FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA ANALÍTICA

UNIVERSIDAD DE GRANADA

EVALUACIÓN DE LA INCORPORACIÓN EN LA DIETA ANIMAL (INGESTA Y METABOLIZACIÓN) DEL PROPIL PROPANO TIOSULFONATO COMO ADITIVO NATURAL DERIVADO DE ALIÁCEAS

TESIS DOCTORAL

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Granada 2017

Editor: Universidad de Granada. Tesis Doctorales
Autora: Paloma Abad Campos
ISBN: 978-84-9163-735-6
URI: http://hdl.handle.net/10481/49075

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EVALUATION OF THE INCORPORATION IN THE ANIMAL DIET (INGESTION AND METABOLISATION) OF PROPIL PROPANE THIOSULFONATE AS A NATURAL ADDITIVE DERIVED FROM ALIÁCEAS

Por

Paloma Abad Campos

DEPARTAMENTO DE QUÍMICA ANALÍTICA

UNIVERSIDAD DE GRANADA

MEMORIA presentada para aspirar al Grado de Doctor en Ciencias, Sección Químicas

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CERTIFICA:

Que el trabajo que se presenta en esta **TESIS DOCTORAL**, con el título de **“EVALUATION OF THE INCORPORATION IN THE ANIMAL DIET (INGESTION AND METABOLISATION) OF PROPIL PROPANE THIOSULFONATE AS A NATURAL ADDITIVE DERIVED FROM ALIÁCEAS”**, ha sido realizado en los laboratorios del citado grupo bajo mi dirección y la de la profesora Dña. Natalia Arroyo Manzanares, en el Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Granada y reúne todos los requisitos para poder optar al Grado de Doctor.

En Granada, a 6 de Noviembre de 2017

La doctoranda Paloma Abad Campos y las directoras de la tesis Dña. Natalia Arrollo Manzanares y Dña. Ana M. García Campaña, garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de las directoras de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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Contenido

OBJETO DE LA MEMORIA

Objeto de la memoria

La presente tesis muestra una alta relevancia para la industria alimentaria al centrar el estudio en un nuevo aditivo natural que se incorpora en la dieta animal, el propil propano tiosulfonato (PTSO), extracto vegetal procedente del género *Allium*, principalmente del ajo (*Allium Sativum*) y de la cebolla (*Allium Cepa*), tradicionalmente conocidos por sus propiedades curativas y antimicrobianas.

El trabajo realizado tiene como objetivo principal el estudio del comportamiento del PTSO en piensos destinados a alimentación animal y también en el propio animal tras la ingesta, haciendo posible la evaluación de su inocuidad con objeto de garantizar su estabilidad como aditivo en piensos así como la seguridad alimentaria de productos y subproductos de animales que han incluido este ingrediente en su dieta habitual. Para ello, en esta tesis se han desarrollado distintas metodologías para el control de PTSO y sus derivados en piensos, así como para controlar sus posibles residuos en alimentos derivados de animales, tales como leche y huevos y garantizar que las características organolépticas de los mismos permanecen inalteradas. Igualmente se han estudiado los beneficios que presenta este aditivo sobre la salud de gallinas ponedoras y su producción, así como sobre la reducción de la producción de metano por parte del ganado vacuno, lo que supone una apuesta eficaz y simple para proteger el medio ambiente.

Otro aspecto considerado ha sido el estudio de la metabolización del PTSO durante el proceso de digestión animal mediante simulación del tracto gastrointestinal del cerdo usando un simulador dinámico (SimuGIT), que reproduce las condiciones en el estómago y el duodeno, permitiendo el estudio de su biodisponibilidad tanto al añadirlo

libre como vehiculizado en diferentes medios, con objeto de permitir la selección de la formulación comercial más eficaz.

Finalmente, se ha estudiado mediante ensayos *in vitro* el efecto del PTSO contra ciertos mohos micotoxigénicos que pudieran afectar a los piensos, usando en medios de cultivo adecuados para diferentes cepas productoras de micotoxinas, y empleando distintas concentraciones de PTSO, confirmando el efecto antifúngico que posee el mismo y pudiendo determinar su dosis letal para cada cepa..

Los estudios se han llevado a cabo usando técnicas cromatográficas con distintos tipos de detección, así como desarrollando métodos de tratamientos de muestra adecuados, juntos con ensayos microbiológicos y organolépticos.

Los contenidos de esta tesis están ampliamente relacionados con la actividad de la empresa granadina Domca, situada en la localidad de Alhendín (Granada), dedicada al desarrollo y fabricación de una amplia gama de compuestos para la industria alimentaria. Domca cuenta con un departamento de I+D, con una amplia experiencia en la investigación y desarrollo de tratamientos antimicrobianos y antioxidantes naturales como alternativa a los tradicionales así como recubrimientos alimentarios. Uno de sus desarrollos principales es un producto considerado como aditivo para alimentación animal, a base de extractos naturales de aliáceas, cuyo principal ingrediente activo es el PTSO.

Los resultados de esta Tesis pretenden aportar un mejor conocimiento de un producto natural que aporta numerosas ventajas para la salud animal y mejora la calidad de los productos derivados, demostrando igualmente las ventajas que supone la colaboración

de la universidad con el mundo empresarial para fomentar la investigación, la innovación y el desarrollo en nuestro entorno.

Resumen

Resumen

Desde la década de los cincuenta, la adición de antibióticos en pequeñas dosis al pienso de los animales de abasto ha venido siendo una práctica habitual para mejorar las producciones. Los antibióticos promotores del crecimiento (APC) presentan beneficios tales como el aumento de la productividad, evitan pérdidas en el valor nutritivo del pienso, y contribuyen a la prevención de infecciones subclínicas y a reducir la mortalidad. Sin embargo, tras años de polémica y numerosos estudios, ha quedado demostrada la relación entre el consumo de APC y la presencia de residuos de los mismos en los tejidos y subproductos de los animales que los consumen, así como la aparición de bacterias resistentes a los antibióticos tras periodos prolongados y continuados de ingesta. La presencia de estos residuos en productos alimenticios representa un peligro potencial para los consumidores, debido a la aparición de reacciones alérgicas y modificaciones en la flora intestinal. Este hecho añadido a la creciente preocupación por la transmisión y la proliferación de bacterias resistentes a través de la cadena alimentaria llevó a la prohibición del uso de APC en la Unión Europea (UE) desde el año 2006.

Después de la prohibición de los APC, con la búsqueda de nuevas alternativas, el uso de productos como los probióticos, prebióticos, ácidos orgánicos, aceites esenciales y extractos vegetales ha aumentado de manera considerable. Dentro de los extractos vegetales, los procedentes del género *Allium*, principalmente del ajo (*Allium Sativum*) y de la cebolla (*Allium Cepa*) son tradicionalmente conocidos por sus propiedades curativas y antimicrobianas. Estos efectos beneficiosos están asociados a la presencia compuestos organosulfurados (fundamentalmente tiosulfinatos y tiosulfonatos) en los

tejidos vegetales y que poseen carácter antimicrobiano, anti-inflamatorio, antioxidante, y antiparasitario.

En los últimos años varios estudios se han centrado en la evaluación de las propiedades beneficiosas del propil propano tiosulfonato (PTSO) a nivel microbiológico. Los estudios realizados (*in vivo* e *in vitro*) demuestran que la inclusión de PTSO en la dieta supone importantes beneficios en la salud y bienestar animal. Sin embargo, dada la novedad de esta molécula, hasta ahora no se han realizado estudios de la presencia y acumulación de PTSO en los diferentes tejidos de los animales que lo consumen, ni en sus subproductos.

La principal novedad de esta tesis, que ha permitido el estudio en profundidad del comportamiento del PTSO, ha sido la descripción por primera vez de la reacción entre el PTSO, y el grupo tiol (-SH) de la cisteína (CYS) y el glutatión (GSH), ambos presentes en la composición de los piensos y en los tejidos intra- y extra-celulares. Los productos resultantes de esta reacción son s-propil mercaptocisteína (CSSP) y s-propil mercaptoglutatión (GSSP), dependiendo que el PTSO haya reaccionado con CYS o con GSH. Este hecho ha sido determinante en el desarrollo de métodos para poder establecer un control de calidad adecuado para el PTSO como ingrediente de un pienso o bien para la determinación de residuos en alimentos procedentes de animales en cuya dieta has sido incorporado el PTSO.

El PTSO se usa ampliamente como ingrediente natural en alimentación animal, añadido a los piensos junto con un agente vehiculizante, y su control de calidad es necesario para garantizar que su uso sea una alternativa eficaz a los APC. Este aspecto se ha abordado en los capítulos 1 y 2 de esta tesis, planteando diversos

métodos analíticos usando cromatografía líquida para su control en piensos destinados a la alimentación de diversos tipos de animales.

Las propiedades características del PTSO (olor y pungencia) podrían alterar los atributos sensoriales de los alimentos procedentes de los animales que lo han ingerido, lo que supondría un aspecto negativo que debería ser controlado con objeto de no modificar las características originales propias del producto. Sin embargo también debido a sus propiedades beneficiosas podría presentar ciertas ventajas en caso de animales productores de alimentos como la leche o los huevos. En este sentido, en el segundo bloque de esta tesis, se han llevado a cabo dos estudios con objeto de evaluar la influencia del uso del PTSO en piensos sobre tales productos, ampliamente consumidos por la población (capítulos 3 y 4). Para ello se ha planteado una metodología analítica para controlar el PTSO y sus derivados en leche y huevo. Igualmente se han llevado a cabo ensayos organolépticos para determinar la concentración de PTSO que no altera las características de la leche y se han llevado a cabo procedimientos para demostrar los beneficios de este producto en la salud de gallinas ponedoras y en la eficiencia de su producción.

En el tercer bloque (capítulo 5) de esta tesis se estudia la biodisponibilidad de PTSO, libre y vehiculizado con diferentes agentes, tras su ingesta mediante la simulación del tracto gastrointestinal del cerdo, usando el simulador llamado SimuGIT, que reproduce las condiciones de la digestión en el estómago y el duodeno, conectado en serie a un reactor equipado con una membrana de microfiltración de cerámica que simula las condiciones y microvellosidades en el intestino. En este caso se han llevado a cabo análisis de los extractos en diferentes momentos de la digestión, mediante un procedimiento analítico adaptado al tipo de muestra.

Finalmente, se ha estudiado el potencial del PTSO como inhibidor del crecimiento de hongos micotoxigénicos (capítulo 6). Estos hongos pueden generar como productos secundarios de su metabolitos compuestos denominados micotoxinas, altamente tóxicos. La presencia de micotoxinas en alimentos y piensos puede provocar efectos adversos en la salud humana y animal, ya que pueden causar cáncer y trastornos estrogénicos y mutagénicos, así como problemas, gastrointestinales y renales e incluso la muerte. La prevención es el mejor método para controlar la contaminación por hongos y micotoxinas, por lo que el uso de PTSO como agente inhibidor de cepas de hongos micotoxigénicos podría evitar importantes problemas de salud animal y humana. En este caso se ha aplicado un método analítico para la determinación conjunta de las micotoxinas más relevantes en los cultivos de hongos estudiados, en función del tipo de cepa, dosis de PTSO y tiempo, pudiéndose determinar la dosis letal del mismo.

Así, el contenido de esta Tesis se ha estructurado en cuatro bloques, que incluyen los distintos capítulos:

1. **Control de calidad del PTSO como aditivo en alimentación animal.**

   Capítulo 1: Determinación mediante cromatografía líquida de alta resolución del derivado de aliáceas propil propano tiosulfonato, aditivo natural utilizado en alimentación animal.

   Capítulo 2: Desarrollo de un método rápido y simple para la determinación de propil propano tiosulfonato y sus derivados en piensos mediante UHPLC-ESI-MS/MS.

2. **Influencia del PTSO en la salud animal y alimentos derivados.**

Capítulo 3: Extracto de cebolla como aditivo en alimentos para rumiantes: determinación de propil propano tiosulfonato como marcador de sus efectos sobre las propiedades sensoriales de la leche.

Capítulo 4: Evaluación del uso de extracto de cebolla como suplemento alimenticio en avicultura: estudio de su influencia en la calidad del huevo y sobre la productividad y el estado de salud de gallinas ponedoras.

3. **Determinación de la biodisponibilidad del PTSO tras su ingesta en cerdos mediante simulación dinámica del proceso digestivo porcino.**

   Capítulo 5: Efectos de diferentes estrategias de vehiculación para el propil propano tiosulfonato durante la simulación dinámica del tracto gastrointestinal del cerdo.

4. **Evaluación de la capacidad antifúngica del PTSO frente a hongos micotoxigénicos.**

   Capítulo 6: Capacidad antifúngica del propyl propano thiosulfonato frente a cepas micotoxigénicas de *Aspergillus parasiticus*, *Aspergillus flavus, Penicillium verrucosum* y *Fusarium graminearum.*

Summary

Summary

Since the fifties, the addition of antibiotics in small doses to animal feed has been a common practice to improve production. The antibiotic growth promoters (AGP) have benefits such as increased productivity, avoid losses in the nutritive value of the feed, and contribute to the prevention of subclinical infections and reduce mortality. However, after years of controversy and numerous studies, it has been demonstrated the relationship between the consumption of AGP and the presence of residues thereof in the tissues and products from animals that consume them, as well as the appearance of resistant bacteria to the AGP after prolonged and continuous periods of intake. The presence of antibiotic residues in food products represents a potential danger for consumers, due to the appearance of allergic reactions, development of bacterial resistance and changes in the intestinal flora. This fact, added to the growing concern about the transmission and proliferation of resistant bacteria through the food chain led to the prohibition of the use of APC in the European Union (EU) in 2006.

After the prohibition of the AGP, with the search of new alternatives, the use of products such as probiotics, prebiotics, organic acids, essential oils and plant extracts has increased considerably. Among the plant extracts, those from the Allium genus, mainly garlic (*Allium sativum*) and onion (*Allium cepa*) are traditionally known for their healing and antimicrobial properties. These beneficial effects are associated to the presence of organosulfur compounds (mainly thiosulfinates and thiosulfonates) in the vegetal tissues which present antimicrobial, anti-inflammatory, antioxidant and antiparasitic character.

In recent years several studies have focused on the evaluation of the beneficial properties of PTSO at the microbiological level. The studies carried out (*in vivo* and *in vitro*) show that the inclusion of PTSO in the diet supposes important benefits in animal health and welfare. However, given the novelty of this molecule, until now, there have not studies conducted to the presence and accumulation of PTSO in the different tissues of the animals that consume it, or in their products.

The main novelty of this thesis, which has allowed the study of PTSO behavior in-depth, has been the description for the first time of the reaction among PTSO, and the thiol (-SH) group of cysteine (CYS) and glutathione (GSH), both present in the composition of the feeds and in the intra- and extra-cellular tissues. Resulting products from this reaction are s-propyl mercaptocysteine (CSSP) and s-propyl mercaptoglutathione (GSSP), depending on whether PTSO has reacted with CYS or with GSH. This fact has been decisive in the development of methods to establish an adequate quality control for the PTSO as an ingredient of a feed or for the determination of residues in foods from animals whose diet has been incorporated into the PTSO.

PTSO is widely used in as natural ingredient in animal feed, which is added incorporated to a vehiculization agent, and its control is necessary to guarantee a its correct use as alternative to AGP, due to its efficiency improving animal health. This point is development in chapters 1 and 2 of this thesis where several analytical methods have been developed for the monitoring of PTSO in feed by liquid chromatography.

PTSO characteristics (odor and pungency) could alter sensory attributes of foods from animals that have ingested it. This fact could suppose a negative aspect that should be controlled to avoid the modification of to the original properties of the product. However due to its known beneficial properties, the use of PTSO could present certain advantages for animals producing foods, such as milk or eggs. In this sense, in the second part of this thesis, two studies have been conducted in order to evaluate the influence of the use of PTSO as feed additive on products from animals (chapters 3 and 4). To this aim, an analytical methodology has been proposed to control the PTSO and its derivatives in milk and eggs. Likewise, organoleptic tests have been carried out to determine the concentration of PTSO that does not alter the characteristics of the milk and procedures have been carried out to demonstrate the benefits of this product in the health of laying hens and in the efficiency of their production.

In the third part (chapter 5), studies have been carried out the estimate the bioavailability of PTSO, after being intake, free and combined with vehiculization agents, by simulation of the gastrointestinal tract pork with the reactor SimuGIT, simulating the conditions in the stomach and duodenum, connected in series to a continuous plug-flow reactor equipped with a ceramic microfiltration membrane, simulating the conditions and microvilli in the intestine. In this case, extracts have been analyzed at different digestion times, using an analytical procedure adapted to the type of sample.

Finally, the potential effect of PTSO against mycotoxigenic fungi has been studied (chapter 6). Mycotoxigenic fungi strains can generate mycotoxins as secondary metabolites, which present a high toxicity. The presence of mycotoxins in food and feed can cause adverse effects on human and animal health, as they can cause cancer and estrogenic and mutagenic disorders, as well as gastrointestinal and renal problems and even death. The prevention is the best method to control fungi and mycotoxins contamination so, the use of PTSO as inhibitor agent of mycotoxigenic fungi strains could avoid important food and feed problem derived from the presence of these types of molds.

This thesis, focused on the study of PTSO as natural additive, is divided in four structural parts:

1. **Quality control of PTSO in animal feed.**

   Chapter 1: Analytical method based on high-performance liquid chromatography and ultraviolet detection for the monitoring of the allium derivative PTSO used as natural additive in animal feed.

   Chapter 2: A rapid and simple UHPLC-ESI-MS/MS method for the screening of PTSO and its derivatives in animal feed.

2. **Influence of the PTSO on animal health and derived foods.**

   Chapter 3: Use of onion extract as dairy cows feed supplement: monitoring of PTSO as marker of its effect on milk attributes.

   Chapter 4: Evaluation of onion extract as feed supplement for poultry: study of its influence on egg quality, productivity and health status of laying hens.

3. **Bioavailability of PTSO after being ingested by simulation of the gastrointestinal tract of the porcine digestive process with a dynamic simulator**.

   Chapter 5: Effects of different vehiculization strategies for PTSO during dynamic simulation of the pig gastrointestinal tract**.**

4. **Effect of PTSO against mycotoxigenic fungi strains.**

   Chapter 6: Antifungal and antimycotoxigenic potency of PTSO against mycotoxigenic strains of *Aspergillus parasiticus*, *Aspergillus flavus, Penicillium verrucosum* and *Fusarium graminearum*.

INTRODUCTION

I1. Introduction

Garlic (*Allium sativum*) and onion (*Allium cepa*) have been used in folk medicine through the centuries for treatment of several health disorders like diabetes, cardiovascular disorders, rheumatism, etc.[1] Recent advances in the fields of immunonutrition, physiology, microbiology and pharmacology have identified their health benefits related to their antioxidant effect, cardiovascular protection by reducing cholesterol and anti-platelet activity and prevention of cancer.[2-6] In addition, they and their respective essential oils have shown antimicrobial effect against Grand Positive and Gram Negative bacteria including *Bacillus cereus, Escherichia coli, Shigella spp., Vibrio spp., Yersinia enterocoliticab, Listeria monocytogenes, Salmonella ssp. and Campylobacter spp.* Also, they present antifungal activity against *Aspergillus spp., Penicillium* spp. and *Fusarium spp*.[7]

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Related to animal feed, several studies have shown positive effects on growth performance in broilers[8,9] and on ruminants' methane emissions reduction.[10] In addition, in aquaculture their use has been proven that increase the survival rate.[11]

Biological and medical functions of garlic and onions are associated to their high content on organosulfur compounds (OSC)[12,13] The number of sulfur atoms, chain length and the presence of double bonds are responsible of each OSC characteristic odor, pungent and biological activities.[14] The main OSC found in all *Allium spp.* and the best studied are thiosulfinates which are generated when garlic and onion are crushed or their tissues are damaged by the reaction between S-alk(en)yl-L-cysteine sulphoxides (flavour precursors) and the enzyme alliinase. The structure and content of the cysteine sulfoxides is responsible for the flavour and odor and depends on the different *Allium spp.* and the enzyme allinase is common to all *Allium spp.*[15]

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Thiosulfinates are very unstable compounds and they decompose quickly at room temperature. In garlic, thiosulfinates decompose to vinyl ditiins, polysulfides and ajoenes. In onion, thiosulfinates react quickly given rise to thiosulfonates which are highly stable.[16,17]

I2. Biosynthesis of OSC

The aroma of bulbs from garlic and onion is largely dominated by volatile sulfur odorants that have a strong odor even at low concentrations.[18] It is well-known that cysteine sulfoxides (CSO), which are mainly present in the bulb, act as flavour precursors generating the typical taste and odor in presence of the enzyme alliinase which catalyze the reaction when alliums vegetable tissues are damaged or cut.[19,20] The most important CSOs in *Allium spp*. are s-methyl-L-cysteine sulfoxide, s-allyl-L-cysteine sulfoxide, s-propenyl-L-cysteine sulfoxide and s-propyl-L-cysteine sulfoxide commonly known as methiin, alliin, isoalliin and propiin, respectively.[17] Their structures are shown in table I1.

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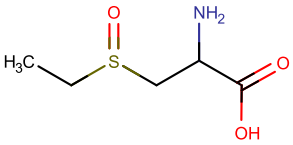
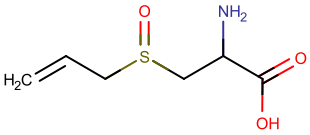
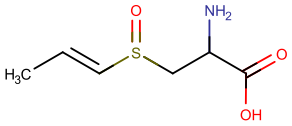
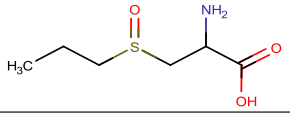
Table I1: *Allium spp.* flavour precursors

Common name	Chemical name	Chemical structure	Representative species
Methiin	S-Methyl-L-cysteine sulfoxide		*Allium cepa.* *Allium sativum.*
Alliin	S-Allyl-L-cysteine sulfoxide,		*Allium sativum.*
Propiin	S-Propenyl-L-cysteine sulfoxide		*Allium cepa.*
Isoalliin	S-Propyl-L-cysteine sulfoxide		*Allium cepa.*

Allium thiosulfinates biosynthetic pathway consisted of CSO transformation to ammonia, sulfenic acids and piruvates catalyzed by the enzyme allinase. Furthermore, the rapid condensation of two sulfenic acids results in the formation of thiosulfinates.

In garlic, alliin reacts catalyzed by the enzyme allinase giving as product diallyl thiosulfinate (allicin), the most studied thiosulfinate. Firstly, alliin decomposes to allylsulfenic acid, ammonia and piruvates by the action of allinase. The combination of two molecules of allylsulfenic acid gives allicin by rapid condensation (see figure I1).

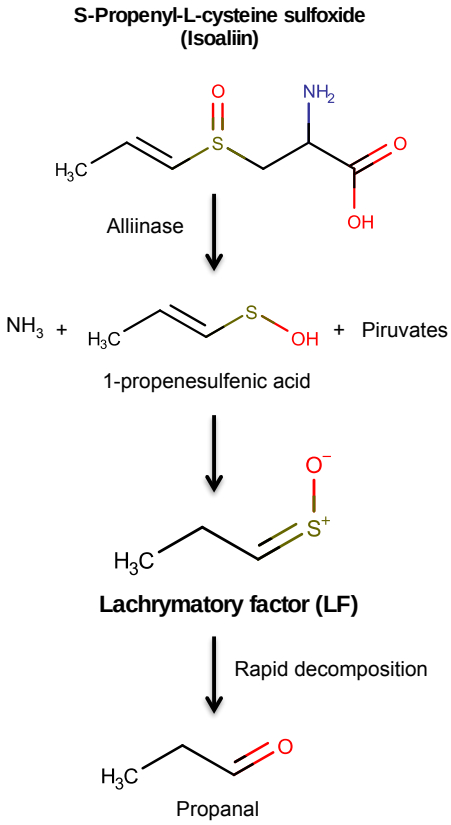


Figure I1. Proposed biosynthetic pathway for allicin in garlic

In onion, the main thiosulfinate is propyl thiosulfinate (PTS), which is generated from the CSO propiin also catalyzed by the enzyme allinase. Similar to allicin, propiin decomposes to propanesulfenic acid, ammonia and piruvates by the action of the enzyme allinase. The combination of two molecules of propanesulfenic acid gives PTS by rapid condensation (figure I2).

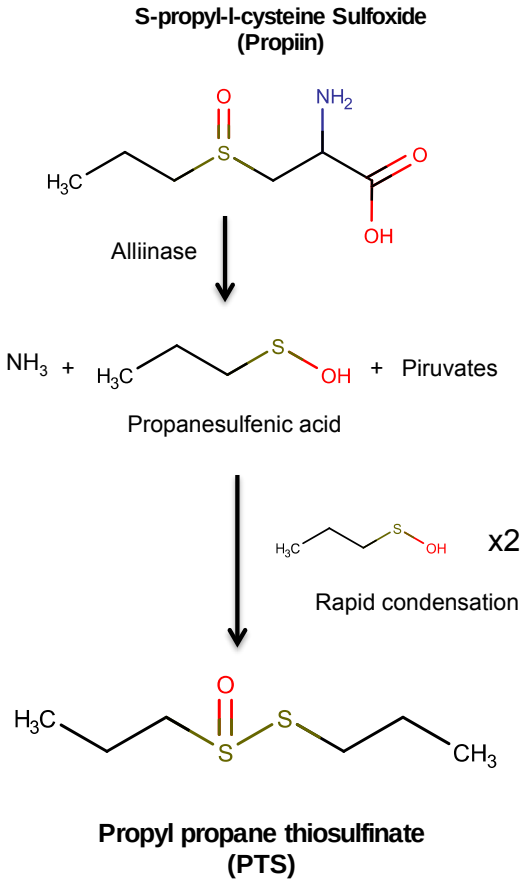


Figure I2. Proposed biosynthetic pathway for PTS in onion

In the case of isoaliin, also present in onion, the reaction catalyzed by allinase (figure I3) gives 1-propenesulfenic acid, ammonia and piruvates. However, in this case, 1-propenesulfenic acid is catalyzed bylachrymatory-factor synthase enzyme, characteristic of onion, giving propanethial s-oxide. Propanethial s-oxide is commonly

known as lachrymatory factor (LF) and it is the mayor responsible of onion pungency and tears apparition in eyes when onion is cut.

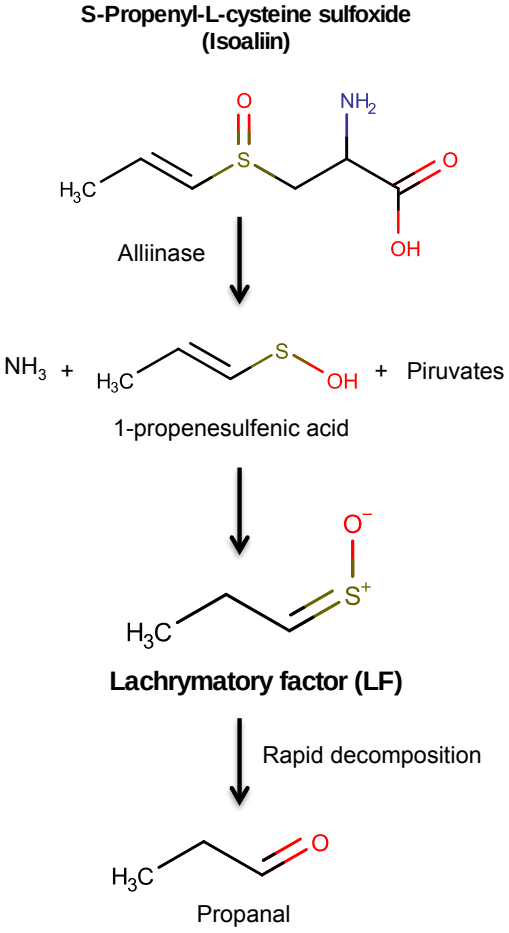


Figure I3. Proposed biosynthetic pathway for LF generation in onion

Mettiin, a minority onion and garlic CSO, gives thiosulfinates according the described standard mechanism. Metiin induced thiosulfinates are minority compounds in *Allium spp*. (garlic and onion) and they have been hardly studied.[1,14]

Thiosulfonates is another typical onion OSC that may be present naturally in traces amounts. Thiosulfonates are mainly generated by disproportion reaction, which consist of rearrangements of thiosulfinates leading to disulfides and thiosulfonates. Finally, disulfides compounds are also converted to thiosulfonates compounds by oxidation reaction.[17] Figure I4 shows the disproportion reaction in onion where the tiosulfinate PTS gives propyldisulfide (PDS) and propyl propane thiosulfonate (PTSO). PDS also is easily transformed to PTSO in presence of O_2.

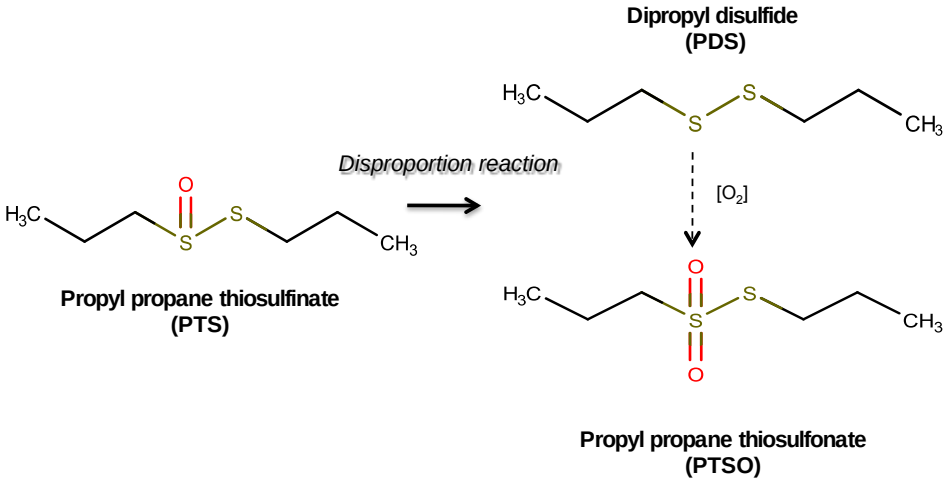


Figure I4. Proposed biosynthetic pathway for PTSO generation in onion

I3. Allium OSC properties

I3.1. Allium thiosulfinates

Allicin finding was first reported by Wertheim (1844)[21] and later by Semmler (1892)[22] who identified the correct disulfide structure as the main component of distilled oil of garlic. Fifty years later, it became clear that these compounds were not present in the intact bulbs because they were formed by enzymatic reaction of the flavour precursors, catalyzed by the enzyme allinase,[23-25] as it has been described in previous section.

Allicin presents a broad range of biological activities including antifungal, antibacterial, anticancer, antiatherosclerotic, antihypertensive, anti-inflammatory, and antithrombotic.[26] It also could be used for improvement shelf life of preshable foods. This function is widely known and moreover, they are a great alternative to chemical additives due to their potential values as food preservatives.[27]

As stated above, the main thiosulfinate is PTS and it is obtained from propiin by the action of allinase. PTS has less known that allicin but recent studies have proven its antimicrobial properties.[28] Also, it has been proven that PTS plays an important role

21. Wertheim, T. Protozoology. London: Bailliére, Tindall & Cassel; 1844.

22. Semmler, F. W. (1892). Arch. Pharm., Berl., 230, 434.

23. Cavallito, C.J.; Bailey, J.H. Am. Chem. Soc., 66 (1944) 1950-1951.

24. Cavallito, C.; Bailey, J.H.; Buck J.S. J. Am. Chem. Soc., 67 (1944) 1032-1033.

25. Stoll, A. & Seebeck, E. (1948). Helv. chim. acta, 31, 189

26. Corzo-Martinez, M., Corzo, N., Villamiel, M. Trends Food Sci. Tech., 18 (2007) 609-625.

27. Benkeblia, N.; Lanzotti, V. Food, 1 (2007) 193-201

28. Ruiz, R.; García, M.; Lara, A., Rubio, L. Vet. Microbiol., 29 (2010) 110-117.

reducing methane emissions in rumen[29] and toxicology studies have demonstrated PTS safety by absence of mutagenic effects. Nevertheless, dose of PTS must be controlled because in spite of PTS does not induce oxidative DNA damage it has induced DNA breaks damage in Caco-2 cells at higher concentrations.[30]

I3.2. Allium thiosulfonates

PTSO, responsible of freshly cut onion odor, is the most studied onion thiosulfonate.[31] PTSO has been used as natural ingredient in animal feed recently. It has shown antimicrobial activity against pathogens like *Enterobacteriaceae*, *Escherichia Coli*, *Salmonella spp*. and *Campylobacter jejuni*. These properties have been described *in vitro*[31] and *in vivo*[32] and some recent studies have proven its anticoccidial properties by its effectivity against *Eimeria acervulina*.[33] Furthermore, dietary supplementation with PTSO could modulate intestinal microbiota composition and improve nutrients digestibility without affecting mucosal enzyme activity in growing broilers[34,35] and controlling methane inhibition by the modulation of rumen fermentation.[29] Additionally, Mylona (2012) suggested that PTSO could show a reduction of fungal growth and

29. Martínez-Fernández, G.; Abecia, L.; Martín-García, A. I.; Ramos-Morales, E.; Hervás, G.; Molina-Alcaide, E.; Yáñez-Ruiz D. R. Animal., 7 (2013) 1925-1934.

30. Mellado-García, P.; Maisanaba, S.; Puerto, M.; Prieto, A.I.; Marcos, R.; Pichardo, S.; Camean, A. M. Food Chem. Toxicol., 99 (2017) 231-240.

31. Pruthi, J.S. Quality assurance in spices and spice products. Modern methods of analysis. Allied Published Limited (1998).

32. Peinado, M.J.; Ruiz, R.; Echávarri, A.; Rubio, L. A. Poultry Sci., 91 (2012) 2148-2157.

33. Kim, D. K., Lillehoj, H. S., Lee, S. H., Lillehoj, E.P., Bravo, D. Brit. J. Nutr. 109 (2012) 1-13.

34. Peinado, M.J.; Ruiz, R.; Echavarri, A.; Aranda-Olmedo, I.; Rubio. L.A. Anim. Feed Sci. Tech., 181 (2013) 87-92.

35. Rubio, L.A., Peinado, M.J.; Ruiz, R.; Suárez-Pereira, E.; Ortiz Mellet, C.; García Fernández, J.M. J. Animal Phys. Animal Nutr., 99 (2015) 418-423.

mycotoxin production in *Fusarium spp*. (ochratoxin A, deoxynivalenol, fumonisins, zearalenone, etc.).[36]

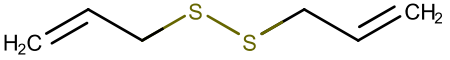
Finally, the use of PTSO as natural ingredient has been supported by recent toxicology studies. Non-mutagenic effects were observed by results, which indicated that PTSO did not induce DNA breaks or oxidative DNA damage. Moreover, it has been confirmed the absence of genotoxicity of PTSO in rats.[30]

I3.3. Other OSC

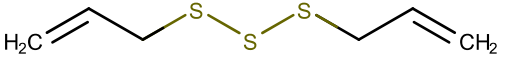
The sulfenic acids intermediate usually produces thiosulfinates by condensation, however these compounds can participate in a variety of reactions like dehydration, rearrangement, condensation, Diels-Alder reaction, hydrolysis or pyrolisis giving other types of OSC.[15,27] Moreover, thiosulfinates are highly unstable and undergo degradation giving ajoenes, vinyldithiins and mono and polysulfides (figure I5). Ajoenes and vinyldithiins appear in the first reactions of thiosulfinates degradation pathway and they have associated properties like the inhibition of platelet aggregation while mono and polysulfides, mainly di and trisulfides, appear with the last transformations of thiosulfinates showing properties like anti-cancer effects.[16] The amount of each product will depend on the allium vegetable composition (CSO, amino acids, etc).

36. Mylona, K. Fusarium species in grains: dry matter losses, mycotoxin contamination and control strategies using ozone and chemical compounds. Doctoral Thesis. University of Cranfield (2012).

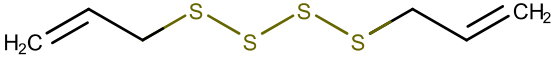
Diallyl sulfide (DAS)



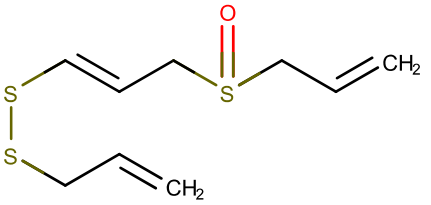
Diallyl disulfide (DAS2)



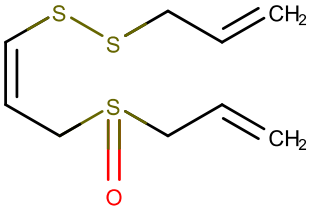
Diallyl trisulfide (DAS3)



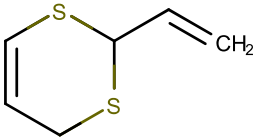
Diallyl tetrasulfide (DAS2)



E-Ajoene



Z-Ajoene



Vinyl dithiins

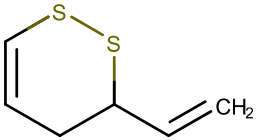


Figure I5. Other *allium sativum* OSC: ajoenes, vinyldithiins and mono and polysulfides

I4. Allium thiosulfinates and thiosulfonates: involved reactions

As it was described in previous section, thiosulfinates react naturally to give thiosulfonates and disulfides. However, thiosulfinates and thiosulfonates can also react spontaneously with some of the compounds present in allium tissues, such as amino acids or other compounds containing sulfhydryl group (also known as thiol group) as glutathione (GSH).[37,38]

I4.1. Thiosulfinates and thiosulfonates in presence of amino acids: colored reactions

One of the most important problems of garlic and onion storage is the appearance of colored pigments in cloves homogenates. This phenomenon is known as “greening” in garlic and “pinking” in the case of onion due to the color of the formed pigments (green, green-blue or blue for garlic pigments and pink or red for onion pigments) and appears as consequence of thiosulfinates decomposition and its reaction with amino acids,[39] so it is believed that greening and pinking have the same nature.[40] Green pigments appear almost instantaneously during storage of minimally processed or pre-peeled garlic, in case of onion, the “pinking” process during storage could extend several hours.[41]

All amino acids except cysteine (CYS), which has a sulfhydryl (SH) group, react with thiosulfinates to form color pigments. However, they do not react in the same way and

37. Cho, J., Lee, E.J.; Yoo, K.S.; Lee, S.K.; Patil, B.S. J. Food Sci., 74 (2009) C11-C16

38. Andrew, B. Thiol-X Chemistries in Polymer and Materials Science. Royal Society of Chemistry, Abingdon (UK), 2013, chapter 4, 76-94.

39. Kubec, R.; Velíšek, J. J. Agric. Food Chem., 55 (2007) 3491–3497

40. Kubec, R.; Hrbácová, M.; Musah, R.A.; Velísek. J.. Agric. Food Chem., 52 (2004) 5089–5094

41. Kucerová, P.; Kubec, R.; Simek, P.; Václavík, L.; Schraml, J. J. Agric. Food Chem., 59 (2011) 1821-1828.

the formed pigment therefore depends on their reactions, the amount of thiosulfinates and/or reactive amino acids. The colors of these pigments could vary from yellow, green, blue, red to pink.

Two steps are necessary for pigment formation: the first step is the thiosulfinates generation (enzymatic stage) and secondly its reaction with free amino acids (non enzymatic stage) present in garlic and onion tissues.[37] Based on this information, it is known that CSO as thiosulfinates precursors are the limiting factor in color pigment formation. Table I2 shows the influence of CSO on pinking and greening pigments formation in presence of glycine. Glycine (GLY) has been usually used in model reaction systems.[42]

Table I2. Influence of CSO in color formation involving glycine in *Allium spp.* (adapted from reference Kucerová et al., 2011)[40]

Isoalliin	Alliin	Methiin	Propiin	GLY	Alliinase	Resulting color
+	-	-	-	+	+	Magenta
+	+	-	-	+	+	Dark blue
+	-	+	-	+	+	Magenta
+	-	-	+	+	+	Magenta
-	+	-	-	+	+	None
-	-	+	-	+	+	None
-	-	-	+	+	+	None
-	-	-	-	+	+	None
-	+	+	+	+	+	None

(+): Presence; (-): Absence

42. Kubec, R., Urajová, P.; Lacina, O.; Hajšlová, J.; Kuzma, M.; Zápal, J. J. Agric. Food Chem., 63 (2015) 10192-10199

As can be seen in table I2, in presence of GLY, reaction of isoalliin catalyzed by alliinase gave magenta pigment. The same color was obtained when isoalliin was combined with methiin or propiin, however a dark blue pigment was yielded by isoalliin and alliin combination. In in absence of isoalliin or when isoalliin was in the presence of a mixture of methiin, propiin and alliin none pigment was found.

Thiosulfonates, sulfoxides, sulfides, and disulfides do not form any colored products when mixed with GLY.[39] Figure I6 summarizes the scheme of general allium coloration process.

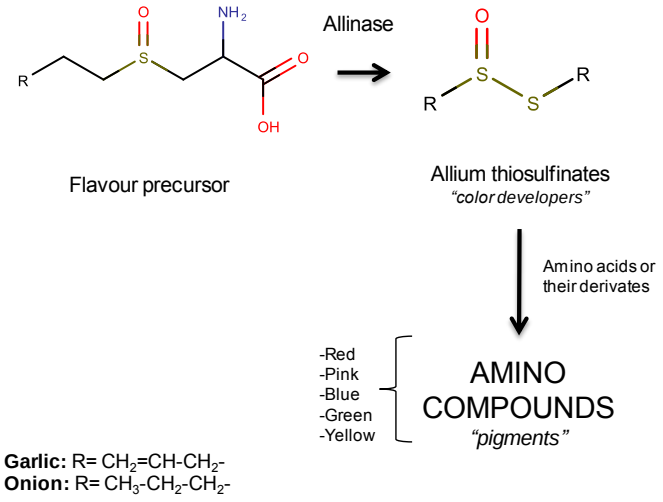


Figure I6. Discoloration reactions involved allium compounds

Recently, the structure of color pigments from onion have been studied[43] but the pathway or its formation are poorly understood because discoloration in *Allium spp.* is a complex process which depend on many factors and make it unpredictable and difficult to control.

I4.2. Thiosulfonates and thiosulfonates behavior in presence of cysteine and glutathione

As it has been described previously, CYS is the only amino acid that does not react with thiosulfinates to form color pigments. However, CYS can also reacts with thiosulfinates and thiosulfonates.[44] Reaction between thiosulfinates and CYS occurs as consequence of the presence of a sulfhydryl (-SH) group in its structure. This reaction is faster and more spontaneous than the reactions with other amino acids and they happen when the molar concentration ratio of CYS to total thiosulfinates is 2:1.[45,44] This phenomenon is known as thiolation and gives mixed disulfide compounds as result.[19] Keeping this ratio, no colour pigments formation occurs and consequently, CYS has been reported to prevent garlic pigmentation.[46]

GSH is, a non-protein tripeptide consisted of three amino acids including CYS and it is also present in cellular tissues. GSH contains therefore a sulfhydryl group within its structure and reacts with thiosulfinates in the same way that CYS.[47,48] Spontaneous

43. Kubec, R., Urajová, P.; Lacina, O.; Hajšlová, J.; Kuzma, M.; Zápal, J. J. Agric. Food Chem., 63 (2015) 10192-10199

44. Shin, Y.K.; Kyung, K.H. Food Chem., 142 (2014) 217-219.

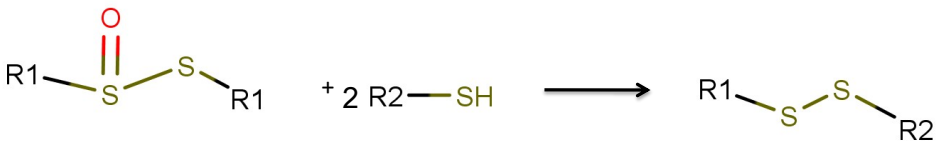
45. Yoo, M.; Lee, S.; Lee, S.; Seog, H.; Shin, D. Food Sci. Biotechnol., 19 (2010) 1619-1626

46. Zang, J.; Wang, D.; Zhao, G. Trends Food Sci. Technol., 30 (2013) 162-173

47. Zhang, G.; Parkin, K.L. J. Agric. Food Chem., 61 (2013) 3030-3038.

reaction between GSH and thiosulfinates, also happen when the molar concentration ratio of GSH to total thiosulfinates is 2:1.[44,45] Thereby, GSH also react with allium compounds to yield mixed disulfide conjugates.[49]

In fact, allicin thiosulfinate is considered a highly reactive sulfur specie and undergoes a redox instantaneous reaction with thiol groups from CYS and GSH according to the reaction shown in Figure I7.[50]



Thiosulfinate | Functional thiol | Disulfide product

Product	Molecular weight (g/mol)	Molecular formula	R1	R2
S-Allyl mercaptocysteine	193	$C_6H_{11}NO_2$	C_3H_5	$C_3H_6NO_2$
S-Allyl mercaptoglutatione	379	$C_{13}H_{21}N_3O_3$	C_3H_5	$C_{10}H_{16}N_3O_3$

Figure I7: Instantaneous reaction between thiosulfinates and the thiol groups of CYS and GSH

48. Rabinov, A.; Miron, T.; Mirelman, D.; Wilchek, M.; Glozman, S.; Yavin, E.; Weiner, L. Biophys. Acta., 1499 (2000) 144-153.

49. Zhang, G.; Li, B.; Lee, C.H.; Parkin, K. J. Agric. Food Chem., 58 (2010) 1564-1571.

50. Borlinghaus, J.; Albrecht, F.; Gruhlke, M.C.H.; Nwachukwu, I.D.; Slusarenko, A.J. Molecules. 19 (2014) 12591-12618.

As result of these reactions s-allylmercaptocysteine and s-allylmercaptogluthatione are obtained as products for CYS and GSH, respectively. These products are not hazardeous to health and show biological activities like hepato-protective,[51] antioxidant,[52] antiproliferative,[53] apoptosis-inducing[54] and antimetastatic,[55] being their biological activities associated to the disulfide bonds.[47] In addition, s-allylmercaptocysteine or s-allylmercaptogluthatione modify the main thiol extra- and intra-cellular redox buffer, which are related to allicin antibacterial properties.[56]

A similar but hardly reaction occurs with thiosulfonates as well as allicin and its proposed mechanism is shown in Figure I8.[38] Thiosulfonates react with sulfhydryl group to yield disulfides and intermediate products (by-products). These intermediates also react with thiol group given also disulfides and water.

51. Xiao, J.; Ching, P.; Liong, E.C.; Nanji, A.A.; Fung, M.F.; Tipoe, G.L. Eur. J. Nutr., 52 (2013) 179-191.

52. Pedraza-Chaverri, J.; Barrera, D.; Maldonado, P.; Chirino, Y.; Macias-Ruvalcaba, N.; Medina-Campos, O.; Castro, L.; Salcedo, M.; Hernandez-Pando, R. Clin. Pharmacol., 4 (2004)5-17.

53. Shirin, H.; Pinto, J.T.; Kawabata, Y.; Soh, J.; Delohery, T.; Moss, S.F.; Murty, V.; Rivlin, R.S.; Holt, P.R.; Weinstein, I.B. Cancer Res., 61 (2001) 725-731.

54. Xiao, D., Pinto. J.T.; Soh. J.; Deguchi. A.; Gundersen, G.G.; Palazzo. A.F.; Yoon, J.; Shirin, H.; Weinstein, I.B. Cancer Res., 63 (2003) 6825-6837.

55. Howard, E.W.; Ling, M.; Chua, C.W.; Cheung, H.W.; Wang, X.; Wong, Y.C. Clin. Cancer Res., 13 (2007) 1847-1856.

56. Müller, A.; Eller, J.; Albretch, F.; Prochnow, P.; Kuhlmann, K.; Bandow, J.E.; Slusarenko, A.J.; Leichert, L.I.O. J. Biol. Chem., 291 (2016) 11477-11490.

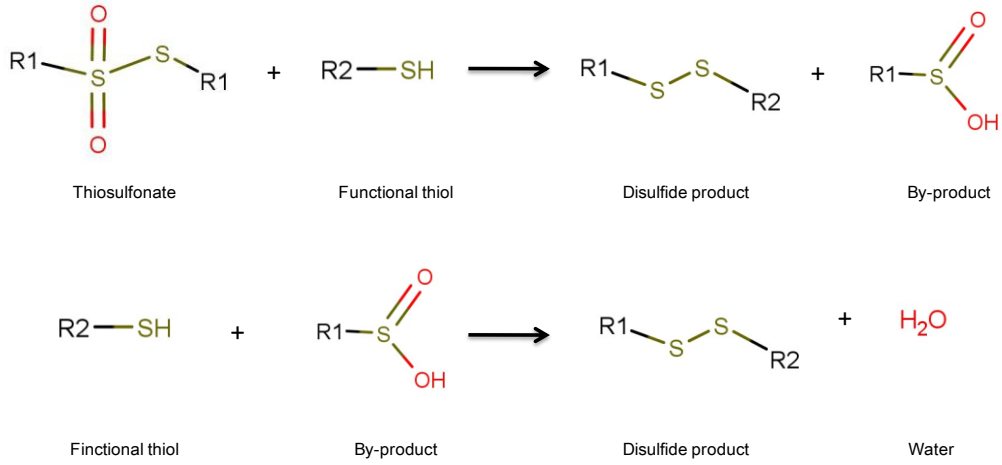


Figure I4. Reaction mechanism of thiosulfonates and compounds with a thiol group

In this thesis PTSO behavior in presence of CYS and GSH in several matrices (animal feed, milk and eggs) have been studied in depth. For this purpose, analytical methods for PTSO determination in the explored matrices have been developed.

I5. Analysis methods of allium thiosulfinates and thiosulfonates

The amount of allicin and other OSC in garlic and onion is highly variable depending on the nature and the location of the plant so many author have try to identify and analyze these compounds in different crops in an effort of evaluate their quality.[57] Consequently, several methods for qualitative and quantitative determination of thiosulfinates and thiosulfonates, specifically for allicin, PTS and PTSO, have been reported. This section summarizes the existing analytical methodologies for these compounds including the

57. Schulz, V. A Physicians' Guide to Herbal Medicine. (1998) 107-125

different sample treatment proposed in several matrices (*Allium spp.*, garlic powder, breath, plasma, simulated gastric fluids, etc.).

Because of their excellent resolution and/or mass identification capabilities, traditionally gas chromatography (GC) coupled to a flame ionization detector (FID) or mass spectrometry (MS) figured prominently in the effort to characterize allium volatiles. However, since nineteen's high performance liquid chromatography (HPLC) was proposed as alternative to GC due to its better resolution,[1] and nowadays, HPLC with ultraviolet detection (UV) is the most commonly used technique for allicin, PTS and PTSO determination. Table I3 summarizes the analytical methods proposed for thiosulfinates and thiosulfonates determination in recent years.

Table I3. Analytical characteristic of several methods proposed for allicin, PTS and PTSO determination

Analyte	Metodology[a]	Matrix	Sample Treatment	LOD/LOQ[b]	Ref.
PTSO	GC-MS	*Allium cepa*	Distillation	NA/NA	18
Allicin	GC-MS	*Allium cepa*	Supercritical fluid extraction	NA/NA	58
Allicin	GC-MS	*Allium Sativum*	SPME	NA/NA	59
Allicin	GC-FID GC-MS	Essential oil	NA	NA/NA	60
Allicin	HPLC-UV	*Allium sativum*	Solid-liquid extraction	NA/NA	1

58. Sinha, N.K.; Guyer, D.E.; Gage, G.D.; Lira, C.T. J. Agric. Food Chem., 40 (1992) 842-845.

59. Calvo-Gómez, O.; Morales-López, J.; López, M.G. J. Chromatogr. A., 1036 (2004) 91-3

60. Mnayer, D.; Fabiano-Tixier, A.S.; Petitcolas, E.; Hamieh, T.; Nehme, N.; Ferrant, C.; Fernandez, X.; Chemat, F. Molecules, 19 (2014) 20034-20053.

