamox can be calculated (Supplementary Figure 5). About 94.7 % of imazamox (66.04 µg/g of fresh weight) was metabolized in the R cultivar at 8 days after treatment with 200 g ai of herbicide h^{-1}, while in the S cultivar only a 0.42% was metabolized (0.29 µg/g of fresh weight).



S-Figure 5. Degradation of the imazamox expressed as the percentage of each compound (imazamox, hydroxylated metabolite and glucose conjugated metabolite) in both cultivars at different times and doses.

These results suggest that the R cultivar possesses a mechanism of resistance complementary to ALS mutation and imazamox absorption, based on the metabolism of imazamox and, considering the degradation percentage, this mechanism is very important as it provides a higher level of resistance to this herbicide.

Concerning the behavior of the R cultivar *versus* doses, it was found that imazamox metabolism occurs mostly at medium and high doses of the herbicide

(100–200 g ai ha^{-1}). After 24 h since application with 50 g ai ha^{-1} dose, imazamox was not metabolized in the R cultivar, detecting metabolism with doses equal or above 100 g ai ha^{-1} of herbicide. After 8 days, 86.6% of imazamox in the R cultivar was metabolized for the dose 50 g ai ha^{-1} *versus* percentages of 96.4 and 94.7 % metabolized for doses 100 and 200 g ai ha^{-1}, respectively. These results suggest that metabolism occurs when the enzyme is saturated with the herbicide, which reduces the amount of intracellular herbicide that can reach the ALS enzyme, thus increasing the tolerance to the herbicide as compared to other populations with a single resistance mechanism (Rojano et al., 2011, Yu et al., 2007).

In all cases the hydroxylated metabolite appeared first and in a higher proportion than the glucose conjugated metabolite. This indicates that the former is a product of a transition step to the final metabolite: the glucose conjugated compound. This would explain why this metabolite appeared also in the S cultivar, even when the plant was fully affected and without survival probability. Then, the hydroxy group in this compound reacts with the anomeric carbon of the glucose group by condensation to form the glucose conjugated metabolite. Therefore, we can conclude that the R cultivar of *Triticum aestivum* var. Pandora presents a key resistance mechanism and may be considered as a crop for the control of weeds susceptible to imazamox.

3. Experimental

3.1. Plants and Growing Conditions

Two cultivars of *Triticum aestivum* var. Pandora with different degrees of tolerance/resistance to imazamox were used for comparative purposes. Seeds of the two cultivars of wheat were obtained from plants cultivated in Chile (INIA-Carillanca) (Mellado et al., 2007). The seeds were germinated in pots (three plants per pot) containing peat and sandy loam potting mixture (1:2, v/v) in a growth chamber at 28/18 °C (day/night) with a 16 h photoperiod under 850 μmol m^{-2} s^{-1} photosynthetic photon-flux density and 80% relative humidity.

3.2. Dose–Response Assays

Treatment with imazamox (PULSAR 40, BASF) was applied to the fifth or sixth of true leaves in both plants by using a laboratory track sprayer furnished with a Tee Jet 80.02.E.VS flat-fan nozzle and delivering a spray volume of 200 l ha^{-1} at 200 kPa. imazamox was applied at doses 0, 17.5, 35, 70, 140, 280, 420 and 560 g ai ha^{-1} to both cultivars. Above-ground fresh weight per pot was determined 21 days after spraying, and data were expressed as percentages of the values for the corresponding control (untreated plants). The ED_{50} (herbicide concentration needed to inhibit plant growth of each species by 50% with respect to the untreated control) were determined according to Menendez et al. (2006). The tolerance/susceptibility ratio was computed as ED_{50} (*cultivar R*)/ED_{50} (*cultivar S*) in terms of fresh weight. Experimental treatments were replicated 5 times and each test was conducted four times. The data were pooled and fitted to a nonlinear log−logistic regression model, using the equation $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where *Y* is the fresh above-ground weight expressed as a percentage of the untreated control; *c* and *d* are coefficients corresponding to the lower and upper asymptotes; *b* is the slope of the curve; *g* is the herbicide rate at the point of inflection halfway between the upper and lower asymptotes; and *x* (independent variable) is the herbicide rate. Regression analysis was conducted by using Sigma Plot 10.0 statistical software according to Cruz-Hipólito et al. (2009).

3.3. ALS Activity Assay

The ALS response to ALS-inhibiting herbicides was determined *in vitro* using crude extracts isolated from R and S cultivars leaves as described by Osuna and De Prado, (2003). In addition to imazamox other imidazolinones herbicides (Imazethapyr, Imazaquin, Imazethabenz-methyl and Imazapyr) were used to study the possible cross-resistance to this class of herbicides. These herbicides were applied following the same protocol described for imazamox. The I_{50}, which is the concentration of herbicide that causes a 50% reduction in ALS activity, was measured in plants treated with the different herbicides and was used for the R/S ratio calculated as $I_{50}(R)/I_{50}(S)$. Total protein content was measured using the Bradford method (Bradford, 1976). The maximum ALS

specific activity (nmol of acetoin mg^{-1} of protein h^{-1}) was measured in the absence of herbicide and the data were pooled and fitted to the log-logistic model (Seefeldt et al. 1995). The experiment was repeated three times using 3 replications per concentration of herbicide.

3.4. Imazamox Metabolism

3.4.1. Samples

Application of imazamox was performed as Dose-Response assays with a relative volume of 200 l ha^{-1} and with three doses of 1.25, 2.5 and 5 l ha^{-1} PULSAR 40 and a 0 dose (control). Plants —both treated with herbicide and control— were cut after 3, 6, 24 h and 8 days since application, and stored at −40 ºC until use. Before extraction, the frozen samples were washed with 60 ml of water to remove traces of imazamox and soil on the leaf surface. The sample was placed in a porcelain mortar and grinded to a fine powder using liquid nitrogen.

3.4.2. *Ultrasound-Assisted Extraction Method*

0.5 g of sample was mixed with 10 ml 90:10 (v/v) methanol–water and extracted using ultrasound that was applied at 70 W irradiation power for 10 min (duty cycle of 0.7 s/s), after which the solid residue was removed by centrifugation (15 min at 2900 g). Then, 6 ml of this extract was taken and evaporated to dryness under an air stream. The solid residue was reconstituted by 0.5 ml of the extractant (90:10 methanol–water) and filtered through a nylon filter syringe (45 µm pore-size and 13 mm i.d. from Millipore, Carrigtwohill, Ireland) before chromatographic analysis.

3.4.3. Clean-up and Preconcentration

6 ml of the supernatant (extract) was taken and evaporated to dryness under a nitrogen stream. The solid residue was reconstituted by 0.5 ml of the extractant (90:10 methanol–water) and filtered through a nylon filter syringe before chromatographic analysis.

3.4.4. Chromatographic Analysis

The same chromatographic method was used for identification of imazamox metabolites and for determination of imazamox and its metabolites in extracts from plants by LC–DAD. A HILIC column (20 cm × 4.6 cm, 3 µm particle size) was used for analysis in both cases. 50 µL of the reconstituted phase was injected into the LC with a 1% (v/v) acetic acid in water as mobile phase A, and pure methanol as mobile phase B. The elution program started with 5% mobile phase B and followed the linear gradient: step 1, 5 to 20% methanol in 10 min; step 2, 20 to 80% methanol in 10 min; step 3, 80% to 100% methanol in 5 min; step 4, 100 to 5% methanol in 10 min. The constant flow rate and column temperature were 1.0 ml min^{-1} and 40 ºC, respectively. Chromatographic grade and LC–MS grade solvents were used for LC–DAD and LC–MS analysis, respectively.

3.4.5. LC–TOF/MS Confirmatory Analysis of Metabolites of imazamox

The metabolites identification was conducted by LC–TOF/MS confirmatory analysis in accurate mode due to the complexity of plants extracts. The analyses were performed in an Agilent 1200 Series LC system interfaced to an Agilent 6540 UHD Accurate-Mass TOF─LC/MS detector (Palo Alto, USA), equipped with an Agilent Jet Stream Technology electrospray ion source operating in the positive ionization mode. The separation conditions were identical with those for the LC–DAD determination, except for the use of the respective LC–MS/MS grade solvents. The plant extracts were injected into the LC–TOF/MS system. The injected sample volume also was 50 µl. The operating conditions were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 l min^{-1}; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 4000 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V. Data acquisition (2.5 Hz) in both the centroid and profile modes was governed via the Agilent MassHunter Workstation software. The mass range and detection window were set at *m/z* 60–1100. Reference mass correction on each sample was performed with a continuous infusion of Agilent TOF biopolymer analysis mixture containing purine (*m/z* 121.0508) and hexamethoxyphosphazyne (*m/z* 322.0481). A resolution of 20 000 was used to examine the isotopic profiles for imazamox and its metabolites. The analytes were identified by accurate mass detection. The

experiment was repeated 7 times, using 3 replications per sample in LC–TOF/MS for comparison of the results.

3.4.6. LC–DAD Analysis of imazamox and its metabolites

After identification and confirmatory analysis by LC–TOF/MS, imazamox and metabolites were determined by LC–DAD analysis at the maximum absorption wavelength (240 nm). A 15 Gold HPLC System from Beckman Coulter (Fullerton, USA) equipped with a 26 System Gold Diode Array detector (DAD, wavelength range 190–600 nm) was used in this case. Chromatographic peaks in LC–DAD were assigned according to retention times using as reference the imazamox peak detected by spiking extracts with the commercial standard. Quantification of imazamox metabolites was based on the calibration model prepared for imazamox and expressing the results as μg of the analyte equivalent to imazamox g^{-1} of plant.

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Capítulo 8

Liquid chromatography–diode array detection to study glufosinate metabolism in Triticum aestivum T-590 and importance of genetic modification on its resistance

Liquid chromatography–diode array detection to study glufosinate metabolism in Triticum aestivum T-590 and importance of genetic modification on its resistance

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Enviado a:

Phytochemistry

Liquid chromatography–diode array detection to study glufosinate metabolism in *Triticum aestivum* T-590 and importance of genetic modification on its resistance

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Abstract

The resistance to glufosinate of two lines —genetically modified (GM) and unmodified (T-590 and T-549, respectively)— of *Triticum aestivum* population has been studied. In the GM line of wheat the *pat* gene was introduced to increase the resistance to glufosinate. Experiments in a controlled growth chamber showed that line T-590 presented a high resistance to glufosinate with an ED_{50} value of 478.59 g active ingredient per hectare (g ai ha^{-1}) *versus* 32.65 g ai ha^{-1} for line T-549. The activity of glutamine synthetase (GS) in leaf extracts from both lines was investigated. The I_{50} for line T-590 was 694.100 μM *versus* 55.46 μM of glufosinate for line T-549, with a resistance factor of 12.51. Metabolism studies showed a higher and faster penetration of glufosinate in line T-549 than in line T-590. LC–TOF/MS analysis of glufosinate metabolism at 48 h after herbicide treatment (300 g ai ha^{-1}) revealed an 83.4% conversion of the herbicide (66.5% in N-acetyl-glufosinate metabolite), while in line T-549 conversion of the herbicide was about 40% (0% in N-acetyl-glufosinate). These results suggest that metabolism of glufosinate by the *pat* gene is a key mechanism of resistance in line T-590 that explains such high levels of herbicide tolerated by the plant, together with other mechanisms due to unmodified pathway, absorption and loss of glufosinate affinity for its target site.

