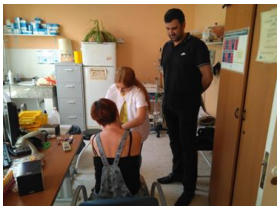
EVALUACIÓN DE LA INFLUENCIA DE LA EXPOSICIÓN LABORAL A FITOSANITARIOS EN MUJERES QUE RECOGEN VERDURAS. POSIBLES CONSECUENCIAS SOBRE SU SALUD






TESIS DOCTORAL. José Martín Reina

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UNIVERSIDAD DE SEVILLA
FACULTAD DE FARMACIA
DEPARTAMENTO DE NUTRICION Y BROMATOLOGÍA,
TOXICOLOGIA Y MEDICINA LEGAL

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“A lo largo de los años hubo muchos momentos en los que el destino me preparó quiebros insospechados, sorpresas y esquinazos imprevistos que hube de afrontar a matacaballo según fueron viniendo. Alguna vez estuve preparada para ellos; muchas otras, no.”

“Por muy duros que fueran los tiempos, jamás se fue de su lado el optimismo con el que apuntaló todos los golpes y al que se acogió para ver siempre el mundo desde el lado por el que el sol luce con más claridad”.

(El Tiempo entre costuras – María Dueñas-)

A mis hijos, Ana y José, mi motivación diaria.

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Me preguntaron en Labinsk (Rusia) de manera despectiva, si “sólo era farmacéutico”, y ese viento avivó el rescoldo que, desde hacía años, habitaba en mí.

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Con "el motor" en marcha y la ruta comenzada, **mis amigos** me apoyaron incondicionalmente y me mostraron su preocupación por cómo se iba desarrollando todo. Tengo la suerte de tener buenos amigos, aquellos que, no teniendo al lado, ni hablando con ellos a diario, están ahí siempre. **José María, Inma, Miguel, Francis, Francisco de Asís, Ana, Juan**, muchas gracias a todos porque he sentido vuestro interés, apoyo y ese aliento tan necesario en muchas ocasiones.

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INDICE DE ABREVIATURAS

ACh.- Acetilcolina

AChE.- Enzima Acetilcolinesterasa

AD.- Alzheirmer´s Diseases

AMPA.- Aminomethylphosphonic Acid

BOE- Boletín Oficial del Estado

DDT.- DiCloro-Difenil-Tricloroetano

DFR.- Residuos Foliares Desprendible

DL_{50}.- Dosis Letal Media

ECHA.- Agencia Europea de Sustancias y Mezclas Químicas

EFSA.- Agencia Europea de Seguridad Alimentaria

ELA.- Esclerosis Lateral Amiotrófica

EPSPS.- Enzima 3-enolpiruvil-shikimato-5-fosfato sintasa

FAO.- Food and Agriculture Organization

FAOSTAT.- Statistics Division of Food and Agriculture Organization

FMOC.- 9-fluorenylmethyl chloroformate

GLY.- Glyphosate

HBM.- Human Biomonitoring

HPLC.- High-Performance Liquid Chromatography

IARC.- Agencia Internacional de Investigación sobre el Cáncer

ILO.- Organización Internacional del Trabajo

IMA.- Informe de Medioambiente de la Junta de Andalucía

INSHT.- Instituto Nacional de Seguridad e Higiene en el Trabajo

INSST.- Instituto Nacional de Seguridad y Salud en el Trabajo

INSSBT.- Instituto Nacional de Seguridad Salud y Bienestar en el trabajo

IRAC.- Insecticide Resistance Action Commitee

KIM-1.- Kidney Injure Molecule 1

LC.- Liquid chromatography

LC-FLD.- Liquid chromatography combitated with fluorescence detection

LC/MS.- Chromatography–mass spectrometry

LMR.- Límite Máximo de Residuos

LPO.- Peroxidación de Lípidos

MAPA.- Ministerio de Agricultura, Pesca y Alimentación

MDA.- Malondialdhido

NAG.- Nacetil-b-D-glucosaminidasa

NGAL.- Lipocaina asociada a la gelatinasa de neutrofilos

NHL.- Non-Hodgkin's Lymphoma

NOE.- Not Occupationally Exposed

NTP.- Nota Técnica de Prevención.

OMS.- Organización Mundial de la Salud

PD.- Parkinson´s Diseases

PPE.- Personal Protective Equipment

PPP.- Plants Products Protection

PRL.- Prevención de Riesgos Laborales

ROESB.- Registro Oficial de Establecimientos y Servicios de Biocidas

ROPO.- Registro Oficial de Proveedores y Operadores (fitosanitarios)

ROS.- Especie Reactiva de Oxígeno

TC.- Coeficiente de Transferencia

TPRL.- Técnico en Prevención de Riesgos Laborales

UE.- Unión Europea

VGSC.- Canales de Calcio voltaje dependientes

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I. INTRODUCCIÓN

La exposición del ser humano a mezclas complejas de contaminantes medioambientales es una realidad en la sociedad actual. Por contaminantes ambientales se entienden aquellos factores exógenos, no esenciales para los humanos, que, cuando se liberan al medio ambiente, pueden ser perjudiciales para la salud humana y/o el medio ambiente **(Vabre et al., 2017**). Dentro de este grupo de contaminantes, destacan los denominados contaminantes emergentes, como los plaguicidas (**Pereira et al., 2015**).

Sin embargo, el uso de plaguicidas por el hombre viene de antaño, así los sumerios ya aplicaban compuestos azufrados para combatir los insectos (**Želježić & Perković, 2011**). Aunque el desarrollo de los plaguicidas y su uso, podrían encuadrarse en tres etapas, a lo largo de la historia:

Una primera etapa, meramente accidental y empírica, basada en la acción plaguicida de algunos compuestos químicos como el azufre, arsénico o sulfato de cobre.

Una segunda etapa, pre-científica, a partir del 1922, cuando en Holanda comienza la utilización de aceites con propiedades insecticidas.

Y una tercera etapa, científica, marcada por el descubrimiento de las propiedades del dicloro-difenil-tricloroetano (DDT), conocido desde años atrás, pero cuyo éxito, al ser efectivo frente a los piojos que transmitían el tifus exantémico en los soldados de la II Guerra Mundial, lo convirtió en el inicio del

desarrollo de nuevos productos frente a distintas plagas (**Casas Maya, 2017**)

A partir de aquí, y hasta nuestros días, numerosos productos químicos se han ido desarrollando, cada vez más específicos, con la finalidad de eliminar las distintas plagas que pudieran aparecer, con dos objetivos fundamentales:

1. Obtener alimentos que duraran más en el tiempo, y así poder llegar a la población mundial, con el consiguiente rendimiento económico del cultivo, y
2. Lograr beneficio para la salud humana porque evita enfermedades transmitidas por insectos (vectores) (**Ortiz Hernández et al., 2017**)

Intentar cubrir estos objetivos ha contribuido a que haya aumentado la variedad de sustancias en el mercado, cada vez más específicas y también a que distintas administraciones lleven a cabo su control, dado su potencial daño para la salud humana y animal.

Pero, no podría haber beneficio sin perjuicio. Hay una peligrosidad intrínseca en los plaguicidas o sus metabolitos, por ser compuestos biocidas y, por tanto, actuar contra los seres vivos (**Doménech, 2004**). Así la población en general, a lo largo de toda su vida, está expuesta a pequeñas concentraciones de plaguicidas a través de la dieta y el medio ambiente (**González-Alzaga et al., 2014**)

Revisemos brevemente, algunos aspectos relacionados con nomenclatura, reglamentación, clasificación y exposición laboral a estos compuestos.

1. Concepto de plaguicida y Reglamentación.

En relación con la nomenclatura, sin duda la terminología utilizada es frecuentemente complicada de entender para el lego en la materia. Así la Food and Agriculture Organization (FAO) define a los plaguicidas como "*cualquier sustancia o mezcla de sustancias destinadas a prevenir, destruir o controlar cualquier plaga, incluyendo los vectores de enfermedades humanas o de los animales, las especies no deseadas de plantas o animales que causan perjuicio o que interfieren de cualquier otra forma en la producción, elaboración, almacenamiento, transporte o comercialización de alimentos, productos agrícolas, madera y productos de madera o alimentos para animales, o que pueden administrarse a los animales para combatir insectos, arácnidos u otras plagas en o sobre sus cuerpos. El término incluye las sustancias destinadas a utilizarse como reguladoras del crecimiento de las plantas, defoliantes, desecantes, agentes para reducir la densidad de fruta o agentes para evitar la caída prematura de la fruta, y las sustancias aplicadas a los cultivos antes o después de la cosecha para proteger el producto contra la deterioración durante el almacenamiento y transporte*" (**FAO, 2014**). Sin embargo, el concepto plaguicida, es definido por el Reglamento Europeo Nº 528/2012 del Parlamento Europeo y del Consejo del 22 de mayo del 2012 relativo a la comercialización y el

uso de biocidas (**UE, 2014**) como un grupo de biocidas, destinados, según el tipo de producto al que pertenezcan, al control de roedores, aves, peces, moluscos, insectos, ácaros, etc.

En la actualidad, el reglamento europeo por el que se regula la normativa de existencia y uso de plaguicidas es la Nº 528/2012 del Parlamento Europeo y del Consejo del 22 de mayo del 2012 relativo a la comercialización y el uso de biocidas (**UE, 2014**). Esta norma establece que los plaguicidas son necesarios para controlar organismos que puedan ser dañinos para animales y humanos y puedan causar daños a los recursos naturales. También expone que estos plaguicidas suponen un riesgo para humanos, debido a sus propiedades y el uso que se hace de ellos. Como es evidente, todos ellos deben ser usados según la normativa y asegurando un alto nivel de protección para la salud de humanos, animales y medio ambiente.

En España, el uso de biocidas (y por tanto de los plaguicidas afectados) y la exposición y los riesgos que estos suponen está controlada por el Ministerio de Sanidad, Servicios Sociales e Igualdad, encargado de publicar las distintas normativas relacionadas con estos. La reglamentación española además de acogerse a la autorización según la normativa europea, también incluye un registro de plaguicidas según un Registro Nacional (Real Decreto 3349/83) y un listado de productos insecticidas y repelentes comercializados para enfermedades como son el Dengue, Chikunguya y Zika (**BOE, 1984**).

A lo mencionado anteriormente, desarrollado por "el canal de salud", hay que unir, por otro lado "el canal de agricultura". Relacionado con la agricultura y la alimentación, el Reglamento (CE) nº 1107/2009 del Parlamento Europeo y del Consejo de 21 de Octubre de 2009 relativo a la comercialización de productos fitosanitarios y por el que derogan las directivas 79/117/CEE y 91/414/CEE del Consejo, junto a la Directiva 2009/128/CE del Parlamento Europeo y del Consejo de 21 de Octubre de 2009 por la que se establece el marco de la actuación comunitaria para conseguir un uso sostenible de los plaguicidas, marcan lo indicado en el RD 1311/2012, de 14 de Septiembre, por el que se establece el marco de actuación para conseguir un uso sostenible de los productos fitosanitarios (**BOE, 2012; EU, 2009**)

Se podría resumir de forma que "un raticida", si se utiliza por el "canal salud", como biocida, se le aplica una normativa y si se utiliza "por el canal agrícola", como fitosanitario, se le aplica otra normativa: siendo el mismo producto. En el Ministerio de Sanidad, y en el Ministerio de Agricultura, se indican las distintas normativas para autorizar los productos y, en las comunidades autónomas, en las consejerías de sanidad y de agricultura se indican las distintas normativas para que una entidad pueda aplicar un producto: Deben estar inscritas en un registro: ROPO (Registro Oficial de Proveedores y Operadores) y ROESB (Registro Oficial de Establecimientos y Servicios de Biocidas) (**BOE, 2002; BOE, 2012**), según la finalidad del producto a emplear.

En cualquier caso, se podría indicar que los plaguicidas, son productos químicos, con función específica, dentro del grupo de los fitosanitarios (cuando se aplican en el mundo agrícola) y dentro del grupo de los biocidas (cuando se aplican en ámbitos distintos al mundo agrícola).

2. Clasificación de plaguicidas

Los plaguicidas se pueden clasificar de diferentes maneras atendiendo a sus características. Normalmente, los plaguicidas se clasifican según el tipo de plaga al que estén destinados, siendo los más importantes de esta clasificación los fungicidas, herbicidas, rodenticidas e insecticidas.(**Aktar et al., 2009; Martín-Reina et al., 2017)**

La Organización Mundial de la Salud (OMS) realiza una clasificación centrada en la capacidad de producir daño tras una exposición aguda a uno de ellos, que también podríamos definir como el daño en la salud de una persona tras una o varias exposiciones en 24 horas. Esta clasificación se realiza basándose siempre en el concepto de dosis letal media (DL_{50}), siendo ésta la estimación estadística de la cantidad de una sustancia tóxica por peso corporal (mg/kg), necesaria para matar al 50% de animales de experimentación (usualmente ratas de laboratorio) en los que se ensaya el efecto letal (Tabla 1).

Tabla 1. Clasificación de los plaguicidas según la OMS, basada en la DL_{50}.

		DL50 para la rata (mg/kg peso)		
Clase		Oral	Dermal	**Ejemplo**
Clase IA	Extremadamente peligrosos	< 5	< 50	Bromadiolona Parathion
Clase IB	Altamente peligrosos	5-50	50-200	Warfarina Methiocarb
Clase II	Moderadamente peligrosos	50-2000	200-2000	Lambda-cyhalothrin Cipermetrina
Clase III	Ligeramente peligrosos	> 2000	> 2000	Glifosato
Clase U	Improbable que presente peligros agudos	>5000		Mancozeb

Otras clasificaciones que se realizan de los plaguicidas son según su estructura química, dando lugar a diversas familias como pueden ser los organoclorados o los organofosforados, siendo estos

algunos de los más conocidos por ser los más utilizados a lo largo de la historia, aunque cada vez están más en desuso por sus propiedades tóxicas. Estas características químicas van a influir en las propiedades fisicoquímicas de los plaguicidas, así los organoclorados pertenecen al grupo de contaminantes orgánicos persistentes debido a su alta liposolubilidad, lo que los hace bioacumularse a lo largo de la cadena alimentaria. Como alternativa a su uso, surgieron los organofosforados, carbamatos y piretroides, considerados insecticidas no persistentes, debido a su rápida hidrólisis en el medio ambiente **(Kim et al., 2017; Pereira et al., 2015**). Por otro lado, the Insecticide Resistance Action Commitee (IRAC) clasifica los plaguicidas, según su mecanismo de acción (**IRAC, 2020**) (tabla 2) .

Tabla 2. Mecanismo de acción de los diferentes plaguicidas

MECANISMO DE ACCIÓN	**Características**
Actúan sobre el sistema nervioso y muscular	Son la mayoría de los plaguicidas. De acción rápida, inhiben encimas de neurotransmisores, canales de iones, o moduladores de receptores, entre otros
Reguladores del crecimiento	Mimetizan hormonas, o inhiben biosintesis de nutrientes, entre otros
Actúan sobre el sistema digestivo	Mediante disruptores microbianos o virus

	patógenos ocluidos
Actúan sobre el metabolismo energético	Inhibiendo el transporte de electrones en el complejo mitocondrial, por lo que se impide el uso de energía.

(Adaptado de IRAC 2020 (**IRAC, 2020**))

3. Efectos tóxicos de los principales plaguicidas

➢ Organofosforados

Los organofosforados son los insecticidas más usados en el mundo. Se trata de compuestos liposolubles y volátiles, resultando en una toxicidad variable debido a que estas características facilitan su absorción. Sus efectos varían dependiendo del grado de toxicidad y de la vía de entrada al organismo (**Fernandez et al., 2010**)

El principal efecto tóxico de los organofosforados se debe a la inhibición de la enzima acetilcolinesterasa (AChE), una enzima encargada de catalizar la hidrólisis de la acetilcolina (ACh) (Figura 1). La consecuencia de la inhibición de la enzima resulta en una acumulación del neurotransmisor en el espacio sináptico, dando lugar a efectos neurotóxicos debido a la hiperexcitabilidad de tejidos, provocando el denominado síndrome colinérgico **(Caro-Gamboa et al., 2020; Martín-Reina et al., 2017**)

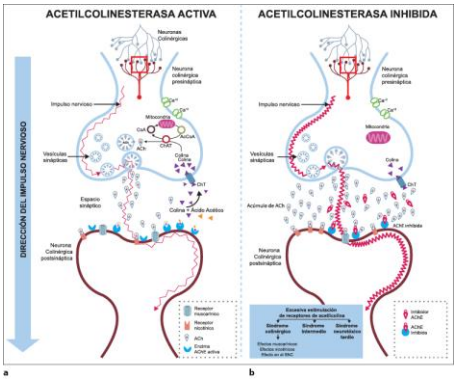

Figura 1. Mecanismo de activación e inhibición de la colinesterasa (Tomado de (Caro-Gamboa et al., 2020)

Diferentes autores han encontrado evidencias de la existencia de intoxicaciones tanto agudas como crónicas en la exposición laboral a plaguicidas. Las intoxicaciones agudas pueden derivar en síntomas que varían de leve a muy grave y pueden ser dolores de cabeza, náuseas, vómitos, bradicardia, miosis, dermatitis o quemaduras. Los problemas neurotóxicos están más relacionados con la exposición crónica, resultando en problemas cognitivos, motores, sensoriales (**Joshaghani et al., 2007; Roldán-Tapia et al., 2005**) y enfermedades neurodegenerativas como pueden ser el Alzheimer, el Parkinson o la esclerosis lateral amiotrófica (ELA) (**Sánchez-Santed et al., 2016**)

Además de la inhibición de la AChE, los organofosforados tienen la capacidad de producir daños oxidativos debido a la capacidad de producir especies reactivas de oxígeno (ROS) que cuando su producción es excesiva conduce a un estado de estrés

oxidativo (**Kemp et al., 2008**). El cerebro es particularmente susceptible a este daño oxidativo debido a su alto contenido en lípidos, alta demanda de energía y menos defensas antioxidantes en relación a otros órganos **(Pearson et al., 2017**)

Los organofosforados también afectan a receptores hormonales, actuando como ligando y dando lugar como consecuencia a una actividad como disruptor endocrino y produciendo alteraciones hormonales (**Combarnous, 2017**)

- **Carbamatos**

La toxicidad de los carbamatos al igual que la de los organofosforados, se caracteriza por su potente actividad anticolinesterasa, que inhibe tanto a la acetilcolinesterasa como a la butirilcolinesterasa, las cuales se usan como biomarcadores de exposición a estos insecticidas (**Gonzalez et al., 2012**).

A diferencia de los organofosforados, la exposición a carbamatos da lugar a una toxicidad más corta y reversible ya que se metabolizan rápidamente (**Silberman & Taylor, 2018**)

La exposición puede dar lugar a una toxicidad tanto aguda como crónica, siendo la piel, los pulmones o la conjuntiva, algunas de las vías de entrada. Los síntomas, muy similares a los de una intoxicación por organofosforados, son hipersalivación, lagrimeo o problemas gastrointestinales. También se puede desarrollar bradicardia o taquicardia y miosis o midriasis (**Silberman & Taylor, 2018**).

- **Piretroides**

Los piretroides iniciales fueron piretrinas naturales, obtenidos del extracto aislado de *Chrysanthemun*, que actualmente se sintetizan a raíz de su origen. Los piretroides son relativamente menos tóxicos para los mamíferos que los organofosforados, por ello y por ser de amplio espectro, son de los plaguicidas más usados en la mayoría de los países como alternativa al uso de organofosforados (**Lei et al., 2017**).

La actividad de los piretroides está basada en su efecto sobre el canal de sodio y cloro (**Bradberry et al., 2005; Scott, 2019**). Los piretroides incluyen efectos tóxicos como neurotoxicidad, toxicidad respiratoria, o efectos tóxicos en la piel, y en el sistema reproductor (**Lei et al., 2017**). Diferentes estudios muestran también que los piretroides juegan un papel como disruptores endocrinos, teniendo un efecto sobre receptores tanto estrogénicos como androgénicos *in vitro*, que podría derivar en una alteración de la salud reproductiva de hombres adultos (**Saillenfait et al., 2016; Ben Slima et al., 2017; Ghosh et al., 2018; Hernández et al., 2020; Singh et al., 2020**).

Dentro del grupo de los piretroides podemos diferenciar los piretroides tipo I, aquellos que tienen una estructura básica de éster carboxílico como ciclopropano y los piretroides tipo II, que además de la estructura básica poseen un grupo ciano (**Bradberry et al., 2005**).

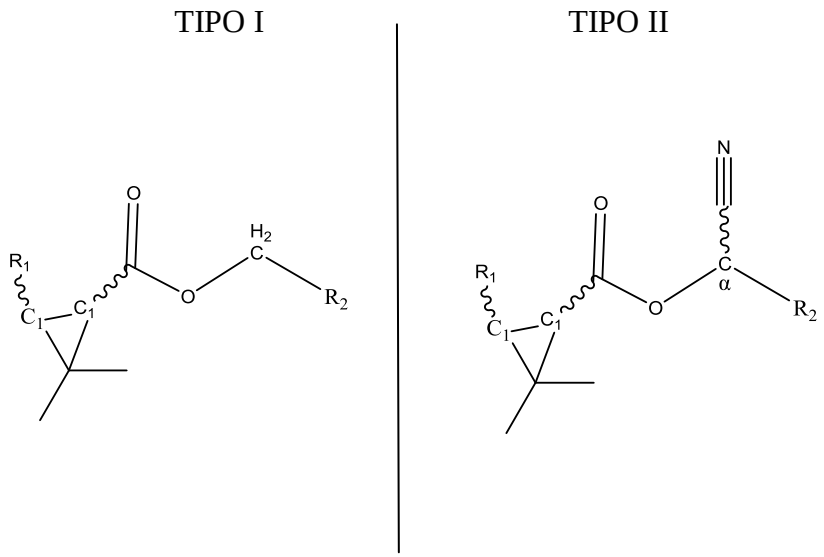

Figura 2. Estructura de los pietroides tipo I y tipo II. Tomado de (**Corcellas et al., 2015**)

Dependiendo del tipo de piretroide, se produce un efecto u otro. Los piretroides tipo I dan lugar a una hiperexcitabilidad por una prolongación de la apertura del canal y en el caso de los tipo II, esta prolongación de apertura que es mayor, resulta en una despolarización de la membrana y en un bloqueo de la conducción.**(Martín-Reina et al., 2017; Wakeling et al., 2012**)

- **Glifosato**

El glifosato ha llegado a ser el herbicida más usado en la historia de la agricultura, se utiliza para eliminar las malas hierbas indeseables en ambientes agrícolas y forestales. Tiene una alta distribución, representando entorno al 43%-51% de la cantidad total

de herbicidas utilizados a nivel mundial. **(Martinez et al., 2018**). Cuando se comenzó a usar en 1974, el glifosato era considerado de bajo peligro para mamíferos, sin embargo, la Agencia Internacional de Investigación sobre el Cáncer (IARC) lo considera desde 2015 un producto probablemente carcinogénico para los humanos (Grupo 2A) ” (**Tarazona et al., 2017**).

A raíz de esta clasificación, la Unión Europea ha evaluado exhaustivamente al glifosato, a través de la Autoridad Europea de Seguridad Alimentaria (EFSA) y de la Agencia Europea de Sustancias y Mezclas Químicas (ECHA) para establecer si su uso puede conducir a efectos inaceptables en la salud humana, animal o ambiental, sin haber llegado a una conclusión definitiva (REFERENCIAS).

Sin embargo, tanto la Agencia Europea para las Sustancias Químicas (ECHA) como la Autoridad Europea en Seguridad Alimentaria (EFSA), han descartado que provoque cáncer en los humanos, por lo que la Unión Europea ha renovado la licencia del glifosato hasta el 2022 (**ECHA, 2017**). Se necesitan más estudios toxicológicos y epidemiológicos para sacar mejores conclusiones sobre la seguridad del glifosato.

En cuanto a su toxicidad en humanos, muchos estudios demuestran que los herbicidas basados en glifosatoto es tóxico para las células humanas placentarias **(Davoren & Schiestl, 2018)**. También está comprobado que puede actuar sobre la actividad de la aromatasa, actuando como disruptor endocrino (**Salazar-López &**

Aldana Madrid, 2011), pero también hay artículos en los que se ha demostrado que el glifosato presenta genotoxicidad por daños en la estructura del ADN y que puede provocar toxicidad *in vivo* en células humanas (**Monroy et al., 2005**).

4. Consumo de plaguicidas

Según datos de la división de estadística de la FAO (FAOSTAT), en el 2017, Europa comercializó un total de 490217 toneladas de plaguicidas en total, siendo España el segundo país europeo que más plaguicidas consumió: 60896 toneladas, por detrás de Francia, con 70604 toneladas. La misma base de datos indica que, en el 2018, Europa consumió un total de 478326 toneladas de plaguicidas en total, siendo España, de nuevo, el segundo país europeo que más plaguicidas consumió: 61343 toneladas, por detrás de Francia, con 85072 toneladas. Sobre la disminución de consumo total de plaguicidas en Europa, es coherente a la normativa mencionada anteriormente. Sin embargo, ocurre lo contrario en los dos primeros países de mayor consumo, donde el número de toneladas se ha visto incrementado.

Centrados en España, en el 2017, se comercializaron 37999 toneladas de fungicidas y bactericidas, 16077 toneladas de herbicidas, y 6663 toneladas de insecticidas. En el 2018, se consumieron 38067 toneladas de fungicidas y bactericidas, 16593 toneladas de herbicidas, y 6488 toneladas de insecticidas. Estos datos, obtenidos inicialmente en la FAOSTAT, coinciden prácticamente con los datos publicados por el Ministerio de

Agricultura, Pesca y Alimentación en sus documentos "Encuesta comercialización productos fitosanitarios. Años 2017 y 2018" (**MAPA, 2018; MAPA, 2017**)

Una vez obtenidos los datos de consumo de productos a nivel europeo, y nacional, hemos consultado los consumos a niveles autonómicos, así como en las diferentes provincias andaluzas, con el fin de tener un conocimiento de la provincia de Sevilla, relativo y comparable. Para ello, se utilizó el Informe Medioambiental de la Junta de Andalucía **(Consejeria Agricultura y Pesca, 2018a, 2018b**) que hacen referencia a los consumos del año 2017:

En esta base de datos, donde se habla de consumo (frente a comercialización en la documentación del ministerio), no se encontraron datos relacionados a 2018, pero, según esta fuente, en 2017 hubo un consumo en España de 124157 toneladas del total de fitosanitarios, de los cuales, 46170 se consumieron en Andalucía (Figura 3).

Lo primero que llama la atención es la cifra que prácticamente es el doble de lo comunicado por el Ministerio de Agricultura y por la FAOSTATS para el 2017. Atendiendo a esta base de datos, en el 2017, Andalucía fue la comunidad autónoma con mayor consumo de Fitosanitarios, siendo los insecticidas y los herbicidas los productos más utilizados, de la totalidad.

Según la base de datos de la Junta de Andalucía, en 2017, Almería fue la primera consumidora de plaguicidas, seguida de

Sevilla la segunda provincia con mayor consumo de producto (Figura 4 y Figura 5).

