



Simultaneous determination of *Allium* compounds (Propyl propane thiosulfonate and thiosulfinate) in animal feed using UPLC-MS/MS

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ABSTRACT

Propyl-propane-thiosulfonate (PTSO) and Propyl-propane-thiosulfinate (PTS) are organosulfur compounds used to supplement the diet of livestock because of their beneficial effects on feed palatability, their antibacterial, anti-inflammatory, and antimethanogenic activities. Besides, antibiotic residues in the environment can be reduced by using these natural bioactive compounds. The objective of this study was to optimize the extraction parameters for the analysis of PTSO and PTS in feed matrices by performing a solid-liquid extraction and quantification by Ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). Optimization was performed using the Response Surface Methodology on a Box-Behnken experimental design, optimizing the following parameters: solvent:sample ratios and evaporation temperature set for the rotary evaporator. The method was validated for 3 concentration levels for both PTSO (100, 500, 1000 ng g⁻¹) and PTS (500, 1150, 2300 ng g⁻¹). The highest recoveries of PTSO and PTS were obtained using 12.5 mL of 100% acetonitrile, stirring for 15 min, and an evaporation temperature of 20 °C. The validated method was further applied to detect and quantify these compounds in different feed matrices. In conclusion, this is the first study to simultaneously analyze PTSO and PTS at low concentrations, employing a sensitive technique such as UPLC-MS/MS.

1. Introduction

The *Allium* genus includes more than 600 plant species, among which the edible garlic (*A. sativum*) and onion (*A. cepa*). These plants contain flavonoids, polysaccharides, and glucosinolates, as well as numerous organic sulfur compounds (Cozzolino et al., 2021). Organic sulfur compounds are phytochemical molecules with sulfur atoms in their structure. They are present as secondary metabolites in many plant species (Poojary et al., 2017) and they are well-known for their beneficial biological properties, such as: antibacterial, antifungal, antioxidants, antiviral, and antiprotozoal activities, among others (Farhat et al., 2021; Kyung, 2012; Putnik et al., 2019). Increasing evidence indicates that a regular ingestion of *Allium* spp. can reduce the risk of various illnesses, like type 2 diabetes mellitus, cancer, and cardiovascular diseases (Cecchi et al., 2020). Propyl-propane-thiosulfonate (C₆H₁₄O₂S₂)

(PTSO) and Propyl-propane-thiosulfinate (C₆H₁₄O₁S₂) (PTS) are two of the compounds obtained by the enzymatic decomposition of the initial compounds present in the cytoplasm of *Allium* cells.

The properties of PTSO and PTS are of great interest for the agri-food industry (Llana-Ruiz-Cabello et al., 2015a), which has recently experienced an increase in the demand for natural additives (Cascajosa-Lira et al., 2020a, 2020b, 2021, 2020b). Recently, the number of patents and research reports on organosulfur derivatives have increased. Examples in this sense include the use of dialkyl thiosulfonate and thiosulfinate to reduce and prevent parasites in both terrestrial and aquatic animals (Bravo and Lillehoj, 2013; Núñez et al., 2016), but also, their use as active compounds in food packaging (Mellado-García et al., 2015; Llana-Ruiz-Cabello et al., 2015b; Maisanaba et al., 2017). Furthermore, these compounds have shown beneficial effects as inhibitors of methanogenesis in goats (Martínez-Fernández et al., 2013, 2015) and

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modulators of the gut microbiota in farm animals (Guillamón et al., 2021; Rabelo-Ruiz et al., 2021a, 2021b). Additionally, they act as a sensory enhancer improving the palatability of feed when used as additives.

The regulation (EC) No 1831/2003 sets the framework on the use of additives in animal nutrition. It prohibits the use of antibiotics as a growth promoter: “[...] promoting agents will be forbidden, while allowing sufficient time for the development of alternative products to replace those antibiotics.”. Hence, there is an increasing demand for new alternative safer products, that guarantee similar production levels, without generating antimicrobial resistance. In this context, PTSO and PTS could be a promising alternative to antibiotic use in farm animals. PTS decreases bacteria resistance in humans, caused, among others, by the excessive use of antibiotics in livestock and the use of their manure as crop fertilizer (Peinado et al., 2012, 2013). Furthermore, the antimicrobial effectiveness of both compounds was demonstrated *in vitro* against multidrug-resistant bacteria isolated from humans (Solórzano-Puerto et al., 2018).

Quality control in the agri-food industry requires rapid, sensitive, and precise analytical methods for the monitoring of feed additives to ensure their safety and efficacy. However, few studies are available on the chemical analysis of PTSO and PTS, and none tackle their simultaneous extraction, identification, and quantification (Abad et al., 2015, 2016; Pastor-Belda et al., 2020). Mass spectrometry (MS) combined with ultra-performed liquid-chromatography (UPLC-MS/MS) is a quick selective analytical method employed to determinate the chemical composition and contamination of both food and feed (Campbell et al., 2017; Guzmán-Guillén et al., 2017; Vera et al., 2018; Díez-Quijada Jiménez et al., 2020; Le et al., 2021; Shehata et al., 2021; Wang et al., 2021). This advanced analytical technique offers shorter analysis periods, it can easily separate a great variety of chemical mixtures, and it has a high level of versatility. These properties are absent in other chromatographic systems such as gas chromatography (GC) and high-performance liquid chromatography with UV detection (HPLC-UV) (Martín-García et al., 2020).

Currently, there is an increase in the use of multivariate optimization strategies as analytical methodologies. They lower the number of experiments necessary to reach optimal conditions and indicate possible influences among tested variables (Gomes et al., 2017). In this regard, Response Surface Methodology (RSM) is a useful approach to multivariate optimization, applicable to an array of experimental designs. The Box-Behnken design (BBD) for multivariate optimization is increasingly being used for analytical procedures because it generates a smaller number of experiments with reliable results (Rodsamran and Sothornvit, 2019; Pal and Jadeja, 2019). BBD has been previously employed for the analysis of contaminants in food and feed additives (Gomes et al., 2017; Pal and Jadeja, 2019; Rambo et al., 2019; Díez-Quijada Jiménez et al., 2020; Güray et al., 2020; Martín-García et al., 2020; Nardelli et al., 2020; Feki et al., 2021).

In this context, the aim of this study was to develop, optimize, and validate an extraction method of PTSO and PTS from feed using a BBD and applying RSM, and to quantify the total amount of these organic sulfur compounds in real feed samples. To the best of our knowledge, this is the first report describing a validated method for the simultaneous determination of PTSO and PTS in feed by employing an optimized extraction technique together with UPLC-MS/MS analysis.

2. Materials and methods

2.1. Chemicals and reagents

PTSO ($C_6H_{14}O_2S_2$) and PTS ($C_6H_{14}O_1S_2$), and the solid support/material sepiolite were kindly provided by DMC Research Center SL (Alhendín, Granada, Spain), as well as all the broiler feed samples. Methanol (MeOH), dichloromethane (DCM), and acetonitrile (ACN) of analytical-grade were purchased from Merck (Darmstadt, Germany). For

UHPLC-MS/MS, LC-MS grade reagents were employed: water (VWR International, Fontenay-sous-Bois, France) and formic acid (FA) (Fluka, Steinheim, Germany). Ultrapure water (18.2 M Ω cm resistivity) purified by the NANOpure Diamond™ (Barnstead, USA) system was used. Additionally, 25 mm syringe filters with 0.2 μ m cellulose acetate membrane were supplied by VWR (International Radnor, Pennsylvania, USA).