Allicin/PTS	HPLC-UV	*Allium sativum*	Solid-liquid extraction	NA/NA	61
Allicin	HPLC-UV	Breath, Plasma or Simulated Gastric Fluids	Hidration	NA/NA	62
Allicin	HPLC-UV HPLC-ED	*Allium sativum*	Solid-liquid extraction Triple extraction distillation	0.1 mg L^{-1}/NA 0.01 mg L^{-1}/NA	63
Allicin	HPLC-UV	*Allium sativum*	Solid-liquid extraction	NA/NA	64
Allicin	HPLC-UV	*Allium sativum*	Solid-liquid extraction	NA/NA	65
Allicin	HPLC-UV	*Allium sativum*	Supercritical fluid extraction	NA/NA	66
Allicin	HPLC-UV	*Allium sativum*	Ultrasonic extraction and maceration with distillated water	NA/NA	67
Allicin	HPLC-UV	Garlic powder and tablets	Vortex-Sonication	0.27 μg L^{-1}/ 81 μg L^{-1}	68
Allicin	HPLC-UV	*Allium sativum*	Simple extraction with dichloromethane and diethyl ether	6.63 μg L^{-1}/20.09μg L^{-1}	45
Allicin	HPLC-UV	*Allium sativum*	Solid-liquid extraction/concentration	NA/NA	69
Allicin	HPLC-UV	*Allium sativum*	SPME	9 mg L^{-1}/21 mg L^{-1}	70

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Allicin	HPLC-UV	Allium sativum	Radiation extraction techniques	NA/NA	[71]
Allicin	UHPLC-UV	*Allium sativum*	Solid-liquid extraction	0.79 μg mL^{-1}/2.63 μg mL^{-1}	[72]
Allicin	UHPLC-UV	*Allium sativum*	Solid-liquid extraction	1μg mL^{-1}/2.5 μg mL^{-1}	[73]
Allicin	HPLC-APCI-MS	*Allium tricoccum*	Supercritical fluid extraction	NA/NA	[74]
Allicin	LC-CIS-MS/MS	*Allium spp.*	Ethanolic extraction (60ºC)	NA/18 μg/mL	[75]
Allicin/PTS	DART-MS	*Allium sativum*	Solid-liquid extraction	NA/NA	[76]
Allicin	HPLC-ESI-MS	*Allium spp.*	Solid-liquid extraction	NA/NA	[77]
Allicin	LC-MS	*Allium spp.*	Maceration	NA/NA	[78]

(a) LC: liquid chromatography; APCI: atmospheric pressure chemical ionization; ED: electrochemical detection; SPME: Solid-phase microextraction; UHPLC: Ultra Performance Liquid Chromatography; CIS: coordination ion spray; DART: direct analysis in real-time; N/A: Non applicable

(b) LOD: Limit of detection; LOQ: limit of quantification

First reported papers related to OSC determination, focused their attention in the identification of different components present in allium extracts.[1,18] These analytical methods tried to identify the most number of OSC in allium extracts. Once volatile allium compounds were well identified and characterized, researchers move their

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attention toward the improvement of analysis techniques in terms of sensitivity, linearity, precision, limits of detection (LOD) and limits of quantification (LOQ).

I5.1. Gas cromatography

GC is a technique used traditionally for volatile compounds analysis mainly CSO. First studies of onion essential oil by GC-MS, date back from 1971.[18] A total of 45 flavour constituents of onion oil including PTSO were identified and described for the first time. Volatile garlic compounds have also been studied SPME coupled to GC-MS.[59] After that, Sinha *et al.*,[60] analyzed more than 34 onion flavours components including allicin and then Mnayer *et al.*, analyzed allium essential oils from Alliaceae family first by GC-FID and subsequently, GC-MS was used to identify their chemical constituents. A total of 27 constituents were identified representing more than 94.63% of the total garlic essential oil, whereas for onion essential oil, 31 constituents were identified representing 82.36 % of the total.[60]

I5.2. Liquid chromatography

The most common technique for allium thiosulfinates and thiosulfonates determination is HPLC coupled to UV detection. First techniques based on liquid chromatography data from 1991 when reversed-phase HPLC-UV was used to separate and quantitate all the detectable alkyl and alkenyl thiosulfinates of garlic homogenates.[64] Since then, several methods combined with different detection systems have been developed. Block (1992) compared normal-phase HPLC-UV with GC-MS for thiosulfinates determination obtaining the best results with HPLC-UV.[1] Althought Block made this comparation using normal phase for thiosulfinates determination, the most part of authors that have studied volatile organosulfur choose reverse phase column (C18) for

HPLC-UV analysis being MeOH and water the most commonly solvents used mobile phases.

Bocchini *et al.* (2001) related an on-line post-column photoreactor to determine allicin comparing HPLC-UV and HPLC-Electrochemical Detection (ED).[63] LODs were 0.1 mg L^{-1} and 0.01 mg L^{-1} for HPLC-UV and HPLC-ED respectively. The use of a post-column photochemical reactor allowed an improvement of 10 times for LODs and LOQs for the analysis of Allicin compared to traditional HPLC-UV.

Rybak *et al.* proposed SC-CO_2 coupled with HPLC-UV, an accurate and precise method for the extraction, separation and detection in garlic sampler providing 96% of recoveries for allicin.[66] Arnault *et al.* also described a HPLC-UV method for the analysis of allicin and other OSC (alliin, deoxyalliin, and dipeptide precursors) in garlic powder.[65] LOD for allicin was 16.2 μg mL^{-1}. Lastly, Baghalian *et al*. analyzed allicin in 24 types of garlic by HPLC-UV.[67] A similar HPLC-UV technique was also used in 2007 for garlic powder and tablets improving Arnault and Baghalian results.[68] For this analysis method LODs and LOQs were 0.27 and 0.81 μg mL^{-1}**,** respectively. Bose *et al*. applied Baghalian methodology in 2014 obtaining LOD lower than 3.33 μg mL.[71]

Some years later, normal-phase HPLC-UV method was proposed again to determine garlic sulfur compounds including allicin from garlic bulbs using n-hexane and 2-propanol as mobile phase.[45] LOD and LOQ were 6.63 and 20.09 μg mL^{-1} respectively, lower than obtained for reverse phase in both cases. At the same time, Al-Dulimyi *et al.*, determined alliin and allicin in different types of garlic extracts by the use of reverse phase.[69]

Lastly, HPLC-UV methodology was extrapolated to Ultra High Performance Liquid Chromatography (UHPLC)-UV system obtaining a reduction of the analysis time compared to HPLC analysis were analysis time are around 20-25 min. UHPLC gives rapid, sensitive and high resolution separations obtaining LOD and LOQ of 0.79 and 2.63 μg mL^{-1} respectively.[72] A similar procedure for UHPLC-UV was described by Khar in 2011.[73] For this analysis method, LODs and LOQs were 1.0 and 2.5 μg mL^{-1} respectively. As can be seen, both methods give very similar results.

The determination of Allicin by HPLC or UHPLC coupled to UV detection is limited because of the lack of sensitivity caused frequently by the apparition of interferences at the typical analysis wavelength. For this reason, other analytical techniques have been developed in order to improve the quantity of compounds which can be analyzed simultaneously as well as the quality of analysis. The coupling of LC system to MS is a good alternative. Reverse phase column (C18) is generally used being MeOH or MeCN and water the most commonly solvents used mobile phases.

Differents ionization sources have also been proposed for OSCs determination. Specifically, atmospheric Pressure Chemical Ionization (APCI) has been used for the analysis of thiosulfinates and other thermolabile OSC (capaenes like 1-(2-propenylsulfenyl) propyl disulde) present in allium vegetables.[74] In addition, a LC-MS/MS method using a Coordination Ion Spray (CIS) has been also developed as alternative of HPLC-UV. In order to enhance the allicin detection by the formation of an ionized coordinated complex, silver nitrate aqueous solution (1mM) at 10 μL min-1 was added post column .[75] Electrospray ionization (ESI) has also been proposed for OSCs determination. HPLC-ESI-MS has been used for *Allium hookeri* root analysis, a plant

whose composition is very similar to garlic. Ten alkyl thiosulfinates including allicin were characterized with this method.[68]

Despite the extensive published works on allium chemistry and OSC analysis, direct observation of intermediates has not proven possible due to rapid kinetic reactions in garlic and onion. A new and novel technique, Direct Analysis in Real-Time (DART)-MS, to observe and measure directly the kinetics of disappearance of garlic and onion intermediates. DART is method, which for most compounds gives simple mass spectra, obtained by briefly holding sample in the gas stream.[76] Allicin and PTS could be identified with this technique.

Allicin has been also determined in breath, plasma and simulated gastric fluids by HPLC-UV. Data indicate that allicin decomposes in stomach acid to release allyl sulfides, disulfides and other volatiles that are postulated to be metabolized by glutathione and/or S-adenosylmethionine to form allyl methyl sulfide.[62] In this thesis the potential of the LC coupled to UV and MS for the determination of OSC, in particular PTSO, in different matrices will continue to be studied.

I.5.3. Sample treatment

Different sample treatments have been proposed for thiosulfinates and thiosulfonates extraction in the last years. The most part of described extraction methods are based on sample treatments consisted of simple extraction at room temperature using water as extraction solvent.[1,61,64,65,72,73,76,77] This procedure has also applied using organic solvens.[45,75] Bochini *et al.*, proposed a triple extraction. First procedure consisted on aqueous extraction at room temperature while the second included the saturation of the sample with sodium chloride (NaCl) and followed by triple extraction with CH_2Cl_2. [63]

Other simple techniques have been proposed for OSC extraction like distillation,[18] maceration,[67,78] vortex-sonication,[68] radiation, [71] supercritical fluid extraction[58,66,74] or SPME.[59,71]

In this thesis, other sample treatments will be proposed for the determination of PTSO in feed (it has been proposed as animal feed additive) and in products derived from animals that eat this feed, such as eggs and milk.

CHAPTER 1

High-Performance Liquid Chromatography Method for the monitoring of the allium derivative propyl propane thiosulfonate used as natural additive in animal feed.

Determinación mediante cromatografía líquida de alta resolución del derivado de aliáceas propil propano tiosulfonato, aditivo natural utilizado como aditivo natural en alimentación animal.

Resumen

En este capítulo se propone un método de análisis por cromatografía líquida de alta resolución en fase inversa y con detección ultravioleta para la determinación de propil propano tiosulfonato (PTSO) en piensos. Este derivado de aliáceas, característico del género *Allium Cepa* (cebolla), se utiliza como alternativa al uso de antibióticos promotores del crecimiento en alimentación animal. El análisis de PTSO implica un tratamiento de muestra previo a su determinación, basado en la extracción sólido-líquido, en piensos de gallinas ponedoras, utilizando acetona como disolvente de extracción. El método se ha caracterizado para este tipo de piensos, obteniendo límites de detección y cuantificación de 11,2 y 37,3 mg kg^{-1}, respectivamente, valores inferiores a las concentraciones esperadas en muestras que contienen este aditivo. La repetibilidad (precisión intradiaria) y la precisión intermedia (precisión interdía), expresadas como desviaciones estándar relativas fueron inferiores al 8,3% en todos los casos y las recuperaciones oscilaron entre 90,5 y 94,6%. Por último, con el fin de comprobar la identificación inequívoca de PTSO, las muestras se sometieron a la detección por espectrometría de masas en tándem. El método propuesto es un procedimiento sencillo para el control del aditivo PTSO en piensos comerciales, siendo posible su implementación en laboratorios de rutina con fines de cuantificación y estudios de estabilidad en los productos comerciales.

Abstract

A new simple analytical method for monitoring propyl propane thiosulfonate (PTSO) in animal feed is presented in this chapter. PTSO is an active ingredient from *Allium cepa* (onion), proposed as a natural additive for feed being an efficient alternative to antibiotics used as growth promoter due to its efficiency improving animal health. Reversed-phase liquid chromatography with ultraviolet detection has been used and a previous sample treatment based on solid-liquid extraction has been developed and optimized in order to extract PTSO from feed for laying hens using acetone as extraction solvent. The method has been characterized for laying hens feed obtaining limits of detection and quantification of 11.2 and 37.3 mg kg^{-1}, respectively, which are lower than the concentrations expected in samples containing this additive. Repeatability (intraday precision) and intermediate precision (interday precision) expressed as relative standard deviations were lower than 8.3% in all the cases, and recoveries ranged from 90.5 to 94.6%. Finally, in order to check the unequivocal identification of PTSO, mass spectrometry detection was applied. The proposed method is a simple procedure for monitoring PTSO in commercial feed, being possible to its implementation in routine laboratories for quantification purposes and stability studies of the distributed products.

1.1. Introduction

The use of antibiotics in animal nutrition to cause improvements in growth and feed conversion and to decrease mortality from clinical diseases has been very common throughout last five decades. The antibiotics mainly used as antibiotics growth promoters (AGP) include tetracyclines, penicillins, sulfonamides, and aminoglycosides. However, the growing concern over the transmission and the proliferation of resistant bacteria via the food chain has led to a ban of the use of AGP in livestock within the European Union (EU) since 2006. Regulation 1831/2003[1] stated that antibiotics, other than coccidiostats and histomonostats, might be marketed and used as feed additives only until December 31, 2005; as from January 1, 2006, those substances would be deleted from the Community Register of Authorized Feed Additives. After the ban of AGP by the EU, the use of natural feed additives such as probiotics, prebiotics, organic acids and plant extracts has been increased in animal diets.

Plant extracts and essential oils have been significantly exploited in animal nutrition, particularly for their antimicrobial, anti-inflammatory and anti-oxidative and anti-parasite properties.[2-6] These natural alternatives to AGP try to enhance production performance

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improving the flavor and palatability in animal feed, among others.[7] The existing data shows that their effect depends on the type of plant extract, diet and appropriate dosage.[8,9] Beneficial effects of natural products coming from different plant extracts like cinnamaldehyde, carvacrol, thymol, and eugenol (major active ingredients of cinnamon oregano and clove oil) have been described.[10-13]

Allium plants such as garlic and onion have been used as food ingredients for thousands of years, but they are also known for their healing properties and few side effects. Antimicrobial properties of garlic are well known since XIX century and in 1944 allicin, the main garlic active principle was isolated and identified.[14] Despite the antimicrobial properties of allicin,[15] its use in animal feed is limited due to its poor stability. In the last decades, other compounds from *Allium spp.*, i.e., propyl propane thiosulfonate (PTSO), have been studied.[16] The company Domca S.A. (Alhendín, Granada, Spain) has proposed PTSO, to be added to feed as a natural alternative to AGP. The product is commercialized under the trademark Garlicon®.

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Several analytical methods for allium derivatives analysis using liquid chromatography with ultraviolet detection (HPLC-UV) have been developed but most of them are focused on the analysis of n-polisulfides compounds and allyl derivatives in raw onion or garlic, and not on thiosulfonates.[17-19] Therefore, it is necessary to develop a method that allows us to quantify PTSO in this type of matrices in order to control the content of this active ingredient in animal feed. This determination presents certain complexity since feed usually contain many other additives such as vitamins and minerals, and it is therefore necessary to pay special attention to the selectivity of the analytical method. Also, simplicity, robustness and low cost should be important characteristics for routine analysis. In this chapter, a new analytical method to determine PTSO in animal feed based on HPLC-UV analysis is proposed. Moreover, a sample treatment method based on solid-liquid extraction has been developed and optimized in order to extract PTSO from animal feed. Both methodologies are simple and easy to use in quality control laboratories. In order to ensure the identification of PTSO quantified by the propose HPLC-UV in this matrix, its confirmation was carried out by mass spectrometry (MS).

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1.2. Materials and methods

1.2.1. Chemicals and reagents

All reagents were of analytical reagent grade. Acetonitrile (MeCN), methanol (MeOH) and water, all HPLC grade, and analytical grade acetone (ACO), ethyl acetate (EtAc), CH_2Cl_2, perchloric acid (70%), phosphoric acid (85%) and hydrochloric acid (HCl) 5 N were supplied by Panreac (Madrid, Spain). Formic acid (FA) used as mobile phase additive and MeOH both for liquid chromatography (LC)-MS grade were obtained from Sigma Aldrich (St Louis, MO, USA) and Merck (Darmstadt, Germany), respectively. FA (analytical grade) was also purchased from Merck.

HPLC mobile phases were filtered using a vacuum system with Millipore nylon membrane (Milford, MA, USA) filters (0.20 μm, 47 mm). Purified samples were filtered through polyethylene (PET) Chromafil® (Macherey-Nagel, Germany) filters (25 mm, 0.20 μm).

PTSO high purity grade (95%) was supplied by Domca S.A. (Alhendín, Granada, Spain). A stock standard solution of PTSO was prepared in ACO (2000 mg kg^{-1}) and stored at -20ºC (solution stable at least 12 months).

1.2.2. Instruments and equipment

The analyses of feed extracts were performed using a HPLC Agilent 1260 Infinity LC (Agilent Technologies, Santa Clara, CA) system consisted of a quaternary pump, on-line degasser, autosampler (injection volume from 0.1 – 900 µL, capacity: 100 vials), and a column thermostat and a Diode Array Detector (DAD). The confirmation of PTSO was carried out using an Agilent 1290 Infinity LC (Agilent Technologies) equipped with a binary pump, on-line degasser, autosampler (5 µL loop), and a column thermostat. The mass-spectrometer measurements were performed on a triple quadruple mass spectrometer API 3200 (AB SCIEX, Toronto, ON, Canada) with electrospray ionization (ESI).

A polytron (Kinematica AG, Luzern, Switzerland), a universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) and an evaporator system (Rotavapor-R 200/205 from BÜCHI Labortechnik AG, Postfach, Switzerland) were also used for sample treatment.

1.2.3. Sample treatment

Sample treatment was carried out with laying hens feed purchased from a local dealer (Santa Fe, Granada, Spain). Table 1.1 shows feed composition.

Table 1.1. Composition of laying hens feed used in the development of the analysis method for PTSO determination.

LAYING HENS FEED	
RAW MATERIALS	Corn (46 %), soybean meal (25 %), wheat (15.1 %), sodium carbonate (10 %), phosphate (1.6 %), soybean oil (1.5 %), sea salt (0.5 %), lechamix chickens (0.2 %), sodium bicarbonate (0.1 %)
ANALYTICAL COMPOSITION	Crude protein (16 %), crude cellulose (14 %), crude fat (3.75 %), calcium (3.75 %), lysine (0.8 %), phosphorus (0.6 %), methionine (0.3 %), sodium (0.2 %)
ADDITIVES	Nutritional additives: vitamin A (E-472), vitamin D3 (E-671), vitamin E (α-tocopherol). Oligoelements: E-4 cupric sulfate pentahydrate (copper, 4.5 mg kg^{-1}), E-1 ferrosdo heptahydrate sulfate (Iron 22.6 mg kg^{-1}), E-8 sodium selenite (16 mg kg^{-1} celenium), E-5 manganese sulfate monohydrate (75 mg kg^{-1}), E-6 zinc oxide (38 mg kg^{-1}), E-3 sulfate heptahydrate cobalt (0.35mg kg^{-1}), E-2 anhydrous calcium iodate (iodine 0.75 mg kg^{-1}) Technological additives: Butyl hydroxytoluene (BHT), E-321, 40 mg kg^{-1}

Finely ground samples of feed (5 g) free of PTSO were placed in a polypropylene centrifuge tube (50 mL). Samples were spiked with proper amounts of PTSO stock solution and homogenized for two minutes with a vortex and a contact time of 1 h was considered before the extraction process. ACO (25 mL) was added into the tube and immediately it was homogenized using the polytron for 1 min at 22000 rpm. Then, the

sample was centrifuged for 5 min at 5000 rpm. The supernatant was transferred to an evaporating flask and the solid residue was re-extracted again with 20 mL of ACO. The final supernatant (from both extractions) was evaporated to dryness (P=-0.8 bar; T=40 ºC). Then, the sample was reconstituted with MeOH (5 mL) and subsequently it was vortexed for 30 s. Finally, the extract was filtered through PET Chromafil® filter and injected into the HPLC-UV system. Figure 1.1 shows a scheme of the proposed sample treatment.

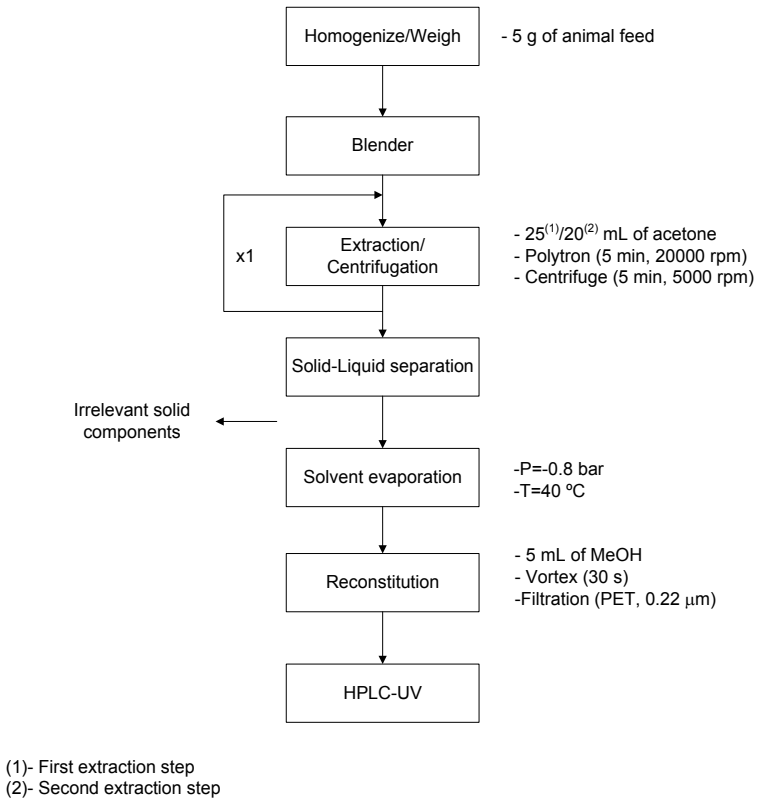

Figure 1.1. Proposed sample treatment for PTSO determination in animal feed

1.2.4. HPLC-UV analysis

The determination of PTSO was accomplished at 25ºC with a Gemini-NX 5u C18 110A New Column (150x4.6 mm, 5 µm) supplied from Phenomenex (Torrance, CA). The flow rate and injection volume were 1 mL min^{-1} and 20 µL, respectively. The mobile phase consisted of (A) perchloric acid 30 mM and (B) MeCN. The gradient elution program was as follows: 0 min - 50 % B; 3 min - 50 % B; 9 min - 100 % B; 19 min - 100 % B. Finally it was back to 50 % B in 6 min and kept for 5 min for column equilibration. The total chromatography run time was 28 min and detection wavelength was 200 nm (Band width = 4 nm).

1.3. Results and discussion

1.3.1. Development of the chromatographic method

Three different columns were tested: a C-8 column from Agilent (Microsorb 100-5, 150 x 436 mm, 5 µm) and two C18 columns: Gemini-NX (150 x 4.6 mm, 5 µm) and Agilent TC18 (250 x 4.6 mm, 5 µm). The mobile phase consisted of two components: (A) Perchloric acid 30 mM and (B) MeCN. Using C8 column, peaks corresponding to interferences from the matrix appeared at the same retention time that PTSO and it was discarded for this analysis. Then, the two C18 columns were compared. For the Agilent TC18 column the gradient elution program was as follows: 0 min - 50 % B; 5 min - 50 % A; 15 min - 100 % B; 31.7 min - 100 % B. Finally it was back to 50 % B in 6.6 min and kept for 8.7 min for column equilibration. PTSO retention time was 10.4 min and the total chromatography run time was 47 min. For Gemini-NX, the gradient was programmed as follows: 0 min - 50 % B; 3 min - 50 % B; 9 min - 100 % B; 19 min -

100 % B. Finally it was back to 50 % B in 4 min and kept for 5 min for column equilibration. The total chromatography run time was 28 min. Gemini-NX column provided a reduction in the analysis time and solvent consumption, showing a retention time for PTSO of 5.4 min. The peak area for PTSO obtained with Gemini-NX column was higher than that obtained with TC18 column, so an increase in the LOD of the method was also achieved. For these reasons Gemini-NX column was selected. PTSO chromatograms from each column are shown in figure 1.2.

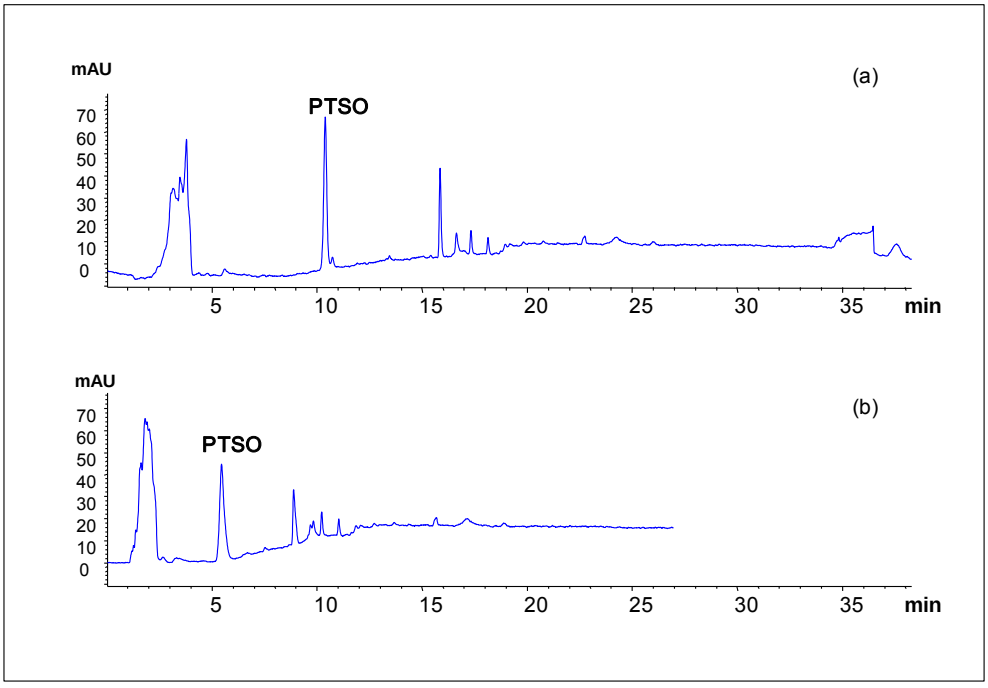

Figure 1.2. Chromatograms from PTSO (50 mg kg^{-1}) determined using (a) an Agilent TC18 column and (b) a Gemini-NX column

FA, hydrochloric acid, perchloric acid, phosphoric acid aqueous solutions, all of them at a concentration of 30 mM, and water without any acid were tested as component A in the mobile phase; using MeCN as component B. Gemini-NX gradient elution program was used. Unstable baseline and a poor signal for PTSO were obtained with formic and hydrochloric acid, so they were discarded. Finally, perchloric acid 30 mM was selected as component B for the mobile phase considering that improves PTSO absorptivity and therefore the sensitivity of the method in UV. Results, in terms of peak area for each kind of component A tested are shown in figure 1.3. PTSO retention time was 5.4 min al all tested solutions.

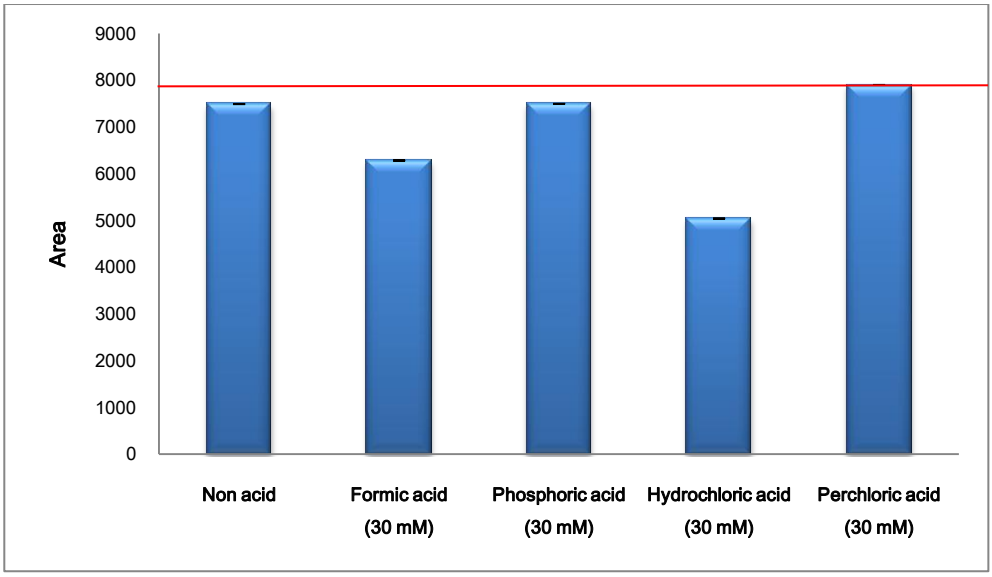

Figure 1.3. Effect of the addition of an acid on the mobile phase component A on PTSO (50 mg kg^{-1}) peak area

Different injection volumes of the extracts of three feed samples spiked with 50 mg kg^{-1} of PTSO were evaluated (10, 20, 40, 60, 80 and 100 µL) using the signal-to-noise ratio (S/N) and peak area as analytical responses. Chromatograms corresponding to each injection volume are shown in figure 1.4 (chromatograms plotted in cascade mode). As can be seen PTSO peak was not symmetric from 40 µL onwards. In addition, interfering substances from the matrix appeared at higher injection volumes, so 20 µL was selected as optimum.

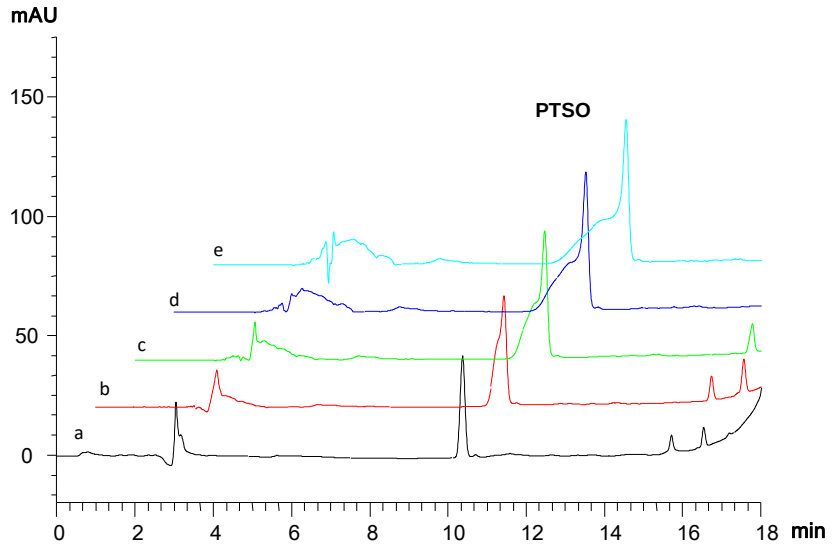

Figure 1.4. Influence of the injection volume in the determination of PTSO (50 mg kg^{-1}): (a) 20 µL, (b) 40 µL, (c) 60 µL, (d) 80 µL and (e) 100 µL

Column temperature was studied between 20 and 35 °C and an optimum of 25 ºC was selected since relative standard derivation (RSD) was minimum at this value and no great differences were observed in the retention time obtained for each analysis. Results are shown in table 1.2.

Table 1.2. Effect of column temperature on PTSO analysis

TEMPERATURE	20 ºC	25º C	30 ºC	35 ºC
H (mAU)	111.6	112.2	111.5	98.2
RSD (%)	2.4	1.3	4.6	6.8
Tr (min)	5.5	5.3	5.1	4.9

Also, mobile phase flow rate was evaluated in the range of 0.750 to 1.250 mL min^{-1}. No signal intensity differences were observed between 0.75 and 0.875 mL min^{-1} but in both cases analysis time was increased considerably. The two higher flow rates checked (1.125 and 1.250 mL min^{-1}) presented feed impurities eluting near to PTSO retention time so 1 mL min^{-1} was chosen as optimum.

1.3.2. Optimization of sample preparation

The optimization was carried out using 5 g of finely ground feed for laying hens spiked with 75 mg kg^{-1} of PTSO. For the extraction, 25 mL of three solvents with different polarity index (PI) were tested: CH_2Cl_2 (PI=3.1), EtAc (PI=4.4) and ACO (PI=5.1). The extraction with CH_2Cl_2 gave a chromatogram with many interfering peaks from endogenous component of the feed matrix. Despite recoveries were nearly 90%, CH_2Cl_2 was discarded as extraction solvent given that an interference peak appeared nearly PTSO retention time. Using ACO as extraction solvent a cleaner extract was obtained with less interferences, compared with EtAc. In addition, using ACO the recoveries were slightly better than with EtAc (95 vs. 92 %) so, ACO was chosen.

Once the extraction solvent was evaporated, 5 mL of MeOH, MeCN: perchloric acid 30 mM (50:50 v/v) and MeCN were checked as reconstitution solvents. MeOH was selected because with perchloric acid 30 mM and MeCN more interference compounds from the matrix were observed. A typical chromatogram corresponding to a blank laying hen feed sample (free of PTSO) and a spiked laying hen feed sample (containing PTSO at 50 mg kg^{-1}), both submitted to the proposed method, are shown in figure 1.5 As can be seen, no interferences from the matrix were coeluting with the analyte.

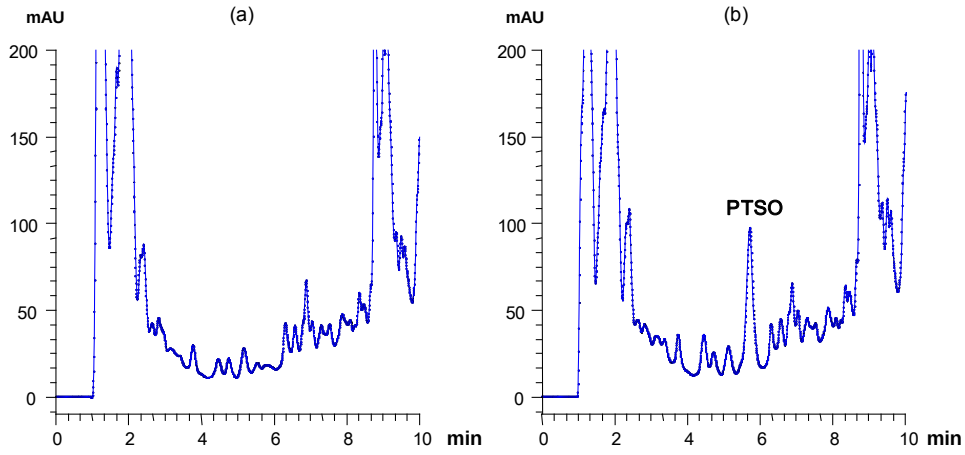

Figure 1.5. Chromatograms from (a) a blank feed sample and (b) a feed sample spiked with PTSO at 50 mg kg^{-1}

1.3.3. Characterization of the method

To check the suitability of the proposed method for the determination of PTSO in animal feed, linear range, LOD and LOQ, precision and trueness were evaluated using laying hens feed as representative matrix.

1.3.3.1. Calibration curve, linearity and detection and quantification limits

Matrix-matched calibration (MMC) was used in this study due to the complexity of the sample (animal feed). For the MMC curves, laying hens feed were spiked at five different concentrations levels of PTSO (between 50 and 150 mg kg^{-1}). Each level was prepared in duplicated and injected in triplicate. Statistical parameters were calculated by least-square regression. LOD (3xS/N ratio) and LOQ (10xS/N ratio) were 11.2 and 37.3 mg kg^{-1}, respectively. It was confirmed that PTSO analytical response was linear over the studied range (R^2 = 0.9926).

1.3.3.2. Precision study

The precision of the whole method was evaluated in terms of repeatability and intermediate precision. For repeatability study, spiked samples with three different concentrations of PTSO were prepared in triplicate (50, 100 and 150 mg kg^{-1}). The extraction procedure for each sample was applied the same day and the extracts were injected in triplicate. Intermediate precision was calculated from three spiked samples at the same concentration above mentioned (50, 100 and 150 mg kg^{-1}) but analyzed during five days and injected in triplicate. The results, expressed as RSD (%) of peak areas are shown in table 1.3. Satisfactory precision was obtained considering that RSD was in all cases lower than 8.3 %.

Table 1.3. Precision (% RSD of peak areas) of the proposed method for the determination of PTSO in laying hens feed

PTSO (mg kg^{-1})	Repeatability (n=9)	Intermediate precision (n=15)
50	1.1	8.3
100	2.4	6.8
150	1.0	5.4

1.3.3.3. Trueness studies

Trueness was evaluated by means of recovery studies. Feed samples were spiked at three concentration levels of PTSO (50, 100 and 150 mg kg^{-1}) processed as described previously and injected and submitted to the proposed HPLC-UV method. Recoveries were calculated using blank samples treated and spiked properly (50, 100 and 150 mg kg^{-1}) just before the reconstitution in the injection solvent. All the samples were injected in triplicate. Recoveries were calculated as (spiked sample signal/spiked blank signal) x 100. The results are shown in table 1.4 showing recoveries for all the studied concentrations of the linear range higher than 90.2 %, which ensures that no significant losses are produced during the procedure, demonstrating the trueness of the proposed method.

Table 1.4. Recovery study (% RDS in parenthesis) of the proposed method for the determination of PTSO in laying hens feed

PTSO (mg kg^{-1})	Recovery (n=9)
50	90.2 (4.4)
100	90.5 (7.0)
150	94.6 (5.2)

1.3.4. Application to different matrixes

In order to ensure the applicability of the proposed method to different feed matrixes, it was carried out on different batches of laying hens, mice trout and swine feed samples. For the MMC, feed samples were spiked at six different concentrations levels of PTSO (between 50 and 150 mg kg^{-1}). It was confirmed that PTSO analytical response was linear over the studied range for all types of feed (laying hens feed (R^2 = 0.997), mice feed (R^2 = 0.996), trout feed (R^2 = 0.997) and swine feed (R^2 = 0.995)). No significant differences in the slopes of the MMC curves for the matrices studied were found ($P \geq 0.05$), so we can confirm that laying hens feed can be a representative matrix to characterize the method in feed and could be used as MMC curve for quantification purposes. Also, control samples for each kind of matrix containing 100 mg kg^{-1} of PTSO were analyzed obtaining recoveries of 85.9% (n=6), 87.0% (n=6) and 84.3% (n=6) for mice, trout and swine feed respectively.

1.3.5. Confirmation of PTSO in feed matrix by mass spectrometry

Animal feed is a complex matrix. For this reason, the peak corresponding to PTSO was identified by LC-MS/MS. This confirmatory analysis was performed in ESI+ mode and multiple reaction monitoring (MRM)™. Initially, in order to study mass fragmentation of PTSO, a standard solution of 200 mg L^{-1} in 0.1% aqueous FA: MeOH (50:50. v/v) was infused into the mass spectrometer. The compound was tested using ESI positive/negative mode. ESI+ showed the best results, being the precursor ion the protonated form $[M+H]^+$, with m/z 183. Figure 1.6 shows the MS/MS spectrum obtained in Q3, showing the fragmentation of PTSO, in which losses of 42 Da and 84 Da were observed, probably corresponding to one or both side chains.

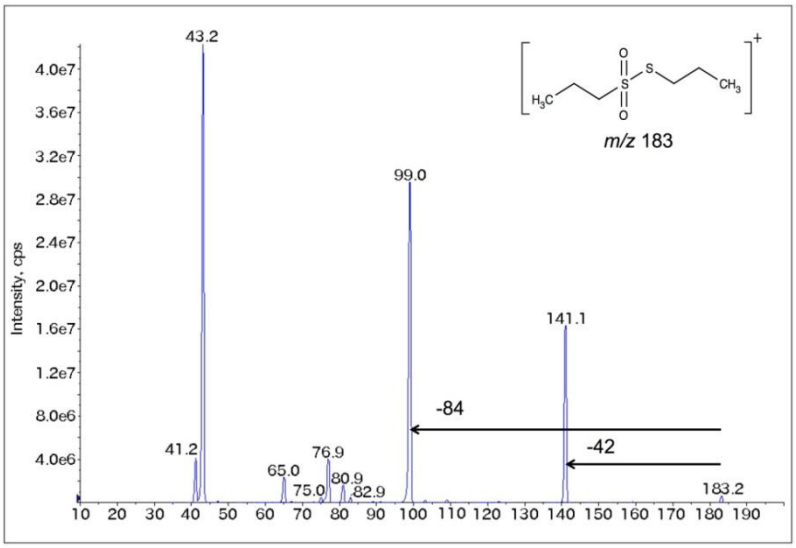

Figure 1.6. MS/MS spectrum of the precursor ion of PTSO (*m/z* 183)

During the infusion, the parameters affecting MS detection were optimised for PTSO in order to obtain the maximum sensitivity, obtaining the values indicated in table 1.5 for declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision cell exit potential (CXP) and collision energy (CE). The target scan time established for each transition was 0.1 s.

Table 1.5. Monitored ions of PTSO and MS/MS parameters

	Precursor ion (m/z)	Molecular ion	DP	EP	CEP	Product ions	CE	CXP
PTSO	183.2	$[M+H]^+$	21	10.5	10	99.0	17	4
						141.1	13	4

Declustering potential (DP), Entrance potential (EP), Collision cell entrance potential (CEP), Collision cell exit potential (CXP) and Collision energy (CE). All expressed in voltage.

Once PTSO LC-MS/MS parameters were optimized, one feed sample spiked at 200 mg kg^{-1} of PTSO was prepared following the whole sample treatment described in section 1.2.3 and analyzed by the HPLC-MS/MS method.

In this study the same column and gradient were used, however the nature of the mobile phase was slightly modified in order to make it compatible with the electrospray formation and MS detection: 0.1% aqueous FA solution (solvent A), and MeCN (solvent B) at a flow rate of 1 mL min^{-1}. The temperature of the column was 25°C and the injection volume was 5 µL (full loop).

The ionization source parameters were: source temperature 500°C; curtain gas (nitrogen) 30 psi; ion spray voltage 5000 V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi. Under these conditions PTSO was determined at 6.5 min being characterized by two precursor-product ion transitions. Figure 1.7 shows the

corresponding HPLC-MS/MS chromatogram of a PTSO standard solution. A small shift in the retention time was observed compared with the UV detector (from 5.8 to 6.5 min) but this is probably due to the longer connection between the HPLC and MS.

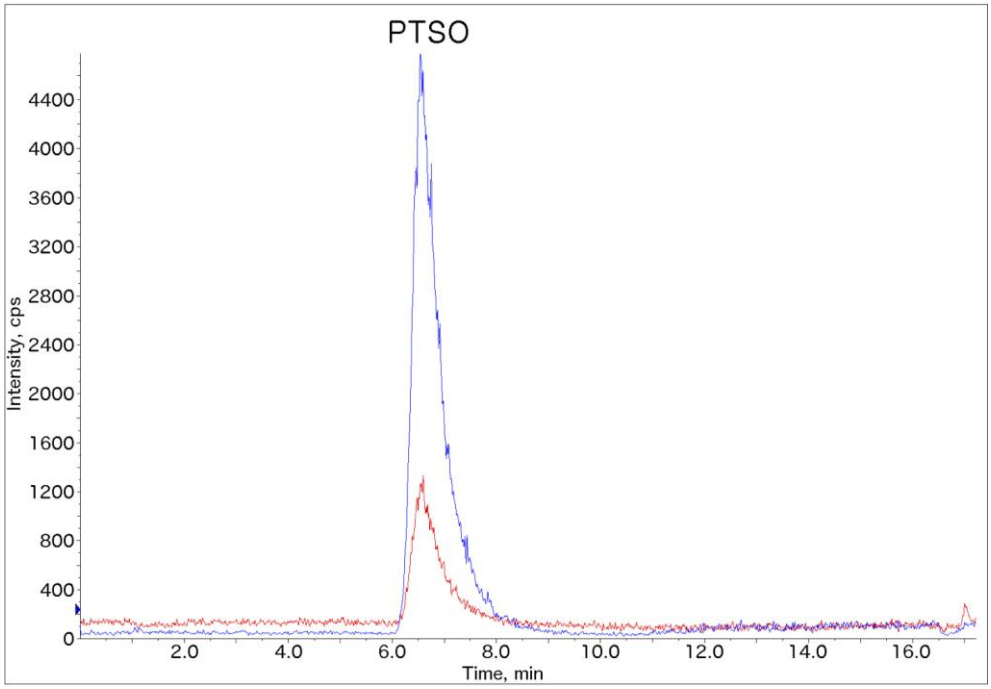

Figure 1.7: HPLC-MS/MS extracted ion chromatogram (XIC) of a feed sample spiked with PTSO at 200 mg L^{-1} (product ions: blue (99.0); red (141.1))

1.4. Conclusions

A new analytical method was developed for the determination of PTSO in animal feed by HPLC-UV detection. This method provided a simple, robust and efficient alternative for the monitoring of this additive, used as natural growth promoter, in feed for different animals. It can be used in the quality control of the feed treated with this product and also to monitor PTSO stability in the feed. Satisfactory linearity was obtained with good sensitivity and accuracy for this kind of matrixes. The unequivocal identification of PTSO was carried out by MS/MS in order to confirm the nature of the monitored PTSO peak by LC-UV in these kinds of complex matrixes. The applicability of this method was demonstrated in different feed samples, being a useful alternative to be implemented in routine analysis for feed quality control laboratories.

This work was published as:

High-performance liquid chromatography method for the monitoring of the allium derivative propyl propane thiosulfonate used as natural additive in animal feed. Abad, P., Lara, F.J., Arroyo-Manzanares, N., Baños, A., Guillamón, E., García-Campaña A.M. Food Anal. Meth., 8 (2015) 916-921.

CHAPTER 2

A rapid and simple UHPLC-ESI-MS/MS method for the screening of propyl propane thiosulfonate and its derivatives in animal feed.

Desarrollo de un método rápido y simple para la determinación de propil propano tiosulfonato y sus derivados en piensos mediante UHPLC-ESI-MS/MS.

Resumen

Con el fin de garantizar la prevención de alteraciones en productos destinados a alimentación animal es necesario el desarrollo de métodos que permitan llevar a cabo un correcto control de calidad de los mismos. Sin embargo, la información disponible sobre el análisis del aditivo propil propano tiosulfonato (PTSO) es escasa y los métodos analíticos utilizados para su control han demostrado un alcance limitado. Además, los métodos analíticos existentes presentan deficiencias cuando se aplican a otras matrices o cuando se trata de medir niveles de concentración bajos.

Este capítulo describe por primera vez la reacción instantánea entre el PTSO con productos que contienen en su estructura el grupo tiol, más concretamente cisteína y glutatión, dando lugar a s-propilmercaptocisteína (CSSP) y s-propilmercaptoglutatión (GSSP) respectivamente. La existencia de esta reacción, explica las limitaciones de métodos anteriores cuando el procedimiento existente se aplica a un pienso que contiene cisteína y/o glutatión. Teniendo en cuenta esta información, se propone un método usando cromatografía liquida de ultra-alta eficacia acoplada con espectrometría de masas en tándem (UHPLC-MS/MS), combinado con un tratamiento de muestra basado en la extracción sólido-líquido con metanol, para el control del PTSO, CSSP y GSSP en una amplia gama de muestras de piensos (cerdos, lechones, pollos de engorde, gallinas, conejos y perdices). Para la validación del método, se establecieron curvas de calibración en presencia de matriz para PTSO, CSSP y GSSP y se obtuvieron límites de detección y cuantificación inferiores a los reportados para PTSO cuando se analizó mediante cromatografía líquida de alta resolución con detección ultravioleta (HPLC-UV). La desviación estándar relativa de todo el método analítico fue inferior al 10% en todos los casos, mientras que las recuperaciones

oscilaron entre 93% y 104%. Este método proporciona una alternativa simple, robusta y eficiente para la determinación de este aditivo.

Abstract

PTSO when is used as feed additive must be control to ensure the prevention of feed alterations. Nevertheless, the information available on PTSO is scarce and the analytical methods used for its monitoring present some limitations. Moreover, the existing analytical methods exhibit shortcomings when they are applied to other matrices or when low concentration levels must be measured.

This chapter describes for the first time the instantaneous reaction of PTSO with cysteine and glutathione, generating s-propyl mercaptocysteine (CSSP) and s-propyl mercaptoglutathione (GSSP), thus explaining the limitations of previous methods when they are applied to feed containing cysteine and glutathione. Therefore, an ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method combined with a sample treatment based on a simple solid-liquid extraction with methanol is proposed for the monitoring of PTSO, CSSP and GSSP in a wide range of animal feed samples including those for pigs, piglets, broilers, hens, rabbits and partridges feed.

Matrix-matched calibration curves were established for PTSO, CSSP and GSSP and limits of detection and quantification were estimated, being lower than the ones previously reported for PTSO when using high performance liquid chromatography with ultraviolet detection (HPLC-UV). The relative standard deviation of the whole analytical method was lower than 10% in all cases, while recoveries ranged from 93% to 104%. This method provides a simple, robust and efficient alternative for the determination of this additive.

2.1 Introduction

Garlic (*Allium sativum*) and onion (*Allium cepa*) have been used in folk medicine through the centuries for treatment of several health disorders like diabetes, cardiovascular problems, rheumatism, etc.[1] Recent advances in the fields of immunonutrition, physiology, microbiology and pharmacology have identified their health benefits, related to their antioxidant effect, cardiovascular protection by reducing cholesterol and anti-platelet activity and prevention of cancer.[2-6] In addition, they and their respective essential oils have shown antimicrobial effect against Grand Positive and Gram Negative bacteria including *Bacillus cereus, Escherichia coli, Shigella spp., Vibrio spp., Yersinia enterocoliticab, Listeria monocytogenes, Salmonella ssp. and Campylobacter spp.* Also, they present antifungal activity against *Aspergillus spp., Penicillium* spp. and *Fusarium spp*.[7]

Lastly, related to animal feed, several studies have shown positive effects on growth performance in broilers and on ruminants' methane emissions reduction.[8-10]

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Biological and medical functions of garlic and onions are associated to their high content on OSCs.[11,12] The number of sulfur atoms, chain length and the presence of double bonds are responsible of each OSCs characteristic odor, pungency and biological activities. The main OSCs found in all *Allium spp.* and the best studied are thiosulfinates which are generated when garlic and onion are crushed or their tissues are damaged by the reaction between S-alk(en)yl-L-cysteine sulphoxides (flavor precursors) and the enzyme alliinase. The structure and content of the cysteine sulfoxides are responsible for the flavor and odor and depend on the different *Allium spp.*; the enzyme allinase is common to all *Allium spp.*[13]

Thiosulfinates are very unstable compounds and they decompose quickly at room temperature. In garlic, thiosulfinates decompose to vinyl ditiins, polysulfides and ajoenes. As well as in onion, they react quickly, given rise to thiosulfonates which are highly stable,[14,15] found among them PTSO.

PTSO antimicrobial, anticoccidial, antifungal and inmunomodulating activity has been already demonstrated.[16-22] Several methods for qualitative and quantitative

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determination of allium thiosulfinates and thiosulfonates have been reported, mainly based on GC-FID or GC-MS.[23]

First reported papers related to allium volatile compounds focused their attention in the identification of different components present in allium extracts, depending on the sample origin and using different sample treatments and analytical techniques. [24-26] In the last 15 years, methods of analysis of these volatile allium compounds have been improved in terms of sensitivity and accuracy.[15]

It is known that allicin (thio-2-propene-1-sulfinic acid S-allyl ester), a compound similar to PTSO, which is the main biologically active component of garlic, reacts with CYS and GSH, both of which are present in animal feed. The result of these reactions is s-allylmercaptocysteine or s-allylmercaptogluthatione for CYS or GSH, respectively. These products are not hazardous to health and show biological activities like hepato-

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protective,[27] antioxidant,[28] antiproliferative,[29] apoptosis-inducing,[30] and antimetastatic[31] activities, all of them associated to the presence of disulfide bonds.[32]

In this chapter the reaction of PTSO with CYS and GSH, giving CSSP and GSSP, respectively, is described for the first time. In addition, to ensure the quality control of PTSO, we have developed an UHPLC–MS/MS method for the monitoring of PTSO, CSSP and GSSP in a wide range of animal feed samples (for pigs, piglets, broilers, hens, rabbits and partridges feed) combined with a sample treatment based on a simple solid-liquid extraction with methanol.

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2.1. Materials and methods

2.1.1. Chemicals and reagents

All reagents were of analytical reagent grade, solvents used as mobile phase were LC-MS grade, while those used during sample treatment were HPLC grade. FA used as mobile phase additive for LC-MS and MeOH was obtained from Sigma Aldrich (St Louis, MO, USA). FA (analysis grade), was supplied by Merck (Darmstadt, Germany). Potassium dihydrogen phosphate, sodium hydroxide and MeCN, MeOH, ACO and EtAc (all of them of HPLC grade), were supplied by Panreac (Madrid, Spain). L-CYS hydrochloride anhydrous (extra pure) was obtained from Scharlab (Barcelona, Spain), L-GSH reduced (quality 97%) from Alfa Aesar (Ward Hill, MA, USA) and L-Lysine (LYS) (>97%), L-Serine (SER), L-Methionine (MET) (>98%, HPLC) and GLY (>99%, HPLC) from Sigma Aldrich (St Louis, MO, USA). L-GSH oxidized (quality >98%, HPLC) and L-Cystine (quality >98%, HPLC) used for blocking SH groups study were also purchased from Sigma Aldrich.