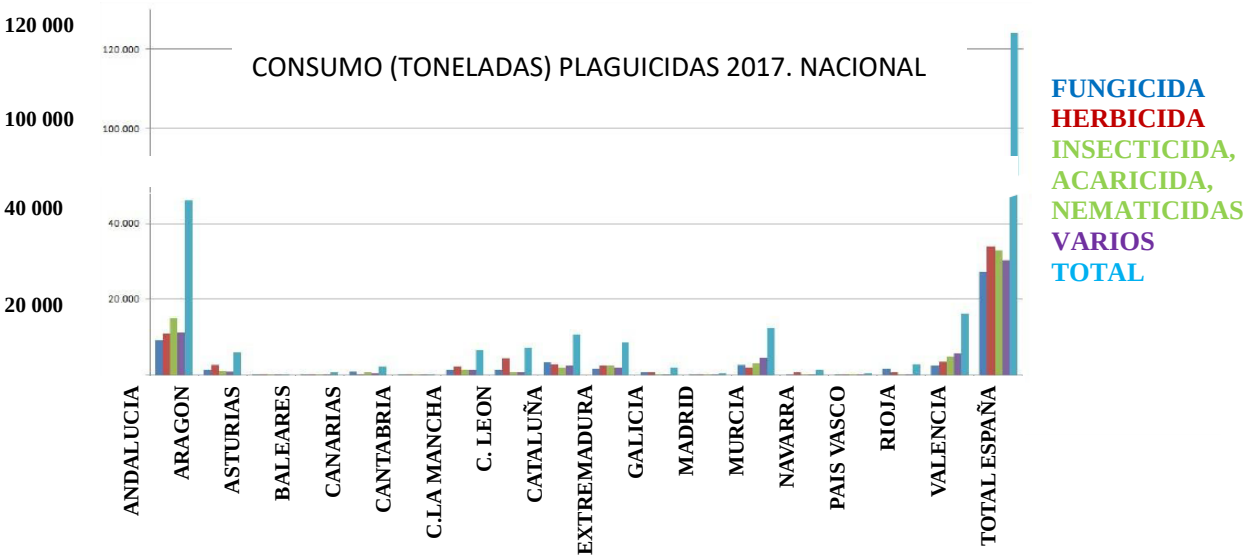

Figura 3. Consumo en Toneladas de Fitosanitarios 2017. Ámbito: España. IMA18

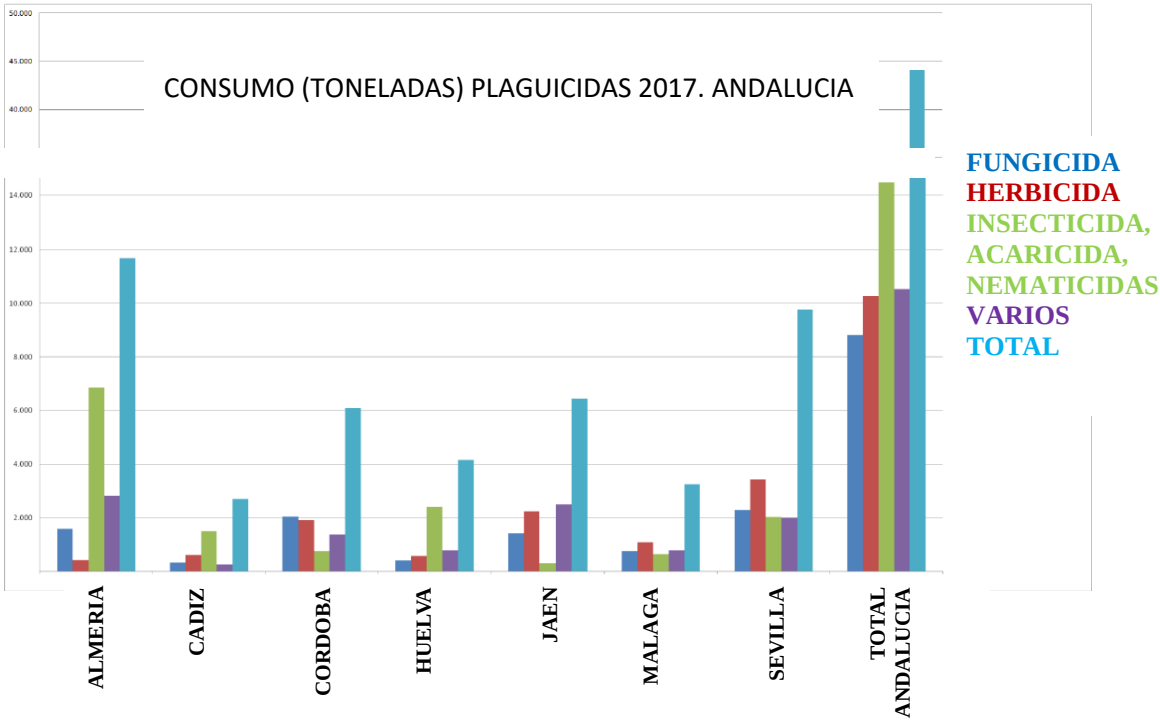

Figura 4. Consumo en Toneladas de Fitosanitarios 2017.

Ámbito: Andalucía. IMA18

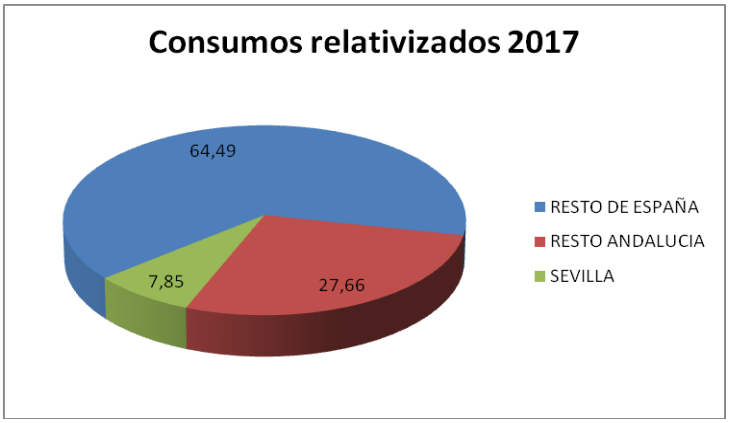

Figura 5. Consumo en la Provincia de Sevilla, en relación con el resto de Andalucía y el territorio nacional. IMA18

5. Exposición laboral a plaguicidas

La exposición prolongada a plaguicidas puede ser perjudicial para el hombre, alterando la función de diferentes órganos en el cuerpo. A este respecto, existe una creciente evidencia sobre el vínculo de la exposición a plaguicidas con la incidencia de enfermedades crónicas humanas, incluyendo cáncer, Parkinson, Alzheimer, esclerosis múltiple, diabetes, envejecimiento, enfermedades cardiovasculares y enfermedad renal crónica (**Mostafalou & Abdollahi, 2013**). Además, la exposición a mezclas de estos contaminantes puede tener múltiples efectos acumulativos, aditivos o sinérgicos en la salud humana. El estrés oxidativo ha sido propuesto como el mecanismo que vincula la exposición a plaguicidas y un mayor riesgo para el desarrollo de estas

enfermedades. Además de un aumento de la producción de radicales libres, la exposición a estos contaminantes y sus mezclas también puede afectar a los niveles de enzimas antioxidantes, así como aumentar la peroxidación lipídica.(**Domingo-Relloso et al., 2019; Hilgert Jacobsen-Pereira et al., 2018**)

Para el ser humano, las vías de entrada más frecuente de los plaguicidas son la cutánea, la respiratoria y la digestiva.

En el mundo laboral, la peligrosidad que supone el uso y la exposición a plaguicidas se encuentran más centrados en los trabajadores encargados de su aplicación, pero podemos considerar una exposición laboral a plaguicidas a las actividades como la fabricación y formulación del plaguicida, su transporte y almacenamiento, su venta o su aplicación, ya sea a pie o máquina en el campo, la aérea o en espacios cerrados. También consideramos exposición laboral a la que sufren los trabajadores de las zonas tratadas, es decir, aquellos trabajadores que se encuentran expuestos a diario a ellos, tanto en la recolección como en la manipulación posterior de los cultivos (**INSHT, 2015**).

El riesgo al que se enfrentan los trabajadores viene definido, además de por el tipo de exposición, por las características de la exposición sufrida. Las características de la exposición pueden ser el tipo de producto, la forma en la que se presenta el mismo, el modo en el que se usa el plaguicida, los hábitos del aplicador como son las medidas de contención o protección usadas y las actividades posteriores al tratamiento (**INSHT, 2015**)

La Organización Mundial de la Salud (OMS) estima que el 70% de las intoxicaciones por plaguicidas en el mundo resultan de una exposición laboral, causando daños a millones de trabajadores agrícolas cada año (**Dalmolin et al., 2020**). De hecho, la Organización Internacional del Trabajo clasificó la actividad agrícola como uno de los tres sectores profesionales más peligrosos junto con la minería y la construcción (**FAO, ILO, UITA, 2007**).

La normativa en materia de Prevención de Riesgos Laborales (PRL) se ha ido desarrollando, y multiplicando, con el fin de conseguir lo que la Ley de PRL describe (**B.O.E., 1995**). De acuerdo con esta ley y dentro de la especialidad de la higiene industrial, y, más concretamente, en lo referido a los agentes químicos, desde el año 1995, se ha desarrollado una batería importante de normativas, con el fin de evitar que la salud de los trabajadores se vea mermada en su puesto de trabajo como consecuencia de una exposición inadecuada a ellos. En base a esta normativa, se han desarrollado nuevos protocolos de protección dirigidos a agricultores. Sin embargo, hay algunos trabajos agrícolas tradicionales como la recolección manual de frutas y verduras que no están suficientemente protegidos por esta legislación. La mayoría de las leyes sobre Salud y Seguridad en el Trabajo se refieren a los trabajadores que transportan, almacenan y aplican plaguicidas (**Martin-Reina et al.**, en prensa).

Pero no solo la legislación, sino también la mayoría de los estudios epidemiológicos se centran en los efectos de los plaguicidas en agricultores que manejan estos químicos, y no en

recolectores de frutas y verduras. Esta situación es aparentemente normal, ya que los fabricantes establecen un período de tiempo de seguridad, tras la aplicación del producto, en el que el trabajador debe evitar entrar en contacto con el mismo. Pasado este tiempo, el fabricante garantiza que el campo, el cultivo tratado y el medio ambiente están libres de residuos de biocidas y que no existe riesgo de exposición. Sin embrago, la EFSA publicó en 2014 la Guía sobre la evaluación de la exposición de operadores, trabajadores, residentes y transeúntes en la evaluación de riesgos para productos fitosanitarios donde indica que los términos "Coeficientes de transferencia" (TC) (la transferencia de residuos de la superficie de la planta a la ropa o la piel del trabajador), "Residuos foliares desplazables" (DFR) (cantidad de residuos que se eliminan del follaje después de ser aplicado) y Reingreso (cuando el trabajador, operador, transeúnte o residente va nuevamente al lugar donde se ha aplicado) deben considerarse para comprender el riesgo de exposición a plaguicidas después de su aplicación (**EFSA, 2014**). Este documento se reflejó posteriormente en los "Criterios de Evaluación de la estimación de la exposición a productos fitosanitarios de los operarios, trabajadores, residentes y transeúntes" (**Ministerio de Sanidad, 2017**) y recogido en "Prevención de Riesgos Durante el uso de productos Fitosanitarios" (**INSSBT, 2017**). Teniendo esto en cuenta y teniendo en cuenta que el Instituto Nacional de Seguridad y Salud en el Trabajo (INSST) demostró la presencia de residuos de plaguicidas en los guantes externos de los agricultores que estaban recolectando tres cultivos

de invernadero una vez pasado el tiempo seguro para la reentrada (**Abril Muñoz, 2017**), se podría concluir que hay riesgo a exposición química de fitosanitarios en personas que re-entran en cultivos para hacer tareas de recolección.

Aquellos trabajadores expuestos en su ambiente laboral a estas sustancias son una población que proteger. Así, existe una abundante legislación relativa a la protección del trabajador que manipula los plaguicidas (transporte, almacenamiento y aplicación), sin embargo, no se le da suficiente importancia a la protección de aquellos trabajadores encargados de la recolección de verduras y frutas. En España, el Instituto Nacional de Seguridad e Higiene en el Trabajo (INSSBT), el órgano científico-técnico especializado en la seguridad y la salud laboral, se encarga de la publicación de documentos denominados notas técnicas de prevención (NTP), en las que se especifican los peligros de los plaguicidas y los elementos necesarios para una protección tanto grupal como personal de los trabajadores. Y son los técnicos de prevención de riesgos laborales (TPRL) los encargados del difícil reto de conseguir que el trabajador no tenga accidentes, ni enferme como consecuencia de su trabajo; en este caso concreto que el trabajador no enferme debido a la exposición a agentes químicos (B.O.E., 1997).

Una de las misiones fundamentales de la figura del TPRL es preguntarse si, en ambientes laborales donde la exposición a distintos tipos de productos (químicos y/o biológicos) es indirecta, dicha exposición está controlada y, lo más importante, si esta

exposición indirecta no afecta a la salud de los trabajadores. Situaciones donde hacernos esta pregunta las encontramos en diferentes campos, como, por ejemplo: en el sector médico, la exposición indirecta de personal médico a productos químicos como el formaldehido, glutaraldehido o anestésicos; o como en el caso del presente estudio donde nos hemos centrado en la exposición laboral indirecta a plaguicidas. Es por ello, que conocedores de la amplia normativa y documentación que ya existe sobre la peligrosidad de la exposición directa a plaguicidas, relativa principalmente a los trabajadores que los aplican, hemos querido extender el estudio a los posibles efectos adversos de la exposición indirecta a plaguicidas en una población de mujeres agrícolas que recogen verduras.

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II. JUSTIFICACION y OBJETIVOS

Como se ha pretendido exponer en la Introducción, podríamos decir que los contaminantes ambientales son una seria amenaza para la salud humana, siendo un problema crítico de salud pública que necesita la implementación de medidas de protección, prevención e información. Atendiendo a lo descrito en la introducción, y partiendo de la hipótesis de que la exposición a plaguicidas es peligrosa para la salud de las personas, tanto en su uso directo, como en la exposición indirecta, hemos querido realizar un estudio de investigación en mujeres que se dedican a la recolección de frutos y verduras, "a cielo abierto", dada su mayor vulnerabilidad, por los siguientes motivos:

i) Porque en la recolección no se tiene la noción de peligrosidad de los plaguicidas aplicados anteriormente. Cuando se recoge, sí se tiene en cuenta el tiempo de espera indicado por el fabricante, pero no se tiene en cuenta el producto químico que se pudiera desprender en la recolección.

ii) Porque de acuerdo con el punto anterior, de manera general no se utilizan equipos de protección individual, como si se estuvieran aplicando biocidas.

iii) Porque, centrándonos en la mujer trabajadora expuesta a estos compuestos, más vulnerable a los posibles efectos perjudiciales de los plaguicidas, estaremos estudiando como afecta esta exposición a la fertilidad, procreación y desarrollo de la descendencia.

iv) Porque en España existen muy pocos estudios epidemiológicos que investiguen la exposición laboral a plaguicidas de manera indirecta, y, los que existen, están centrados en trabajadores de invernaderos en Almería, expuestos durante los períodos de aplicación **(García García et al., 2016; Lozano-Paniagua et al., 2018)**.

v) Para intentar ayudar a los profesionales de la salud a detectar mejor a

los pacientes en riesgo de sufrir algunas de estas patologías y poder prevenir y/o limitar los factores agravantes y tratarlos lo antes posible.

El estudio se va a llevar a cabo en mujeres trabajadoras de Marinaleda (Sevilla). Marinaleda es un pueblo situado en la provincia de Sevilla, en la cuenca del Genil en la comarca de Sierra Sur. Tiene una extensión de 24,8 km^2 y una población total de 2.665 habitantes. De la población total, un 65,2% se encuentran en edades comprendidas entre los 20 y los 65 años, de los cuales 1.299 son mujeres, representando un 49% de la población.

El motivo de esta elección se fundamenta en que Marinaleda presenta una organización política y social, que la hacen única e idónea para la consecución de los objetivos de este trabajo. Algunas de sus características principales son:

a) La población de Marinaleda es eminentemente agrícola, en las que las mujeres son las encargadas de recolectar las frutas y verduras.

b) Se trata de un municipio eminentemente agrícola, cuya economía se basa en la producción agropecuaria.

c) El sector primario se basa en el trabajo en la finca “El Humoso”.

d) El sector secundario está basado en la elaboración de productos agrícolas en la cooperativa “Marinaleda SCA” (https://www.marinaleda.coop/) .

e) Toda la población tiene un puesto de trabajo.

f) Existe igualdad de salario para todos, sin importar el puesto que ocupe.

Nota aclaratoria:

La cooperativa “Marinaleda SCA” supone uno de los ejes centrales de actividad en el pueblo. Se trata de una cooperativa existente desde finales de los años 70, controlada por los propios trabajadores.

La finca o cortijo “el Humoso” tiene una extensión de 1.200 hectáreas, situado en la comarca de Sierra Sur, a 11,5 Km de Marinaleda, donde se desarrolla la agricultura.

Según datos de 2015, la agricultura supone una de las principales actividades económicas. Hay tanto cultivos herbáceos, que suponen una superficie de 812 hectáreas, como cultivos leñosos, que tiene una superficie de 1.207 hectáreas. En el caso del cultivo leñoso, el principal cultivo es la aceituna. También hay otros tipos de cultivo, como el pimiento o la alcachofa.

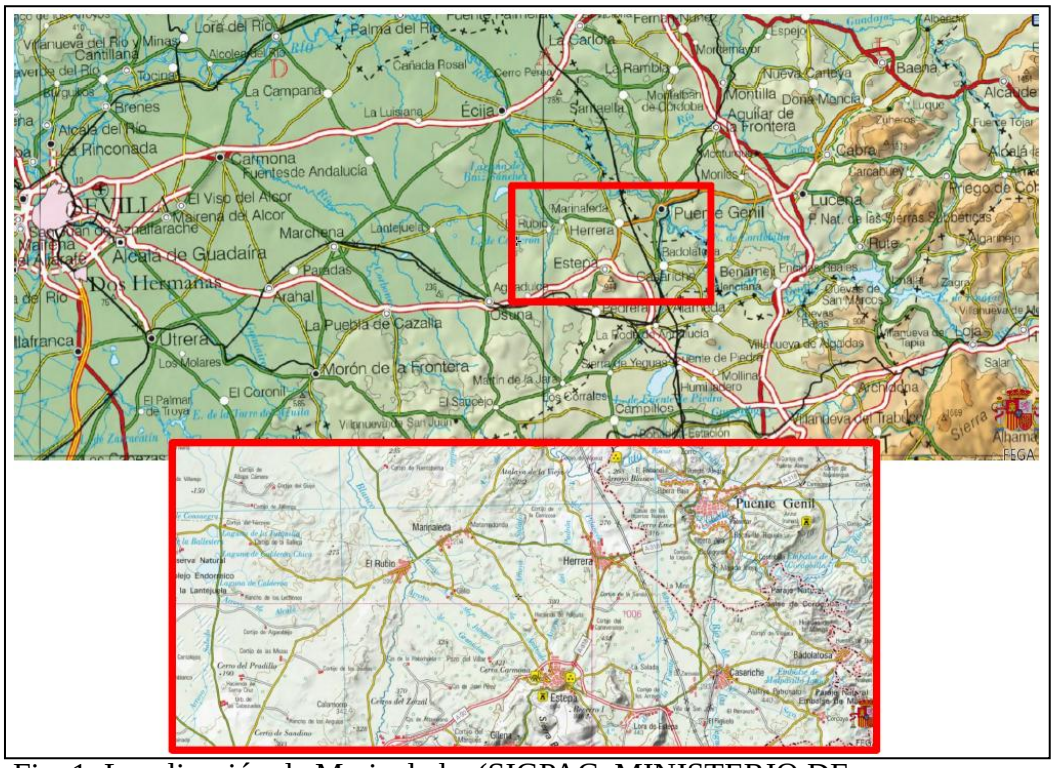

Fig. 1. Localización de Marinaleda. (SIGPAC. MINISTERIO DE AGRICULTURA PESCA Y ALIMENTACION)

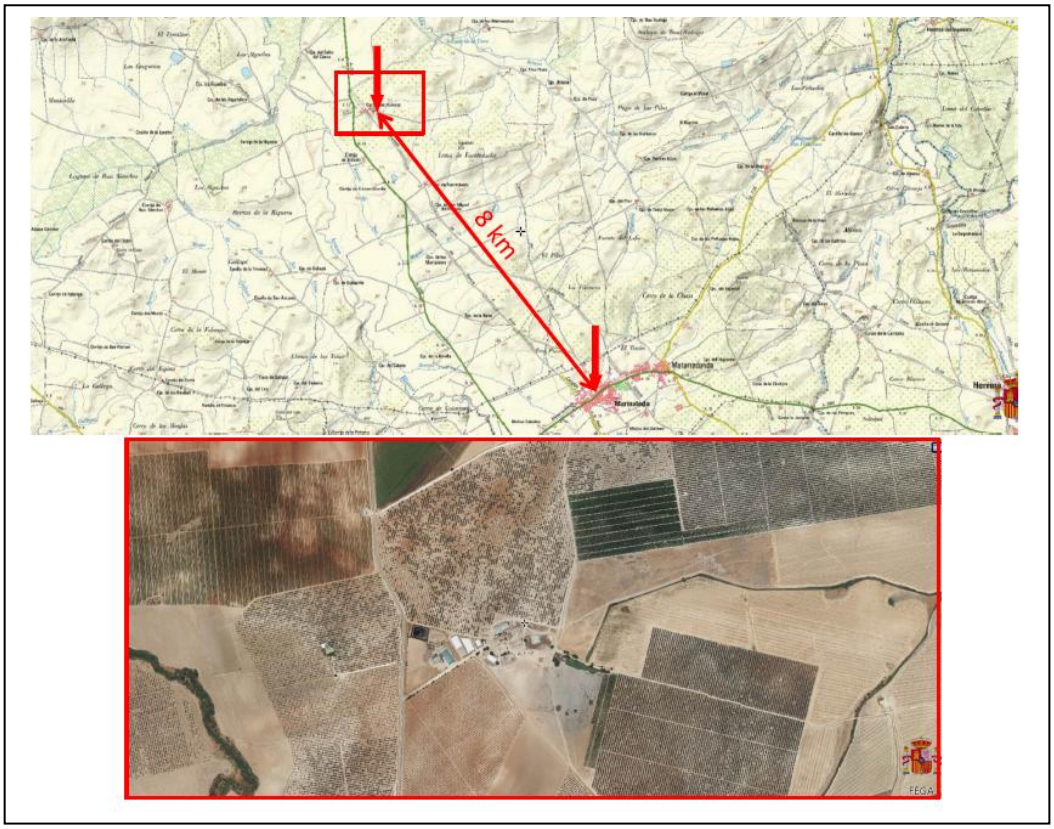

Fig. 2. Distancia Marinaleda-El Humoso y detalle en satélite de las parcelas agrícolas. (SIGPAC. MINISTERIO DE AGRICULTURA PESCA Y ALIMENTACION)

OBJETIVOS

El objetivo principal de este estudio ha sido el de comprobar si las trabajadoras que recogen verduras y frutas en el campo a “cielo abierto” ven alterados sus parámetros biológicos, y en consecuencia su estado de salud, a consecuencia de la aplicación previa de fitosanitarios en los productos recogidos.

Para conseguir este objetivo principal nos hemos apoyado en varios objetivos secundarios:

a) Realizar un análisis sobre los mecanismos tóxicos de los plaguicidas más utilizados, así como los efectos que pueden generar en trabajadores con exposición indirecta, poniendo especial atención a mujeres y su reentrada en cultivos, tras la aplicación para la recogida de futas y vegetales.

b) Comprobar, mediante análisis, en qué estado se encontraban algunos valores clínicos de las trabajadoras con las que hemos llevado a cabo el estudio: marcadores de la función renal, marcadores de la función hepática, hemos analizado la colinesterasas, las hormonas tiroideas, las hormonas relacionadas con la función tiroidea y las hormonas relacionadas con la reproducción: FSH, LH y AMH. Todo esto acompañado de una encuesta epidemiológica.

c) Estudiar los marcadores de estrés oxidativo en lípidos, proteínas y ADN.

d) Profundizar en el análisis de los valores renales, buscando posibles daños renales tempranos, relacionados con la ocupación y el medioambiente en el que se encuentran las mujeres.

e) Validar un método para la determinación de glifosato y su metabolito (AMPA), en orina, mediante UPLC MS/MS

f) Determinar el grado de exposición a glifosato, analizando las muestras de orina del grupo de mujeres.

g) Determinar el valor de metales y metales pesados en sangre en el grupo de mujeres con el que se ha llevado a cabo el estudio.

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III. MATERIAL Y MÉTODO

1. Selección de la población de estudio

La población incluida en el presente estudio ha consistido en un grupo de mujeres trabajadoras (40 mujeres) del pueblo sevillano de Marinaleda dedicadas a la recogida y manipulación de frutas y verduras.

Marinaleda es un pueblo situado en la provincia de Sevilla, en la cuenca del Genil en la comarca de Sierra Sur. Tiene una extensión de 24,8 km^2 y una población total de 2.665 habitantes. De la población total, un 65'2% se encuentran en edades comprendidas entre los 20 y los 65 años, de los cuales 1.299 son mujeres, representando un 49% de la población.

La elección del pueblo se basa en su organización política y social, que la hacen única e idónea para la consecución de los objetivos de este trabajo. Algunas de sus características principales son:

- Se trata de un municipio eminentemente agrícola, cuya economía se basa en la producción agropecuaria.
    - El sector primario se basa en el trabajo en la finca "El Humoso".
    - El sector secundario está basado en la elaboración de productos agrícolas en la cooperativa "Marinaleda SCA".
- Toda la población tiene un puesto de trabajo.

- Existe igualdad de salario para todos, sin importar el puesto que ocupe.

La cooperativa “Marinaleda SCA” supone uno de los ejes centrales de actividad en el pueblo. Se trata de una cooperativa existente desde finales de los años 70, controlada por los propios trabajadores.

La finca o cortijo “el Humoso” tiene una extensión de 1.200 hectáreas, situado en la comarca de Sierra Sur a 11’5 km de Marinaleda donde se desarrolla la agricultura.

Según datos de 2015, la agricultura supone una de las principales actividades económicas. Hay tanto cultivos herbáceos, que suponen una superficie de 812 hectáreas, como cultivos leñosos, que tiene una superficie de 1207 hectáreas. En el caso del cultivo leñoso, el principal cultivo es la aceituna. También hay otros tipos como el pimiento o la alcachofa.

En este estudio, concretamente, contamos con la participación de una muestra (n = 40) de mujeres de esta población, comprendidas en un rango de edad entre 18 y 45 años, que trabajan en la recolección de futas y verduras en la finca “El Humoso” y en la cooperativa “Marinaleda SCA”.