2.2. Preparation of stock material

Standard stock solutions containing both PTSO and PTS were prepared in three different solvents to test the most adequate for the best elution and resolution of the peaks. The solvents used (50% MeOH in ultrapure water, 100% MeOH, and 100% ACN) were selected based on previous reports (Abad et al., 2016; Pastor-Belda et al., 2020). Pure MeOH was finally selected as the resuspension solvent because it enabled the best elution and recovery for both compounds. Dilutions of the stock solution (10 mg L⁻¹) were performed in MeOH to obtain the different working solutions at the desired concentrations (100–2500 ng mL⁻¹). These concentrations were chosen so that the highest was the minimum employed in previous validated extraction methods of PTSO (Abad et al., 2015, 2016; Pastor-Belda et al., 2020). However, to the best of our knowledge, no previous studies reported a validated method to extract PTS. Thus, the 100–2500 ng mL⁻¹ range was selected to improve the sensitivity of the previous methods for PTSO and to propose a new method for PTS.

Broiler feed samples were chosen as the experimental feedstuff matrix. Both PTSO and PTS were absorbed into the solid carrier sepiolite (from DMC Research Center SL) to ensure a homogeneous addition of the organosulfur compounds following the method used in the food industry. Then, the enriched sepiolite was incorporated into the feed-stuff to obtain the desired concentrations. A prior analysis was performed on the solid carrier spiked with the organosulfur compounds to confirm the initial concentrations of 13 μ g PTSO g⁻¹ and 30 μ g PTS g⁻¹.

2.3. PTSO and PTS analysis

2.3.1. Preliminary assay of extraction solvent

In the first assay, pure DCM, MeOH, and ACN were tested to determine the most suitable solvent to simultaneously extract both compounds (Abad et al., 2015, 2016). Broiler feed was spiked with a stock solution containing 2.5 mg L⁻¹ of each PTSO and PTS. These tests showed that ACN yielded the highest recovery of 87%, versus 81% for DCM and 69% for MeOH. Thus, ACN was selected as the extraction solvent for further assays.

2.3.2. Preliminary assay of extraction conditions

For the preliminary assay a BBD was used to analyze the optimal extraction conditions testing 2 independent variables at 3 levels: solvent:sample ratios (X_1) (5.0, 12.5, and 20.0) and stirring time (X_2) (15, 30, and 45 min). These levels were selected based on previous results obtained in our laboratory and the scientific literature (Ramírez et al., 2017). The extraction yield of PTSO (Y_1) and PTS (Y_2) were the response variables. The BBD generated 7 experimental runs for the extraction conditions needed for the Response Surface Methodology (RSM) that were randomized and carried out in an unblocked design. A set of 3 replicates were forced at the center of the experimental design to adequately estimate the pure error sum of squares (Ferreira et al., 2007; Díez-Quijada Jiménez et al., 2020; Bodea et al., 2021).

2.4. Experimental design for the optimization study and target optimal levels for the response parameters

For the optimization study the independent variables were chosen based on the preliminary assay. The variation of solvent:sample ratios (X_1) showed an influence on the yield of PTSO (Y_1) and PTS (Y_2)

modelled by a quadratic equation within the 5–20 interval, thus appropriate for RSM optimization procedure. The stirring time (X_2) showed no influence upon extraction yield and was, therefore, eliminated as independent variable (data not shown). Thus, the second independent variable was changed, and the temperature of evaporation (X_3) was included instead, as most of the organic sulfur compounds of the genus *Allium* are thermolabile (Ilić et al., 2012; Yoo et al., 2014; Ramirez et al., 2017). The three chosen values were 20, 35, and 50 °C because the organic sulfur compounds seem to degrade when exposed to temperatures higher than 35 °C (Ilić et al., 2012; Yoo et al., 2014). Prior to performing the method validation, the stability of these compounds was checked in the resuspension solvent (100% MeOH) spiked into the matrix. The compounds proved to be stable for at least 6 h at 4 °C. So, the samples were extracted, injected, and analyzed by UPLC-MS/MS within this period.

Thus, another BBD with 7 experimental runs was performed with solvent/sample ratios (X_1) and temperature of evaporation (X_3) as independent variables (Table 1). The experimental design was randomized and carried out in an unblocked design, with 3 replicates at the center, similar to the preliminary study. These experiments were carried out by spiking the feed samples at 500 $\mu\text{g g}^{-1}$ of each compound.

2.5. PTSO and PTS extraction protocol

The optimization of the UPLC-MS/MS method for PTSO and PTS analysis was performed by acquiring mass spectra for the working solutions obtained as explained in section 2.2 and adjusting the mobile phase strength. Six calibration standards (100, 250, 500, 750, 1000, and 2500 $\mu\text{g L}^{-1}$) were prepared with both compounds, to obtain their calibration curves using 100% MeOH. Afterwards, matrix-matched calibration curves were prepared at the same concentrations from the broilers feed extracts, obtaining linear ranges of 100–2500 $\mu\text{g L}^{-1}$.

Control broilers feed samples (1 g) supplied by DMC Research Center SL were homogenized and extracted with 19.5 mL ACN, followed by shaking in an orbital shaker (15 min), sonication (15 min), and centrifugation (3700 rpm, 15 min, 20 °C). The extracts were evaporated to dryness in a rotary evaporator at 20 °C and resuspended in 1 mL 100% MeOH. Prior to UPLC-MS/MS analysis, the extracts were filtered through a syringe filter (0.22 μm). The extraction solvent:sample ratio and the temperature of evaporation were set according to the BBD in Table 1.

2.6. Instrumentation and UPLC-MS/MS analysis

Chromatographic separation was performed using a UPLC Acquity (Waters) coupled to a Xevo TQ-S micro (Waters) consisting of a triple quadrupole mass spectrometer equipped with an electrospray ion source operated in positive mode. The separation was performed on an Acquity

Table 1

The extraction yields of PTSO and PTS based on a Box–Behnken design for Response Surface Methodology (RSM).

Sort order	Independent Variables		Response—Dependent Variables				desir
	X_1 solvent: sample ratio (w/ v)	X_3 temperature of evaporation (°C)	Y ₁ PTSO recovery yield (%)		Y ₂ PTS recovery yield (%)		
			exp	pred *	exp	pred *	
1	5.00	20.00	70.23	69.29	68.61	67.33	0.00
2	20.00	20.00	99.64	98.70	91.64	92.93	0.93
3	5.00	50.00	82.89	81.95	40.83	42.12	0.09
4	20.00	50.00	94.03	93.09	69.00	67.71	0.60
5	12.50	35.00	89.87	85.76	99.08	90.14	0.67
6	12.50	35.00	82.26	85.76	72.09	90.14	0.67
7	12.50	35.00	81.41	85.76	99.24	90.14	0.67
p-value **			1.000		0.619		

UPLC BEH C18 1.7 μm (2.1 \times 100 mm) column. The injection volume was 5 μL and the flow rate was 0.4 mL min^{-1} . Two different solvents were used as a mobile phase: solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid). The following gradient was used: 0.0–0.5 min 10% B, 0.5–5.0 min from 10% to 100% B, 5.0–7.0 min 100% B, then 10% B up to 10.0 min. Multiple Reaction Monitoring (MRM) was applied, where the parent and fragments ions were monitored at Q1 and Q3, respectively.