Keywords: *Triticum aestivum*, GMO crops, glufosinate, resistance, metabolism, degradation, LC–DAD.

1. Introduction

A new amino acid was isolated in 1972 in a German laboratory from one species of the soil bacterium: *Streptomyces viridochromogenes*. The name given to this amino acid [butanoic acid, L-2-amino-4-(hydroxymethylphosphinyl)-] by Bayer was phosphinothricin (Bayer et al., 1972; Hoerlein, 1994; OECD, 1999). Later, this substance showed herbicidal activity and received the name of glufosinate ammonium, which is the active ingredient in the commercial herbicide all over the world. Glufosinate inhibits the activity of the enzyme glutamine synthetase (GS) being competitively fixed in the substrate (glutamate) binding site (Bayer et al., 1972; Lea et al., 1984; Hoagland, 1999). This herbicide inhibits the synthesis of L-glutamine decreasing the levels of aspartic acid, asparagine and other amino acids precursors in the chemical synthesis of nucleic acids and proteins. The synthesis of L-glutamine also functions as a mechanism for incorporating ammonia (NH_3) in plants (Hoerlein, 1994). Treatment with glufosinate causes accumulation of ammonia (Tachibana et al., 1986b) and decrease of photosynthesis (Sauer et al., 1987). Because of its nonselective behavior, glufosinate is not used in all crops, but in postemergence treatments.

Thanks to advances in genetic engineering, recombinant DNA technology has been used to obtain and commercialize new varieties of genetically modified crops with preset properties which offer significant advantages to farmers who grow them, facilitating, among other things, better control of weeds.

The introduction of a gene to produce herbicide resistance in plants can follow two approaches. The first consists of inserting into the plant the gene of an enzyme which is not inhibited by the herbicide, in which glyphosate resistance is based; in the second, a gene of an enzyme that modified the herbicide in which is based the glufosinate resistance.

At the end of the 80's of the past century, the glufosinate resistance gene was identified and designed as pat, because of the function of the enzyme (Strauch et al., 1988; Thompson et al., 1987; Wohlleben et al., 1998). Its origin was based on identification of glufosinate in *Streptomyces* which suggested that these bacteria had a biochemical mechanism to maintain endogenous GS activity.

This enzyme —Pat (Phosphinotricine-N-acetyltransferase)— consists of 183 amino acids (Wehrmann et al., 1996; Wohlleben et al., 1998), acetylated to phosphinothricin (glufosinate) in the N-terminus. The generated product, butanoic acid, 2-(acetylamino)-4-(hydroxymethylphosphinyl)-, known as N-acetyl-glufosinate, does not present herbicidal activity by modification of the herbicide to this harmless compound (Thompson et al., 1987; Wehrmann et al., 1996; Wohlleben et al., 1998; Wohlleben et al., 1988; Broer et al., 1989; Dröge et al., 1992). The movement of transgenes to wild relatives takes place by pollen, and the production of viable hybrids depends on the physical proximity and synchrony of flowering in GM plants (genetically modified) with sexually compatible species. There is not evidence that Pat protein expression in a variety of plant species has resulted as a consequence of altered genes flowing naturally. However, the introgression of glufosinate tolerance in sexually compatible weed populations is possible and has the potential to cause control problems (Mallory-Smith and Zapiola, 2008; Warwick et al., 2007).

Glufosinate metabolism has been extensively studied both in soil (Tebbe and Reber, 1988, 1991; Smith, 1988, 1989; Behrendt et al., 1990; Gallina and Stephenson, 1992) and plants (Haas, 1986; Droge et al., 1992; Dröge et al., 1994) using a large number of analytical techniques for separation and identification of such metabolites. These studies showed that glufosinate in soil is rapidly transformed into butanoic acid, 4-(hydroxymethylphosphinyl)-2-oxo- (PPO), which becomes propanoic acid, 3-(hydroxymethylphosphinyl)- (MPP) by decarboxylation; then, PPO suffers a reduction with lost of oxygen in position 2 and formation of butanoic acid, 4-(hydroxymethylphosphinyl)- (MPB) (Behrendt et al., 1990).

Glufosinate metabolism has also been studied in unmodified plants, which presented natural resistance, and in genetically modified plants. These studies showed that the metabolites described in soil also appear in unmodified plants (Dröge et al., 1992; Dröge et al., 1994) in addition to the known metabolite butanoic acid, 2-hydroxy-4-(hydroxymethylphosphinyl)- (MHB) (Dröge et al., 1994). The studies were performed on crops as soybean (*Glycine max*), wheat (*Triticum aestivum*), and maize (*Zea mays*) (Komossa and Sandermann, 1992)

that presented even some tolerance to glufosinate. In addition to the above metabolites, the compound resulting from acetylation of glufosinate by the Pat enzyme (N-acetylphosphinothricine), which produces a rapid decrease in the content of glufosinate in the plant, was found in GM plants. This and the other metabolites are transported at the top of the plant, as demonstrated by Dröge et al. (1994) and Beriault et al. (1999).

The economic importance of wheat at the global level results from its use for a large number of aliments for humans and animals. This importance relies on the viscoelastic properties of wheat flour dough, which allow wheat to be used for making bread and many other food products such as cake, biscuits, pasta and noodles. The transgenic line T-590 studied in this work showed an improved breadmaking quality, as a consequence of the expression of the 1Dy10 high molecular weight glutenin subunit gene (Leon et al., 2009). Glufosinate resistance was incorporated in this line along with the 1D10 gene. Glufosinate is one important tool for weeds control in this crop, but proper function requires genetically modification in this crop.

The aim of this research was to study the effectiveness of introduction of the pat gene in wheat as compared to the unmodified geneotype, and to show the relative importance of the involved patways using two lines of the same genotype of *Triticum aestivum* wheat (an unmodified line and other genetically modified *pat* gene). The unavailability of some of the metabolites led to plan identification of them by liquid chromatography–time-of-flight mass spectrometry (LC–TOF/MS) for subsequent determination of the precursor and metabolites by liquid chromatography–absorptiometry detection by a diode array detector (LC–DAD).

2. **Results and Discussion**

2.1. Dose−Response Assays

The shoot biomass production in both line T-549 and line T-590 wheat plants was reduced by application of glufosinate (Fig. 1).

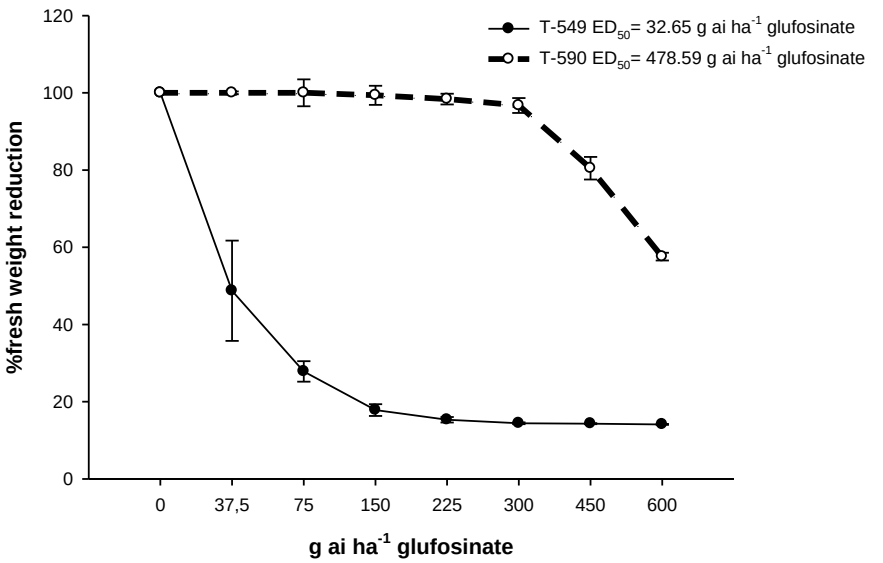

Fig. 1. Shoot biomass in lines T-590 and T-549 of *Triticum aestivum* plants treated with glufosinate at different doses.

The ED_{50} (herbicide concentration required for a 50% reduction of plants fresh weight) for line T-590 was 478.59 grams of active ingredient per hectare (g ai ha^{-1}) *versus* 32.65 g ai ha^{-1} for line T-549 (Table 1). These results, obtained from the whole plant dose–response bioassay, show that the line T-590 was 14.66 times more resistant than line T-549; therefore, the former is highly resistant to glufosinate.

Table 1. Parameters of the Log–Logistic equation used to calculate the glufosinate dose required for 50% reduction of fresh weight (ED_{50}) in both lines of *Triticum aestivum.*

	C	***d***	***b***	**ED_{50}[a]**	***P*[b]**	**RF[c]**
Line T-549	1.42 ± 0.61	98.35 ± 3.87	2.47 ± 0.33	32.65 ± 3.72	<0.001	
Line T-590	1.68± 0.29	100.03 ± 5.32	2.94 ± 0.67	478.59 ± 6.04	<0.001	14.66

Equation $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where Y is the percent plant injury; x (independent variable) the herbicide dose; c and d are the lower and upper asymptotes, respectively; b is the slope of the curve; and ED_{50} is the effective dose required for 50% plant injury. Data were pooled and fitted to a nonlinear regression model. All are the means of four replicates

[a]Expressed in g ai ha^{-1}

[b]Probability level of significance of the nonlinear model

[c]RF, resistance factor = ED_{50} of line T-590/ED_{50} of line T-549.

2.2. Glutamine Synthetase Activity Assays

The specific *in vitro* activity of GS obtained from shoot tissue of lines T-549 and T-590 was similar (364.68 ± 29.21 and 378.14 ± 15.07 nmol of glutamine mg^{-1} of protein h^{-1}, respectively). Line T-549 presented an I_{50}=55.46 µM *versus* T-590 with an I_{50}=694.10 µM. The resistance factor for *in vitro* glufosinate was 12.52 (Table 2).

Table 2. Parameters of the Equation[a] used to calculate the herbicide concentration required for 50% reduction of the GS activiy (I_{50}) of line T-590 and line T-549 of *Triticum aestivum.*

	A	**c**	**D**	**b**	**I50 (µM)**	**Pseudo R**	**P**	**RF**
Glufosinate	**S**	1.170	100.067	0.255	55.460	0.998	<0.0001	
	R	4.837	100.191	1.274	694.100	0.997	<0.0001	12.515

[a]Y = c + {(d - c)/[1 + (x/g)b]}, where Y is the percentage of plant injury, x (independent variable) is the herbicide concentration, c and d are the lower and upper asymptotes, b is

the slope of the line, and I_{50} is the effective dose required for 50% reduction in GS activity. Data were pooled and fitted to a nonlinear regression model.

b Approximate coefficient of determination of nonlinear models with a defined intercept calculated as pseudo r^2 = 1 - (sums of squares of the regression/corrected total sums of squares). [c]Probability level of significance of the nonlinear model. RF = resistant factor = I_{50} of resistant /I_{50} of susceptible line.