Los criterios de inclusión que se utilizaron fueron: mujeres cuya edad estuviera comprendida entre 18 y 45 años; residentes en Marinaleda durante al menos los últimos 10 años, y que hubieran firmado el informe de consentimiento y completado el cuestionario

epidemiológico. Se excluyeron del estudio aquellas mujeres con hepatopatías o en tratamiento con anticoagulantes orales, ya que pueden interaccionar con los plaguicidas. Además de aquellas que usen anticonceptivos hormonales y las que no tengan un ciclo menstrual regular. Nos centramos en limitar la edad para focalizar el objetivo de centrarnos en el periodo fértil de estas trabajadoras expuestas de forma crónica a cantidades traza de plaguicidas

Las participantes (n = 40) se dividieron en dos grupos, aquellas que durante el estudio trabajan en la cooperativa (n = 20) y las que trabajan en la finca (n = 20), encargadas de la recolección. Durante los doce meses en los que se desarrolla el estudio, dividido en cuatro tomas de muestras, una inicial y otras tres de forma trimestral, algunas de ellas han pasado de trabajar en la finca a hacerlo en la cooperativa o al revés.

Inicialmente, se realizó una entrevista con las mujeres en la que se les informa sobre los objetivos del proyecto y se procedió a la firma del consentimiento informado. Posteriormente, se realizaron cuatro extracciones de sangre y toma de muestra de orina. La primera de las extracciones se realizó en octubre del 2017, la segunda, tercera y cuarta se realizaron en 2018 (febrero, junio y octubre).

En cada una de las tomas de muestras y en la entrevista inicial se llevaron a cabo encuestas epidemiológicas sobre la salud general de las mujeres. Estas encuestas fueron desarrolladas por el equipo de investigación para este proyecto en concreto, con el

asesoramiento de la Dra. Horno, médico del trabajo y colaboradora del mismo.

En la primera de las encuestas se buscaba establecer un conocimiento base sobre cada una de ellas, desde lugar de procedencia y residencia, hasta problemas de salud, pasando por hábitos alimenticios y datos laborales que pudieran tener relevancia en el estudio. Los otros cuatro cuestionarios, realizados durante la toma de muestras, estaban más enfocados a problemas de salud tanto de carácter reciente como pasados o antecedentes familiares de algún tipo, así como posibles cambios observados entre tomas, que se pudieran relacionar con la exposición a los plaguicidas.

2. Análisis hematológicos

Los análisis hematológicos se han llevado a cabo en sangre total. La extracción de sangre se realizó en tubos de extracción por el sistema de vacío BD Vacutainer®: El tubo con contenido de ácido etilendiaminotetraacético (EDTA), el anticoagulante de elección para las pruebas de hematología, se destinó para las determinaciones de hemograma, con sangre total.

La hematología se llevó a cabo con un analizador hematológico automatizado Sysmex XN-100^{TM} (Sysmex España, Barcelona) en el laboratorio del Hospital Comarcal de Osuna (Servicio Andaluz de Salud), según el método optimizado por el hospital. Los parámetros analizados han sido:

Porcentaje de Linfocitos y recuento (%)

Porcentaje de Hematíes y recuento (%)

Hemoglobina (g/dl)

Hematocrito (%)

Volumen Corpuscular medio

Hemoglobina Corpuscular media

Hematocrito Corpuscular medio

Plaquetas (x 10^3/µl).

Volumen plaquetario medio

Distribución plaquetaria

3. Análisis bioquímicos

Los análisis bioquímicos se han llevado a cabo en suero. Al igual que en el estudio anterior, la extracción de sangre se realizó en tubos extracción por el sistema de vacío BD Vacutainer®, pero en este caso los tubos no contienen anticoagulante (EDTA), conteniendo en su lugar un activador de la coagulación, lo cual nos permite obtener el suero tras un periodo de 30 min para la coagulación y centrifugación a 3.000 x g durante 5 min.

El suero así obtenido se destinó para el análisis bioquímico general (Transaminasas hepáticas (GPT/ALT y GOT/AST), marcadores de la función renal (urea y creatinina), y marcadores de daño muscular (creatina quinasa).

Estas pruebas fueron realizadas mediante técnicas espectrofotométricas visible-ultravioleta en un equipo automatizado Vital Scientific-Selectra XL (Vital Scientific, Brentwood, USA), en colaboración con el laboratorio de Análisis Clínicos del Hospital Comarcal de Osuna (Servicio Andaluz de Salud) acorde a los métodos validados en dicho hospital.

La transaminasa hepática (GPT/ALT) se ha determinado de acuerdo con el método de Murray R. Alanine aminotransferase. Kapplan A et al Clin Chem The CV Mosby Co. St Louis. Toronto. Princeton 1984; 1088-1090 (L. Kaplan et al., 1984) La alanina aminotrasferasa (ALT) inicialmente llamada transaminasa glutámico pirúvica (GPT) cataliza la transferencia reversible de un grupo amino de la alanina al α-cetoglutarato con formación de glutamato y piruvato. El piruvato producido es reducido a lactato en presencia de lactato deshidrogenasa (LDH) y NADH:

Alanina + α-cetoglutarato ----(1)--→Glutamato+Piruvato

Piruvato + NADH+ H^+ --------(2)---→ Lactato + NAD^+

(1): ALT; (2): LDH

La velocidad de disminución de la concentración de NADH en el medio, determinado fotometricamente, es proporcional a la concentración catalítica de ALT en la muestra ensayada

La GOT/AST se ha determinado de acuerdo con el método de Murray R. Aspartate aminotransferrase. Kapplan A et al Clin Chem The CV Mosby Co. St Louis. Toronto. Princeton 1984; 1112-

116 (L. Kaplan et al., 1984). La aspartato aminotransferasa (AST) inicialmente llamada transaminasa glutamato oxaloacética (GOT) cataliza la transferencia reversible de un grupo amino del aspartato al α-cetoglutarato con formación de glutamato y oxalacetato. El oxalacetato producido es reducido a malato en presencia de malato deshidrogenasa (MDH) y NADH:

Aspartato + α-cetoglutarato -(1)--→Glutamato + Oxalacetato

Oxalacetato + NADH+ H^+ --------(2)---→ Malato + NAD^+

(1): AST; (2): MDH

La velocidad de disminución de la concentración de NADH en el medio, determinada fotometricamente, es proporcional a la concentración catalítica de AST en la muestra ensayada

- Urea

Los niveles de urea en suero se ha analizado por el método de la ureasa descrito por Kaplan (**A. Kaplan, 1969**), con las correspondientes modificaciones para su adaptación al autoanalizador Vital Scientific-Selectra XL (Vital Scientific, Brentwood, USA). El método se fundamenta en la descomposición de la urea por la ureasa en dióxido de carbono (CO_2) y amoníaco (NH_3). El amoniaco formado se une al α-cetoglutarato por acción de la glutamato-deshidrogenasa (GLDH) con la oxidación paralela del NADH a NAD^+:

Urea + H_2O + $2H^+$ --------(1)--------→2 NH_3 + CO_2

2 NH_3 + α-cetoglutarato + NADH -(2)-→

-(2)-→ H_2O + NAD^+ + L-Glutamato

(1): Ureasa; (2): GLDH

La disminución de la concentración de NADH o el aumento de la concentración de NAD^+ en el medio de reacción es proporcional a la concentración de urea de la muestra ensayada.

- Creatinina

Los niveles de creatinina en suero se han analizado por el método de Jaffe de 1971 y Kaplan (**Delanghe & Speeckaert, 2011; L. Kaplan et al., 1984)** adecuadamente modificado para su adaptación al Vital Scientific-Selectra XL (Vital Scientific, Brentwood, USA). El método se fundamenta en la reacción de la creatinina en condiciones alcalinas (pH > 12) con los iones picrato y la consiguiente formación de un complejo rojizo. La velocidad de formación del complejo medido a través del aumento de la absorbancia a λ = 492 nm, en un intervalo de tiempo prefijado es proporcional a la concentración de creatinina en la muestra analizada.

Creatinina + Ácido pícrico ----------→ Complejo de adición rojo

(pH > 12; temperatura: 37ºC)

- Creatina quinasa

Se determinó por el método de (**L. Kaplan et al., 1984**) adecuadamente modificado para su adaptación al equipo. La creatina quinasa (CK) cataliza la transferencia reversible de un grupo fosfato de la fosfocreatina al ADP. Esta reacción se acopla con otras catalizadas por la hexoquinasa (HK) y por la glucosa-6-fosfato deshidrogenasa (G6F-DH):

Fosfocreatina+ ADP ---(1)-----→Creatina + ATP

ATP+ Glucosa----(2)---→ ADP + Glucosa-6-fosfato

Glucosa-6-P + $NADP^+$-(3)-→ 6Fosfogluconato + NADPH + H^+

(1): CK; (2): HK; (3): G6F-DH

La velocidad de formación de NADPH, determinado fotometricamente, es proporcional a la concentración catalítica de CK en la muestra ensayada.

4. Análisis de hormonas

Al igual que los estudios anteriores, la extracción de sangre se realizó en tubos extracción por sistema de vacío BD Vacutainer®. Uno de los tubos contenía un activador de la coagulación, por lo que, posteriormente se pudo separar el suero del resto de componentes sanguíneos por centrifugación. Este tubo, se destinó para el análisis de hormonas.

Las hormonas tiroideas (TSH y T4L) y hormonas gonadotrópicas (FSH y LH), se analizaron con equipo automático, autoanalizador CL-1000i (Mindrai, Shenzhen, China), mediante

inmunoensayo-quimioluminiscencia. La hormona antimulleriana AMH se determinó mediante técnica ELISA con el kit AMH gen II (Beckman Coulter, USA) de acuerdo con las instrucciones del fabricante.

5. Peroxidación lipídica

La extracción de sangre destinada a este estudio se realizó en tubos BD Vacutainer® que contenían un gel coagulante separador, que se destinó a la separación de la sangre con la finalidad de obtener suero. Para ello las muestras se centrifugaron hasta su completa separación, obteniendo una fase de suero y otra de paquete globular y una interfase, en la que se encontraban el gel. De las muestras de suero obtenidas, se hicieron dos alícuotas de 2ml, en tubos eppendorf. Todas las muestras fueron conservadas en un congelador a -78ºC hasta su utilización.

Para llevar a cabo la determinación de la oxidación lipídica, se siguió el protocolo de Esterbauer y Cheeseman (**Esterbauer & Cheseman, 1990**), el cual mide los niveles de malondialdehido (MDA) mediante el método del ácido tiobarbitúrico. Los reactivos utilizados en esta prueba fueron: ácido acético al 20%, una disolución acuosa 40 µM de TEP (1,1,3,3-tetraetoxy-propano, 97%), SDS (dodecilsultafo sódico) al 8%, butilhidroxitolueno (BHT) al 1% y ácido tiobarbitúrico (ATB), preparado al 0,8% (extemporáneo). Los resultados obtenidos se expresan en nmol/mg proteínas.

6. Determinación del grado de exposición a glifosato.

Las muestras de las participantes fueron recogidas de su primera orina de la mañana. Los envases fueron entregados por parte de nuestro equipo de investigación, de una toma para su utilización en la siguiente. Las muestras de orina fueron congeladas hasta la validación del método

7. Validación de método de determinación de glifosato y AMPA en orina por UPLC-MS/MS

Para llevar a cabo la validación del método se utilizaron muestras de orina sintética libre de glifosato y su metabolito AMPA, ambos objeto de este estudio. Se ha adaptado el método en agua de la nota técnica de Waters y el validado por (**Demonte et al., 2018**) por UPLC-MS/MS en muestras de agua.

Fase de extracción y derivatización

Tanto el glifosato como su metabolito AMPA son compuestos muy polares que requieren de una derivatización previa para poder ser detectados por UPLC-MS/MS. En la Figura 8 se puede observar la reacción de derivatización con FMOC-Cl, el cual es el reactivo más usado por reaccionar a temperatura ambiente tanto con aminas primarias como aminas secundarias, sin necesidad de oxidación previa.

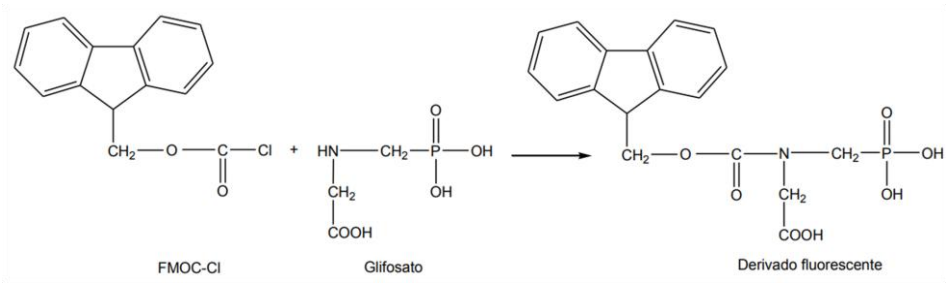

Figura 1. Reacción de derivatización entre glifosato y FMOC.

Debido a este mecanismo de reacción, uno de los parámetros que tuvimos que optimizar fue la concentración de FMOC, debido a la presencia en la orina de una alta concentración de compuestos con grupos amino en su estructura (urea y ácido úrico). Además se optimizaron la cantidad de orina y el estado de la muestra (líquido *vs* liofilizado).

La metodología adoptada finalmente consta de dos etapas y se describe a continuación:

1. *Pre–tratamiento de la muestra:*

Se toma 1 ml de muestra de orina previamente homogenizada y se le adicionan las concentraciones correspondientes de cada uno de los analitos (glifosato y AMPA) como patrones internos. Posteriormente se liofiliza durante 24 horas.

2. ***Etapa de derivatización:***

Esta etapa comienza con la adición de buffer borato 5% para lograr un pH de 9 en el cual se desarrolla la reacción de derivatización, seguidamente el reactivo derivatizante, FMOC a una concentración optimizada de 8 mg/mL. Se deja reaccionar treinta minutos a 60ºC. Una vez pasado el tiempo, se añaden 250 µL de HCl 6M, pH 1 para terminar la reacción de derivatización. A continuación, se centrifugan las muestras y se toma una alícuota y se filtra con filtros de jeringa de 0,2 µm. Tras este paso, las muestras están en condiciones de ser inyectadas en el equipo UHPLC-MS/MS empleado.

Condiciones Cromatográficas

El análisis LC-MS/MS se realizó utilizando un cromatógrafo líquido de ultra alta resolución (ACQUITY UPLC™, Waters, Milford, MA, EE.UU.) acoplado a un espectrómetro de masa triple cuadrupolo (TQD, Waters Micromass, UK) equipado con una fuente de ionización por electrospray (ESI) capaz de operar en modo positivo y negativo.

Para la separación de los analitos se utilizó una columna Acquity UPLC BEH® C18 (tamaño de partícula 1,7 µm, 2,1 x 50 mm); la fase móvil consiste en agua (A) y H_2O:ACN (95:5, B) utilizando acetato de amonio como modificadores; y se comparó

ESI en modo positivo y negativo. La velocidad de flujo fue de 0,5 mL/min y la temperatura de la columna fue 40°C.

Se seleccionó ESI en modo positivo ya que se obtuvo mayor sensibilidad. En la Tabla 1 se muestran las condiciones de operación de la fuente de ionización.

Tabla 1. Condiciones de operación de la fuente de ionización

PARÁMETROS FUENTE DE IONIZACIÓN	
Temperatura de la fuente	140 ºC
Temperatura de desolvatación	500 ºC
Caudal de gas de desolvatación	600 L/h
Caudal de gas de cono	15 L/h
Voltaje de capilar	1 kV
Voltaje de extractor	1 V

La selección de las condiciones del voltaje del cono para generar el ion precursor y las energías de colisión para obtener cada fragmento específico como así también los iones seleccionados se realizó empleando ensayos de infusión y datos bibliográficos.

El ion precursor, los iones fragmento para cada compuesto específico, junto con sus respectivos voltajes de cono y energías de colisión se muestran en la Tabla 2.

Tabla 2. Ion molecular, valores de m/z de los fragmentos, voltajes de cono y energías de colisión para cada compuesto específico.

Analito m/z	Ion molecular (Cono)	Producto 1 m/z (CE)	Producto 2 m/z (CE)
Glifosato-FMOC	392.0 (20V)	88.1 (30V)	214.1 (10V)
AMPA-FMOC	334.0 (20V)	112.1 (15V)	179.1 (20V)

Una vez desarrollado el método se pasó a validarlo para verificar que cumple con los requisitos necesarios para su posterior aplicación. Así, el método propuesto fue validado tomando en cuenta las pautas para la validación de métodos analíticos (ICH, 2005) con respecto a los parámetros de linealidad, sensibilidad, precisión y recuperación.

8. Determinación de Colinesterasa Eritrocitaria y Plasmática.

La actividad colinesteresa se llevó a cabo en sangre total (eritrocitaria) y en plasma (Plasmática), mediante el método colorimétrico de (**Ellman et al., 1961**) adaptado a microplaca por Clemente et al (2010), utilizando para ello un lector de placas visible- ultravioleta.

9. Determinación de Oxidación Proteica.

La determinación del nivel de oxidación de proteínas se ha llevado a cabo en plasma mediante el método de Levine y colaboradores (**Levine et al., 1990**), cuantificándose

espectrofotométricamente la cantidad de grupos carbonilo (grupos carbonilos/mg proteína).

En este método, la 2,4-Dinitrofenilhidrazina (DNPH) – también llamado Reactivo de Brady- se transforma en 2-4Difenilhidrazona cuando reacciona con los grupos carbonilos de las proteínas en medio acidificado.

El análisis se lleva a cabo en un espectrofotómetro uv, aplicando la ley de Lambert-Beer para determinar las concentraciones de grupos carbonilos/mg proteína.

10. Determinación de Oxidación de ADN

La oxidación de ADN se ha llevado a cabo en sangre total (anticoagulante EDTA) mediante el kit OxiselectTM de cuantificación de daño oxidativo de ADN (Cells Biolabs Inc San Diego CA, EEUU) siguiendo el protocolo, optimizado de matera previa en nuestro laboratorio se obtienen los sitios apurinicos/apirimidinicos por 100000 pares de bases. Los análisis se llevaron a cabo, pero no pudimos obtener resultados, quizás porque fueron los últimos ensayos practicados y la sangre, aunque congelada a muy baja temperatura, pudo haber sufrido daño en el ADN.

11. Daño renal temprano

El daño renal se ha determinado en muestras de orina, en colaboración con la Universidad de Salamanca. Determinándose como marcadores de daño renal temprano, las actividades N-acetil-

β-D-glucosaminidasa (NAG), y lipocalina asociada a la gelatinasa de neutrofilos (NGAL) así como los niveles de Albumina, mediante métodos optimizados por los colegas de la Universidad de Salamanca.

11. Determinación de Metales

Estas determinaciones se realizaron a partir de la sangre total. Las determinaciones se han realizado en el laboratorio de la Universidad de La Laguna (Tenerife). Brevemente, las muestras se han tratado con Nítrico y peróxido de hidrogeno, previamente a la incineración en microondas. Una vez incineradas las muestras, se analizaron por espectrometría de emisión óptica de acoplamiento inductivo a plasma (Inductively Coupled Plasma-Optical Emission Spectrometry, ICP-OES) para determinar los metales, utilizando un equipo de Thermo Scientific, modelo ICAP 6300 Duo. Las condiciones del equipo fueron las siguientes: potencia, 1150 W; flujo de gas nebulizado 0.5 L/min; inyección de la muestra a la bomba de flujo: 50 rpm; tiempo de estabilización: tiempo, 0 s.

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IV.RESULTADOS

CAPÍTULO 1. RELACION ENTRE SALUD Y PLAGUICIDAS EN MUJERES AGRICULTORAS

1. Introduction

A pesticide, according to the Food and Agriculture Organization of the United Nations (FAO) is defined as "*any substance, or mixture of substances of chemical or biological ingredients intended for repelling, destroying or controlling any pest, or regulating plant growth*" (FAO, 2014). The European regulation considers these biocidal products as chemical products, and its use are regulated and controlled by the European Chemical Agency (ECHA).

Phytosanitary, are defined as "*a pesticide product intended for preventing, destroying or controlling any pest causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products*" (**FAO, 2006**). They are considered as an exception, and are regulated by the European Union (EU) document Regulation 1107/2009 (**EU, 2009**). Although FAO definition clearly links phytosanitary and pesticides, the terms are not synonymous because pesticides are a broader category that includes biocides, used to control organisms not involved in plant or crop production.

All these chemicals, once applied, not only affect different pests, but also may have certain risks to humans, animals and the

environment, due to their intrinsic properties and associated use patterns (**EU, 2012**). For this reason, and taking into account the increased use of these products in the last years, new restriction laws and standards about its use including farmer's protections have been developed. However, there are some traditional jobs such as manual collection of fruits and vegetables that are not protected enough by this legislation, because most of the mandatory laws about health and safety in workers are related only to who apply pesticide.

The Spanish National Institute for Health, Safety at Work (Instituto Nacional de Seguridad e Higiene en el Trabajo, INSHT) has published a series of documents on the danger to health that involves the use of biocidal products, as well as regulations related to their use, in order to protect workers both individually and collectively. Some examples are: i) Prevention technical note nº 1033 "Biocidal products: Prevent risk during their use" This document describes how to prevent risk during the use of pesticides (**INSHT, 2015**); ii) "Prevention technical note nº 660 and nº661 "Biological control of workers exposed to pesticides". These documents describe what kind of biological controls are necessary to carry on workers exposed to pesticides to guarantee their health

(**INSHT, 2006a**); iii) "Working exposure to pesticides depending on applying equipment" This document describes the different possibilities of exposure to pesticides when the equipment changes (**INSHT, 2006b**); iv) "Clothes for protection versus pesticides" This document describes the importance of the different kind of clothes based on the pesticides used (**INSST, 2019**).

Summarizing, most of the actual legislations are about the workers that handle the pesticides, *ergo*, people who transport, storage and apply pesticides to the crop. But not only legislation, also most of the epidemiologic studies are focused on the effects of pesticides in farmers who handle these chemicals but not in people who collected the fruits and vegetables. This situation is apparently normal, because the pesticides products have a secure time in which, the worker must avoid to get in contact with the product. But, after the secure time, manufacturer guaranty that the field, treated crop and environment are free of biocidal residues and there is not exposition risk. The European Food Safety Authority (EFSA) published in 2014 the *Guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products* where the terms "Transfer Coefficients" (TC) (the transfer of residues from the plant surface to the clothes or

skin of the worker), “Dislodgeable Foliar Residue” (DFR) (amount of residue that take away from the foliage after being applied) and Re-entry (when the worker, operator, bystander o resident go again to the place that were applied) have to be considered to understand the risk of exposure to biocides after being applied. Taking this into consideration, it was demonstrated that in three crops studied by the Spanish INSST (Instituto Nacional de Seguridad y Salud en el Trabajo), the secure time was enough for the re-entry. For example, the pyridaben was under the quantification limit in ten days (this chemical product has fifteen days of secure time in its safety data sheet). The results were similar with the others two products (**EFSA, 2017**)

However, the EFSA publishes yearly a report with pesticide residues in food (**EFSA, 2019**); in the last report it concluded that the percentage of samples with residues below the limit of quantification (LOQ) remained stable in the EU (52.8% in 2013 to 52.3% in 2016), demonstrating that the overall situation remained stable compared to previous years. From 2299 samples taken in Spain in 2016 63% were under LOQ, 33% between the LOQ and the Maximum Residue Level (MLR) and a 5.4% above the MLR. Taking into account only fruits and vegetables (1491 samples), 50%

were below LOQ, 46% were between the LOQ and the MLR and the 6.8% remaining were above the MLR.

Among farm workers, women are considered by the European legislation (Council Directive 92/85/EEC of 19 October 1992) as "*a specific risk group in many respects and measures must be taken with regard to their safety and health"*. According to this directive is recommended the introduction of measures to encourage improvements in the safety and health at work for pregnant workers and workers who have recently given birth or are breastfeeding. This directive is a specific development of Directive 89/391/EEC art 16 (**EU, 1989**).

Fundamentally, the health consequences of pesticide exposure in females are influenced and dictated by biological factors such as menarche, pregnancy, lactation and menopause which are absent when evaluating risk assessment and toxicological profiles simply considering male representatives. The tendency to develop disorders related to breast cancer, fibroids or reproduction is also unique to women's health, so these chemicals can affect the future generations (**Wahlang, 2018**)

The aim of the present review is to analyze/describe the toxic mechanism of most used pesticides and the deleterious effects that

may appear in workers with indirect exposure to them, specially, women that re-enter in the crop ground to collect fruits and vegetables after the application of plants protection products.

2. Methodology

At first, a specific search was undertaken to locate and review articles related with toxic effects of pesticides and occupational safety. In three different engines searches (Scopus, Web of Science and Pubmed), were introduced the parameters for finding the articles were:

"Carbamate toxics effects women", "Glyphosate toxics effects women", "Mancozeb toxics effects women", "Pyrethroids toxics effects women", "Organophosphates toxics effects women".

From this first step, when we obtained only 49 papers, we widened the search to the bibliography of each document found, according to the purpose of this review.

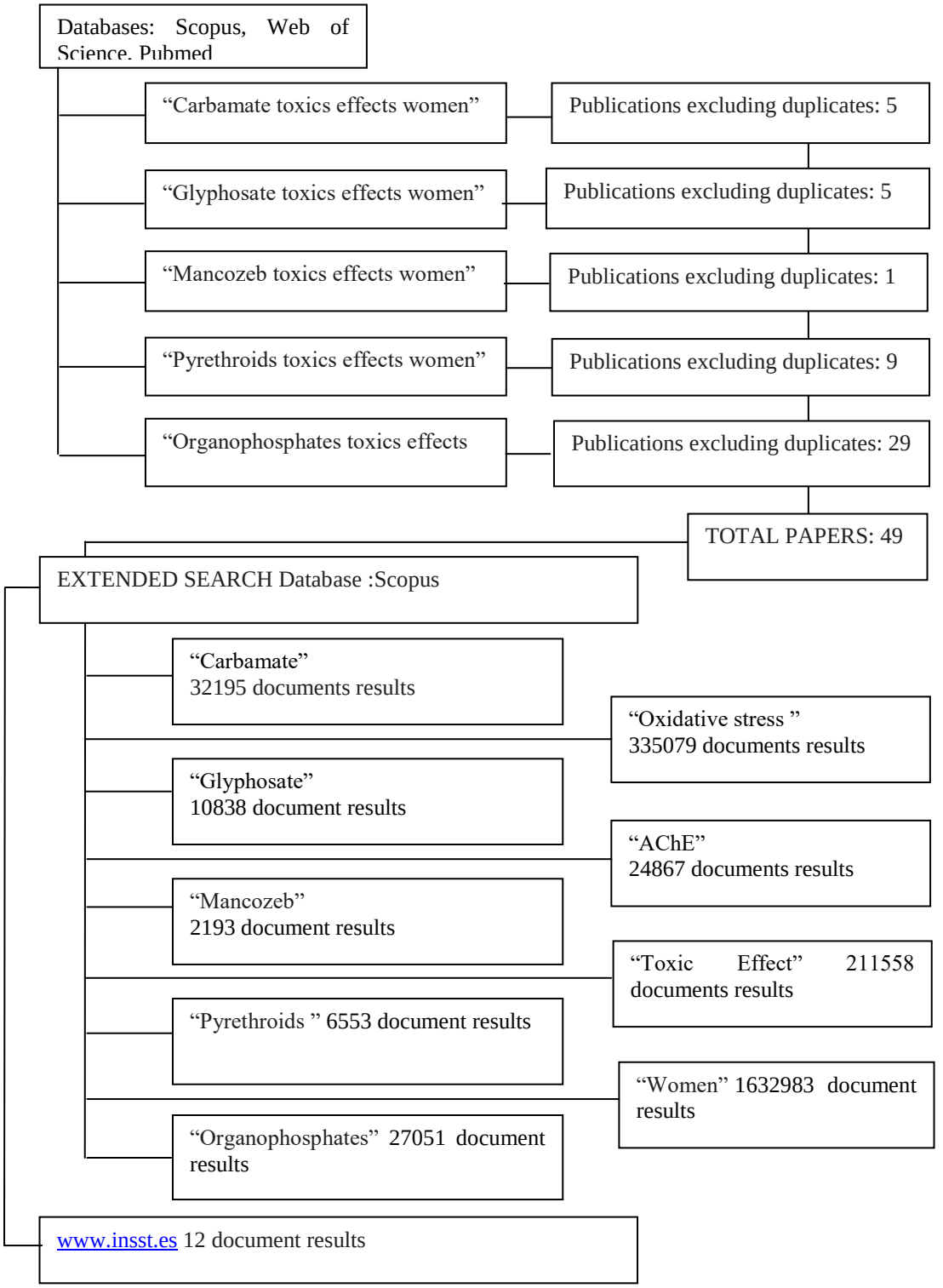
According to the study we are researching, the second step was to widen the search and use different questions in the database web related with pesticides using a combinations of the following terms:

Glyphosate// Organophosphates// Pyrethroids//Carbamates// Mancozeb// Oxidative Stress// AChE//Toxic effects// women //

Also, in the Spanish National Institute of Health and Safety at Work, we had a searching for documents related with pesticides exposure and occupational health in female workers.