The transitions employed for PTSO were 183.1/42.8, 183.16/76.9, and 183.1/140.9 and for PTS they were 167.1/72.8, 167.1/42.8, and 167.1/124.9, choosing the first for quantitation and the others for confirmation (Fig. 1). For UPLC-ESI-MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: capillary voltage: 2.40 kV, desolvation temperature: 500 °C, source desolvation gas flow: 650 L h^{-1} and source cone gas flow: 50 L h^{-1} .

2.7. Validation of the method

The following parameters were studied to validate the method: selectivity, limits of detection and quantification, working range, trueness, and precision, following different analytical guidelines (González and Herrador, 2007; Magnusson and Örnemark, 2014; AOAC, 2016).

Then, 7.7 mg, 38.5 mg, and 77 mg of spiked sepiolite were added to 1 g of broilers feed sample to obtain the three concentrations needed for validation: 100, 500, and 1000 ng g^{-1} for PTSO and 500, 1150, and 2300 ng g^{-1} for PTS.

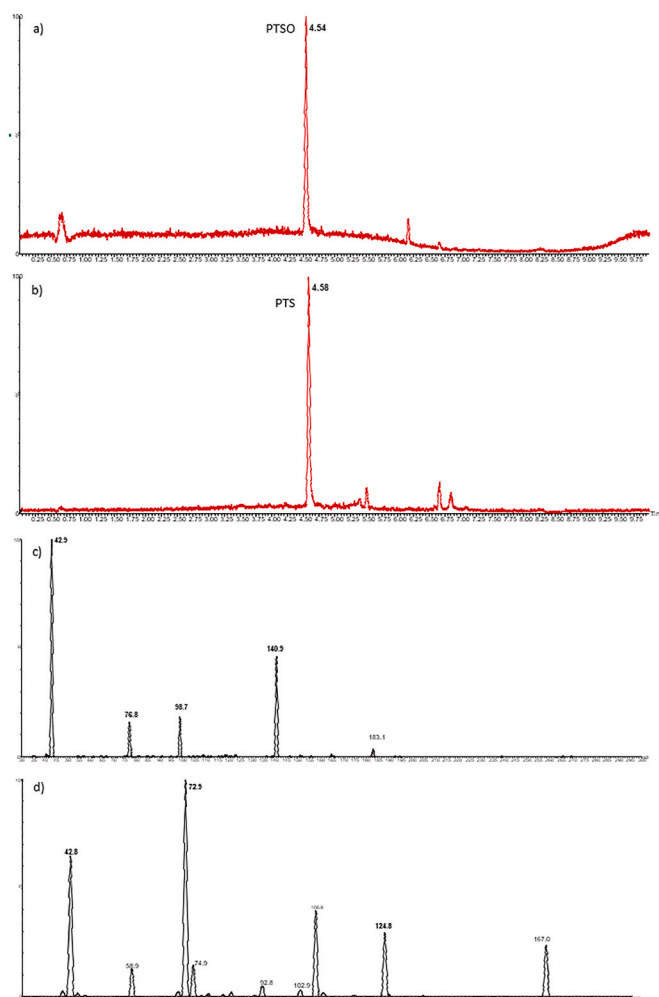


Fig. 1. MRM chromatograms by UPLC-MS/MS of a) PTSO and b) PTS spiked in feed matrix; c) mass spectra of PTSO; d) mass spectra of PTS.

The extractions were performed for each concentration in triplicate per day, on 3 different days, covering the working range, following the protocol described in section 2.5. Finally, the results were compared with the respective tabulated reference values at each concentration level, according to AOAC (2016). The detection and quantification limits (LOD and LOQ) were obtained using the standard deviation (SD) of the response and the slope of the calibration curves, following the equation $3xSD/slope$ and $10xSD/slope$, for LOD and LOQ, respectively. The SD was calculated from the calibration curve using the residual standard deviation of the regression line (ICH Harmonised Tripartite Guideline, 2005; Miller and Miller, 2000).

2.8. Application of the validated method to feed samples

At the end of the validation assays, a set of 6 feed samples containing PTSO and PTS was analyzed in triplicate by the validated method. Four samples contained only PTSO (ruminants, broilers, lactating pigs, and hens (1) feed), and two samples contained both PTSO and PTS (hens (2) and fish feed).

2.9. Statistical analyses and model fitting

XLSTAT (version 2021.3.1) and Minitab® (version 19.2020.1) statistical programs were used to create and analyze the Box–Behnken experimental design (BBD). Response Surface Methodology (RSM) was used on a BBD for the preliminary and optimization study. Two continuous explanatory variables (X_1 : solvent:sample ratio, X_2 : stirring time) each with 3 levels were chosen for the preliminary study, while in the optimization study only X_1 was kept, and another continuous explanatory variable was introduced (X_3 : temperature of evaporation). The effect of the independent variables was modelled on the recovery yields of the 2 compounds (Y_1 : yield of PTSO and Y_2 : yield of PTS). The linear, interaction, and squared coefficients of the RSM were determined by least squares regression. The terms for the mathematical model were selected by a stepwise approach with an $\alpha \leq 0.15$ for a term to enter the model. Additional terms were added in the final step to maintain the hierarchical model because the method intended for a simultaneous extraction of the 2 compounds with interlinked subsequent steps. Two-dimensional response surface charts and desirability functions were developed after obtaining the model equations. The fitting of the model was performed by analysis of variance (ANOVA) and Mann–Whitney two-tailed test. The recovery yields were set to maximum for the optimization procedure. The statistical validation of the optimum extraction conditions was done by comparing the theoretical values with new experimental runs by an independent sample *t*-test and regression analysis. Precision and recovery for the validation of the extraction and quantification method were obtained by applying a one-way ANOVA; statistical significance was considered at $p < 0.05$.

3. Results and discussion

3.1. Optimization study

The recovery yields of PTSO and PTS obtained with varying solvent: sample ratios and temperatures set for the rotary evaporator, as generated by BBD, were analyzed by RSM. The experimental and the predicted yields are presented in Table 1. The Mann–Whitney two-tailed test showed that the experimental and predicted values are statistically similar at a significance level of $\alpha = 0.001$.

Where: exp—experimental values; pred—values predicted by the RSM model; desir—overall desirability (0 ... 1); * the predicted value resulted from the model optimizing the extraction of PTSO and PTS; ** Mann–Whitney two-tailed test ($\alpha = 0.001$) of the experimental data versus the values predicted by the model optimizing the PTSO and PTS yields.

The model generated by RSM consisting of 2 polynomial equations

was a good fit for the experimental recovery yields (Table 2). The lack-of-fit of the obtained model was not significant for either compound at levels higher than $p = 0.619$.