2.3. Glufosinate Metabolism

Extracts from T-549 and T-590 wheat plants were injected into both the LC–TOF/MS and LC–DAD arrangements for separation–identification and separation–quantification, respectively. As shows Figure 2, the metabolites were correctly identified in line T-590 as glufosinate, PPO, MHB, MPP, MPB and N-acetyl-glufosinate. The precursor ions at *m/z* 402.1112, 420.1218, 151.0166, 179.0115, 181.0271 and 165.0322, corresponding to $[M–H]^+$ adducts with theoretical formula $C_{20}H_{22}NO_6P$, $C_{22}H_{24}NO_7P$, $C_4H_9O_4P$, $C_5H_9O_5P$, $C_5H_{11}O_5P$ and $C_5H_{11}O_4P$, respectively.

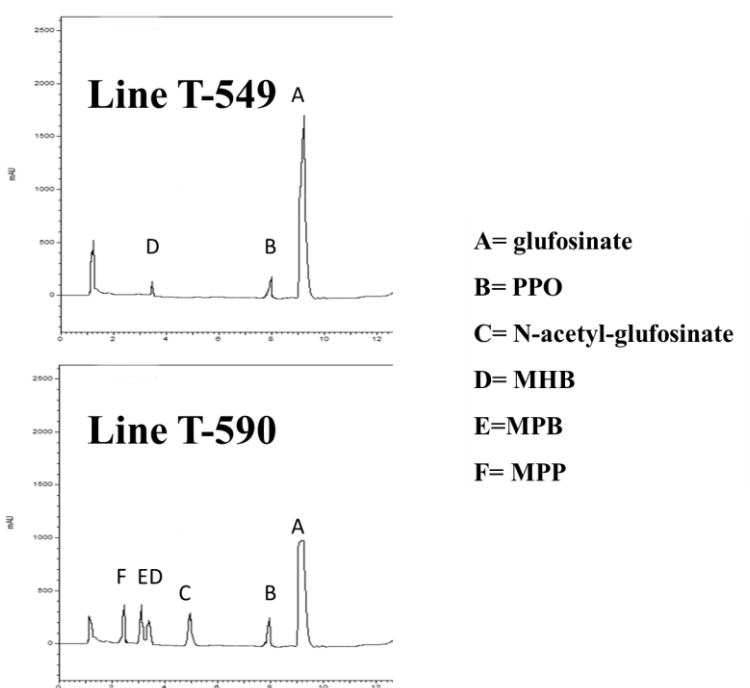

Fig. 2. Chromatogram of line T-590 of *Triticum aestivum* plants as compared with line T-549 plants 48 h after treatment with 300 g ai ha^{-1} glufosinate.

These peaks were identified as 2-amino-4-(hydroxymethylphosphinyl)-butanoic acid (glufosinate), 2-(acetylamino)-4-(hydroxymethylphosphinyl)-butanoic acid (N-acetyl-glufosinate), 3-(hydroxymethylphosphinyl)-propanoic acid (MPP), 4-(hydroxymethylphosphinyl)-2-oxo-butanoic acid (PPO), 2-hydroxy-4-(hydroxymethylphosphinyl)-butanoic acid (MHB) and 4-(hydroxymethylphosphinyl)-butanoic acid (MPB), and their structures appear in Figure 3.

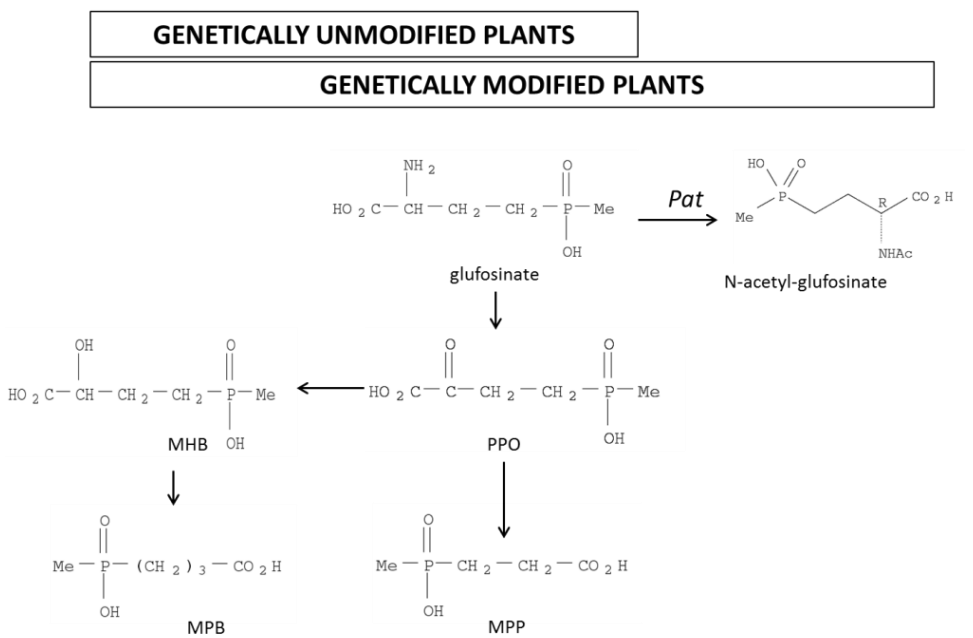

Fig. 3. Structures of glufosinate and its metabolites according to the possible mechanism described by Wolfgang Drögue et al. (1994)

The retention times of the target compounds were 9.046, 5.299, 2.624, 8.680, 3.661 and 3.137 min for glufosinate, N-acetyl-glufosinate, MPP, PPO, MHB and MPP, respectively. The results obtained by LC–DAD show a clear difference between cultivars with respect to the exposure time and different doses used (Table 3). Regarding metabolism, line T-590 undergone metabolism (at 6 h) by the two pathways described by Dröger et al. (1994) in contrast to line T-549, which presents only the natural pathway described in soils (at 24 h). Metabolism appeared at medium and high doses in both lines (300 to 600 g ai ha^{-1}). Table 3 shows as at short times (3 to 6 h) and a dose of 50 g ai ha^{-1} metabolism does not exist.

Table 3. Total glufosinate and its metabolites (derivatized with FMOCl) in samples of plants T-590 and T-549 lines of wheat at different times and at different doses, (metabolites expressed as glufosinate µg g-1 fresh weight, average values obtained by LC–DAD and LC–TOF/MS ± standard deviation).

Time	Line	Dose (g ai ha^{-1})	d-Glufosinate	d-N-Acetyl-glufosinate	MPP	PPO	MPB	MHB
0 h	T-549	150	–	–	–	–	–	–
		300	–	–	–	–	–	–
		600	–	–	–	–	–	–
	T-590	150	–	–	–	–	–	–
		300	–	–	–	–	–	–
		600	–	–	–	–	–	–
3 h	T-549	150	0.95 ± 0.10 C	–	–	–	–	–
		300	1.12 ± 0.07 B	–	–	–	–	–
		600	1.48 ± 0.03 A	–	–	–	–	–
	T-590	150	–	–	–	–	–	–
		300	0.69 ± 0.03 DE	–	–	–	–	–
		600	0.74 ± 0.06 D	–	–	–	–	–
6 h	T-549	150	2.62 ± 0.03 A	–	–	–	–	–
		300	2.73 ± 0.06 A	–	–	–	–	–
		600	2.54 ± 0.04 AB	–	–	–	–	–
	T-590	150	0.84 ± 0.08 E	–	–	–	–	–
		300	1.23 ± 0.04 DE	–	–	–	–	–
		600	1.48 ± 0.03 D	–	0.39 ± 0.06 F	0.41 ± 0.03 F	–	–
24 h	T-549	150	4.14± 0.05 C	–	–	1.04 ± 0.02 F	–	–
		300	5.09 ± 0.07 B	–	–	1.52 ± 0.05 EF	–	–
		600	6.13 ± 0.09 A	–	–	1.69 ± 0.03 EF	–	0.43 ± 0.02 G
	T-590	150	1.84 ± 0.07 EF	0.19± 0.03 G	0.38 ± 0.02 G	0.63 ± 0.01 FG	–	–
		300	1.76 ± 0.02 EF	0.30 ± 0.02 G	0.42 ± 0.05 G	0.78 ± 0.03 FG	–	–
		600	1.98 ± 0.05 E	0.56 ± 0.03 G	0.97 ± 0.08 F	0.71± 0.03 FG	–	0.32 ± 0.01 G
48 h	T-549	150	7.68 ± 0.12 D	–	–	1.30 ± 0.06 G	–	–
		300	9.47± 0.04 CD	–	–	2.45 ± 0.02 F	–	3.86 ± 0.07 EF
		600	14.73 ± 0.03 AB	–	0.92 ± 0.04 G	3.04 ± 0.08 F	–	1.52 ± 0.06 FG
	T-590	150	1.62 ± 0.05 FG	2.03 ± 0.05 FG	0.66 ± 0.05 G	0.79 ± 0.06 G	–	–
		300	1.86 ± 0.03 FG	7.48 ± 0.08 D	0.79 ± 0.07 G	0.84 ± 0.02 G	0.08 ± 0.02 G	0.19 ± 0.03 G
		600	1.92 ± 0.02 FG	15.99 ± 0.03 A	1.47 ± 0.03 G	0.99 ± 0.03 G	0.29 ± 0.04 G	0.24 ± 0.03 G

[a] Means within a file (the same hours) followed by the same letter were not significantly different at the 5% level as per Tukey's test. Mean values ± standard errors of the means.

This permits to conclude that metabolism acts in the presence of a certain amount of intracellular glufosinate (in our case 300 and 600 g of ai ha-1) because high doses of glufosinate can change the characteristics of plants as the photosynthesis, the activity of other enzymes, and the amount of ammonium, as the most important changes (Ay et al., 2012). N-Acetyl-glufosinate represents between 40 and 76.5% of the total metabolites in line T-590 at 48 h; therefore, degradation by acetylation is considered the most important degradation pathway, because it degrades *ca.* 5 times more amount of glufosinate than the natural pathway. Therefore, we can conclude that the pat gene metabolism is a key mechanism of resistance in pat-modified plants.

3. Experimental

3.1. Plants and Growing Conditions

Transgenic line (T-590) and control line (T-549) are both derived from *Triticum aestivum* L. *cv Anza.* Transgenic line T590 was obtained as described (Leon et al., 2009). Line T-590 was transformed with bar gene, which confers tolerance to phosphinorthricin (PPT) under the control of maize ubiquitin promoter (Christensen and Quail, 1996). Seeds of two lines of wheat were obtained from the Institute for Sustainable Agriculture (CSIC), then germinated in pots (three plants per pot) containing peat and sandy loam potting mixture (1:2, v/v) in a growth chamber at 28/18 °C (day/night) with a 16 h photoperiod under 850 μmol m^{-2} s^{-1} photosynthetic photon-flux density and 80% relative humidity.