By selecting the papers, we considered the main activity of a women group working in the crop and the pesticides used before collecting. Based on this, we selected the fittest papers for this target.

Full-length experimental articles related to pesticides and occupational safety effects related to the activity that the team is researching, were obtained. We used the newest and whose author had other scientific publications about this area. A final number of 78 studies were found relevant that constituted the main structure of the present review.


Databases: Scopus, Web of Science, Pubmed
"Carbamate toxics effects women"
Publications excluding duplicates: 5
"Glyphosate toxics effects women"
Publications excluding duplicates: 5
"Mancozeb toxics effects women"
Publications excluding duplicates: 1
"Pyrethroids toxics effects women"
Publications excluding duplicates: 9
"Organophosphates toxics effects
Publications excluding duplicates: 29
TOTAL PAPERS: 49
EXTENDED SEARCH Database :Scopus
"Carbamate"
32195 documents results
"Oxidative stress "
335079 documents results
"Glyphosate"
10838 document results
"AChE"
24867 documents results
"Mancozeb"
2193 document results
"Toxic Effect" 211558 documents results
"Pyrethroids " 6553 document results
"Women" 1632983 document results
"Organophosphates" 27051 document results
www.insst.es 12 document results

3. Toxicity Mechanism of pesticides most common used

The use of pesticides in Europe increased from 440,804.68 to 483,253.72 tonnes during the period 2000 to 2016. In Spain, during the last year, 61,895 tonnes of active pesticides ingredients were used; of which, 15,225 tonnes of herbicides, 2,280 tonnes of organophosphates, 118 tonnes of pyrethroids and 26 tonnes of carbamates (FAOSTAT website)

Depending on the organism pesticide can be classified in different groups. Thus, the Biocidal Products Regulation (BPR) (**EU, 2012**) bases it classification on the pest target, but pesticide can be also classified by the target organ or their mechanism of action (IRAC website). In this work, as shown in Table 1.

TYPE	PEST TARGET	IRAC Classification	Target Organ
GLYPHOSATE (CLYCINES)	Herbicide	2A	Aromatic Aminoacid route in weeds
PYRETHROIDS (Permethrins)	Insecticide	3A	Nerve Action. Sodium channel modulators
ORGANPHOSPHATES	Insecticide	1B	Nerve Action. AChE inhibitors
CARBAMATES	Insecticide	1A	Nerve Action. AChE inhibitors
MANCOZEB	Fungicide	Not classified	Energetic way of fungi

TABLE 1 Pesticide classification according to BPR and IRAC

3.1. Glyphosate (N-(phosphonomethyl) glycine

Glyphosate (GLY) is the most used herbicide in the world (**Devoren and Schiestl, 2018**). This product is used for removing a wide range of weeds. The exposure to glyphosate occurs when

humans get in contact with this product through the skin, breathing the aerosol or eating contaminated products with glyphosate and/or glyphosate-based formulations products. Acute oral toxicity of technical grade glyphosate is low (LD_{50} ranging from 800 to 5000 mg/kg), however, there is an increasing interest in potential chronic effects of formulated glyphosate and its degradation products as they accumulate in the environment (**Van Bruggen et al., 2018**). In the last years, the controversy about the human safety from glyphosate has grown in Europe. While an assessment carried out by the World Health Organization's International Agency for Research on Cancer, concluded that glyphosate "probably" causes cancer (classified as 2A). Both the European Food Safety Authority (EFSA) and the European Chemicals Agency (ECHA), by contrast, determined the chemical was safe. For this reason in 2017 the European Union decided to renew the license of this herbicide for the next five years.

Glyphosate exerts its action, once absorbed by the weed, acting as a potent inhibitor of 5-enolpyruvyl-shikimate-3-phospate synthase (EPSPS). It binds and blocks the activity of this enzyme which can't act in the synthesis of aromatic amino acids and many other important plant metabolites (**Martinez et al., 2018**). Shikimate

pathway is present in various micro-organisms, plants, and parasites, but is absent in mammals. Thus, the lack of this pathway is the basis for underlying low toxicity of glyphosate in animals (**Agostini et al., 2020; Meftaul et al., 2020**). However, glyphosate-based formulations exhibited different toxic effects to terrestrial organism. In particular, the surfactant, polyethoxylated tallow amine (POEA), present in Roundup formulations is responsible for the observed toxicity toward human health. It appears that POEA is 10,000 times more toxic than GLY in different human cell lines (**Mesnage et al., 2015**). Thus, Benachour and Seralini (**Benachour and Seralini, 2009**) concluded that glyphosate-based formulations induce apoptosis and necrosis in three different human cell types (HUVEC primary neonate umbilical cord; 293 embryonic kidney and JEG3 placental cells). Also, Gasnier et al. (**Gasnier et al. 2009**) concluded that glyphosate based herbicides damage human DNA in an *in vitro* study with human hepatoma cells (HepG2), where endocrine disruption, inhibition in estrogens receptors and transcriptional activities and cytotoxic effects were observed too. This endocrine disruption activity could be associated with the GLY carcinogenetic effects (**Davoren and Schiestl, 2018**). However, only a few studies reported association between GLY exposure and

cancer-related complications but only in univariate analyses. No association was found between GLY and prostate cancer or multiple myeloma and only was suggested a possible correlation between GLY exposure and colon, colorectal, rectal and pancreatic cancer (**Acquavella et al., 2016; Agostini et al., 2020**). Another plausible association between GLY exposure and cancer development was observed with non-Hodgkin's lymphoma (NHL). Some authors such as Acquavella et al. (**Acquavella et al. 2016**) after a systematic review where seven studies about NHL and GLY exposure were evaluated, concluded that there was no relationship between these two variables. Also, Chang and Delzell (**Chang and Delzell, 2016**) reached the same conclusion after a systematic review and meta–analysis of the relationship between GLY exposure and the risk of lymphohematpoietic cancers, including NHL. However, recently, a compelling link between exposures to glyphosate-based herbicides and increased risk for NHL has been suggested in accordance with findings from experimental animal and mechanistic studies and a meta-analysis of human epidemiological studies (**Zhang et al., 2019**).

In order to clearly define the seriousness of glyphosate exposure to carcinogenicity and genotoxicity new studies and independent

research must be performed. In fact, all the discrepancies between the opinions of the various scientific institutions are mainly because of their different economic and social interests (**Torreta et al., 2018**).

3.2. Pyrethroids

Pyrethroids are a group of synthetic insecticides derived from natural pyrethrins, obtained from pyrethrum extract, isolated from the *Chrysanthemun*. These insecticides have been used for more than 50 years and are included in 25% of the world-wide insecticide market (**Shafer et al., 2005**). The activity of pyrethroid insecticides, according to some authors is due to its effect on sodium channel, and has been extensively reviewed (**Scott, 2019**). The principal target of pyrethroids is the sodium channels encoded by voltage-sensitive sodium channel gen codes (**Soderlund et al., 2002; Soderlund, 2012**).

Pyrethroids are classified in different groups or types: type I or type T: without the *α-cyano-3-phenoxybenzyl* group in the molecule (pyrethrin I, resmethrin and permethrin) and type II or type CS: with *α-cyano-3-phenoxybenzyl group* in the molecule (cypermethrin, deltamethrin and fenvalerate) (**Soderlund, 2012**)

Depending on the type, the sodium channel will be open for longer. Type I pyrethroids keeps the voltage-dependent sodium channel open for less time causing disordered cell function. On the other hand, type II pyrethroids maintains the voltage-gated sodium channel (VGSC) open for longer periods of time, causing cell excitation which could lead to membrane depolarization and blockage (**Gammon, 2019**) Therefore, it can be concluded that qualitative differences in sodium channels can be modified depending on pyrethroids types (I or II). These are generally correlated with the production of different intoxication syndromes, suggesting that actions on sodium channels are sufficient to explain the acute toxicity of this insecticide class.

On the other hand, several authors have showed that some type II pyrethroids have the voltage gate chloride channel (VGCC) as target (**Ensley, 2018**), they confirmed that deltamethrin and cypermethrin suppress the state of VGCC. But not only some types II pyrethroids affect the VGCC, also some type I pyrethroids such as bioallethrin can affect these channels.

Several studies have also proved that some pyrethroids can act as endocrine disruptor (**Garey and Wolff, 1998; Jin et al., 2010; Slima, 2017; Singh et al., 2020**). Thus, Garey and Wolff

(**Garey and Wolff, 1998**) probed for the first time that fenvalerate, sumithrin, d-trans allethrin and permethrin were endocrine disruptors *in vitro*, using two cancer cells lines (Ishikawa Var-I human endometrial cancer line and the T47D human breast cancer line). Sumithrin and fenvalerate demonstrated significant estrogenicity activity, comparable to 17-alfa-ethynylestradiol further both d-trans-allethrin and fenvalerate as well, antagonized the action of progesterone. The estrogenic activity of cypermethrin and permethrin, is similar to 17B-estradiol in the MCF-7 human breast carcinoma cell line, was also probed by Jin et al. (**Jin et al., 2010**). In the same way, the estrogenic activity and the disruptive effects on the thyroid function of pyrethroids was demonstrated (**Ghosh, 2018; Hernandez et al., 2020**)

3.3 Organophosphates

Organophosphates are insecticides used in agricultural, homes, garden, veterinary practices and everywhere (EPA/GOV). Exposure to organophosphates usually takes place when people and/or animals get in contact with these products through the skin, breathing the aerosol or eating contaminated foods with organophosphates (**Roberts, 2013**).

The organophosphates most important mechanisms are:

a) Inhibition of acetylcholinesterase (AChE)

The IRAC published in May 2018 the last classification of pesticides based on their mode of action and the organophosphates are classified in Group 1 as Acetylcholinesterase (AChE) inhibitors. Fareed et al. (**Fareed et al., 2017**) described all the symptoms related with the inhibition of AChE observed in exposed workers to organophosphates, such as: nervous system (dizziness, headache and convulsions), ocular system (blurred vision, refractive error, watering and pain in eyes, etc.), gastro-intestinal system (hyperacidity, pain in abdomen and constipation) and others (burning sensation of skin and itching, body aches, etc.).

b) Oxidative stress and apoptosis

Pesticides are not only toxic themselves, they are also implicated in the production of free radicals related with the pathophysiology of others chronic diseases in humans, related with oxidative damages of lipids, carbohydrates, proteins and nucleic acids, including changes of DNA structure (**Ojha et al., 2011**). Thus, Lukaszewicz-Hussain et al. (**Lukaszewicz-Hussain et al., 2010**) described the production of reactive oxygen species (ROS) by the conjugation between organophosphate and cytochrome P450s or by inhibiting

the oxidative phosphorylation inducing a decrease in ATP levels what implicate an increase of ROS production. Later, Fareed et al. (**Fareed et al., 2017**) confirmed that workers, who presented inhibition in AChE and butyrylcholinesterase (BChE), also had alterations in parameters related with the oxidative stress. In this study they found high activity of blood catalase and glutathione peroxidase, as well as low level of blood glutathione and high level of blood malondialdehyde, representing a subclinical state of oxidative stress.

c) Endocrine disruptors

Organophosphates, carbamates, pyrethroids and organochlorines can mimic the estrogenic function, acting as an agonist and promoting a transcriptional activation of estrogen-responsive genes (**Mostafalou and Abdollahi, 2017**). Organophosphates induce endocrine disruption through a multitargeted process at the level of hypothalamo–hypophyseal–gonadal axis (Senthilkumaran, 2015).

3.4 Carbamates

Carbamates are classified as inhibitors of the cholinesterases and IRAC classified them in the Group 1 with organophosphates (IRAC website). The mechanism of AChE Inhibition by carbamates occurs

in a similar way of that described for organophosphates, but in this case the inhibition induced by carbamates is reversible in 30 minutes, while the organophosphates inhibition can be active during hours to days, depending upon the nature of groups attached by the organophosphate (**Fukuto, 1990**).

Bajda et al. (**Bajda et al., 2018**) published that Novel carbamates derivates (Butyl-carbamic acid 3-(4-phenyl-piperazin-1-ylmethyl)-phenylester hydrochloride, Isopropyl-carbamic acid 3-(4-phenyl-piperazin-1-ylmethyl)-phenyl ester hydrochloride, Ethyl-carbamic acid 3-(4-phenyl-piperazin-1-ylmethyl)-phenylester hydrochloride, Dimethyl-carbamic acid 3-(4-phenyl-piperazin-1-ylmethyl)-phenyl ester hydrochloride) are also moderately potent inhibitors of other cholinesterases such as BuChE.

So, the carbamates influence not only the levels of the neurotransmitters but are also responsible for diseases related to the nervous system.

But, on the other hand, carbamates could play an important role in the treatment of Alzheirmer´s diseases (AD) and Parkinson´s Diseases (PD) because they could be used as potential dual inhibitor of AChE and BChE (**Wu et al., 2020**). One of the most characteristic features of AD and PD is the decreasing level of

acetylcholine (**Arumugasamy et al., 2020**). AChE and BuChE hidrolyses acetylcholine, but their inhibition, increases the level of acetylcholine in the brain (**Nordberg et al., 2013**). This is the reason why new carbamates are being studied and compared with rivastigmine as therapy of AD (**Fallah et al., 2020**).

3.5 Mancozeb

Mancozeb is a biocide widely used as fungicide. It is a subclass of dithiocarbamates pesticides that has been probed to induce oxidative stress, apoptosis and DNA-damage in rat cells (**Calviello et al., 2006**). Evidence has shown that pesticides based in mancozeb induce genetic damage in fishs, oxidizing purines and pyrimidines bases in DNA chains (**Marques et al., 2016**). This fungicide can disrupt the normal function of adrenal cortical cells, even triggering the apoptosis in cells (**Bisson and Hontela, 2002**). It has also been described how Mancozeb reduces the T4 plasma levels in a dose-dependent manner (**Kackar et al., 1997; Axelstad et al., 2011**). Paro et al. (**Paro et al., 2012**) described mancozeb toxic effects on mammalian granulose cells (mice, and human) at low concentrations. This description could be the first step to study the effect of the Mancozeb in the human reproductive system.

4. Effects on women farmers health

Agricultural workers represent a potentially vulnerable population, due to a combination of unique social and cultural risk factors as well as exposure to hazards inherent in agricultural work. Most of the researches are focused on operators or workers handling concentrated pesticides, loading, mixing, spraying, and cleaning equipment. However, some authors have been probed that the exposed population is larger than that, and not restricted to applicators, thus long-term exposure to low levels of pesticides can also have chronic effects on health (**Nassar and Ribeiro, 2020**).

Pesticide exposure among agricultural workers have been linked to certain cancers, DNA damage, oxidative stress, neurological disorders, and respiratory, metabolic, and thyroid effects (**Curl et al., 2020**). Specifically, organophosphates and carbamates chronic effects are weight loss, anemia, anorexia, impaired liver function, and delayed neuropathy (**Yushananta et al., 2020**). Among farm workers, women are considered as a risk population due the relationship between agrochemicals and breast cancer, reproductive disorders, birth defects and developmental neurotoxicity in new born (**Girard et al., 2020; Kass et al., 2020**). Thus, organophosphates can induce a reduced head circumference in

infants and a reduced short term memory and attention, among others effects. A cephalic malformation with severe mental retardation in infants whose parents were exposed to the chlorophenoxy herbicide has been reported (**Bjørling-Poulsen et al., 2008**). Shirangi et al. (**Shirangi et al., 2020**) after an epidemiological study in pregnant women who were on paid employment during pregnancy, concluded that maternal occupational exposure to estimated concentrations of pesticides and phthalates were associated with impaired fetal growth. Anaemia during pregnancy increases the risk of bleeding, premature birth, infant death in the womb, stunted fetal growth, giving birth to babies with low birth weight and stunting, perinatal death and reduced body defense (**Eskenazi et al., 2004; Jaacks et al., 2019; Yushananta et al., 2020**).

Also, after a different epidemiologic studies, has been described the relationship between pesticide exposure and chronic diseases such as, cancer, neurodegenerative diseases, cardiovascular and respiratory diseases, diabetes, renal diseases, autoimmune diseases, genetic and epigenetic modifications, endocrine disruption, oxidative stress. Carcinogenicity is the most reported toxicity effect in relation to each class of pesticides, being insecticides the most

related with the incidence of cancer (**Mostafalou et al., 2013; 2017**).

The effects in the female reproductive system have been studied, describing the mechanisms by which pesticides are involved in ovarian cycle disturbance, causing toxicity and fertility problems. The pesticides have a direct damage to the cell´s structure, interfering with the biochemical processes necessary for normal cell function, and generating toxic metabolites. Thus, the pesticides have effect on fertility, spontaneous abortion, stillbirths, premature birth, low weight birth and developmental defects, developmental inmunotoxicity and childhood cancer. Additionally, the pesticides allow the disruption hormonal function (**Bretveld et al., 2006; Frazier, 2008**). A comparison between pesticide exposure and their effects on the reproduction in two groups of women: 281 with a diagnosis of infertility and 216 who had a successful delivery, concluding that working in the agricultural industry had an elevated risk of infertility (**Ma et al., 2019**).

The relationship between the exposure to pesticides and the risk of genotoxicity was also observed. Thus, Dhananjayan et al. (**Dhananjayan et al., 2019**) described the relationship between occupational exposure to pesticides (insecticides, acaricides,

fungicides and herbicides) with low activity of AChE and BChE, as well as an elevated level of DNA damage in a group of women that worked in tea garden. Kori et al. (**Kori et al., 2018**) added a new point of view in farm workers exposed to pesticides. They concluded that cholinergic and non-cholinergic systems where linked to depression, impulsivity and mood disturbances have also found to be affected following pesticide exposure in humans, which could explain an elevated association of pesticide exposure with suicidal ideation and other behavioural alterations. In this sense, a few studies have linked self-reports of exposure to organophosphates with increased depression and anxiety, suggesting that associations may be stronger in women, though the mechanism of this gender difference is unclear (**Suarez-Lopez et al., 2020**).

The study of the relationship between pesticides exposition and effects on endocrine system during pregnancy showed no significant association between 3-phenoxybenzoic acid (3PBA) (pyrethroid metabolite) concentrations and most of the thyroid hormones, only a negative association between pyrethroids exposure and (free triiodothyronine) (FT3) serum level was found (**Hu et al., 2019**). Similarly, Mulder et al. (**Mulder et al., 2019**)

studied the relationship between organophosphates exposition and thyroid hormone levels in cord blood, showing that the organophosphate metabolites in urine were not associated with maternal thyroids hormones.

Congenital anomalies linked to maternal occupational exposure have been described, thus, neural tube defects, congenital heart defects, orofacial clefts, cleft lips or palate and hypospadias are related with pesticides occupational exposure (**Spinder et al., 2019**).

In summary, most of the studies published in the last years confirm the relationship between the occupational exposure to pesticides and health issues. It has been probed that exposure depends on the intensity, frequency, and duration of pesticide contact and that chronic exposure to small concentrations also can produce chronic effects in workers. For these reason not just applicators but all agricultural workers must be trained for safe handling and self-protective of pesticides. Taking into account the increasing number of women active in agricultural and horticultural tasks many will work during their reproductive years and likely be exposed to a variety of pesticides during pregnancy.

Further research is warranted to better understanding of the mechanisms of action of these chemicals and their behavior in humans after a chronic exposure of small concentrations or in an indirect exposure when women farmers collecting fruits and vegetables. Also, epidemiologic studies and animal research are need in order to elucidate the impact on exposure and health effects of the different routes of exposure (dermal, inhalation), interaction between different pesticides, duration of the exposure. All these information will help to identify the needs and challenges faced by this population, with special emphasis on women in their reproductive years.

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CAPÍTULO 2. BIOMARCADORES DE ALTERACIÓN DE DAÑO RENAL TEMPRANO Y SU RELACIÓN CON LA ACTIVIDAD DE LA COLINESTERASA, ESTRÉS OXIDATIVO Y PARÁMETROS HEMATOLÓGICOS Y BIOQUÍMICOS EN MUJERES AGRICULTORAS

1. Introduction

Agrochemicals, including pesticides and fertilizers, are extensively used in agriculture practices to kill pests that harm crops, enhancing agricultural productivity. These chemicals are potentially toxic to some organisms, including humans, and need to be safely used and properly disposed (**Cestonaro et al. 2020**). However, due to their indiscriminate use in a number of applications there is a high risk of exposure to these pesticides through occupational and non-occupational settings (**Kori et al., 2019**). Agricultural workers are among the most vulnerable working populations due to social and cultural risk factors frequently associated with their ethnicity, immigration status, social class, and rural location. In addition, these potential risk factors can be exacerbated by occupational hazards associated with agricultural work (**Curl et al., 2020**). The three major routes of pesticide entry into the farmer's organism are inhalation, ingestion and dermal absorption. Exposure to low-levels of pesticides is known to produce a variety of biochemical changes, some of which may be responsible for the adverse biological effects reported in humans (**Wafa et al., 2013**). Further when in the real life situation is commonly observed the use of mixtures of pesticide which are related to higher incidence of pesticide poisonings and deaths. The interaction of two or more chemicals may exhibit synergistic effects that could potentially cause damage to various organ systems of the body. The type and severity of adverse health effects of pesticides are determined by the individual chemical category, the dose and

the duration of exposure, the exposure route and the use of personal protective equipments—PPE (**Hernandez et al., 2013; Garcia-Garcia et al., 2016; Curl et al., 2020**).

Chronic and acute exposures to pesticides are assessed by the levels of their biomarkers, which are cholinesterase enzymes, acetylcholinesterase activity (AChE) in red bloods cells and butyrylcholinesterase activity (BChE) in plasma. BChE is reduced more rapidly and intensely than AChE, reflecting acute exposure to toxic agents. AChE is, in fact, a more accurate biomarker of chronic and low-intensity exposures (**Fareed et al., 2017; Hilgert Jacobsen-Pereira et al et al., 2018**). Both enzymes levels in blood are considered as biomarkers of the exposure to organophosphate and carbamate pesticides (**Cestonaro et al., 2020**). However, oxidative stress plays an important role in toxicity of a wider range of pesticides including pyrethroid and carbamate insecticides and herbicides as glyphosate (**Fareed et al., 2017; Campuzano-Cortina et al., 2017**). In addition to increasing the production of free radicals, exposure to these pesticides can also affect anti-oxidant capacity and defense mechanisms, as well as increase lipid peroxidation. The by-product most often measured is malondialdehyde (MDA), one of the main lipid hydroperoxides produced by the peroxide degradation of polyunsaturated fatty acids (**Hilgert Jacobsen-Pereira et al., 2018; Lozano-Paniagua et al., 2018**). Thus, oxidative stress has been proposed as a mechanism linking exposure to pesticides to increased risk for the development of diseases such as cancer, renal and neurodegenerative diseases

(**Mostafalou et al., 2013; 2017; Gunatilake et al., 2019; Kass et al. 2020**) and reproductive disorders (**Bretveld et al., 2006; Frazier, 2008; Ma et al., 2019**). Further, alterations in the hematological parameters such as decrease size of red blood cells, higher platelets and white blood cell (WBC) counts and increased activities of gamma-glutamyltransferase (GGT), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin has been shown to be linked with hepatic cell damage in human occupationally exposed to the pesticide (**Arnal et al., 2011; Wafe et al, 2013; Manfo et al., 2020**). Conversely, nephrotoxic changes as evidenced by elevated levels of plasma urea, uric acids and creatinine in workers occupationally exposed to pesticides have also been reported (**Johnson et al., 2019, Kori et al., 2019**). Other hematological parameters such as biomarkers of the thyroid function have been less studied but there is some increasing evidence that occupational exposure to agricultural pesticides may affect thyroid function. Thus, it has been observed a significant decreased in serum levels of thyroid-stimulating hormone (TSH) and significant increases in free thyroxine (FT4) and total triiodothyroxine (TT3) in farmer from Brazil (**Bernieri et al., 2019; Kori et al., 2020**).

It is necessary to delve into the evaluation of the impact of occupational exposure to agrochemicals in order to estimate the risk and develop effective strategies for the prevention of these health problems. The increased utilization of pesticides other than organophosphates makes it necessary to look for new biomarkers

beyond the classics used in agricultural health surveillance protocols. Most of the epidemiological studies about pesticides exposition are focused on agricultural workers but there are hardly any studies about indirect exposition of workers who collect the fruit and vegetables and are chronically exposed indirectly to pesticides trace levels (**Curl et al., 2020**). Alterations in fertility, procreation and development of offspring caused by chronic pesticides exposure made women one of the most vulnerable populations; however, there are only few epidemiologic studies about it.

In Spain, the health of pesticide applicators is evaluated by a number of clinical laboratory tests, including markers of hepatic function (AST and ALT) and cholinesterase activities, and these tests are not mandatory for farmers who collect the fruits and vegetables. The few published epidemiologic studies are focused on greenhouse pesticides sprayers (**Hernández et al., 2003; 2004; 2013; García-García et al., 2016; Lozano-Paniagua et al., 2018**). Taking these premises in consideration, this study was conducted with the aim of ascertaining if also the indirect exposure to pesticides leads to some biochemical parameter changes. Thus, cholinesterase activities, oxidative stress status (lipid peroxidation and protein oxidation), hepatic function (AST and ALT levels), renal function (serum creatinine and urea; and urinary proteins, NAG, NGAL, KIM-1, albumin and transferrin) and hormonal function (TSH, T4, FSH, LH and AMH) were evaluated in farmer women who collect fruits and vegetables comparing with a group of

women non- occupational exposed to pesticides but living in the same rural environment.