Where: the explanatory variables were coef—coded coefficients. A stepwise selection of terms was used with $\alpha \leq 0.15$ for a hierarchical model; NA—not applicable, the parameter was removed from the model.

The model equations within the interval (X_1 : 5 ... 20 and X_2 : 20 ... 50 °C) in uncoded units for both compounds are presented in equations (1) and (2).

$$Y_1 = 47.000 + 2.773 \cdot X_1 + 0.625 \cdot X_3 - 0.040 X_1 \cdot X_3 \quad (1)$$

$$Y_2 = 35.400 + 11.760 \cdot X_1 - 0.840 \cdot X_3 - 0.402 \cdot X_1^2 \quad (2)$$

where: Y_1 : recovery yield of PTSO; Y_2 : recovery yield of PTS; X_1 : solvent: sample ratio; X_3 : extraction temperature (°C).

The RSM contour plots (Fig. 2) show the effect of the 2 continuous predictors (X_1 : solvent:sample ratio and X_3 : evaporation temperature) upon the recovery yield of each of the 2 compounds. The solvent:sample ratio seemed to positively influence the yields of both PTSO and PTS, with a greater impact on the latter ($b_{1PTS} > b_{1PTSO}$). But, while the effect appeared linear ($p = 0.036$) for PTSO, it was quadratic for PTS ($p = 0.105$, at a significance level of $\alpha = 0.15$). This means that the negative coefficient ($b_{11} = -0.402$) for the quadratic variable created a parabolic curve with one maximum, within the tested range. These tendencies were reported only in the present study, as it is the first time that PTSO and PTS are determined simultaneously and support the optimization procedure within the selected range. On the other side, the data show that the evaporation temperature affects differently the 2 compounds: it seemed to positively influence the recovery yield of PTSO ($b_3 = 0.625$), while negatively affecting, and to a higher extent, the yield of PTS ($b_3 = -0.840$). This might explain why the optimization of the simultaneous extraction was so tedious and difficult to accomplish.

By using the RSM, a desirability function (equation (3)) was generated that permitted determining the mathematical optimum of the 2 independent variables, within the tested ranges. The optimization procedure was run against the RSM model taken as a system of 2 equations with only one possible solution. By doing this, the values of the independent variables that jointly optimize the recovery yields of both compounds were determined.

$$D = -0.933 + 0.197 \cdot X_1 + 7.843 \cdot 10^{-3} \cdot X_3 - 4.665 \cdot 10^{-3} \cdot X_1^2 - 9.440 \cdot 10^{-3} \cdot X_1 \cdot X_2 \quad (3)$$

Thus, after the optimization analysis, the model suggested that the optimum solvent:sample ratio is 19.5 and the optimum evaporation temperature 20 °C, with a composite desirability $D = 0.931$. Although other authors have previously managed to extract PTSO using a solvent: sample ratio of around 10 (Abad et al., 2015, 2016), this study shows that a ratio of 19.5 is needed for the simultaneous extraction of PTSO and PTS. The desirability of each run in the BBD experimental design

Table 2

Model parameters (coded coefficients), p-values, and goodness of fit statistics obtained by Response Surface Methodology (RSM) for each of the 2 response variables (Y_i).

Coefficient		Y_1 PTSO recovery		Y_2 PTS recovery	
		coef	p	coef	p
Intercept	b0	85.76	0.000	90.14	0.001
Linear	b1	10.14	0.016	12.80	0.140
	b3	1.76	0.458	-12.61	0.145
	interaction	b13	-4.57	0.115	NA
Quadratic	b11	NA	NA	-22.62	0.105
	b33	NA	NA	NA	NA
R^2		0.91	–	0.81	–
Lack-of-fit		–	0.602	–	0.884
The model		–	0.046	–	0.128

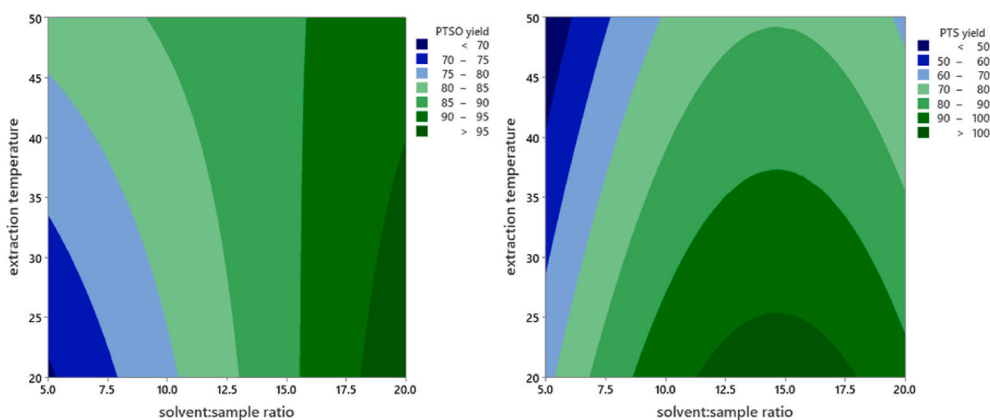


Fig. 2. Response Surface Methodology (RSM) contour plots of extraction parameters on the recovery yield of PTSO and PTS.

was also assessed by using equation (3) (Table 1).

The new validation experimental results were within an error of prediction of 14.88% (Table 3). Additionally, a Mann–Whitney two-tailed test showed no significant difference between the values predicted by the proposed model and new experimental data. Thus, the model was validated for both PTSO and PTS.

3.2. Calibration characteristics

3.2.1. Linearity

Two 6-point calibration curves were obtained for PTSO and PTS by plotting the responses as a function of concentrations of the various standards obtained for both compounds spiked in broiler feed extracts. The linear range was between 100 and 2500 ng g⁻¹. The obtained regression equations were: $y = 163.49x - 2160.7$ for PTSO ($R^2 = 0.9926$), and $y = 3177.8x + 231716$ for PTS ($R^2 = 0.9970$) (Fig. 3).

The corresponding Huber plots were obtained with these calibration points (Fig. 4), representing the relationship between the signal response/analyte concentrations against the analyte concentrations (Huber, 1998). The target line in Fig. 4 represents the median signal/concentration calculated for all the assayed concentrations, and the two action lines represent 95 and 105% of this median value. The obtained values for all tested concentrations were within the median value $\pm 5\%$ for both PTSO and PTS. Thus, the method demonstrates its linearity within the range 100–2500 ng g⁻¹ for both compounds.

3.2.2. Limits of detection and quantification

The values obtained for LOD and LOQ are shown in Table 3. Both values for PTSO (0.082 and 0.272 ng g⁻¹, respectively) were much lower than those reported by Abad et al. (2016) using UHPLC-MS/MS (130 and 440 ng g⁻¹, respectively), and by Pastor-Belda et al. (2020) using GC-MS

(6–100 and 20–340 ng g⁻¹, respectively, depending on the extraction technique). Moreover, the present study is the first of its kind because it shows the simultaneous detection and quantification by UPLC-MS/MS of individual PTSO and PTS compounds, with good sensitivity for PTS as well (LOD and LOQ of 0.095 and 0.317 ng g⁻¹, respectively). Thus, the current study builds upon the method proposed by Abad et al. (2016) by lowering the LOD and LOQ and manages to validate it for PTS as well.