PTSO high purity grade (95%) was kindly provided by Domca S.A. (Alhendín, Granada, Spain).

Ultrapure water (18.2 MΩ cm^{-1}, Milli–Q Plus system, Millipore Bedford, MA, USA) was used throughout all the trials.

Clarinert™ 13mm syringe filters with 0.22 µm nylon membrane (Agela Technologies Inc., Wilmington, DE, USA) were used for sample filtration prior to injection into the chromatographic system.

Kits SampliQ QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) consisting of buffered QuEChERS extraction packet buffered (4 g $MgSO_4$, 1g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate) and extraction packet non-buffered QuEChERS (4 g $MgSO_4$, 1g NaCl) were supplied by Agilent Tecnologies Inc. Materials for dispersive solid phase extraction (dSPE) in the clean-up step, such as endcapped C18 and primary secondary amine (PSA) were both of them from Agilent Tecnologies Inc. (Wilmington, DE, USA) and Supelclean LC Alumina-N and SupelTM QuE Z-Sep (Z-Sep) from Supelco (Bellefonte, PA, USA).

2.1.2. Preparation of standard solutions

Individual stock standard solution containing 2000 mg L^{-1} was prepared by addition of PTSO in MeOH and stored in a dark bottle at -20ºC. It was stable for at least 3 months. Working solutions were prepared diluting the stock solution in MeOH to the desired concentration prior to use.

Individual stock solutions of CYS, L-cystine, L-GSH oxidized, MET, SER, LYS and GLY at 10 mM were prepared by dissolving the appropriate amount of each compounds in water and stored refrigerated. They were stable for 7 days.

2.1.3. Instrumentation and equipment

All experiments were carried out using an Agilent 1290 Infinity LC (Agilent Technologies, Waldbron, Germany) equipped with a binary pump, on-line degasser, autosampler (5 µL loop), and a column thermostat. Mass-spectrometer measurements were performed on a triple quadrupole mass spectrometer API3200 (AB Sciex, Toronto, ON, Canada) with electrospray ionization (ESI). A Zorbax Eclipse Plus RRHD (50 x 2.1 mm, 1.8 µm) chromatographic column was used for the separation. The

instrumental data were collected using the Analyst Software version 1.5 with Schedule MRM™ Algorithm (AB Sciex).

A Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), an oven with natural air convection (Raypa, Barcelona, Spain) and an evaporator system (System EVA-EC, from VLM GmbH, Bielefeld, Germany) were also used in the sample treatment procedure.

2.1.4. Sample treatment

Animal feed samples (for pigs, piglets, hens, broilers, rabbits and partridges), kindly provided by Piensos Pisur (Albolote, Granada, Spain) were stored at room temperature. A portion of 1 g of was placed into a 50 mL screw cap test tube with conical bottom. Subsequently, 10 mL of MeOH were added to the tube and it was shaken by vortex for 3 min and centrifuged at 5000 rpm for 5 min. Then, 2 mL of the upper MeOH layer was transferred to a vial, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 500 µL of MeOH:H_2O (50:50, v/v). The extracts were filtered with a 0.22 µm filter before injection.

2.1.5. UHPLC-MS/MS analysis

UHPLC separations were performed in a C18 column (Zorbax Eclipse Plus RRHD 50 x 2.1 mm, 1.8 µm) using a mobile phase consisting of 0.05 % aqueous FA solution (solvent A), and MeOH (solvent B) at a flow rate of 0.4 mL min^{-1}. The eluent gradient profile was as follows: 0 min - 10 % B; 0.5 min - 10% B; 2.5 min - 100% B; 3 min - 100 % B. Finally, it was back to 10% B in 0.5 min and kept for 2 min for column equilibration. The temperature of the column was set at 25 °C and the injection volume

was 5 µL (full loop). The MS was working with ESI^+ under MRM conditions obtaining the values indicated in Table 2.1. The target scan time established for each transition was 0.1 s. The ionization source parameters were: source temperature 500 ºC; curtain gas (nitrogen) 30 psi; ion spray voltage 5500V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi. Under these conditions CSSP, GSSP and PTSO were determined in just 4.0 min, all of them being characterized by two precursor-product ion transitions.

Table 2.1. Monitored ions of the target analytes and MS/MS parameters

	Retention time (min)	Precursor ion (m/z)	Molecular ion	DP[a]	EP[a]	CEP[a]	Product ions[b]	CE	CXP[a]
CSSP	1.7	196.0	$[M+H]^+$	36.0	6.0	12.0	107.0 (Q) 179.0 (I)	11.0 17.0	4.0
GSSP	1.9	382.0	$[M+H]^+$	31.0	9.5	38.0	130.0 (Q) 150.0 (I)	24.0 27.0	4.0
PTSO	4.0	183.0	$[M+H]^+$	21.0	10.5	10.0	141.1 (Q) 99.0 (I)	13.0 19.0	4.0

[a] Declustering potential (DP), Entrance potential (EP), Collision cell entrance potential (CEP), Collision cell exit potential (CXP) and Collision energy (CE). All expressed in voltage.

[b] Product ions: (Q) Transition used for quantification, (I) Transition employed to confirm the identification.

2.2. Results and discussion

2.2.1. PTSO behavior in presence of CYS and GSH

It is known that during garlic and onion storage, intensely coloured pigments are often formed.[33-35] This phenomenon is a consequence of the reaction between garlic and onion thiosulfinates and the amino acids present in their cloves. CYS and GSH, a non-protein tripeptide consisted by three amino acids including CYS, do not form pigments with thiosulfinates in the same way as other pigment-formed amino compounds because CYS and GSH have the sulfhydryl (SH) group within their structure.[36,37] Reactions between thiosulfinates and CYS/GSH are spontaneous and more faster than the reactions with other amino acids and happen when the molar concentration ratio of CYS/GSH to total thiosulfinates is 2:1.[38] For that reason, coloured reactions do not appear when thiosulfinates are added to feed at low concentrations. This phenomenon could also appear in cell membranes and in extra cellular fluids where GSH and CYS are the dominant thiols suggesting that the aqueous medium enhance the reaction[39].

33. Kubec, R.; Hrbáčova, M.; Musah, R.A.; Velíšek. J. J. Agr. Food Chem., 52 (2004) 5089-5094.

34. Kubec, R.; Veliäsÿek. J.J. Agr. Food Chem., 55 (2007) 3491-3497.

35. Kučerová, P.; Kubec, R.; Šimek, P.; Václavík, L.; Schraml. L.J. Agr. Food Chem., 59 (2011) 1821-1828.

36. Zhang, G.; Parkin. K.L. J. Agr. Food Chem., 61 (2013) 3030-3038.

37. Rabinov, A.; Miron, T.; Mirelman, D.; Wilchek, M.; Glozman, S.; Yavin, E.; Weiner. L. Biochim. Biophys. Acta., 2000, (2000) 144-153.

38. Yoo, S.K.; Lee, K.S.; Patil. B.S. J. Food Sci., 74 (2009) C11-C16.

39. Miron, T.; Rabinkov, A.; Mirelman, D.; Wilchek, M.; Weiner, L. Bioch. Biophys. Acta., 1463 (2000) 20-30.

Both of them react quickly in presence of thiosulfinates to form mixed-disulfide conjugates.[40]

In the case of allium thiosulfonates, there is no information about their reaction in the presence of amino acids. Consequently, this paper tries to demonstrate that allium thiosulfonates in general and PTSO in particular have a similar behavior to other allium thiosulfinates in the presence of CYS and GSH when used as feed additives.[36,37,38] For that purpose, 1 mL of PTSO standard solution (5 mM) was mixed with 1 mL of the standard solution 10 mM of CYS or GSH, to satisfy the molar concentration ratio (1:2). The presence of PTSO was immediately monitored after mixing by MS, demonstrating that PTSO disappears completely in the presence of CYS and GSH.

Product reactions were also identified by MS using direct infusions of each mix solution. Precursor ion $[M+H]^+$ of each compound was observed in full scan and MS^2 spectrum was studied in order to confirm the identification of CSSP and GSSP. MS^2 of CSSP was characterized by abundant ions at *m/z* 179, corresponding to the loss of NH_3 (-17 Da), *m/z* 150, corresponding to loss of COOH (-46 Da) and *m/z* 107 assigned as two sulfurs bonded to propyl group. On the other hand, MS^2 of GSSP was characterized by the abundant ion at *m/z* 236, corresponding to the loss of chain COOH-CH(NH_2)-CH_2-CH_2-CO. Ion with *m/z* 236 underwent further loss of two sulfurs bonded to propyl group yielding *m/z* 130. In addition, ion with *m/z* 150 was also found in GSSP spectrum, suggesting that both CSSP and GSSP have a part of their structure in common. As expected, loss of NH_3 (-17 Da) yielding *m/z* 365 ion was also observed in GSSP MS^2 spectrum. Finally, MS^2 of PTSO was characterized by the abundant ions *m/z* 149,

40. Zhang, G.; Li, B.; Lee, C.H.; Parkin., K. J. Agr. Food Chem., 58 (2010) 1564-1571.

corresponding to the loss of a propyl group and m/z 99, matched with the loss of two propyl groups. MS^2 spectra of CSSP and GSSP are shown in figure 2.1.

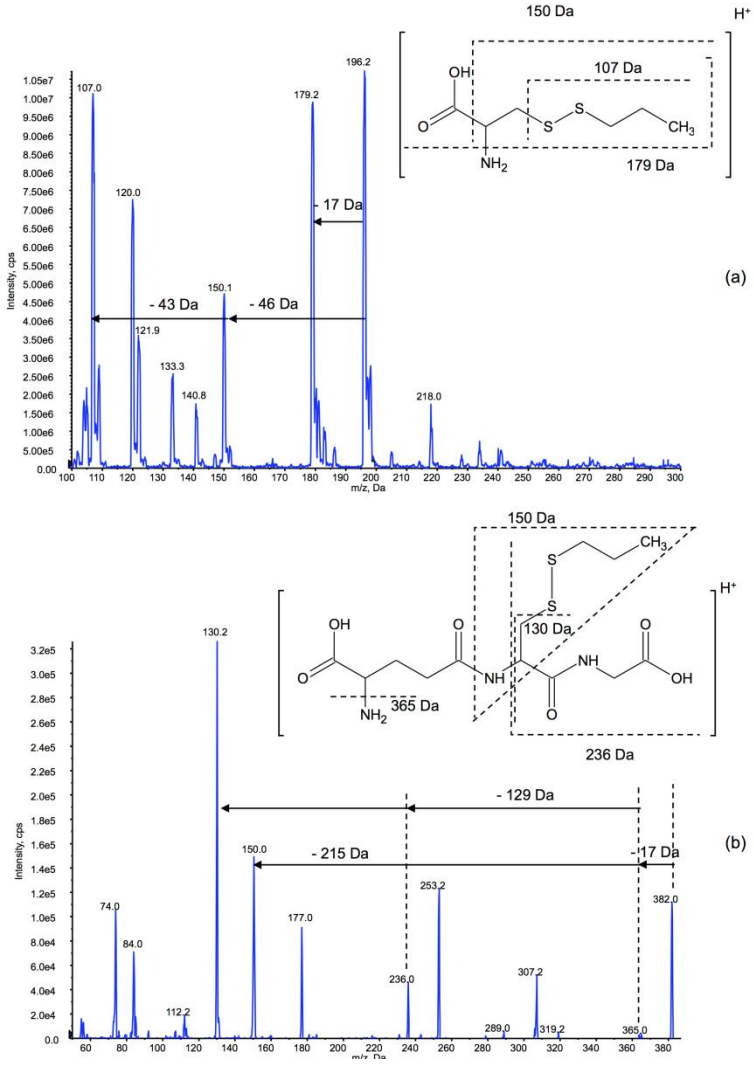

Figure 2.1. MS^2 spectra acquired for (a) CSSP and (b) GSSP

Once the products of reaction were identified, the mechanism of reaction based on typical reactions between thiosulfonates and functional thiols[41] and reaction mechanisms previously described for thiosulfinates and CYS,[42] was proposed (figure 2.2).

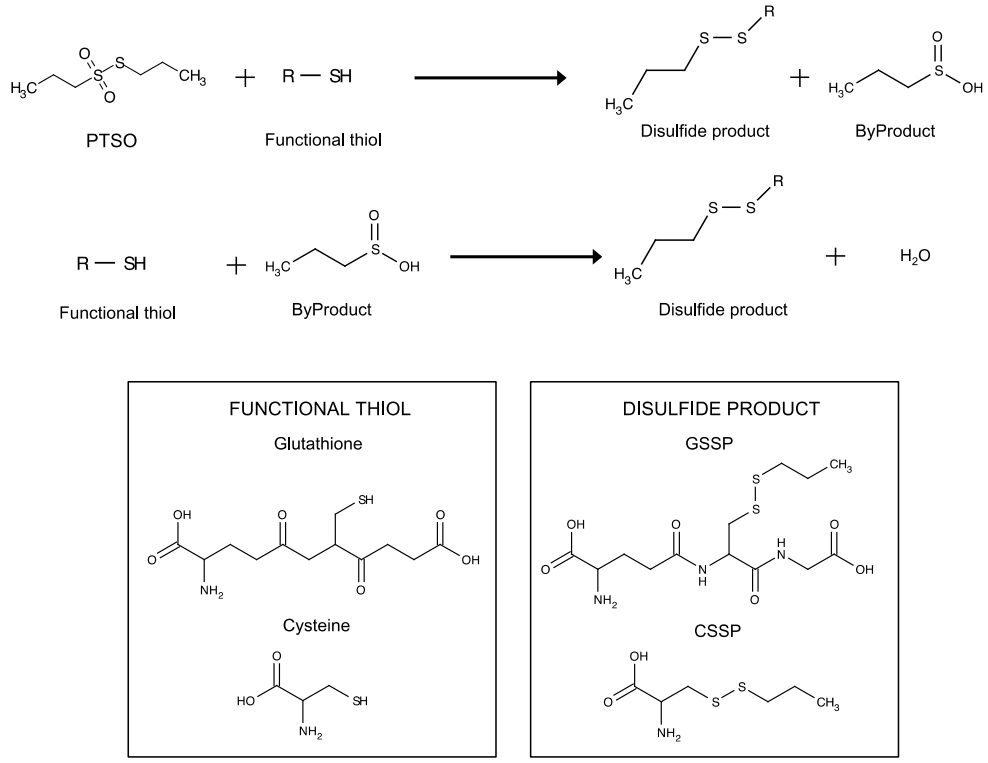

Figure 2.2. Mechanism proposed for the reactions between PTSO and the thiol group of CYS and GSH giving disulfide products CSSP and GSSP

41. Andrew, B. Thiol-X Chemistries in Polymer and Materials Science. Royal Society of Chemistry, Abingdon (UK), 2013, chapter 4, 76-94.

42. Shin, Y.K., Kyung, K.H. Food Chem., 142 (2014) 217-219.

Lastly, in order to confirm the proposed reaction, 1 mL of PTSO standard solution (5 mM) was mixed with 1 mL of 10 mM standard solution of cystine or GSH oxidized (CYS and GSH with –SH groups blocked) and the presence of PTSO was also monitoring by MS. In this case, PTSO remained unchanged, confirming that the reaction occurs by the presence of the -SH group. In addition, the same procedure was performed with MET, SER, LYS and GLY, in order to demonstrate the behavior of PTSO in presence of other amino acids without –SH group. As expected, PTSO does not react with these amino acids. Table 2.2 summarizes the studied amino acid including recovery values of PTSO (% Rec PTSO) calculated as (signal of standard solution of PTSO/ signal of mixture of PTSO with each amino acid) × 100.

Table 2.2. Behaviour of PTSO in presence of CYS, cystine, GSH, GSH oxidized, and some amino acids without –SH group (MET, SER, LYS and GLY)

PTSO (mM)	Compound	Molecular formula	Molecular weight	Concentration (mM)	Molar ratio	-SH group	% Rec PTSO
5	CYS	$C_3H_7NO_2S$	121.16	10	1:2	√	0
5	Cystine	$C_6H_{12}N_2O_4S_2$	240.3	10	1:2	X	99.9
5	GSH	$C_{10}H_{17}N_3O_6S$	307.32	10	1:2	√	0
5	GSH oxidized	$C_{20}H_{32}N_6O_{12}S_2$	612.63	10	1:2	X	99.9
5	MET	$C_5H_{11}NO_2S$	149.21	10	1:2	X	100.0
5	SER	$C_3H_7NO_3$	105.09	10	1:2	X	99.9
5	LYS	$C_6H_{14}N_2O_2$	146.19	10	1:2	X	100.0
5	GLY	$C_2H_5NO_2$	75.04	10	1:2	X	99.9

2.2.2. Optimization of MS detection and chromatographic separation

In order to get the highest sensitivity, MS/MS detection was optimized for CSSP, GSSP and PTSO. For this purpose, standard solutions of 20 mg L^{-1} (GSSP and CSSP standards were obtained from the reaction indicated above) in 0.1% aqueous FA/MeCN (50/50; v/v) of each analyte were individually infused into the mass spectrometer. All the compounds were tested using ESI positive/negative mode. ESI+ showed the best results in terms of sensitivity for the compounds, so it was selected for the rest of the work. Precursor ions were protonated molecules.

During the infusion, the parameters declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision cell exit potential (CXP) and collision energy (CE), were optimized for the three compounds in order to obtain the maximum sensitivity (see results in table 2.1). Each compound was characterized by its retention time and by two precursor-product ion transitions. The dwell-time established for each transition was 0.1 s. The most intense product ion was used for quantification, while the second one was used to complete the identification.

In order to optimize the chromatographic parameters, mobile phases consisted of water with 0.1% FA (solvent A) and MeCN or MeOH (solvent B) were tested. MeOH without acid as solvent B provided better results in terms of analytical signal, so it was selected for subsequent studies. Results are shown in figure 2.3.

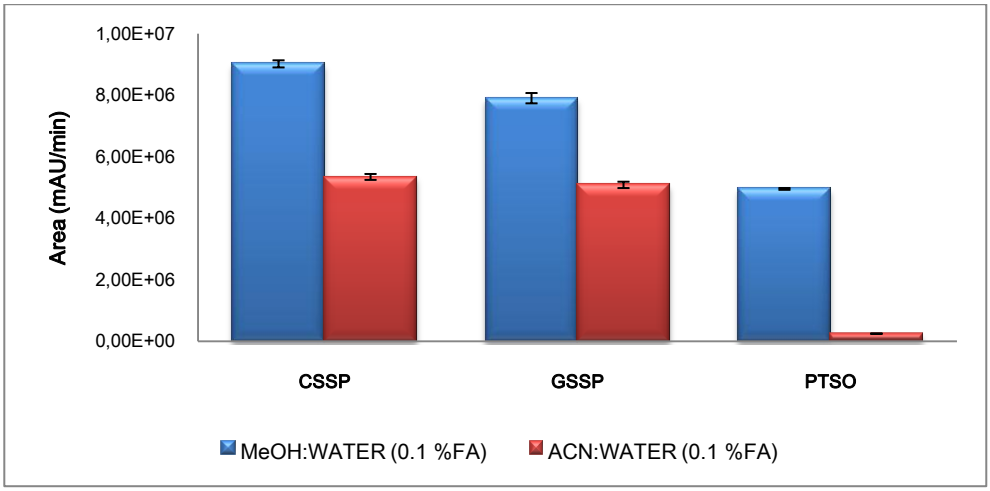

Figure 2.3. Influence of mobile phase B composition on peak area of the studied compounds (n=6)

The gradient was studied in order to get the best separation, peak shape and sensitivity in the shortest time and the following gradient was selected: 0 min - 10 % B; 0.5 min - 10% B; 2.5 min - 100% B; 3 min - 100 % B. Finally, it was back to 10% B in 0.5 min and maintained for 2 min for column equilibration. Although the run time for each injection was 5.5 min (including the cleaning and conditioning of the column), under optimum conditions all the analytes were eluted in about 2 min.

The use of acid in the solvent A is required to improve the ionization step in ESI+ so different percentages of FA were tested (0 to 0.2%) (figure 2.4). A concentration of 0.05% FA gave the highest signals and best peak shape. Also, the addition of ammonium formate in the mobile phase was studied (0 to 5 mM); however, its presence produces lower peak intensity, probably due to the formation of ammonium adducts.

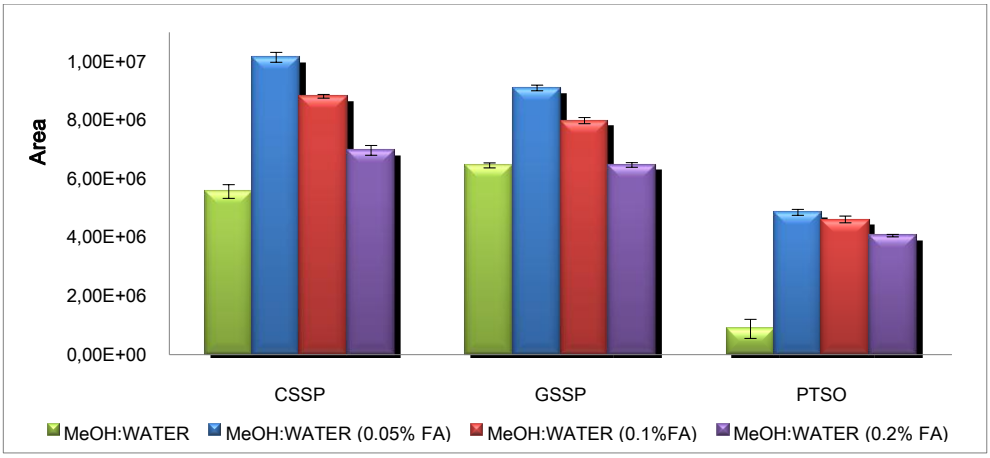

Figure 2.4. Influence of the FA concentration in the mobile phase A on peak area of the studied compounds (n=6)

The use of acid in the solvent B was also studied. MeOH without acid as solvent B provided the best results in terms of analytical signal, so it was selected as optimum. Results are shown in figure 2.5.

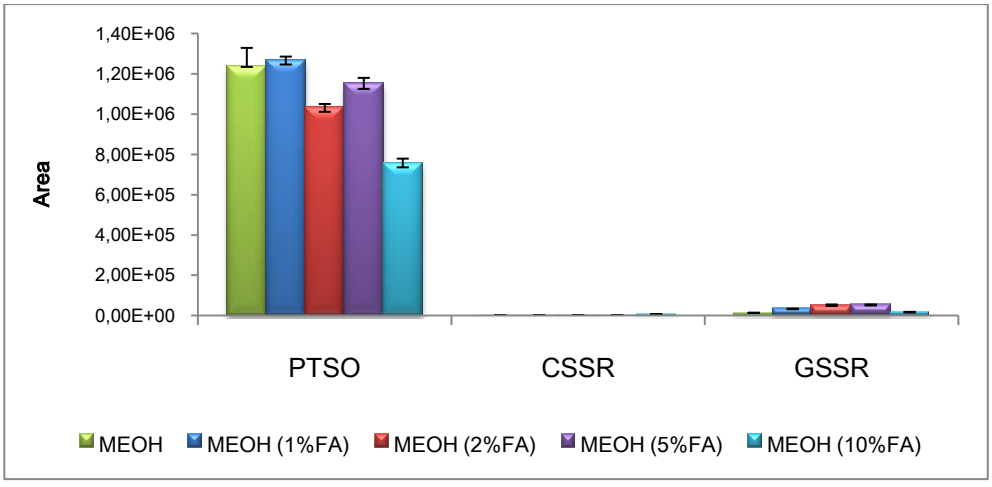

Figure 2.5. Influence of the FA concentration in the mobile phase B on peak area of the studied compounds (n=6)

The flow rate was studied from 0.3 mL min^{-1} to 0.5 mL min^{-1} and 0.4 mL min^{-1} was selected as a compromise between signal intensity, peak shape and run time. The temperature of the column was studied between 25°C and 45°C and 25°C was selected (figure 2.6). The injection volume was 5 µL (full loop) for all the experiments.

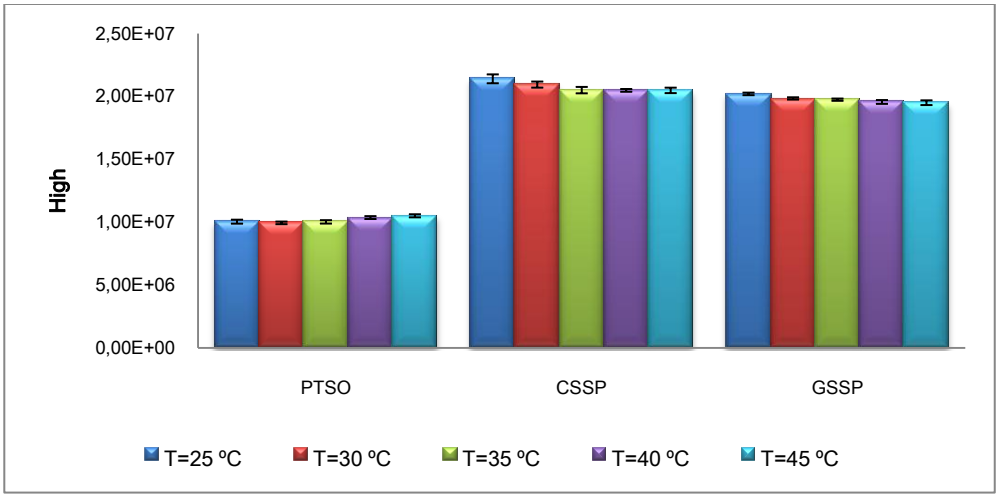

Figure 2.6. Influence of the ionization source on peak intensity of the studied compounds (n=6)

The ionization source parameters were optimized following the recommendations of the manufacturer: source temperature was tested between 300 and 600ºC and 500°C was selected as optimum (figure 2.7).

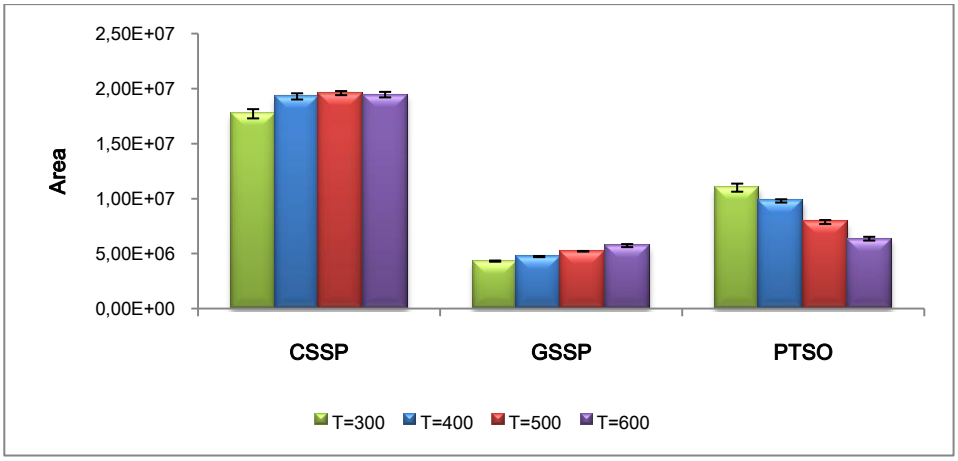

Figure 2.7. Influence of the ionization source on peak area of the studied compounds (n=6)

The curtain gas (nitrogen) was studied between 25 and 45 psi. No significant differences were found, so 30 psi was selected.

Ion spray voltage was evaluated from 4500 to 5500 V and finally 5500 V was selected as optimum (figure 2.8) and GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi.

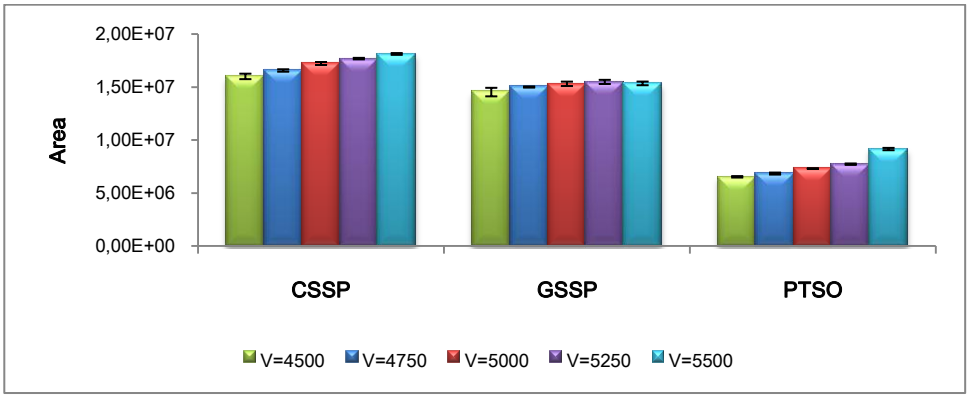

Figure 2.8. Influence of the ion spray voltage on peak area of the studied compounds (n=6)

2.2.3. Optimization of sample preparation

Optimization was carried out using 1 g of hens feed samples as representative matrix spiked with 100 µL (200 mg kg^{-1}) of PTSO stock solution at 2000 mg L^{-1} in MeOH. In order to achieve full homogenization, the tube was shaken using a vortex for 1 min after PTSO addition. Preliminary analyses of samples were performed in order to check that blank feed samples were free of analytes (PTSO, CSSP and GSSP).

The first step of the QuEChERS procedure based on an extraction/partitioning process was initially checked due to the good results that this methodology had reported in different fields related with the monitoring of residues and organic compounds.[43] Thus, different solvents and different Agilent SampliQ QuEChERS extraction kits were tested: (a) 8 mL of H_2O + 10 mL of MeOH + Extraction packed non-buffered (4 g $MgSO_4$, 1 g NaCl), (b) 8 mL H_2O + 10 mL MeOH + Extraction packed buffered (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate), (c) 8 mL 30 mM NaH_2PO_4 buffer pH 7.1 + 10 mL MeOH + Extraction packed buffered (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate) and (d) 10 mL of MeOH.

Results showed that the presence of water promoted the reaction between PTSO and CYS/GSH, so the previous options were discarded and a simple solid–liquid extraction was tested, using four different extraction solvents (figure 2.9): 10 mL of MeCN, 10 mL of MeOH, 10 mL ACO and 10 mL of EtAc.

43. Bruzzoniti, M.C.; Checchini, L.; De Carlo, R.M.; Orlandini, S.; Rivoira L.; Del Bubba., M. Anal. Bioan. Chem., 406 (2014) 4089-4116.

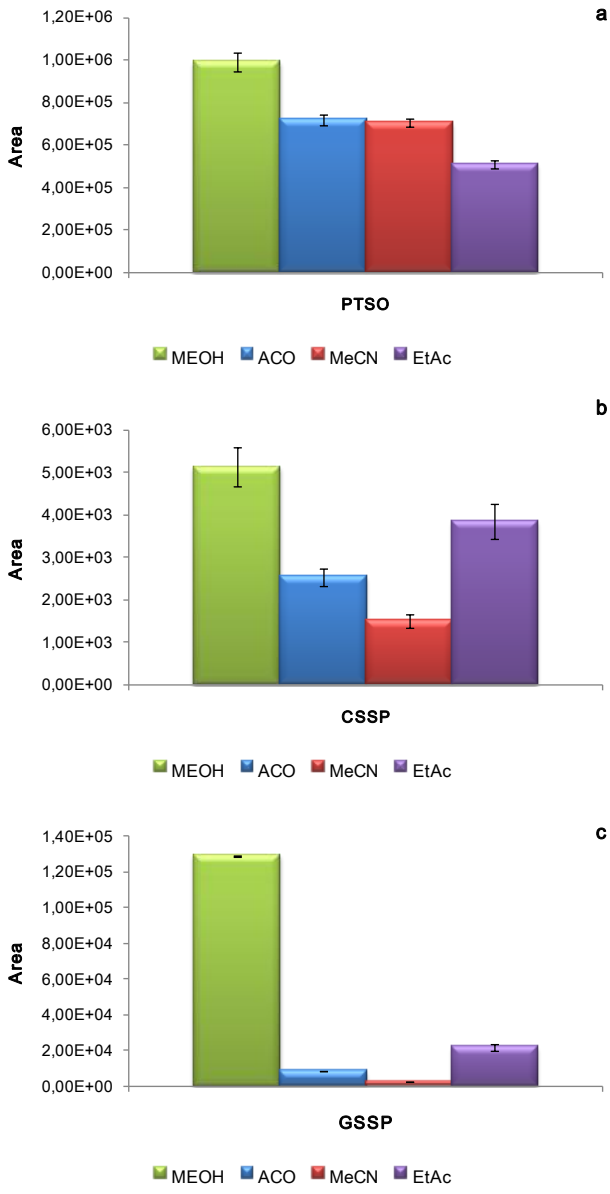

Figure 2.9. Influence of the extraction solvent on peak area of the studied compounds: (a) PTSO, (b) CSSP and (c) GSSP (n=6)

The best results in terms of peak area were obtained with MeOH. Also, efficiency of the extraction was evaluated in terms of recovery percentages for PTSO (figure 2.10), achieving satisfactory values with MeOH. Therefore, those conditions were selected for further experiments.

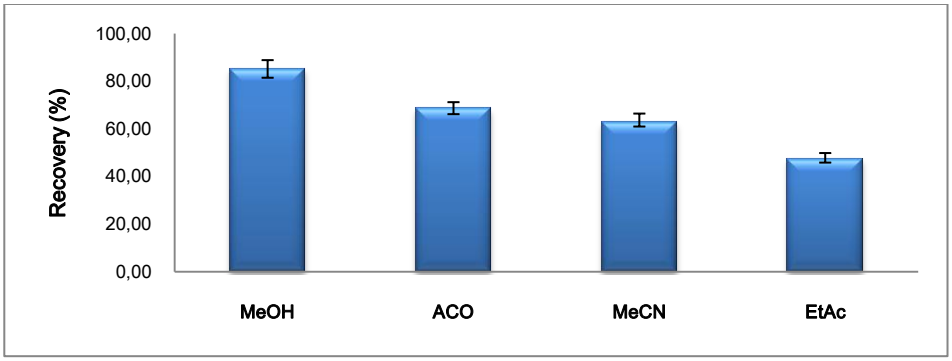

Figure 2.10. Influence on the extraction efficiency for PTSO of the extraction solvent (n=6)

Then, the percentage of FA in the extraction solvent was studied between 0 and 5%. The best results were obtained when FA was not used as additive in the extraction solvent (figure 2.11).

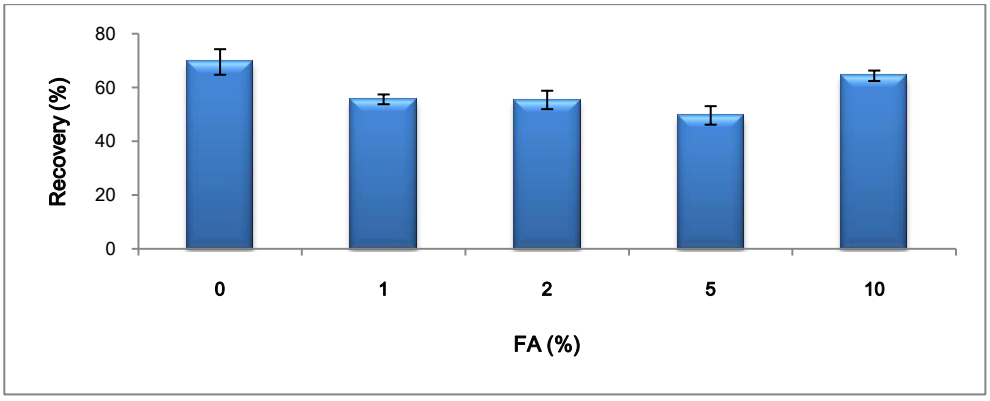

Figure 2.11. Influence on the extraction efficiency of PTSO of the addition of FA on the extraction solvent (MeOH) (n=6)

Also, the amount of sample was studied between 1 and 5 g, obtaining 1 g as optimum because as sample size increases, the content of CYS and GSH is increasing for the same amount of PTSO, so the efficiency in the extraction of PTSO is lower than that expected in the optimum conditions (figure 2.12).

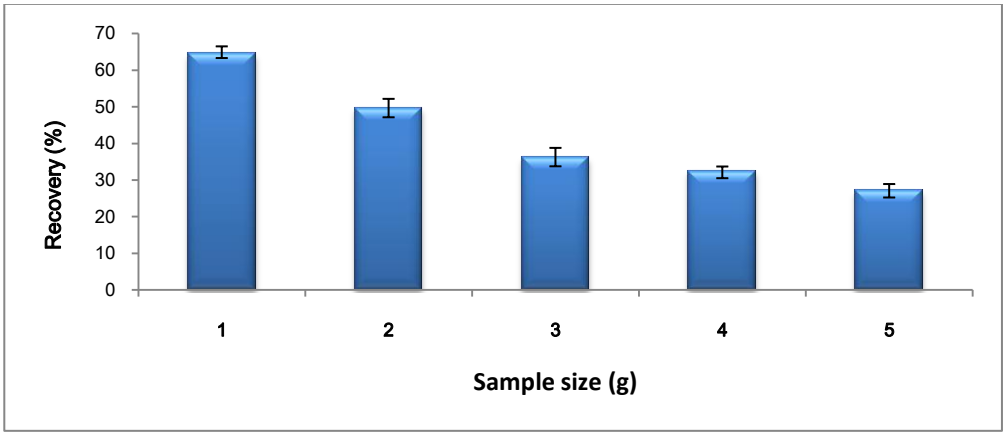

Figure 2.12. Influence on the extraction efficiency of PTSO of the sample size (n=6)

The extraction volume was studied between 2.5 and 20 mL obtaining 10 mL as optimum (figure 2.13).

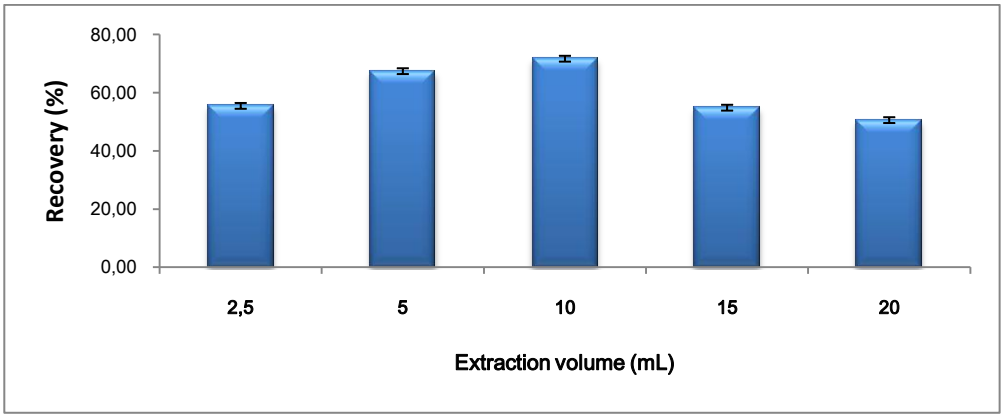

Figure 2.13. Influence on the extraction efficiency of PTSO of the extraction volume

Finally, a second step, involving sample clean-up by dSPE was evaluated using 150 mg of different sorbents, such as alumina-N, C18, PSA and Z-Sep on 2 mL of solvent extract (figure 2.14).

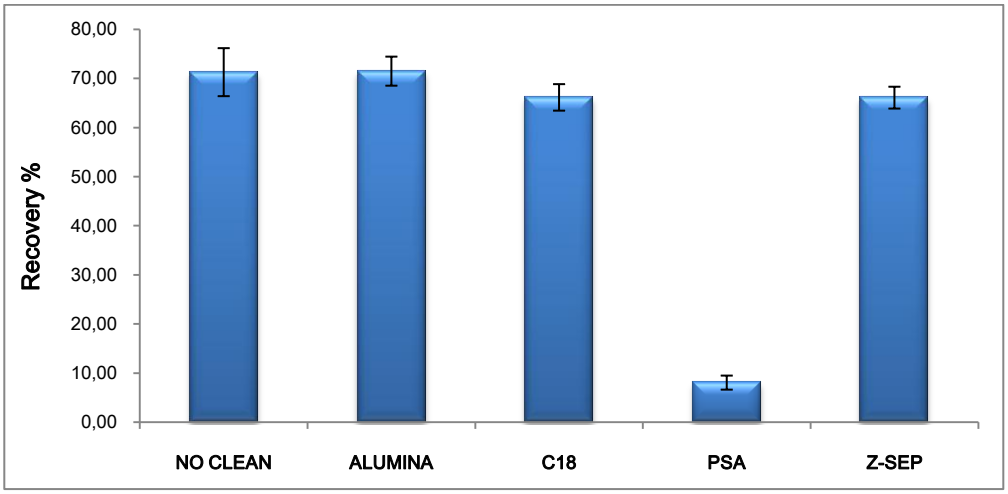

Figure 2.15. Influence on the extraction efficiency of PTSO of the cleaning step using different sorbents (n=6)

As it possible to see in figure 2.15, no cleaner extracts were obtained and no improvements on recovery values were observed, thereby the introduction of a clean-up step was discarded. Finally, sample procedure is summarized in Section 2.4 and a typical chromatogram corresponding to a spiked pig's feed sample submitted to the proposed method is shown in figure 2.16.

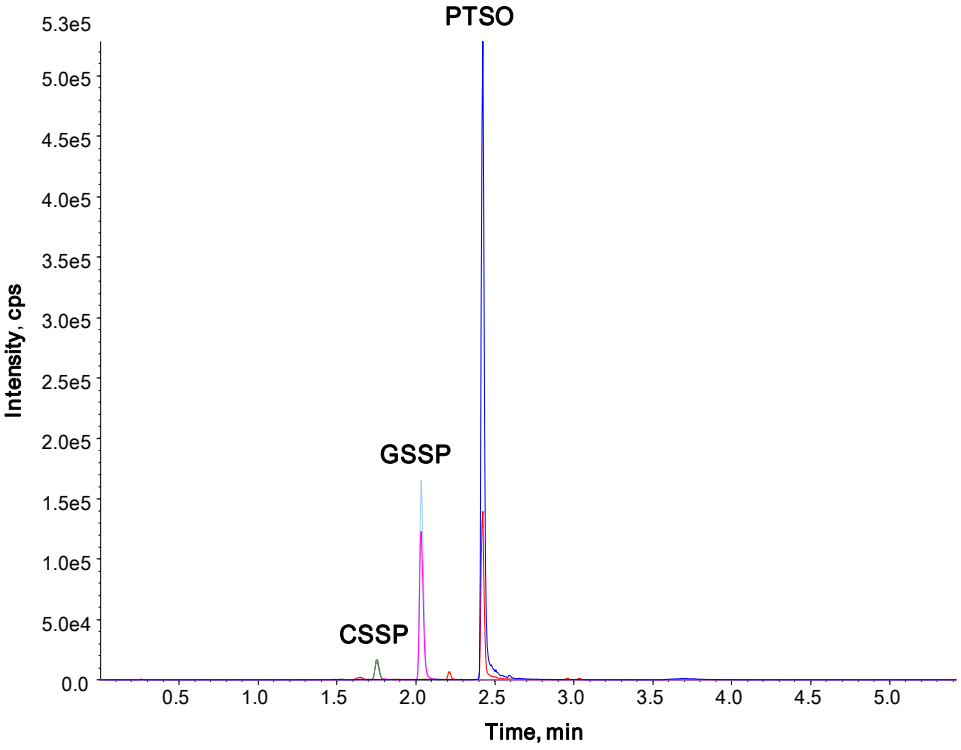

Figure 2.16. UHPLC-ESI-MS/MS total ion chromatogram of a blank pig feed sample spiked with 75 mg kg $^{-1}$ of PTSO

2.2.4. Characterization of the method

To check the suitability of the proposed method for the determination of PTSO, CSSP and GSSP in animal feed, the linear dynamic range and LODs and LOQs were evaluated for six different feedstuffs (for pigs, piglets, hens, broilers, rabbits and partridges). In addition, studies of matrix effect (ME), precision and recovery for PTSO in each matrix were carried out.

2.2.4.1. Calibration curves and performance characteristics

Matrix-matched calibration curves were established by spiking feed blank samples at six different concentration levels of PTSO (ranging from 0.75 to 75 mg kg^{-1} for pigs, hens and rabbits, from 1 to 100 mg kg^{-1} for piglets and broilers and from 1.5 to 150 mg kg^{-1} for partridges). Each concentration level was prepared in duplicate and injected in triplicate. Statistical parameters were calculated by least-squares regression. LODs were considered as (3xS/N ratio) and LOQ (10xS/N ratio).

PTSO was determined as sum of CSSP, GSSP and remaining PTSO, using peak areas as analytical signals in order to determine the initially amount of PTSO added to the feed. In the same way, matrix-matched calibration curves were established for CSSP and GSSP by spiking feed blank samples at six different concentration levels of each compound (ranging from 1 to 100 mg kg^{-1}). Table 2.3 summarizes the results. The satisfactory determination coefficients confirm that PTSO, CSSP and GSSP analytical responses were linear over the range studies.

Table 2.3. Statistical and performance characteristics of the proposed method

Matrix	Analyte	Linear dynamic range (mg kg^{-1})	R^2	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Piglets	PTSO	0.76–100	0.993	0.23	0.76
	CSSP	0.11–100	0.994	0.03	0.11
	GSSP	0.14–100	0.994	0.04	0.14
Pigs	PTSO	0.39-100	0.997	0.12	0.39
	CSSP	0.04–100	0.999	0.01	0.04
	GSSP	0.44–100	0.992	0.13	0.44
Broilers	PTSO	0.44-100	0.993	0.13	0.44
	CSSP	0.20–100	0.994	0.06	0.20
	GSSP	0.32–100	0.991	0.10	0.32
Hens	PTSO	0.85–100	0.992	0.25	0.85
	CSSP	0.20–100	0.993	0.06	0.20
	GSSP	0.03–100	0.999	0.01	0.03
Rabbits	PTSO	0.57-100	0.994	0.17	0.57
	CSSP	0.10–100	0.999	0.03	0.10
	GSSP	0.51–100	0.992	0.15	0.51
Partridges	PTSO	1.45-100	0.991	0.44	1.45
	CSSP	0.09–100	0.995	0.03	0.09
	GSSP	0.34–100	0.994	0.10	0.34

In addition, table 2.4 shows the values of the ME at three PTSO concentration levels for each type of animal feed, calculated as 100 x [(PTSO signal of spiked extract/ PTSO signal of standard solution)], considering as analytical signal the peak area. A value close to 100% indicates that there is no significant ME, while values >100% and <100% indicate signal enhancement and signal suppression due to the presence of the matrix, respectively. As can be seen, a significant ME was found (lower than 80%) for most of matrices and concentration levels.

Table 2.4. ME (%) from different types of animal feed samples, calculated as 100 x [(PTSO signal of spiked extract/PTSO signal of standard solution)]

Matrix	Level 1	Level 2	Level 3
Piglets	82.0	74.1	70.3
Pigs	78.1	70.9	69.2
Broilers	73.8	71.7	66.8
Hens	73.9	84.4	62.8
Rabbits	77.0	77.3	76.3
Partridges	66.8	65.7	74.2

Level 1: Pigs, hens and rabbits: 0.750 mg kg^{-1}; piglets and broilers: 1 mg kg^{-1}; partridges: 1.5 mg kg^{-1}
Level 2: 25 mg kg^{-1}
Level 3: 100 mg kg^{-1}

2.2.4.2. Precision study

The precision of the whole method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was assessed by applying the whole procedure to six feed samples on the same day (experimental replicates) spiked at three different concentration levels of PTSO. Each sample was injected in triplicate (instrumental replicates). Intermediate precision was evaluated with a similar procedure, with five samples per concentration level analyzed in different days. The results, expressed as RSD of the sum of the peak areas of PTSO, CSSP and GSSP are shown in table 2.5. Satisfactory precision was obtained, with RSD lower than 10% in all the cases.

Table 2.5. Precision study (% RSD of peak areas) of the proposed method for the determination of PTSO in feed samples

	Repeatability (*n* = 9)			**Intermediate precision** (*n* = 15)		
Matrix	**Level 1**	**Level 2**	**Level 3**	**Level 1**	**Level 2**	**Level 3**
Piglets	5.1	4.9	7.7	9.9	10.0	9.4
Pigs	8.8	7.2	2.9	9.4	7.4	8.3
Broilers	9.9	8.1	7.8	9.6	9.5	9.2
Hens	6.9	8.3	3.5	7.3	10.0	8.7
Rabbits	7.2	7.2	3.1	9.0	8.7	9.9
Partridges	9.3	4.3	1.7	9.3	8.5	9.3

Level 1: Pigs, hens and rabbits: 0.75 mg kg^{-1}; piglets and broilers: 1 mg kg^{-1}; partridges: 1.5 mg kg^{-1}

Level 2: 25 mg kg^{-1}

Level 3: 100 mg kg^{-1}

2.2.4.3. Recovery studies for PTSO in feed samples

The trueness was evaluated through recovery experiments as apparent recovery. [44] Since a suitable sample with certified concentrations of PTSO was not available, artificially fortified PTSO-free samples were used. Recovery studies have been assessed under repeatability conditions and samples from the six studied matrices were spiked at three different concentration levels, processed as described previously and injected in triplicate into the UHPLC-MS/MS system. The measured concentration was determined using the obtained matrix-matched calibration curves, and the recovery was calculated as [measured concentration / actual (added) concentration] x 100. The results are shown in Table 2.6 and as can be seen, very good recoveries were obtained (between 93% and 104%).

Table 2.6. Recovery study (% RDS in parenthesis) of the proposed method for the determination of PTSO in feed samples

	Recoveries (*n=9*)		
Matrix	**Level 1**	**Level 2**	**Level 3**
Piglets	93.5 (8.5)	97.5 (4.9)	101.3 (4.9)
Pigs	94.4 (8.7)	99.5 (7.2)	97.8 (2.9)
Broilers	94.6 (9.1)	95.5 (8.4)	94.0 (7.9)
Hens	96.5 (9.5)	103.9 (8.1)	105.3 (6.5)
Rabbits	95.6 (7.6)	97.5 (7.3)	99.6 (3.1)
Partridges	97.4 (4.3)	101.6 (1.7)	103.9 (3.3)

Level 1: Pigs, hens and rabbits: 0.75 mg kg^{-1}; piglets and broilers: 1 mg kg^{-1}; partridges: 1.5 mg kg^{-1}

Level 2: 25 mg kg^{-1}

Level 3: 100 mg kg^{-1}

44 D. T. Burns, K. Danzer, A. Townshend, A. *Pure Appl. Chem.,* 2002, **74**, 2201-2205.

2.2.5. Influence of the moisture on PTSO reaction

During sample treatment optimization, it was observed that the presence of water might influence the reaction; so this phenomenon was evaluated in feed samples. For this purpose, feed samples (for pigs, piglets, hens, broilers, rabbits and partridges) were dried in oven at 120 ºC for 2 h. Wet and dried samples of each feedstuff were spiked with PTSO at two different concentration levels (25 mg kg^{-1} and 100 mg kg^{-1}) and PTSO, CSSP and GSSP were quantified using the obtained calibration curves.

Table 2.7 summarizes the results, showing that moisture affects the PTSO reaction. Dried samples show higher concentrations of PTSO and therefore lower concentrations of GSSP and CSSP than wet samples. Moreover, samples with higher humidity percentage show lower concentration of PTSO, confirming that the proposed reaction is favoured by the presence of water in the sample.