3.2. Dose–Response Assays

Treatments with glufosinate (Finale®, Bayer, 150 g ai L^{-1}) were applied to the fifth or sixth of true leaves in both plants by using a laboratory track sprayer furnished with a Tee Jet 80.02.E.VS flat-fan nozzle and delivering a spray volume of 300 L ha^{-1} at 200 kPa. Glufosinate was applied at doses of 0, 37.5, 75, 150, 225, 300, 450 and 600 g ai ha^{-1} to both lines. Above-ground fresh weight

per pot was determined 504 h (21 days) after spraying, and the data were expressed as percentage of the value for the corresponding control (untreated plants). The ED_{50} (herbicide rates needed to inhibit plant growth of each species by 50% with respect to the untreated control) were determined according to Menéndez et al. (2006). The tolerance/susceptibility ratio was computed as ED_{50} (line T-590)/ED_{50} (line T-549) in terms of fresh weight. Experimental treatments were replicated 5 times and each test was repeated three times. The data were pooled and fitted to a nonlinear log−logistic regression model, using the equation $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where Y is the fresh above-ground weight expressed as a percentage of the untreated control; c and d are coefficients corresponding to the lower and upper asymptotes; b is the slope of the curve; g is the herbicide rate at the point of inflection halfway between the upper and lower asymptotes: and x (independent variable) is the herbicide rate. Regression analysis was conducted by using Sigma Plot 10.0 statistical software, according to Cruz-Hipólito et al. (2009).

3.3. GS Activity Assay

The GS response to glufosinate was *in vitro* determined using crude extracts isolated from T-590 and T-549 lines leaves as described by Rhodes et al. (1975). The I_{50} is the concentration of herbicide that caused a 50% reduction in GS activity and it was used for the R/S ratio calculated as $I_{50}(R)/I_{50}(S)$. Total protein content was measured using the Bradford method (Bradford, 1976). The maximum GS specific activity (nmol of glutamine mg^{-1} of protein h^{-1}) was determined in the absence of herbicide. The data were pooled and fitted to the log-logistic model (Seefeldt et al., 1995). The experiment was repeated three times, using 4 replications per concentration of herbicide.

3.4. Glufosinate Metabolism

3.4.1. Chemicals and reagents

Preparation of the chromatographic mobile phases for LC–diode array detection (DAD) analyses required chromatographic grade acetone, formic acid, and acetonitrile that were from Panreac (Barcelona, Spain). LC–MS required

acetonitrile and formic acid in MS grade from Scharlab (Barcelona, Spain), and deionized water (18 MΩ·cm) provided by a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA). Sodium hydroxide (purity 99.9%) and FMOC-Cl (purity 99.0%) were used for derivatization, glufosinate standard and its MPP metabolite standard were from Sigma (St. Louis, USA). N-Acetyl-glufosinate was synthesized using a mixture of 5 mM glufosinate solution (pH 8) and an excess of acetic anhydride from Sigma–Aldrich (St. Louis, USA), under vigorous agitation (by a magnetic stirrer) at room temperature for 1 h. The stock standards solutions were prepared by dissolving 0.1 g of glufosinate, N-acetyl-glufosinate or MPP in 100 mL in 90:10 (v/v) water–acetonitrile. The commercial herbicide used for plant treatment was Finale® (15% w/v of glufosinate) from Bayer (Frankfurt, Germany).

3.4.2. Samples

Commercial glufosinate was applied to 90% of the plants when them have an averaged five–six leaves, and the remainder 10% was used as control. Application was performed by spraying at 200 kPa in a closed chamber calibrated at 0.5 m height above the target surface, with a relative volume of 300 L ha^{-1} and with one dose of 2 L ha^{-1} Finale® (300 g ai ha^{-1}). Plants —both treated with herbicide and controls— were cut at 0, 3, 6, 24 and 48 h after herbicide application, and stored at −40 ºC until use.

Before extraction, the frozen samples were washed with 60 mL of water to remove traces of herbicide and soil on the leaf surface. The sample was placed in a porcelain mortar and grinded to a fine powder using liquid nitrogen.

3.4.3. Ultrasound-assisted extraction method

One gram of sample powder was placed into the extraction vessel with 10 mL 90:10 (v/v) water–acetonitrile. The ultrasonic probe was immersed into the extraction mixture for sonication at 280 W irradiation power for 10 min with a duty cycle of 70% (0.7 s/s irradiation). After that, the extract was isolated by centrifugation (15 min at 2.900 × g).

3.4.4. Clean-up and preconcentration

The method used was proposed to remove polysaccharides and peptides. 5 mL of the supernatant (extract) was taken and 1:1 (v/v) mixed with acetone with subsequent centrifugation (4.000 × g). The liquid phase was evaporated to dryness under a nitrogen stream. The solid residue was reconstituted by 1 mL of water–acetonitrile (90:10, v/v) and filtered through a nylon filter syringe before chromatographic analysis.

3.4.5. Derivatization

The method for derivatization of the target herbicide and its acetylated metabolite was followed to obtain products which allow a better chromatographic separation between them and from other glufosinate metabolites. Briefly, 250 µL of 5 mM FMOC-Cl and 250 µL of water were added to 3 mL of the reconstituted phase and subjected to agitation by magnetic stirrer for 1 h at 30 ºC.

3.4.6. Chromatographic analysis

The gradient chromatographic method was developed according to the following steps: 50 µL of the reconstituted phase was injected into the chromatograph using a 0.1% (v/v) formic acid in water as mobile phase A, and pure acetonitrile as mobile phase B. The elution program started with 10% mobile phase B and followed the linear gradient: step 1, 10 to 45% acetonitrile in 32 min; step 2, 45 to 70% methanol in 3 min; step 3, 70% to 10% acetonitrile in 5 min. The constant flow rate and column temperature were 1.0 mL min^{-1} and 30 °C, respectively.

3.4.7. Identification of glufosinate metabolites by LC–TOF/MS

Glufosinate metabolites were identified by LC–TOF/MS in high resolution mode. The samples were analyzed both with positive and negative ionization modes. The operating conditions for the mass spectrometer were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L min^{-1}; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L min^{-1}; capillary voltage, 3000 and 4000 V for negative and positive ionization modes; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V.

Data acquisition (2.5 Hz; mass range 60 to 1100 *m/z*) in both the centroid and profile modes was governed via the Agilent MassHunter Workstation software. The instrument gave typical resolution 15000 FWHM (Full Width at Half Maximum) at *m/z* 112.9856 and 30000 FWHM at *m/z* 1033.9881. To assure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses with the use of signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3H tetrafluoropropoxy) phosphazine or HP-921] in positive ion mode. In negative ion mode, ions with *m/z* 119.0362 (proton abstracted purine) and *m/z* 1033.988109 adduct of HP-921 were used. The analytes were identified by accurate mass detection and formula elucidation based on isotopic distribution.

Once the samples were analyzed by LC−TOF/MS, the molecular features were extracted from the raw data files prior to formula generation. Ions with identical elution profiles and related *m/z* values (representing different adducts, ions generated after specific neutral losses or isotopic forms from the monoisotopic ions) were extracted as molecular features (MFs) in a matrix characterized by retention time (RT) and accurate mass and containing intensity in apex of chromatographic peaks as data. The isotope model corresponded to common organic molecules with peak spacing tolerance of *m/z* 0.0025 ± 6.0 µg L^{-1}. The MF extraction algorithm limited extraction to ions exceeding 5000 counts with charge state limited to a maximum of two. The allowed negative ions were deprotonated species and formate adducts, while allowed positive ions were protonated species and sodium adducts. Dehydratation, glucosidation and phosphate neutral losses were also allowed. Candidate formula generation was supported on C, H, O, N, S and P elements by setting a cut-off value of mass accuracty at 6 µg L^{-1}.

3.4.8. Determination of glufosinate and its metabolites by LC–UV

Glufosinate and its metabolites were determined by LC–UV analysis at the absorption wavelength 270 nm for underivatized compounds and 340 nm for derivatized compounds. Chromatographic peaks in LC–UV were assigned according to their retention times taking as reference the peaks of glufosinate and

N-acetyl-glufosinate derivatives (d-glufosinate and d-N-acetyl-glufosinate since now) and that of MPP located by spiking extracts with the commercial and synthesized standards. Quantification of non commercial glufosinate metabolites was based on the calibration model run for the available compound with structure more similar to them (MPP) and the results expressed as µg of the analyte equivalent to MPP g^{-1} of fresh plant.

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DISCUSIÓN DE LOS RESULTADOS

La normativa vigente en la Universidad de Córdoba para laelaboración de una Memoria de Tesis Doctoral en la modalidad en la que seincluyen los artículos (publicados o próximos a su publicación) como talesrequiere una discusión conjunta de los resultados, lo que puede ser más omenos factible dependiendo de la homogeneidad de la investigaciónrealizada. Teniendo en cuenta que la investigación recogida en esta Memoria abarca: (A) Desarrollo de plataformas analíticas para diferentes herbicidas y su validación; (B) la subsiguiente aplicación de cada plataforma al estudio del metabolismo del herbicida en cuestión y su comportamiento a través de las rutas metabólicas en las que está implicado, la discusión de los resultados requiere considerar cada plataforma analítica junto con la(s) aplicación(es) a que ha dado lugar como forma más adecuada de evaluar cada estudio. Por tanto, la estructura de la Memoria en la que se han considerado plataformas y aplicaciones separadamente con objeto de poner de manifiesto las aportaciones analíticas y las agronómicas, se rompe ahora para una discusión más clara, que se ha dividido en cuatro secciones que corresponden a las 4 plataformas analíticas, considerando en cada caso sus aplicaciones y los resultados de éstas.

Sección I. **Puesta a punto de un método para la determinación de glifosato y sus metabolitos basado en electroforesis capilar de polaridad invertida y detección por absorción indirecta. Aplicación del método al estudio del metabolismo del herbicida en diferentes plantas**

Las malas hierbas suponen un importante problema para la agricultura de muchos países. Este problema se agrava cuando este tipo de hierbas muestran resistencia a herbicidas que hasta el momento eran capaces de controlarlas. Éste es el caso del *Lolium multiflorum*, la *Digitaria insulares* y la *Conyza canadensis,* entre las especies que en los últimos años han venido presentando una más fuerte resistencia a glifosato, produciéndose su descontrol e infectando grandes áreas de cultivo. En otros casos se han buscado especies de leguminosas que fuesen tolerantes a glifosato y que pudieran ser fácilmente controladas, como *Mucuna pruriens* y *Clitoria ternantea,* para utilizarlas como cubiertas vegetales. Esta situación de descontrol y control de especies vegetales plantea la necesidad de

estudiar la causa de la resistencia que provoca la ineficacia del glifosato sobre estas especies.