In addition, UHPLC-MS/MS is a powerful analytical technique which provides shorter times of analysis, acquires a high degree of versatility, and it has the ability to easily separate a wide variety of chemical mixtures (Martín-García et al., 2020). Other chromatographic systems, such as GS-MS, need a prior sample pre-treatment (Talhaoui et al., 2015).

3.2.3. Selectivity and matrix effects

The proposed method determines PTSO and PTS by the optimization of MRM transitions characteristic of each compound, in addition to the corresponding area ratio between them, as it is described in section 2.6. In this way, PTSO and PTS can be determined without interferences from other components of similar behaviour in the feed matrix (feedstuff) where the analytes were spiked, as it can be seen in Fig. 1a and b. Following Barwick (2016), the method selectivity is considered appropriate.

The matrix effect (ME) can be affected by multiple factors such as: the properties of the analytes, the matrix composition, and the amount of sample (Li et al., 2014). ME was calculated considering the solvent and matrix calibrations curves for each compound, as: $100 \times [(slope \text{ matrix/slope solvent}) - 1]$, expressed in percentage. The values obtained were -27.40 for PTSO and -38.60 for PTS, showing in both cases a medium matrix effect ($|20| < ME < |50|$). Although no strong matrix effects were observed in the present study, the decreases in PTSO and PTS signals highlight the need to use matrix-matched calibration curves

Table 3

Recovery estimations (experimental, predicted, and error (%)); within-day repeatability (S_w); between-day repeatability (S_B); intermediate precision (S_{IP}); and its relative standard deviation ($\%RSD_{IP}$) for PTSO and PTS. Limits of detection (LOD) and quantification (LOQ).

	Concentration level (ng g ⁻¹)	Validation parameters									
		Exp Recovery (%)	Pred Recovery (%)	% Error of pred	P value*	S_w	S_B	S_{IP}	RSD_{IP} (%)	LOD (ng g ⁻¹)	LOQ (ng g ⁻¹)
PTSO	100	109.35	97.71	-11.64	0.26	7.84	3.64	6.74	6.16	0.082	0.272
	500	105.83	97.71	-8.12		34.41	46.05	38.68	7.31		
	1000	107.65	97.71	-9.94		44.82	92.92	64.94	6.03		
PTS	500	109.48	95.00	-14.48	0.70	21.94	6.82	18.34	3.35	0.095	0.317
	1150	109.99	95.00	-14.88		83.75	110.96	93.70	7.42		
	2300	105.91	95.00	-10.91		236.49	237.57	255.14	10.47		

Acceptable ranges for RSD_{AOAC} (%): 15% for 100 ng g⁻¹, 11–15% for 500 ng g⁻¹ and 7.5–11% for 1000, 1150 and 2300 ng g⁻¹. Acceptable ranges for $Recovery_{AOAC}$ (%): 80–110% for all concentration levels. Where: exp - experimental values; pred - values predicted by the RSM model, resulted from the model optimizing the extraction of PTSO and PTS; * - Mann–Whitney two-tailed test ($\alpha = 0.001$) of the experimental data versus the values predicted by the model optimizing the PTSO and PTS extraction.

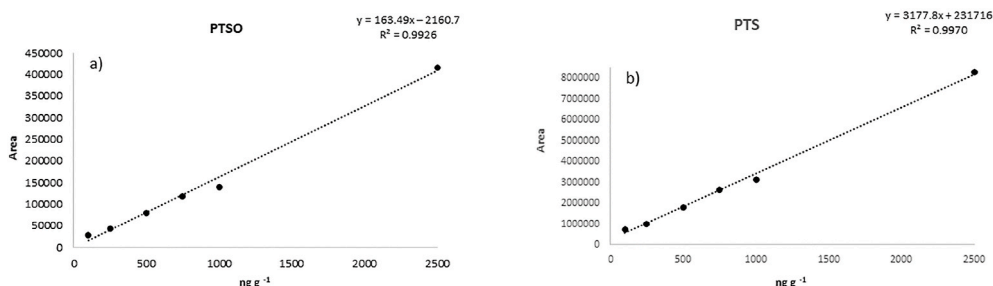


Fig. 3. Calibration curves for (a) PTSO and (b) PTS spiked in broilers feed extracts, with a linear range within 100 and 2500 ng g^{-1} .

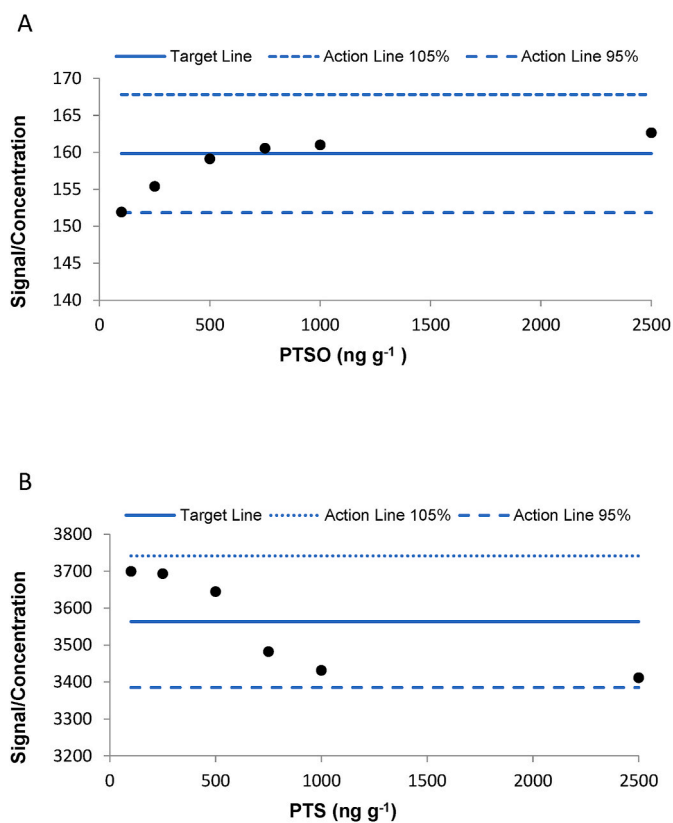


Fig. 4. Response linearity in broilers feed by the Huber plots for (a) PTSO and (b) PTS.

for quantification to obtain more accurate results. These signals suppressions were also reported by Abad et al. (2016) for PTSO by the significant ME reported in most of the matrices and concentrations tested, due to the alteration of ionization efficiency.

3.3. Precision, trueness and recovery studies

The measurement uncertainty assayed in the present study included repeatability and intermediate precision (Barwick, 2016). Repeatability expresses the precision evaluated under the same experimental conditions over a short time interval, and intermediate precision corresponding to the values obtained in different days, different analysts or equipment (inter-assay precision) (González et al., 2010). Moreover, trueness represents the closeness of agreement between a test result and the accepted reference value of the measured analyte, and it can be assessed by spiking and recovery (González et al., 2010; Magnusson and Örnemark, 2014). Table 3 shows the values obtained for repeatability (within-day repeatability, S_w , or between-day repeatability, S_B), intermediate precision (S_{IP}), and S_{IP} relative standard deviations (%RSD_{IP}).