Table 2.7. Effect of the moisture in the PTSO reaction among CYS and GSH

Matrix (Moisture)*	Added PTSO concentration (mg kg^{-1})	Sample type	Measured concentration (mg kg^{-1})		
			PTSO	CSSP	GSSP
Piglets (7.4%)	25	Wet	18.3	4.5	19.0
		Dry	21.8	4.3	5.1
	100	Wet	77.7	5.1	83.7
		Dry	96.6	4.3	5.6
Pigs (7.5%)	25	Wet	22.0	0.2	12.4
		Dry	22.1	0.1	11.9
	100	Wet	94.2	0.2	23.6
		Dry	95.9	0.2	16.8
Broilers (9.6%)	25	Wet	8.0	6.5	58.3
		Dry	19.3	4.5	15.0
	100	Wet	75.0	8.0	88.7
		Dry	92.9	4.9	20.2
Hens (6.5%)	25	Wet	18.9	5.2	15.2
		Dry	22.5	4.56	1.4
	100	Wet	93.5	5.5	16.4
		Dry	98.1	4.5	1.5
Rabbits (5.2%)	25	Wet	16.5	5.3	25.1
		Dry	21.0	4.9	7.0
	100	Wet	88.4	7.1	34.6
		Dry	95.4	5.0	9.5
Partridges (7.4%)	25	Wet	23.7	0.5	4.4
		Dry	24.7	0.3	0.9
	100	Wet	95.0	0.9	19.0
		Dry	99.2	0.3	2.7

*Moisture content was determined by oven drying at 120 ºC for 2 h

2.3. Conclusions

This chapter describes for the first time the reaction of PTSO with GSH and CYS (both of them present in feed samples) and the impact of this reaction on analysis/analytical methods. In chapter 1, HPLC-UV methodology allowed PTSO determination and quantification in feed samples with low content in CYS and GSH but this methodology is not valid when these compounds are present in high amounts or when lower concentration levels of PTSO must be measured.

The rapid and spontaneous reaction among PTSO and CYS and GSH has been demonstrated and the products of this reaction have been characterized and identified using MS. In addition, it has been demonstrated that this reaction is favored in presence of water, so its use should be avoided during extraction. Thus, a UHPLC–MS/MS method combined with a sample treatment based on a simple methanolic extraction has been proposed for the screening of PTSO, CSSP and GSSP in animal feed samples (for pig, piglet, broiler, hen, rabbit and partridge). This method allows the quantification of PTSO as a sum of CSSP, GSSP and the unreacted PTSO with LOQs much lower (0.5-1.5 mg kg^{-1}) than those obtained by HPLC-UV methodology (30 mg kg^{-1}), and achieving an 80% reduction in analysis time. It provides a simple, robust and efficient alternative for monitoring this additive when used as natural growth promoter in different animal feeds and could be easily implemented in feed quality control.

This work was published as: A rapid and simple UHPLC-ESI-MS/MS method for the screening of propyl propane thiosulfonate, a new additive for animal feed. Abad, P., Arroyo-Manzanares, N., García-Campaña, A.M. Anal. Meth., 8 (2016) 3730-3739.

CHAPTER 3

Use of onion extract as dairy cows feed supplement: monitoring of propyl propane thiosulfonate as marker of its effect on milk attributes

Extracto de cebolla como aditivo en alimentos para rumiantes: determinación de propil propano tiosulfonato como marcador de sus efectos sobre las propiedades sensoriales de la leche

Resumen

El extracto de cebolla se utiliza como complemento alimenticio para la dieta de las vacas lecheras, actuando como inhibidor de la producción de metano. Sin embargo sus propiedades características (olor y pungencia) podrían alterar las propiedades sensoriales de la leche. En este capítulo, se propone un método para evaluar la influencia del extracto de cebolla sobre las propiedades de la leche, utilizando como marcador el principio activo propil propano tiosulfonato (PTSO). El PTSO se extrae de la leche mediante un procedimiento QuEChERS y se controla por cromatografía líquida con detección ultravioleta (HPLC-UV). Una vez desarrollado, este método, se aplicó a las muestras de leche obtenidas de 100 vacas lecheras alimentadas durante dos meses con alimento enriquecido con extracto de cebolla. Además, se llevó a cabo una cata mediante la realización de un panel sensorial, con objeto de establecer la concentración máxima de PTSO que no alteraba las propiedades organolépticas de la leche, obteniendo un valor máximo de 2 mg kg^{-1}. Este capítulo, ayuda a demostrar que el uso de extracto de cebolla como suplemento alimenticio puede ser una alternativa interesante para controlar las emisiones de metano sin ninguna influencia en los atributos de la leche.

Abstract

Onion extract is used as feed supplement for dairy cows diet, acting as inhibitor of methane production; however, its characteristic properties (odor and pungency) could alter sensory attributes of milk. In this chapter, a method to evaluate the influence of this extract on milk properties, using propyl propane thiosulfonate (PTSO) as marker, has been proposed. PTSO is extracted using a QuEChERS procedure and monitored by liquid chromatography with ultraviolet detection (HPLC-UV). The method was applied to milk samples obtained from 100 dairy cows fed during two months with enriched feed with onion extract. In addition, a milk-tasting panel was established to evaluate the PTSO residue that should not be exceeded in order to guarantee milk sensory attributes. It was obtained that PTSO concentration lower than 2 mg kg^{-1} does not alter milk organoleptic properties. This fact makes onion extract an interesting alternative as feed supplement to control the methane emissions without any influence on milk attributes.

3.1. Introduction

Agricultural animal production is a source of gases that has been on the rise in recent years, thus becoming a potential ecological risk.[1] Global emissions of methane (CH_4), a potent greenhouse gas, represent 18% of total emissions to the atmosphere, contributing to global warming. The main sources of CH_4 emissions are natural sources like wetlands, oceans, lakes, rivers and anthropoids followed by agricultural sources such as manure or enteric fermentation.[2-4]

Recent studies indicate that changes in rumen ecosystem as a mitigating strategy could reduce CH_4 emission as well as improve the efficiency of converting plant material into milk and meat.[5] Ruminants in different production systems have access to different types and quantities of feed so the spatial distribution of produced greenhouse gases is expected to vary considerably depending on their location. In Europe, most of livestock-related CH_4 emissions arise from fermentation in the digestive tract of herbivorous animals. The main actions for the reduction of ruminant CH_4 emissions can be classified into three broad categories: addition of rumen modifiers (using specific substances that inhibit methanogenesis or biological control directed at reducing methanogens), use of diets that decrease H_2 production which would be converted to

1. Hartung, J.; Phillips, V.R. J. Agric. Eng. Res., 57 (1994) 173-189.

2. GHG sources. URL (http://www.climate-change-knowledge.org/ghg_sources.html)

3. Knapp, J.P.; Laur, G.L.; Vadas, P.A.; Weiss, W.P.; Tricarico, J.M. J. Dairy Sci., 97 (2014) 3231-3261.

4. Morgavi, D.P.; Forano, E.; Martin, C.; Newbold, C.J. Animal, 4 (2010) 1024-1036.

5. Hook, E.S.; Wright, A.D. G.; McBride, B.W. Archaea (2010) Article ID 945785.

CH_4 (feeds, feeding management and nutrition) or increase of animal production and efficiency (fewer cows are needed to produce the same amount of milk).[6]

The reduction of enteric CH_4 production in ruminants is of environmental and a nutritional interest. The most promising strategy, an important objective for ruminant nutritionists, is the development of new feed additives that act as rumen modifiers.[7] The correct choice of feed ingredients could modify microbial fermentation and hence the produced amount of CH_4 and volatile fatty acids (VFA) such as propionate, acetate or butyrate. VFA generation also affects the amount of CH_4 produced, since propionate formation consumes hydrogen, whereas acetate and butyrate formation generate it for methanogenesis.[8] Thus, a ruminant diet that increases propionate production will reduces CH_4 generation and consequently atmospheric emissions, whereas a shift in favor of acetate and butyrate production will increase ruminant CH_4 production.[9,10]

In recent years, essential oils or plant extracts have been widely studied as dairy cows feed supplement and they have proven to act on the reduction of CH_4 emissions by alteration of VFA (acetate, propionate and butyrate) generation. Moreover, it has been demonstrated that their use improves feed efficiency and milk production.[11,12] As a consequence, the interest towards natural products like garlic, onion, cinnamon, yucca,

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11. Patra, A. K.; Yu, Z. Environ. Microbiol. Appl., 78 (2012) 4271-4280.

12. Castillejos, L.; Calsamigliaa, S.; Ferret, A.; Losa, R. Anim. Feed Sci. Tech., 132 (2007) 186-201.

anise, oregano or capsicum extracts has increased. These natural alternatives previously showed antimicrobial activity and antioxidant properties and present an important effect in a positive alteration of rumen fermentation by VFA modification.[13,14] Specifically, *Allium spp.* extracts, including onion extract (OE), have shown an important reduction of CH_4 emissions by alteration of rumen VFA and this fact has been associated with the presence of OSCs characteristic of the alliaceous family.[15-17]

Despite the beneficial effects of the use OE,[18,19] its dosage in cattle feed should be controlled in order to preserve organoleptic properties (mainly odor and flavor) of milk or its derivative products as yoghurt and cheese. Several studies have demonstrated the transmission of onion flavor to milk after the intake indicating that it can be transferred to the udder via vascular routes from the digestive system.[20,21] In this sense,

13. Cowan, M. M. Clin. Microbiol., 12 (1999) 564-582.

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19. Martínez-Fernández, G.; Abecia, L.; Ramos-Morales, E.; Martin-García, A.I.; Molina-Alcaide, E.; Yáñez-Ruiz, D.R. Anim. Feed Sci. Tech., 191(2014) 16-25.

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PTSO, which is responsible of the odor of freshly cut onion[22], has been proposed in this work as an OE residue marker in milk samples. In the previous chapters, we have developed analytical methods for PTSO analysis in animal feed[23,24] but there is not method for its control in milk samples or dairy products.

Considering the presence of PTSO in milk samples could alter its organoleptic properties, such as taste and flavor, in this chapter we propose an analytical method for the monitoring of PTSO and its derivatives in milk after the ingestion of OE supplement in feed. The method consists of a sample treatment based on the previous extraction of PTSO with a QuEChERS procedure and its analysis by HPLC-UV. The method was applied for the control of milk samples from 100 dairy cows that ate feed enriched with OE for two months. In addition, a milk- tasting panel was applied in order to determine the maximum content of PTSO residue in milk that should not be exceeded to guarantee its quality in relation to preserve its organoleptic properties.

22. Pruthi, J.S. Quality assurance in spices and spice products: Modern methods of analysis. Allied Published Limited: New Delhi. (1998).

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24. Abad, P.; Arroyo-Manzanares, N.; García-Campaña, A.M. Anal. Method., 8 (2016) 3730-3739.

3.2. Materials and methods

3.2.1. Chemicals and reagents

All reagents were of analytical reagent grade. HPLC-grade solvents MeOH and MeCN were purchased from VWR BDH Prolabo (West Chester, Pennsylvania, USA). Perchloric acid (70%), NaCl and magnesium sulfate ($MgSO_4$) were supplied by Panreac (Madrid, Spain). FA and sodium phosphate monobasic monohydrate were obtained from Sigma Aldrich (St Louis, MO, USA). HPLC mobile phases were filtered using a vacuum system with Millipore (Milford, MA, USA) filters (nylon, 0.20 μm, 47 mm). Samples were filtered through PET Chromafil® (Macherey-Nagel, Germany) filters (polyester, 25 mm, 0.20 μm).

Kits of SampliQ QuEChERS consisted of buffered QuEChERS extraction packet (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate) and non-buffered QuEChERS extraction packet (4 g $MgSO_4$, 1 g NaCl) were supplied by Agilent Tecnologies Inc (Wilmington, DE, USA). C_{18} and primary secondary amine (PSA) sorbents were also supplied by Agilent Tecnologies Inc. and Supelclean LC Alumina-N (Al) and Supel™ QuE Z-Sep (Z-Sep) sorbents by Supelco (Bellefonte, PA, USA).

Ultrapure water (18.2 MΩ cm^{-1}, Milli–Q Plus system, Millipore Bedford, MA, USA) was used throughout the trials.

PTSO standard (95% of purity) and Garlicon®, an onion commercial extract containing PTSO as main active ingredient, were kindly provided by Domca S.A. (Alhendín, Granada, Spain).

Individual stock standard solution containing 2 g L^{-1} of PTSO was prepared in MeOH and stored in a dark bottle at -20 ºC. It was stable for at least 3 months. Working solutions were prepared by diluting the stock solution in MeOH to the desired concentration prior to use.

3.2.2. Instrumentation and equipment

The Agilent 1260 Infinity HPLC (Agilent Technologies Inc) system was used for the proposed HPLC method, which includes a quaternary pump, an online degasser, an autosampler (injection volume from 0.1 - 900 μL, capacity: 100 vials), a column thermostat and a DAD. A C18 column Zorbax BONUS RP Analytical (150 x 4.6 mm, 5 μm) from Agilent Tecnologies Inc. was used.

Additionally, confirmation of the presence of trace amounts of PTSO was carried out using an UHPLC system (Agilent 1290 Infinity LC, Agilent Technologies Inc) equipped with a binary pump, on line degasser, autosampler (5 μL loop) and a column thermostat. MS measurements were performed on a triple quadrupole mass spectrometer API3200 (AB Sciex, Toronto, ON, Canada) with ESI+. A Zorbax Eclipse Plus RRHD (50 x 2.1 mm, 1.8 μm) chromatographic column from Agilent Tecnologies Inc was used. The instrumental data were collected using the Analysts Software version 1.5 with Schedule MRM™ Algorithm (AB Sciex).

A Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) and a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) were also used in the sample treatment procedure.

3.2.3. Chromatographic conditions

3.2.3.1. HPLC-UV analysis of PTSO

The determination of PTSO was performed in a C18 column (150 x 4.6 mm, 5 µm), using a mobile phase consisting of 30 mM aqueous perchloric acid solution (solvent A) and MeCN (solvent B), at a flow rate of 0.7 mL min^{-1}. The injection volume was 20 µL and the gradient elution program was as follows: 50% B (0-3 min), 50-100% B (3-9 min), 100% B (9-14 min). The initial conditions were re-established by 2 min of linear gradient, followed by equilibration time of 4 min. The total chromatographic run time was 20 min and detection wavelength was set at 200 nm (bandwidth = 4 nm).

3.2.3.2. UHPLC-MS/MS analysis of PTSO and its derivatives

The sensitive quantification and confirmation of the presence/absence of PTSO and its typical derivatives (CSSP and GSSP) in milk samples was carried out by UHPLC-MS/MS. The analysis was performed according to the method previously development in chapter 2.[24] Under these conditions PTSO, CSSP and GSSP were determined in 5.5 min, being characterized by two precursor-product ion transitions.

3.2.4. Sample treatment for PTSO extraction

A 5 ± 0.05 g milk sample was placed into a 50 mL conical bottom screw tube. Subsequently, a volume of 10 mL of 6.4% of FA in MeCN was added to the tube and vortexed for 1 min. A non-buffered QuEChERS extraction packet was added, shaken vigorously for 1 min and then, the tube was vortexed for 1 min. The sample was centrifuged at 5000 rpm for 5 min and afterwards an aliquot of the supernatant was

filtered through a PET Chromafil® syringe filter and transferred to an Agilent CrossLab Vial. Finally, it was injected into the HPLC-UV system.

3.2.5. Experimental study

The experiment was carried out at a local farm located in Reus, Tarragona (Spain). A total of 200 cows (11-months-old) were selected and isolated from the rest. All cows were maintained for 90 days with *ad libitum* access to feed and water. The feed intake was antibiotic-free.

At the beginning of the experiment, cows were randomly allocated to two groups: 100 cows were selected as control group (CG) and the other 100 cows were considered as treated group (TG). The diet of TG was supplemented with OE containing PTSO. OE was introduced gradually in feed. Dosage was increased by 5 g per animal and per day during a period of 5 days. During the experiment, the intake per day and per animal was established in 25 g of commercial OE (Garlicon®) to avoid rumen alterations derived from high concentrations of PTSO.[15] The diet of CG had not any additional supplement. The field trial was extended for 2 months. In this period, the access of CG and TG to feed was controlled whereas the access to water was *ad libitum*.

The presence of off-flavor depends on the time elapsed between cow consumption and milking. Previous studies indicated that onion flavors can persist in milk for longer than

12 h, moreover, the stronger taste and flavor appears within 2 and 4 h after onion intake so milk samples were collected 3 h after eating cattle feed.[25]

Once per week, milk samples from both groups were collected, homogenized and immediately pasteurized in two different tanks. Milk samples were stored at -20 ºC before analysis.

3.2.6. Procedure for sensory evaluation of real milk samples containing PTSO

Milk must have a good flavor and an attractive appearance, among other nutritional parameters in order to be acceptable to consumers. Organoleptic properties of milk may be affected by several external factors, such as feeds and weeds consumed or odors inhaled by the cow, internal infections of the cow or microbiological or chemical alterations of the milk. Consequently, color, odor and flavor of milk containing OE residues were evaluated in order to establish the maximum level of PTSO residue that could be present without causing any alteration to the organoleptic properties of milk. Sensory evaluation was carried out using a triangle test.

3.2.6.1. Triangle test

The triangle test is a discriminating test consisting of checking whether a difference exists between two products. Panelists taste a similar amount of three milk samples at the same temperature and they should recognize the differences. In a triangle test, the

25. Álvarez, V.B. Sensory evaluation of milk products. In dairy processing and quality assurance, second edition; John Wiley & Sons Ltd: River Street, Hoboken, USA (2016) 467.

probability of getting the right result is 1/3. This methodology was applied following the methodology described in the ISO 4120:2004.[26]

3.2.6.2. Panelist selection

A total of 26 candidates were pre-selected as panelists and 16 of them were chosen for the triangle tests. Subsequently, panelists underwent a training session. Training for the formation of sensory memory was carried out by direct contact with PTSO (the evaluated attributes were odor, taste and flavor). The panel was trained in two sessions of 0.5 h at room temperature (20 ± 1 ºC). The panelists (53% female and 47% male, from Granada, Spain) were daily consumers of milk all of them and the ages were between 25 and 60 years old (average age of 37 years old).

3.2.6.3. Samples evaluation

The tests were carried out at Domca S.A. laboratories, simulating the conditions described in the general guidance for the design of test rooms (ISO 8589:2007).[27] The design and analysis (using Thurstonian model) of sensory discrimination tests were carried out using XLSTAT software version 2016.1.

26. ISO 4120:2004 - Sensory analysis - Methodology - Triangle test. URL: (http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=33495) (Friday, October 13, 2017).

27. ISO 8589:2007 - Sensory analysis - General guidance for the design of test rooms. URL: (https://www.iso.org/standard/36385.html) (Friday, October 13, 2017).

3.3. Results and discussion

3.3.1. Influence of the presence of CYS and GSH on the PTSO content in milk

In chapter 2 it has been demonstrated that PTSO reacts with CYS and GSH (a tripeptide with antioxidant properties which contains CYS), both present in a wide range of feed and food, resulting in CSSP and GSSP as products.[24] The most abundant proteins in milk are casein (α_{s1}-casein, α_{s2}-casein, β_{s1}-casein and κ-casein), α–lactalbumin and β–lactoglobulin. Although some of these proteins contribute to the presence of small amounts of CYS in milk, it is in its oxidized form, namely cystine ($C_6H_{12}N_2O_4S_2$), which does not trigger a reaction with PTSO (Figure 3.1).[28,29]

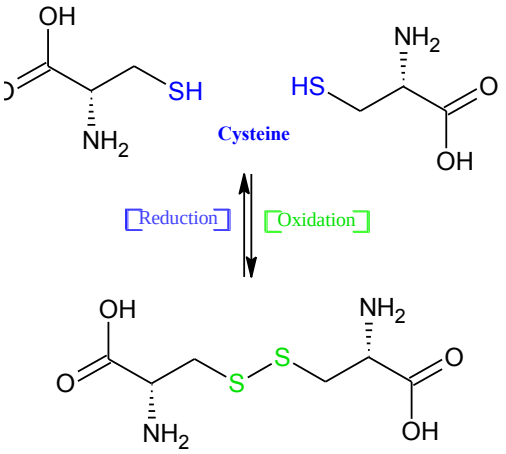


Figure 3.1. CYS/Cystine equilibrium redox reaction

28. Kailasapathy, K.; Pritchard, S.R. Chemical, physical and functional characteristic of dairy ingredients. In Dairy ingredients for food processing, first edition; Blackwell Publishing: Ames, Lowa, USA. 2011.

29. Belitz, H.D.; Grosch, W.; Schieberle, P. Milk and dairy products, in Food Chemistry, fourth edition; Springer-Verlag: Berlin-Heidelberg, Germany (2009) 498.

The amount of PTSO could also be affected by the presence of GSH which is found in milk but it is expected that PTSO will not react with GSH because it is converted in oxidized GSH by the reaction with GSH peroxidase.[30,31] This is an important aspect to be taken into account when considering this molecule as a marker for OE residues in milk. Regardless of this issue, the absence of derivative compounds from PTSO (CSSP and GSSP) in milk was tested by applying the previously proposed UHPLC-MS/MS method in chapter 2.[24] As it can be seen in Figure 3.2 which shows a chromatogram from a cow whole milk sample spiked with 20 mg kg^{-1} of PTSO, neither CSSP nor GSSP are detected.

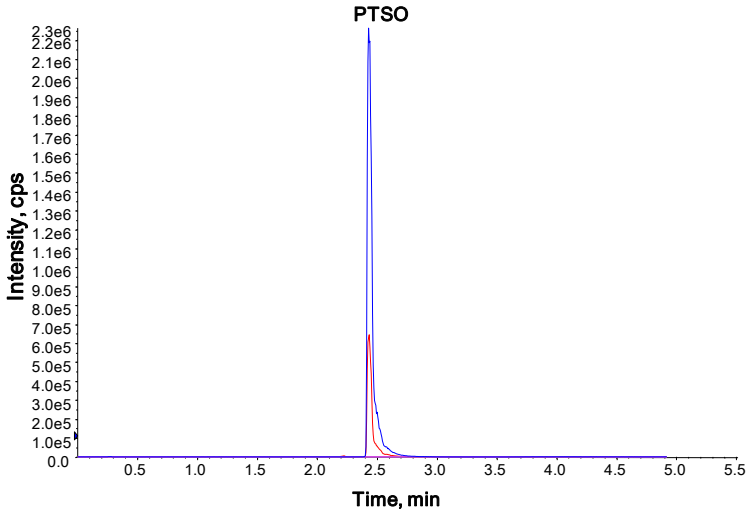


Figure 3.2. Chromatogram of a milk sample spiked with 20 mg kg^{-1} of PTSO extracted with the optimized QuEChERS procedure and analyzed by UHPLC-MS/MS

30. Lindmark-Månsson, H.; Åkesson, B. Brit. J. Nutr., 84 (2000) 103-110.

31. Stagsted, J. Int. Dairy J., 16 (2006) 662–668.

3.3.1.1. Development of the HPLC-UV method

HPLC-UV chromatographic method was adapted from the analytical method developed in chapter 1 for PTSO determination in feed.[23] Gradient program, flow rate, injection volume and column temperature were re-optimized for PTSO determination in milk. All the experiments were performed using whole dairy milk samples spiked with 50 mg kg^{-1} of PTSO.

The mobile phase consisted of 30 mM aqueous perchloric acid solution (A) and MeCN (B) and the final gradient program was as follows: 50% B (0-3 min), 50-100% B (3-9 min), 100% B (9-14 min). The initial conditions were re-established by 2 min of linear gradient, followed by an equilibration time of 4 min. The total run time was 20 min.

Different injection volumes (5, 10, 20 and 30 μL) were evaluated in order to select the optimum for obtaining the highest signal-to-noise ratio (S/N) and satisfactory peak efficiency. It was observed that peaks were not symmetrical from 30 μL, so a volume of 20 μL was chosen. Results are shown in table 3.1.

Table 3.1. Study of the injection volume on the area and S/N ratio for the proposed HPLC-UV method for monitoring PTSO residues in milk. RSD (%) is given in parenthesis (n=6)

Injection volume (μL)	Peak area (mAU/min)	S/N	Peak symmetry
5	1179.8 (0.4)	247.8	Yes
10	2370.4 (0.2)	497.9	Yes
20	**4676.1 (0.8)**	**982.5**	**Yes**
30	4697.2 (1.1)	986.8	No

Column temperature was studied between 20 °C and 40 °C and an optimum of 25 ºC was selected as a compromise between retention time and column shelf life, since no significance differences were observed among all tested temperatures (see table 3.2).

Table 3.2. Study of column temperature for the proposed HPLC-UV method for monitoring PTSO residues in milk. RSD (%) is given in parenthesis (n=6)

Temperature,ºC	Peak high (mAU)	Retention time, min
20	167.1 (0.9)	5.4
25	**169.6 (0.9)**	**5.3**
30	168.1 (0.4)	5.1
35	166.9 (0.2)	4.9
40	164.9 (0.1)	4.7

Finally, flow rate was evaluated from 0.6 to 1.2 mL min^{-1} and 0.7 mL min^{-1} was selected (see table 3.3) as a compromise between signal intensity and run time.

Table 3.3. Study of the flow rate of the mobile phase for the proposed HPLC-UV method for monitoring PTSO residues in milk. RSD (%) is given in parenthesis (n=6)

Flow rate (mL min^{-1})	Peak area (mAU/min)	Peak width (min)	Retention time (min)	Co-eluted interferences
0.6	8089.1 (0.5)	0.41 (0.07)	7.4	No
0.7	**6853.2 (0.6)**	**0.36 (1.37)**	**7.2**	**No**
0.8	5725.8 (0.7)	0.37 (0.30)	5.8	Yes
0.9	5204.9 (0.6)	0.39 (0.44)	5.6	Yes
1.0	4605.3 (0.9)	0.38 (0.47)	5.3	Yes
1.1	4389.4 (1.9)	0.34 (0.25)	4.7	Yes
1.2	4013.0 (0.1)	0.31 (1.62)	4.4	Yes

3.3.1.2. Optimization of the sample treatment

Sample treatment based on QuEChERS procedure has already been developed for the extraction of several food contaminants like antibiotics[32], mycotoxins[33] or pesticides[34] in milk but to the best of our knowledge, this is the first time that a QuEChERS procedure is used for the extraction of PTSO in *Allium spp*. The optimization was carried out using 5 g of whole dairy milk (ultra-high temperature, UHT) spiked at 50 mg kg^{-1} with PTSO. Different extraction solvents were evaluated: MeOH, ethyl acetate, acetone, and MeCN (10 mL in all cases). Using MeOH, no protein precipitation was observed, with ethyl

32. Arroyo-Manzanares, N.; Gámiz-Gracia, L.; García-Campaña, A.M. Food Chem., 143 (2014) 459-464.

33. Aguilera-Luiz, M.M.; Plaza-Bolaños, P.; Romero-González, R.; Martínez-Vidal, J.L.; Garrido- Frenich, A. Anal, Bioanal. Chem., 399 (2011) 2863-2875.

34. Jeong, I.S.; Kwak, B.M.; Ahn, J.H.; Jeong, S.H. Food Chem., 133 (2012) 473-481.

acetate no PTSO was detected and recovery values for acetone and MeCN were 19.8 and 76.2 % respectively. The best results in terms of recovery values were obtained with MeCN; therefore it was selected for the rest of experiments.

Thus, the extraction/partitioning process of the QuEChERS procedure was optimized for the analysis of PTSO in milk by using different solvents and Agilent SampliQ QuEChERS extraction kits: (a) 10 mL of MeCN, (b) 10 mL of MeCN + non- buffered QuEChERS extraction packet, (c) 8 mL of H_2O + 10 mL of MeCN + non- buffered QuEChERS extraction packet, (d) 10 mL of MeCN + buffered QuEChERS extraction packet, (e) 8 mL of H_2O + 10 mL of MeCN + buffered QuEChERS extraction packet, and (f) 8 mL of 30 mM NaH_2PO_4, buffer pH 7.1 + 10 mL of MeCN + buffered QuEChERS extraction packet. The best recovery percentages and lower losses of analyte were obtained for the option (b) (see figure 3.3).

The second step of the QuEChERS procedure, based on a dSPE for cleaning up, was also evaluated and different commercial sorbents (C_{18}, Al, Z-Sep and PSA) were tested. However, this step did not show any improvement on the analytical signal intensity so it was discarded.

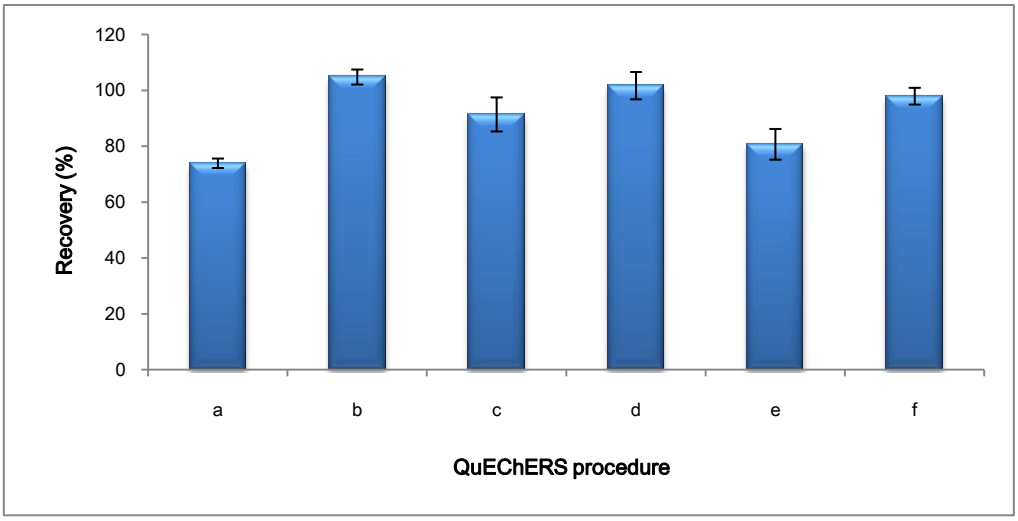


Figure 3.3. Study of different QuEChERS procedures checked for the analysis of PTSO in milk samples (n=6): (a) 10 mL of MeCN, (b) 10 mL of MeCN + non- buffered QuEChERS extraction packet, (c) 8 mL of H_2O + 10 mL of MeCN + non- buffered QuEChERS extraction packet, (d) 10 mL of MeCN + buffered QuEChERS extraction packet, (e) 8 mL of H_2O + 10 mL of MeCN + buffered QuEChERS extraction packet, and (f) 8 mL of 30 mM NaH_2PO_4, buffer pH 7.1 + 10 mL of MeCN + buffered QuEChERS extraction packet

Once the sample treatment was selected, the amount of each salt ($MgSO_4$ and NaCl) in the extraction kit and the influence of the presence of acid (FA) in the extracting solvent (10 mL of MeCN) were optimized by a multivariable approach to improve the PTSO recovery, using an experimental design and taking into account possible interactions among the chosen variables. A central composite design (2^3+ star, faced centered) with three spaced central points (17 runs) was used to generate the response surface, using the recovery percentage of PTSO in the extraction process as an analytical response (table 3.4). These variables were studied in the following ranges: amount of NaCl (0-2 g), amount of $MgSO_4$ (0−5 g), and percentage of FA in MeCN (0-10 %).

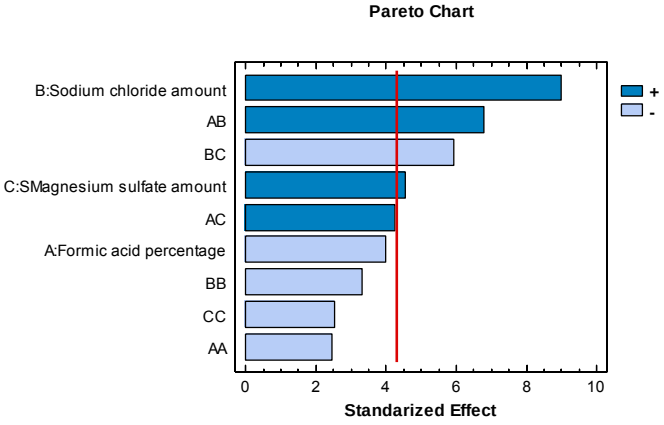
Table 3.4. Experimental design runs used to generate the response surface in the optimization of the sample treatment

Run	FA in MeCN (%)	NaCl (g)	$MgSO_4$ (g)
1	5	1	2.5
2	0	0	0
3	10	0	0
4	0	2	0
5	10	2	0
6	0	0	5
7	10	0	5
8	0	2	5
9	5	1	2.5
10	10	2	5
11	0	1	2.5
12	10	1	2.5
13	5	0	2.5
14	5	2	2.5
15	5	1	0
16	5	1	5
17	5	1	2.5

A Pareto chart (Figure 3.4 a) was obtained from the screening experimental design showing that some of the studied variables and/or the interactions between them have a significant effect (positive or negative) on the response when their value changed inside the selected experimental domain. The length of each bar is proportional to the value of the corresponding effect (main effects or interactions between factors) statistically estimated by STATGRAPHICS. The vertical line shows the limit of decision to consider the significance of the factors and/or of the interactions between them (based on the standardized effect = estimated effect/standard error; with a p-value 0.05 at 95% of confidence level).

In this case, two main significant effects were observed: NaCl and $MgSO_4$ amounts (B and C). Both of them have a positive effect on the response. There is also a significant interaction between NaCl amount and FA percentage (AB). This interaction causes a positive effect on PTSO recovery. Another interaction, with negative effect in this case, is that observed between NaCl and $MgSO_4$ amount (BC).

a



b

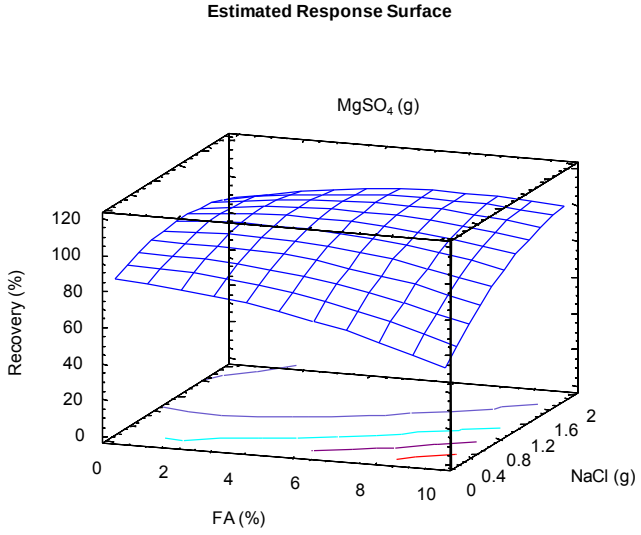


Figure 3.4. (a) Pareto chart showing the effects of the studied variables on the recovery percentage. Vertical line shows the limit of decision to consider the significance of the factors (based on the effect = estimated effect/standard error, p-value = 0.05 at 95% of confidence); (b) Response surface plot showing the effect on PTSO recovery of the FA concentration in MeCN and of the NaCl and $MgSO_4$ amounts

Based on the information obtained from the Pareto chart, the three selected variables (amount of NaCl and $MgSO_4$ and FA percentage in MeCN) were simultaneously optimized by a response surface design. Optimum conditions were obtained (Figure 3.4 b): NaCl = 1.9 g, $MgSO_4$= 2.5 g and FA in MeCN = 6.4%. The p-value of the lack-of-fit test was 0.089 and the determination coefficient (R^2) was 84.1%, proving the suitability of this design. To verify the selected optimum values for the studied variables, several milk samples (n=5) were treated with the proposed method. A mean value of 97.5 ± 1.1% was obtained for PTSO recovery.

Finally, the extraction volume (MeCN with 6.4 % FA) was evaluated in the range of 2.5 to 10 mL and a volume of 10 mL was chosen as optimum since a decrease of the volume amount involved lower recovery percentages (see figure 3.5).

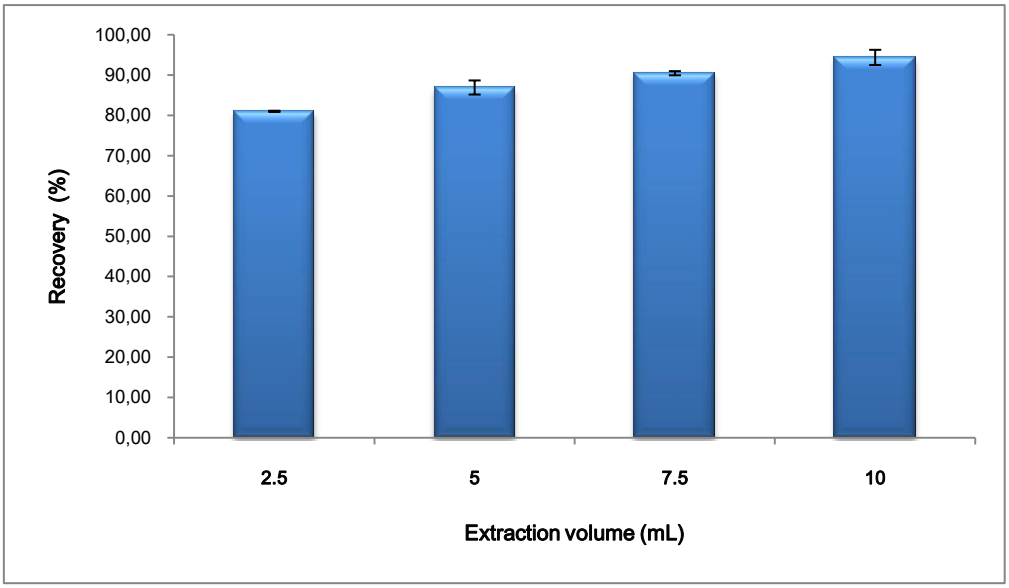


Figure 3.5. Effect of the extraction volume on PTSO recovery value (n=6)

Sample size was also evaluated between 1 and 10 g, selecting 5 g in order to obtain better signal intensity (see figure 3.6) and therefore lower LOD.

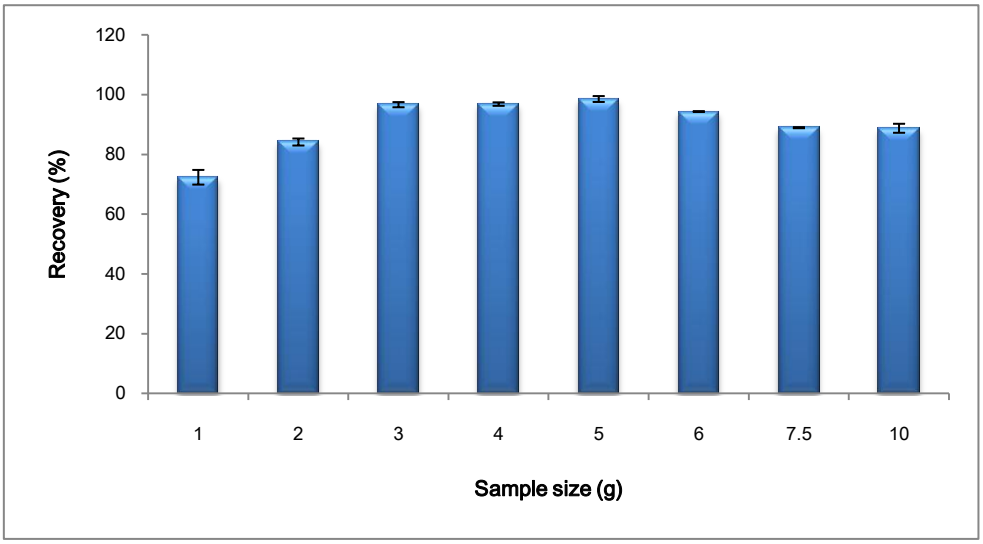


Figure 3.6. Effect of sample size on PTSO recovery percentage (n=6)

3.3.1.3. Characterization of the method

In order to check the suitability of the proposed method, linear dynamic range, LODs and LOQs were evaluated for milk samples from different ruminants: pasteurized dairy milk, UHT dairy milk (whole, semi-skimmed and skimmed), UHT semi-skimmed goat milk and UHT semi-skimmed sheep milk. In addition, studies of precision and recovery for PTSO content in each matrix were carried out. Figure 3.7 shows a chromatogram of a whole milk blank sample and a whole milk sample spiked with 50 mg kg^{-1} of PTSO.

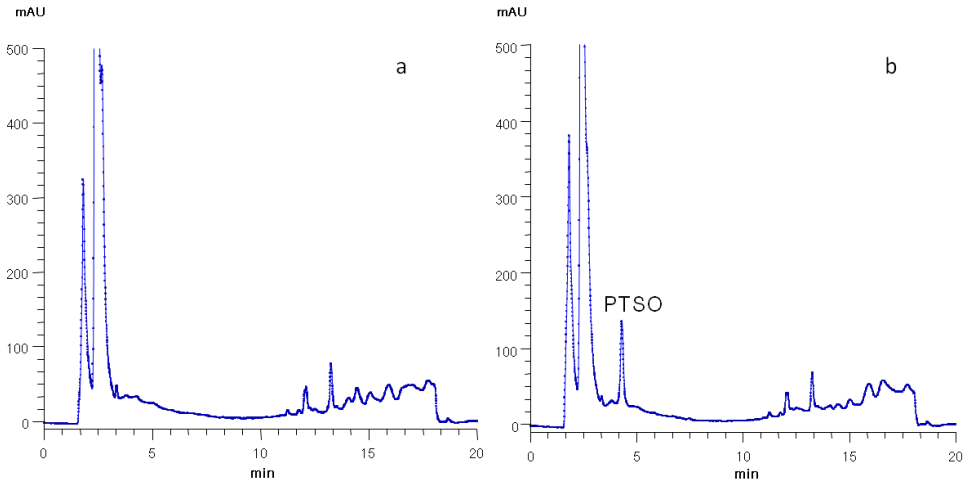


Figure 3.7. Chromatograms from (a) a blank whole milk sample and (b) a whole milk sample spiked with PTSO at 50 mg kg^{-1}

3.3.1.4. Calibration curves and performance characteristics

Matrix-matched calibration curves were established by spiking milk blank samples at six different concentration levels of PTSO (ranging from 5 to 500 mg kg^{-1} for pasteurized dairy cow milk, UHT semi-skimmed goat milk and UHT semi-skimmed sheep milk and from 10 to 500 mg kg^{-1} for whole, semi-skimmed and skimmed dairy cow milk). Each level was prepared in duplicate and injected in triplicate. Statistical parameters were calculated by least-square regression. Obtained values for LOD (3xS/N ratio) and LOQ (10xS/N ratio), linear dynamic range and linearity are shown in table 3.5.

Table 3.5. Statistical and performance characteristics of the proposed HPLC-UV method

Matrix	Linear dynamic range (mg kg^{-1})	R^2	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
A	6.0-500	0.9957	1.8	6.0
B	6.3-500	0.9987	1.9	6.3
C	6.0-500	0.9997	1.8	6.0
D	4.7-500	0.9996	1.4	4.7
E	2.7-500	0.9995	0.8	2.7
F	3.7-500	0.9994	1.1	3.7

A: UHT skimmed dairy milk; B: UHT semi-skimmed dairy milk; C: UHT whole dairy milk; D: Pasteurized whole dairy milk; E: UHT semi-skimmed goat milk; F: UHT semi-skimmed sheep milk

3.3.1.5. Precision study

The precision of the whole method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was assessed by applying the whole procedure to six types of milk samples on the same day (experimental replicates) spiked at three different concentration levels of PTSO: 10, 300 and 500 mg kg^{-1} and each sample was injected in triplicate (instrumental replicates). Intermediate precision was evaluated with a similar procedure, but in this case, five samples of each concentration level were analyzed five different days. The results, expressed as RSD of peak areas, are shown in table 3.6. Satisfactory precision was obtained, with RSDs lower than 10% in all cases.

Table 3.6. Precision study (% RSD of peak areas) of the proposed HPLC-UV method

	Repeatability (n = 9)			Intermediate precision (n = 15)		
Matrix/Level	**Level 1**	**Level 2**	**Level 3**	**Level 1**	**Level 2**	**Level 3**
A	3.0	1.0	2.6	7.9	1.6	3.2
B	8.6	1.8	1.2	8.7	2.1	4.5
C	4.5	0.8	1.7	6.6	1.5	1.3
D	5.4	0.7	0.3	6.8	1.5	1.6
E	5.0	1.1	0.4	7.3	3.6	1.0
F	5.3	3.2	1.0	5.8	1.7	1.0

Level 1: 10 mg kg^{-1}; level 2: 300 mg kg^{-1}; level 3: 500 mg kg^{-1}

A: UHT skimmed dairy milk; B: UHT semi-skimmed dairy milk; C: UHT whole dairy milk; D: Pasteurized whole dairy milk; E: UHT semi-skimmed goat milk; F: UHT semi-skimmed sheep milk.

3.3.1.6. Recovery study

Three milk samples were spiked at three different concentration levels of PTSO, submitted to the QuEChERS procedure and injected per triplicate. Recoveries were calculated as 100 × [(signal of a spiked sample / signal of spiked extract]; additionally, in all cases blank samples were previously analyzed and none of them gave a positive result for PTSO. Results are shown in table 3.7, demonstrating the trueness of the proposed method (all of the recovery values were higher than 82%).

Table 3.7. Recovery study of the HPLC-UV proposed method (RSD given in parenthesis)

	Recovery (%) (n = 9)		
Matrix/Level	**Level 1**	**Level 2**	**Level 3**
A	82.5 (8.8)	93.1 (1.8)	92.5 (1.2)
B	82.4 (4.5)	92.2 (0.8)	95.7 (1.7)
C	84.0 (5.4)	92.0 (0.7)	96.3 (0.3)
D	84.3 (5.3)	93.3 (3.2)	91.9 (1.0)
E	82.5 (8.8)	93.1 (1.8)	92.5 (1.2)
F	89.4 (5.0)	96.5 (1.1)	97.5 (0.4)

Level 1: 10 mg kg^{-1}; level 2: 300 mg kg^{-1}; level 3: 500 mg kg^{-1}

A: UHT skimmed dairy milk; B: UHT semi-skimmed dairy milk; C: UHT whole dairy milk; D: Pasteurized whole dairy milk; E: UHT semi-skimmed goat milk; F: UHT semi-skimmed sheep milk.

3.3.1.7. Analysis of real milk samples

The validated method was applied for the analysis of milk samples from CG and TG. All samples were prepared following the proposed sample treatment and analyzed in triplicate by the HPLC-UV method. None of them gave a positive result of PTSO levels above the LODs. To confirm the absence of PTSO, a more sensitive and confirmatory UHPLC-MS/MS method previously developed in our lab was applied to all samples (LOQ=250 µg kg^{-1}).[24] As it was expected, negative results were obtained from samples belonging to CG. However, in all samples from TG, the presence of PTSO was detected and confirmed. Thus, a sensory evaluation was carried out in order to confirm that the detected amount of PTSO did not affect to organoleptic properties of the milk samples.

3.3.1.8. Sensory evaluation of real milk samples containing PTSO

Three different tests were performed in three consecutive days. Test 1 and test 2 were performed in order to establish a sensorial limit for PTSO in milk samples whereas test 3 was performed to check organoleptic properties in real milk samples. In all cases, 100 g of milk were offered to the panelists at a temperature of 20 ± 1 °C in a transparent plastic vessel coded with a two-digit number. The first test consisted of free-PTSO milk samples versus milk samples containing 10 mg kg^{-1} of PTSO. Test samples for each panelist were randomly selected by XLSTAT program. Milk samples containing PTSO were correctly identified by 88% of panelists, and therefore, it can be said that this concentration of PTSO modifies milk organoleptic properties (p-value < 0.0001). The second test consisted of the comparison of milk samples containing 2 mg kg^{-1} of PTSO versus free-PTSO milk samples. In this case, 87.5% of panelist did not find any significant differences among the tested samples showing that a concentration of 2 mg kg^{-1} of PTSO does not alter organoleptic properties of milk (p-value = 0.661). All samples were prepared with pasteurized dairy milk.

Finally, the test 3 was carried out with real milk samples from TG at the end of the experiment (results are shown in table 3.8 and 3.9). In this case, samples from CG were faced to samples from TG. None of the panelists found significant differences among the tested samples (p-value= 0.661).

Table 3.8. Code of samples assigned to each panelist for the analysis of milk real samples (CG, Control Group; TG, Test Group) performed by the triangular test using XLSTAT software

Judge (J)	Code of samples for each panelist		
	Sample 1	Sample 2	Sample 3
J1	CG	TG	TG
J2	CG	TG	TG
J3	CG	TG	TG
J4	TG	TG	CG
J5	TG	CG	TG
J6	TG	CG	TG
J7	CG	CG	TG
J8	TG	CG	CG
J9	TG	CG	TG
J10	TG	TG	CG
J11	CG	TG	CG
J12	CG	TG	CG
J13	CG	TG	TG
J14	TG	CG	TG
J15	TG	CG	CG
J16	CG	CG	TG
J17	TG	TG	CG

CG: Control group; TG: Treated group

Table 3.9. Results of sensory analysis of real milk samples from CG and TG

Judge (J)	ONION TASTE			ONION FLAVOR			DIFFERENT SAMPLE
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	
J1	No	No	No	No	No	No	Not found
J2	No	No	No	No	No	No	Not found
J3	No	No	No	No	No	No	Not found
J4	No	No	No	No	No	No	Not found
J5	No	No	No	No	No	No	Not found
J6	No	No	No	No	No	No	Not found
J7	No	No	No	No	No	No	Not found
J8	No	No	No	No	No	No	Not found
J9	No	No	No	No	No	No	Not found
J10	No	No	No	No	No	No	Not found
J11	No	No	No	No	No	No	Not found
J12	No	No	No	No	No	No	Not found
J13	No	No	No	No	No	No	Not found
J14	No	No	No	No	No	No	Not found
J15	No	No	No	No	No	No	Not found
J16	No	No	No	No	No	No	Not found
J17	No	No	No	No	No	No	Not found

Hence, although small traces of PTSO were found in milk samples from TG by UHPLC-MS/MS, the identified concentration does not alter organoleptic properties of milk and Garlicon® can, therefore, be used as supplement feed at doses of 25 g per day. This positive result allows us to draw a strategy to reduce CH_4 emissions without compromising milk quality. Moreover, the proposed analytical method is suitable for controlling the presence of residues of PTSO in milk as marker of OE, at concentrations that could alter organoleptic properties of milk (above 2 mg kg^{-1}).

3.4. Conclusions

In this chapter PTSO has been proposed as a residue marker in milk samples of the use of OE as feed supplement for milk-producer animals. Among the advantages of PTSO added in the dairy cow's diet is the inhibition of the methane production; however, its odor and pungency could alter sensory attributes of milk. For this reason, we have proposed an analytical method to evaluate the influence of OE-based feed supplements on milk properties, by monitoring PTSO using HPLC with UV detection, previous extraction by a QuEChERS procedure. The method has been applied to dairy cows fed with OE-enriched feed in order to check the presence of the marker in the produced milk and with the aim to correlate the PTSO content with any alteration in the organoleptic properties. UHPLC-MS/MS has been used as confirmatory technique to ensure also the absence of the derivative compounds from PTSO (CSSP and GSSP). By means of a milk-tasting panel, a PTSO concentration of 2 mg kg^{-1} has been established as limit to guarantee the original milk sensorial characteristics, demonstrating that OE can be used as feed supplement to control the methane emissions, which involved important consequences for the environment, without any alteration of milk attributes.

This work was published as: Use of onion extract as dairy cows feed supplement: monitoring of PTSO as marker of its effect on milk attributes. Abad, P., Arroyo-Manzanares, N., García-Campaña, J. Agric. Food Chem., 65 (2017) 793-799.

CHAPTER 4

Evaluation of onion extract as feed supplement for poultry: study of its influence on egg quality, productivity and health status of laying hens

Evaluación del uso de extracto de cebolla como suplemento en avicultura: estudio de su influencia en la calidad del huevo y sobre la productividad y el estado de salud de gallinas ponedoras

Resumen

En este capítulo, se ha evaluado la utilización del propilpropano tiosulfonato (PTSO), como suplemento en piensos de gallinas ponedoras, con el fin de demostrar sus efectos positivos en la producción y calidad de los huevos, así como en el estado de salud de las gallinas. El estudio se llevó a cabo con 90 gallinas ponedoras cuya alimentación se suplementó con PTSO durante 28 días a una dosis de 24 mg kg^{-1} por animal y día. Tras realización del ensayo, se observó un aumento de la productividad de las aves traducido en un aumento del número y tamaño de los huevos. Así mismo, para la dosis utilizada, se demostró que no se alteraban las propiedades nutricionales de los huevos. Además, se confirmó un efecto modulador sobre la microbiota intestinal por el aumento de *Lactobacillus spp*. y *Bifidobacterium spp* y por la reducción de las poblaciones de Enterobacterias. Por último, se verificó que las propiedades organolépticas del huevo no se alteraban tras el uso de PTSO como aditivo en piensos. Para ello, se propuso un nuevo método analítico basado en extracción en fase sólida y espectrometría de masas en tándem de cromatografía líquida de ultra-alta resolución (UHPLC-MS / MS) para la determinación de posibles residuos de PTSO en huevo.