En la actualidad existen varios mecanismos conocidos que podrían explicar la resistencia de una hierba dada al glifosato; sin embargo, la ausencia de métodos analíticos adecuados ha impedido el estudio fehaciente de los mecanismos a través de los cuales tiene lugar el metabolismo del herbicida. Previamente al estudio que se recoge en esta Memoria no se habían determinado en plantas y de forma simultánea el glifosato y todos sus metabolitos.

El planteamiento de la investigación fue utilizar una técnica simple de manejo, de bajo coste de mantenimiento —especialmente de bajo coste de reactivos con vistas a una aplicación masiva a diferentes tipos de hierbas en muy distintas condiciones de crecimiento— y con buenas selectividad y sensibilidad dadas las altas concentraciones de proteínas, aminoácidos y polisacáridos en los extractos de plantas en general y la baja concentración a la que pueden encontrarse los metabolitos al comienzo de la degradación del herbicida. Con estas premisas se seleccionó la electroforesis capilar como técnica rápida y de bajo consumo y la planta *Lolium spp*. como hierba en la que se había detectado la resistencia al herbicida. La modalidad de detección mediante medida indirecta de la absorbancia evitó la derivatización de los analitos, con el consiguiente ahorro de reactivos y tiempo, además de la dificultad o imposibilidad de encontrar un reactivo común para el precursor (glifosato) y sus metabolitos (ácido aminometilfosfónico, glioxilato, sarcosina y formaldehído). Una vez optimizadas las variables que influyen en el método, se consiguieron unos valores excelentes de sus características analíticas, tales como rango lineal de las curvas de calibrado entre 5 y 500 µg/mL para todos los metabolitos, con coeficientes de regresión entre 0.999 y 0.998, precisión expresada como desviación estándar relativa entre 81.4 y 108.1%, un tiempo de análisis de 20 min con muy buena selectividad, como se puso de manifiesto al aplicar el método a extractos de plantas no tratadas y tratadas (comportamiento previsible dado el modo de inyección utilizado, que impide la introducción en el capilar de las grandes cantidades de proteínas, aminoácidos y polisacáridos que se lixivian junto con los analitos). El método se completó mejorando la preparación de la muestra

(lixiviación de la planta en cuestión) mediante la asistencia de ultrasonidos y se aplicó, para su validación, a 5 biotipos de *Lolium spp*. tratados y no tratados con glifosato y a dos concentraciones, con recogida de las plantas a diferentes tiempos desde la aplicación del herbicida (desde el tiempo 0 a las 96 h). Los ensayos de recuperación completaron el desarrollo del método que se aplicó a estudios que han dado lugar a las siguientes publicaciones:

1. Limited uptake, translocation and enhanced metabolic degradation contribute to glyphosate tolerance in *Mucuna pruriens* var. *utilis* plants.
2. Glyphosate tolerance by *Clitoria ternantea* and *Neonotonia wightii* plants involves differential absorption and translocation of the herbicide.
3. Pool of Resistance Mechanisms to Glyphosate in *Digitaria insularis*.
4. Two non-target mechanisms are involved in glyphosate resistant horseweed (*Conyza canadensis* L. Cronq.) biotypes.
5. Glyphosate resistance in a *Digitaria insularis* biotype is due entirely to differential metabolism.

En todos ellos se ha estudiado el metabolismo del glifosato encontrándose un mayor o menor grado de metabolismo. Esta conversión ha influenciado la mayor o menor resistencia a este herbicida junto con otros mecanismos de resistencia anteriormente descritos, siendo en ocasiones la causante exclusiva de la resistencia al herbicida.

La relevancia de estos estudios ha tenido resonancia en los lectores de las revistas científicas en las que se han publicado, ya que algunos de ellos han estado en la lista de los 20 más citados en las revistas de agronomía durante 2 cuatrimestres consecutivos (de enero a agosto de 2012): En concreto, el estudio número 3 ha ocupado el puesto número 1 de esa lista, mientras que el estudio número 1 ha ocupado el número 8.

Sección II. **Optimización de un método espectrofotométrico para la detección y confirmación de glioxilato como un biomarcador de la resistencia de plantas a los herbicidas**

Tras el desarrollo y aplicación del método recogido en el Capítulo 2 para la determinación de glifosato y como parte del estudio metabolómico, se comprobó

que uno de los metabolitos de este herbicida (el glioxilato) mostraba variaciones según la planta presentara o no resistencia al herbicida. Este comportamiento llevó a la búsqueda de un método para la determinación individual de este metabolito en plantas. Se desarrolló un método colorimétrico de cribado basado en la formación de una fenilhidrazona, que se oxida a 1,5-difenilformazan por la acción del $K_3Fe(CN)_6$en HCl dando lugar a un producto de color rojo intenso. La optimización simultánea de la extracción asistida por ultrasonidos de glioxilato en plantas y su derivatización mediante un diseño multivariante ha permitido la determinación de este analito en plantas frescas sin interferencias de feofitina y de compuestos con grupos carbonilo.Los límites de detección y cuantificación del método (0.05 mg/mL y 0.17 mg/mL, respectivamente) y su excelente precisión (del 3.3% para la repetitibidad y del 5.6% para la reproducibilidad en el laboratorio entre días, expresadas ambas como desviación estándar relativa) hacen que sólo se requieran 50 mg de planta para la determinación de glioxilato, que se realiza en 32 min. El error relativo que presentó este método comparado con el método basado en CE mencionado en la Parte I sobre muestras de *Lolium spp*. tratadas con glifosato fue siempre inferior a 7%. La ausencia de interferencias propias de la matriz vegetal, e incluso de los herbicidas,elimina las etapas largas y tediosas de limpieza y, por tanto, reduce el tiempo total de desarrollo; lo que redunda en beneficio de la instrumentación y de la precisión del método, haciéndolo apropiado para su uso en análisis sistemático. Estas características lo convierten en un firme candidato para la proposición de un test rápido para el análisis en campo.

En la actualidad se llevan a cabo experimentos con este método en los que se estudia la respuesta del glioxilato como biomarcador de diversos factores bióticos y abióticos de estrés que pudieran afectar de alguna forma a los niveles de glioxilato que puedan presentar las plantas sometidas a esos factores. Estos estudios no se recogen en la Memoria puesto que aún no se han extraído conclusiones definitivas.

Sección III. **Puesta a punto de un método para la identificación mediante LC–TOF/MS y determinación mediante LC–UV de imazamox y sus metabolitos en plantas con extracción asistida por ultrasonidos. Aplicación del método al estudio del metabolismo del herbicida en diferentes plantas**

Desde su descubrimiento en 1998 la importancia del imazamox ha ido creciendo gracias a sus propiedades, entre las que destaca su baja toxicidad, característica de este herbicida y en general de todos los miembros pertenecientes a la familia de las imidazolinonas. La necesidad de controlar las malas hierbas resistentes a los herbicidas muy usados en trigo, maíz, arroz, etc. llevó a la búsqueda de nuevos cultivos con mutaciones que pudieran conferirle resistencia/tolerancia a las imidazolinonas. En el año 2000 estos cultivos comenzaron a comercializarse con el nombre de Clearfield® (BASF Corporation). Se les consideraba entonces como un control integrado de malas hierbas basado en el desarrollo de variedades tolerantes a las imidazolinonas utilizando mutaciones tradicionales de inducción y de cultivo. Un ejemplo de estos cultivos es la variedad de trigo Pandora resistente al imazamox, muy usada en Chile, pero de la que se conoce poco acerca del metabolismo del herbicida y de la importancia que puede tener este proceso en esta variedad de trigo.

La investigación recogida en esta Memoria sobre este herbicida se ha dirigido al desarrollo de un método para la determinación simultánea de imazamox y sus metabolitos en plantas. Teniendo en cuenta la ausencia de estándares comerciales de los metabolitos, se realizó su identificación así como la confirmación mediante LC–TOF/MS de ellos y de su precursor utilizando extractos de plantas tratadas con el herbicida, para proponer finalmente la cuantificación de los metabolitos identificados en relación con su precursor.

La preparación de la muestra se llevó a cabo por extracción asistida con ultrasonidos, para lo que se utilizóuna potencia de 70 W y un ciclo útil de 0.7 s/s durante 10 min, con posterior limpieza por filtración de los extractos y concentración antes de la separación cromatográfica y la detección por absorción a 240 nm. El tipo de extracción (lixiviación) se optimizó y comparó con otros modos de extracción como la maceración y la extracción asistida por microondas. Aunque con auxilio de esta última energía el tiempo de lixiviación resultó más

corto (5 min), la rápida degradación de los compuestos daba lugar a una etapa cuya robustez con respecto al máximo de lixiviación era muy pequeña. La lixiviación asistida por ultrasonidos requirió un tiempo ligeramente más largo (10 min), pero con una meseta más amplia de máxima eficiencia de la etapa (de 3 min), por lo que se adoptó esta vía de aceleración de la lixiviación como la más adecuada. El análisis cromatográfico se completó en 30 min usando una columnade interacción hidrofílica (HILIC). Las características analíticas del método para imazamox mostraron un amplio rango dinámico lineal de la curva de calibración entre 0.27 y 600 µg/mL, con un coeficiente de correlación de 0.998 y una precisión, expresada como desviación estándar relativa y a dos niveles de concentración (0.1 y 2 µg/mL), de 2.9 y 5.0% para la repetitibidad, y de 4.7 y 6.9% para la reproducibilidad dentro del laboratorio, respectivamente. Estas características hacen que el método permita una buena identificación y confirmación de la presencia de imazamox y sus metabolitos en los extractos de plantas tratadas. El método se validó mediante el análisis de muestrasde trigo (*Triticum aestivum*) de la variedad Pandora (biotipos resistente, R, y susceptible, S) tratados con imazamox siguiendo las prácticas convencionales agronómicas (200 g de ingrediente activo/Ha ó 200 g ia/Ha).

El método se utilizó para el estudio recogido en el Capítulo 7 (**Importance of Imazamox metabolism in Clearfield *Triticum aestivum*)**. El análisis mediante LC–TOF/MS de plantas sometidas a la acción del imazamox a los 8 días del tratamiento con el herbicida (200 g ia/Ha) reveló un metabolismo intenso, ya que la conversión del herbicida en su hidroximetabolito fue del 67%, y un 27.7% en el metabolito glucosilado para el cultivar R. Por el contrario, en elbiotipo S se detectaron trazas de un único metabolito (el hidroxilado). Los resultados obtenidos sugieren que el metabolismo del imazamox en el cultivar R es el mecanismo fundamental de la resistencia que explica los altos niveles de herbicida tolerados por la planta, junto con un segundo mecanismo debido a la pérdida de afinidad del imazamox por su sitio diana.