To our best knowledge, no study has been done on these all parameters together by taking into account this indirectly exposed population of harvest farmer women.

2. Materials and Methods

2.1 Study population

A longitudinal study was conducted on a cohort of 39 women in fertile age from Marinaleda (Sevilla, Spain). Marinaleda is a small town with an area of 24.8 km^2 and a total population of 2,665 inhabitants. Of the total population, 65.2% are between 20 and 65 years of age, of which 1,299 are women, representing 49% of the population. Its political and social organization make it unique and suitable for the achievement of this study. The economic activity of this town is based on agriculture where women collect the fruits and vegetables in a field or work in the factory where these fruits and vegetables are canned. Total of 39 women (age between 18 and 45 years) were asked to be part of this longitudinal study. 22 of them, directly involved in collection of fruits and vegetables in the field, were classified as farmers, and the 17 remaining participants who work in the canned factory and live in the same rural environment were classified as non-occupational exposed (NOE) group. The survey was divided in four periods with sample collection every three months along one year. The

chronogram of sampling, the crops collected at this time and the pesticides most used for these crops are listed in Table 1. Women with a clinical diagnosis of chronic diseases were excluded. A preliminary questionnaire was specifically designed for the study to record the personal and occupational information of the participants. Ethical clearance was obtained from the Coordinating Committee of Ethics of Biomedical Research of Andalusia (Spain) and was in agreement with the Declaration of Helsinki for International Health Research. Written informed consent was also obtained from all the participants after being informed about the objectives of the study and the right to drop out of the study at any time.

Table 1.- Pesticides most used for these crops are listed in

Date of sampling	Crop collected in the previous three months	Pesticides applied	Target Pest
05/10/2017	Pepper	Pendimethalin	Herbicide
	Garlic	Fluazifop-P-butyl	Herbicide
	Artichoke	λ-Cyhalothrin	Insecticide
08/02/2018	Garlic	Bromoxynil	Herbicide
	Artichoke	Fluazifop-P-butyl	Herbicide
	Broccoli	Glyphosate	Herbicide
	Green beans	Dimethylamine	Herbicide
	Olives	Diflufenican	Herbicide
	Wheat	Chlortoluron	Herbicide
		Tritosulfuron	Herbicide
		Imidacloprid	Insecticide
		λ-Cyhalothrin	Insecticide
		Mancozeb	Fungicide

		Azoxystrobin	Fungicide
		Copper oxychloride	Fungicide
06/06/2018	Pepper	Cycloxydim	Herbicide
	Garlic	Fluazifop-P-butyl	Herbicide
	Artichoke	Pendimethalin	Herbicide
	Broccoli	Glyphosate	Herbicide
	Green beans	Imazamox	Herbicide
	Olives	Dimethylamine	Herbicide
	Wheat	Fluometuron	Herbicide
	Cotton	Ethofumesate	Herbicide
	Zucchini	Fluroxypyr	Herbicide
	Chickpeas	Napropamide	Herbicide
	Sunflowers	Tritosulfuron	Herbicide
	Chamomile	Bromoxynil	Herbicide
		Pinoxaden	Herbicide
		λ-Cyhalothrin	Insecticide
		Chlorpyrifos	Insecticide
		Deltamethrin	Insecticide
		Betacyfluthrin	Insecticide
		Propanocarb	Fungicide
		Chlorthanolil	Fungicide
		Copper oxychloride	Fungicide
		Azoxystrobin	Fungicide
		Tebuconazole	Fungicide
10/10/2018	Pepper	Pendimethalin	Herbicide
	Garlic	Fluazifop-P-butyl	Herbicide
	Artichoke	λ-Cyhalothrin	Insecticide

2.2 Sample collection

Blood samples (≈15 mL of venous blood) were obtained from all the participants every three months along the study period. The blood samples were kept in heparinized and nonheparinized tubes and kept in an ice box until transported to the laboratory. Both plasma and serum were separated from the blood. The whole blood samples were used for the hematological test while plasma and serum samples were used for the biochemical analysis.

2.3 Assay of cholinesterase's activities

Red blood cells AChE activity was measured as described by Ellman et al. (1961) and adapted for microplates, as described by Guimarães et al. (2007). Briefly, acetylthiocholine iodide (AcSCh) 9 mM was used as substrate and 5,5'-dithio-bis (2-nitrobenzoic) 0.75 mM (DTNB) acid as chromogen. The optical density at 415 nm was measured each 30 seconds for 3 min using an ELISA plate reader (Synergy HTX, BIO-TEK, Winooski, Vermont, U.S.A). Enzyme activity is expressed as micromoles of AcSCh hydrolyzed per minute per milligram of protein.

Plasma cholinesterase (BChE) was determined by measuring the rate of hydrolysis of butyrylthiocholine (BuSCh) instead of AcSCh according to the same method mentioned above. Enzyme activity is expressed as micromoles of BuSCh hydrolyzed per minute per milligram of protein.

2.4 Hematological and biochemical analysis

The following hematological parameters were determined using a hematology analyzer (Sysmex XN-1000™) in whole blood collected in heparinized tubes: red blood parameters (number of red blood cells, $\times 10^6/\mu L$), hemoglobin (g/dl), hematocrit (%), white blood parameters (total number of leukocytes, $\times 10^3/\mu L$, total number ($\times 10^3/\mu L$) and percentage of neutrophils, lymphocytes, eosinophils, monocytes and basophils), and platelet count ($\times 10^3/\mu L$).

Biochemical parameters were measured on fresh serum samples using a clinical analyzer (Vital Scientific - Selectra XL) following standard procedures for clinical biochemistry. The measured parameters included: glucose, total proteins (g/dl), lipid profile: total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides; liver function test: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) (units for all these test are U/l); and renal function test: creatinine (mg/dl), urea (mg/dl), uric acid (mg/dl).

2.5 Hormonal analysis

Thyroid hormone levels were measured on fresh serum samples by paramagnetic particles, chemiluminescent immunoassay using a CL-1000i analyzer (Mindrai, Shenzhen, China). The measured hormones were levels of thyroid-stimulating hormone (TSH) (µUI/ml) and free thyroxine (FT4) (ng/dl). The reference

values were 0.270-5.500 µUI/ml for TSH and 0.93-1.70 ng/dl for FT4.

Sex hormone levels [luteinizing hormone (LH), folliclestimulating hormone (FSH)] were assayed in serum using a clinical chemiluminescence immunoassay analyzer (CL-1000i, Mindrai). Units for these tests are µUI/ml. Anti-Müllerian hormone (AMH) levels were measured on serum samples using the Beckman Coulter AMH Gen II ELISA according to the manufacturer's instructions. All assays were measured using an ELISA plate reader (Synergy HTX, BIO-TEK, Winooski, Vermont, USA). The minimal detectable AMH concentration with the AMH Gen II assay is 0.9 pmol/L (as advised by the manufacturer) and samples with undetectable levels were recorded as 0.9 pmol/L.

2.6 Oxidative stress markers

Lipid peroxidation products were quantified by the thiobarbituric acid (TBA) method (**Esterbauer and Cheeseman, 1990**). Malondialdehyde (MDA) is formed as an end-lipid peroxidation product which reacts with the TBA reagent under acidic conditions to generate a pink colored product. Values were presented as nmol TBARS/g tissue.

Protein carbonyl content, a biomarker of protein oxidation, was assayed with the method described by Levine et al. (1990) using 2,4-dinitrophenylhydrazine (DNPH) prepared in 2MHCl, 20% trichloro acetic acid (w/v), and 6 M guanidine hydrochloride.

Results are expressed as nmol carbonyl/mg protein, using the extinction coefficient 22,000 M^{-1} cm^{-1}.

2.7 Urinary early kidney damage and predisposition to kidney damage biomarkers

Proteinuria was measured with the Bradford assay (Bradford, 1976). NAG activity was quantified using a commercial kit [“N-Acetyl-β-D-glucosaminidase (NAG) assay kit®”, Diazyme®, Poway, CA, USA] following the manufacturer’s instructions. NGAL was measured by commercial ELISA (“Human NGAL ELISA Kit 036CE®”, BioPorto Diagnostics®, Hellerup, Denmark), according to the manufacturer’s instructions; and for the quantification of KIM-1, the kit “KIM-1 (human) ELISA Kit #ADI-900-226®” (Enzo Life Sciences®, Farmingdale, NY, USA) was used. Albumin was quantified using the “Human Albumin ELISA Quantitation Set E80- 129®” kit, and the “Human Transferrin ELISA Quantitation Set E80-128®" kit was used to determine transferrin, both from Bethyl Laboratories®, Montgomery, TX, USA. Both procedures require the “ELISA Starter Accessory kit E101®" kit, which provides the necessary reagents for the determination of both proteins. All biomarkers values in humans were factored by urinary creatinine concentration with the objective of normalizing the effect of urine concentration (**Adejeji et al., 2019**). The urinary creatinine required for the normalization of all biomarkers was measured using the commercial kit “Quantichrom® cratinine assay kit” (BioAssay Systems®, Haywar, CA, USA).

Because these biomarkers do not have reference values established as "normal" or "physiological", a battery of urinary samples from a Control group consisting of healthy women (non-working in any of the activities described in point 2.1 and non-residents in Marinaleda) with demographic, anthropometric and biochemical characteristics statistically similar to the women in the groups “Farmers” and “NOE” (data not shown) was included.

2.8 Statistical analysis

Descriptive statistics were generated for demographic parameters in the Farmers group and NOE group. Frequencies and percentages for all the categorical parameters were compared between both groups using the Fisher's exact test. In the case of continuous quantitative variables, firstly, it was studied whether the data in the groups followed a normal distribution, applying Shapiro–Wilk's test. After that, an unpaired student's t test was used to compare the mean values of the quantitative characteristics (demographic parameters, oxidative stress biomarkers, cholinesterase activity, biochemical and hematological parameters) between the Farmers and NOE groups. On the other hand, the comparison of the urinary excretion of the biomarkers of kidney damage described in point 2.7 between the groups was carried out with an ANOVA test coupled with a Scheffé test (for normal data) or a Kruskal-Wallis test (for non-normal data). Finally, and with the aim of establishing the possible relationship between exposure to pesticides and the development of subclinical kidney damage in Marinaleda workers and residents, Spearman correlation studies (for

non-normal data) were carried out between excretion of the different biomarkers evaluated and the blood levels of cholinesterase, acetylcholinesterase, lipoperoxidase and protein oxidation.

The criterion for significance was set at $p<0.05$. All the statistical analysis was performed using the IBM SPSS statistics software version 24.0 (International Business Machines, Armonk, NY, USA). Microsoft Office Excel 2016 and Powerpoint 2016 (Microsoft, Redmond, WA, USA) were used to create the artwork and illustrations presented.

3. Results

3.1 Characteristics of the studied population

Characteristics of the study population are shown in Table 2. Twenty two women farmers involved in recollection of vegetables and fruits were evaluated in comparison with the non-exposed group formed by 17 women. Nonsmokers were the majority of the women from both groups. Most of the participants in both groups are non- or sporadic alcohol consumers, only 22% and 36% were weekend alcohol consumers in farmers and non-exposed groups, respectively. In relation to the time that participants are working at that job, most of them have been in the job for more than 10 years (≈89% in farmers and 73% in no exposed group).

Table 2.- Characteristics of the study population

	Farmers (n = 22)	**NOE (n = 17)**	**p-value**
Age	(%)	(%)	
18-28 years	13.6	17.6	n.s.
29-38 years	13.6	35.3	
39-45 years	72.7	47.1	
Smoking habits	(%)	(%)	
Smokers	36.4	35.3	n.s.
Non-smokers	63.6	64.7	
Alcohol consumption	(%)	(%)	
Non-consumer	45.5	64.7	<0.05
Sporadic	36.4	--	
Weekend	18.1	35.3	
Number of years working at that job	(%)	(%)	
< 5	9.1	--	<0.01
5-10	--	29.4	
> 10	90.9	70.6	
Use of PPE	(%)	(%)	
Yes	100	88.2	n.s.
No	--	11.8	
Type of PPE	(%)	(%)	
Mask	--	--	
Gloves	100	90.9	n.s.
Glasses	--	--	

Health status and neurological endpoints were assessed taking into account the self-reported symptoms collected through the epidemiological questionnaire. A variety of neurological symptoms, including headache (25%) and dizziness (25%), gastrointestinal and respiratory symptoms (35%) have been reported in women farmers. These symptoms were significant as compared to non exposed participants. The health status was assessed along the monitoring period through three more questionnaires. There were no significant differences in neurological, gastrointestinal or respiratory symptoms collected between the same participants and only 5% of farmers informed about a weight gain after the first sample collection.

3.2 Effects on cholinesterase's activities

The activities of AChE in erythrocyte and BuChE in serum have been estimated and represented in a Figure 1. No statistically significant alterations in cholinesterase activity, neither AChE nor BuChE, were found in women farmers when compared to the non exposed women group. However, it can be observed that AChE activity was slightly lower in farmers than in NOE group in the last three monitoring dates (February, June and October 2018). Thus, when comparing the AChE activity detected in the farmer group in these three dates there was a statistically significant decrease (≈18%) ($p<0.05$ and $p<0.01$) in relation with those detected in the first sampling date (October 2017). These results suggest a low exposure to cholinesterase inhibitor insecticides.

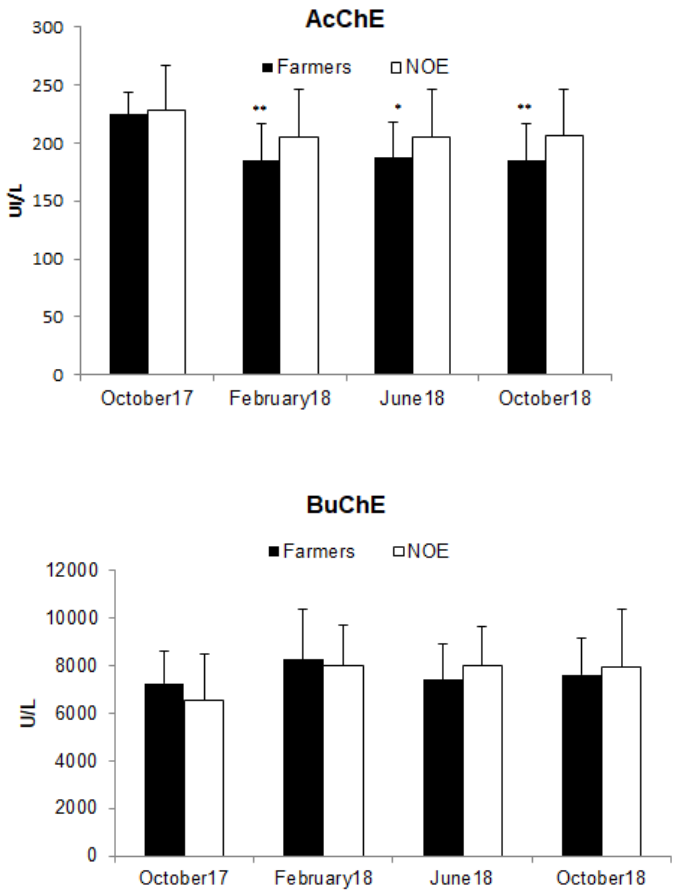

Figure 1. Activities of AChE in erythrocyte and BuChE in serum

3.3 Effects on hematological and biochemical parameters

No significant differences were found in results from the hemograms when comparing farmers and non-exposed women groups along the all monitoring period.

Biochemical parameters are shown in Table 3. Women farmers presented a significant increase in serum levels of glucose

when compared to non-exposed groups in October 17 ($p<0.05$) and October 18 ($p<0.001$). On the other hand, the levels of total proteins in women farmers were significantly reduced in relation to non-exposed women groups only in February 18 ($p<0.001$). Regarding lipid profile, a very significant increase in HDL ($p<0.01$ and $p<0.001$) along all the studied monitoring periods were observed in farmers women as compared with non-exposed groups. Interestingly, a decrease in LDL (June 18 and October 18) and triglycerides (February 18 and June 18) ($p<0.01$ and $p<0.001$) was also observed, being the non-exposed group the population that presents a significant increase in lipid parameters even with values above of the reference values, suggesting abnormal accumulation of lipids. The liver function biomarkers assessed, AST, ALT and LDH didn't show significant differences between both groups of population (farmers and non-exposed) in none of the four periods studied. However, when comparing the renal function markers in farmer and non-exposed women, a significant increase in urea ($p<0.001$) along the four monitoring periods and in creatinine in October 17 ($p<0.001$) and February 18 ($p<0.05$) were observed.

Table 3:Biochemical Parameters

	October 2017		February 2018		June 2018		October 2018		Reference values
	Farmers	**NOE**	**Farmers**	**NOE**	**Farmers**	**NOE**	**Farmers**	**NOE**	
	Mean±SD	***Mean±SD***	***Mean±SD***	***Mean±SD***	***Mean±SD***	***Mean±SD***	***Mean±SD***	***Mean±SD***	
Glucose (mg/dL)	91.59±13.28*	81.71±7.17	79.00±14.65	76.69±6.52	75.80±7.08	77.44±6.45	82.09±23.03***	61.13±8.07	75-110
Total proteins (g/dL)	6.99±0.34	6.94±0.32	7.45±0.36	7.43±0.14	6.99±0.39***	7.41±0.14	7.59±0.28	7.44±0.21	6.5-8
Lipid Profile									
Total cholesterol (mg/dL)	187.59±15.92	174.65±49.99	193.26±22.64	199.86±57.03	186.62±20.33	200.36±56.53	185.34±30.91	218.34±70.60	90-220
HDL (mg/dL)	54.95±12.41**	43.29±10.61	59.30±9.67***	42.43±11.50	60.22±10.67***	42.29±11.50	58.01±10.81**	46.79±6.62	35-65
LDL (mg/dL)	119.36±15.13	116.82±41.37	125.74±18.41	144.14±48.64	105.26±15.92**	144.21±48.42	100.35±23.94**	142.13±48.25	<129
Triglycerides (mg/dL)	137.50±52.50	128.24±41.10	135.87±42.54**	198.14±60.81	106.66±38.45***	198.21±60.69	136.39±45.10	148.93±82.67	50-200
Hepatic Function Biomarkers									
LDH (U/L)	401.77±54.	376.71±65.	371.04±50.30	373.25±59.12	355.70±47.47	373.94±59.03	412.70±51.62	437.69±54.61	230-460

	43	50							
AST (U/L)	20.55±11.15	19.06±6.36	22.96±10.74	19.31±3.84	17.18±5.97	19.38±4.00	18.34±5.30	20.96±4.58	10-37
ALT (U/L)	20.05±13.81	18.29±13.03	23.65±16.76	18.44±11.99	17.73±8.93	18.81±11.67	15.56±7.08	14.63±4.37	10-40
Renal Function Biomarkers									
Urea (mg/dL)	33.82±9.09***	22.00±5.07	30.13±3.05***	21.19±2.51	33.53±8.03***	21.63±2.92	33.99±5.56***	23.94±5.56	15-50
Creatinine (mg/dL)	0.83±0.15***	0.67±0.09	0.71±0.06*	0.63±0.08	0.68±0.04	0.62±0.09	0.71±0.07	0.70±0.06	0.60-1.20
Uric acid (mg/dL)	4.07±0.82	3.89±0.80	3.86±0.78	3.78±0.73	3.72±0.70	3.82±0.71	4.31±0.97	3.98±0.58	2.4-7.0
Creatinine kinase (U/L)	156.09±95.79	126.88±77.18	108.78±62.29	110.56±33.67	97.39±26.26	110.75±33.77	107.76±22.74	90.54±10.54	10-195

3.4 Effects on hormonal analysis

Results for hormonal analysis (TSH, FT4, LH, FSH and AMH) are shown in Table 4. There is a great variability in results of all of these parameters in both studied groups (farmers and non-exposed) and between the different periods of the study (October 17, February 18, June 18 and October 18). Compared with non-exposed group, only levels of FT4 and LH were significantly decreased in farmer's women in June and October 2018, respectively. Levels of FT4 and TSH were within the reference values in both populations (farmers and non-exposed). Also levels of AMH in three monitoring periods measured were within normal levels of ovarian reserve.

Table 4: Results for hormonal analysis

	October 2017		February 2018		June 2018		October 2018		Reference values
	Farmers	**NOE**	**Farmers**	**NOE**	**Farmers**	**NOE**	**Farmers**	**NOE**	
	Mean±SD	***Mean±SD***	***Mean±SD***	***Mean±SD***	***Mean±SD***	***Mean±SD***	***Mean±SD***	***Mean±SD***	
TSH (mUI/L)	1.33±0.74	1.86±1.13	1.87±0.80	1.61±0.67	1.41±0.56	1.62±0.68	1.88±0.85	2.70±3.23	0.27-5.5
FT4 (ng/dL)	1.16±0.07	1.27±0.26	1.18±0.12	1.22±0.29	1.04±0.10**	1.23±0.29	1.19±0.08	1.23±0.15	0.93-1.70
LH (U/L)	24.19±31.86	38.71±45.22	31.48±47.34	56.30±60.24	25.34±34.27	55.60±59.59	18.48±30.87***	97.76±51.05	
FSH (U/L)	19.11±22.14	51.83±116.93	21.64±25.54	29.30±22.69	16.96±19.39	29.20±22.71	12.75±15.12	42.61±17.55	
AMH (ng/ml)			0.99±1.26	1.08±1.32	0.88±0.88	1.09±1.31	1.10±1.10	1.39±2.84	

3.5 Effects on lipid and protein oxidation

The activity of the biomarkers of oxidative stress studied in farmers and non-exposed women at the four study periods (October 2017, February 2018, June 2018 and October 2018) are shown in Figure 2. As compared to non-exposed groups, women farmers had significantly higher levels of TBARS and carbonyl groups in the two first sampling (October 17 and February 18) ($p<0.01$ and $p<0.001$). In the last two samples were observed a decrease in levels of TBARS and carbonyl groups in women farmers without significant differences in comparison to the values of those non-exposed. There were no significant differences in levels of TBARS and carbonyl groups between all the non-exposed groups.

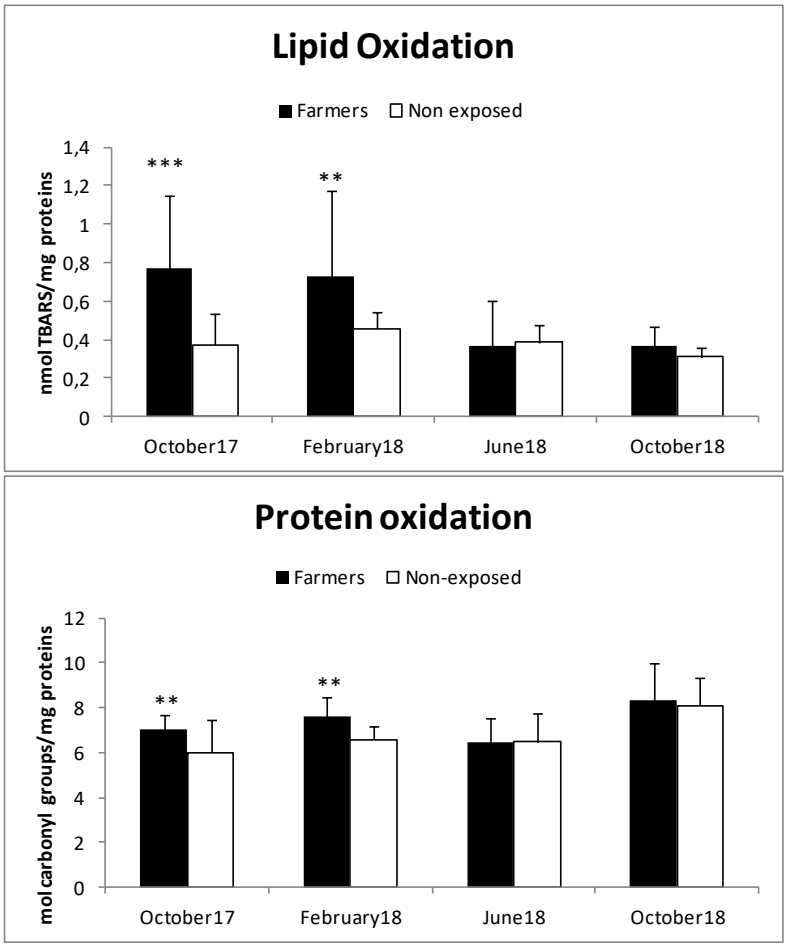

Figure 2 The activity of the biomarkers of oxidative stress studied

3.6 Effects on early renal function markers

Urinary levels of the biomarkers of early kidney damage and predisposition to kidney damage are shown in Figure 3. As can be observed, all the evaluated biomarkers presented at least at some of the sampling times high levels in their excretion with respect to the control group, on many occasions with high statistical

significance ($p < 0.001$). Although, in general, no statistically significant differences were detected between the farmers and NOE groups, a greater excretion of KIM-1 (in October 2017 and February and October 2018) and transferrin (in February 2018) was observed in the second group.