Abstract

The use of propyl propane thiosulfonate (PTSO) as feed supplement has been evaluated to demonstrate its positive effect on egg production and healthy status of laying hens. The study was carried out on 90 laying hens whose feed was supplemented with PTSO for 28 days at a dose of 24 mg kg^{-1} per animal and day. Nutritional properties of eggs were not affected at this dose whereas an improvement of fowl productivity was observed based on the increase in egg quantity and weight. In addition, a modulator effect on intestinal microbiota was confirmed by the increase of *Lactobacillus spp.* and *Bifidobacterium spp*. as well as by the reduction of Enterobacteriae populations. Finally, the preservation of egg organoleptic properties was checked by monitoring PTSO content by means of a new analytical method which consisted of solid phase extraction and ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS).

4.1. Introduction

As it has been explained in previous chapters, AGP have been commonly used as supplements in animal feed due to their properties related to the improvement of digestion efficiency and animal health status until their ban in 2006.[1,2] Since then, researchers and nutritionists have shifted their attention toward the search of viable alternative feed additives for supplement use.[3]

Plant extracts used on livestock and poultry are excellent alternatives due to their proven positive effects. Some studies have revealed that this type of products could improve the response of the immunity system or even enhance productivity.[4-9]

For this reason, this chapter proposes for first time the use of PTSO as feed supplement for laying hens. PTSO, as it has been said previously, is a natural ingredient that comes from the natural degradation of the main onion flavour

1. Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L268, 29-43.

2. Sojoudi, M.R.; Dadashbeiki, M.; Bouyeh, M. ROAVS., 2 (2012) 243-248.

3. Dibner, J.J.; Richards, J.D. Poultry Sci., 84 (2005) 634-643.

4. Greathead, H. P. Nutr. Soc., 62 (2003) 279-290.

5. Calsamiglia, S.; Cardozo P.W.; Ferret A.; Bach A. J. Anim. Sci., 86 (2008) 702-711.

6. Benchaar, C.; McAllister, T.A.; Chouinard, P.Y. J. Dairy Sci., 91 (2008) 4765-4777.

7. Hernández, F.; Madrid, J.; Garcia, V.; Orengo, J.; Megias, M.D. Poultry Sci., 83 (2004) 169-174.

8. Kim, D. K.; Lillehoj, H. S.; Lee, S. H.; Lillehoj, E.P.; Bravo, D. Brit. J. Nutr., 109 (2013) 76-88.

9. Lee, S.H.; Lillehoj, H.S.; Hong, Y. H.; Jang, S.I.; Lillehoj, E.P.; Ionescue, C.; Mazuranok, L.; Bravo, D. British Poultry Sci., 51 (2010) 213-221.

precursor: propiiin.[10]. For broilers, antimicrobial activity of PTSO has been demonstrated *in vitro* and *in vivo* against Enterobacteriaceae like *E.coli* and *Salmonella spp*. as well as against *Campylobacter jejuni*.[11-13] In addition, PTSO could modulate intestinal microbiota composition and improve nutrients digestibility without affecting mucosal enzyme activity in growing broilers.[14]

The aim of this study was therefore to evaluate the influence of PTSO intake of on laying hens by monitoring egg quality and productivity as well as laying hen health status. For this purpose, a field experiment with 180 laying hens was carried out during 28 days. One half of laying hens (treated group) were supplemented with PTSO whereas the other half had a PTSO-free diet (control group). Egg productivity from both groups was compared by the studied of egg quantity and weight. In addition, egg quality was determined by the amino acid content and the proximate analysis results. Health status was evaluated by feces analyses from each group of microbiota probiotic population (*Lactobacillus spp*. and *Bifidobacterium spp*.), Enterobacteriae, total anaerobic bacteria, *Clostridium* spp. and *Enterococcus spp.* Lastly, an analytical method was developed to control egg organoleptic properties (odour and flavour) by monitoring PTSO residues. This analytical method was based on the application of UHPLC-

10. Bravo D.; Lillehoj, H. WO 2013124441 (A1) (2013).

11. Ruiz, R.; García, M., Lara, A.; Rubio, L.Vet. Microbiol., 29 (2010) 110-117.

12. Peinado, M.; Ruiz, R.; Echávarri, A.; Rubio, L.A. Anim. Feed Sci.Tech., 144 (2010) 110-117.

13. Peinado, M.J.; Ruiz, R.; Echávarri, A.; Rubio, L. A. Poultry Sci., 91 (2012) 2148-2157.

14. Peinado, M.J.; Ruiz, R.; Echavarri, A.; Aranda-Olmedo, I.; Rubio. L.A. (2013). Anim. Feed Sci.Tech., 181 (2013) 87-92.

MS/MS technique,[15] using solid phase extraction (SPE) as sample treatment with Oasis mixed-mode cation exchange (MCX) cartridges.

4.2. Materials and methods

4.2.1. Chemicals and reagents

All reagents were of analytical reagent grade. Solvents used as mobile phase were LC-MS grade. MeOH used as mobile phase was supplied by VWR BDH Prolabo (Leuven, Belgium). FA (both analytical and MS grade), ammonium hydroxide 30% (NH_4OH), potassium sulfate (K_2SO_4), sodium chloride (NaCl), potassium chloride (KCl), GSH reduced 98 % and CYS hydrochloride 97 % were purchased from Sigma Aldrich (St Louis, MO, USA). MeOH, ACO, MeCN, EtAc and trichloroacetic acid (TCA) were supplied by Panreac (Barcelona, Spain). Finally, sodium phosphate dibasic (Na_2HPO_4), monopotassium phosphate (KH_2PO_4) and peptone solution 1 % was purchased from Scharlab (Barcelona, Spain). Ultrapure water (18.2 MΩ cm^{-1}, Milli-Q Plus system, Millipore Bedford, MA, USA) was used throughout all the work.

Clarinert™ 13 mm syringe filters with 0.22 µm nylon membrane (Agela Technologies, Wilmington, DE, USA) were used for filtration of samples prior to the injection into the chromatographic system. Extraction cartridges containing a MCX polymeric sorbent (Oasis MCX 150 mg, 6 cc, 30 µm particle size; Waters,

15. Abad, P.; Arroyo-Manzanares, N.; García-Campaña, A.M. (2016). Anal. Methods, 8 (2016) 3730-3739.

Milford, MA, US) were used for SPE. Also, silica cartridges (Strata SI-1 500 mg, 6 cc, 55 µm particle size, Phenomenex, Torrance, CA) and reversed-phase hydrophilic-lipophilic balance polymer cartridges (Oasis HLB 500 mg, 6 cc; 60 µm particle size Waters) were tested in the optimization of the SPE procedure.

A standard of pure PTSO (95%) and Garlicon®, a feed premix containing PTSO (17 %) and were kindly provided by the company DOMCA S.A. (Alhendín, Granada, Spain). A 2 g L^{-1} stock standard solution of PTSO was prepared by dissolving the appropriate amount of PTSO in MeOH. This standard solution was stored in a dark bottle at -20 ºC. It was stable for at least 12 months. Working solutions were prepared diluting the stock solution in MeOH to the desired concentration prior to use.

Individual stock solutions of CYS and GSH at concentrations of 10 mM were prepared by dissolving the appropriate amount of each compound in water and stored refrigerated. They were stable for 7 days.

CSSP and GSSP standard solution were prepared mixing 1 mL of PTSO methanolic solution (10 mM) and 2 mL of a CYS or GSH aqueous solution (10 mM), respestively, in order to achieve the stoichiometric ratio (1:2).[16] These solutions were prepared at the time of use.

16. Abad, P.; Arroyo-Manzanares, N.; García-Campaña, A.M. (2016). Anal. Methods, 8 (2016) 3730-3739.

4.2.2. Culture media used for bacterial growth

Culture media used for bacterial growth analysis were as follows: Slanetz Bartley agar for the enumeration of *Enterococcus*, MacConkey agar for Enterobacteriaceae total count; Wilkins Chalgren agar for the general growth of anaerobes and Sulfite-Polymyxin-Sulfadiazine agar for enumeration of sulfite-reducing clostridia. All media were provided by Biokar Diagnostics S.L. (Beauvais, France) and subjected to a sterilization process in autoclave at 121 °C for 20 minutes. Furthermore, a modified Columbia agar medium was used for enumeration of *Bifidobacterium spp.*[17] and a LAMVAB medium was used for *Lactobacillus spp.* recount.[18]

4.2.3. Instrumentation and equipment

PTSO analyses were performed using an Agilent 1290 Infinity LC (Agilent Technologies, Waldbron, Germany) equipped with a binary pump, on line degasser, autosampler (5 µL loop) and a column thermostat. MS measurements were performed on a triple quadrupole mass spectrometer API3200 (AB Sciex, Toronto, ON, Canada) with ESI. A Zorbax Eclipse Plus RRHD (50 x 2.1 mm, 1.8 µm, Agilent Technologies) chromatographic column was used for the separation. Instrumental data were collected using the Analysts Software version 1.5 with Schedule MRM™ Algorithm (AB Sciex).

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17. Beerens, H. Lett. Appl. Microbiol., 11 (1990) 155-157.

18. Hartemink, R.; Domenech, V.R.; Rombouts, F.M. J. Microbiol. Methods, 27 (1997) 77-84.

Amino acid analyses were performed using an Agilent 1260 Infinity LC (Agilent Technologies, Waldbron, Germany) system consisted of a quaternary pump, online degasser, autosampler (injection range 0.1 – 900 µL), a column thermostat and a DAD. Amino acid separation was performed using an AccQ-Tag chromatographic column (150 x 3.9 mm, 4 µm, Waters).

A Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), a polytron (Kinematia AG, Luzern, Switzerland), an oven with natural air convection (Raypa, Barcelona, Spain), a muffle furnance (Selecta, Barcelona, Spain) and an evaporator system (System EVA-EC, from VLM GmbH, Bielefeld, Germany) were also used for sample treatment.

Statistical data treatment was performed using STATGRAPHICS Centurion XVI Version 16.2.04 (32 bits). ANOVA simple factor analysis was performed and data were compared by Fisher's test LSD (Least significant difference). Confidence interval was established in 95 %.

4.2.4. Experimental design

The field experiment was carried out in a local farm located in Alhendín (Granada, Spain). Hens were in battery cages with a rate of six animals per cage and their eggs were therefore classified as type 3. 90 days prior to the beginning of the experiment, all hens were given *ad libitum* access to water and antibiotic-free feed.

Housing temperature was maintained at 20 ± 2 ºC, the average relative humidity was 78 ± 3% and the photoperiod was 16 hours. A total of 180 hens (36-weeks-old) were randomly allocated in two groups of 90 animals: control group (CG) and treated group (TG). Both groups were fed with a standard basal diet (Piensos Pisur, Peligros, Spain). Nutritional information of the used feed is included in table 4.1.

Table 4.1. Nutritional information of feed used as laying hen diet in the study

COMPOSITION	%
Crude protein	16.0
Crude fiber	3.9
Oils and crude oils and fats	3.5
Crude ash	13.0
Calcium	3.9
Phosphorous	0.6
Sodium	0.2
Methionine	0.5
Lysine	1.0
Carbohydrates	57.6

In addition, TG diet was supplemented with 140 mg kg^{-1} of Garlicon® per day and per fowl that equals 24 mg kg^{-1}. Statistical analysis was performed by comparison of analyzed variables in the study (number and size of collected eggs, nutritional and organoleptic properties and studied microorganism) between CG and TG at different times (0, 7, 14, 21 and 28 days) against the whole data obtained for CG along the field trial.

4.2.5. Egg productivity

Eggs from both groups were collected every week. They were counted and weighed. Eggs were classified into four different groups according to the Regulation (EC) Nº 589/2008:[19] Very large (XL), weight > 73 g; Large (L), weight between 63 and 73 g; Medium (M), weight between 53 and 63 g and Small (S), weight lower than 53 g.

4.2.6. Egg quality

Proximate analysis consisted of determining total fat, moisture, ash, total protein, total carbohydrates and energy content. Total fat in egg samples was determined using the official method of analysis AOAC 925.32-1925.[20] Moisture was calculated by taking a homogenized portion of egg (5 ± 0.1 g) and heating it to 102°C for 1 h. The percentage of moisture was calculated as [(initial mass-final mass)/initial mass] x 100.[21] Ash content was determined gravimetrically. An amount of 5 ± 0.1 g of beaten egg was kept in a muffle furnace at 550 ºC for 18 h.[22] Ash content was calculated as (ash mass /initial sample mass) x 100. Total protein content was determined by nitrogen determination by Kjeldahl

19. Regulation (EC) No 589/2008 of 23 June 2008 laying down detailed rules for implementing Council Regulation (EC) No 1234/2007 as regards marketing standards for eggs. OJ L164 (2008) 14-15.

20. Official Methods of Analysis of AOAC International. (2015). URL: (http://www.aoacofficialmethod.org/index.php?main_page=product_info&products_id=1755) (Friday, October 27, 2017)

21. Bradley, R.L. Compositional analysis of food (2010) 87-104.

22. Marshall, M.R. Compositional analysis of food, (2010) 107-114.

procedure[23] using a conversion factor of 6.25 equivalent to 0.16 g of nitrogen per gram of protein.[24] Total carbohydrates were obtained gravimetrically as the difference between 100 and the sum of the percentage of total fat, moisture, ash and total protein content.[25] Last, energetic value (kcal) was obtained by multiplying the number of grams of carbohydrates, proteins and fat by 3.68, 4.36, and 9.02 respectively. Sum of all values correspond to total egg calories.[26]

Amino acids were analyzed by HPLC-UV using the equipment described in section 4.2.3. The chromatographic method for the determination of amino acids in feed samples was performed according to an analytical method from Waters®.[27]

4.2.7. Microbiological determinations

Fecal samples (1 ± 0.1 g per sample) were collected weekly in sterile plastic containers classified and labeled for analysis. They were diluted with 9 mL of phosphate buffered saline, pH 7.3, prepared as a mixture of different salts at the following concentrations: 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.15 g L^{-1} Na_2HPO_4 and 0.2 g L^{-1} KH_2PO_4, and containing of CYS hydrochloride in a concentration of 0.5 g L^{-1} to favor anaerobic bacteria survival. Viable counts of bacteria in the feces

23. Owusu-Apenten, R.K. Food Proteins Analysis, (2002) 1-45.

24. Chang, S.K. Compositional analysis of food, (2010) 135-146.

25. BeMiller, J. Compositional Analysis of Food, (2010) 149-178.

26. Merril, A.L., Watt, B.K. Agriculture Handbook Nº 74 (1973).

27. Waters Corporation. URL: http://www.waters.com/webassets/cms/library/docs/4acqtag.pdf (Friday, October 27, 2017)

samples were then estimated by plating serial 10-fold dilutions in the peptone solution. Media and culture conditions to determine each bacterial group are shown in table 4.2.

Table 4.2. Culture conditions and growth media used for bacterial growth analysis

Growth media	Bacterial group	T[a] (ºC)	Culture conditions
MacConkey	Enterobacteriaceae	35 ± 1 ºC	18-24 h aerobic atmosphere
Wilkins-Chalgren	Total anaerobic bacteria	35 ± 1 °C	24-48 h anaerobic atmosphere
SPS agar	*Clostridium* spp.	35 ± 1 ºC	24 h anaerobic atmosphere
LAMVAB	*Lactobacillus* spp.	35 ± 1 ºC	72-120 h aerobic atmosphere
Slanetz Bartley	*Enterococcus* spp.	37 ± 1 ºC	24-48 h aerobic atmosphere
MB agar	Total bifidobacteria	37 ± 1 ºC	48-72 h anaerobic atmosphere

4.2.8. Analysis of PTSO residues in egg samples

As it was mentioned in Chapter 2, PTSO reacts with thiol group (-SH) of CYS and GSH, yielding CSSP and GSSP, respectively. Both CYS and GSH are present in eggs and therefore, in this chapter, PTSO and their derivatives CSSP and GSSP were monitored in egg samples. An amount of 5 ± 0.1 g of beaten egg was placed into a 50 mL conical bottom screw tube. Protein precipitation was forced using 10 mL of MeCN with 5% FA and shaken by vortex for 3 min.

Samples were centrifuged for 5 min at 5000 rpm and 3 mL of the supernatant solution was percolated through a MCX SPE-cartridge previously conditioned with 3 mL of MeOH. A washing step was carried out with 4 mL of water. In order to guarantee a total lack of washing solution in the cartridge, vacuum was applied for a few minutes until no drops were observed falling from the cartridge. The elution step was performed with 4 mL of MeOH solution containing 5% of NH_4OH. Then, 500 μL of the recollected extract were evaporated to dryness under a gentle nitrogen current and it was reconstituted with 1 mL of MeOH:water (1:1, v/v) solution. Finally, samples were analyzed by UHPLC-MS/MS using a mobile phase consisting of 0.05 % aqueous FA solution (solvent A), and MeOH (solvent B) at a flow rate of 0.4 mL min^{-1} (gradient mode). Column temperature was set at 25 ºC and the injection volume was 5 μL (full loop). The eluent gradient profile was as follows: 10 % B (2.5 min) and 100% B (3 min). It was then back to 10% B in 0.5 min and kept for 2 min for column equilibration. The MS was working with ESI^+ under MRM conditions shown in table 4.1. The target scan time established for each transition was 0.1 s. The ionization source parameters were: source temperature 500 ºC; curtain gas (nitrogen) 30 psi; ion spray voltage 5500V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi.[28] Under these conditions CSSP, GSSP and PTSO were determined in 4.0 min. A scheme of the whole sample procedure is shown in figure 4.1

28. Abad, P.; Arroyo-Manzanares, N.; García-Campaña, A.M. (2016). Anal. Methods, 8 (2016) 3730-3739.

Table 4.3. Monitored ions of the target analytes and MS/MS parameters

	Retention time (min)	Precursor ion (m/z)	Molecular ion	DP[a]	EP[a]	CEP[a]	Product ions[b]	CE	CXP[a]
CSSP	1.7	196.0	$[M+H]^+$	36.0	6.0	12.0	107.0 (Q) 179.0 (I)	11.0 17.0	4.0
GSSP	1.9	382.0	$[M+H]^+$	31.0	9.5	38.0	130.0 (Q) 150.0 (I)	24.0 27.0	4.0
PTSO	4.0	183.0	$[M+H]^+$	21.0	10.5	10.0	141.1 (Q) 99.0 (I)	13.0 19.0	4.0

[a] Declustering potential (DP), Entrance potential (EP), Collision cell entrance potential (CEP), Collision cell exit potential (CXP) and Collision energy (CE). All expressed in voltage.

[b] Product ions: (Q) Transition used for quantification, (I) Transition employed to confirm the identification.

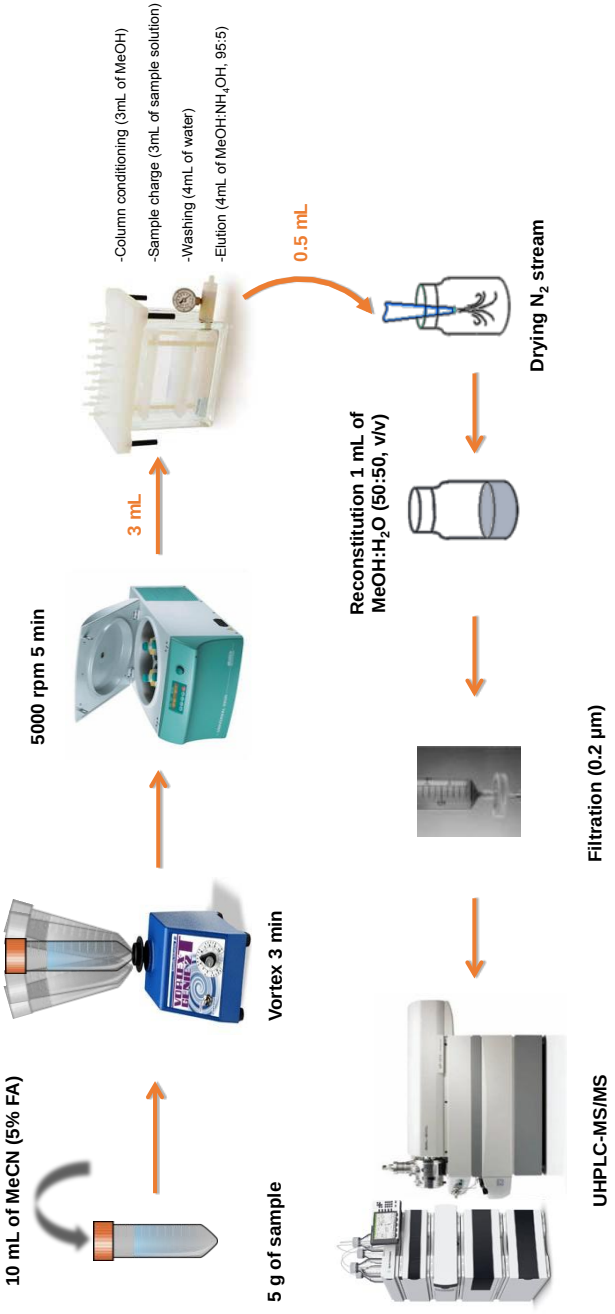

Figure 4.1. Proposed sample treatment for PTSO determination in eggs

4.3. Results and discussion

4.3.1. Egg productivity

Eggs from CG and TG groups were weekly collected, counted and weighed. A total of 847 eggs were collected (411 from CG and 436 from TG) and classified as described in section 4.2.5.[19] Results of eggs number classified by size are summarized in table 4.4.

Table 4.4. Number of eggs classified by size

Time (days)	Total eggs		M size		L size		XL size	
	CG	TG	CG	TG	CG	TG	CG	TG
0	81	84	4	6	61	64	16	14
7	83	83	3	7	72	66	8	10
14	83	90	9	5	59	59	15	26
21	85	90	7	2	60	63	18	25
28	79	89	5	1	67	62	7	26
Total	**411**	**436**	**28**	**21**	**319**	**314**	**64**	**101**

CG: Control group; TG: Treated group: XL size: > 73 g; L size: 63-73 g; M size: 53-63 g

The number of eggs type XL in TG suffered an increase of 57.8 % compared to the number of these eggs in CG throughout the field trial, whereas the number of M and L size eggs were reduced by 25 and 1.6 %, respectively. As consequence of the increase in the number of XL size eggs, average size of eggs of TG increased during this experiment.

In the interest of compare weight results obtained for TG and CG, a single factor or one-way ANOVA was carried out. CG behavior along the field trial has been considered as reference to be compared to TG, therefore the average of the results of CG was set as control/reference value. Results are shown in figure 4.2 and as can be seen, a significant increase of egg size was observed for TG from 14th day (P-value of 0.001, 0.011 and 0.034 for 14th, 21st and 28th day). Therefore, an increase of average weight was obtained as result of use PTSO as feed supplement.

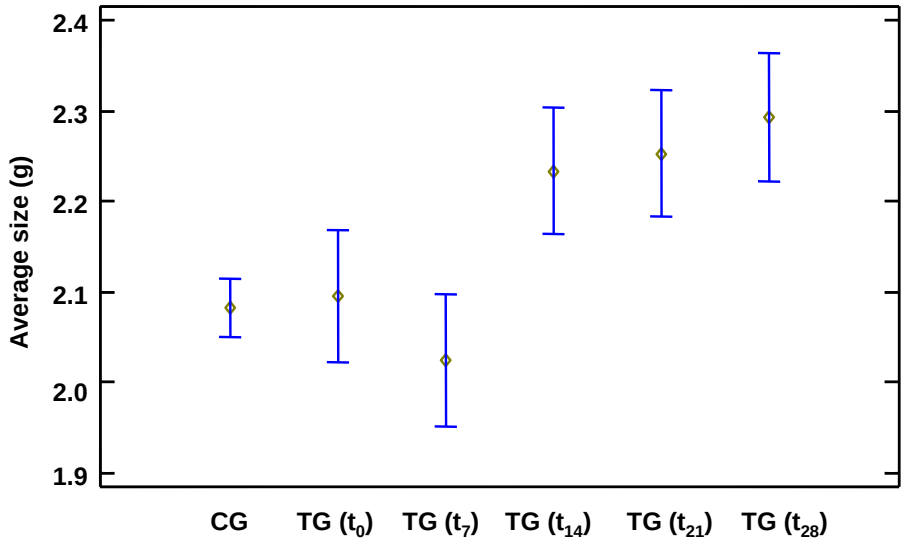

Figure 4.2. Effect of PTSO used as feed supplement on egg size. Results of single factor or one-way ANOVA average being CG: control group; TG: treated group; t_0= 0 days; t_7= 7 days; t_{14}= 14 days; t_{21}= 21 days; t_{28}= 28 days

4.3.2. Egg quality

The influence of PTSO on egg nutritional parameters was studied by analyzing energetic value, total fat, moisture, ash, total protein and total carbohydrates. Results are shown in table 4.5.

Table 4.5. Results of the proximate analysis of eggs (n=20) for TG and CG, collected at different times along the field trial

Hen's group	Time	Energetic value (Kcal)	Moisture (%)	Ash (%)	Total Proteins (%)	Total fat (%)	Total carbohydrates (%)
CG	t_0	134.9 ± 0.2	77.1 ± 0.2	0.9 ± 0.1	11.2± 0.1	8.7 ± 0.1	2.1 ± 0.1
	t_7	131.1 ± 0.1	77.1 ± 0.1	0.9 ± 0.1	11.0 ± 0.1	8.0 ± 0.1	3.1 ± 0.1
	t_{14}	132.8 ± 0.1	76.9 ± 0.1	0.9 ± 0.1	11.1 ± 0.1	8,1 ± 0.1	3.1 ± 0.1
	t_{21}	140.2 ± 0.2	76.6 ± 0.1	0.9 ± 0.1	11.1 ± 0.1	9.3 ± 0.1	2.2 ± 0.1
	t_{28}	137.9 ± 0.3	76.7 ± 0.1	0.9 ± 0.7	10.9 ± 0.1	9.0 ± 0.1	2.5 ± 0.1
TG	t_0	131.4 ± 0.1	77.1 ± 0.1	0.9 ± 0.1	11.2 ± 0.1	8.0 ± 0.1	2.8 ± 0.1
	t_7	133.3 ± 0.1	76.8 ± 0.1	0.9 ± 0.1	11.2 ± 0.1	8.1 ± 0.1	3.1 ± 0.1
	t_{14}	135.4 ± 0.1	77.1 ± 0.1	0.9 ± 0.1	11.0 ± 0.1	9.0 ± 0.1	2.1 ± 0.1
	t_{21}	138.9 ± 0.1	76.8 ± 0.1	0.8 ± 0.1	11.2 ± 0.1	9.1 ± 0.1	2.4 ± 0.1
	t_{28}	132.8 ± 0.1	76.9 ± 0.1	0.9 ± 0.1	11.0 ± 0.1	8.1 ± 0.1	3.0 ± 0.1

CG: Control group; TG: Treated group; t_0: 0 days; t_7: 7 days; t_{14}: 14 days; t_{21}: 21 days; t_{28}: 28 days

In order to compare the obtained results for TG and CG, single factor or one-way ANOVA was also carried out for each parameter using the average of the results of CG as control/reference value. The results are shown in figure 4.4. As can be seen, no significant differences were observed for egg nutritional parameters, obtaining P-values higher than 0.05 in all the cases.

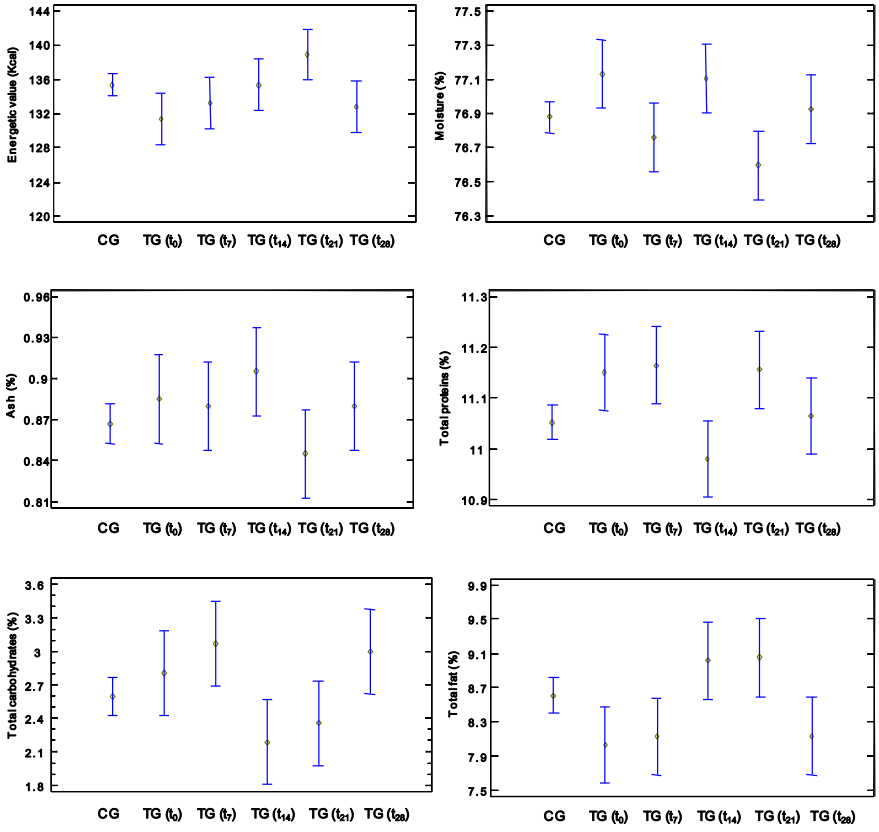

Figure 4.4. Effect of PTSO used as feed as supplement on the egg nutritional profile. Results of single factor or one-way ANOVA average being CG: control group; TG: treated group; t_0= 0 days; t_7= 7 days; t_{14}= 14 days; t_{21}= 21 days; t_{28}= 28 days

Egg amino acids content was also monitored in both experimental groups (TG and CG) as quality control parameter. Egg is characterized by the presence of 17 amino acids (figure 4.5): Aspartic acid (ASP), SER, GLY, Glutamic acid (GLU), Histidine (HIS), Arginine (ARG), Threonine (THR), Alanine (ALA), Proline (PRO), CYS, Tyrosine (TYR), Valine (VAL), MET, LYS, Isoleucine (ILE), Leucine (LEU), Phenylalanine (PHE).[29] Therefore, all this amino acid were analyzed according to the Waters methodology indicated in section 4.2.6.[26]

Results are shown in table 4.6. Results of ANOVA indicated that there were not significant differences between CG and TG (P-value>0.05 in all cases).

29. Ministry of Agriculture, Food and Environment (Spain). (2017). Huevo.org. URL. http://www.huevo.org.es/huevo_salud_composicion_aminoacidos.asp. (Friday, October 27, 2017).

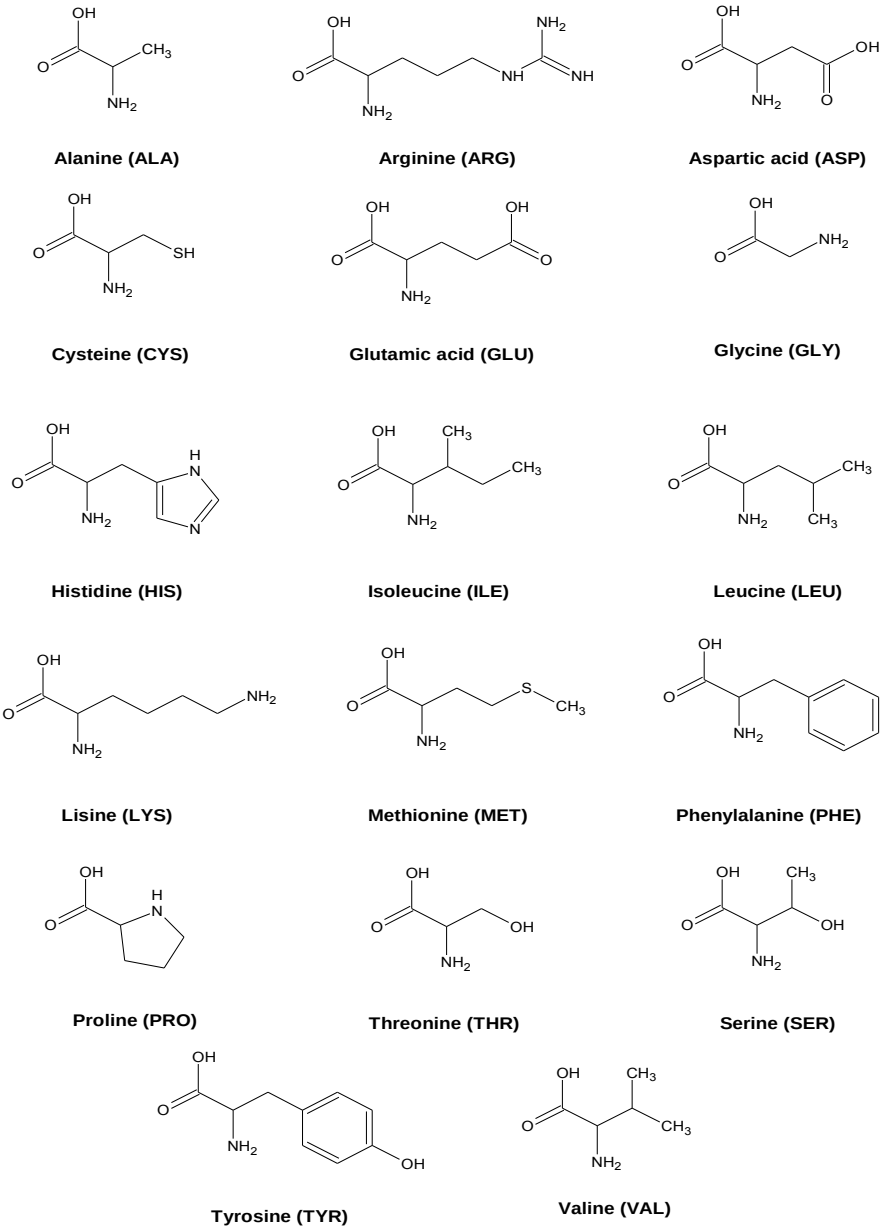

Figure 4.5. Chemical Structure of typical amino acids present in egg

Table 4.6. Content of amino acids for eggs of the control and treated groups, collected at different times along the field trial

	Time	PHE	LEU	ILE	LYS	MET	VAL	TYR	CYS	PRO	ALA	THR	ARG	HIS	GLU	GLY	SER	ASP
CG	t_0	8.6± 0.2	8.8 ± 0.1	5.4 ± 0.1	6.0 ± 0.1	3.8 ± 0.1	7.2 ± 0.1	3.9 ± 0.1	4.2 ± 0.1	6.9 ± 0.2	8.1 ± 0.1	5.5 ± 0.1	9.2 ± 0.1	4.3 ± 0.3	7.04 ± 0.2	10.1 ± 0.2	9.3 ± 0.1	8.7 ± 0.2
	t_7	6.4± 0.1	8.5 ± 0.2	5.2 ± 0.1	5.5 ± 0.1	3.8 ± 0.1	7.4 ± 0.1	3.8 ± 0.1	4.3 ± 0.1	6.8 ± 0.1	7.9 ± 0.1	6.0 ± 0.1	8.5 ± 0.1	4.5 ± 0.1	10.1 ± 0.3	11.0 ± 0.2	10.1 ± 0.1	9.4 ± 0.2
	t_{14}	6.7 ± 0.1	7.7 ± 0.1	4.7 ± 0.1	5.0 ± 0.1	3.4 ± 0.1	6.5 ± 0.1	3.5 ± 0.1	3.8 ± 0.1	6.5 ± 0.1	6.8 ± 0.1	5.3 ± 0.1	8.1 ± 0.1	4.0 ± 0.2	7.9 ± 0.2	8.9 ± 0.2	8.9 ± 0.1	7.8 ± 0.2
	t_{21}	6.7 ± 0.1	7.0 ± 0.2	4.3 ± 0.1	4.2 ± 0.1	3.2 ± 0.1	5.8 ± 0.1	3.3 ± 0.1	3.6 ± 0.1	5.7 ± 0.1	6.1 ± 0.1	4.8 ± 0.1	7.5 ± 0.1	3.7 ± 0.1	7.5 ± 0.2	6.8 ± 0.2	7.9 ± 0.1	6.0 ± 0.2
	t_{28}	8.0 ± 0.1	10.0 ± 0.1	5.9 ± 0.1	6.2 ± 0.3	4.3 ± 0.1	8.4 ± 0.2	4.2 ± 0.1	4.7 ± 0.1	8.2 ± 0.1	9.0 ± 0.2	6.7 ± 0.1	10.4 ± 0.1	5.1 ± 0.1	10.3 ± 0.2	11.2± 0.3	11.3 ± 0.1	10.0 ± 0.3
TG	t_0	8.5 ± 0.1	8.8 ± 0.2	5.4 ± 0.2	6.0 ± 0.3	3.8 ± 0.1	7.3 ± 0.2	3.9 ± 0.1	4.1 ± 0.1	6.9 ± 0.1	8.6 ± 0.2	5.3 ± 0.1	9.3 ± 0.1	4.2 ± 0.1	7.2 ± 0.2	10.0 ± 0.2	9.2 ± 0.1	8.6 ± 0.2
	t_7	7.8 ± 0.1	9.0 ± 0.2	5.4 ± 0.1	6.3 ± 0.1	3.8 ± 0.1	7.6 ± 0.1	3.5 ± 0.1	4.2 ± 0.1	7.2 ± 0.1	8.2 ± 0.1	5.7 ± 0.1	10.9 ± 0.1	4.5 ± 0.1	7.4 ± 0.2	10. ± 0.2	9.6± 0.1	9.9 ± 0.2
	t_{14}	9.3 ± 0.2	12.7 ± 0.3	7.5 ± 0.2	9.0 ± 0.5	5.1 ± 0.1	10.6 ± 0.1	4.9 ± 0.1	5.6 ± 0.2	9.8 ± 0.2	11.8 ± 0.3	7.7 ± 0.1	12.3 ± 0.1	5.7 ± 0.2	6.7 ± 0.2	15.1 ± 0.4	13.1 ± 0.1	13.8 ± 0.4
	t_{21}	8.5 ± 0.1	9.8 ± 0.2	5.9 ± 0.1	7.0 ± 0.3	4.0 ± 0.1	8.3 ± 0.1	3.6 ± 0.1	4.3 ± 0.1	7.7 ± 0.1	8.7 ± 0.1	5.9 ± 0.1	11.6 ± 0.1	4.7 ± 0.1	6.2 ± 0.1	11.4 ± 0.2	10.1 ± 0.1	10.6 ± 0.2
	t_{28}	8.8 ± 0.1	9.9 ± 0.2	5.8 ± 0.1	6.4 ± 0.2	4.2 ± 0.1	8.1 ± 0.1	4.2 ± 0.1	4.5 ± 0.1	7.7 ± 0.1	8.5 ± 0.1	6.1 ± 0.1	9.9 ± 0.1	4.7 ± 0.1	7.4 ± 0.1	11.9 ± 0.1	10.1 ± 0.1	9.1 ± 0.2

4.3.3. Microbiological analysis

PTSO effect on intestinal microbiota was studied since it plays a key role in animal health and may be influenced by various factors such as diet, environment, stress or the presence of pathogens.[30]

Intestinal microbiota (Enterobacteriacae, *Clostridium spp.*, *Lactobacillus spp.* and *Bifidobaterium spp.*) from CG and TG was analyzed weekly. A single factor or one-way ANOVA was carried out for each intestinal microbiota parameter using the average of the results of CG as control/reference value, in order to compare TG and CG. Results (table 4.7) confirmed that the use of PTSO as feed supplement has a significant effect on the intestinal microbiota by reducing of Enterobacteriacae in hen's feces after 14 days of treatment. This trend observed for 14th day (P-value=0.005), 21st (P-value=0.016) and time 28th (P-value=0.001). No significant effects derived of the intake of PTSO were detected for anaerobic total bacteria, *Clostridium spp.* and *Enterococcus spp*. Enterobacteriacae include *Salmonella spp.* whose prevention is one of the major challenges in poultry production. PTSO supplementation reduces this pathogen which could translate in a decrease of associated animal pathologies such as salmonellosis. The reduction of the fecal contmination in eggs ensures animal and food safety.[31]

30. Gabriel, I. ; Mallet, S. ; Sibille, P. INRA Production Animals, 18 (2005) 309-322.

31. Chambers, J.J.; Gong, J. Food Res. Int., 44 (2011) 3149-3159.

Table 4.7. Microbiological analysis results expressed as Log UFC/g (RSD in parenthesis)

Hen's group	Time	*Bifidobacterium spp.*	*Enterococcus spp.*	*Lactobacillus spp.*	*Clostridium spp.*	*Anaerobic total bacteria*	*Enterobacteriaceae*
CG	t_0	5.7 ± 0.2	7.5 ± 0.1	8.5 ± 0.2	7.4 ± 0.2	8.5 ± 0.1	6.8 ± 0.3
	t_7	5.3 ± 0.2	7.5 ± 0.1	8.4 ± 0.1	7.7 ± 0.2	8.5 ± 0.2	6.8 ± 0.1
	t_{14}	5.7 ± 0.2	7.6 ± 0.1	8.6 ± 0.1	7.7 ± 0.1	8.6 ± 0.1	7.2 ± 0.3
	t_{21}	5.9 ± 0.1	7.4 ± 0.3	8.6 ± 0.1	7.6 ± 0.2	8.9 ± 0.1	6.8 ± 0.1
	t_{28}	5.7 ± 0.1	7.3 ± 0.1	8.6 ± 0.1	7.9 ± 0.	8.6 ± 0.1	7.2 ± 0.1
TG	t_0	5.6 ± 0.1	7.4 ± 0.2	8.6 ± 0.1	7.7 ± 0.1	8.5 ± 0.1	6.6 ± 0.2
	t_7	5.8 ± 0.2	7.6 ± 0.1	8.9 ± 0.1	7.7 ± 0.1	8.6 ± 0.1	6.2 ± 0.1
	t_{14}	6.9 ± 0.3	7.5 ± 0.3	10.0 ± 0.6	8.0 ± 0.1	9.0 ± 0.1	5.7 ± 0.2
	t_{21}	6.7 ± 0.2	7.6 ± 0.1	10.1 ± 0.2	7.9 ± 0.5	8.9 ± 0.1	5.8 ± 0.2
	t_{28}	6.4 ± 0.2	7.5 ± 0.1	9.8 ± 0.3	7.5 ± 0.5	8.7 ± 0.3	5.4 ± 0.1

CG: Control group; TG: Treated group; t_0: 0 days; t_7: 7 days; t_{14}: 14 days; t_{21}: 21 days; t_{28}: 28 days

Related to intestinal microbiota, there was an increase from TG respect to CG of *Lactobacillus spp.* at 14th day (P-value<0.0001), 21st day (P-value<0.0001) and 28th day (P-value=0.001) and the same behaviour was observed for total bifidobacteria population at 14th day (P-value=0.000), 21st (P-value=0.000) and 28th day (P-value=0.022). Statistical results are shown in figure 4.5.

Lactobacillus spp. and *Bifidobaterium spp.* population are associated to the inhibition of pathogen growth; they are also closely related to the increase in nutrient absorption efficiency as well as the improvement on the inmunostimulatory effect.[32,33] Both *Lactobacillus spp.* and *Bifidobaterium spp.* are increased by diet supplement with PTSO therefore it could be said that the inclusion of PTSO in laying hens diet have a positive intestinal microbiota modulation.

32. Shah, N.P. J. Dairy Sci., 4 (2000) 894-907.

33. Pan, D.; Yu, Z. Gut Microbes, 5 (2014) 108-119.

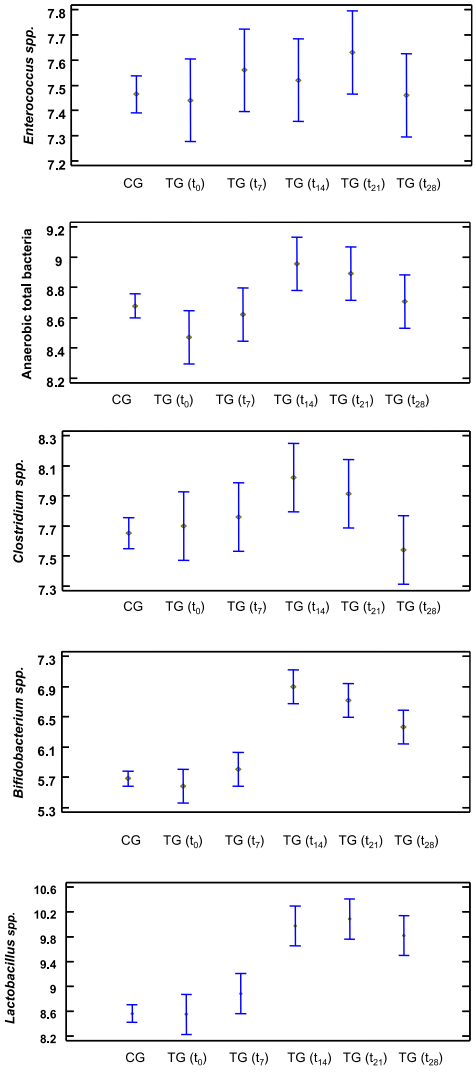

Figure 4.5. Intestinal microbiota ANOVA results for Enterobacteriacae, anaerobic total bacteria, *Clostridium spp.*, *Lactobacillus spp.* and *Bifidobaterium spp*. CG: Control group; TG: Treated group**;** t_0: 0 days; t_7: 7 days; t_{14}: 14 days; t_{21}: 21 days; t_{28}: 28 days

4.3.4. Analysis of PTSO residues in egg samples

In order to establish a methodology to control the sensory attributes of eggs, a new analytical method to determine PTSO and/or its derivatives is mandatory. In this chapter, the analytical method for determining PTSO residues in egg was developed taking into account that PTSO reacts with the thiol group (-SH) of CYS and GSH (both present in egg), producing CSSP and GSSP, respectively.[33]

4.3.4.1. Optimization of sample treatment

All the experiments were performed using samples of 5 ± 0.1 g of beaten eggs from battery cage farming hens spiked with 200 mg kg^{-1} of PTSO. In order to achieve full homogenization, the sample was placed in a conical bottom screw tube and it was vortexed for 1 min after PTSO addition. Numerous methods have been development for protein precipitation in egg samples in order to remove interferences in the sample treatment.[34] Acids like TCA or organic solvents are well-known protein-precipitating agents.[35] In this chapter, for PTSO, CSSP and GSSP determination, protein precipitation was carried out by sample centrifugation upon adding a protein precipitation agent. Firstly, several precipitation solvents were tested using a volume of 10 mL in all cases: 20% of TCA in water, MeOH: H_2O (1:1) with 20% of TCA, MeOH, ACO, MeCN and EtAc. Results are shown in figure 4.6 and as can be seen the best results in terms of signal intensity were obtained for MeCN. In any case, PTSO was found

34. Trziszka, T.; Różański, H.; Polanowski, A. J. Life and Sci., 8 (2013) 862-877.

35. Sivaraman, T.; Kumar, T. K. S.; Jayaraman, G.; Yu, C. J. Protein Chem., 16 (1997) 291-297.

in the final extract, demonstrating that PTSO reacts completely with the amino acids giving CSSP and GSSP, and optimization of sample treatment was therefore focused on the extraction of these two corresponding derivatives.

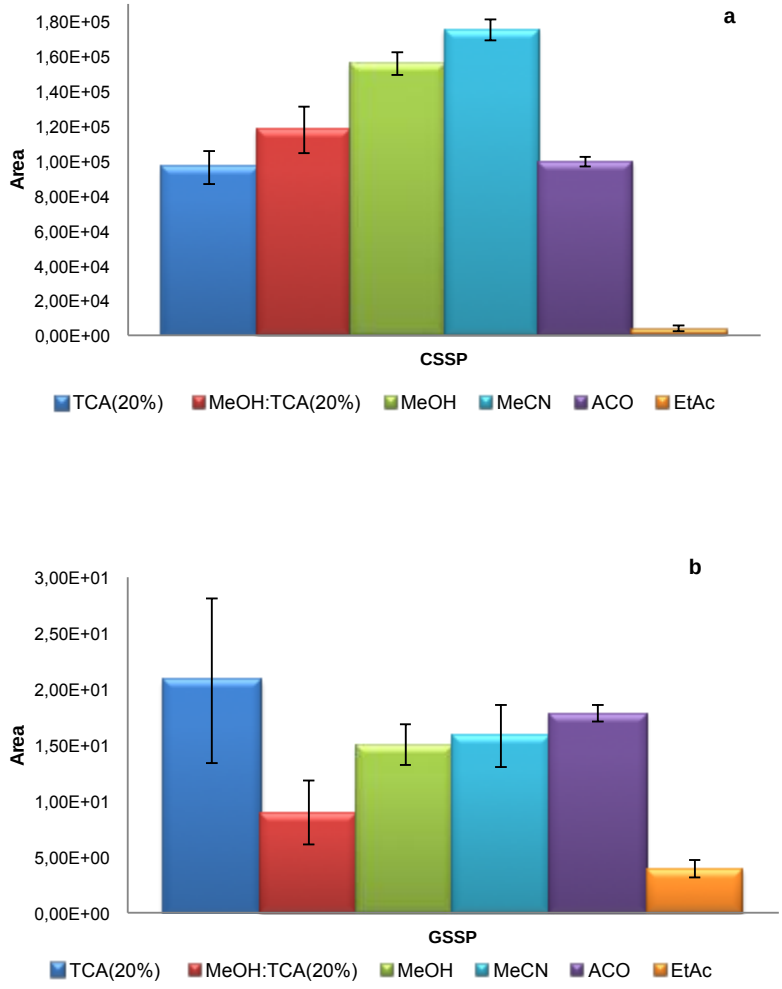

Figure 4.6. Effect of precipitation solvent on (a) CSSP and (b) GSSP peak area

Once, precipitation solvent was added to the egg samples, they were shaken before centrifuging. Vortex (1-3 min) and a polytron (1-3 min) were checked as agitation mechanisms and the most efficient in terms of signals was vortex for 3 min (figure 4.7).

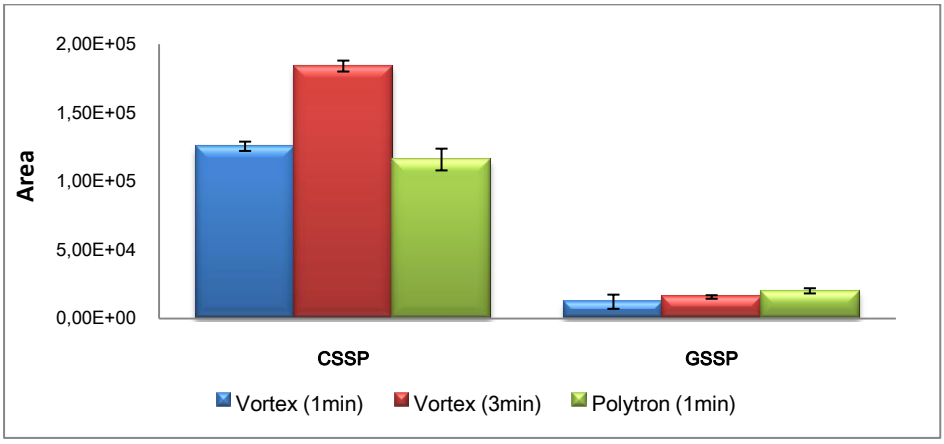

Figure 4.7. Effect of shaking mode on CSSP and GSSP peak area

Egg is a very complex matrix, hence a SPE procedure has been proposed for sample clean-up. Different SPE cartridges were tested in order to evaluate the best option to retain CSSP and GSSP: Silica, MCX and HLB. The applied protocols of conditioning, loading, and elution are shown in table 4.7.

Table 4.7. Study of the SPE procedure for the extraction of residues of PTSO derivatives(CSSP and GSSP) in egg using different sorbents

	Silica	**MCX**	**HLB**
Conditioning (3 mL)	Hexane	MeOH	MeOH/H_2O
Loading (4 mL)	Sample solution in MeCN	Sample solution in MeCN (5 % FA)	Sample solution in MeCN (5 % FA)
Elution (4 mL)	Cl_2CH_2	MeOH:NH_4OH (95:5)	MeOH
Total recovery (CSSP+GSSP) (%)	14.9	95.8	38.3

In order to evaluate the SPE extraction efficiency, a standard solution of CSSP and GSSP at 200 mg kg^{-1} was prepared in MeCN for Silica and in MeCN with 5% FA for MCX y HLB. After elution, in all cases, 1mL was evaporated to dryness under a gentle nitrogen current and reconstituted with 1 mL of injection solvent (MeOH:water, 1:1, v/v). Since no dilution or preconcentration were carried out in any of SPE protocols, recoveries were calculated as [(sum of signal of CSSP and GSSP in the final extract / sum of a standard solution of CSSP and GSSP at 200 mg kg^{-1} in injection solvent (MeOH:water, 1:1, v/v)]$\times$100.