Sección IV. **Puesta a punto de un método para la identificación mediante LC–OF/MS y determinación mediante LC–UV de glufosinato y sus metabolitos en plantas previa extracción asistida por ultrasonidos y derivatización con FMOC-Cl. Aplicación del método al estudio del metabolismo del herbicida en diferentes plantas**

El glufosinato es un herbicida de origen natural que presenta una limitada utilización en cultivos, ya que es un herbicida no selectivo. Estas características han propiciado la búsqueda de cultivos resistentes a este herbicida para poder controlar las malas hierbas que presentan resistencia a los herbicidas que comúnmente se utilizaban en esos cultivos. A diferencia de los cultivos resistentes a imidazolinonas, los resistentes a este herbicida se consiguen mediante la introducción de un gen denominado *pat* que provoca la acetilación del glufosinato evitando que este llegue a la enzima diana (glutamina sintetasa). La ausencia de herramientas analíticas eficaces y fiables para la determinación del herbicida y de los productos de su metabolismo ha ocasionado que en la mayoría de los estudios publicados hasta la fecha se haya recurrido a la determinación de los analitos de interés basada en la emisión de un compuesto radiactivo y en los tiempos de retención. Considerando que las señales que se obtienen con estos métodos pueden no ser debidas a los metabolitos en estudio, estos métodos no constituyen herramientas apropiadas para una completa e inequívoca identificación.

Por los motivos anteriores, el objetivo de la investigación en este caso fue el desarrollo de una doble estrategia (cualitativa y cuantitativa) para la identificacióny el análisis de glufosinato y sus metabolitos en plantas. Teniendo en cuenta que no existen estándares de algunos metabolitos del glufosinato (sólo el glufosinato y el el 3-metilfosfínico-propanoico o MPP son comerciales, mientras que el N-acetil-glufosinato puede sintetizarse de forma relativamente fácil), se planificó la identificación de aquéllos para los que no existen patrones y el análisis confirmatorio de todos los analitos. Se utilizaron para ello extractos de plantas tratadas con glufosinato y como herramienta analítica un equipo LC–TOF/MS en el modo de alta resolución, lo que supuso la primera parte de la estrategia. La segunda parte fue el desarrollo de un método cuantitativo para la

determinación de glufosinato y sus metabolitos en plantas basado en una etapa de lixiviación de los analitos acelerada por ultrasonidos, la separación individual mediante LC y la detección fotométrica.

Con las anteriores premisas se sintetizó el metabolito acetilado (el N-acetil-glufosinato) y se adquirió el metabolito comercial (MPP), además del propio herbicida. Se optimizó la etapa de lixiviación, asistida también en este caso mediante ultrasonidos, así como una posterior etapa de limpieza–preconcentración basada en evaporación del lixiviante y filtración (se eliminó así la acetona utilizada para la limpiar el extracto de polisacáridos y proteínas, que provocan burbujas de aire en el LC, además de las sustancias insolubles en la fase reconstituyente —agua–acetonitrilo, 90:10), realizando el seguimiento del estudio mediante LC–UV. Se comprobó una mejor separación de los analitos (conocidos y potenciales) tras la derivatización de los dos compuestos con grupos amino (el glufosinato y su metabolito acetilado) con cloruro de fluorenilmetoxicarbonilo (FMOC-Cl) debido a que la disminución de la polaridad de los productos modifica sus tiempos de retención. El glufosinato y sus metabolitos para la que están disponibles los estándares (N-acetil-glufosinato y 3-metilfosfínico-propanoico o MPP) se cuantificaron; mientras que los restantes metabolitos (PPO, MPB y MHB) identificados mediante LC–TOF/MS se cuantificaron de forma relativa utilizando como referencia el estándar más similar a ellos (el MPP). La detección se llevó a cabo a 270 nm para los analitos no derivatizados y a 340 nm para los productos de la derivatización. El análisis cromatográfico se completó en 40 min utilizando una columna cromatografía basada en interacción hidrofílica (HILIC). Las características analíticas del método fueron un amplio rango dinámico lineal de las curvas de calibración entre 0.047 y 700 µg/mL, con un coeficiente de correlación de 0.999 para el glufosinato; entre 0.077 y 700 µg/mL con un coeficiente de correlación de 0.998 para el N-acetil-glufosinato; y entre 0.116 y 600 µg/mL con un coeficiente de correlación de 0.998 parael MPP. La precisión de la determinación del glufosinato —estudiadaados niveles de concentración, 0.1 y 5 µg/mL—fue de 2.7 y 6.0% para la repetibilidad, y de 4.7 y 7.2% para la reproducibilidad dentro del laboratorio, respectivamente. El método fue validado utilizando muestras de trigo

(*Triticum aestivum*) modificado genéticamente y no modificado tratadas con glufosinato en ambos casos. De esta forma se comprobó que la doble estrategia cualitativa/cuantitativa constituía una alternativa muy ventajosa a los métodos que utilizan radiactividad como herramienta clave para el análisis de glufosinato y sus metabolitos en plantas ya que la identificación y la confirmación realizadas mediante LC–TOF/MS proporcionan una mayor fiabilidad. Además, se pretendió que el método fuera lo suficientemente rápido para su validación mediante el análisis sistemático practicado en un número de muestras suficientemente grande.

El método se aplicó al estudio que se recoge en el Capítulo 8 (**Liquid chromatography–diode array detection to study glufosinate metabolism in *Triticum aestivum* T-590 and importance of genetic modification on its resistance)** sobre dos líneas de trigo, uno modificado genéticamente (T-590) y otro no modificado (T-549). El estudio puso de manifiesto que al cabo de 48 horas de la aplicación del herbicida su conversión fue del 83.4% (66.5% en el metabolitoN-acetil-glufosinato que proviene de la degradación originada por el gen *pat*, y del 16.8% en otros metabolitos pertenecientes a la vía de degradación natural), mientras que en la línea T-549 sóloel 40% del herbicida se convirtió en PPO, MPP y MHB (0% en N-acetil-glufosinato).Estos resultados sugieren que el metabolismo del glufosinato promovido por el gen *pat* es el mecanismo fundamental de la resistencia en la línea T-590, que explica los altos niveles de herbicida tolerados por la planta, junto con otros mecanismos que corresponden a la vía natural de degradación metabólica común en plantas no modificadas.

De forma conjunta puede considerarse que la investigación realizada proporciona unas herramientas útiles para los estudios de metabolismo de herbicidas en plantas de diferentes características y naturaleza.

CONCLUSIONES

Una revisión en profundidad de la bibliografía existente sobre el metabolismo de herbicidas en plantas ha permitido evaluar críticamente el estado de los métodos utilizados actualmente (los tipos de detección, las etapas de selección de la muestra, muestreo, conservación y preparación que preceden al análisis) y los resultados conseguidos, mostrando sus deficiencias o carencias y proporcionando pautas para la investigación en esta área de la malherbología, a la que se ha contribuido con el diseño de plataformas analíticas simples y fiables, cuya validación y aplicación se ha realizado y se continua en la actualidad.

Las conclusiones sobre la investigación realizada son las siguientes:

(i) Se ha desarrollado una plataforma basada en electroforesis capilar con detección indirecta por absorción UV que constituye una valiosa herramienta analítica para la determinación de glifosato y sus metabolitos en maleza. Su interés deriva de la simplicidad de las etapas de preparación de la muestra, de la que está ausente la derivatización, y de la etapa de separación individual, que es simple, rápida y de bajo coste. Su aplicación a la determinación del herbicida y sus metabolitos en plantas tratadas con glifosato, en donde las altas concentraciones de proteínas, aminoácidos y polisacáridos generalmente dificultan los análisis, avalan su utilidad. Las características analíticas de esta plataforma, tales como repetibilidad, reproducibilidad y límites de cuantificación permiten la determinación a nivel de trazas de los analitos de interés.

La importancia de esta plataforma se ha puesto de manifiesto en las publicaciones sobre la aplicación de esta herramienta analítica, tales como la realizada en *Digitaria insularis* (Anexo 1) que ocupa el número 1 en la lista de los 20 trabajos más citados durante durante 2 cuatrimestres consecutivos (de enero a agosto de 2012), o la que ha tenido por objeto la *Mucuna pruriens* que forma parte del cuerpo de Tesis (Capítulo 6) y ocupa el número 8 en misma lista (http://wipimd.com/?wmdc243=22175446DND20724TJTk7kPccNz1345063129&cmpgn89116=DND20724TJTk7kPccNz).

(ii) Se ha puesto a punto una plataforma para cribado ("screening") —Capítulo 3— de respuesta sí/no rápida sobre la presencia de glioxilato que permite también su cuantificación utilizando instrumentación simple como es un espectrofotómetro. Además de su simplicidad y rapidez, la plataforma está dotada de una buena reproducibilidad y sensibilidad, por lo que se puede utilizar para el seguimiento de los cambios en concentración de glioxilato en plantas, relacionando las variaciones en el nivel de este metabolito con la aplicación de herbicidas. La ausencia de etapas de preparación de la muestra y el rápido desarrollo (32 min) permite su aplicación simultánea a un gran número de muestras en un tiempo corto.

La aplicación de la plataforma para obtener información del modo de acción de los herbicidas en plantas (tanto resistentes como susceptibles a los herbicidas), que constituirá la demostración de su utilidad para estudios agronómicos, es objeto de nuestra actual investigación.

(iii) Se ha diseñado una estrategia para la determinación mediante LC–UV de imazamox y sus metabolitos (compuestos no comerciales) —Capítulo 4. El método permite la cuantificación relativa de los metabolitos expresados como imazamox tras su identificación y del análisis confirmatorio mediante LC–TOF/MS de su presencia en plantas resistentes tratadas con el herbicida. La utilidad de esta plataforma se ha demostrado al aplicarla a estudios de evaluación del metabolismo de imazamox en plantas realizados en dos biotipos de *Triticum aestivum* (trigo). El Capítulo 7 recoge este estudio, en el que se pone de manifiesto la existencia e importancia del metabolismo del imazamox en la resistencia de la planta a este herbicida.

(iv) Se ha desarrollado una plataforma analítica para la determinación de glufosinato y sus metabolitos mediante LC–DAD tras una etapa de

derivatización que supone un avance en el estudio metabolómico de este herbicida ya que evita el uso de métodos basados en ^{14}C. Se consigue así la cuantificación relativa de los metabolitos del glufosinato que no se encuentran disponibles comercialmente, después de su identificación y la confirmación de su presencia en las plantas resistentes tratadas con el herbicida por LC–TOF/MS. Las características analíticas de esta plataforma permiten su uso en estudios de evaluación del metabolismo de glufosinato en plantas. Uno de esos estudios, recogido en el Capítulo 8, se ha dedicado a trigo modificado genéticamente y no modificado, en el que se pone de manifiesto que la resistencia a este herbicida depende de la ruta de degradación seguida.

(v) En todos los casos la etapa de preparación de la muestra (lixiviación) se ha auxiliado mediante ultrasonidos, lo que ha acelerado enormemente su desarrollado y ha aumentado de forma notable la eficiencia respecto al uso de la lixiviación convencional.