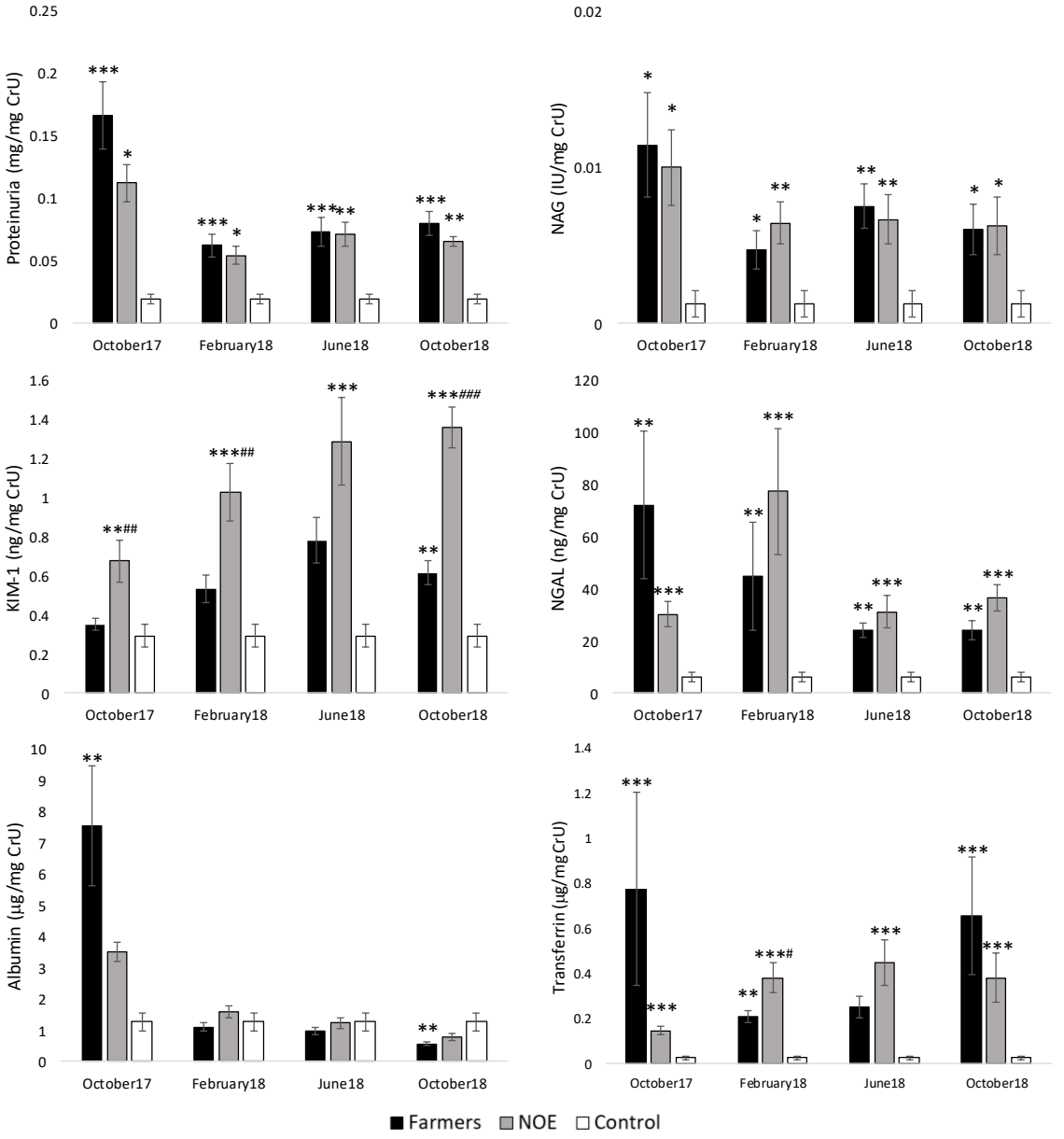

Figure 3.- Urinary levels of the different biomarkers of early kidney damage and predisposition to kidney damage evaluated at different sampling times

The results of the correlation analysis performed between the excretion of the evaluated urinary biomarkers and some of the blood parameters related to exposure to pesticides are presented in Table 5. First, the possible existence of this correlation was evaluated in all the women belonging to the same population (Table 5A). In this analysis, highly significant correlations were identified between some of the urinary biomarkers (proteinuria, albumin, and transferrin) and some blood parameters, such as the enzymes cholinesterase and acetylcholinesterase. To a lesser extent, significant correlations were also detected between KIM-1, NGAL and transferrin with some of these blood parameters.

When the workers were subdivided into the groups farmers (Table 5B) and NOE (Table 5C), it was found that, although urinary albumin was the biomarker whose excretion was related to a greater extent with the exposure parameters studied in the two groups, the second marker that was best associated with these parameters in the NOE group was KIM-1; while in the farmers group it was transferrin. Besides, in both groups the correlation profile was different, which could suggest that depending on the type of agent to which the individual is exposed, the type of subclinical damage caused by it could be marked by different urinary molecules.

A) Marinaleda (Farmers + NOE groups)						
	Proteinuria	**Urinary NAG**	**Urinary KIM-1**	**Urinary NGAL**	**Urinary Albumin**	**Urinary Transferrin**
Blood proteins	-0.268**	-0.140	0.245**	0.060	-0.441***	0.323***
Blood cholinesterase	-0.237**	0.001	0.338**	0.001	-0.291**	-0.028
Blood acetylcholinesterase	0.033	-0.043	-0.187*	-0.341***	0.212**	-0.299***
Blood lipoperoxidase	-0.032	-0.097	-0.098	-0.067	0.151	-0.265**
Blood protein oxidation	-0.137	-0.104	-0.079	-0.032	-0.165	0.118
B) Farmers group						
Blood proteins	-0.276*	-0.101	0.141	0.126	-0.424***	0.327**
Blood cholinesterase	-0.286*	0.137	0.227	0.141	-0.258*	-0.180
Blood acetylcholinesterase	0.078	0.011	-0.426***	-0.331**	0.384**	-0.271*
Blood lipoperoxidase	-0.092	-0.030	-0.090	-0.024	0.212	-0.468***
Blood protein oxidation	-0.219	-0.148	0.063	0.144	-0.090	0.124
C) NOE group						
Blood proteins	-0.208	-0.173	0.471***	0.013	-0.481***	0.295*

Blood cholinesterase	-0.143	-0.201	0.611***	-0.164	-0.360***	0.134
Blood acetylcholinesterase	0.054	-0.084	-0.034	-0.364**	0.035	-0.349**
Blood lipoperoxidase	0.087	-0.156	0.078	0.046	0.198	0.079
Blood protein oxidation	-0.080	-0.017	0.086	-0.141	-0.273*	0.169

Table 5. Results of the correlation study carried out between the urinary biomarkers evaluated and some blood parameters of exposure to pesticides. Data are expressed as Spearman's correlation coefficient (ρ). Significance of the correlation: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. KIM-1: kidney injury molecule 1; NAG: N-acetyl-β-D-glucosaminidase; NGAL: neutrophil gelatinase-associated lipocalin; NOE: non-occupational exposed.

4. Discussion

This study assessed alterations in the function of biomarkers of exposure to ChE inhibitor pesticides and oxidative stress biomarkers with changes in biochemical, hematological and hormonal parameters in two populations of rural women residents all of them in Marinaleda (Sevilla, Spain). This town is surrounded by the fields where most of the population work, either sowing, applying pesticides or collecting the fruits. One group consisting of women farmers indirectly exposed to pesticides during harvesting and the other one formed by women non-occupationally exposed to these compounds. Considering the low number of studies investigating the health of harvest farmers, especially women, our study provides important data on the effects associated with this indirect exposure to pesticides in this population group in comparison with the NOE group, and about the influence of the rural environment when comparing these two rural groups with a non-rural control group. The strength of the present study lies in the fact that all the parameters studied were assessed four times along the study in order to relate the observed effects to a chronic exposure.

During the sampling, farmer women were in the middle of the harvest time, collecting different cultures along the study. Our data showed that most of these women (88.9%) were chronically and indirectly exposed to pesticides during more than 10 years. All of them reported the use of PPE but only gloves because none of

them reported the use of masks or glasses. No significant differences were observed in BuChE activities along the four sampling when farmer and NOE are compared. However, AChE activity was found to be lower (10%, 8% and 10%) in the second, third and fourth sampling, in women farmers as compared to the NOE group, but was not found to be statistically significant. The inhibition observed in AChE activities along the sampling period, is very far from the Biological Exposure Index (BEI) that establish a 70% of AcChE activity an individual's baseline as a reference value for exposure control, what means a 30% of decreased in the enzymatic activity (**INSST, 2019**). Although both enzymatic activities are markers of early biologic effects related to OPs and carbamates exposure, AChE inhibition is more sensitive and preferred than BuChE, since it reflects the biological effects on the nervous system and shows a lower recovery rate, representing the inhibition of the neural AChE in a more realistic manner (**Cestonaro et al., 2020**). The inhibition of cholinesterases activities has been associated with cholinergic dysfunctions in pesticides-exposed workers (**Kori et al., 2019**), mainly in pesticide sprayers who are directly exposed to these compounds. Thus, significant reduction in the enzymatic activity (17%-26%) were observed in pesticide sprayers and in women plucking leaves in tea plantations from India (**Fareed et al., 2017; Kori et al., 2019; Dhananjahan et al., 2019**). Similar results were reported by Centonaro et al. (2020) in farmers from Brazil with a decreased enzymatic activity of 21% for AChE in comparison with NOE group. On the other

hand, and more in agreement with our results, Vikkey et al. (2017) observed that 88% of the cotton farmers from Benin displayed less than 20% AChE inhibition. Some authors have concluded that despite AChE activity is used to biomonitor exposure to pesticides in human population, in some cases no differences in this enzymatic activity are found among exposed populations and NOE groups (**Zepeda-Arce et al., 2016; Jacobsen-Pereira et al., 2018**). Although weakly, AChE was significantly and inversely associated with glucose, showing that the chronic exposure to pesticides can also contribute to the increase in glucose levels (**Cestonaro et al., 2020**). Our results showed a significant increase in glucose levels in women farmers in comparison with the NOE group in two of the four sampling dates. However, despite the chronic exposure of this group, the measured levels of glucose did not represent a real hyperglycemia, probably since the pesticides employed are not only OPs and carbamates and that the exposure to these compounds is indirect. Further, our study also showed a significant increase in HDL levels as compared to NOE group, however, a trend of decreased in the levels of lipid profile including cholesterol, triglycerides and LDL comparing to NOE group indicating that the indirect exposure to pesticides in this population of women does not lead to an abnormal accumulation of lipid, altered metabolism, hepatic dysfunction or cardiac problems as it has been observed in previous studies in pesticide sprayers (**Kori et al. 2019**). The long-term exposure to pesticides may increase the levels of AST, ALT and LDH, causing damage to liver function. In the present study no

significant differences were observed in these parameters when comparing both groups (farmers *vs* NOE). This fact with the significant increase in HDL levels lead us to conclude that there is no liver damage in this indirectly exposed population. Some pesticides may interfere with the female hormonal function, which may lead to negative effects on the reproductive system through disruption of the hormonal balance necessary for proper functioning (**Bretveld et al., 2006**). In the present study slightly higher levels in FT4 and LH were observed in farmers in comparison with the NOE group, but only in June and October 18 were statistically significant, respectively. Despite these differences, levels of FT4 both in farmer and NOE groups along the study were all within the normal reference values. Some authors have observed a relationship between significant increases in FT4 levels and pesticide exposure, suggesting that occupational exposure to pesticides may affect thyroid function (**Bernieri et al., 2018; Curl et al. 2020**). In relation with LH levels, all the values detected in farmers are higher than 30 U/L considered as menopause indicator in elder women or a signal of ovarian malfunction or an early menopause in younger women. Considering the age range of all the participants in the present study, it can be thought that the chronic exposure to trace levels of pesticides could result in a disruption of the hormonal balance.

Oxidative stress as a possible mechanism of toxicity for pesticides has become a focus of toxicological research because it is considered as a critical pathophysiological mechanism in different

human pathologies associated with pesticide exposure (**Zepeda-Arce et al., 2016**). Oxidative damage may result in cellular adaptation, damage to cellular lipids, DNA, proteins and/or cellular death (**Curl et al., 2020**). In the present study, lipid peroxidation and protein oxidation as biomarkers of oxidative stress were investigated among women farmers who are indirectly exposed to a mixture of pesticides and women who are not occupationally exposed to these compounds. MDA and TBARS as direct measure of MDA have been used as a biomarker of lipid peroxidation and is one of the most reliable markers to determine oxidative stress in clinical situations (**Zepeda-Arce et al., 2016; Lozano-Paniagua et al., 2018**). Several studies reported increased MDA concentrations in applicators, farmers and sprayers exposed to different pesticides (**Wafa et al., 2013; Jacobsen-Pereira et al., 2018; Lozano-Paniagua et al., 2018; Kori et al, 2019**). In this study MDA levels were higher in women farmers than in the NOE group, but only in two of the sampling dates (October 17 and February 18). Similarly, levels of protein oxidation measures as nmol of carbonyl groups were higher in farmers than in NOE group in samples from October 17 and February 18. It has been demonstrated that oxidative stress is the main mechanism of acute intoxication from some pesticides, either individually or in a mixture, although the underlying molecular mechanisms are not clear (**Lukaszewicz-Hussain,2010; Lozano-Paniagua et al., 2018**). This fact could explain the recovery observed in both in the farmer's biomarkers to the control levels along the study.

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CAPÍTULO 3. VALIDACIÓN DE UN MÉTODO SIMPLE PARA LA DETERMINACIÓN DE GLIFOSATO Y AMPA EN ORINA HUMANA POR UPLC-MS/MS

Introduction

Human exposure to complex mixtures of environmental pollutants is a reality in today's society. Environmental pollutants refer to all of the exogenic, non-essential factors for humans, which, when released into the environment, can be detrimental to human health and/or to the environment [**1]**. Within this group of pollutants, those that are called emerging pollutants such as pesticides and metals are standing out [**2, 3**].

The use of pesticides in agriculture has allowed us to obtain safer food by reducing vector-borne diseases. However, their stability in the environment and their excessive use may produce toxicity through various mechanisms [**3**]. General population is exposed to small concentrations of pesticides through diet and the environment throughout their lives, being more important this pesticide exposure in those population using them in a professional manner [**4**].

Pesticides for conventional use are classified as insecticides, herbicides and fungicides. Regarding to herbicides, glyphosate (N-phosphonomethyl-glycine) is a broad-spectrum herbicide with a high worldwide distribution, being estimated that glyphosate (GLY) accounts for 43% to 51% of the total amount of herbicides used. GLY is a systemic and non-selective herbicide intended for use against undesirable and deep-rooted perennial species, and also biennial and annual broad-leaved, grass and sedge species in agricultural and forestry environments and also in non-agricultural areas such as water systems, parks, road verges and

gardens [5]. Concerning its toxicity mechanism, there is much controversy. Its mechanism of action is through the inhibition of the enzyme 3-enolpiruvil-shikimato-5-phosphate synthase (EPSPS), avoiding the production of three key aromatic amino acids for the growth and survival of the plant. However, this route does not exist in humans, being thought that its true toxicity is caused by the surfactant that decouples elements of oxidative phosphorylation, which causes oxidative stress [6]. GLY have come under international debate since the International Agency for Research on Cancer (IARC) classified the chemical as a 'Group 2A – probably carcinogenic to humans' [7]. However, the Unites States Environmental Protection Agency has been classified GLY as category IV, the least toxic category (practically non-toxic and not an irritant) [8]. In the European Union, GLY has been thoroughly assessed by Member States, the European Food Safety Authority (EFSA) and the European Chemicals Agency (ECHA) to establish whether its use results in any unacceptable effects on human, animal or environmental health. These international agencies have classified GLY as non-carcinogenic to humans [9, 10]. There is not robust evidence of cytotoxicity, genotoxicity, DNA damage, carcinogenicity or reproductive toxicity from GLY [11-13]. In 2017, the European Commission authorized the use of GLY for a further 5 years [14]. Nonetheless, further toxicological and epidemiological studies are needed to draw better conclusions about the safety of GLY.

Human biomonitoring (HBM), which involves the measurement of a chemical in biological substances such as blood,

urine, hair or milk is considered the gold standard for exposure assessment and provide a very useful tool in public health [**15**]. The non-persistent substances such as GLY tend not to accumulate in the human body and are consequently quickly metabolized and excreted via urine within 4–72 h after exposure [**16**]. Studies conducted with rats suggest that nearly all absorbed GLY is rapidly excreted unchanged in urine, because is poorly metabolized being aminomethyl phosphonic acid (AMPA) its major metabolite [**17**]. The HBM of GLY and its metabolite in urine is a useful strategy in order to obtain valuable information about recent internal exposure to pesticides (1–3 days) [**16**]. Also, HBM allows identification and elimination of possible sources of exposure, studying relationships between pollutants and health effects, identifying groups of populations vulnerable to these pollutants and setting priorities in environmental and health research [**18**].

Both GLY and AMPA are polar and amphoteric compounds that are very soluble in water. Also, their difficult evaporation, poor solubility in common organic solvents, low volatility, low mass and favored complexing behavior hinder their analysis, as does the difficulty in detecting them [**19, 20**]. Thus, it is necessary to employ tedious and time consuming cleaning procedures, frequently involving the use of ion-exchange solvents and further derivatization, in order to facilitate their chromatographic separation by gas GC, GC–MS, LC or LC-MS [21]. Several methods for GLY and AMPA analysis in different matrices, mainly in environmental and food samples, are reported in the literature. Liquid chromatography (LC) is the most suitable

method to detect GLY [22]. Normally LC has been used in combination with fluorescence detection (LC-FLD) mainly in waters [23]. Chromatography–mass spectrometry (LC/MS), or alternatively HPLC/MS, is the most common method to detect GLY in environmental samples due to its higher sensitivity [19, 24]. Sensitivity can be significantly improved by LC/MS–MS or ultra performance liquid chromatography-triple quadrupole tandem mass spectrometry (UPLC-MS/MS) [25-28]. Another approach based on high-performance liquid chromatography (HPLC) is those coupled to inductively coupled plasma with triple quadrupole tandem mass spectrometry (HPLC-ICP/MS-MS) [29]. Most of these methods include numerous steps for the purification and derivatization of the compound. Derivatization procedures can be made both pre-column and post-column. Pre-column procedures are based mainly on derivatization with 9-fluorenylmethyl chloroformate (FMOC) [30-32, 22, 23, 26] to form fluorescent derivatives (improve detection) and/or to reduce the polar character of the analytes facilitating the chromatographic retention [33]. Development of new methods to reduce the steps in the analysis of these compounds is the objective of the scientific community, thus, Strava et al. [34] developed a method using a White Light Reflectance Spectroscopy Immunosensor that reduced the time of analysis and allowed the fast on-site determination of GLY in drinking water samples. Although there are many studies about the determination of GLY and AMPA in environmental samples, human biomonitoring studies of these compounds are scarce and have not been widely developed, reflecting the problems associated with this analysis. Thus, Jensen

et al. [35] validated a direct determination of GLY and AMPA method without derivatization in human milk and urine by LC-MS/MS. These authors found no traces of these compounds in human milk or human urine. Later, McGuire et al. [17] did not detect them either in human milk and urine using the same method. However, GLY was detected in over 90% of pregnancies from a cohort (Indiana, US) in urine samples employing also a LC-MS/MS method without derivatization [36]. Connolly et al. [37] developed a new LC-MS/MS method to detect GLY in urine without derivatization but including a previous solid phase extraction step. This method was applied to detect this compound in amenity horticulturalists first [37, 38] and in general young population later [39].

Analytical method validation strategies used to compare a performance measure with a reference value, not reflecting the population's needs, and they rarely consider the robustness study, which is essential for the "method transfer", following harmonization purposes [40]. Besides, the assessment of intra-laboratory accuracy is a fundamental stage in method validation [41]. Following all these considerations, in the present study, we aimed to develop an analytical procedure based on a derivatization step with FMOC-Cl, followed by UPLC-MS/MS for GLY and AMPA detection and quantification in human urine samples. Optimization and validation of the proposed method was carried out according to the holistic approach [40, 41]. The present procedure has been intended for routine determination in urine samples from farmers whose handle GLY.

Material and methods

Chemicals and reagents

Glyphosate (GLY) and Aminomethylphosphonic acid (AMPA) and Fluorenylmethoxycarbonyl chloride (FMOC-Cl) were supplied by SIGMA–Aldrich Chemie GmbH (Steinheim, Germany). Standard solutions of GLY and AMPA were prepared in Milli-Q water (100 mg/mL) and diluted as required for their use as working solutions (0.5 – 20 ng/mL). All chemicals and reagents used in this study were analytical grade materials. Dichloromethane and acetonitrile were purchased from Merck (Darmstadt, Germany). Deionized water (418 MΩ/cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, USA).

Derivatization procedure

Several alternatives for derivatization of GLY and AMPA with FMOC-Cl were submitted to a preliminary evaluation, designing different procedures adapted to the aim of this work and based on the method from Demonte et al. [26] with different modifications and adapting it to our samples (human urine). Thus, different aspects were evaluated: volume and conditioning of the sample, concentration of the FMOC-Cl and buffer volume (Table 1).

Table 1. Detailed steps of the procedures under study.

	Option A	**Option B**
Sample volume	5 mL	1 mL
Condition of the sample	Without lyophilization	Lyophilized
Derivatization	1 mL borate buffer 5% 500 µL FMOC-Cl 1.5 g/L	2 mL borate buffer 5% 2 mL FMOC-Cl 8 g/L

From the initial evaluation of the proposed approaches, the following method was finally selected for further optimization and validation: Blank urine samples (1 mL), previously homogenized, were spiked with different amounts of standards for calibration, between 0.5 and 20 ng/mL GLYP and AMPA. Each quality control (QC) sample was prepared using blank urine samples (1 mL) spiked with the same standard stocks and working solutions of GLYP and AMPA used in the calibration studies. The concentrations of the different QC samples were as follows: low QC level—1 ng/mL (GLYP) and (AMPA); medium QC level—5 ng/mL of each compound; and high QC level—20 ng/mL of each compound. Once spiked all the urine samples, they were submitted to lyophilization for 24 hours. After that, the derivatization stage started with the

addition of 5% borate buffer to achieve a pH 9, in which the derivatization reaction occurs, followed by the derivatization reagent, FMOC-Cl at an optimized concentration of 8mg/mL. This reaction took place at a 60ºC for 30 minutes. Afterwards, 250 µL de HCl pH 1 was added to stop the reaction and the samples were centrifuged (8 min, 8000 rpm). Finally, samples were passed through 0.2 µm filter before injection into the UPLC-MS/MS system.

Chromatographic conditions

Chromatographic separation was performed using an ultra high resolution liquid chromatograph (ACQUITY UPLC™, Waters, Milford, MA, EE.UU) coupled to a Xevo TQ-S micro (Waters) consisting of a triple quadrupole mass spectrometer equipped with an electrospray ion source operated in positive mode, which was selected due to its higher selectivity. LC analyses were performed on an Acquity UPLC BEH® C18 column (particle size 1.7 µm, 2.1 x 100 mm). Injection volume was 10 µL and flow rate was 0.5 mL/min. Two different solvents were used as a mobile phase: Solvent A (Water 10 mM ammonium acetate) and solvent B (Acetonitrile:Water 95:5 10 mM ammonium acetate), the following gradient was used: 0.0-1.0 min 5% B, 1.0-7.0 min from 5% to 70 % B, 7.0-9.0 min from 70% to 100 %B, 9.0-10.0 min 100% B, then 5% B up to 12.0 minutes. Multiple Reaction Monitoring (MRM) was applied where the parent ions and fragments ions were monitored at Q1 and Q3, respectively. The m/z values for the precursor and fragment for each specific compound along with their

respective cone voltages and collision energy values are shown in Table 2.

Table 2 The m/z values for the precursor and fragment for each specific compound along with their respective cone voltages and collision energy values

Compound	Cone voltage (V)	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)[a]	Collision energy (eV)
Glyphosate-FMOC	20	392.0	*Q* 87.8	30
			q 179.1	10
AMPA-FMOC	20	334.0	*Q* 155.8	20
			q 179.1	15

[a] *Q*: transition used for quantification; *q*: transition used for confirmation.

For UPLC-ESI-MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: capillary voltage: 2.90 kV, source temperature: 150ºC, desolvation temperature: 350 ºC, source desolvation gas flow: 650 l/h and source cone gas flow: 50 l/h. Chromatographic and mass spectrometry data handling was performed using MassLynx software v 4.1 (Waters).

Statistical criteria for method validation

Once we have developed the method, the next step was the validation to verify that it meets the needed requirements for its posterior application. Thus, the proposed method was validated taking into account the guidelines for the validation of analytical methods [**42**] regarding their linearity, sensitivity, precision and recovery parameters.

A calibration curve was prepared for each compound, spiked with internal standard of glyphosate and AMPA in different concentrations 0.5, 1, 2, 10 and 20 μg/L and injected in triplicate. To assess the matrix effect, we developed calibration curves in urine free of glyphosate and AMPA.

Three validation standards covering the optimal working range were used, which were measured in triplicate for three different days. Three concentration levels (0.5, 2 and 20 μg/L) of GLY and AMPA were spiked into urine samples in order to determinate the repeatability and the precision through recovery assays.

Application of the validated method in human urine samples

Human subjects

All procedures used in this study were approved by the Coordinating Committee of Ethics of the Biomedical Investigation of Andalucía (protocol number: 0231-N-17), and informed consent was obtained from each subject. A total of 20 women farmers were recruited from Marinaleda, Sevilla (Spain), which was part of a larger investigation about the possible toxic effects of the indirect pesticides exposition in women who collect fruits and vegetables in the field. This geographic location was targeted because it is an eminently agricultural municipality, whose economy is based in agricultural production.

Urine collection and preservation

Urine collection was carried out by the participants themselves, who were provided with a 125 mL polypropylene urine container and the urine collection instructions. A first-morning urine sample was collected from each volunteer. Samples were transported to the laboratory and stored at -80ºC, until analysis.

Results and discussion

Pre-column derivatization with FMOC-Cl

The concentration of sodium tetraborate has long been known to be critical because an excess may interfere with the solvents of the chromatographic system and at lower concentrations

the buffering capacity may be insufficient to complete the derivatization [**26**]. For that reason, in our study two different proportions of the borate buffer were evaluated (Table 1). The final concentrations of the buffering reagent tested with reference to the starting volume of the sample were 2.3 g/L for Option A and 6 g/L for option B. This concentration range is in agreement with others reported in literature [**26, 43**]. In our case the option B was that achieve the good results obtained.

Both GLY and AMPA are very polar compounds that require a previous derivatization procedure to be detected by UPLC-MS/MS. FMOC-Cl is the most used compound to react at an ambient temperature with primary and secondary amines without the need of a previous oxidation method. The required amount of this derivatizing reagent is also critical to ensure a complete and reproducible reaction, due to the presence of a very high level of amines on urine (in the forms of uric acid and urea). An excess of reagent must be used for the complete derivatization of the analytes present in the samples. This amount was experimentally determined in a range of concentrations between 1.5 and 8 g/L of FMOC-Cl and in a range of volume added between 0.5 and 2 mL referred to the initial volume of sample considered in each tested procedure (Table 2). The optimization of this excess of FMOC-Cl is important since the unreacted reagent becomes an undesirable impurity and the formation of by-products occurs during the reaction [**26**]. In our case, 2 mL of FMOC-Cl 8 g/L was used (method B). The selected method performed adequately no introducing problems with the excess of reagent but reaching the optimum concentration to

derivatize the GLY and AMPA present in urine samples and also the natural amines present in human urine.

Method validation

In order to develop the UPLC–MS/MS for the detection of GLY and AMPA, commercially available standards solutions of these compounds were assayed to acquire mass spectra and adjust mobile phase strength. The first experiment carried out was to select the optimal ESI-MS parameters and the appropriate ions analyzing individual solutions of GLY and AMPA derivatized with FMOC-Cl to monitor the MS intensity. Although these compounds are usually analyzed in negative ion mode [**25, 31, 35**] also it has been reported the positive ion mode analyzing [**21, 26, 44**]. In our case, though, GLY and AMPA were analyzed in both negative and positive ion modes, greater sensitivity was obtained here in the positive mode and hence, this positive ion mode was selected. A major ion for each compound was evident in the mass spectra of the compounds, m/z 392 for GLYP–FMOC and 334 for AMPA–FMOC, corresponding to the protonated molecular ions $[M+H]^+$. Multiple reactions monitoring (MRM) mode was used to obtain the maximum sensitivity for quantitative analysis (Table 2). To check how the matrix influenced the ionization, the peak areas of GLY and AMPA in standard solutions were compared with those obtained in blank urine samples.

The responses as a function of concentration were calculated from GLY and AMPA standards prepared in blank urine samples, and were measured by a 5-point calibration curve with a

linear range within 0.5–20 µg/L. The regression equations obtained were y = 4611x + 302.64 (R^2 = 0.9998) and y = 1574.7x + 53.664 (R^2 = 0.9992) for GLY and AMPA, respectively.