The highest recovery percentages (table 4.6) were obtained using MCX, which was therefore selected as optimum. Subsequently, the optimization of the different parameters affecting the SPE extraction process was carried out in egg sample in order to get the best efficiency in the extraction of CSSP and GSSP. Conditioning solvent volume of MeOH was studied from 3 to 5 mL no significant

influence on the recovery percentage was observed, so 3 mL (minimum volume recommended by the supplier) of MeOH was chosen as optimum.

The influence of FA addition in the extraction solvent (MeCN) was also studied between 0-10% and better retention was obtained for 5% of FA, so it was selected as optimum (figure 4.8).

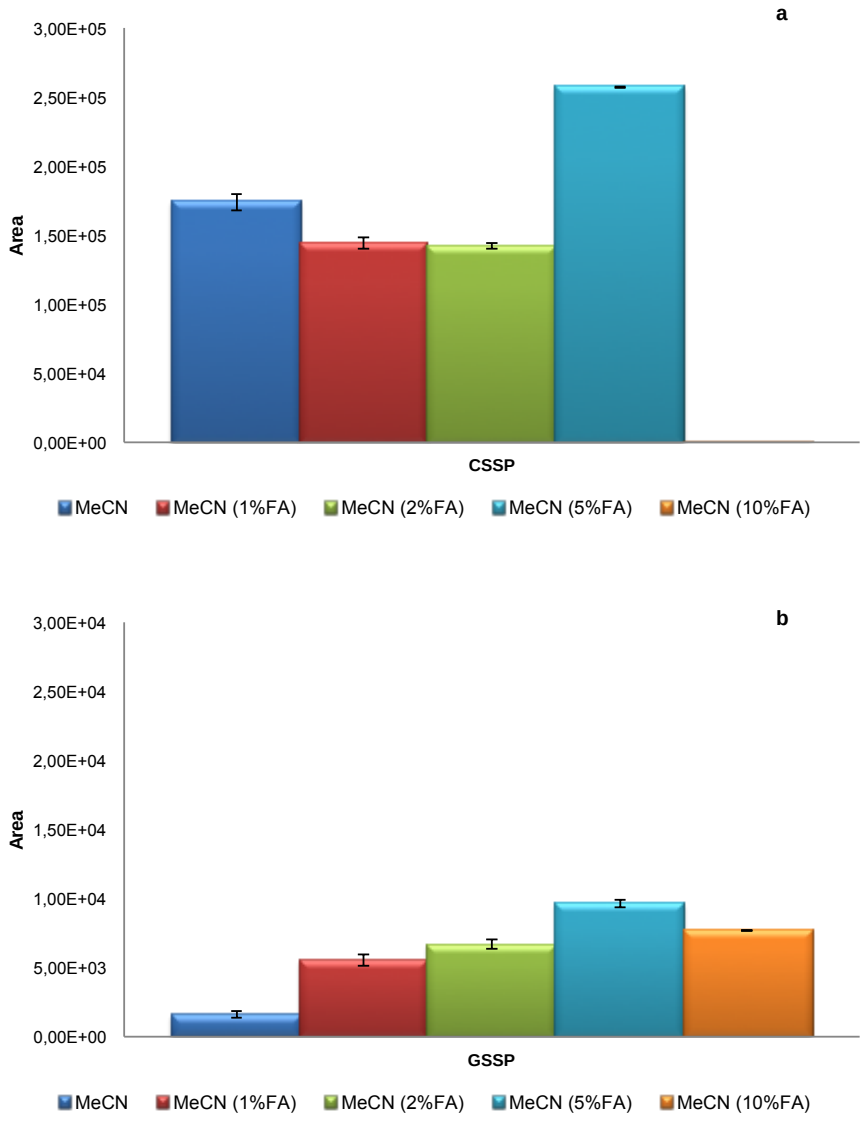

Figure 4.8. Effect of solvent acidification with FA on (a) CSSP and (b) GSSP peak area

Lastly, load sample volume was investigated between 3-5 mL. It was observed that higher sample loading volumes, worse results in terms of recovery percentages were obtained (figure 4.9). This could be due to the presence of matrix components which could saturate the MCX column. Therefore, best signal intensity was obtained for 3 mL, and this volume was used as optimum.

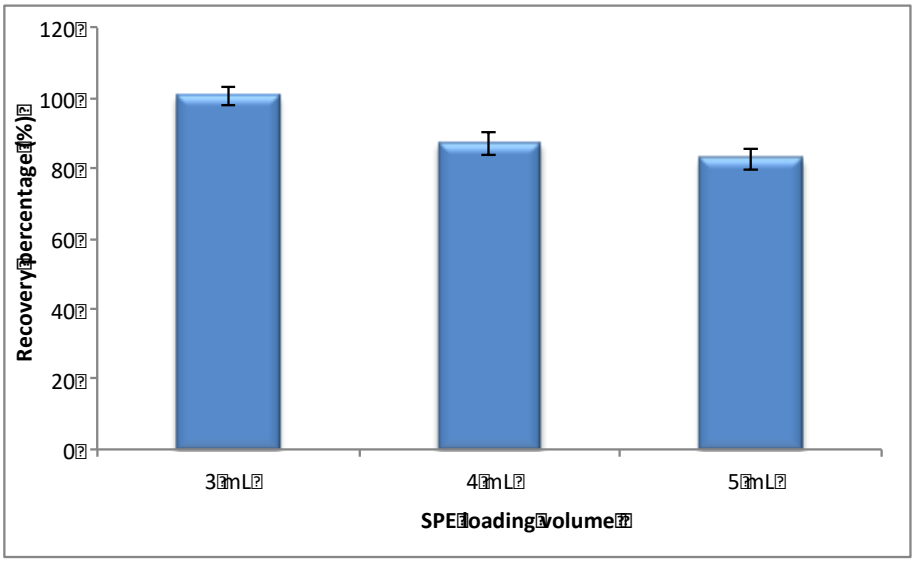

Figure 4.9. Effect of SPE loading volume on recovery values expressed as sum of CSSP and GSSP signals

The introduction of a washing step was evaluated in order to obtain a cleaner extract and consequently a reduction the matrix effect. Aliquots of 3 mL of water with different concentrations of FA (0, 1, 2 and 3%) were tested and a decrease in the analytical signal was observed when the percentage of FA was increased. This inversely proportional relationship between the acid percentage and CSSP and GSSP retention may be associated with a less efficient clean-up of the

interferences retained providing an extract, which could produce lower signals due to ion suppression, therefore, acid was avoided.

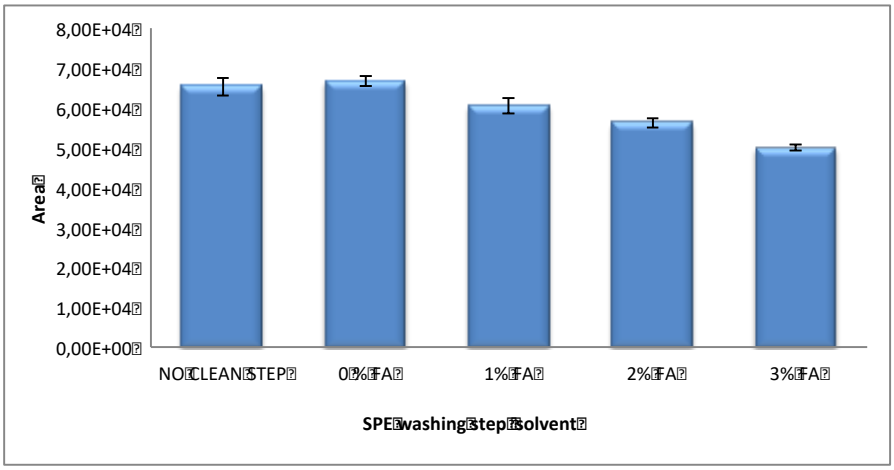

Figure 4.10. Effect of the use of FA in washing step on peak area expressed as sum of CSSP and GSSP

To conclude, the volume of water used in the washing step was evaluated between 2-5 mL, and 4 mL were selected (figure 4.11). Because of the high cartridge volume, it was necessary to apply vacuum after the washing and elution steps in order to remove the washing solvent from the cartridge before elution.

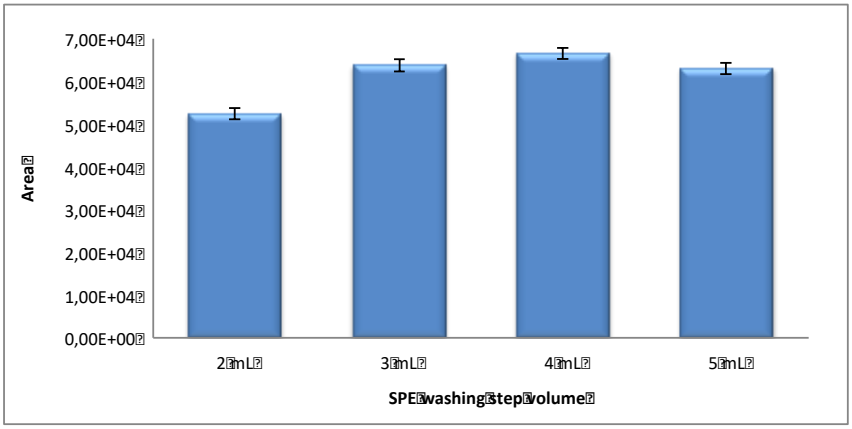

Figure 4.11. Effect of washing step water volume on peak area expressed as sum of CSSP and GSSP

Also, the influence of the elution solvent volume was studied between 1-4 mL, and 4 mL of MeOH:NH_4OH (95:5) were selected as the best option in order to elute efficiently all the retained compounds (figure 4.12).

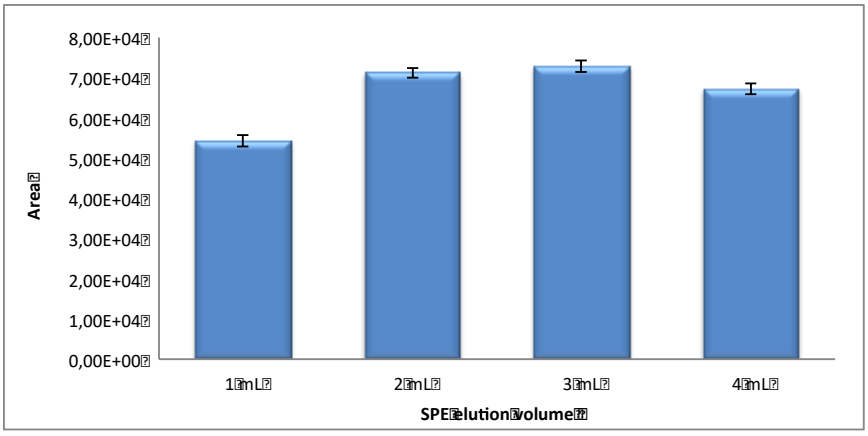

Figure 4.12. Effect of MeOH:NH_4OH (95:5) volume (elution solvent) on peak area expressed as sum of CSSP and GSSP

Finally, the volume of dried organic phase was studied between 0.5 and 2 mL. In all the cases, the residue was reconstituted with 1 mL of MeOH:water (50:50). The best results were obtained using 0.5 mL, since higher volumes involve an increase in the matrix effect.

A summary of the final sample treatment is included in section 4.2.8 and a chromatogram of an egg sample submitted to the optimized sample treatment is shown in figure 4.13.

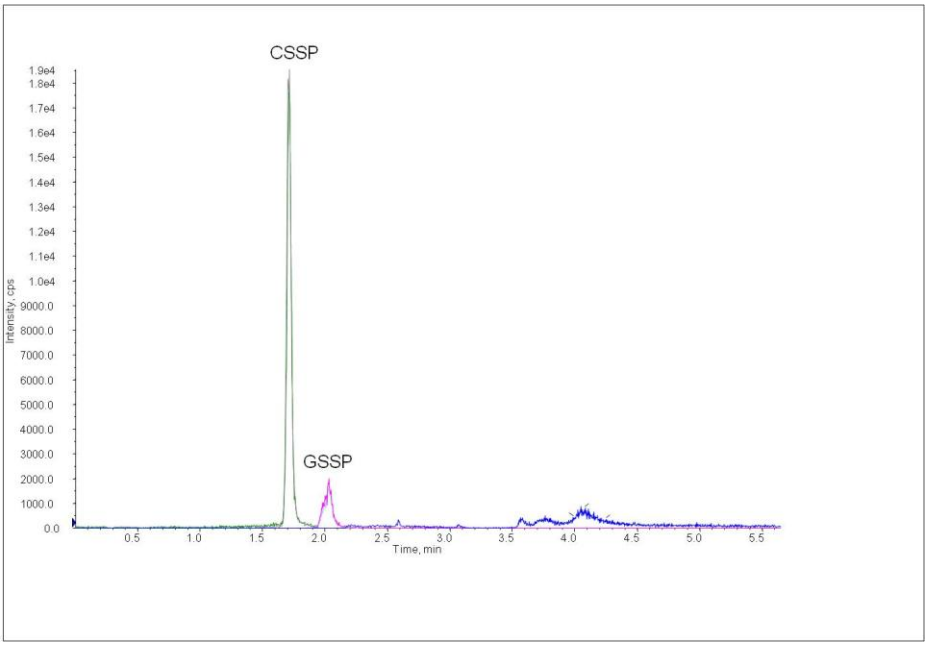

Figure 4.13 Chromatogram of an egg sample from a free farming hen spiked with 2.5 mg kg^{-1} of PTSO and subjected to the proposed method

4.3.4.2. Characterization of the SPE-UHPLC-MS/MS method in egg sample

In order to check the suitability of the method for the monitoring of possible residues of PTSO, a full characterization was carried out for eggs of category 3 (battery cage farming), type of eggs obtained from the animal trial. However, with a view to future experiments, the developed method was also characterized for eggs of category 2 (free-farming). Since, feed for hens that produce eggs of categories 1 and 0 could not be supplemented with PTSO; method characterization was not considered for these kind of egg.

For characterization, different parameters such as linear dynamic range, R^2, LOD and LOQ and precision were evaluated for each matrix. PTSO residues were monitored as sum of its derivatives (CSSP and GSSP).

Matrix-matched calibration curves were obtained using egg samples spiked at six different concentrations of PTSO (from 0.250 to 25 mg kg^{-1}). Each concentration level was prepared in duplicate, submitted to the subsequent sample treatment and injected in triplicate. Sum of CSSP and GSSP peak area was considered as analytical signal. Prior to that, blank egg samples from laying hens whose diet was PTSO-free were analyzed in order to check a possible presence of PTSO, CSSP or GSSP and none of them gave a positive result. Statistical parameters were calculated by least-square regression, while LODs and LOQs were calculated as 3·S/N ratio and 10·S/N ratio, respectively. The results are summarized in table 4.8. The satisfactory determination coefficients confirm that PTSO analytical responses were linear over the studied range. As can be seen, low LOQs were obtained for both types of eggs.

Table 4.8. Statistical and performance characteristics for the SPE-UHPLC-MS/MS proposed method for the determination of PTSO residues.

Matrix	Linear dynamic range ($\mu g\ kg^{-1}$)	R^2	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)
Free farming eggs	237-25000	0.995	71.7	237.0
Battery cage farming eggs	211.3-25000	0.994	63.4	211.3

The precision of the whole method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was assessed for samples from the two studied egg types by application of the whole procedure on the same day to three samples of each kind (experimental replicates) spiked at three concentrations levels of PTSO (0.250, 2.5 and 25 mg kg^{-1}). Each extract was injected in triplicate (instrumental replicates). Intermediate precision was evaluated with a similar procedure, by spiking and analyzing samples injected in triplicate, during five different days. The results, expressed as relative standard deviation (%RSD) of peak areas are shown in table 4.9. As it can be seen, satisfactory precision with RSD lower than 10 % was obtained.

Table 4.9. Precision (% RSD of peak areas) of the proposed method for the monitoring of PTSO residues in eggs

	Repeatability (n=9)		Intermediate precision (n=15)	
Level	**Free-farming**	**Battery cage farming**	**Free-farming**	**Battery cage farming**
0.25 mg kg^{-1}	5.1	3.9	9.3	5.9
2.5 mg kg^{-1}	2.2	5.2	8.2	7.1
25 mg kg^{-1}	6.2	4.1	8.6	7.7

4.3.4.3. Monitoring of PTSO residues in egg samples

According to the procedure described in section 4.2.8, collected eggs were treated and analyzed by the proposed UHPLC-MS/MS method. Twenty egg samples from CG and TG respectively were analyzed weekly. The presence of PTSO or its derivatives (CSSP and GSSP) was not detected in any sample; in other words, PTSO residues that could affect organoleptic properties of eggs (odour and flavour) were not detectable when using the recommended and tested doses.

4.4. Conclusions

In this chapter, we have demonstrated the effect of the intake of PTSO as feed supplement on the productivity of laying hens, increasing the size and weight of eggs. In addition, healthy effects were also observed. Evaluation of intestinal microbiota has shown that PTSO reduced Enterobateriacae population, which could trigger a decrease of animal pathologies such as salmonellosis. Moreover, the increase in *Lactobacillus spp.* and *Bifidobacterium spp.* populations populations may imply an improved immune system in hens. Lastly, this study has proven that the use of PTSO as does not alter egg nutritional profile nor egg organoleptic properties, since residues of PTSO or its derivatives were not found in egg samples using a proposed SPE-UHPLC-MS/MS method. In light of all of the above, the beneficial effects of PTSO as feed supplement for laying hens have been established.

This work has been submitted for publication as:

Evaluation of onion extract as feed supplement for poultry: study of its influence on egg quality, productivity and health status of laying hens. Abad, P., Arroyo-Manzanares, N., Ariza, J.J., Domínguez B., Baños, A., García Campaña, A.M. J. Agric. Food Chem.

CHAPTER 5

Effects of different vehiculization strategies for propyl propane thiosulfonate during dynamic simulation of the pig gastrointestinal tract.

Efectos de diferentes estrategias de vehiculación para el propil propano tiosulfonato durante la simulación dinámica del tracto gastrointestinal del cerdo.

Resumen

Este capítulo evalúa la biodisponibilidad del propil propano tiosulfonato (PTSO) en el tracto gastrointestinal del cerdo por medio de un sistema de simulación gastrointestinal dinámico *in vitro* (GITSS) basado en un biorreactor de membrana que simula las condiciones en del estómago y el duodeno. El sistema consta de un reactor continuo tipo tanque agitado (CSTR), conectado en serie a un reactor tubular de flujo continuo (PFR) equipado con una membrana tubular de microfiltración de cerámica que simula las condiciones y microvellosidades a lo largo del intestino. Los valores de biodisponibilidad han sido evaluados para diferentes estrategias de vehiculación del PTSO, incluyendo monooleato de sorbitán de polietilenglicol (un tensioactivo no iónico comúnmente conocido como tween 80) y matrices de encapsulación (β-ciclodextrina frente a mono- y diglicéridos de ácidos grasos comestibles mezclados con aceite de girasol hidrogenado) y comparados con la absorción de PTSO libre. Tras la simulación, se demostró que la cantidad neta de PTSO absorbido en el intestino simulado cuando se usa tween 80 como vehiculizante (calculada como la cantidad neta en la corriente de permeado después de la simulación de estómago/duodeno/intestino dividida por la cantidad total alimentada) es casi tres veces mayor que la obtenida para el PTSO libre. Sin embargo, los valores de absorción obtenidos para el PTSO encapsulado en β-ciclodextrina o en mono- y diglicéridos de ácidos grasos mezclados con aceite vegetal no mejoraron respecto a los de PTSO libre. Estos prometedores resultados, indican que el PTSO soportado con tween 80 muestra una alta e interesante resistencia a las condiciones digestivas simuladas en el estómago y, por lo tanto, favorece la absorción de PTSO a lo largo del intestino.

Abstract

This chapter evaluates the bioavailability of allium derivative propyl propane thiosulfonate (PTSO) in the pig gastrointestinal tract by means of an *in-vitro* dynamic gastrointestinal tract simulator system (GITSS). GITSS is based on a membrane bioreactor comprising a continuous stirred tank reactor (CSTR), simulating the conditions in the stomach and duodenum, connected in series to a continuous plug-flow tubular reactor (PFR) equipped with a tubular ceramic microfiltration membrane simulating the conditions and microvilli along the intestine. Bioavailability values have been evaluated for different vehiculization strategies, including mere carriers such as polyethylene glycol sorbitan monooleate (a nonionic surfactant also known as tween 80), and encapsulation matrices (β-cyclodextrin vs mono- and diglycerides of edible fatty acids mixed with hydrogenated sunflower oil) and compared with the absorption of free PTSO. The net absorbed amount of PTSO in the GITSS when tween 80 was used as a carrier (calculated as the net amount in the permeate stream after the stomach / duodenum / intestine simulation divided by the total fed amount) was found to be over almost 3 times higher than the one for free PTSO. Neither the encapsulated PTSO in β-cyclodextrin nor by means of mono- and diglycerides of fatty acids plus a vegetable oil succeeded to improve absorption values for free PTSO. These promising results indicate that tween 80 provides to the PTSO molecule an interesting and high resistance against the simulated digestive conditions in the stomach and thus enables favorably the subsequent absorption process of PTSO along the intestine.

5.1. Introduction

The effects of essential oils on pig performance, nutrient utilization, immune response and intestinal health are widely known.[1] Recently, the positive effects derived of the use of PTSO as feed supplement in animal feed has been studied in pigs.[2,3] PTSO has been proposed as alternative to AGP due its proven beneficial effects on animals.[4-6] As it has been described in previous chapters, onion is a product naturally enriched in volatile sulfur compounds, as well as the rest *Allium spp.*, and is characterized by its pungency and strong odor. PTSO comes from the natural reaction between the flavor precursor s-propyl-L-cysteine sulfoxide (propiin) characteristic of onion bulbs and the enzyme alinase, common to all *Allium spp.* when onion tissues are cut or damaged.[7] The whole proposed biosynthesis pathway, detailed in the Introduction of the present thesis, is shown in figure 5.1.

PTSO has shown antibacterial activity against pigs predominant bacterial groups (total aerobes, total anaerobes, lactobacilli, bifidobacteria, coliforms, enterobacteria, bacteroides and clostridia) including *Escherichia coli* and *Salmonella typhimurium*, two common pathogens of pigs.[2] Also, inmunomodulating effect of PTSO on pig immune

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7. Block, E. Garlic and Other Alliums: The Lore and the Science. Royal Society of Chemistry, Cambridge (2010) 100-223.

system has been proven *in vivo*.[3] To exert its beneficial effects, the PTSO must be able to tolerate the conditions of the stomach as well as in the gut.

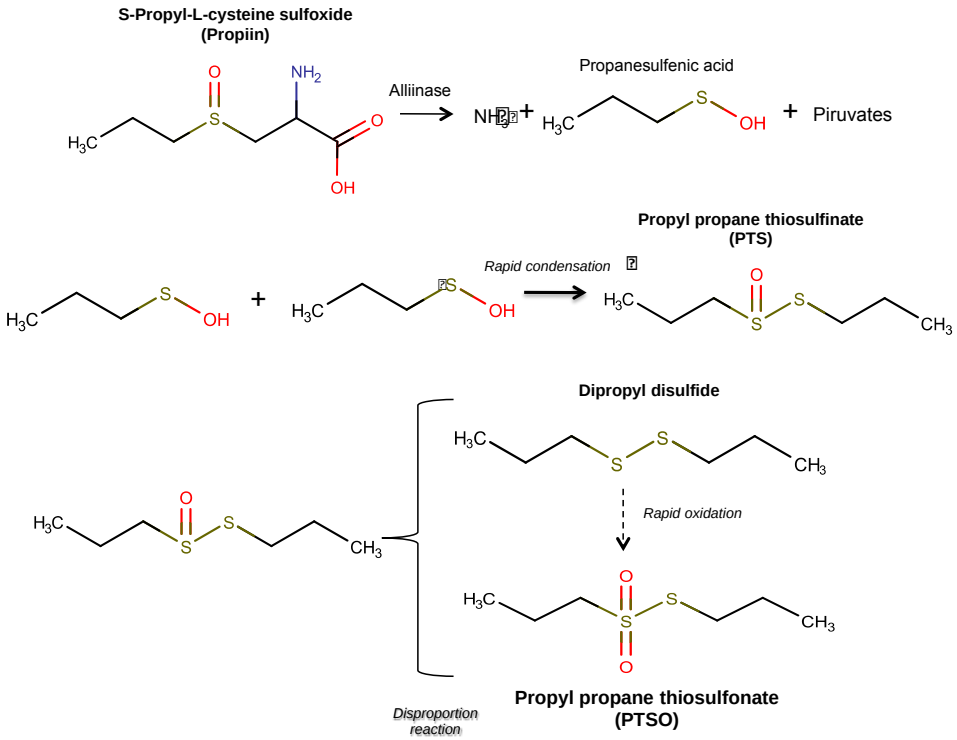

Figure 5.1. Proposed PTSO biosynthesis pathway

However, PTSO presents the inconvenience of its low solubility in aqueous medium. Octanol-water partition coefficient (K_{ow}) measures intrinsic lipophilicity (how hydrophilic or hydrophobic) of a chemical substance. K_{ow} is used to know the affinity to animal tissues of one chemical product and is useful in estimating the distribution of drugs

within the body.[8] PTSO K_{ow} value is 2.285 (lower affinity to cellular tissues), thus the development of a controlled-release delivery system by a carried material is necessary to increase PTSO cellular permeability and bioavailability.

In animal nutrition research, the methodology for the evaluation of nutrient digestion is crucial because it allows the estimation of the nutritive value of particular feedstuffs[9] but also the bioavailability of drugs[10] and feed supplements.[11,12] *In vitro* digestion models are widely used to study structural changes, digestibility, and release of food components under simulated gastrointestinal conditions in humans and animals. Nevertheless, since the complexity of human and animal digestive tracts, these *in vitro* results do not reliably represent *in vivo* models.[13] As alternative to these systems, dynamic simulators adapted to the pig gastrointestinal digestion system constitutes a good option.. Dynamic simulation uses data obtained from *in vivo* studies in pigs, such as the pepsin activity, stomach digest pH curves and secretion rates of gastric fluid into the system to establish the work program.[14] Therefore, there is still a clear need to improve *in vitro* models in order to avoid discrepancies with posterior *in vivo* experiments. In the present chapter, a dynamic gastrointestinal tract simulating system

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10. Hébrard, G.; Blanquet, S.; Beyssac, E.; Remondetto, G.; Subirade, M.; Alri, M. J. Biotechnol. 127 (2006) 151-160.

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13. Hur, S.H.; Lim, B.O.; Decker, E.A.; McClements, J. Food Chem., 125 (2011) 1-12.

14. Chiang, C.C.; Croom, J.; Chuang, S.T.; Chiou, P.W.S.; Yu, B. Asian-Austr.J. Anim. Sci., 21 (2008) 1522-1528.

(GITSS) is used to study the stomach tolerance and *in vitro* bioavailability of PTSO (free vs. different vehiculization strategies) in pigs. To the best of our knowledge, this is the first time that bioavailability of PTSO is studied in a pig GITSS.

5.1. Materials and methods

5.1.1. Chemicals and reagents

All reagents were of analytical reagent grade. Formic acid used as mobile phase additive for LC-MS and MeOH were obtained from Sigma Aldrich (St Louis, MO, USA). HPLC-grade solvents MeOH and MeCN were purchased from VWR BDH Prolabo (West Chester, Pennsylvania, USA). Perchloric acid (70%) was supplied by Panreac (Madrid, Spain). HPLC mobile phases were filtered using a vacuum system with Millipore (Milford, MA, USA) filters (nylon, 0.20 μm, diameter 47 mm). Samples were filtered through PET Chromafil® (Macherey-Nagel, Germany) filters (polyester, diameter 25 mm, 0.20 μm).

Ultrapure water (18.2 MΩ cm^{-1}, Milli–Q Plus system, Millipore Bedford, MA, USA) was used throughout the trials.

PTSO and PTS standards (95% of purity in both cases), a blend of 40 % of PTSO supported on tween 80 (Garlicon), an encapsulated product containing 12 % of PTSO in β-cyclodextrin (Proallium) and a 8 % of PTSO encapsulated product in mono- and diglycerides of edible fatty acids mixed with hydrogenated sunflower oil (from now abbreviated as “fatty acids”) via spray-chilling (Garlicaps) were kindly provided by Domca S.A. (Alhendín, Granada, Spain).

Reagents used for the simulation of the gastrointestinal tract (GIT) fluids are detailed follow: hydrochloric acid (HCl) and sodium bicarbonate ($NaHCO_3$) both 1 M, supplied by Panreac. Pepsin from porcine gastric mucosa, lipase from porcine pancreas and bile salts were purchased from Sigma-Aldrich.

5.1.2. Instruments and methods

5.1.2.1. GITSS - SimuGIT

The dynamic *in vitro* GITSS (SimuGIT), schematically shown in figure 5.2, consists of a CSTR and a continuous PFR equipped with a tubular ceramic MF membrane, connected in series. This model was previously satisfactorily applied to study the stomach tolerance and effective delivery enhancement of nanoencapsulated macelignan through the simulated human gastrointestinal tract.[15]

15. Rivas-Montoya, E; Ochando-Pulido, J.M.; López-Romero, J.M.; Martinez-Férez, A. Chemical Eng. Sci., 140 (2016) 104-113.

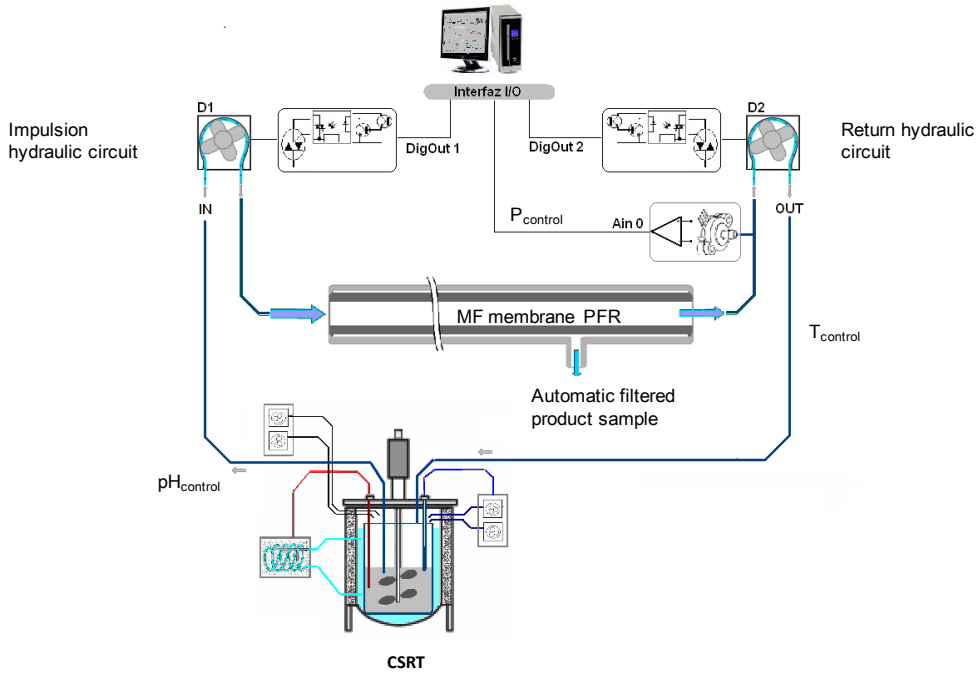

Figure 5.2. Work scheme of the dynamic *in vitro* GITSS (SimuGIT) adapted from Rivas-Montoya et al.[15]

The CSTR used to simulate the pig stomach is a fermenter (model Biostat B) supplied by Braun Biotech International. It comprises a reactor vessel (2 L) equipped with an impeller stirrer (180W, model Rushton) and a proportional-integral-derivative (PID) unit control system for the temperature (T), pH, dissolved oxygen (D_{O2}), foam and reactor level (V), all equipped with their corresponding sensors. The control system unit includes a RS-422 interface, which enables the control of the Biostat B CSTR with a computer. The CSTR is provided with a jacket to maintain the temperature constant, such that the temperature in the vessel is measured by means of a Pt-100 digital sensor and accurately controlled ($T_{set\ point}$ ± 0.1 °C) by the PID loop connected to a

cooler/chiller. The stirring rate ranged between 50 and 150 rpm. The CSTR system is also provided with sampling and reagents addition inlets by means of peristaltic pumps (Eyela, modelMP-3). The pH inside the CSTR could be measured by a crystal pH electrode provided by Hamilton (model Easyferm Plus K8) immersed in the vessel and adjusted by an own-made pH control system based in data acquisition modules, which controls two peristaltic pumps that serve to add the gastric solutions (HCl or $NaHCO_3$). Finally, the PID control system acts on impulsion and return pumps, such that varying these pumps' flow rates it is possible to regulate the pressure inside the hydraulic circuits, as well as the product filtration rate. The continuous PFR connected in series to the CSTR served for the simulation of the conditions in the intestine. It consisted of a cylindrical tube made of stainless steel (provided by Prozesstechnik GmbH, Basel, Switzerland) equipped with a monochannel ceramic MF membrane in tubular configuration provided by Atech Innovations GmbH. The MF membrane used for the experiments was an inorganic one of α-Al_2O_3 active surface with a mean pore diameter (Dp) equal to 0.2 μm and molecular weight cut-off (MWCO) in the range 1.2 kDa, on α-Al_2O_3 support (model 20N). The dimensions of the selected membrane were 1000 mm length, 6 mm duct diameter and 2 mm ± 0.5 thickness. This type of MF membrane also ensures a series of advantageous characteristics, such as high thermal and mechanical stability, resistance against aggressive chemical reagents, good washability and permeability recovery, as well as high selectivity and performance, low cost and long lifetime service.

The PFR was integrated with the CSTR by means of a drive and return system made of chemically-resistant polyethylene tubes. The digested solution exiting the CSTR was continuously driven to the PFR also by means of a peristaltic pump (Eyela, modelMP-3), maintaining the tangential velocity inside the membrane module constant. The

operating pressure could be adjusted accurately ($P_{set\ point}$ ± 0.01bar) with a spring-loaded pressure-regulating valve (SS-R4512MM-SP model, Swagelok) and monitored by a digital pressure gauge (Endress + Hauser, model Ceraphant PTC31). A precision electronic mass balance with USB connectivity (Sartorius, model Quintix 5102, accuracy equal to 1 mg) was coupled to an automatic sampling and data registration system.

5.1.2.2. HPLC-UV analysis of PTSO in samples from the GITSS

To evaluate the bioavalilability of PTSO, samples obtained from the GITSS were monitored in relation to the PTSO content. An Agilent 1260 Infinity HPLC (Agilent Technologies Inc) system was used for the proposed HPLC method, which includes a quaternary pump, an online degasser, an autosampler (injection volume from 0.1-900 µL, capacity: 100 vials), a column thermostat and a DAD.

The determination of PTS and PTSO in the samples obtained from the GITSS, based on a methodology developed in previous chapters,[4,5] was performed in a C18 column (150 x 4.6 mm, 5 µm), using a mobile phase consisting of 30 mM aqueous perchloric acid solution (solvent A) and MeCN (solvent B), at a flow rate of 0.7 mL min^{-1}. The injection volume was 20 µL and the gradient elution program was as follows: 0 min - 50 % B; 3 min - 50 % B; 9 min - 100 % B; 14 min - 100 % B. The initial conditions were re-established by 2 min of linear gradient, followed by an equilibration time of 4 min. The total chromatographic run time was 20 min and detection wavelength was set at 200 nm (bandwidth = 4 nm). Samples from GITSS were directly filtered and injected into the HPLC-UV.

5.1.2.3. UHPLC-MS/MS analysis of PTSO and derivatives in samples from the GITSS

UHPLC-MS/MS analyses of PTSO and its derivatives (CSSP and GSSP) in samples taken from the GITSS, were carried out using an Agilent 1290 Infinity LC (Agilent Technologies, Waldbron, Germany) equipped with a binary pump, on-line degasser, autosampler (5 µL loop), and a column thermostat. MS measurements were performed on a triple quadrupole mass spectrometer API3200 (AB Sciex, Toronto, ON, Canada) with ESI. The instrumental data were collected using the Analyst Software version 1.5 with Schedule MRM™ Algorithm (AB Sciex).

UHPLC analyses were performed, according to the methodology developed in chapter 2,[5] using a C18 column (Zorbax Eclipse Plus RRHD 50 x 2.1 mm, 1.8 µm) and a mobile phase consisting of 0.05 % aqueous FA solution (solvent A), and MeOH (solvent B) at a flow rate of 0.4 mL min^{-1}. The eluent gradient profile was as follows: 0 min - 10 % B; 0.5 min - 10% B; 2.5 min - 100% B; 3 min - 100 % B. Finally, it was back to 10% B in 0.5 min and kept for 2 min for column equilibration. The temperature of the column was set at 25 °C and the injection volume was 5 µL (full loop). The MS was working with ESI^+ under MRM conditions obtaining the values indicated in Table 5.1. The target scan time established for each transition was 0.1 s. The ionization source parameters were: source temperature 500 ºC; curtain gas (nitrogen) 30 psi; ion spray voltage 5500V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi. Under these conditions CSSP, GSSP and PTSO were determined in just 4.0 min, all of them being characterized by two precursor-product ion transitions. All collected samples from the GITSS were diluted at 50 % (v:v) with MeOH and injected in triplicate into the equipment.

Table 5.1. Monitored ions of the target analytes and MS/MS parameters

	Retention time (min)	Precursor ion (m/z)	Molecular ion	DP[a]	EP[a]	CEP[a]	Product ions[b]	CE	CXP[a]
CSSP	1.7	196.0	$[M+H]^+$	36.0	6.0	12.0	107.0 (Q) 179.0 (I)	11.0 17.0	4.0
GSSP	1.9	382.0	$[M+H]^+$	31.0	9.5	38.0	130.0 (Q) 150.0 (I)	24.0 27.0	4.0
PTSO	4.0	183.0	$[M+H]^+$	21.0	10.5	10.0	141.1 (Q) 99.0 (I)	13.0 19.0	4.0

[a] Declustering potential (DP), Entrance potential (EP), Collision cell entrance potential (CEP), Collision cell exit potential (CXP) and Collision energy (CE). All expressed in voltage.

[b] Product ions: (Q) Transition used for quantification, (I) Transition employed to confirm the identification.

5.2. GITSS conditions

All GITSS experiments were performed using an initial dose of PTSO of 40 mg kg^{-1}. The dose of each vehiculization or encapsulated product was calculated taking into account the PTSO percentage in each one of them.

5.2.1. Stomach conditions

Gastrointestinal porcine conditions in the stomach were simulated in the CSTR. Temperature was established in 38.8 ± 1.0 ° C during the whole process[16] and the stirring speed of the reactor was fixed in 100 rpm to simulate the continuous peristaltic movement of stomach walls. Acid secretions of the gastric glands were simulated with porcine pepsin (stock solution of 5.3 mg L^{-1}) added at a rate of 4 mL min^{-1} and HCl (1 M) and pH was controlled in all moment by the pH control system described above. To simulate empty stomach, each testing product has been added to the artificial stomach

16. Ingram, D.L., Legge, K.F. J Physiol., 210 (1970) 989-998.

(reactor containing 855 mL of water) at pH 1.5[17] and subsequently pepsin was added. Samples from the bioreactor were taken at 10 min (just after pepsin addition) and 30 min after feeding the system.

5.2.2. Duodenum simulation

The postprandium has been simulated in the same bioreactor, but reproducing the conditions of the chyme in the duodenum. The pH regulation system has been configured to dose $NaHCO_3$ 1M, emulating the action of pancreatic juices to a pH of 6.5 similar to that achieved in the pig duodenum: 6.1 in the proximal duodenum and 6.8 in the distal duodenum.[18,19] Next, a set of auxiliary pumps have been configured to dose bile salts and porcine pancreatic lipase (stock solution of 4 g L^{-1} and 1 g L^{-1}, respectively) at a rate of 4 mL min^{-1}. A sample from the bioreactor was taken just after the addition of biliary and pancreatic fluids (approximately 50 min after feeding the system).

17. deRouchey, J.; Goodband, B.; Tokach, M. North American Veterinary Community Conference (2009) 375-376.

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19. Stevens, C.E.; Hume I.D. Comparative physiology of the vertebrate digestive system. Second edition Press Syndicate, New York (1995). 188-228

5.2.3. Intestinal absorption

In order to simulate intestinal absorption, bioreactor fluids are pumped to flow through the modular filtration system described previously. The cut-off size of the membrane has been selected based on previous experience and permeability of the molecules to be tested, depending of molecular weight (182.3 g mol^{-1} for PTSO). Samples were taken after 10, 30, 60, 90, 120, 150 and 180 minutes of intestinal simulation of both, the fluids absorbed through the membrane (permeate side) and the non-absorbed ones that will end up forming part of the colonic residue (retentate side). In summary, each simulation process had a total duration of 240 min: stomach conditions (0-30 min); duodenum simulation (30-60 min) and finally intestinal absorption (jejunum and illeum) (60-240 min).

Each experiment was performed in triplicate and each sample taken at simulated stomach, duodenum and from the intestinal absorption, was analyzed per duplicate. All generated date of the studied variables have been registered in a control system based on data acquisition modules and computer connected actuators that have allowed the programming, control and supervision of all elements of the simulator.

5.3. Results and discussion

5.3.1. HPLC-UV analysis of PTSO in samples from the GITSS

All samples were analyzed in triplicate by the proposed HPLC-UV method. It is worth to note that PTSO molecule was only detected in samples corresponding to the stomach phase, previously to the biliary salts secretion step (same results were obtained by UHPLC-MS/MS, see below). This result agrees with previous research works revealing that sulfates compounds can be reduced under digestion conditions.[20] In our simulation study, PTSO was reduced to PTS after the addition of biliary salts, thus bioavailability results from this point onwards are expressed as PTS percentage. Figure 5.3 shows chromatograms corresponding to PTSO and PTS standard solutions.

PTS amount was calculated by establising a previous calibration curve at six different concentration levels (ranging from 2 to 200 mg kg^{-1}). Each level was prepared in duplicated and injected in triplicate. Statistical parameters were calculated by least-square regression. LOD (3xS/N ratio) and LOQ (10xS/N ratio) were 0.4 and 1.6 mg kg^{-1}, respectively. It was confirmed that PTS analytical response was linear over the studied range (R^2 = 0.9996).

PTS as well as PTSO has antimicrobial activity against pig predominant bacterial groups (total aerobes, total anaerobes, lactobacilli, bifidobacteria, coliforms, enterobacteria, bacteroides and clostridia) including *E. coli* and *S. typhimurium*, two common pathogens of pigs.[2]

20. Suttlel, N.F. Natural Nutrition of Livestock, CABI, Edition nº 4 (2010) 206-222.

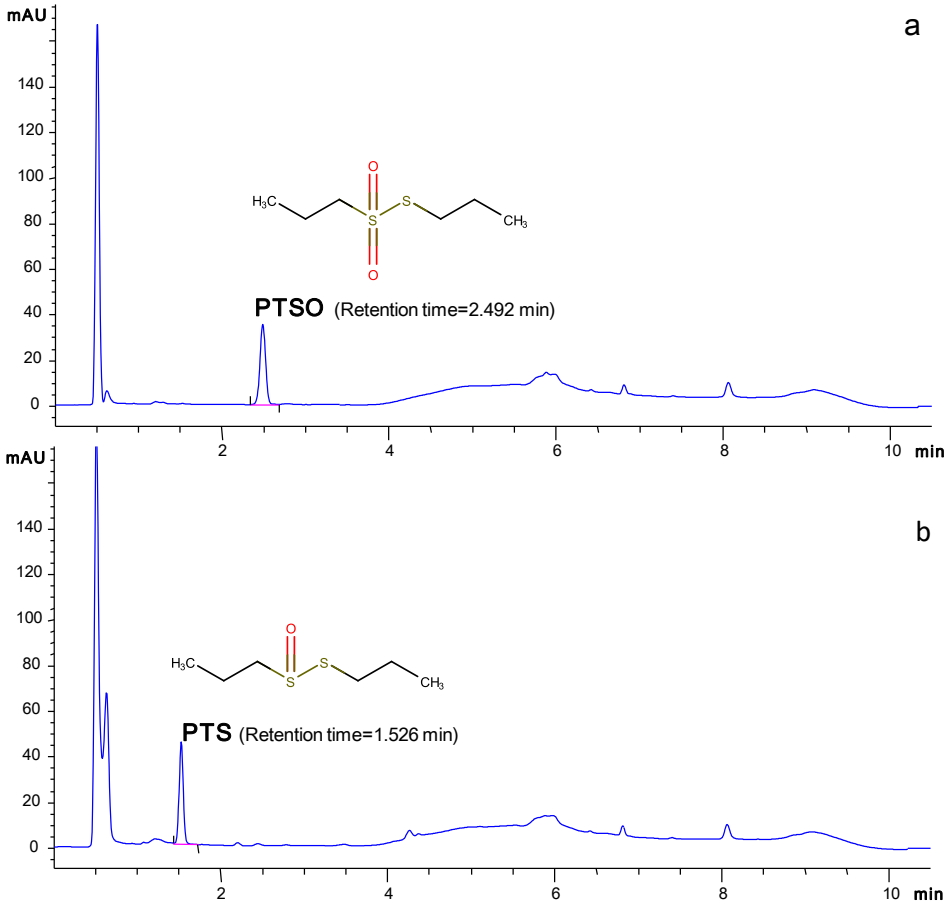

Figure 5.3. Chromatogram of (a) PTSO and (b) PTS standard solution both at a concentration of 50 mg Kg^{-1}

5.3.2. UHPLC-MS/MS analysis of PTSO and derivatives in samples from the GITSS

PTSO reacts spontaneously in presence of the thiol group (-SH) characteristic from CYS and GSH giving CSSP and GSSP, respectively. Both, CYS and GSH are present in extra and intra cellular animal tissues. For this reason, the relevance of these reactions inside the GITSS must be studied according to the methodology developed in chapter 2.[5]

Confirmation of the secondary reactions among PTSO, CYS and GSH were studied by UHPLC-MS/MS analysis. Neither CYS nor GSH derivatives (CSSP and GSSP) were detected in any analysed samples. PTSO was only detected in samples corresponding to the stomach conditions before bile salt secretion. This phenomenon is detailed in section 5.1.2.2.

5.3.3. Gastrointestinal simulation of PTSO resistance/absorption in the GITSS

To exert its beneficial effects, PTSO must be able to tolerate the strong acidic conditions of the environment in the stomach as well as of the bile secreted into the duodenum in the small intestine. It has been previously demonstrated that encapsulation in the form of microparticles is able to protect active ingredients which are chemically or biologically non-stable against degradation, thus improving their efficacy. Moreover, encapsulation can also enhance the transport, manipulation and intake of pharmacological products that would otherwise be water-insoluble or present irritating effects. With this goal, several carriers for PTSO (Garlicon, Proallium, Garlicaps) have been tested and compared with the absorption of free PTSO in a dynamic GITSS model. Bioavailability values for PTSO have been measured by HPLC-UV (expressed as PTS percentage along the artificial intestine) and it has been

calculated taking into account the PTS amount found in each step of the process divided by PTS initial amount (PTSO initial amount/(PTSO molecular weight) x PTS molecular weight).

The conditions in the stomach and the duodenum were correctly simulated by the CSTR, where the relevant variables (agitation, temperature, reactor level, pH kinetics adjustment by addition of HCl simulating the gastric juices, subsequent addition of $NaHCO_3$ simulating the pancreatic juices and alkali secretion, as well as dosage of pepsin, pancreatic lipase and bile juices) should be accurately controlled. Figure 5.5 shows the pH evolution in the stomach *vs* digestion time and during intestine simulation, whereas figure 5.6 depicts the accurate temperature values obtained during the whole simulation process. The simulation of the gastric process started with the addition of gastric pepsin and HCl 1M in the CSTR simulating the gastric juices, until a pH equal to 1.5 was achieved. After stomach simulation, 86.6 % of the PTSO resisted the gastric fluids when tween 80 was present, whereas only 72.75 % and 28.04 % were found for the encapsulating matrixes (Proallium and Garlicaps respectively) and 49.71 % for free PTSO (see table 5.2).

Table 5.2. Simulated digestion results for free PTSO, Garlicon, Proallium and Garlicaps

	Free PTSO	GARLICON	PROALLIUM	GARLICAPS
Resistant to stomach	49.71	86.60	72.75	28.04
Resistant to stomach and duodenum	33.62	50.19	32.79	11.23
Bioavailable in duodenum	1.11	0.72	2.22	0.01
Bioavailable in duodenum + jejunum	4.49	14.20	5.71	3.14
Bioavailable in duodenum + jejunum +ileum	7.44	17.82	8.44	18.88

After 26 min, the pH in the GITSS became progressively increased with the addition of $NaHCO_3$ 1 M until a pH up to 6.5 was attained, simulating the passage into the duodenum. Subsequently, bile salts and lipase were added into de CSTR vessel, modifying naturally the pH (without artificial regulation) up to values around 7.5 according to scientific literature (pH for pig proximal and distal jejunum=7.4, ileum=7.5).[18] With regard to figure 5.6, it can be seen the optimal temperature control achieved by the external jacket connected to a cooler/chiller, the Pt-100 digital sensor and the PID loop.

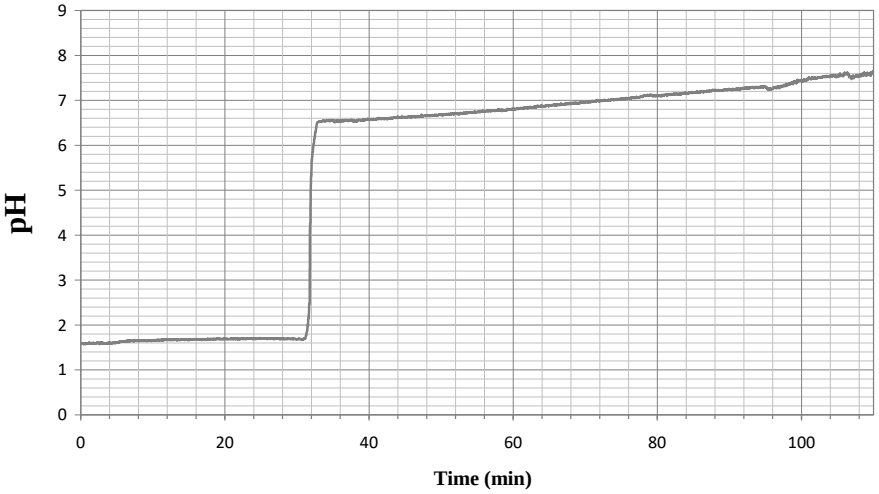

Figure 5.5. pH evolution *vs* time during the digestion process

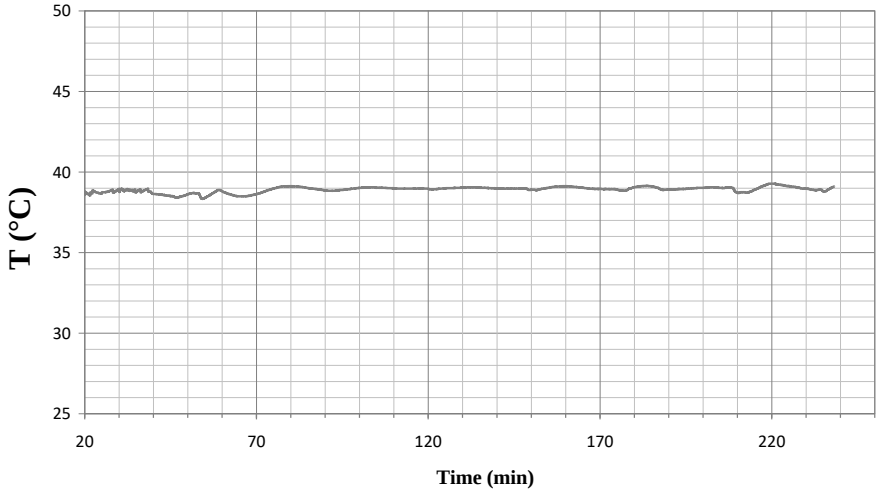

Figure 5.6. Temperature evolution *vs* time during the complete simulation process

On the other hand, the upper and lower GIT was successfully reproduced by the PFR, equipped with a ceramic MF membrane in tubular configuration, in which the main variables (pressure and tangential velocity) were, as well, appropriately controlled. The use of peristaltic pumps in the dynamic GITSS model permitted the transport of reagents and products while avoiding their contact with the impulsion elements, thus preventing their contamination. Furthermore, during the absorption process the concentration of product inside the CSTR vessel increased in time, simulating the water loss of the chyme during its advance inside the porcine intestine. Finally, the CSTR served as reservoir, simulating the large intestine and finally the colonic residue.