ANEXO I

Glyphosate tolerance by *Clitoria ternantea* and *Neonotonia wightii* plants involves differential absorption and translocation of the herbicide

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Glyphosate tolerance by *Clitoria ternatea* and *Neonotonia wightii* plants involves differential absorption and translocation of the herbicide

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Abstract

Glyphosate tolerance by *Clitoria ternatea, Neonotonia wightii* and *Amaranthus hybridus* was studied in whole plants from Mexico. Experiments in a controlled growth chamber showed both legumes to be highly tolerant of glyphosate, with and ED_{50} values of 600.18 g ae ha^{-1} for *C. ternatea* and 362.94 g ae ha^{-1} for *N. wightii*. On the other hand, *A. hybridus* was highly susceptible to the herbicide (ED*50*=42.22 g ae ha^{-1}). Shikimate accumulation peaked 96 h after treatment in the tolerant plants and the susceptible weed under 500 g ae ha^{-1} glyphosate. The shikimic acid content of whole leaves was 4.0 and 5.0 times higher in the susceptible weed than in *N. wightii* and *C. ternatea*, respectively. ^{14}C-glyphosate absorption and translocation tests showed *A. hybridus* to absorb 30% more herbicide than the legumes 24 h after glyphosate foliar application. ^{14}C-glyphosate translocation as measured by quantified autoradiography revealed increased translocation of the herbicide to untreated leaves and roots in *A. hybridus* relative to the two legumes. The cuticular surface of *A. hybridus* exhibited very low wax coverage relative to the epicuticular surface of *N. wightii* and, especially, *C. ternatea*. No significant degradation of glyphosate to aminomethylphosphonic acid and glyoxylate metabolites was detected among the tolerant leguminous plants or the susceptible weed population. These results indicate that the high glyphosate tolerance of *Clitoria ternatea* and *Neonotonia wightii* is mainly a result of poor penetration and translocation of the herbicide to apical growing points in their plants.

Pool of Resistance Mechanisms to Glyphosate in *Digitaria insularis*

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ABSTRACT

Digitaria insularis biotypes resistant to glyphosate have been detected in Brazil. Studies were carried out in controlled conditions to determine the role of absorption, translocation, metabolism, and gene mutation as mechanisms of glyphosate resistance in *D. insularis*. The susceptible biotype absorbed at least 12% more ^{14}C-glyphosate up to 48 h after treatment (HAT) than resistant biotypes. High differential ^{14}C-glyphosate translocation was observed at 12 HAT, so that >70% of the absorbed herbicide remained in the treated leaf in resistant biotypes, whereas 42% remained in the susceptible biotype at 96 HAT. Glyphosate was degraded to aminomethylphosphonic acid (AMPA), glyoxylate, and sarcosine by >90% in resistant biotypes, whereas a small amount of herbicide (up to 11%) was degraded by the susceptible biotype up to 168 HAT. Two amino acid changes were found at positions 182 and 310 in EPSPS, consisting of a proline to threonine and a tyrosine to cysteine substitution, respectively, in resistant biotypes. Therefore, absorption, translocation, metabolism, and gene mutation play an important role in the *D. insularis* glyphosate resistance.

Two non-target mechanisms are involved in glyphosate-resistant horseweed (*Conyza canadensis* L. Cronq.) biotypes

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ABSTRACT

The physiological and biochemical bases for glyphosate resistance and susceptibility in horseweed (*Conyza canadensis* L. Cronq.) populations collected from Córdoba, Huelva, Málaga, Jaén and Seville in southern Spain were investigated. Screening 25 populations treated with glyphosate (238g acid equivalentha^{-1})at the rosette stage (BBCH 14-15) revealed reductions in fresh weight (fw) of 9-99%. The resistant biotype (R C004) was 6.1 times more resistant than the susceptible biotype (S). Shikimate accumulation in both biotypes increased until 72h after treatment (HAT), and then continued to increase (to 61.2%) in the S biotype, but decreased by 40% in the R (C004) biotype. Differential glyphosate spray retention and foliar uptake of applied ^{14}C-glyphosate between the R (C004) and S biotype had no effect on resistance to this herbicide. Quantitative and qualitative tests showed greater ^{14}C-glyphosate mobility in the S biotype than in the R (C004) biotype. Glyphosate was metabolized faster in the R (C004) biotype than in the S biotype. The herbicide disappeared completely from the R (C004) biotype by conversion into glyoxylate, sarcosine and aminomethylphosphonic acid within 96 HAT. On the other hand, 41.43nmolg-^{1}fw of all glyphosate applied remained in the S biotype and glyoxylate was its only non-toxic metabolite. These results suggest that glyphosate resistance in horseweed is due to two different non-target mechanisms, namely: (a) impaired glyphosate translocation and (b) glyphosate metabolism to other compound.

Glyphosate resistance in a Digitaria insularis biotype is due entirely to differential metabolism

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Glyphosate resistance in a *Digitaria insularis* biotype is due entirely to differential metabolism

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Two metabolic pathways are known for glyphosate degradation by soil microorganisms. In plants, a first degradation pathway involves the herbicide degradation by glyphosate oxidoreductase (GOX) enzyme action, producing AMPA and glyoxylate. AMPA is the main metabolite originated from glyphosate degradation, whereas glyoxylate, despite being a metabolite derived of glyphosate degradation, is also a plant endogenous metabolite involved in different metabolic pathways. AMPA is degraded to methylamine by C–P lyase enzyme action, and by methylamine dehydrogenase enzyme action, methylamine generates formaldehyde. A second pathway is glyphosate degradation to sarcosine by direct C–P lyase enzyme action. Therefore, the appearance of these metabolites indicates that both pathways can be involved in the metabolism of glyphosate in plants. Thus, the relative percentage of both glyphosate and these metabolites can be used as an indicator of glyphosate metabolism in plants

ANEXO II

Use of wheat (Clearfield) Resistant to imazamox in control of *Lolium* spp with multiple resistance to herbicides

6th Workshop of the EWRS working group: CROP-WEED INTERACTIONS

The results obtained show that imazamox-resistant clearfield wheat is anexcellent tool for the control of herbicide-resistant gramineae with different modes of action to the inhibitors of acetolactate synthase (ALS, EC 4.1.3.18).However, the selection of biotypes of *Lolium multiflorum* and L. r*igidum* resistant to herbicides of the sulphonylurea group, and, to a lesser degree, to imidazolines, will create a control problem in the future. It is of prime importance for the farmer to know about herbicide-resistant biotypes, as well as the mechanisms implicated, before using this new chemical control option. Thus, farmers should follow the recommendations made inIntegrated Weed Management (IWM), in order to forecast and/or delay theappearance of herbicide-resistant biotypes.

Uso de trigo resistente a Imazamox (Clearfield) en el control de *Lolium* spp con resistencia múltiple a herbicidas

XXVI Congreso Brasileiro de Ciência das Plantas Daninhas y XVIII Congreso de la Asociación Latinoamericana de Malezas

Los resultados obtenidos muestran que el trigo clearfield resistente a imazamox es una excelente herramienta para el control de gramíneas que son resistentes a herbicidas con diferentes modo de acción a los inhibidores de la acetolactato sintasa (ALS, EC 4.1.3.18). Sin embargo, la selección de biotipos de *Lolium multiflorum* y *L. rigidum* resistentes a herbicidas del grupo de las sulfonilureas y en menor nivel a imidazolinonas presentara problema de control en el futuro. Es primordial que el agricultor conozca la existencia de biotipos resistentes a herbicidas, así como los mecanismos involucrados antes de utilizar esta nueva opción de control químico. De esta forma el agricultor debe seguir las recomendaciones previstas en el manejo integrado de malezas (IWM), con el fin de prevenir y/o retrasar la aparición de biotipos resistentes a herbicidas.

Can Clearfield Wheat Control *Lolium Spp* with Multiple Resistance?

5th International Weed Science Congress

Lolium rigidum and *Lolium multiflorum* are the most injurious weed species in winter wheat World-wide. Their chemical control is currently becoming increasingly difficult as they have a cross and multiple resistance to a Wide range of selective and non selective herbicides in winter wheat. At the beginning of this decade, the firm BASF presented different varieties of wheat resistant to imidazolinones (clearfield), a group of herbicides belonging to ALS-inhibitors (acetolactate synthase), the first enzyme involved in the synthesis of ramified amino acids, valine, leucine and isoleucine, which are essential for the synthesis of proteins in plants. Clearfield was developed by means of classic plant improvement methods, in which there is no insertion of imidazolinone-resistant genes, so that they are not considered as being genetically modified organisms (GMO). The herbicide most used is imazamox, which is absorbed via leaf and/or root and travels through the plants. Imazamox effectively controls gramineae like *Lolium* spp, *Bromus* spp, *Phalaris* spp, *Alopecurus myosuroides*, etc, and dicotyledons such as *Amaranthus* spp, *Abutilon theophrasti*, *Chenopodium album*, etc.

The objective of this work was the use of imazamox-resistant wheat (clearfield) in the control of five populations of *Lolium* spp. With a resistance to diclofop-methy, iodosulfuron, imazamox and glyphosate.

The results obtained show that imazamox-resistant Clearfield wheat is an excellent tool for the control of herbicide-resistant gramineae with different modes of action to inhibitors of acetolactate synthase (ALS). However, the selection of biotypes of *Lolium multiflorum* and *L. rigidum* resistant to herbicides of the sulphonylurea group, and, to a lesser degree, to imidazolines, will create a control problem in the future.

It is of prime importance for the farmer to know about herbicide-resistant biotypes, as well as the mechanisms implicated, before using this new chemical control option. Thus, farmers should follow the recommendations made in Integrated Weed Management (IWM), in order to forecast and/or delay the appearance of herbicide-resistant biotypes.

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Cross resistance of Sinapis alba to ALS- Inhibiting herbicides: first case in the world

XIIIth International Conference on Weed Biology

Over the past 2 decades many grass and broadleaf weed species in the world have been identified as resistant to herbicides that inhibit acetolacte synthase (ALS). To this list, white mustard (Sinapis alba) has been added. Three crop fields containing S. alba with suspected resistance to tribenuron methyl were inspected near Ronda, Malaga, southern Spain. Plant control had been unsatisfactory during the two previous years. Assays to determine the ED50 for tribenuron methyl in two populations were carried out, using field collected seeds. Additionally, the resistance factor (RF), a ratio of the ED50 for resistant divided by susceptible plants, was calculated. Results showed resistance factors of 5 to 7 for the two populations, confirming low level resistance to ALS inhibitors. This is the first case of ALS resistance described worldwide in this species. The effect of several ALS inhibitors on ALS (target site) activity was measured in leaf extracts from both biotypes. Only for tribenuron, mesosulfuron and iodosulfuron was the ED50 value higher for the R compared to the S biotype [I50 (R)/I50(S) values of 3969, 900 and 835, respectively]. For the other herbicide families, we found the lowest resistance factor levels as: 36, 24, 2 with Flucarbazone, Florasulam, Imazamox, respectively. These data suggest that resistance to sulfonylurea herbicides found in the R biotype of Sinapis alba results primarily from an altered target site.