Linearity

Five different concentrations of GLY and AMPA were spiked to blank human urine (0.5–20 µg/L), submitting them to the proposed method. The calibration plot (signal response/analyte concentration against their concentrations) was established according to Huber [45] by replicate analysis (n=3) at all concentration levels (Fig. 1). The target line has zero slopes and the intercept represents the median of the response factors obtained in a fashion similar to the action limits of control charts. Both parallel horizontal lines on the graphic represent the 0.95 and 1.05 times the median value. As can be observed, no intersections with the lines were found in the case of AMPA, so the linear ranges of the methods apply to the full ranges studied, however on the GLY representation, the 0.5 value cannot be adjusted to the lineal ranges, discarding this concentration as lineal for our method.

Sensitivity

For validation purposes it is normally sufficient to provide an indication of the level at which detection becomes problematic and quantitation is acceptable in terms of repeatability precision and trueness. For this purpose, the limits of detection (LOD) and quantitation (LOQ) were determined based on the standard deviation of the blank, by measuring 10 independent sample blanks once each, and were estimated according to the equation Y_{LOD} or

$_{LOQ} = Y_{blank}+nS_{blank}$, where Y_{blank} and S_{blank} are the average value of the blank signals and its corresponding standard deviation. In these expressions, n=3 in the case of LOD and n=10 in the case of LOQ. Afterwards, Y_{LOD} and Y_{LOQ} values are converted in concentration units by using the calibration function. The LOD and LOQ obtained are 0.5 and 1 µg/L for GLY and 0.1 and 0.5 µg/L for AMPA, respectively (Table 3). Other authors obtained values of 0.02 and 0.1 µg/L for GLY and 0.03 and 0.1 µg/L for AMPA for LOD and LOQ, respectively or 1 µg/L for GLY LOD in urine using LC-MS [**17, 46**], also 0.1 µg/L for GLY LOD by LC-MS/MS [**36**]. Our values are similar to those obtained by Connoly et al. [**15**] in human urine (GLY LOD: 0.5 µg/L) by LC-MS/MS.

Table 3. Estimations of within-condition repeatability (S_w), between-condition repeatability (S_B), intermediate precision (intra-laboratory reproducibility, S_{IP}) and its relative standard deviations ($\%RSD_{IP}$), and recoveries of GLY and AMPA assayed in human urine, at three concentration levels, in three different days. Reference RSD values and recovery percentages by AOAC. Limits of detection (LOD) and quantitation (LOQ) for both compounds.

	GLY-AMPA VALIDATION					
	0,5 ppb		**5 ppb**		**20 ppb**	
	GLY	**AMPA**	**GLY**	**AMPA**	**GLY**	**AMPA**
S_W	-----	0.03	0.81	0.15	3.05	0.79
S_B	-----	0.08	0.28	0.39	1.19	2.40
S_{IP}	-----	0.05	0.68	0.26	2.59	1.53
RSD_{IP}(%)	-----	9	12.70	4.8	11.90	7.3
RECOVERIES (%)	-----	119	108	107	109	104
RSD_{AOAC} (%)	-----	30	21-30	21-30	15-21	15-21
ACEPTABLE RECOVERY RANGE (%)	-----	40-120	40-120	40-120	60-115	60-115
	GLY		**AMPA**			
LOD (ppb)	0.5		0.1			
LOQ (ppb)	1		0.5			

Precision

Precision refers to the closeness of agreement between independent test results obtained under stipulated conditions and, according to the International Conference on Harmonisation Guidelines [**42**]. Thus, precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The first one expresses the precision evaluated under the same experimental conditions over a short time interval, and it is termed as intra-assay or within-run. On the other hand, intermediate precision applies to within-laboratory variations: different days, different analysts or equipments, and is sometimes called between-run or inter-assay precision [**41**]. The third level, reproducibility, expresses the between-laboratories precision like in collaborative studies, and it will not be considered in this work.

To assess the precision study, we spiked blank urine samples at three concentrations of GLY and AMPA standards (0.5, 2 and 20 /L), in triplicate (n=3) within the same day, as recommended by the ICH guidelines, and over a period of three days; afterward, they were subjected to the proposed method and results were obtained. Considering three different days as the main source of variation, an analysis of variance (ANOVA) was performed for each concentration, obtaining estimations of within-day (S_w), also known as repeatability, and between-day (S_B). Also, the intra laboratory reproducibility or intermediate precision (S_{IP}) was obtained [**40, 41**]. All these parameters are shown in Table 3.

From these data, the corresponding relative standard deviations, RSD_{IP} are calculated, and were compared with the acceptable RSD percentages obtained from the AOAC Peer Verified Methods (PVM) program [**41, 45**]. As a quick rule [**41**], the RSD_{IP} results should be compared with one-half the corresponding RSD values tabulated. Our results, at the three concentration levels considered, were lower or the same order than the one-half $\%RSD_{AOAC}$ tabulated, so the proposed method can be considered as precise (Table 3).

Trueness and recovery

The trueness of an analytical procedure expresses the closeness of agreement between the mean value obtained from a series of measurements and the value which is accepted, either a conventional value or an accepted reference value like validation standard [**41**]. It can be obtained from the same ANOVA results previously described for the intermediate precision, and it is normally expressed in terms of bias or recovery obtained for each validation standard considered [**47**]. These recoveries are defined as the ratio between the mean on centration of analyte measured in the fortified sample and the concentration of analyte added ("true" reference value, not determined by method) in the fortified sample, expressed as a percentage. The recoveries obtained for the three validation standards are shown in Table 3. They have been checked for suitability by comparison with the published acceptable recovery range as a function of the analyte concentration [**41**]. In our case, two concentration of the three validation standards ranged

between 0.5 and 5 µg/L, the recovery range (%) could oscillate between 40 and 120% for the two of them (GLY and AMPA). The third one validation standard was 20 µg/L and the recovery range (%) could oscillate between 60-115% in both cases (GLY and AMPA). The recoveries obtained oscillated between these values 119% for 0.5 µg/L (AMPA), 108 and 107% for 5 µg/L (GLY and AMPA, respectively) and 109 and 104% for 20 µg/L (GLY and AMPA, respectively). Thus, the method can be considered as acceptable in terms of recoveries. These recoveries are higher than those obtained by Bernal et al. [**21**] in rat serum samples by liquid chromatography–fluorescence–mass spectrometry method. This may be due to the nature of the samples, as urine has less interferences than plasma. However, recently, Lopez-Ruiz et al. [**48**] have optimized a method to determine polar pesticides in human blood by LC-MS, obtaining recoveries of the same order than ours. Also, similar recoveries were obtained in human samples (milk and urine) by Jensen et al. [**35**] when a direct determination of GLY and AMPA by LC-MS/MS were applied.

Taking into account these considerations, the analytical procedure developed in this work can be considered suitably validated.

3.2 GLY and AMPA determination in women farmers

A total of 20 samples were analyzed using the proposed method. A blank sample, and two quality control were prepared and injected with the samples. Only one of the samples showed GLY positive results (Figure 2) and all of them were negative in relation

to AMPA (concentrations below LOQ). The GLY concentration detected was 2 µg/L. This result is similar to those detected previously in American farmers when urine is collected the same day of pesticides application (3.2 µg/L) [**49**]; after two days since application in French farmers (2 µg/L) [**46**] or post-application in Irish farmers (1.72 µg/L) [**37**].

Conclusions

In this study, an UPLC–MS/MS method was developed and validated for the determination of GLY and its metabolite AMPA in human urine, proving to be sensitive, reproducible, accurate and robust. Its recoveries (108-109% GLY and 104-119% AMPA) and intermediate precisions obtained (11.90-12.70% GLY and 4.8-9% AMPA) permit its validation. Moreover, it has been possible to apply the present method for detection and quantification of both compounds in human urine from women farmers, showing the presence of GLY in urine from one of the participants. For this reason, we can conclude that developed method can be used in routine laboratory analysis to monitor GLY and AMPA residues in human urine samples.

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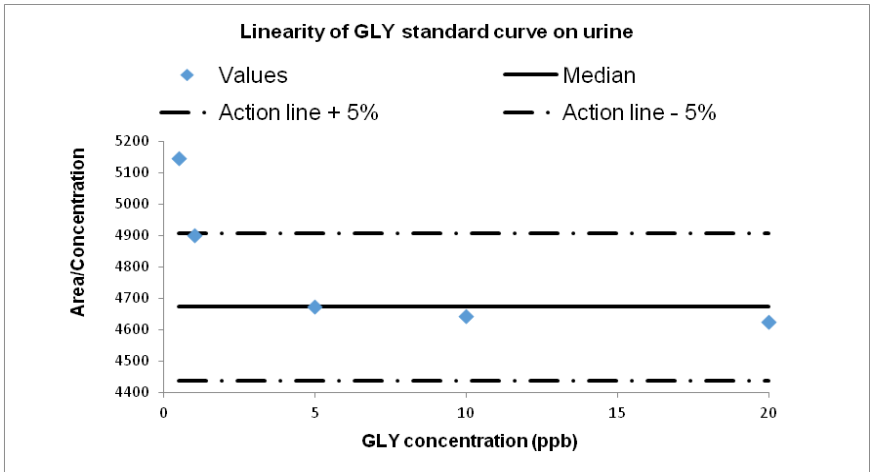
Figure Captions

Figure 1. Huber plot for assessing the linear range of GLY (a) and AMPA (b) in urine

Figure 2. UPLC-MS/MS chromatogram for GLY from one urine sample extract

Figure 1.


Linearity of GLY standard curve on urine
Values
Median
Action line + 5%
Action line - 5%
Area/Concentration
5200
5100
5000
4900
4800
4700
4600
4500
4400
0
5
10
15
20
GLY concentration (ppb)

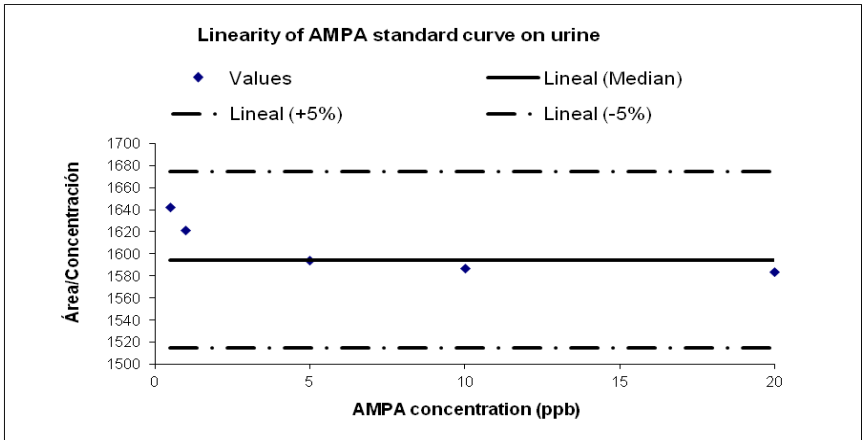
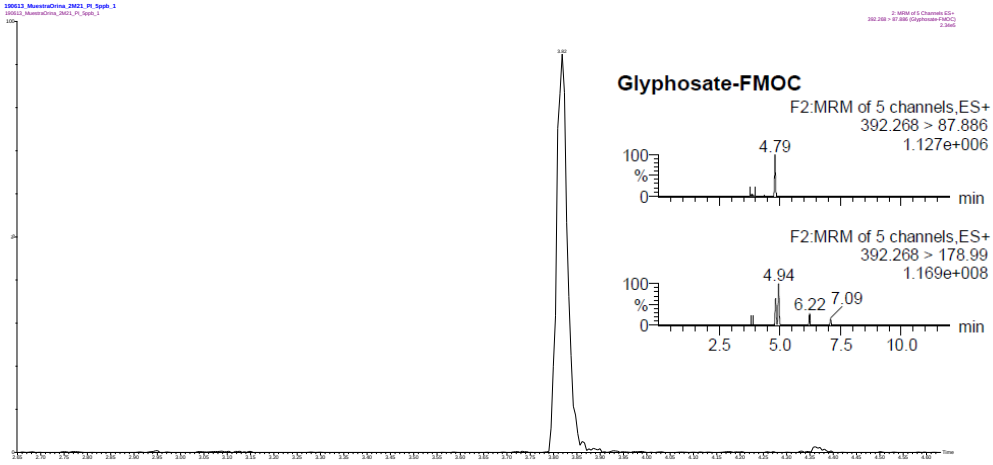

Linearity of AMPA standard curve on urine
Values
Lineal (Median)
Lineal (+5%)
Lineal (-5%)
Área/Concentración
1700
1680
1660
1640
1620
1600
1580
1560
1540
1520
1500
0
5
10
15
20
AMPA concentration (ppb)

Figure 2.


Glyphosate-FMOC
F2:MRM of 5 channels,ES+
392.268 > 87.886
1.127e+006
4.79
100
%
0
min
F2:MRM of 5 channels,ES+
392.268 > 178.99
1.169e+008
4.94
6.22
7.09
100
%
0
min
2.5
5.0
7.5
10.0

CAPÍTULO 4. NIVELES EN SANGRE DE METALES EN MUJERES AGRICULTORAS EN LA PROVINCIA DE SEVILLA

1.Introduction

Workers are exposed to a wide variety of chemicals through the air in the workplace. Every work environment has its own characteristics, being the ideal situation that it was like pure air, with the objective that the health of workers was not altered by chemical substances external to the air. The general Spanish law about health and safety in work (**B.O.E., 1995**), mentions the special care about women workers in pregnancy and workers that feed their children with maternal milk. This law also describes about the special care for keeping the fertility in both sexes. For farmers, the main chemicals products in the environment are the pesticides and the different formulations applied in the fruits and vegetables. A wide range of reports describe the effects of using and applying pesticides, and more specific are the publication about the health risks in the reentry in the crops after applying pesticides (EFSA, 2014a). Some Plants Protection Products (PPP) include in their formulation heavy metals (**Defarge et al., 2018**). In farmer women who harvest the fruits and vegetables, toxics metals can be introduced in the organism through the air (added to PPP´s formulations) causing toxic effects such as disrupting thyroid

function. Also, in the case of pregnant women, metals, might pass through the placenta and reach the fetus (**X. Wang et al., 2020**).

Women farmers who collect fruits and vegetables previously irrigated with different PPP are indirectly exposed to these chemicals. According to Rahayu et al (**Yekti Sri Rahayu, Tatik Wardiyati, 2020**) heavy metals are present in fertilizers and agrochemical, and, as some occupational health documents suggest, the chemical risk in this indirect exposition exist (EFSA, 2014a; Limón et al., 2017).

Several authors have been reported the relationship between metals exposure and the potentially toxic effects. Thus, has been showed that prenatal exposure to Pb is associated with reduce birth weight, birth length, mental retardation, autism, dyslexia, brain damage. Also, infertility, spontaneous abortions, and fetal and neonatal death have reported after occupational exposure to Pb. Mn exposition is associated mainly with increased infant mortality, alteration in cardiovascular function, and Parkinson disease (**Cerrillos et al., 2019; Okereafor et al., 2020**). Monteiro Fernandez et al (**Fernandes et al., 2020**) show the relationship between Al exposure neurodegenerative diseases.

On the other hand, human organism has requirements of appropriate amounts of essential metals such as K, Ca, Co, Cr, Cu, Mg, Mn, and Zn to maintain optimum health, though outside of

beneficial intake ranges, either elevated or reduced levels of these metals can adversely affect health (**Cerrillos et al., 2019**). Thus, low levels of Ca, outcomes colon, rectum and prostate cancer and high levels of Na induces systolic blood pressure, and other cardiovascular problems (**Lim et al., 2012**) .Also, K, is related with effects in the cardiac frequency when there is hypokalaemia or hiperkalaemia (**Halperin & Kamel, 1998**).

It is therefore important to study the degree of exposure of women farmers who collect fruits and vegetables previously irrigated with PPP because is the exposure of the unborn child in case of pregnancy or may be cause of alteration of the fertility. Other factors may have a significant influence in the levels of metals detected in biological samples, e.g. smoking or diet habits. The aim of this study was the simultaneous determination by ICP-OES of Al, Cu, K, Mn, Na, Pb, and Zn in blood samples taken once a quarter along one year in women farmers indirectly exposed to PPP, in order to compare with levels detected in women resident in the same rural environment but not occupationally exposed to PPP (NOE group).

2. Material and methods

2.1 Study population

A longitudinal study was conducted on a cohort of 39 women in fertile age from Marinaleda (Sevilla, Spain). Marinaleda

is a town with an area of 24.8 km^2 and a total population of 2,665 inhabitants, located between 37º22´00.6´´North 4º58´01.3´´ West and 37º22´47.0´´North 4º56´26.8´´ West. Of the total population, 65.2% are between 20 and 65 years of age, of which 1,299 are women, representing 49% of the population. Its political and social organization makes it unique and suitable for the achievement of this study. The economic activity of this town is based on agriculture where women are who collect the fruits and vegetables in field or work in the factory where these fruits and vegetables are canned. Total of 39 women (age between 18 and 45 years) were asked to be part in this longitudinal study. A group of 22 directly involved in collection of fruits and vegetables in field and 17 not involved in this activity but worker in the canned factory was taken as control group. The survey was divided in four periods with sample collection every three months along one year. Women with a clinical diagnosis of chronic diseases were excluded. Ethical clearance was obtained from Coordinating Committee of Ethics of Biomedical Research of Andalusia (Spain) and agreed with the Declaration of Helsinki for International Health Research. Written informed consent was also obtained from all the participants after being informed about the objectives of the study and the right to drop out of the study at any time.

2.2 Chemical and Reagents

Panreac (Barcelona, España) standard solutions of about 1000 mg L^{-1} were used as stock solution of each element for calibration. Other reagents used where of analytical grade. Milli-Q treated water was used throughout.

2.3 Analytical procedure

Peripherally blood samples were obtained from the studied women, by BD Vacutainer ® system, and collected in a container with EDTA for avoiding the coagulation. The blood samples with EDTA were kept frozen at -80 °C until analysis.

1 ml of homogenized whole blood was pre-treated, adding 4 ml of H_2NO_3 concentrated and 2 ml of H_2O_2 (30%), before introducing them in the Microwave Digestion System. After 15 minutes and 180ºC the incinerated samples were moved to the ICP for the next step.

The metals were determined by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) technique; model ICAP 6300 Duo Thermo Scientific. The instrumental conditions were as follows: approximate RF power, 1150 W; gas flow (nebulizer gas flow, auxiliary gas flow), 0.5 L/min; injection n of the sample to the pump flow, 50 rpm; stabilization time, 0 s.

2.4 Quality controls

Detection and quantification limits under reproducibility conditions, which were estimated as three and ten times the standard deviation (SD) resulting from the analysis of 15 blanks (**IUPAC, 1995**) were the lowest for Pb LOD: 3 µg/Kg and LOQ: 8 µg/Kg and the highest for Na: LOD: 9142 µg/Kg and LOQ: 30460 µg/Kg, and K: LOD: 4708 µg/Kg and LOQ: 15700 µg/Kg. Quality controls were performed to verify the accuracy of the analytical procedure. These controls were based on the study of the recovery percentage obtained with blank samples and reference material measured under reproducible conditions. Reference Materials were: ERM-CE195 (Trace metals in bovine blood), BCR-634 (Trace metals in human blood), were processed by digestion as the same way as blood samples.

2.5 Statistical analysis

Statistical analysis was performed using the statistical package IBM Statistics SPSS 24.0 (Statistical Package for the Social Sciences). To describe the characteristics of our participants, descriptive statistical parameters were initially computed. Arithmetic mean ± standard deviation, geometric mean, minimum and maximum values of concentration above the limit of detection were calculated for potential continuous variables. Categorical variables were described using frequencies and percentages.

3. Results and Discussion

The characteristics of population studied are presented in table 1. All the participants were from Marinaleda and Matarredonda (Seville). Most of women had been working more than 10 years in their present job in both studied groups (≈89% in farmers and 73% in NOE group). Most of the women from both groups were nonsmokers (66.66% in farmers and the 54.54% NOE group). All the participants in both groups consumed fruits every day and consumed wide variety of fish frequently (1-3 times/week).

Table 1.- Main Characteristics of studied population

	FARMERS (N=22)	NOE (N=17)
AGE	(%)	(%)
18-24 years	11.11	9.09
25-30 years	11.11	27.27
31-40 years	11.11	27.27
>40 years	66.66	36.36
YEARS WORKING AT THAT	(%)	(%)
WORK	11.11	0
<5 years	0	27.27
5-10 years	88.88	72.72
>10 years		
SMOKING HABITS	(%)	(%)
Non smokers	44.44	36.36
He/She Was smokers	22.22	18.18
Passive smoker	0	0
Smokers	33.33	45.45
FRUIT CONSUMPTION	(%)	(%)
>3 times/day	11.11	18.18
1-2 times/day	88.88	81.81
0 times/day	0	0
VEGETABLES	(%)	(%)
CONSUMPTION	12.50	36.36
>2 times/day	37.50	54.54

1 times/day 0 times/day	50.00	9.09
FISH CONSUMPTION	(%)	(%)
>4 times/week	0	0
1-3 times/week	88.88	100
0 times/week	11.11	0
KIND OF FISH	(%)	(%)
Blue Fish (BF)	12.50	50.00
White Fish (WF)	37.50	0
ShellFish/Mollusk (S/M)	0	0
BF+WF	25.00	10.00
BF+WF+S/M	12.50	20.00
BF+S/M	12.50	10.00
WF+S/M	0	10.00

The concentration and descriptive statistics for each metal in blood along the four sampling are reported in tables 2-5. All the results obtained were over the LOD and the LOQ and are in µg/kg. The 100% of blood samples contained quantifiable amounts of each metal studied.

In the present study, levels of Na were within the normal range for natremia (140 mmol/L) (**Sumit & Berl, 1998**) along the four sampling in both groups studied. No significant differences between NOE and farmers women were detected. However, when comparing the different values within NOE group along the monitoring period, it was detected significant differences between levels in women in the first sampling in relation with levels in third ($p<0.05$) and fourth ($p>0.001$) sampling. These observed differences may be due to

many variables that can affect the levels of Na in blood, mainly consumption of salt though diet. Blood levels of K can be influenced by the diet but in case of farmers the exposition is also though the environment when fertilizers with high concentration in macronutrients such as K are employed. That is the case in the present study, where NPK fertilizer was employed along the period between the second and third sampling. Even so, levels of K in both studied groups were under the normal values (5mmol/L) (**Sterns et al., 2016**), and no significant difference was observed between famers and NOE groups along the study. But when comparing concentrations of K along the study in the farmer group, a significant difference was detected between measured levels in sampling 2 and 4 ($p<0.001$), with higher levels of K in samples from the second (4.58 mmol/L) and third (4.35 mmol/L) monitoring when NPK fertilizer was employed.

In relation with Al levels in serum, values <56.6 µg/Kg are considered normal and when are higher than 481 µg/Kg can be related with some kind of disease (**Fernández-Maestre, 2012**). Our results are higher than this value (481 µg/Kg) mainly in farmers women along the four sampling period. But only in the third sampling were found significant difference between farmers and NOE groups ($p<0.001$) with higher values in farmers than in NOE

participants. The highest values in NOE group along all the period studied was detected in the first monitoring (1554.7 µg/Kg) with a great significant difference when comparing with the rest of monitoring ($p<0.001$). Aluminium is the third common element found in the crust earth. When the pH decreases, the aluminum toxicity increases. The soil with pH acid for low quantity of rain, can allow the crop get high levels of Al (**Jaishankar et al., 2014**) and workers that handle these crops can indirectly be exposed to this metal. On the other hand, women from NOE group are working mainly in a canning factory where Al is commonly used (**Kremser et al., 2021**), that may be the reason for the higher levels detected in this group.

Mn is associated to some pesticides. In this context, different degradations are estimated in Mn-containing pesticides products (**Dórea, 2021**). In the present study Mn 3% was employed as fertilizer in broad beans, artichokes, broccoli and zucchini cultures along the period of second and third sampling. Normal levels of this metal are 3.77-14.15µg/Kg in whole blood. In our study, all the samples are over the 80 µg/Kg of blood. There were not significant differences between farmers and NOE women in samples from the four-monitoring period, neither when comparing within famers group or comparing within NOE group along the

study. The Mn is also related to exposure with steel, alloys and welders (**Park et al., 2015**), so it is possible that workers could handle metallic bins or cans with Mn.

In relation with Zn levels in human blood, normal limits are about 1 mg/Kg, all the participants in the present study had Zn concentrations over 4 mg/Kg. It was observed a higher levels of Zn in NOE group than in farmers along the study, but only in samples from the fourth monitoring significant differences ($p>0.05$) were detected between both groups (5588.12 µg/Kg in NOE group and 4472.17 µg/Kg in farmers). On the other hand, Zn concentration in samples from famers in the first sampling (5584.54 µg/Kg) was significantly higher ($p<0.05$) in relation with those detected in farmers in the rest of the sampling (4426.95-4540.00 µg/Kg). In NOE group also significant differences were detected when comparing samples from the first and second sampling ($p<0.001$). Zn are present in a wide variety of food at very different concentrations (**EFSA, 2014b**), differences observed between groups may be due to this point.

Cu levels in both studied groups along the four sampling were within normal range (666-1462 µg/Kg) (**Llorente Ballesteros et al., 2017**), despite of employing copper oxychloride (70%) as fertilizer in chickpeas cultures and olive grove along all the study. No significant difference between NOE and farmer women were

detected along the period studied. However, significant differences ($p< 0.001$) were observed within farmers group when comparing samples from the first and second monitoring (≈930 µg/Kg) with samples from the third one (622 µg/Kg). Also, in NOE group samples significant differences ($p< 0.001$) were detected between levels of Cu in samples from the first and second monitoring (≈1000 µg/Kg) in relation with values detected in samples from the third and fourth ones (≈660 µg/Kg). differences observed in Cu levels in blood may due to the intake of this element through diet, both through food and contaminated water (**K. Wang et al., 2021**).

There is not a normal range of Pb in blood due its toxic characteristics, but there is a threshold level of Pb in blood for workers uploaded every year. The threshold limit in 2019 was 660 µg/kg (**INSST, 2019**). In the present study, all the results obtained in both groups along the study were under this value. No significant differences were detected in both groups studied along the four monitoring dates. However, significant variations were observed within the groups along the monitoring period. Thus, samples from the second and third sampling were significantly lower ($P<0.05$) than those from the fourth sampling (11 and 14 µg/Kg *vs* 111.73 µg/Kg). In NOE group also were observed significantly differences ($p<0.01$) between samples from the second

and third sampling (12 and 11.89 µg/Kg) in relation with those from the first one (148.83 µg/Kg). Exposure to this metal is more related to environmental pollution, industries workplace or smoking habits than to PPP exposure (**Ali et al., 2019**), this may be the reason why NOE women presents higher levels than farmers one.

We can conclude that the results of our study suggest that levels of blood metals in women farmers are not significantly influenced by the indirect exposure to PPP that contain these elements in their formulation. These findings are important because it would probe that diet is the main source of these metals in blood samples.