Figure 5.7 represents the internal transmembrane pressure evolution, precisely controlled around 50 mmHg during the complete intestinal absorption, thus inhibiting the pressure-dependent absorption mechanisms and making possible to compare the bioavailability (as PTSO in the permeate against the total PTSO fed to the system) of the different vehiculization strategies of PTSO. The pressure in the GITSS could simulate reliably the physiologic pressure in real gastrointestinal tracts as reported by several authors.[21-23] Moreover, the membrane surface microporosity also helped to simulate the microvilli present in the porcine intestinal tube, and also its geometry, which makes this model closer to reality than other multi-vessel systems reported in the literature.

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23. Sarna, S.K.; Shi, X. Function and regulation of colonic contractions in health and disease, In: Physiology of the gastrointestinal tract, vol. I, Fourth ed. Johnson, J.R., (Ed.). Elsevier Academic Press, (2006) 965-993.

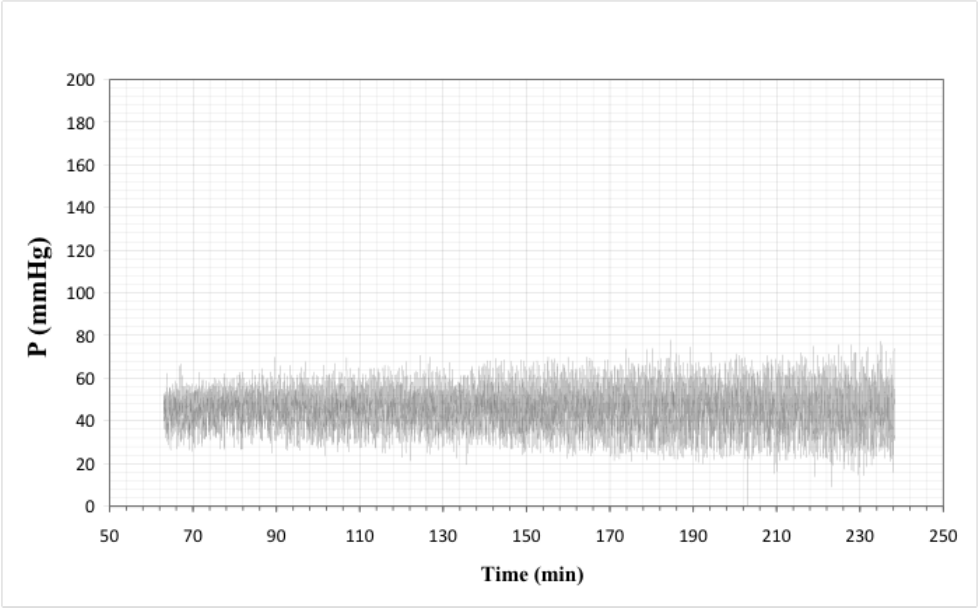

Figure 5.7. Pressure plot during the GITSS experiments

Figure 5.8 shows the PTSO bioavailability (%) expressed as PTS during the intestine simulation. Bioavailability values in duodenum, duodenum + jejunum and duodenum + jejunum + ileum are calculated as the amount of PTSO in the corresponding permeate stream divided by the total amount of PTSO to fed the system. The obtained results clearly highlight the effective adsorption of Garlicon through the proposed GITSS in contrast with free PTSO. The absorbed net amount of PTSO in the GITSS was found to be over 3.5 times higher in the filtered product than the absorption observed for free PTSO (figure 5.8) and/or other encapsulation matrices (Proallium and Garlicaps). In this sense, it is important to note that, from the total bioavailable PTSO when using tween 80 (Garlicon), 0.72 % was absorbed along the duodenum, 14.20 until jejum and 17.82 after ileum, the rest was found in the colonic residue. Results are summarized in table 5.2.

The performance of tween 80 as carrier for PTSO has shown its capability to enhance the PTSO resistance to the strong acidic conditions of the digestion process and also to improve its passage through the intestine. Its effective delivery was thus favored, enhancing the total bioavailability. Indeed, tweens are stable to electrolytes and weak acids and bases, producing a soft gradual saponification with strong acids and bases,[24] which most probably have contributed to help PTSO to resist stomach conditions. In addition, tween 80 (Garlicon), has been recently described as a potential option to create self-emulsifying drug delivery system,[25] being able to enhance the solubility of poorly aqueous soluble drugs (e.g. resveratrol), and thus the total intestinal permeability.

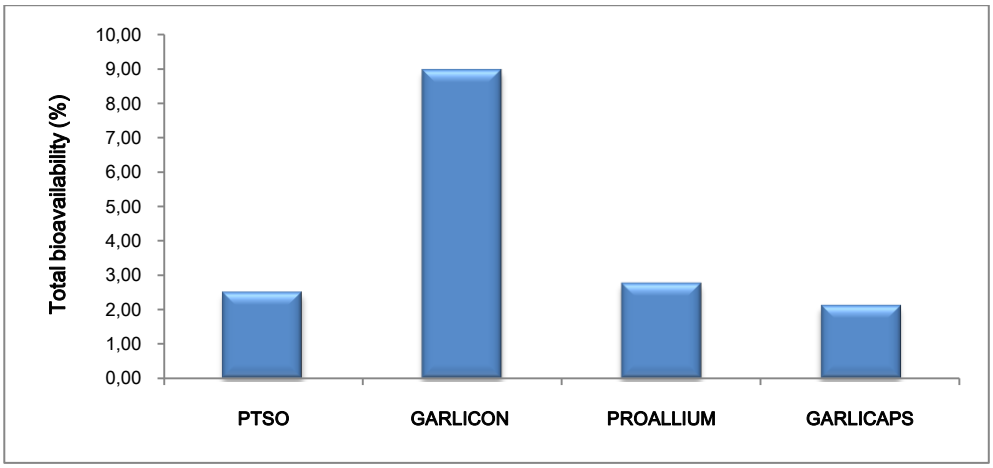

Figure 5.8. PTSO bioavailability during small intestinal simulation (duodenum + jejunum + ileum) using different vehiculization options for PTSO

24. PubChem CID: 86289060 (https://pubchem.ncbi.nlm.nih.gov/compound/86289060 - section=Top) (Monday, October 30, 2017)

25. Balata, G.F.; Essa, E.A.; Shamardl, H.A.; Zaidan, S.H.; Abourehab, M.A.S. Drug Des Devel Ther., 10 (2016) 117-128.

5.4. Conclusions

The development of a controlled-release delivery system by a carried material is necessary to increase PTSO cellular permeability and bioavailability. With this aim, different vehiculization strategies have been tested with regard to the bioavailability values and compared with the absorption of free PTSO. The net amount of PTSO within the absorbed products in the GITSS was found to be over almost 3 times higher when tween 80 was used as carrier (Garlicon) than the one for free PTSO. Neither the encapsulated PTSO in β-cyclodextrin (Proallium) nor by means of mono- and diglycerides of fatty acids plus a vegetable oil (Garlicaps) succeeded to improve absorption values for free PTSO.

The performance of tween 80 as carrier for PTSO has shown its capability to enhance the PTSO resistance to the strong acidic conditions of the digestion process and also to enhance its passage through the artificial intestine.

The proposed dynamic *in vitro* gastric digestion model effectively mimics the *in vivo* pig physiological conditions. The parameters in the stomach and the duodenum were correctly simulated by the CSTR, whereas the upper and lower GIT, jejunum, ileum and colonic residue were also successfully reproduced by the PFR.

This work has been submitted for publication as: On the effects of different vehiculization strategies for allium derivative propyl propane thiosulfonate during dynamic simulation of the pig gastrointestinal tract. Abad, P., Arroyo-Manzanares, N., Rivas-Montoya, E., Guillamón, E., García-Campaña, A.M., Martinez-Ferez, A. LWT-Food Science and Technology.

CHAPTER 6

Antifungal effect of PTSO against mycotoxigenic strains of *Aspergillus parasiticus*, *Aspergillus flavus, Penicillium verrucosum* and *Fusarium graminearum*

Capacidad antifúngica del PTSO frente a cepas micotoxigénicas de *Aspergillus parasiticus*, *Aspergillus flavus, Penicillium verrucosum* y *Fusarium graminearum*

Resumen

Los compuestos organosulfurados característicos del genero allium, incluyendo propil propano tiosulfonato (PTSO), son compuestos antimicrobianos potencialmente útiles para aplicaciones alimentarias. En este estudio, se ha evaluado la actividad antifúngica del PTSO en distintas cepas de hongos productores de micotoxinas, en concreto, en dos cepas de hongos aflatoxigénicos (*Aspergillus parasiticus* y *Aspergillus flavus*), una productora de ocratoxina A (*Penicillium verrucosum*) y una productora de zearalenona (*Fusarium graminearum)* empleando un ensayo en medio sólido. Se ha podido establecer la dosis letal de del PTSO frente a las cepas estudiadas, obteniéndose valores de 10.0, 4.0, 7.5 y 17.5 mg kg^{-1} para *Penicillium verrucosum*, *Fusarium graminearum*, *Aspergillus parasiticus* y *Aspergillus flavus*, respectivamente.

Igualmente, considerando que la producción de micotoxinas está asociada al crecimiento de hongos, en este estudio se ha evaluado la influencia del PTSO sobre dicha producción, observándose que aunque el PTSO presenta una actividad antifúngica, no es posible atribuirle la capacidad de inhibir la producción de ciertas micotoxinas, de modo que la presencia de éstas (ocratoxina A (OTA), zearalenona (ZON), aflatoxina B1 (AFB1), aflatoxina B2 (AFB2), aflatoxina G1 (AFG1) o aflatoxina G2 (AFG2)) se ha detectado en las muestras estudiadas en presencia de una concentración de PTSO por debajo de la letal, donde los hongos habían crecido, usando un método analítico mediante UHPLC-MS/MS. Sin embargo, se observó que la producción de ZON y AFB2 (procedente de *Aspergillus flavus*) se ha reducido significativamente por la presencia de PTSO.

Abstract

Allium organosulfur compounds including propyl propane thiosulfonate (PTSO) are potentially useful antimicrobial compounds for food applications. In this study, the antifungal activity of PTSO was evaluated on two fungi strains producer of mycotoxins, such us, two aflatoxigenic (*Aspergillus parasiticus* and *Aspergillus flavus*), one ochratoxigenic (*Penicillium verrucosum*) and *Fusarium graminearum,* producer of zearalenone, employing an assay on solid medium. The PTSO lethal dose against studied strains has been established, obtaining 10.0, 4.0, 7.5 and 17.5 mg kg^{-1} for *Penicillium verrucosum, Fusarium graminearum, Aspergillus parasiticus* and *Aspergillus flavus,* respectively.

Mycotoxins production is associated to fungal growth and for this reason the capability of PTSO for inhibiting this production has been evaluated. The study has demonstrated that, although PTSO presents antifungal properties, it is not possible to attribute him the property of reduction of mycotoxin production, in relation to ochratoxin A (OTA), zearalenone (ZON), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) or aflatoxin G2 (AFG2), observing their production only in samples treated with PTSO content blow the lethal doses, were studied fungi had grown. This fact has been probed by UHPLC-MS/MS analysis. Nevertheless, ZON and AFB2 (from *Aspergillus flavus*) production has been significantly reduced by the presence of PTSO.

6.1. Introduction

The European Food Safety Authority (EFSA) defines mycotoxins as toxic compounds produced by different types of fungus, belonging mainly to the *Aspergillus*, *Penicillium* and *Fusarium* genera. Under favorable environmental conditions of temperature and moisture, these fungi proliferate, producing mycotoxins as secondary metabolites. They commonly enter the food chain through contaminated food and feed crops, mainly cereals. In the European Union strict rules and legislative limits defined by the European Commission have been set for all of the above mentioned toxins in certain food and feedstuff to protect animals and humans.[1]

The presence of mycotoxins in food and feed may affect human and animal health, as they may cause many different adverse effects such as estrogenic, gastrointestinal, and kidney dis- orders, induction of cancer, and mutagenicity. Furthermore, some mycotoxins are also immunosuppressive and reduce resistance to infectious diseases.[2] Mycotoxins grow under a wide range of climatic conditions and the Food and Agriculture Organization (FAO) has estimated that they affect 25% of the world crops.[3]

1. European Food Safety Authority (EFSA) https://www.efsa.europa.eu/en/topics/topic/mycotoxins (Friday, October 27, 2017).

2. Marin, S.; Ramos, A.J.; Cano-Sancho, G.; Sanchis, V. Food Chem. Toxicol,. 60 (2013) 218–237.

3. Food and Agriculture Organization (FAO). http://www.fao.org/fileadmin/templates/ess/documents/meetings_and_workshops/GS_SAC_2013/Improving_methods_for_estimating_post_harvest_losses/Final_PHLs_Estimation_6-13-13.pdf (Monday. November 6. 2017)

Among the known mycotoxins, one of the most important groups is aflatoxins, which are produced by *Aspergillus flavus and Aspergillus parasiticus.* Other *Aspergillus* species are known to produce aflatoxins but their toxicological significance is lower.[4,5] Aflatoxins are considered as the most dangerous mycotoxins because of their occurrence, toxicological effects and their consequences on crop trade.[6] The four major naturally produced aflatoxins are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2).[7] The terms 'B' and 'G' refer to the blue and green fluorescent emissions produced by these toxins under UV light during the thin layer chromatography (TLC) plate visualization. Their toxicity is ranking in order of AFB1 > AFG1 > AFB2 > AFG2, being AFB1 considered as a potent human carcinogen (group 1) by the International Agency for Research on Cancer (IARC).[8] On the other hand, exposure to aflatoxins in animals results in impairment of liver function and also reduce the food intake, which might also explain the reduced milk production in dairy cattle exposed to aflatoxins.[9]

Ochratoxin A (OTA) is a potent nephrotoxic mycotoxin that has been linked to kidney problems in both livestock and human populations. It has also carcinogenic, genotoxic and immunotoxic properties. *Penicillium verrucosum* is the mayor producer of OTA in

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7. Kumar, P.; Mahato, D.K.; Kamle, M.; Mohanta, T.K.; Kang, S.G. Front Microbiol., 7 (2016) 2170.

8. International Agency for Research on Cancer. Monographs on the evaluation of carcinogenic risks to humans. In traditional herbal medicines, some mycotoxins, napthalene and styrene. International Agency for Research of Cancer, 82 (2002) 171-274.

9. Fink-Gremmels, J. Food Addit. Contam. Part A, 25 (2008) 172-180.

cereals such as wheat and barley in temperate and cold climates.[10] IARC has classified OTA as a possible human carcinogen (group 2B) but the mode of carcinogenic action by OTA is still unknown.[11]

Fusarium toxins encompasses toxins produced by more than 50 species of *Fusarium*, mainly occurring in the grain of developing cereals such as wheat, maize and others.[12,13] Among them, we can distinguish those produced mainly by *Fusarium verticilloides* (fumonisin B1 (FB1) and fumonisin B2 (FB2)), and those produced mainly by *Fusarium graminearum*, including the so-called estrogenic mycotoxins (being the most important zearalenone (ZON)) and the non-estrogenic mycotoxins or trichothecenes (such as deoxynivalenol, fusarenon-X (F-X) and T2 and HT2 toxins).[14]

Figure 6.1 shows the structure of some of the most important mycotoxins.

10. Cabañes, F.J.; Bragulat, M.R.; Castellá, G. Toxins, 2 (2010) 1111-1120.

11. Pfohl-Leszkowicz, A.; Manderville, R.A.; Mol. Nutr. Food Res., 51 (2997) 61-99

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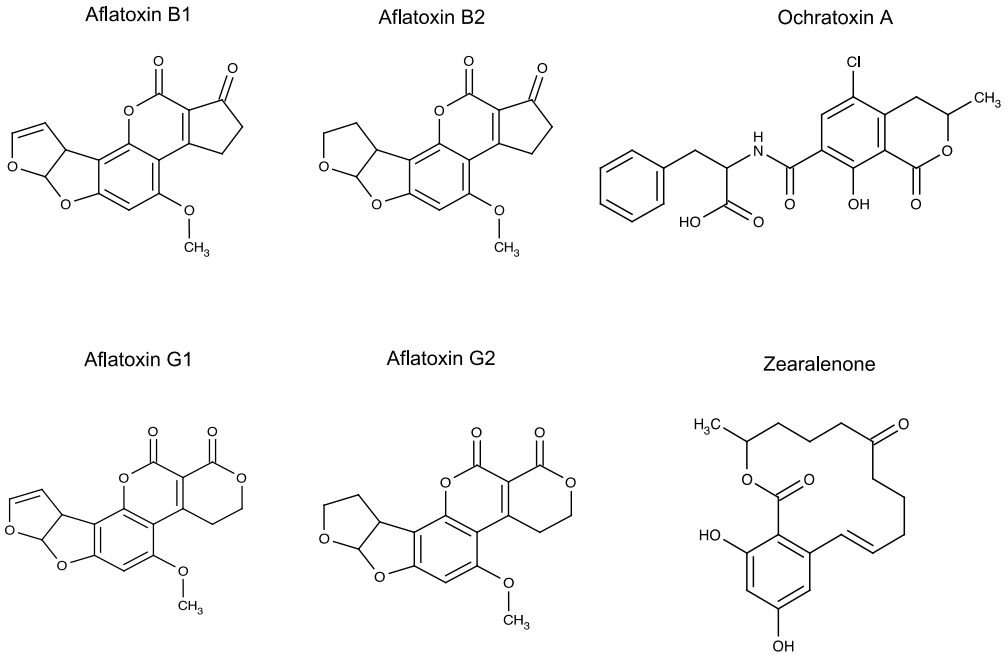

Figure 6.1. Chemical structure of AFB1, AFB2, AFG1, AFG2, OTA and ZON

In relation to the control of mycotoxins in food and feed, Directive 2002/32/EC establishes the maximum levels of undesirable contaminants, including mycotoxins, permitted in feed, but only aflatoxin B1 was regulated in it.[15] Few years later, in 2006, maximum levels for mycotoxins together with certain other contaminants in food were firstly set in Regulation (EC) 1881/2006 and subsequent amendments[16]. Also, scientific opinions about mycotoxins which are continuously published are collected in the

15. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. OJ L140 (2002) 10-22.

16. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. OJ L314M (2007) 558-577.

contaminants catalogue of the European Union.[17] Also, several recommendations have been established in order to complement current regulation for the control of mycotoxins in feed. For example, monitoring of ergot alkaloids in feed and food is contemplated in the Commission Recommendation 2012/154/EU[18] and recommended guidelines for action on the presence of DON, ZON, OTA, T-2 and HT-2 toxins and fumonisins in products intended for animal feeding are reflected in Recommendation 2006/576/EC.[19] Also, provisions for methods of sampling and analysis for the official control of mycotoxins in foodstuffs were introduced in Regulation (EC) 401/2006.[20]

In Europe, the Rapid Alert System for Food and Feed (RASFF) allow member states to notify one another in the case of an identified health threat, either through border rejections, market official control inspections or other means. In 2015, there were 475 notifications on mycotoxins in food, most related to the presence of aflatoxins (421 notifications). This is a significant increase of notifications compared to 2014 (359 in 2014), mainly due to notifications on aflatoxins (107 more in 2015 compared to 2014). In the same way, there were 19 notifications on mycotoxins in feed, of which 17 were on aflatoxins and two on ZON.[21] This trend is maintained in 2016 because the

17. European Commision (2017). Mycotoxins. URL: http://ec.europa.eu/food/safety/chemical_safety/contaminants/catalogue/mycotoxins_en (Monday, November 6, 2017)

18. 2012/154/EU: Commission Recommendation of 15 March 2012 on the monitoring of the presence of ergot alkaloids in feed and food Text with EEA relevance. OJ L77 (2012) 20-21.

19. Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. OJ L 229 (2006) 7-9.

20. Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. OJ L330M (2006) 228-250.

21. The Rapid Alert System for Food and Feed 2015 annual report (https://ec.europa.eu/food/sites/food/files/safety/docs/rasff_annual_report_2015.pdf) (Monday, October 09, 2017)

preliminary 2016 RASFF report indicates that mycotoxins are the second risk for food and feed consumer.[22]

As it was mentioned before, the presence of mycotoxins in food and feed may affect human and animal health as they may cause many different adverse health effects such as induction of cancer and mutagenicity, as well as estrogenic, gastrointestinal and kidney disorders. Some mycotoxins are also immunosuppressive reducing resistance to infectious disease.[23] The prevention is the best method for controlling fungi and mycotoxins contamination. To prevent mycotoxin contamination in grain-based foods and feeds, control of growth of mycotoxigenic moulds is necessary.

Due to their non-toxicity, in the last years essential oils (EOs) from different plants have been used in the prevention of fungal growth and mycotoxins production.[24] EOs are mixtures of volatile constituents obtained from plants, which mainly include terpenes, terpenoids, aromatic and aliphatic constituents, all characterized by low molecular weight. EOs are expected to be more advantageous than the synthetic agents due to their biodegradability and low toxicity.[25]

22. RASFF preliminary annual report 2016 (https://ec.europa.eu/food/sites/food/files/safety/docs/rasff_annual_report_2016.pdf) (Monday, October 09, 2017)

23. European Food Safety Authority (EFSA) https://www.efsa.europa.eu/en/topics/topic/mycotoxins. (Monday, October 09, 2017).

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Table 6.1 shows a summary of the inhibitory *in vitro* effect of EOs on the production of mycotoxins. Minimum inhibitory concentration by *in vitro* studied was obtained for each EO. Cinnamon has the wide activity spectrum, mainly against species of the genera *Aspergillus, Penicillium* and *Fusarium*. Anise, thyme, spearmint, basil, coriander have shown its effect against species of the genera *Aspergillus* and *Penicillium* and clove and oregano against *Aspergillus* and *Fusarium.*

Laurel, geraniol, cumin, turmeric, eugenol, eucalyptus, thyme, rosemary have shown antifungal effect against species of the genera *Aspergillus* as well as mint, oregano and sage against *Penicillum* and shallot against *Fusarium.*

Table 6.2. Use of EOs in preventing the formation of mycotoxins (Adapted by Tanackov and Dimić [26])

Mycotoxin	EOs/components	Applied concentrations	Nutrient medium[a]	Ref.
AFG1	Anise, cinnamon, thyme, spearmint	2 %	Wheat grains	27
	Basil, coriander, laurel	5 %	PKB	28
AFB1	Anise	2 %, 150 mg kg^{-1}, 1000 μg g^{-1}	Wheat grains, MMEA, corn grains	27,29-31
	Basil	5%, 0.2 μL mL^{-1}	PKB, SMKY	32, 33
	Cinnamon	2 %, 250 μg mL^{-1}	Wheat grains, SMKY	34, 27
	Geraniol	0.6 μL mL^{-1}	SMKY	35
	Coriander, laurel	5 %	PKB	33
	Clove	250 μg mL^{-1}, 500 μg g^{-1}, 1000 μg g^{-1}, 5 g kg^{-1}	SMKY, MMEA, corn grains, rice grains	29-31, 34, 36

26. Tanackov, S.D.K.; Dimić, GR. Antifungal activity of essential oils in the control of food-borne fungi growth and mycotoxin biosynthesis in food. In Microbial pathogens and strategies for combating them: science, technology and education (A. Méndez-Vilas, Ed.), Formatex Research Center (2013) 838-849.

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	Cumin	1000 μg g^{-1}	PDB, YES	37-39
	Turmeric	5 g kg^{-1}	Rice grains	36
	Eugenol	0.1 μL mL^{-1}	SMKY	40
	Eucalyptus	50 μL mL^{-1}	YES	41
	Thyme	500 μg g^{-1}, 1000 μg g^{-1}, 250 mg kg^{-1}, 2%	MMEA, corn grains, YES, wheat grains	27, 29-31, 42, 43
	Oregano	7000 mg kg^{-1}, 0.6 μL mL^{-1}	MMEA, SMKY	29, 44, 45
	Rosemary	450 mg kg^{-1}	YES	42
OTA	Anise, cinnamon, thyme, spearmint	2%	Wheat grains	27
	Basil, coriander, mint, oregano, sage	1000 mg kg^{-1}	YES	46
ZON	Cinnamon, clove, oregano, shallot	500 μg g^{-1}, 1000 μg g^{-1}	Corn grains	[47], [48]

(a) PKB: Broth of palm seeds; MMEA: Meal extract agar; SMKY; Agar with yeast extract and sucrose; YES: broth with yeast extract and sucrose; PDB: Potato dextrose broth;

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PTSO, an onion derivative, has antimicrobial activity against *Enterobacteriaceae*, *E.Coli*, *Salmonella spp*. and *Campylobacter jejuni*. It has been studied *in vitro*[49] and *in vivo*[50]. In the same way, anticoccidial properties against *Eimeria acervulina* have also been demonstrated[51]. Furthermore, dietary supplementation with PTSO can modulate intestinal microbiota composition and improve nutrient digestibility without affecting mucosal enzyme activity in growing broilers[52,53] and controlling methane inhibition by the modulation of rumen fermentation.[54] In addition, Mylona[55] suggested that PTSO could show a reduction of fungal growth in *Fusarium spp*. and in mycotoxin production (DON, fumonisins, ZON, etc).

Considering PTSO antifungal activity described above, and the problem arises by the contamination of feed with mycotoxins produced by fungi, in this chapter we have propose an strategy to study the minimal fungicidal concentration doses of PTSO against different mycotoxigenic fungi strains (*Aspergillus flavus, Aspergillus parasiticus, Penicillium verrucosum*, *Fusarium graminearum)* and its effect on mycotoxin production.

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6.2. Materials and methods

6.2.1. Chemicals and reagents

All reagents were of analytical reagent grade. Solvents used as mobile phase were LC-MS grade. MeOH, CH_2Cl_2 and EtAc from Panreac and FA and ammonium formate were purchased from Sigma-Aldrich (St Louis, MO, USA). MeOH was supplied by VWR BDH Prolabo (West Chester, Pennsylvania, USA).

Ultrapure water (18.2 MΩ cm^{-1}, Milli–Q Plus system, Millipore Bedford, MA, USA) was used throughout the work.

Individual standards of each mycotoxin were obtained from Sigma-Aldrich. When standards were provided in dry powder form, the correct amount of solvent was injected through the septum vial. From these stock solutions, mycotoxins intermediate working solutions (used thorough all this work) were prepared at the following concentrations in MeCN: 1 · g mL^{-1} of AFB1, AFB2, AFG1, AFG2 and OTA and 10 · g mL^{-1} of ZON. These solutions were stored at 20 ºC and were stable at least three months. Since mycotoxins are highly toxic compounds, some general precautions should be followed for their manipulation and solutions preparation. Thus, safety glasses and disposable gloves were used thorough the work. Decontamination of laboratory glassware and laboratory surface was carried out by swabbing with 10% hypochlorite solution using disposable paper towels. Contaminated disposable material was properly stored and processed as biohazard residues.

Clarinert™ 13 mm syringe filters with 0.22 µm nylon membrane (Agela Technologies, Wilmington, DE, USA) were used for filtration of samples prior to the injection into the chromatographic system.

A standard of pure PTSO (95%) was kindly provided by the company DOMCA S.A. (Alhendín, Granada, Spain). PTSO 20 used in section 6.2.4 was prepared by dilution of PTSO on tween 80 at a concentration of 20 %.

6.2.2. Instrumentation and equipment

Mycotoxin analyses were performed using an Agilent 1290 Infinity LC (Agilent Technologies, Waldbron, Germany) equipped with a binary pump, on line degasser, autosampler (5 µL loop) and a column thermostat. MS measurements were performed on a triple quadrupole mass spectrometer API3200 (AB Sciex, Toronto, ON, Canada) with ESI. A Zorbax Eclipse Plus RRHD (50 x 2.1 mm, 1.8 µm, Agilent Technologies) chromatographic column was used for the separation. Instrumental data were collected using the Analysts Software version 1.5 with MRM™ Algorithm (AB Sciex).

A Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), a mechanical shaker (model 384 from Vibromatic, Noblesville, USA) and an evaporator system (System EVA-EC, from VLM GmbH, Bielefeld, Germany) were also used for sample treatment.

6.2.3. Fungal strains

Aspergillus parasiticus (CECT 2681) producer of AFB1, AFB2, AFG1 and AFG2 and *Aspergillus flavus* (CECT 2687) producer of AFB1 and AFB2 were obtained from the Spanish Type Culture Collection (Valencia, Spain) and *Penicillium verrucosum* (DSMZ 12639) producer of OTA and *Fusarium graminearum* (DSMZ 1095) producer of ZON were obtained from the German Collection (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Alemania). These test fungal strains were maintained on Sabouraud dextrose agar (SDA), and 5 days old cultures were used for further assays.

6.2.4. Antifungal sensitive tests for PTSO on solid medium

Two tests were performed in order to evaluate the PTSO antifungal activity. Test 1 measures the inhibition halo generated by PTSO applied in the center of the Petri dish, in which the studied mycotoxigenic fungi are grown, being the size related with the PTSO antifungal activity. It is a rapid screening test whose results are obtained in 48 h. In Test 2, PTSO is added to the culture medium and the studied fungi are inoculated in the center of the Petri dishes. In this case, it is possible to estimate the lethal dose of PTSO (minimum inhibition concentration) against the fungi, studied in a period of 21 days.

6.2.4.1. Test 1

A disc diffusion assay was performed to determine the sensitivity of the four fungal strains to PTSO by measuring the inhibition zone diameters (in mm). Petri dishes used throughout the test had a diameter of 90 mm. The assay was carried out according to the methodology proposed by Salie et al.,[56] using potato dextrosa agar (Scharlau, Barcelona, Spain). Diffusion discs of approximately 6 mm diameter were prepared from WHATMAN NO. 1 filter paper. Separate sterile discs were impregnated with PTSO solutions at the desired final concentrations and applied just in the center of the Petri dishes. All the plates containing each fungal strain were prepared in triplicates and incubated at 21 ± 1 °C for 48 h. At the end of the incubation periods the diameter of the zone of inhibition in each of the inoculated plates was measured to evaluate the antifungal activity.

6.2.4.2. Test 2

To evaluate the influence of PTSO on the mycelial growth, different concentrations of PTSO were prepared by mixing different amounts of PTSO 20 with 10 mL of malta agar (Scharlau, Barcelona. Spain). After this process, it is necessary to wait until the medium solidifies and the plates would be ready for use. This mixture was then poured into sterile Petri dishes with a diameter of 90 mm. The center of each solidified medium was inoculated upside down with 15 µL of each target fungal strain suspended in sterile saline solution (0.8% NaCl) with a final titrated concentration of 10^5 spores mL^{-1}. Positive controls were simultaneously run with saline solution and without PTSO. Plates

56. Salie, F.; Eagles, P.F.K.; Leng H.M.J. J Ethonopharmacol. 52 (1996) 27–33.

were incubated at 21 ± 1 °C. The radial mycelial growth of the isolates was determined daily by measuring the length of fungal colonies for 21 days.

6.2.5. Sample treatment for mycotoxin control

The whole content of the Petri plate were cut into small pieces with a scalpel and these were subsequently transferred to a 500 mL screw cap Erlenmeyer flask. Mycotoxins were extracted with 20 mL MeOH:CH_2Cl_2:EtAc (17:33:50, v/v/v) containing 1% (v/v) of FA. The content was shaken during 60 min on mechanical shaker and then centrifuged at 5000 rpm for 5 min. A total of 4 mL of the extract was transferred to a glass tube and evaporated till dryness under a stream of nitrogen. The residue was reconstituted with 1 mL of MeOH:H_2O (50:50. v/v).[57]

6.2.6. UHPLC-MS/MS analysis of mycotoxins

The presence of mycotoxins in the extracts from test 2 was detected by means of the application of an UHPLC-MS/MS method previously developed in our laboratory.[58] UHPLC analysis was performed using a C_{18} column (Zorbax Eclipse Plus RRHD 50 x 2.1 mm, 1.8 μm) with a mobile phase consisting of 0.3% aqueous FA solution with 5 mM ammonium formate (solvent A), and MeOH with 0.3% formic acid and 5 mM ammonium formate (solvent B) at a flow rate of 0.4 mL min^{-1}.

57. Arroyo-Manzanares, N.; Di Mavungu, J.D.; Uka, V.; Malysheva, S.V.; Cary, J.W.; Ehrlich, K.C.; Vanhaecke, L.; Bhatnagar, D.; De Saeger, S. Food Aditt. Contam., 32 (2015) 1656-1673.

58. Arroyo-Manzanares, N.; García-Campaña, A.M.; Gámiz-Gracia. L. J. Chromatogr. A, 1282 (2013) 11-19

The eluent gradient profile was as follows: 0 min: 5 % B; 1 min: 50 % B; 4 min: 80 % B; 6 min: 90 % B. Finally it was back to 5 % B in 0.2 min and kept for 2 min for column equilibration. Column temperature was set at 35 °C and the injection volume was 5 µL (full loop).

The MS was working with ESI in positive mode under the MRM conditions shown in table 6.2. The ionization source parameters were: source temperature 500 °C; curtain gas (nitrogen) 30 psi; ion spray voltage 5000 V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi.

Table 6.2. Monitored ions of the target analytes and MS/MS parameters for the determination of mycotoxins in samples in malta agar medium.

	Retention time (min)	Precursor ion (m/z)	Molecular ion	DP[a]	EP[a]	CEP[a]	Product ions[b]	CE[a]	CXP[a]
AFB1	2.18	313.1	$[M+H]^+$	46.0	12.0	26.0	241.0 (Q)	41.0	4.0
							284.9 (I)	39.0	4.0
AFB2	2.07	315.1	$[M+H]^+$	81.0	4.0	34.0	286.9 (Q)	33.0	6.0
							259.0 (I)	39.0	8.0
AFG1	1.95	329.0	$[M+H]^+$	76.0	9.5	16.0	243.1 (Q)	39.0	6.0
							311.1 (I)	29.0	6.0
AFG2	1.86	331.1	$[M+H]^+$	61.0	6.0	42.0	245.1 (Q)	39.0	6.0
							313.1 (I)	27.0	6.0
OTA	3.59	404.0	$[M+H]^+$	41.0	7.5	16	238.9 (Q)	31.0	6.0
							102.1 (I)	91.0	6.0
ZEN	3.53	319.0	$[M+H]^+$	26.0	8.0	20.0	282.9 (Q)	19.0	4.0
							301.0 (I)	15.0	10.0

(a) Declustering potential (DP), Entrance potential (EP), Collision cell entrance potential (CEP), Collision cell exit potential (CXP) and Collision energy (CE). All expressed in voltage.

(b) Product ions: (Q) Transition used for quantification, (I) Transition employed to confirm the identification.

6.3. Results and discussion

6.3.1. Evaluation of fungal growth inhibition by PTSO (test 1)

Fungal growth inhibition was studied firstly by the use of the technique described in section 6.2.4.1 for PTSO doses of 200, 20000, 50000, 100000 and 200000 mg kg^{-1}, in order to check PTSO antifungal activity against different mycotoxin producer strains. The results of the fungal growth inhibition (expressed in mm) at the tested doses of PTSO are shown in table 6.3. The fungi grows concentrically so that, fungal growth inhibition was determined by the measure of the diameter of the zone of inhibition.

Table 6.3. Fungal growth inhibition (expressed in mm) for test 1 at the tested doses of PTSO.

PTSO (mg kg^{-1})	*Penicillium verrucossum*	*Fusarium graminearum*	*Aspergillus parasiticus*	*Aspergillus flavus*
200	5	5	9	5
20000	46	20	56	45
50000	62	35	60	54
100000	70	48	65	58
200000	>85	>85	68	66

A slight inhibition of fungal growth was observed for 200 mg kg^{-1} and was increasing at higher doses of PTSO, showing the antifungal activity of PTSO for the tested strains. Figure 6.2 shows a picture of the inhibition growth for each strain at the different doses tested. Once it had been demonstrated the PTSO antifungal activity, test 2 was

performed in order to evaluate the minimum PTSO concentration (lethal dose, LD) to produce the fungi growth inhibition.

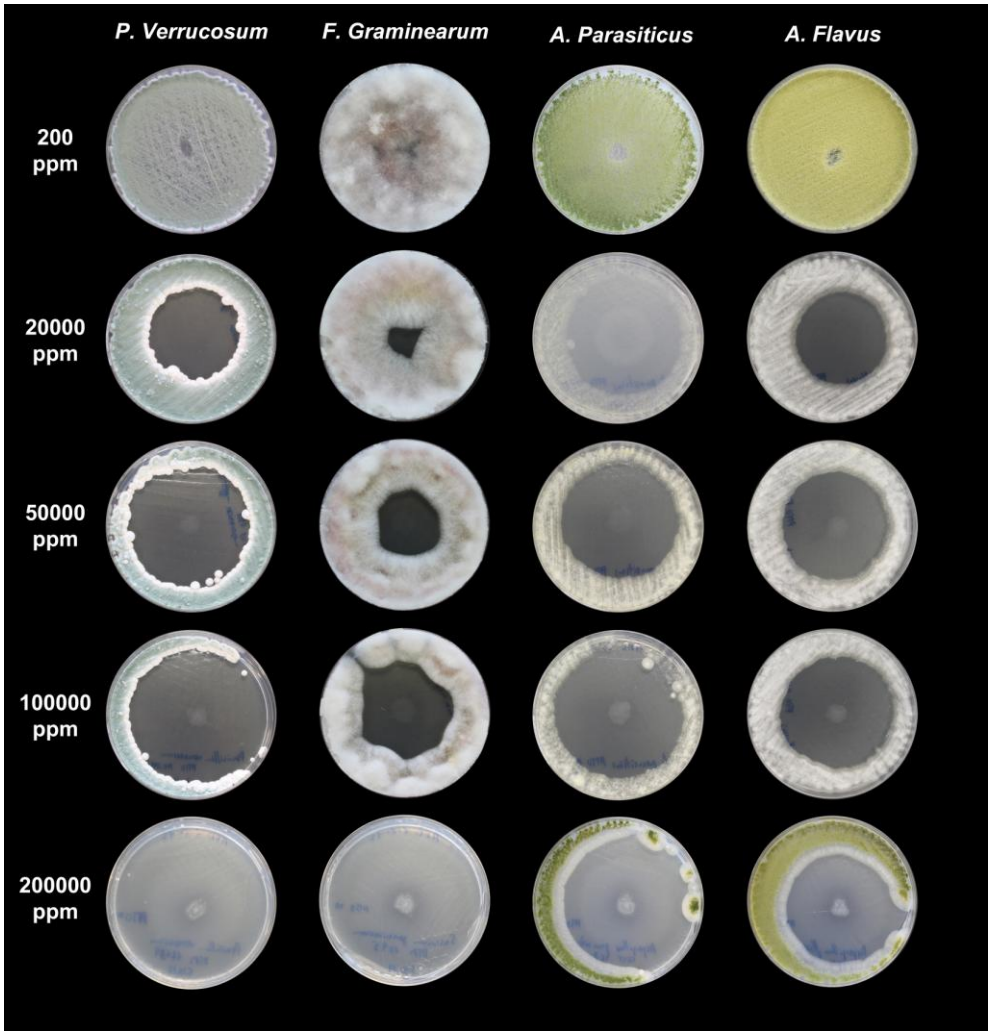

Figure 6.2. Fungal growth inhibition by PTSO for test 1

6.3.2. PTSO LD estimation and mycotoxins production (test 2)

Petri dishes spiked at different concentrations of PTSO (0, 1.875, 3.25, 7.5, 15, 30 and 60 mg kg^{-1}) were prepared for each fungal strain. This procedure is described in section 6.2.4.1. The study consisted in the evaluation, in presence of different concentrations of PTSO, of the mycelial growth in order to estimate the minimum concentration needed to produce the growth inhibition of the studied fungi. Also, the influence on the production of mycotoxins during a total of 21 days was studied. Three replicates of each PTSO concentration were considered for each strain and for each sampling time (7, 14 and 21 days). The codes used for the samples in this test are shown in table 6.4. Then, samples were treated according to the methodology described in section 6.2.5 and analyzed according to the methodology described in sections 6.2.6.

Table 6.4. Codes used for samples analyzed in the fungal growth evaluation (test 2)

P		F		AP		AF	
Code	**mg kg^{-1}**	**Code**	**mg kg^{-1}**	**Code**	**mg kg^{-1}**	**Code**	**mg kg^{-1}**
P0	0	**F0**	0	**AP0**	0	**AF0**	0
P1	1.875	**F1**	1.875	**AP1**	1.875	**AF1**	1.875
P2	3.125	**F2**	3.125	**AP2**	3.125	**AF2**	3.125
P3	7.5	**F3**	7.5	**AP3**	7.5	**AF3**	7.5
P4	15	**F4**	15	**AP4**	15	**AF4**	15
P5	30	**F5**	30	**AP5**	30	**AF5**	30
P6	60	**F6**	60	**AP6**	60	**AF6**	60

P: *Penicillium verrucosum*; F: *Fusarium graminearum*; AP: *Aspergillus parasiticus*; AF: *Aspergillus flavus*

6.3.2.1. Penicillium verrucosum

6.3.2.1.1. Fungal growth inhibition by PTSO

Table 6.5 and figures 6.3 and 6.4 show mycelial growth results for *Penicillium verrucosum* in presence of PTSO. Mycelial growth was measured weekly and it was determined that *Penicillium verrucosum* growth was inhibited at a dose of 15 mg kg^{-1} of PTSO (P3). Petri dish photographs for each concentration level and studied time are included in figure 6.4.

Table 6.5. Mycelial growth (expressed in mm) of *Penicillium verrucosum* in different days and in presence of different PTSO concentrations

Time (days)	0	7	14	21
P0	-	3.1	5.8	7.1
P1	-	3.2	5.3	7.1
P2	-	3.2	5.6	6.8
P3	-	2.2	3.9	6.8
P4	-	-	-	-
P5	-	-	-	-
P6	-	-	-	-

As can be seen in table 6.5, samples P3 shows a slightly minor fungal growth compared to P0, P1 and P2, at 7 and 17 days. At 21 days no significant differences in mycelial growth were observed among P0, P1, P2 and P3, so P4 was therefore considered as PTSO inhibition dose against *Penicillium verrucosum.*

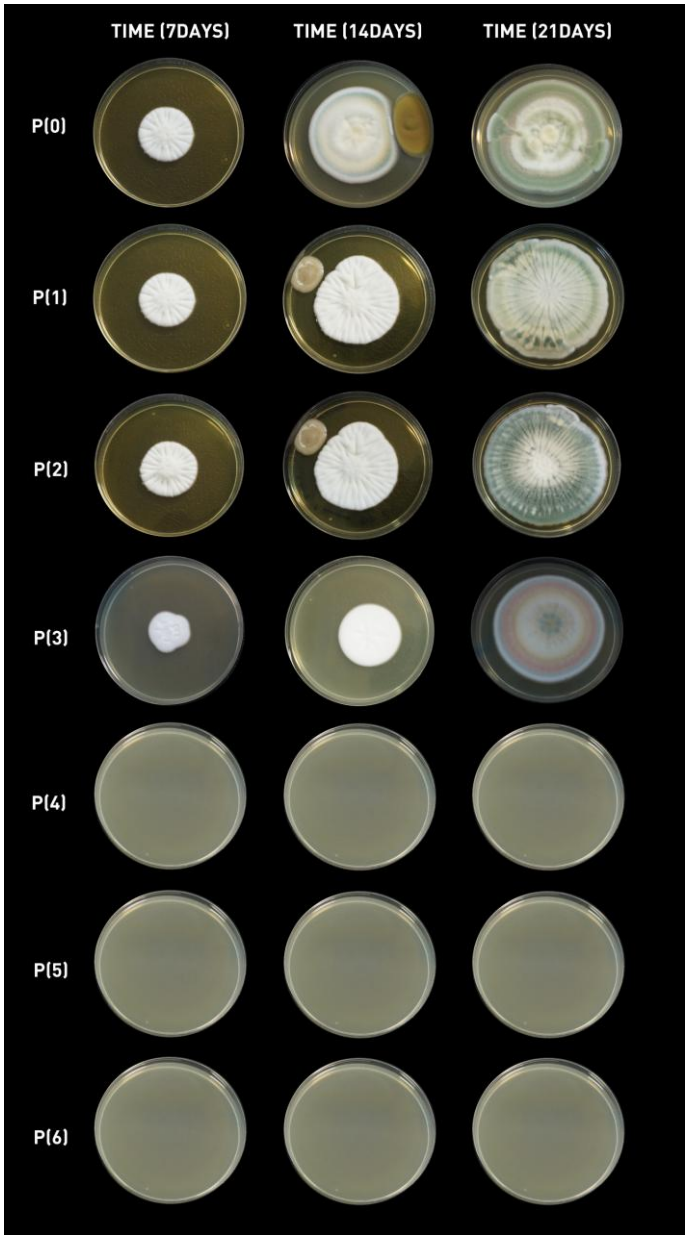

Figure 6.3. Mycelial growth of *Penicillium verrucosum* in presence of different PTSO concentrations in different days

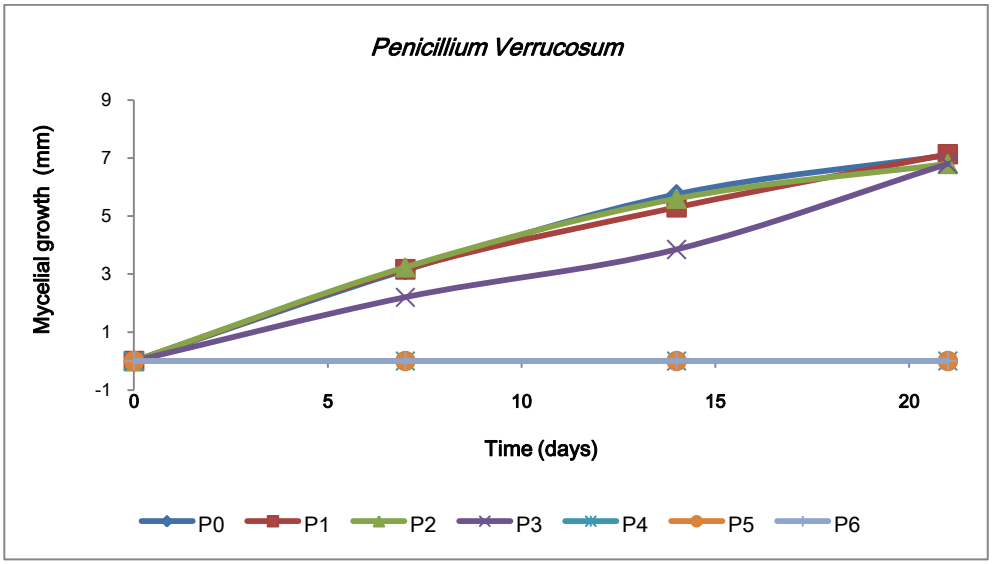

Figure 6.3. Evolution of the mycelial growth of *Penicillium verrucosum* (expressed in mm) in presence of different PTSO concentrations

6.3.2.1.2. Influence of PTSO on OTA production

No influence of PTSO on OTA production was established. Figure 6.4 shows a chromatogram of an extract from the sample P1, analyzed by UHPLC-MS/MS. OTA production was observed only in samples were *Penicillium verrucosum* had grown. OTA production was evaluated in terms of peak area, showing that values obtained for P3 at 7 and 14 days were slightly lower than P0, P1 and P2 at the same time interval, due to a lower growth of the fungus for those times. At 21 days, when the growth of the fungus was the same for P0, P1, P2 and P3, there were not significant differences in the production of OTA (see figure 6.5).

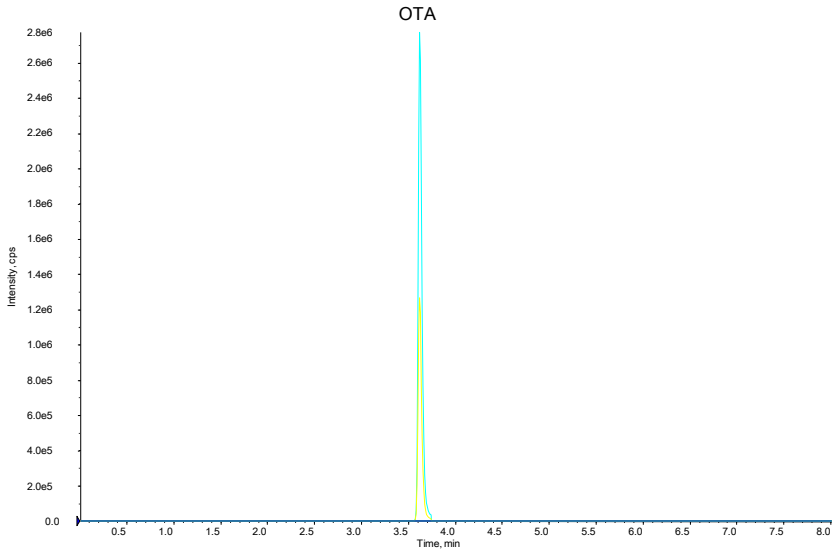

Figure 6.4. Chromatogram of sample P1. OTA is detected.

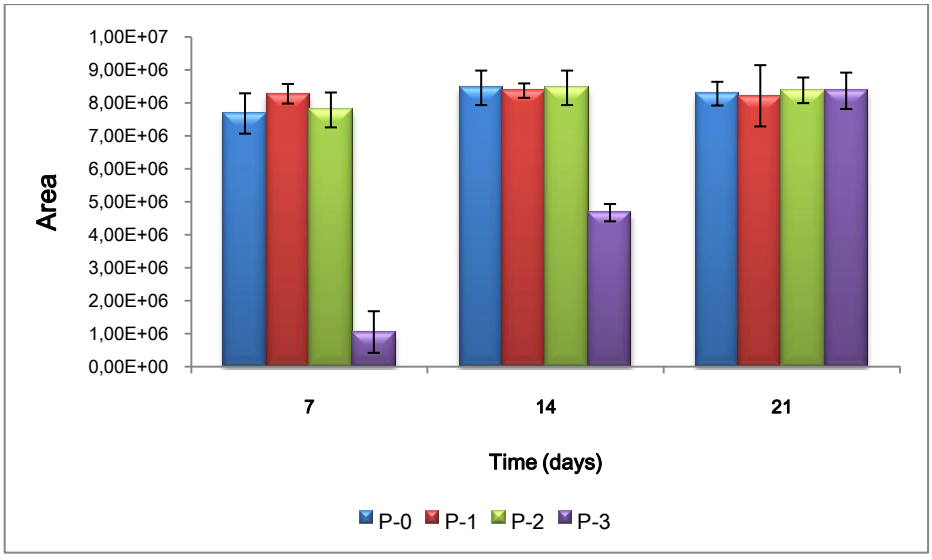

Figure 6.5. Influence of PTSO concentration on OTA production (expressed as peak area) in relation to the interval time

6.3.2.2. Fusarium graminearum

6.3.2.2.1. Fungal growth inhibition by PTSO

Table 6.6 and figures 6.6 and 6.7 show the evolution of the mycelial growth for *Fusarium graminearum* in presence of PTSO. Mycelial growth was measured weekly and it was determined that *Fusarium graminearum* growth was inhibited at a dose of 7.5 mg kg^{-1} of PTSO (F3). Petri dish photographs for each concentration level and studied time are included in figure 6.6.