Three replicates of broilers feed extracts were spiked with standard solutions containing both PTSO (100, 500, and 1000 ng PTSO g^{-1}) and PTS (500, 1150, and 2300 ng PTS g^{-1}) at different concentrations on the same day and on three different days following the ICH guidelines (ICH Harmonised Tripartite Guideline, 2005). Afterwards, an ANOVA was performed for each validation standard according to González et al. (2010) and González and Herrador (2007). The relative standard deviations (%RSD_{IP}) obtained for each compound were compared with the expected RSD values obtained from the AOAC Peer Verified Methods Program (AOAC, 2016) depending on the assayed concentration level as follows: 15% for 100 ng g^{-1} ; 11–15% for 500 ng g^{-1} ; 11% for 1000 ng g^{-1} ; and 7.3–11% for 1150 and 2300 ng g^{-1} . In all cases and at all assayed concentrations the % RSD_{IP} values calculated for PTSO and PTS were lower or in the range specified by the AOAC expected values; thus, the proposed method can be considered as precise (Table 3).

Trueness can be expressed as the bias or recovery obtained for each calibration standard assayed. The total recovery for each calibration standard is defined as the ratio between the observed estimation of each concentration, and the “true” value T, expressed as percentage or as fraction. The recoveries (%) computed for the three calibration standards for each compound considered (100, 500 and 1000 ng g^{-1} for PTSO, and 500, 1150 and 2300 ng g^{-1} for PTS) are shown in Table 3. The recovery yields obtained in the present study (106–109% for PTSO and 106–110% for PTS) agree with those proposed by AOAC (2016) and González and Herrador (2007), and in all cases they are within the range 80–110% (Table 3). Thus, the method can be considered acceptable in terms of trueness and recoveries. These results are a bit higher than those obtained by Abad et al. (2016) for PTSO in broilers feed spiked at 1000 ng g^{-1} (94.6%).

3.4. Application to different matrices

The proposed method was applied to extract and quantify PTSO and/or PTS in several feed samples provided by DMC Research Center SL, as explained in section 2.8, to ensure its applicability to different feed matrices. The sensitivity obtained in this method, with adequate LOD and LOQ values, has allowed the quantification of these compounds in the tested matrices, showing the recoveries presented in Table 4.

Table 4

Recovery study of real feed samples used in agri-food industry. Mean recoveries (%) for PTSO and PTS and their standard deviation (SD, %).

Matrix (feed)	PTSO Mean recovery (%)	PTSO SD (%)	PTS Mean recovery (%)	PTS SD (%)
Ruminants	100.21	2.85	–	–
Broilers lactating	104.72	4.13	–	–
pigs	102.20	4.63	–	–
hens 1	101.46	0.51	–	–
hens 2	87.45	4.48	100.06	1.39
Fish	100.90	4.04	96.67	1.46

4. Conclusions

This study presents for the first time a method for the simultaneous determination and quantification of PTSO and PTS in feed samples. The parameters of the extraction method were optimized by RSM using a Box–Behnken design. The method was validated using solid-liquid extraction and UPLC-MS/MS and showed adequate linearity and sensitivity, precision, and recovery yields. Additionally, the method was applied to real feed samples spiked by the agri-food industry successfully. This suggests the possible application of the proposed method in the industry and monitoring programs of feed additives, to ensure safety and efficacy.

CRedit authorship contribution statement

Antonio Cascajosa-Lira: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. **Ana Isabel Prieto Ortega:** Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Remedios Guzmán-Guillén:** Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Giorgiana M. Cătunescu:** Formal analysis, Methodology, Software, Writing – original draft. **José M. de la Torre:** Resources, Formal analysis, Writing – review & editing. **Enrique Guillamón:** Resources, Funding acquisition, Writing – review & editing. **Ángeles Jos:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing. **Ana M^a Cameán Fernández:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- Abad, P., Lara, F.J., Arroyo-Manzanares, N., Baños, A., Guillamón, E., García-Campana, A.M., 2015. High-performance liquid chromatography method for the monitoring of the *Allium* derivative propyl propane thiosulfonate used as natural additive in animal feed. *Food Anal. Methods* 8, 916–921. <https://doi.org/10.1007/s12161-014-9952-1>.
- Abad, P., Arroyo-Manzanares, N., García-Campana, A.M., 2016. A rapid and simple UHPLC-ESI-MS/MS method for the screening of propyl propane thiosulfonate, a new additive for animal feed. *Anal. Methods* 8, 3730–3739. <https://doi.org/10.1039/c6ay00219f>.
- Barwick, V. (Ed.), 2016. Eurachem/CITAC Guide: Guide to Quality in Analytical Chemistry: an Aid to Accreditation, third ed., p. 978 Available from: www.eurachem.org/0-948926-32-7.
- Bodea, I.M., Beteg, F.I., Pop, C.R., David, A.P., Dudescu, M.C., Vilău, C., Stănilă, A., Rotar, A.M., Cătunescu, G.M., 2021. Optimization of moist and oven-dried bacterial cellulose production for functional properties. *Polymers* 13, 1–25. <https://doi.org/10.3390/polym13132088>.
- Bravo, D., Lillehoj, H., 2013. Use of at least one dialkyl thosulfonate or thosulfinate for reducing the number of apcomplexan an animal. *United States. Pat. Appl. Publ. US, 2013/00794.02 A1 1*.
- Campbell, K., Ferreira Cavalcante, A.L., Galvin-King, P., Opladowska-Stachowiak, M., Brabet, C., Metayer, I., Montet, D., Haughey, S.A., Elliott, C.T., 2017. Evaluation of an alternative spectroscopic approach for aflatoxin analysis: Comparative analysis of