Conflict of interest

The authors declare that there is no conflict of interest.

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Table 2.- First Samples (October 17). Descriptive statics of metal concentrations in blood. Concentrations are in µg/Kg unless otherwise noted

	WOMEN FARMER N= 22			NOE WOMEN N=17		
Group/ Element	Mean±SD	Geometric Mean	Min-Max	Mean±SD	Geometric Mean	Min-Max
Alkali Metals						
Na (mmol/L)	141.23±2.01	141.44	138-146	140±2.34	113.34	135-144
K (mmol/L)	4.35±0.30	4.31	3.6-4.8	4.41±0.34	3.84	3.8-4.9
Post Transition Metal						
Al	963.53±439.19	895.06	420-1890	1554±1054	1195.89	650-3940
Pb	57.14±8.60	57.14	45-67.7	148.83±235.77	57.59	45-630
Transition Elements						
Mn	77.8±7.49	80.00	62-107	79.68±13.76	69.49	72-91
Cu	940.11±152.46	928.60	754-1300	982.99±117.95	886.06	840-1240.9
Zn	5429.41±2070.54	4981.78	350-9890	6203.5±821.70	6149.23	4800-7050

Table 3.- Second Sample (February 18). Descriptive statics of metal concentrations in blood. Concentrations are in µg/kg unless otherwise noted

	WOMEN FARMER N= 22			NOE WOMEN N=17		
Group/ Element	Mean±SD	Geometric Mean	Min-Max	Mean±SD	Geometric Mean	Min-Max
Alkali Metals						
Na (mmol/L)	141.00±1.54	141.17	139-145	140.75±1.57	114.13	140-143
K (mmol/L)	4.62±0.35	4.58	4.1-5.2	4.42±0.24	3.95	4.1-4.8
Post Transition Metal						
Al	571.14±72.58	578.14	459-688	559.95 ±81.51	511.23	405-676
Pb	11.38±3.88	10.63	9-20	12.07±4.48	10.63	9-20
Transition Elements						
Mn	77.37±1.62	77.38	74-81	77.05±1.46	63.96	74-80
Cu	922.35±287.89	900.86	713-1790	1053.82±363.27	916.03	766-1199
Zn	4471.65±805.13	4493.44	350-9890	4572.28±490.31	4145.72	3776-5324

Table 4.- Third Sample (June 18). Descriptive statics of metal concentrations in blood. Concentrations are in µg/kg unless otherwise noted

	WOMEN FARMER N= 22			NOE WOMEN N=17		
Group/ Element	Mean±SD	Geometric Mean	Min-Max	Mean±SD	Geometric Mean	Min-Max
Alkali Metals						
Na (mmol/L)	141.07±1.50	141.23	138.5-143.6	142.06±1.91	114.35	140-146
K (mmol/L)	4.35±0.25	4.35	4.06-4.75	4.42±0.22	3.84	4.1-4.8
Post Transition Metal						
Al	672.94±910.77	568.35	210-4120	125.62±10.93	195.16	120-150
Pb	14.11±6.18	13.10	10-30	12.5±4.47	11.48	10-20
Transition Elements						
Mn	80.00±0.00	80.00	80-80	80.00±0.00	80.00	80-80
Cu	592.35±158.76	586.34	100-830	685.62±153.49	564.40	540-980
Zn	4472.94±438.43	4417.46	3450-4940	5371.25±972.93	4551.13	3950-6820

Table 5.- Fourth Sample (October 18). Descriptive statics of metal concentrations in blood. Concentrations are in µg/kg unless otherwise noted

	WOMEN FARMER N= 22			NOE WOMEN N=17		
Group/Element	Mean±SD	Geometric Mean	Min-Max	Mean±SD	Geometric Mean	Min-Max
Alkali Metals						
Na (mmol/L)	141.34±1.39	141.26	137.5-142.6	143.28±1.52**	114.68	141.4-145.7
K (mmol/L)	4.07±0.22	4.08	3.67-4.53	3.88±0.29	3.41	3.54-4.29
Post Transition Metal						
Al	817.64±457.95	747.15	200-1900	500.62±242.33	480.46	120-800
Pb	117.05±139.94	55.43	10-400	113.75±147.37	59.09	10-420
Transition Elements						
Mn	80.00±0.00	80.00	80-80	80.00±0.00	80.00	80-80
Cu	795.29±217.77	777.23	600-1120	672.5±81.52	642.07	520-790
Zn	4554.70±1326.45	3072.74	30-5400	5588.12±795.35	3801.37	4240-6640

** $p<0.01$

V. DISCUSION

Teniendo en cuenta que nos marcamos un objetivo principal, apoyado en varios objetivos secundarios, vamos a llevar a cabo la discusión de estos secundarios para concluir con el objetivo principal

En nuestro análisis sobre los mecanismos tóxicos de los plaguicidas más utilizados, así como los efectos que pueden generar en trabajadores con exposición indirecta, poniendo especial atención a mujeres y su reentrada en cultivos, tras la aplicación para la recogida de futas y vegetales, deberíamos considerar que las trabajadoras agrícolas representan un grupo potencialmente vulnerable de acuerdo a la combinación de factores sociales y peligros inherentes al trabajo en sí. Aunque la mayoría de las investigaciones se han centrado en trabajadoras que manipulan, mezclan, aplican y limpian equipos con plaguicidas, queda probado que la población expuesta es mayor, no pudiéndose limitar a trabajadores con contacto directo, sino que también la exposición a bajas concentraciones puede tener efectos adversos sobre la salud **(Monteiro Nassar and Gerardo Ribeiro, 2020**). Un ejemplo de esto puede ser el presente estudio.

En nuestro trabajo hemos pretendido estudiar la posible relación de exposición laboral y de un entorno rural, al estado de salud de mujeres trabajadoras agrícolas frente a un grupo de mujeres residentes en el mismo entorno rural, pero, laboralmente, no expuestas a estos contaminantes.

Desde el punto de vista ambiental, se ha medido en orina concentraciones de glifosato, como herbicida ampliamente utilizado

(**Davoren and Schiestl, 2018**) y su metabolito AMPA. En este sentido, también se han determinado la concentración de metales en sangre. Metales, que se pueden encontrar en formulaciones de fitosanitarios, como en el suelo del que se obtienen los cultivos o de algún producto fertilizante.

Para ello **se ha llevado a cabo la validación de un método para la detección de glifosato y su metabolito, AMPA, en orina por UPLC-MS/MS**, mediante una derivatización previa con FMOC. Un método rápido y sensible que se aplicó a las muestras de sangre obtenidas de orina y, mediante la cual se detectó solamente en una de ellas en uno de los muestreos, aunque el metabolito AMPA no fue detectado.

Una vez aplicado el método validado a la determinación de ambos compuestos a las muestras reales, se ha podido comprobar que, una de ella tenía niveles de glifosato en la orina suficiente para ser cuantificado, siendo este valor detectado, del mismo orden de los detectados en otros países en personas que no se encuentran expuestas directamente al producto (**Nova, Calheiros and Silva, 2020**) . Estos datos coinciden con publicaciones que indican que este producto es rápidamente metabolizado y excretados por la orina en un periodo entre 4-72 horas tras la exposición (**Fernández *et al.*, 2020**). En otros estudios se concluye que el GLY es rápidamente excretado y prácticamente no se metaboliza en AMPA (**Mcguire *et al.*, 2016**) Esto nos lleva a ratificar la posibilidad de una posible exposición ambiental de estas trabajadoras a través de la

aplicación de los plaguicidas en los cultivos aledaños.

En relación a los metales se han detectado niveles por encima del límite de cuantificación de todos los elementos estudiados (Na, K, Al, Mn, Zn, Cu, Pb). Respecto a metales influenciados por la dieta, los niveles de Sodio estuvieron dentro de los rangos normales (140 mmol/L) (**Sumit and Berl, 1998**) en los cuatro muestreos llevados a cabo. No se encontraron diferencias significativas entre el grupo NOE y el grupo de mujeres expuestas. Los niveles de K, que también se ven influenciados por la dieta, estuvieron por debajo de los valores normales en ambos grupos (5mmol/L) (**Sterns, Grieff and Bernstein, 2016**), Sin embargo, en el grupo de mujeres expuestas, en el periodo comprendido entre el segundo y tercer muestreo, se empleó el fertilizante NPK. Justamente en este periodo hay diferencias significativas en los niveles medidos en los muestreos 2 y 4 ($p<0.001$), con mayor nivel de K en muestreos de la segunda (4.58 mmol/L) y tercera (4.35 mmol/L) monitorización, cuando el fertilizante NPK se utilizó.

En relación con los niveles de Aluminio en suero, los valores <56.6 µg/Kg se consideran normales y cuando son mayores a 481 µg/Kg pueden estar relacionados con algún tipo de enfermedad (**Fernández-Maestre, 2012**). Nuestros resultados son mayores a estos valores (481 µg/Kg) principalmente en trabajadoras agrícolas a lo largo del periodo de los cuatro muestreos. Pero solamente en el tercer muestreo se encontraron diferencias significativas entre

trabajadoras expuestas y el grupo NOE ($p<0.001$) con mayores valores en los agrícolas que en el grupo NOE. El mayor valor en los participantes NOE a lo largo de todo el periodo de muestreo fue detectado en la primera monitorización (1554.7 µg/Kg) con una gran diferencia significativa cuando se compara con el resto de monitorización ($p<0.001$). Pero, teniendo en cuenta que, diariamente podemos tener un consumo de Aluminio en la dieta de, entre 5-10 mg (**Nordberg *et al.*, 2007**) , que el aluminio es el tercer elemento encontrado en la corteza de la tierra, (**Jaishankar *et al.*, 2014**) y que, el material metálico de envasando comúnmente contiene aluminio, (**Kremser *et al.*, 2021**), nos impide justificar estos valores obtenidos, dado que en estudios donde los trabajadores estuvieron expuestos a diversos plaguicidas, los niveles de los trabajadores expuestos directamente a plaguicidas fueron mayores que los obtenidos en nuestro caso (**Alves *et al.*, 2016**)

El Mn se asocial a algunos plaguicidas, como, entre otros, el Mancozeb (**Rocha *et al.*, 2015; Dórea, 2021**). Este metal, se asocia al acero, soldaduras y aleaciones (**Park *et al.*, 2015**). Los niveles normales de este metal en sangre, se encuentran entre 3.77-14.15µg/Kg. En nuestro estudio, todas las muestras estuvieron por encima de los 80 µg/Kg de sangre. No hubo diferencias significativas entre agricultoras y mujeres no expuestas en los 4 muestreos llevados a cabo, ni en la comparación entre expuestas y NOE, pero sí cabe reseñar que estos valores son mucho mayores que otros valores determinados en estudios similares (**Rocha *et al.*,**

2015), en los que, agricultores de viñedos presentaron alrededor de 2 µg/Kg en su exposición indirecta a plaguicidas.

En relación a los niveles de Zn en sangre humana, los límites normales se encuentran sobre 1 mg/Kg. Todos los participantes del estudio tuvieron concentraciones de este metal sobre 4 mg/Kg. En estudios de exposición directa a plaguicidas, se observó concentraciones de Zin en sangre, en torno a los 200 mg/Kg (**Alves *et al.*, 2016**). Y, en estudios de exposición indirecta, las concentraciones de este metal rondaron el 1-1.5 mg/Kg (**Rocha *et al.*, 2015**). Además, el Zn está presente en una amplia variedad de alimentos a muy diferentes concentraciones (**EFSA, 2014**), y hay estudios donde se puede comprobar que en alimentos españoles, puede llegar a haber una concentración de Zn de hasta 6.44 mg/Kg (**Rai *et al.*, 2019**) por lo que, las diferencias observadas entre grupos, pudieran deberse a multiples factores.

Los niveles de Cu en ambos grupos de estudio a lo largo de los cuatro muestreos estuvieron dentro del rango normal (666-1462 µg/Kg) (**Llorente Ballesteros *et al.*, 2017**), a pesar de emplear Oxicloruro de Cobre (70%) como fertilizante en cultivos de garbanzos y olivar a lo largo de todo el estudio. El Cu es un metal que se encuentra frecuentemente en la dieta (**Wang *et al.*, 2021**). En alimentos procesados procedentes de España, nos podemos encontrar niveles de, hasta 7 mg/Kg de Cu (**Rai *et al.*, 2019**). No se encontraron diferencias significativas entre el grupo de mujeres expuestas y el grupo NOE durante el periodo estudiado.

No hay un rango normal de plumbemia en sangre de acuerdo a sus características toxicas, pero hay un Valor Limite Biológico de plomo en sangre para trabajadores, que se actualiza cada año. El valor limite en 2019 fue de 660 µg/kg (**INSST, 2019**). La exposición a este metal está más relacionada con la contaminación medioambiental, lugares de trabajo industrial y hábitos de consume de tabaco que la exposición a biocidas o fitosanitarios (**Ali, Khan and Ilahi, 2019**), En comida procesada procedente de España, se ha obtenido niveles de Pb, hasta 7 mg/Kg (**González-Martín *et al.*, 2018**). En este estudio, todos los resultados obtenidos en ambos grupos a lo largo del estudio estuvieron por debajo de este Índice Biológico de Referencia para el plomo, pero por encima de valores en estudios similares (**Rocha *et al.*, 2015**) No se detectaron diferencias significativas en los grupos estudiados a lo largo de los diferentes muestreos.

En un estudio realizado en Grecia **(Leotsinidis and Kondanis, 1990**), en 1990, en un medio rural para evitar contaminación ambiental, se obtuvieron concentraciones de 3840 µg/Kg de Pb, 187.6 g/Kg de Zn y 10610 µg/Kg de Cu en pelo del cabello. No son comparables los valores, dado que han pasado muchos años y la concentración obtenida en el cabello no es comparable a la concentración en sangre. Pero, en un estudio publicado en 2016 (**Fierens *et al.*, 2016**), llevado a cabo en Ath (Bélgica) se observó sobre 20 µg/Kg de Pb, lo que nuestros resultados son mejores en la segunda y tercera toma y peores en la

primera y la cuarta.

Una vez determinados los contaminantes ambientales que nos propusimos en los objetivos, el siguiente paso era hacer **un estudio del estado de salud de las participantes de nuestro estudio**. A las trabajadoras agrícolas que realizan la recolección de vegetales y verduras, no se les aplica protocolo "plaguicidas" porque en su reentrada al lugar de trabajo ha transcurrido el tiempo de espera que indica el fabricante del producto y, además, ellas no aplican, por lo que, en teoría, no deberían estar expuestas a plaguicidas. . A pesar de esto, para nuestro estudio, medimos los parámetros recogidos en Protocolo de Vigilancia de la salud "plaguicidas" **(Ministerio de Sanidad y Consumo, 1999**) de aplicación a cualquier trabajador que tras la evaluación de riesgos resulte estar expuesto a plaguicidas, donde se piden los valores de colinesterasa plasmática, eritrocitaria, GGT y GPT y también empleamos la guía para vigilar la salud de los trabajadores del sector agrario **(Ministerio de Sanidad Servicios Sociales e Igualdad, 2013**). En esta guía se recomienda que, al trabajador agrícola, se le aplique el protocolo "Productos Fitosanitarios y Biocidas", que, no existe: entendemos que hace referencia al protocolo "Plaguicidas" mencionado anteriormente.

Además de los parámetros concretos señalados en el Protocolo, incluimos otros parámetros que nos parecieron interesantes como, han sido parámetros clínicos en sangre, biomarcadores de estrés oxidativo (LPO y oxidación proteíca) y

marcadores de daño renal temprano (Nacetil-b-D-glucosaminidasa (NAG), Lipocaina asociada a la gelatinasa de neutrofilos (NGAL) y Albumina, Proteinuria, Kidney Injure Molecule 1 (KIM-1), y Transferrina).

En relación a los biomarcadores estipulados por los protocolos de Vigilancia de la salud publicados en nuestro país, observamos que la función hepática no se ve afectada en ninguno de los grupos estudiados. Las transaminasas ALT y AST se encontraban, entre 15- 20 U/I. a lo largo del periodo de estudio, dentro de los valores de referencia. En otros estudios realizados a aplicadores de plaguicidas, se manifestó que estos parámetros están influenciados por la exposición a plaguicidas, aunque en los resultados, no hay diferencias significativas entre el grupo control y el grupo expuesto (**Awad *et al.*, 2014**) También se ha publicado recientemente un estudio, también a aplicadores (**Tsagué Manfo *et al.*, 2020**) donde, estando todos los valores entre los 15-25 U/I, se encuentra diferencia significativa entre el grupo aplicador y el control en el parámetro ALT, pero no en el AST.

En las colinesterasas, no se encontraron diferencias significativas en la actividad de la BuChE, a lo largo de las cuatro tomas de muestra entre las trabajadoras rurales expuestas y no expuestas (NOE) a plaguicidas. Sin embargo, la actividad de la AChE fue menor (10%, 8% and 10%) en la segunda, tercera y cuarta toma de muestra en mujeres agrícolas frente a las mujeres NOE, aunque,

estadísticamente no fueran diferencias significativas. La inhibición observada en la actividad de la AChE durante el periodo de muestreo, está lejos del Indice de exposición Biológica, que establece el 70% de actividad AcChE, lo que significa una actividad menor al 30% en la actividad enzimática.(**INSST, 2019**).

En estudios de aplicadores de varios plaguicidas (9% organofosforados) se encontró una disminución de la actividad de la AChE del 16.5% (**Shentema *et al.*, 2020**). En otro estudio llevado a cabo en Brasil (**Nerilo *et al.*, 2014**) donde del total de plaguicidas, el 5.9% eran organofosforados y el 0.6% Carbamatos, mostraron una disminución >30% de actividad, lo que el mismo autor contrasta con otros estudios que difieren de sus resultados. En otros estudios, se puede constatar una reducción de la actividad enzimática (17%-26%) en pulverizadores de pesticidas y en mujeres que arrancaban hojas en plantaciones de té en India **(Fareed *et al.*, 2017; Kori *et al.*, 2018; Dhananjayan *et al.*, 2019**). También, Cestonaro et al. (**Cestonaro *et al.*, 2020**) presenta un estudio en agricultoras de Brasil, con una actividad enzimática de AChE disminuida en un 21% en mujeres agrícolas, comparadas con NOE. Más acorde a nuestros resultados, Vikkey et al. (**Vikkey *et al.*, 2017**) observó que un 88% de algodoneros de Benin mostraron menos del 20% de inhibición en la actividad de la AChE.

También hay autores, que han concluido que, a pesar de que la actividad de la AChE se usa como marcador biológico para la exposición a pesticidas en hombres, en algunos casos no se han

encontrado diferencias en la actividad enzimática entre los trabajadores expuestos y NOE **(Zepeda-Arce *et al.*, 2017; Hilgert Jacobsen-Pereira *et al.*, 2018**).

La actividad de la AChE está significativamente e inversamente asociada con la glucosa, mostrando que la exposición crónica a pesticidas pueden contribuir a un incremento de los niveles de la glucemia (**Juntarawijit and Juntarawijit, 2018; Cestonaro *et al.*, 2020**). En nuestro resultado se puede comprobar un incremento significante en los niveles de glucosa en trabajadoras agrícolas con comparación con el grupo NOE en dos de los cuatro muestreo, aunque no supusieron una hiperglucemia, seguramente porque, dentro de los pesticidas estudiados que incrementan la glucemia **(Juntarawijit and Juntarawijit, 2018**) no se emplean solamente OP´s y Carbamatos, sino que se emplean más, y la exposición a estos compuestos fue indirecta.

Con respecto a los marcadores de estrés oxidativo, pudimos observar, que los niveles de MDA fueron mayores en mujeres expuestas que en el grupo NOE, pero solamente en dos muestreos (Octubre 2017 y Febrero 2018). De manera similar, niveles de oxidación proteica en mmol de grupos carbonilos fueron mayores en trabajadoras agrícolas que en el grupo NOE en los mismos muestreos. En estudios de exposición directa a plaguicidas (**Ogut *et al.*, 2011**) se encontraron diferencias significativas en los niveles de MDA, sin embargo, la actividad de la AChE no se vio afectada. Un estudio reciente llevado a cabo en Sicilia (**Ledda *et al.*, 2021**)

también constata el incremento de TBARS en trabajadores expuestos a mezclas de plaguicidas, lo que consolida los estudios previos llevados a cabo **(Wafa *et al.*, 2013; Hilgert Jacobsen-Pereira *et al.*, 2018; Kori *et al.*, 2018; Lozano-Paniagua *et al.*, 2018**). La justificación de los marcadores a lo largo del tiempo, podría justificarse a que el mecanismo molecular por el que se produce el estrés oxidativo no está aun claro (**Lukaszewicz-Hussain, 2010; Lozano-Paniagua *et al.*, 2018)**.

Cabe destacar los resultados obtenidos con respecto a los marcadores tempranos de daño renal, los cuales se han visto claramente alterados en las trabajadoras agrícolas. En nuestro estudio, aparecen diferencias significativas de creatinina en sangre en el primer muestreo y en segundo, donde las trabajadoras expuestas tienen mayores niveles que las trabajadoras no expuestas, lo que coincide con lo publicado en otros estudios **(Yassin and Al-Shanti, 2016; Tsagué Manfo *et al.*, 2020**). Profundizando en el daño renal, se realizaron determinaciones en Proteinuria, Kidney Injure Molecule 1 (KIM-1), N-acetyl-β-D-glucosaminidase NAG, , neutrophil gelatinase-associated lipocalin NAGL, Albumina y Transferrina. No encontramos que la KIM-1 presenta de las mujeres NOE se encuentra significativamente aumentada respecto a las trabajadoras expuestas. Con el NGAL, ocurre lo mismo , salvo en la primera toma, donde las trabajadoras expuestas tenían el marcador aumentado sobre las NOE, y en el resto de parámetros, entre las NOE y las trabajadoras expuestas, hubo muestreos en los que un grupo tenía los parámetros en mayor nivel que el otro.

Para poder comprobar una posible contaminación medioambiental y no solamente la exposición laboral, se introdujo otro grupo control de mujeres no pertenecientes a zona rural, ni de la comunidad autónoma andaluza. Y fueron estos resultados los que mostraron que tanto las trabajadoras expuestas, como las trabajadoras no expuestas, tenían los niveles de daño renal temprano (salvo la albumina) significativamente elevados respecto al grupo control no rural, mostrándose una clara diferencia entre los tres grupos de estudio.

A pesar de que no se han encontrado estudios donde se muestre los niveles de marcadores temprano en exposiciones indirectas a plaguicidas, los resultados obtenidos en nuestro estudio, son coherentes y coinciden con los publicados, donde se hace referencia a la aplicación de plaguicidas y el daño renal (**Mejía *et al.*, 2014; Yassin and Al-Shanti, 2016; Valcke *et al.*, 2017)**

Otros parámetros estudiados fueron las funciones hormonales, ya que algunos pesticidas pueden interferir con la función hormonal femenina **(Bretveld *et al.*, 2006**). En el presente estudio, se observaron niveles ligeramente más altos en FT4 y LH y FSH de mujeres NOE frente a las agrícolas, pero solamente en Junio y Octubre del 2018 fue estadísticamente significativo la comparación entre la FT4 y la LH, respectivamente.. Algunos autores sugieren que la exposición a pesticidas puede afectar a la función tiroidea (**Bernieri *et al.*, 2019; Curl *et al.*, 2020**). En relación con los niveles de LH, todos los niveles detectados fueron mayores a 30 U/L, considerados como un indicador de menopausia

en mujeres mayores o una señal de malfunción ovárica o una menopausia temprana en mujeres jóvenes. Teniendo en cuenta el rango de edad de todas las participantes en el presente estudio, se podría considerar que la exposición crónica a trazas de pesticida podría provocar una disrupción en el balance hormonal.

En un estudio realizado, con una exposición directa moderada a plaguicidas **(Recio *et al.*, 2005**) se observó también la alteración de las hormonas LH y FSH. Por otro lado, un estudio realizado con trabajadores de granja (a cielo abierto), y de invernadero, comparadas con grupo control, **(Slimani, Boulakoud and Abdennour, 2011**) mostró, de manera similar a nuestro estudio que, las trabajadoras de invernadero tenían disminuida la LH y la FT4 respecto a las trabajadoras de granja, y estas, a su vez, disminuida frente al grupo control. Respecto a la FSH, estaban en ambos casos disminuidas respecto al grupo control, pero no hay diferencias significativas entre los dos grupos estudiados, a diferencia de lo que ocurre en nuestro caso.

De manera global, el estudio manifiesta que las mujeres expuestas indirectamente a plaguicidas presentan:

-Menor actividad en las colinesterasas

-Mayores niveles sanguíneos en las transaminasas hepáticas

-Mayor nivel de estrés oxidativo

-Elevados niveles de algunos metales en sangre (Zn, Al, Mn)

-Mayor nivel de daño renal temprano

-Alteraciones hormonales en las funciones tiroideas y reproductoras.

Y, además, los resultados del estudio de correlación entre los marcadores renales y los parámetros medidos en sangre, así como las actividades de las Acetilcolinesterasa, estrés oxidativo y metales, ponen de manifiesto que, la Acetilcolinesterasa en sangre, está relacionada con la proteinuria, el marcador de daño renal temprano KIM-1, la albumina en Orina y transferrina en orina. También que, los niveles de oxidación lipídica, están relacionados con la transferrina en orina

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VI. CONCLUSIONES

De los resultados obtenidos en el presente estudio de investigación, podemos obtener las siguientes conclusiones:

PRIMERA.- Que los parámetros biológicos de seguimiento de la salud a las trabajadoras agrícolas que se recogen actualmente en la normativa y protocolos españoles, son insuficientes para garantizar la salud las trabajadoras, así como también son insuficientes para garantizar una fertilidad y desarrollo adecuado de la descendencia de las trabajadoras, como la ley de PRL **(BOE, 2014**) recoge en su articulado.

SEGUNDA.- Que, en los trabajos a cielo abierto, podría haber menor contaminación laboral debido al producto químico desprendido por la vegetación, pero mayor contaminación medioambiental si se aplican productos en otros cultivos cercanos, en contraposición a los trabajos en invernaderos, donde la propia construcción puede favorecer más la contaminación por exposición laboral frente a la medioambiental.

TERCERA.- Que se hace necesario un mayor hincapié en la formación de las mujeres que llevan a cabo estas tareas de recolección, para poder cambiar ciertos hábitos higiénicos en su trabajo diario, con el fin de disminuir la contaminación por estos agentes químicos.

CUARTO.- Que se hace necesario un mayor hincapié en el uso de Equipos de Protección Individual específicos frente a plaguicidas.

QUINTO.- Que se hace necesario ampliar y profundizar en el estudio otros parámetros alternativos como los que aquí presentamos, en un número mayor de participantes y en distintos tipos de exposición (recolección de verduras, almacenamiento de plaguicidas, transporte de fitosanitarios etc..).

Completarían esos estudios las mediciones ambientales y personales de los productos químicos estudiados a los que las trabajadoras están expuestas. Incluso, realizar los estudios con trabajadores llevando los EPI´s recomendados frente a otros trabajadores que mantuvieran las condiciones actuales, complementarían esta línea inicial de investigación.

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VII. ANEXOS

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