Table 6.6. Mycelial growth (expressed in mm) of *Fusarium graminearum* in presence of PTSO

Time (days)	0	7	14	21
F0	-	9.0	9.0	9.0
F1	-	9.0	9.0	9.0
F2	-	9.0	9.0	9.0
F3	-	-	-	-
F4	-	-	-	-
F5	-	-	-	-
F6	-	-	-	-

As can be seen in table 6.7, no differences among F0, F1 and F2 were observed at 7, 14 and 21 days. *Fusarium* growth is very quick, so the whole diameter of Petri dish is occupied by the fungi since the day 7. F3 is therefore considered as PTSO inhibition dose against *Fusarium graminearum.*

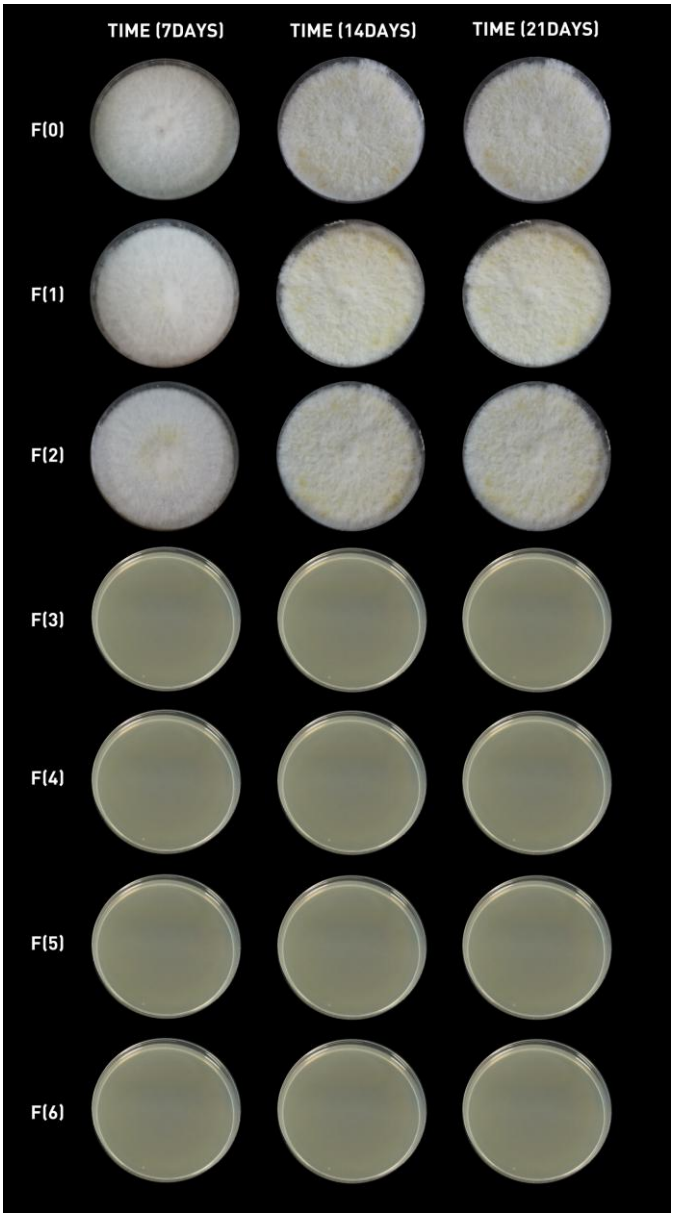

Figure 6.6. Mycelial growth of *Fusarium graminearum* in presence of different PTSO concentrations in different days

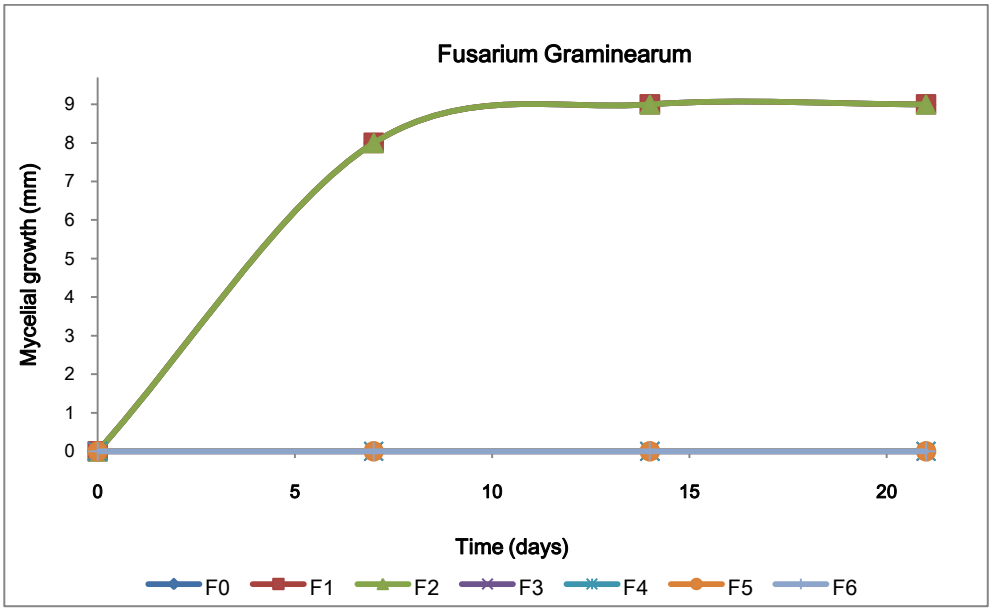

Figure 6.7. Evolution of the mycelial growth (expressed in mm) of *Fusarium graminearum* in presence of different PTSO concentrations

6.3.2.2.2. Influence of PTSO on ZON production

A significant reduction on ZON production in samples treated with PTSO was observed. This reduction was increased at higher PTSO concentrations. Figure 6.8 shows a chromatogram corresponding of an extract from sample F1 analyzed by UHPLC-MS/MS. ZON production was observed only in samples were *Fusarium graminearum* had grown (see figure 6.9).

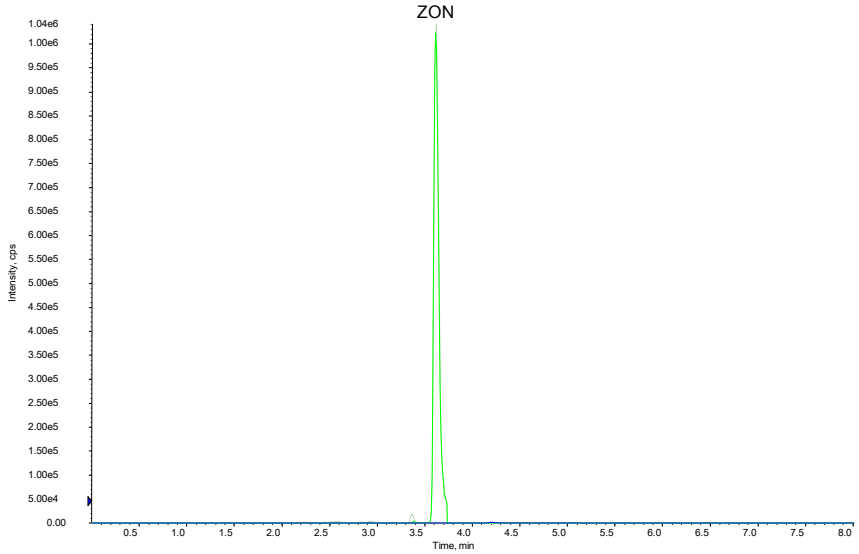

Figure 6.9. Chromatogram of sample F1. ZON is detected.

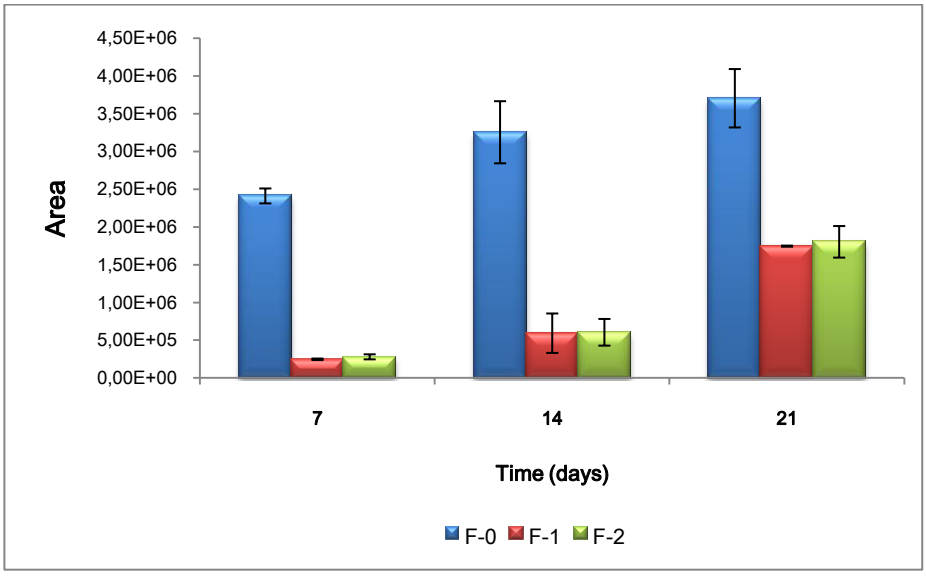

Figure 6.5. Influence of PTSO concentration on ZON production (expressed as peak area) in relation to the interval time

6.3.2.3. Aspergillus parasiticus

6.3.2.3.1. Fungal growth inhibition by PTSO

Table 6.7 and figures 6.8 and 6.9 show mycelial growth results for *Aspergillus parasiticus* in presence of PTSO. Mycelial growth was measured weekly and it was determined that *Aspergillus Parasiticus* growth was inhibited at a dose of 7.5 mg kg^{-1} of PTSO (AP3). Petri dish photographs for each concentration level and studied time are included in figure 6.3.

Table 6.7. Myceliall growth (expressed in mm) of *Aspergillus parasiticus* in presence of different concentrations of PTSO at different time intervals

Time (days)	0	7	14	21
AP0	-	5.4	9.0	9.0
AP1	-	6.3	9.0	9.0
AP2	-	6.4	9.0	9.0
AP3	-	-	-	-
AP4	-	-	-	-
AP5	-	-	-	-
AP6	-	-	-	-

As can be seen in table 6.8, no differences among AP0, AP1 and AP2 were observed at 7, 14 and 21 days. *Aspergillus parasiticus* growth takes up the whole diameter of petri dish from 14 days. AP3 was therefore considered as PTSO inhibition dose against *Aspergillus parasiticus.*

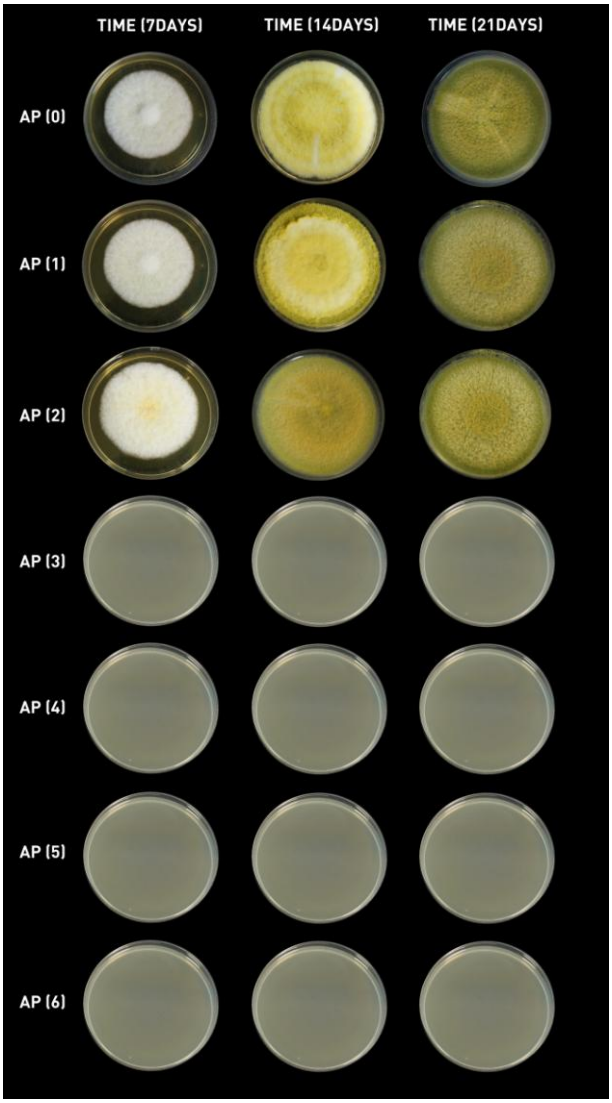

Figure 6.8. Mycelial growth of *Aspergillus parasiticus* in presence of different PTSO concentrations at different days

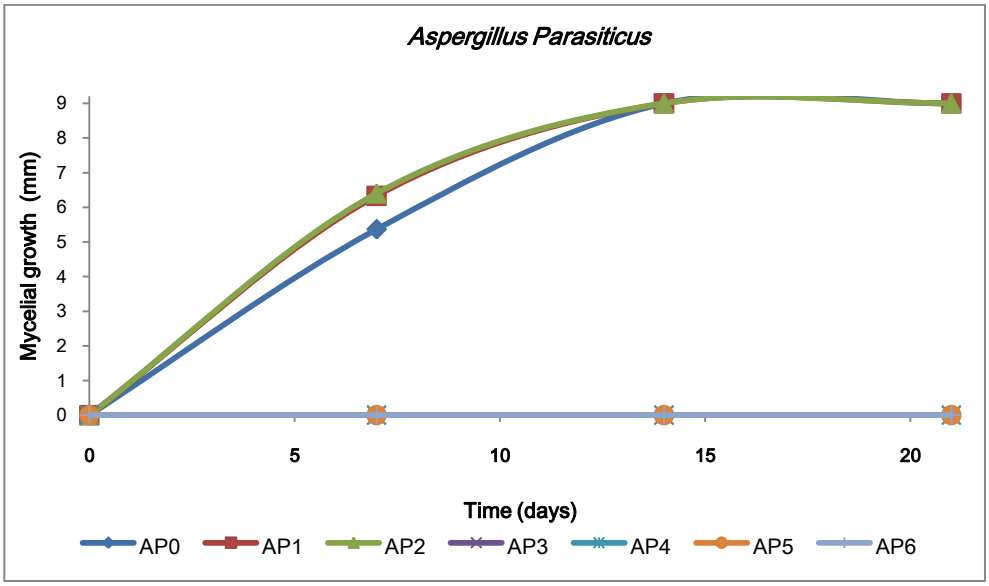

Figure 6.9. Evolution of mycelial growth (expressed in mm) of *Aspergillus parasiticus* in presence of PTSO

6.3.2.3.2. Influence of PTSO on aflatoxin production

No influence of PTSO on AFB1, AFB2, AFG1 and AFG2 production was observed. Figure 6.10 shows a chromatogram corresponding of an extract from AP1 and analyzed by UHPLC-MS/MS. AFB1, AFB2, AFG1 and AFG2 production (expressed as peak area) was observed only in samples where *Aspergillus parasiticus* had grown and no significance differences were found among different analyzed samples (see results for AFB1, AFB2, AFG1 and AFG2 are shown in figures 6.11, 6.12, 6.13 and 6.14, respectively.

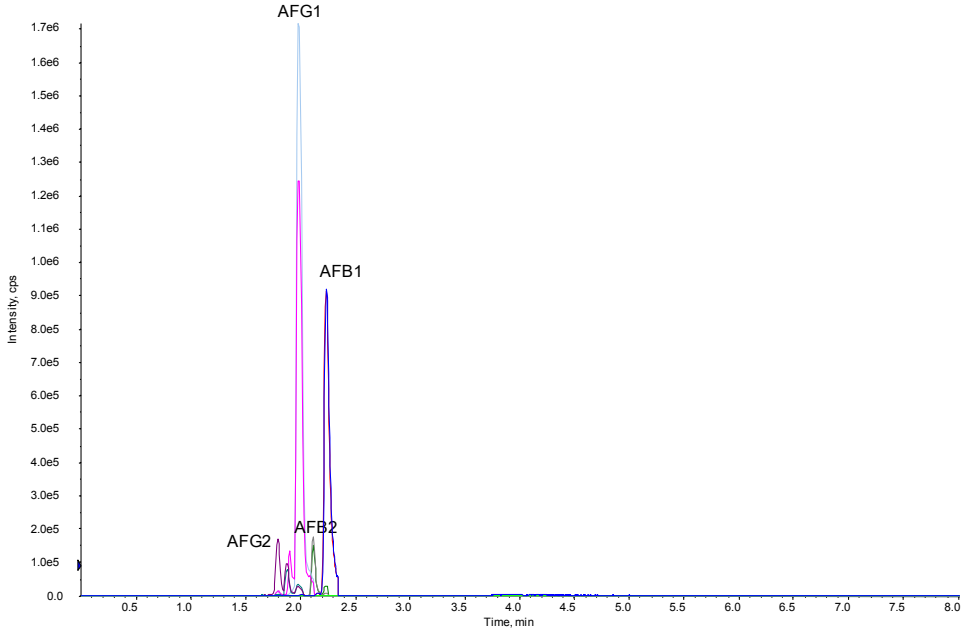

Figure 6.10. Cromatogram of sample AP1, AFB1, AFB2, AFG1 and AFG2 are detected

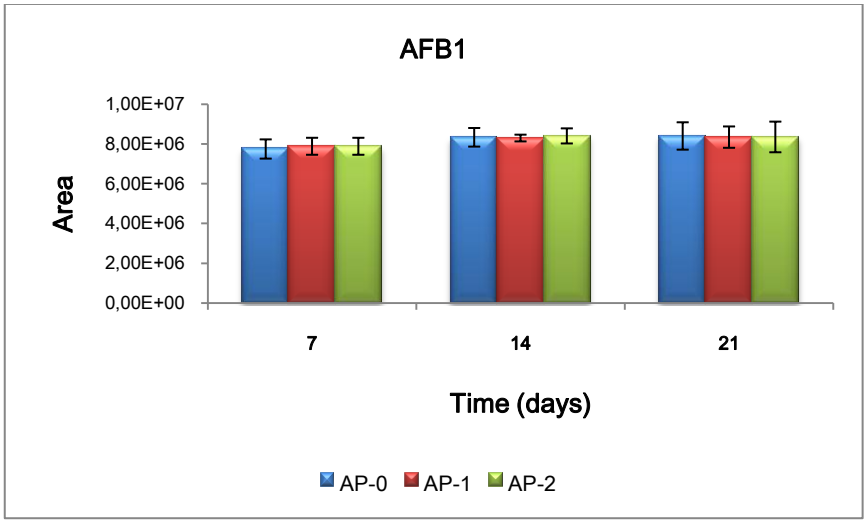

Figure 6.11. Influence of PTSO concentration on AFB1 production (expressed as peak area) in relation to the interval time

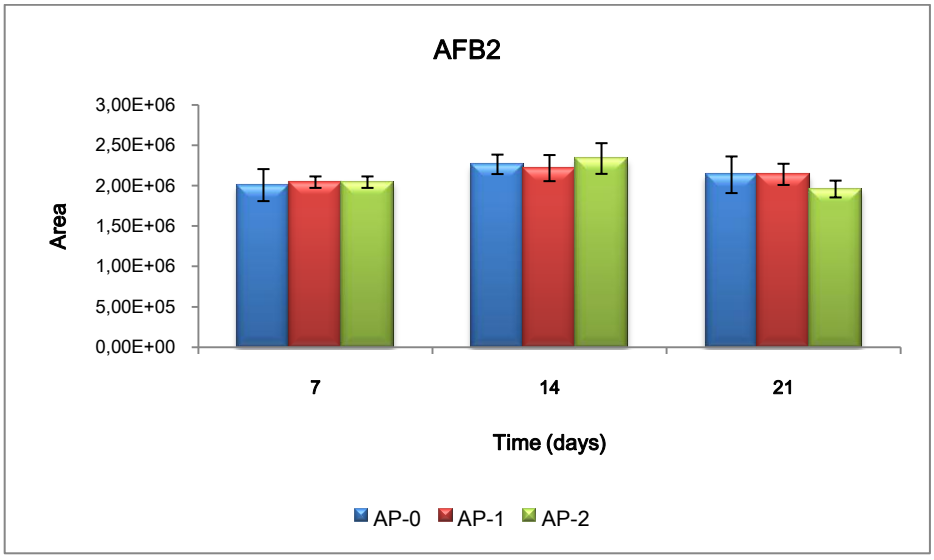

Figure 6.12. Influence of PTSO concentration on AFB2 production (expressed as peak area) in relation to the interval time

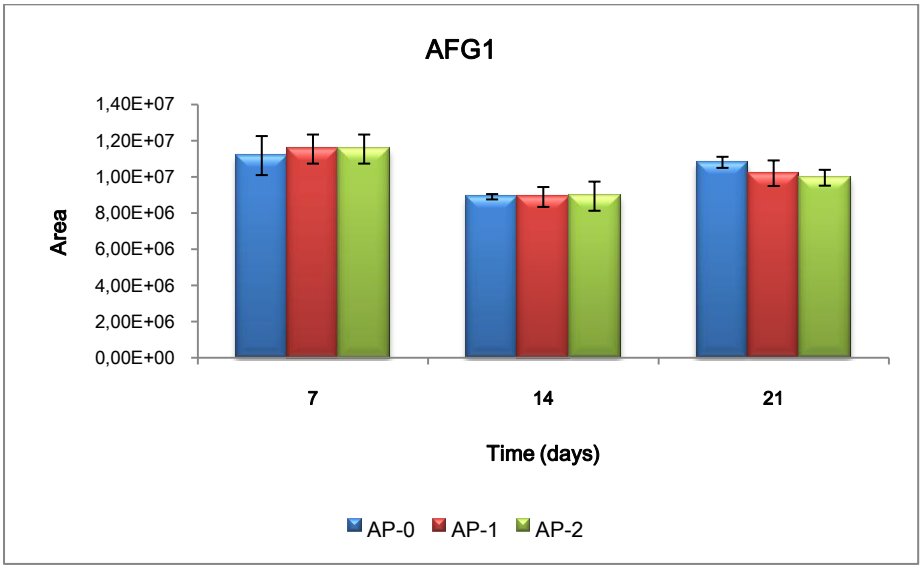

Figure 6.13. Influence of PTSO concentration on AFG1 production (expressed as peak area) in relation to the interval time

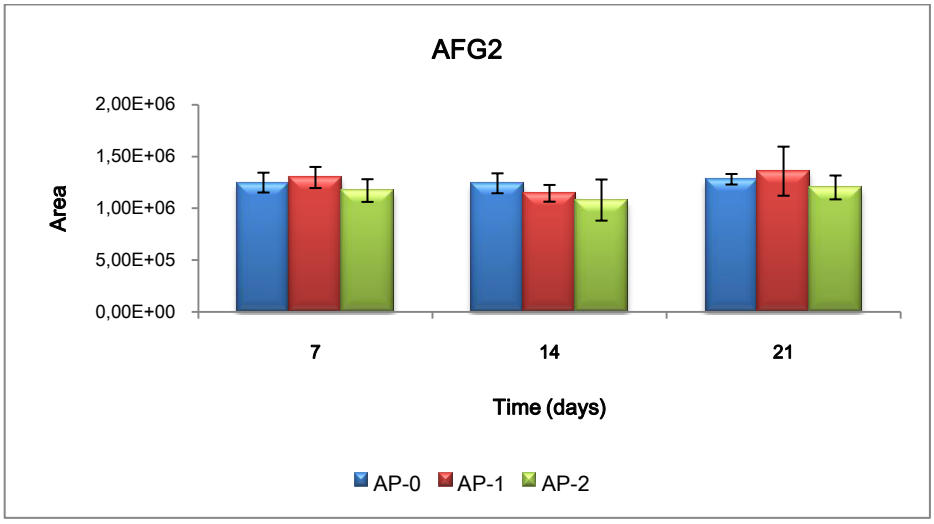

Figure 6.14. Influence of PTSO concentration on AFG2 production (expressed as peak area) in relation to the interval time

6.3.2.4. Aspergillus flavus

Table 6.8 and figures 6.15 and 6.16 show mycelial growth results for *Aspergillus flavus* in presence of PTSO. Mycelial growth was measured weekly and it was determined that *Aspergillus flavus* growth was inhibited at a dose of 30 mg kg^{-1} of PTSO (AF5). Petri dish photographs for each concentration level and studied time are included in figure 6.12.

6.3.2.4.1. Fungal growth inhibition

Table 6.8. Mycelial growth (expressed in mm) of *Aspergillus flavus* in presence of PTSO

Time (days)	0	7	14	21
AF0	-	7.1	9.0	9.0
AF1	-	7.0	9.0	9.0
AF2	-	6.9	9.0	9.0
AF3	-	6.2	9.0	9.0
AF4	-	-	6.7	9.0
AF5	-	-	-	-
AF6	-	-	-	-

As can be seen in table 6.9, fungal growth was slowed down by the presence of PTSO for AF4 compared to AF3, AF2, AF1 and AF0. Nevertheless, this dose was not enough and finally *Aspergillus flavus* grew until it filled the whole plate. At 21 day no differences in mycelial growth are observed among AF4, AF3, AF2, AF1 and AF0. AF4 is therefore considered as PTSO inhibition dose against *Penicillium verrucosum.*

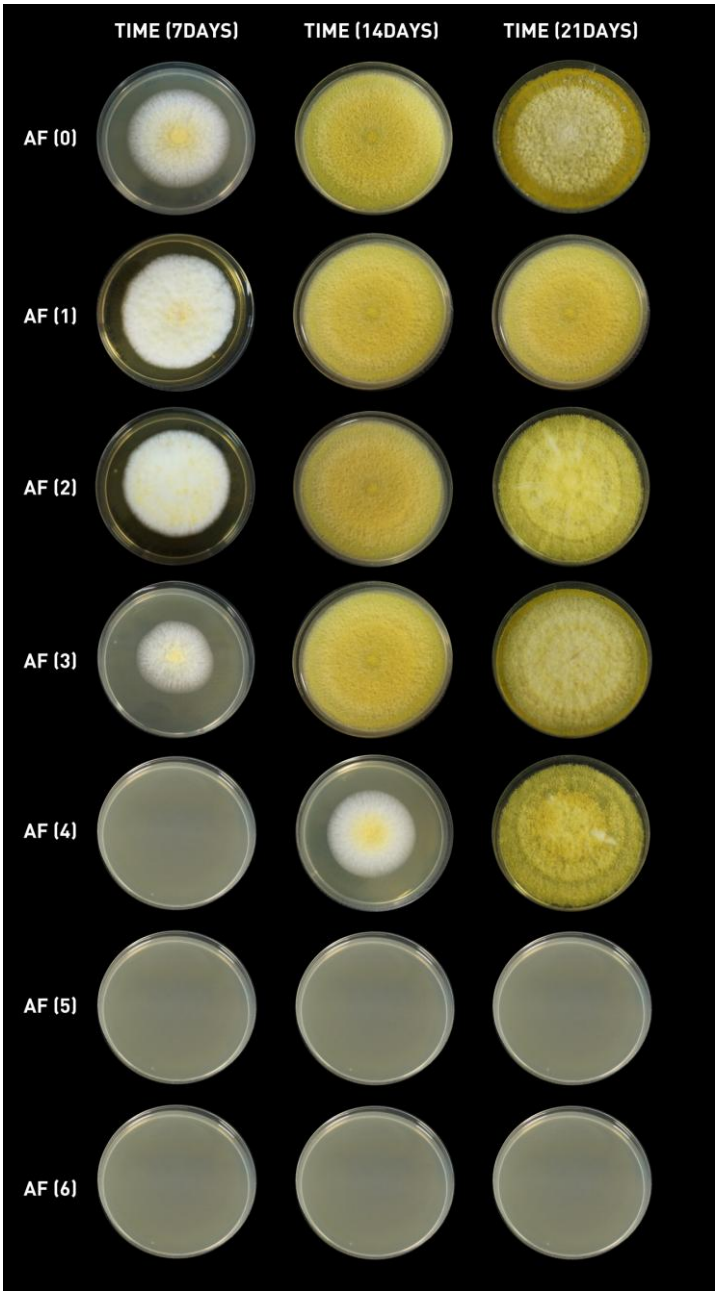

Figure 6.15. Mycelial growth of *Aspergillus Flavus* in presence of of different PTSO concetrations at different days

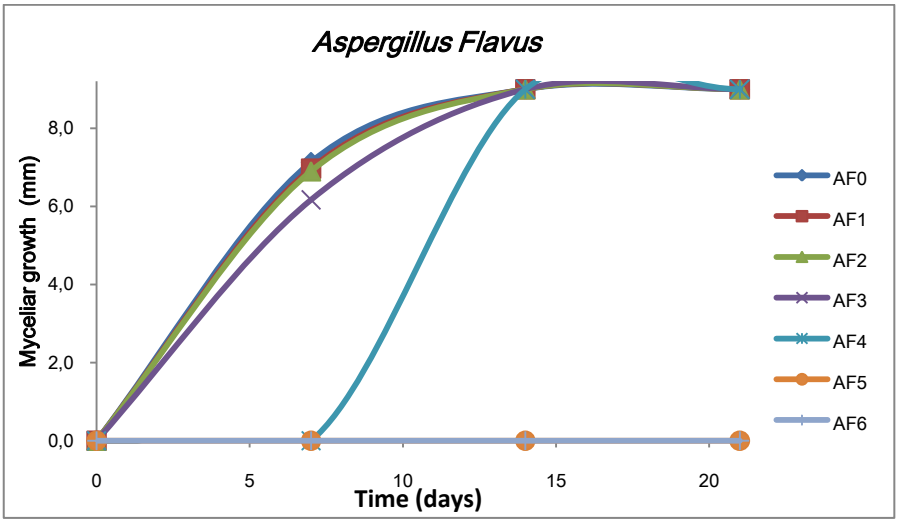

Figure 6.16. Evolution of mycelial growth (expressed in mm) of *Aspergillus Flavus* in presence of PTSO

6.3.2.4.2. Influence of PTSO on aflatoxin production

Figure 6.17 shows a chromatogram extracted from sample AF1, analyzed by UHPLC-MS/MS. AFB1 and AFB2 production was observed only in samples were *Aspergillus flavus* had grown. No significance differences were found among different analyzed samples in relation to AFB1 production. AFB1 production in samples AF3 (time 4 days) and AF4 (time 21 days) was lower than the rest of samples due to the fungal growth had been slowed down. At 21 day, when the growth of the fungus was the same for AF0, AF1, AF2, AF4 and AF5, there were not significant differences in the amount of mycotoxins produced.

In the case of AFB2, it seemed that there was a decrease on fungal mycotoxin production when *Aspergillus flavus* was in contact PTSO at the doses corresponding to AF3 and AF4 samples, although AF5 sample corresponds to the PTSO grow inhibition dose. The results are shown in figures 6.18 and 6.19.

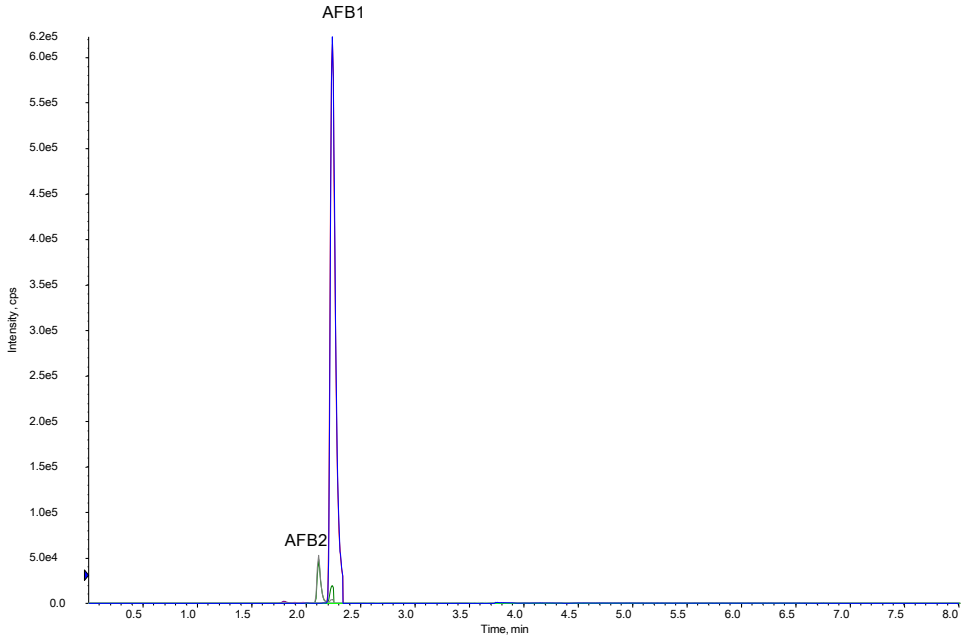

Figure 6.17. Chromatogram of sample AF1. AFB1 and AFB2 are detected

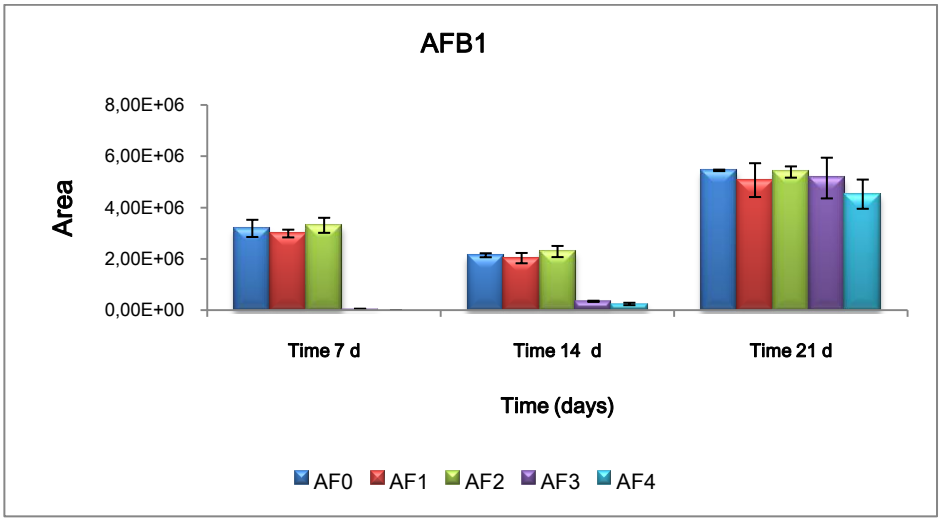

Figure 6.18. Influence of PTSO concentration on AFB1 production (expressed as peak area) in relation to the interval time

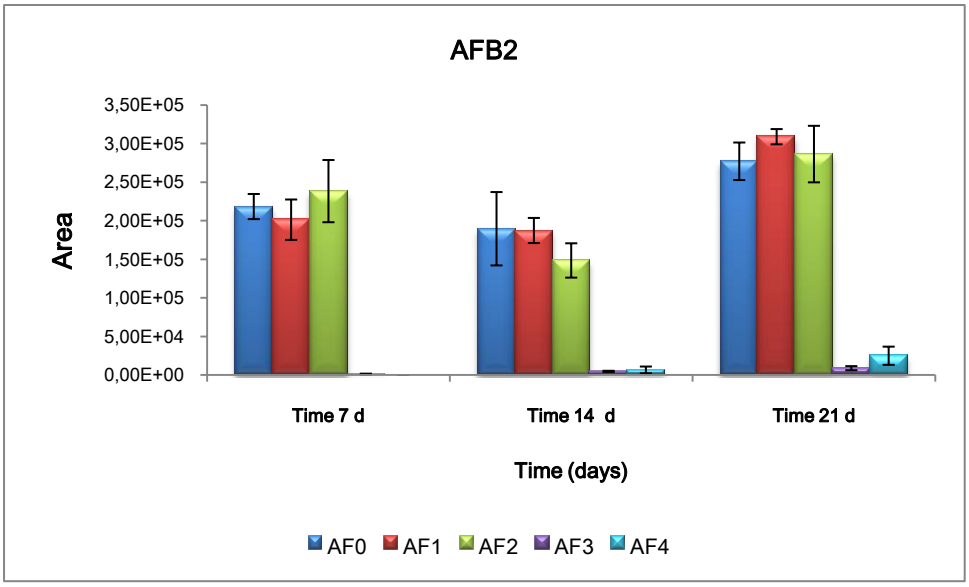

Figure 6.19. Influence of PTSO concentration on AFB2 production (expressed as peak area) in relation to the interval time

6.3.3. Lethal dose of PTSO for the studied mycotoxigenic fungi.

This study was performed to determine the minimum concentration of PTSO needed to inhibit the growth of each strain. Based on the results obtained in section 6.3.2, the following concentrations were determined for the evaluation of the PTSO lethal dose (LD) for each tested fungus. LD was defined as the minimum dose to which fungi growth is inhibited. Samples were prepared according to section 6.2.4. The codes used for the samples considered in this test are shown in table 6.10.

Table 6.10. Codes used for the samples in relation to PTSO concentration, used to determine the PTSO lethal dose for each tested strain.

P		F		AP		AF	
Code	**mg kg^{-1}**	**Code**	**mg kg^{-1}**	**Code**	**mg kg^{-1}**	**Code**	**mg kg^{-1}**
LD-P0	0l	**LD-F0**	0	**LD-AP0**	0	**LD-AF0**	0
LD-P1	10.0	**LD-F1**	4	**LD-AP1**	4	**LD-AF1**	17.5
LD-P2	12.5	**LD-F2**	6	**LD-AP2**	6	**LD-AF2**	20.0
						LD-AF3	22.5
						LD-AF4	25.0

P: *Penicillium verrucosum*; F: *Fusarium graminearum*; AP:*Aspergillus parasiticus*; AF: *Aspergillus flavus*

Fungi growth was observed for 21 days at the tested doses (days 0, 2, 4, 7, 14 and 21). Table 6.11 shows the LD values (10.0, 4.0, 7.5 and 17.5 mg kg^{-1} for *Penicillium verrucosum, Fusarium graminearum, Aspergillus parasiticus* and *Aspergillus flavus,* respectively).

Table 6.11. Mycelial growth of mycotoxigenic tested strains in presence of PTSO and LD obtained for each one.

Time (days)	0	2	4	7	14	21
LD-P0	-	+	+	+	+	+
LD-P1	-	-	-	-	-	-
LD-P2	-	-	-	-	-	-
Penicillium verrucosum LD=10 mg kg^{-1}						
LD-F0	-	+	+	+	+	+
LD-F1	-	-	-	-	-	-
LD-F2	-	-	-	-	-	-
Fusarium graminearum LD=4 mg kg^{-1}						
LD-AP0	-	+	+	+	+	+
LD-AP1	-	+	+	+	+	+
LD-AP2	-	+	+	+	+	+
Aspergillus parasiticus LD=7.5 mg kg^{-1}						
LD-AF0	-	+	+	+	+	+
LD-AF1	-	-	-	-	-	-
LD-AF2	-	-	-	-	-	-
LD-AF3	-	-	-	-	-	-
LD-AF4	-	-	-	-	-	-
Aspergillus flavus LD=17.5 mg kg^{-1}						

(+) : positive fungal grow ; (-) : negative fungal grow.

6.4. Conclusions

In this chapter PTSO is shown as a potential biocontrol agent, helping to prevent the grow of some mycotoxigenic fungi strains, such as *Penicillium verrucosum, Fusarium graminearum, Aspergillus parasiticus* and *Aspergillus flavus* by its capability of inhibiting fungal growth.

Antifungal capacity of PTSO has been demonstrated, establishing LD for *Penicillium verrucosum, Fusarium graminearum, Aspergillus parasiticus* and *Aspergillus flavus* (10, 4.0, 7.5 and 17.5 mg kg^{-1}, respectively). However, PTSO has not a general effect in the inhibition of mycotoxin production on all the studied strains, which is associated to fungal growth. In this sense, OTA, ZON, AFB1, AFB2, AFG1 or AFG2 were observed only in the studied samples in which the fungi had grown, in presence of PTSO concentrations lower than the LD. The presence of mycotoxins was detected by UHPLC-MS/MS.

Penicillium verrucosum and *Aspergillus parasiticus* mycotoxins production (OTA, AFB1, AFB2, AFG1 and AFG2) was not affected by the presence of PTSO in the growth medium whereas in the case of *Fusarium graminearum*, a significant reduction on ZON production was observed. This reduction was increased at higher doses of PTSO. Lastly, related to *Aspergillus flavus*, AFB1 was not affected by the presence of PTSO whereas AFB2 suffer a decrease on its production in presence of PTSO.

Conclusiones finales

Conclusiones finales

Este Tesis ha abordado el estudio de un compuesto natural de gran interés en la industria alimentaria, específicamente en el ámbito de la alimentación animal, el propil propano tiosulfonato (PTSO). La problemática principal abordada en esta Tesis, ha sido el estudio del comportamiento del PTSO en piensos destinados a alimentación animal y también en el propio animal tras la ingesta, haciendo posible la evaluación de su inocuidad con objeto de garantizar su estabilidad como aditivo en piensos así como la seguridad alimentaria de productos y subproductos de animales que han incluido este ingrediente en su dieta habitual.

Dada la novedad de esta molécula y su empleo como aditivo en alimentación animal por sus interesantes propiedades y considerando la falta de métodos analíticos que permitiesen su determinación, tanto en piensos, así como en productos derivados de animales que lo han ingerido, donde pudiera aparecer como residuo, en la presente Tesis se han conseguido los siguientes logros:

- Se ha desarrollado por primera vez un método analítico usando cromatografía líquida de alta resolución con detección UV (HPLC-UV) para la determinación de PTSO en piensos con bajo contenido en cisteína (CYS) y glutatión (GSH) que permite, de manera sencilla y económica, llevar a cabo un control de calidad rutinario en piensos enriquecidos con este aditivo.

- La metodología desarrollada inicialmente presentó el inconveniente de no ser efectiva para piensos con alto contenido en CYS y GSH. Para este tipo de piensos se observó, durante el control de calidad rutinario, que el contenido de

PTSO estimado era muy inferior al contenido real. En este sentido se ha estudiado por primera la reacción del PTSO con el grupo tiol de CYS y GSH presentes en los piensos, generando s-propil mercaptocisteína (CSSP) y s-propil mercaptoglutatión (GSSP) respectivamenye. Teniendo esto en cuenta se ha desarrollado una nueva metodología usando cromatografía líquida de ultra-alta eficacia con espectrometría de masas en tándem (UHPLC-MS/MS) con objeto de analizar simultáneamente de PTSO, GSSP y CSSP en piensos con objeto de controlar su calidad, teniendo en cuenta las posibles interacciones que puede haber con los distintos ingredientes del pienso.

- El PTSO se utiliza como aromatizante/saborizante en piensos destinados a la alimentación de rumiantes (vacas, cabras y ovejas) actuando como inhibidor en la producción de metano. Con objeto de controlar los residuos de este aditivo en leche y estudiar sus posibles efectos en las propiedades organolépticas de la misma, se ha desarrollado un método analítico usando HPLC-UV. En el caso de la leche no se ha determinado en su composición la presencia de elementos que puedan reaccionar con el PTSO. Gracias a este nuevo método, es posible controlar sus residuos en leche garantizando que los atributos sensoriales de la misma no se ven afectados por la presencia de PTSO.

- También se ha desarrollo de un método analítico para la determinación PTSO en huevos obtenidos de gallinas alimentadas con pienso enriquecido con PTSO, mediante UHPLC-MS/MS. En el caso del huevo, debido a su elevado contenido en cisteína y a la presencia del glutatión, el PTSO reacciona rápidamente dando lugar a CSSP y GSSP, de modo que su cuantificación se realizó como la suma de

GSSP y CSSP. Gracias a esta nueva técnica, es posible establecer un control de calidad en huevo que permita garantizar que sus propiedades organolépticas y nutricionales no ve ven afectadas por la ingesta de PTSO. Adicionalmente, se ha demostrado que el uso de PTSO como aditivo mejora la productividad de las gallinas ponedoras (aumento en la cantidad y el peso del huevo) y posee efecto inmunomodulador sobre la microbiota intestinal (aumento de la población de *Lactobacillus spp*. y *Bifidobacterium spp.*) Así mismo, el uso de PTSO permite la reducción de las poblaciones de Eneterobacterias, un patógeno responsable de la aparición de enfermedades tan peligrosas para el ser humano, como la Salmonella.

- Se ha evaluación la biodisponibilidad del PTSO (fracción de PTSO que es absorbida y metabolizada después de ser ingerido) en cerdos y la influencia de la inclusión de agentes de vehiculizacion y/o encapsulación como agentes de mejora de la misma. Se ha estudiado tanto el PTSO como principio activo puro, como PTSO soportado en diversos agentes vehiculizantes (tween 80, ciclodextrina y ácidos grasos) obteniéndose los mejores resultados (aumentado hasta 3.5 veces respecto al principio activo puro) para el PTSO soportado en tween 80.

- Finalmente se ha evaluado la capacidad antifúngica de PTSO frente a cuatro hongos micotoxigénicos: *Penicillium verrucosum* (Ocratoxina A), *Fusarium graminearum* (Zearalenona)*, Aspergillus parasiticus* (Aflatoxinas B1, B2, G1 y G2*)*, *Aspergillus flavus* (Aflatoxinas B1 y B2*).* Se ha comprobado el carácter antifúngico del PTSO frente a las 4 cepas ensayadas. En general, el PTSO actúa como inhibidor del crecimiento micelar, y por tanto como un agente de prevención

en la formación de micotoxinas (su presencia no influye en la producción de micotoxinas una vez que el hongo ha crecido). Sin embargo, en el caso de producción de zearalenona procedente de *Fusarium graminearum* y de AFB2 procedente de Aspergillus flavus, se ha observado una reducción significativa en la producción de micotoxinas independientemente del crecimiento del hongo.

FINAL CONCLUSIONS

Final conclusions

This thesis has studied a natural compound of great interest in the food industry, specifically in the field of animal feed, propyl propane thiosulfonate (PTSO). The main problem presented in this Thesis, has been the study of the behavior of PTSO in animal feed and also its effect in the animal after being ingested, making possible the evaluation of PTSO safety and stability as an additive in animal feed and food (products from animals which have ingested PTSO).

Given the novelty of this molecule, its use as an additive in animal feed for its interesting properties and considering the lack of analytical methods that would allow PTSO determination, both in animal feed and in products derived from animals that have ingested it, where it could appear as residue, in this thesis the following achievements have been achieved:

- An analytical method has been developed for the first time using high performance liquid chromatography with UV detection (HPLC-UV) for the determination of PTSO in feeds with low cysteine content (CYS) and glutathione (GSH) that allows, in a simple and ecomomic way, carry out a routine quality control in feed enriched with this additive.
- The methodology developed initially presented the disadvantage of not being effective for feed with high content in CYS and GSH. For this type of feed it was observed, during the routine quality control, that the estimated PTSO content was much lower than the real obtained content. In this sense, the reaction of the PTSO with the thiol group was studied. CYS and GSH present in the feed reacted with PTSO giving s-propyl mercaptocysteine (CSSP) and s-propyl mercaptoglutathione

(GSSP) respectively. With this in mind, a new methodology has been developed using ultra-high efficiency liquid chromatography with tandem mass spectrometry (UHPLC-MS / MS) in order to analyze PTSO, GSSP and CSSP simultaneously in feed.

- PTSO is used as flavoring in ruminants feed intended (cows, goats and sheep) acting as an inhibitor in the production of methane. In order to control PTSO residues in milk and its possible effects on the organoleptic properties, an analytical method using HPLC-UV has been developed. In the case of milk, the presence of elements that can react with the PTSO has not been determined in its composition. Thanks to this new method, it is possible to control its residues in milk, guaranteeing that the sensorial attributes of it are not affected by the presence of PTSO.
- An analytical method has also been developed for the determination of PTSO in eggs by of UHPLC-MS/MS. In the case of the egg, due to its high content of cysteine and the presence of glutathione, the PTSO reacts rapidly giving rise to CSSP and GSSP, so that its quantification was performed as the sum of GSSP and CSSP. Thanks to this new technique, it is possible to establish an egg quality control that ensures that its organoleptic and nutritional properties are not affected by the intake of PTSO. Additionally, it has been demonstrated that the use of PTSO as an additive improves the productivity of laying hens (increase in the amount and weight of the egg) and has immunomodulatory effect on the intestinal microbiota (increase in the population of *Lactobacillus spp.* and *Bifidobacterium spp.*) Likewise, the use of PTSO allows the reduction of populations of Eneterobacteria, a pathogen responsible for the occurrence of diseases so dangerous to humans, such as Salmonella.

- The bioavailability of PTSO (fraction of PTSO that is absorbed and metabolized after being ingested) has been evaluated in pigs and the influence of the inclusion of vehiculization and/or encapsulation agents as agents for improving it. Both PTSO and PTSO supported in various vehicle agents (tween 80, cyclodextrin and fatty acids) have been studied obtaining the best results (increased up to 3.5 times compared to the pure active principle) for the PTSO supported in tween 80.
- Finally, the antifungal capacity of PTSO has been evaluated against four mycotoxigenic fungi: *Penicillium verrucosum* (Ochratoxin A), *Fusarium graminearum* (Zearalenone), *Aspergillus parasiticus* (Aflatoxin B1, B2, G1 and G2), *Aspergillus flavus* (Aflatoxin B1 and B2). Antifungal properties of PTSO have been verified against the 4 strains tested. In general, PTSO acts as inhibitor of micellar growth, and therefore as a prevention agent in the formation of mycotoxins (its presence does not influence the production of mycotoxins once the fungus has grown). However, in the case of production of Zearalenone from *Fusarium graminearum* and AFB2 from *Aspergillus flavus*, a significant reduction in the production of mycotoxins has been observed independently of the growth of the fungus.

Abreviaturas y acrónimos

Abreviaturas y acrónimos

AFB1:	Aflatoxin B1
AFB2:	Aflatoxin B2
AFG1:	Aflatoxin G1
AFG2:	Aflatoxin G2
AGP:	Antibiotic Growth Promoter
Al:	Alumina-N
ALA:	Alanine
ARG:	Arginine
ASP:	Aspartic acid
CE:	Collision Energy
CEP:	Collision Cell Entrance Potential
CIS:	Coordination Ion Spray
CG:	Control Group
CH_4:	Methane
CH_2Cl_2:	Dichloromethane
CSO:	Cysteine sulfoxides
CSSP:	S-Propylmercaptocysteine
CSTR:	Continuous Stirred Tank Reactor
CXP:	Collision Cell Exit Potential
CYS:	Cysteine
DAD:	Diode Array Detector
DART:	Direct Analysis In Real-Time
DDW:	Distilled Deionized Water
D_{O2}:	Dissolved Oxygen

Dp: Pore Diameter

DP: Declustering Potential

dSPE: Dispersive Solid-Phase Extraction

EI: Electrospay Ionization

EP: Entrance Potential

ESI: Electrospray Ionization

EtAc: Ethyl Acetate

EU: European Union

FID: Flame Ionization Detector

FA: Formic acid

FAO: Food and Agriculture Organization

GC: Gas Chromatography

GITSS: Gastrointestinal Tract Simulating System

GLY: Glycine

GLU: Glutamic acid

GSH: Glutathione

GSSP: S-Propylmercaptogluathione

HIS: Histidine

HPLC: High Perfomance Liquid Chromatography

HCl: Hydrochloric Acid

IARC: International Agency for Research on Cancer

ILE: Isoleucine

KCl: Potassium Chloride

KH_2PO_4: Monopotassium Phosphate

K_2SO_4: Potassium Sulfate

LC: Liquid Chromatography

LF: Lachrymatory factor

LEU: Leucine

LOD: Limit of Detection

LOQ: Limit of quantification

LYS: Lysine

MCX: Mixed-Mode Cation Exchange **ME:** Matrix Effect

MeCN: Acetonitrile

MMC: Matrix-Matched Calibration

MeOH: Methanol

MET: Methionine

$MgSO_4$: Magnesium sulfate

MRM: Mode and Multiple Reaction Monitoring

MS: Mass Spectrometry

MWCO: Molecular Weight Cut-Off

NaCl: Sodium Chloride

Na_2HPO_4: Sodium Phosphate Dibasic

NH_4OH: Ammonium Hydroxide

OE: Onion extract

OSCs: Organosulfur Compounds

OTA: Ochratoxin A

PDMS: Polydimethylsiloxane

PET: Polyethylene

PFR: Plug-Flow Tubular Reactor

PHE: Phenylalanine

PI: Polarity Index

PID: Proportional–Integral–Derivative

PRO: Proline

PSA: Primary Secondary Amine

PTS: Propyl thiosulfinate

PTSO: Propyl Propane Thiosulfonate

QuEChERS: Quick, Easy, Cheap, Effective, Rugged and Safe

SC-CO2: Supercritical carbon dioxide extraction

SDA: Sabourarud Dextrose Agar

R2: Determination coefficient

RASFF: Rapid Alert System for Food and Feed

RSDs: Relative Standard Deviations

SER: Serine

SPE: Solid Phase Extraction

SPME: Solid-phase microextraction

T: Temperature

TCA: Trichloroacetic Acid

TG: Treated Group

THR: Threonine

TLC: Thin Layer Chromatography

TYR: Tyrosine

Z-Sep: Supel™ QuE Z-Sep

UHPLC: Ultra-high Performance Liquid Chromatography

UHT: Ultra-High Temperature

UV: Ultraviolet

V: Level

VAL: Valine

VFA: Volatile Fatty Acids

XIC: Extracted Ion Chromatogram

ZON: Zealalenone