- food and feed samples with UPLC–MS/MS. *Sensors Actuators, B Chem.* 239, 1087–1097. <https://doi.org/10.1016/j.snb.2016.08.115>.
- Cascajosa-Lira, A., Prieto, A.I., Puerto, M., Baños, A., Valdivia, E., Jos, Á., Cameán, A.M., 2020a. Mutagenicity and genotoxicity assessment of a new biopreservative product rich in Enterocin AS-48. *Food Chem. Toxicol.* 146 <https://doi.org/10.1016/j.fct.2020.111846>.
- Cascajosa Lira, A., Prieto, A.I., Baños, A., Guillamón, E., Moyano, R., Jos, A., Cameán, A. M., 2020b. Safety assessment of propyl-propane-thiosulfonate (PTSO): 90-days oral subchronic toxicity study in rats. *Food Chem. Toxicol.* 144 <https://doi.org/10.1016/j.fct.2020.111612>.
- Cascajosa-Lira, A., Puerto, M., Prieto, A.I., Pichardo, S., Jiménez, L.D.Q., Baños, A., Guillamón, E., Moyano, R., Molina-Hernández, V., Jos, Á., Cameán, A.M., 2021. Genotoxicity evaluation of propyl-propane-thiosulfonate (PTS) from *Allium* genus essential oils by a combination of micronucleus and comet assays in rats. *Foods* 10. <https://doi.org/10.3390/foods10050989>.
- Cecchi, L., Ieri, F., Vignolini, P., Mulinacci, N., Romani, A., 2020. Characterization of volatile and flavonoid composition of different cuts of dried onion (*Allium cepa* L.) by HS-SPME-GC-MS, HS-SPME-GC×GC-TOF and HPLC-DAD. *Molecules* 25, 1–14. <https://doi.org/10.3390/molecules25020408>.
- Cozzolino, R., Malorni, L., Martignetti, A., Picariello, G., Siano, F., Forte, G., De Giulio, B., 2021. Comparative analysis of volatile profiles and phenolic compounds of Four Southern Italian onion (*Allium cepa* L.) Landraces. *J. Food Compos. Anal.* 101, 103990. <https://doi.org/10.1016/j.jfca.2021.103990>.
- Díez-Quijada Jiménez, L., Cătunescu, G.M., Campos, A., Vasconcelos, V., Jos, Á., Cameán, A.M., 2020. A new method for the simultaneous determination of cyanotoxins (Microcystins and Cylindrospermopsin) in mussels using SPE-UPLC-MS/MS. *Environmen* 185, 109284. <https://doi.org/10.1016/j.envres.2020.109284>.
- Farhat, Z., Hershberger, P.A., Freudenheim, J.L., Mammen, M.J., Hageman Blair, R., Aga, D.S., Mu, L., 2021. Types of garlic and their anticancer and antioxidant activity: a review of the epidemiologic and experimental evidence. *Eur. J. Nutr.* <https://doi.org/10.1007/s00394-021-02482-7>.
- Feki, F., Klisurova, D., Masmoudi, M.A., Choura, S., Denev, P., Trendafilova, A., Chamkha, M., Sayadi, S., 2021. Optimization of microwave assisted extraction of simmondsin and polyphenols from Jojoba (*Simmondsia chinensis*) seed cake using Box-Behnken statistical design. *Food Chem.* 356, 129670. <https://doi.org/10.1016/j.foodchem.2021.129670>.
- Ferreira, S.L.C., Bruns, R.E., Ferreira, H.S., Matos, G.D., David, J.M., Brandão, G.C., da Silva, E.G.P., Portugal, L.A., dos Reis, P.S., Souza, A.S., dos Santos, W.N.L., 2007. Box-Behnken design: an alternative for the optimization of analytical methods. *Anal. Chim. Acta* 597, 179–186. <https://doi.org/10.1016/j.aca.2007.07.011>.
- Gomes, S.V.F., Portugal, L.A., dos Anjos, J.P., de Jesus, O.N., de Oliveira, E.J., David, J. P., David, J.M., 2017. Accelerated solvent extraction of phenolic compounds exploiting a Box-Behnken design and quantification of five flavonoids by HPLC-DAD in *Passiflora* species. *Microchem. J.* 132, 28–35. <https://doi.org/10.1016/j.microc.2016.12.021>.
- González, A.G., Herrador, M.A., 2007. A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *Trends Anal. Chem.* 26, 227–238. <https://doi.org/10.1016/j.trac.2007.01.009>.
- González, A.G., Herrador, M.A., Asuero, A.G., 2010. Intra-laboratory assessment of method accuracy (trueness and precision) by using validation standards. *Talanta* 52, 1995–1998. <https://doi.org/10.1016/j.talanta.2010.07.071>.
- Guillamón, E., Andreo-Martínez, P., Mut-Salud, N., Fonollá, J., Baños, A., 2021. Beneficial effects of organosulfur compounds from *Allium cepa* on gut health: a systematic review. *Foods* 10 (8), 1680. <https://doi.org/10.3390/foods10081680>.
- Güray, T., Menevşe, B., Yavuz, A.A., 2020. Determination of optimization parameters based on the Box-Behnken design for cloud point extraction of quinine yellow using Brij 58 and application of this method to real samples. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* 243 <https://doi.org/10.1016/j.saa.2020.118800>.
- Guzmán-Guillén, R., Maisanaba, S., Prieto Ortega, A.I., Valderrama-Fernández, R., Jos, A., Cameán, A.M., 2017. Changes on cylindrospermopsin concentration and characterization of decomposition products in fish muscle (*Oreochromis niloticus*) by boiling and steaming. *Food Control* 77, 210–220. <https://doi.org/10.1016/j.foodcont.2017.02.035>.
- ICH Harmonised Tripartite Guideline, 2005. Validation of Analytical Procedures: Text and Methodology Q2(R1). ICH Working Group Available online: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1_Guideline.pdf.
- Ilić, D., Nikolić, V., Stanković, M., Nikolić, L., Stanojević, L., Mladenović-Ranisavljević, I., Šmelcerović, A., 2012. Transformation of synthetic allicin: the influence of ultrasound, microwaves, different solvents and temperatures, and the products isolation. *Sci. World J.* <https://doi.org/10.1100/2012/561823>, 2012.
- Kyung, K.H., 2012. Antimicrobial properties of *Allium* species. *Curr. Opin. Biotechnol.* 23, 142–147. <https://doi.org/10.1016/j.copbio.2011.08.004>.
- Le, H.T.N., Roy, E. Van, Dendooven, E., Peeters, L., Theunis, M., Foubert, K., Pieters, L., Tuentner, E., 2021. Phytochemistry alkaloids from *Lepidium meyenii* (Maca), structural revision of macaridine and UPLC-MS/MS feature-based molecular networking. *Phytochemistry* 190, 112863. <https://doi.org/10.1016/j.phytochem.2021.112863>.
- Li, Y.W., Zhan, X.J., Xiang, L., Deng, Z.S., Huang, B.H., Wen, H.F., Sun, T.F., Cai, Q.Y., Li, H., Mo, C.H., 2014. Analysis of trace microcystins in vegetables using solid-phase extraction followed by high performance liquid chromatography triple-quadrupole mass spectrometry detection. *J. Agric. Food Chem.* 62, 11831–11839.
- Llana-Ruiz-Cabello, M., Pichardo, S., Maisanaba, S., Puerto, M., Prieto, A.I., Gutiérrez-Praena, D., Jos, A., Cameán, A.M., 2015a. *In vitro* toxicological evaluation of essential oils and their main compounds used in active food packaging: a review. *Food Chem. Toxicol.* 81, 9–27. <https://doi.org/10.1016/j.fct.2015.03.030>.