Resistencia cruzada de trigo Clearfield a imidazolinonas

XII Congreso de la Sociedad Española de Malherbología. XIX Congreso Asociación Latinoamericana de Malezas. II Congreso IBCM

Los datos obtenidos en los experimentos realizados han demostrado que el *Triticum aestevum* var. Pandora-R es más resistente a las Imidazolinonas estudiadas que T. aestevum var. Pandora-S; registrándose un alto nivel de resistencia a Imazametabenz-metil. Los valores de I50 calculados para la variedad Pandora-R mostraron el siguiente orden de resistencia: Imazametabenz-metil ≥ Imazamox ≥ Imazapir > Imazaquin > Imazetapir. No obstante, en la variedad Pandora-S el orden resultó diferente: Imazapir > Imazamox ≥ Imazaquin ≥ Imazametabenz-metil > Imazetapir. El alto valor del factor de resistencia a las Imidazolinonas presentado por la variedad Pandora-R es un rasgo que podría ser utilizado como un método eficaz para el control de malezas resistentes a otros herbicidas en cultivos de trigo en Chile.

Determinación de glifosato y sus metabolitos en plantas mediante electroforesis capilar

XII Congreso de la Sociedad Española de Malherbología. XIX Congreso Asociación Latinoamericana de Malezas. II Congreso IBCM

Se desarrolló un método de electroforesis capilar (CE) que permite la determinación simultánea de glifosato y sus metabolitos [ácido aminometilfosfónico (AMPA), glioxilato, sarcosina y formaldehído] en plantas. Este método se usó en varias muestras de una población de *Lolium multiflorum* tratado con 200 g. ha^{-1} de m.a. de glifosato y recogidas a las 96 horas después del tratamiento. Tras la etapa de extracción-limpieza y determinación se obtuvieron los siguientes valores: 20.794±0.257µg/ml (t_m 9.8min), 0.830±0.030µg/ml (t_m 10.9min), 2.986±0.025µg/ml (t_m 11.3min), 9.175 ± 0.211µg/ml (t_m 13.5min) y 0.152 ± 0.014µg/ml (t_m 17.1min) para glifosato, AMPA, glioxilato, sarcosina y formaldehído, respectivamente. Este método rápido y eficaz nos permitirá estudiar si el metabolismo de glifosato está involucrado en la aparición de malezas resistentes, que es hoy día uno de los grandes problemas que se está encontrando en la agricultura mundial.

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Metabolism of Aryloxiphenoxypropionate herbicides in Echinochloa colona found in Colombia

62nd International Symposium on Crop Protection

The aryloxyphenoxypropionates are an important family of higly selective post-emergence herbicides used to control monocotyledonous weeds. At present, 38 weed species have been described showing resistance to some of these herbicides. In rice crops, different biotypes of E. colona have been reported to be resistant to different herbicides due to different mechanisms. The objective of this work was to study the mechanism of resistance of a Clumbian population of E. colona to the herbicides fenoxaprop-p-methyl and cyhalofop-butyl.

Status of Weed Resistance to Glyphosate in Europe

III Simpósio Internacional sobre Glyphosate- uso sustentável

Three diclofop-methyl (DM) resistant biotypes of Lolium rigidum (R1, R2, and R3) were found in different winter wheat fields in Spain, continuously treated with DM, DM + chlortoluron, or DM + isoproturon. Herbicide rates that inhibited shoot growth by 50% (ED50) were determined for DM. There were found that the different biotypes exhibited different ranges of resistance to this herbicide; the resistant factors were 7.2, 13, and 36.6, respectively. DM absorption, metabolism, and effects on ACCase isoforms were examined in these biotypes of L. rigidum. The most highly resistant, biotype R3, contained an altered isoform of ACCase. In biotype R2, which exhibited a medium level of resistance, there was an increased rate of oxidation of the aryl ring of diclofop, a reaction most likely catalyzed by a cytochrome P450 enzyme. In the other biotype, R1, DM penetration was significantly less than that observed in the resistant (R2 and R3) and susceptible (S) biotypes. Analysis of the leaf cuticle surface by scanning electron microscopy showed a greater epicuticular wax density in the leaf cuticles of biotype R1 than in the other biotypes.

Differential Tolerance to Glyphosate in Three Legominous Species

III Simpósio Internacional sobre Glyphosate- uso sustentável

The data obtained showed that Clitoria ternantea, Mucuna pruriens and Neonotonia wightii were glyphosate-tolerant. The ED_{50} values were of 600.18 g ae ha^{-1} for *C. ternatea*, 403.78 for M. pruriens and 362.94 g ae ha^{-1} for *N. wightii*, compared with *A. hybridus* was highly susceptible to the herbicide (ED_{50} = 42.22 g ae ha^{-1}). ^{14}C-glyphosate absorption and translocation tests showed the legumes to absorb 30% less herbicide than the *A. hybridus* 24 h after glyphosate foliar application. ^{14}C-glyphosate translocation as measured by quantified autoradiography revealed increased translocation of the herbicide to untreated leaves and roots in *A. hybridus* relative to the three legumes. The cuticular surface of *A. hybridus* exhibited very low wax coverage relative to the epicuticular surface of *three legumes*, especially, *C. ternatea*. These results indicate that the high glyphosate tolerance of *Clitoria ternatea*, *Mucuna pruriens* and *Neonotonia wightii* is mainly a result of poor penetration and translocation of the herbicide to apical growing points in their plants among other things.

Glyoxylate: A new biomarker to detect glyphosate resistance weeds

52nd Meeting, Weed Science Society of America

The evidence that glyoxylate is a biomarker of tolerance or susceptibility to the action of herbicides belonging to the glycine family makes necessary to develop simple methods for the determination of this metabolite. Glyoxylate level allows both to know the presence/absence of members of the glycine family in plants and plant response to these herbicides. With this aim, a colorimetric-screening method has been developed for determination of glyoxylate based on formation of a phenylhydrazone, then oxidised to red coloured 1,5-diphenylformazan. Simultaneous optimization of ultrasound-assisted extraction of glyoxylate from plants and derivatization by a multivariate design has allowed the determination of the target analyte in fresh plants without interferences from pheophytines and compounds with carbonyl groups. Limits of detection and quantification are 0.05 μg ml^{-1} and 0.17 μg ml^{-1}, respectively, with precision, expressed as relative standard deviation, of 3.3% for repeatability and 5.6% for the within-day laboratory reproducibility. Only 50 mg of plant is necessary for determination of glyoxylate within 32 min. Confirmatory analysis by capillary electrophoresis-diode array detection in samples of *Lolium spp.* subjected to treatment with glyphosate shows that the relative error of the proposed method is always lower than 7%.

Glyphosate metabolism in *Digitaria insularis* biotypes

International Workshop on"Glyphosate Weed Resistance:European Status and Solutions

Some weeds are invading large areas of crops in Brazil, among them is *Digitaria insularis*, which is highly resistant to glyphosate. One of the mechanisms involved in resistance is the metabolism. The glyphosate is degraded to glyoxylate, AMPA, sarcosine and formaldehyde. In this study, we studied two biotypes of *D. insularis*, hereafter designed as susceptible (S) and resistant (R), whose previously dose-response study indicated ED_{50}`s of 212.8 g a.i. ha^{-1} and 830.1 g a.i. ha^{-1}, respectively.

The aim of this study was to compare the degree of glyphosate metabolism in both biotypes.

The resistance was confirmed by testing shikimic acid accumulation at different times (24, 48, 72, 96 and 168 hours after appliying glyphosate), when shikimic acid accumulation varies from 1.14 up 5 times higher in S biotype than R biotype. For further metabolism study, glyphosate metabolites were detected in leaf tissues of both biotypes at 48, 96 and 168 hours after applying glyphosate. It was observed that in the susceptible biotype only 10% of glyphosate was degraded to metabolites AMPA, glyoxylate and sarcosine, while in the resistant biotype that percentage increased up to 96 hours after treatment when 90% of the absorbed glyphosate had been metabolized.

This may explain why the resistant biotype of *D. insularis* can withstand high doses of glyphosate compared with the susceptible biotype.

New biomarker to detect glyphosate resistance in weeds

International Workshop on"Glyphosate Weed Resistance:European Status and Solutions

For many years, we have used the method proposed by Singh & Shaner in 1998 (Weed Technol. 12: 527–530) based on the levels of shikimate, which allows to differentiate between resistant and sensitive plants to glyphosate. In 2011, Rojano et al. (Talanta 82: 1757–1762) proposed another method based on the levels of glyoxylate with the same finality than the previous method.

In this study we try to compare the response time and application rate of the herbicide required of both methods. Two biotypes of Conyza bonariensis were used for the estudy, susceptible (S) and resistant (R) to glyphosate, whose ED50 were 45.00 and 289.36 g a.e. ha-1, respectively.

Two experiments were conducted in which, either varying the response time (3, 6, 9, 12, 24 and 48 HDT) using a fixed dose of application at 222 g a.e. ha-1 and the other experiment with different doses (18.5, 37, 74, 148, 222, 296, 444 and 592 g a.e. ha-1) maintaining the response time fixed at 24 HDT. The results indicate that although both methods can differentiate between R and S biotypes, when the herbicide application rate was 222 the response time was 48 hours for shikimate and only 3 hours for glyoxylate, whereas when the time application was 24 HDT the herbicide dose required was 222 g a.e. ha-1 for shikimate and 37.00 g a.e. ha-1 for glyoxylate. Thus we have concluded that the analysis of glyoxylate could be an interesting procedure to detect glyphosate resistance in plants, as it presents advantages compared to the method based on shikimate, because it requires less time for the assay, and a lower dose of glyphosate application.

Clearfield® wheat *vs.* Imazamox: Metabolism study by LC–DAD and LC–TOF/MS

16th European Weed Research Society Symposium

The Clearfield® Production System for wheat is a novel technology that allows wheat producers to control many problematic grass and broadleaf weeds. It was developed using enhanced, traditional plant breeding techniques and is certainly among the most promising and innovative tools for weed control. This production system allows to control several herbicide resistant weeds which invade a large number of crops, such as annual ryegrass (*Lolium multiflorum Lam.*) diclofop-resistant populations in wheat fields. The herbicide blocks the production of the essential amino acids valine, leucine and isoleucine by inhibiting the enzyme acetolactate synthase (ALS), while in Clearfield® wheat, tolerant to Imazamox, has an altered ALS enzyme. However, there is a lack of understanding on the way that Clearfield® wheat plants metabolize the herbicide at several rates.

A method based on LC–DAD and LC–TOF/MS with a HILIC (20 cm × 4.6 cm) analytical column was used for determination and identification of Imazamox and its metabolites. The mobile phases were 1% acetic acid in water (mobile phase A) and 100% methanol (mobile phase B). Fifty µL of extract from the target treated plant was injected and eluted according to an elution gradient by using initial concentrations of 95% of A and 5% of B.

Our results showed that metabolism of Imazamox in Clearfield wheat begins from medium to high doses. Two metabolites: 3-pyridinecarboxylic acid, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-(hydroxymethyl)- and 3-pyridinecarboxylic acid, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-[(β-D-glucopyranosyloxy)methyl]- were found at high concentrations, confirming that this pathway is the major mechanism of resistance to Imazamox in the Clearfield® wheat.