- Llana-Ruiz-Cabello, M., Pichardo, S., Bãnos, A., Núñez, C., Bermúdez, J.M., Guillamón, E., Aucejo, S., Cameán, A.M., 2015. Characterisation and evaluation of PLA films containing an extract of *Allium* spp. to be used in the packaging of ready-to-eat salads under controlled atmospheres. *LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft - Technol.)* 64, 1354–1361. <https://doi.org/10.1016/j.lwt.2015.07.057>.
- Maisanaba, S., Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Pichardo, S., Prieto, M., Prieto, A.I., Jos, A., Cameán, A.M., 2017. New advances in active packaging incorporated with essential oils or their main components for food preservation. *Food Rev. Int.* 33, 447–515. <https://doi.org/10.1080/87559129.2016.1175010>.
- Martín-García, B., Pimentel-Moral, S., Gómez-Caravaca, A.M., Arráez-Román, D., Segura-Carretero, A., 2020. Box-Behnken experimental design for a green extraction method of phenolic compounds from olive leaves. *Ind. Crop. Prod.* 154, 112741. <https://doi.org/10.1016/j.indcrop.2020.112741>.
- Martínez-Fernández, G., Abecía, L., Martín-García, A.I., Ramos-Morales, E., Denman, S. E., Newbold, C.J., Molina-Alcaide, E., Yáñez-Ruiz, D.R., 2015. Response of the rumen archaeal and bacterial populations to anti-methanogenic organosulphur compounds in continuous-culture fermenters. *FEMS Microbiol. Ecol.* 91, 1–11. <https://doi.org/10.1093/femsec/fiv079>.
- Martínez-Fernández, G., Abecía, L., Martín-García, A.I., Ramos-Morales, E., Hervás, G., Molina-Alcaide, E., Yáñez-Ruiz, D.R., 2013. *In vitro-in vivo* study on the effects of plant compounds on rumen fermentation, microbial abundances and methane emissions in goats. *Animal* 7, 1925–1934. <https://doi.org/10.1017/S1751731113001699>.
- Mellado-García, P., Maisanaba, S., Prieto, M., Llana-Ruiz-Cabello, M., Prieto, A.I., Marcos, R., Pichardo, S., Cameán, A.M., 2015. Genotoxicity assessment of propyl thiosulfinate oxide, an organosulfur compound from *Allium* extract, intended to food active packaging. *Food Chem. Toxicol.* 86, 365–373. <https://doi.org/10.1016/j.fct.2015.11.011>.
- Nardelli, V., D'Amico, V., Della Rovere, I., Casamassima, F., Marchesiello, W.M.V., Nardiello, D., Quinto, M., 2020. Box Behnken design-based optimized extraction of non-dioxin-like PCBs for GC-ECD and GC-MS analyses in milk samples. *Emerg. Contam.* 6, 303–311. <https://doi.org/10.1016/j.emcon.2020.08.002>.
- Núñez, C., Baños Arjona, A., Guillamón, E., Valero, A., Navarro, C., Sanz, A., Brotman, K. A., 2016. Use of propyl propane thiosulfinate and propyl propane thiosulfonate for the prevention and reduction of parasites in aquatic animals. *United States Patent. US 9, 271–947. B2 2*.
- Pal, C.B.T., Jadeja, G.C., 2019. Microwave-assisted deep eutectic solvent extraction of phenolic antioxidants from onion (*Allium cepa* L.) peel: a Box-Behnken design approach for optimization. *J. Food Sci. Technol.* 56, 4211–4223. <https://doi.org/10.1007/s13197-019-03891-7>.
- Pastor-Belda, M., Arroyo-Manzanares, N., Yavir, K., Abad, P., Campillo, N., Hernández-Córdoba, M., Vinãs, P., 2020. A rapid dispersive liquid-liquid microextraction of antimicrobial onion organosulfur compounds in animal feed coupled to gas chromatography-mass spectrometry. *Anal. Methods* 12, 2668–2673. <https://doi.org/10.1039/d0ay00632g>.
- Peinado, M.J., Ruiz, R., Echávarri, A., Aranda-Olmedo, I., Rubio, L.A., 2013. Garlic derivative PTS-O modulates intestinal microbiota composition and improves digestibility in growing broiler chickens. *Anim. Feed Sci. Technol.* 181, 87–92. <https://doi.org/10.1016/j.anifeedsci.2013.03.001>.
- Peinado, M.J., Ruiz, R., Echávarri, A., Rubio, L.A., 2012. Garlic derivative propyl propane thiosulfonate is effective against broiler enteropathogens in vivo. *Poultry Sci.* 91, 2148–2157. <https://doi.org/10.3382/ps.2012-02280>.
- Poojary, M.M., Putnik, P., Bursac Kovačević, D., Barba, F.J., Lorenzo, J.M., Dias, D.A., Shpigelman, A., 2017. Stability and extraction of bioactive sulfur compounds from *Allium* genus processed by traditional and innovative technologies. *J. Food Compos. Anal.* 61, 28–39. <https://doi.org/10.1016/j.jfca.2017.04.007>.
- Putnik, P., Gabrić, D., Roohinejad, S., Barba, F.J., Granato, D., Mallikarjunan, K., Lorenzo, J.M., Bursac Kovačević, D., 2019. An overview of organosulfur compounds from *Allium* spp.: from processing and preservation to evaluation of their bioavailability, antimicrobial, and anti-inflammatory properties. *Food Chem.* 276, 680–691. <https://doi.org/10.1016/j.foodchem.2018.10.068>.
- Rabelo-Ruiz, M., Teso-Pérez, C., Peralta-Sánchez, J.M., Ariza, J.J., Martín-Platero, A.M., Casabuena-Rincón, O., Vázquez-Chas, P., Guillamón, E., Aguinaga-Casañas, M.A., Maqueda, M., Valdivia, E., Baños, A., Martínez-Bueno, M., 2021a. *Allium* Extract implements weaned piglet's productive parameters by modulating distal gut microbiota. *Antibiotics* 10 (3), 269. <https://doi.org/10.3390/antibiotics10030269>.
- Rabelo-Ruiz, M., Ariza-Romero, J.J., Zurita-González, M.J., Martín-Platero, A.M., Baños, A., Maqueda, M., Valdivia, E., Martínez-Bueno, M., Peralta-Sánchez, J.M., 2021b. *Allium*-based phytobiotic enhances egg production in laying hens through microbial composition changes in ileum and cecum. *Animals* 11 (2), 448. <https://doi.org/10.3390/ani11020448>.
- Rambo, D.F., Biegelmeier, R., Toson, N.S.B., Dresch, R.R., Moreno, P.R.H., Henriques, A. T., 2019. Box-Behnken experimental design for extraction optimization of alkaloids from *Erythrina verna* Vell. trunk barks and LC Method Validation. *Ind. Crop. Prod.* 133, 250–258. <https://doi.org/10.1016/j.indcrop.2019.03.030>.
- Ramirez, D.A., Locatelli, D.A., González, R.E., Cavagnaro, P.F., Camargo, A.B., 2017. Analytical methods for bioactive sulfur compounds in *Allium*: an integrated review and future directions. *J. Food Compos. Anal.* 61, 4–19. <https://doi.org/10.1016/j.jfca.2016.09.012>.
- Rodsamran, P., Sothornvit, R., 2019. Extraction of phenolic compounds from lime peel waste using ultrasonic-assisted and microwave-assisted extractions. *Food Biosci* 28, 66–73. <https://doi.org/10.1016/j.fbio.2019.01.017>.
- Shehata, M.G., Awad, T.S., Asker, D., El Sohaimy, S.A., Abd El-Aziz, N.M., Youssef, M. M., 2021. Antioxidant and antimicrobial activities and UPLC-ESI-MS/MS polyphenolic profile of sweet orange peel extracts. *Curr. Res. Food Sci.* 4, 326–335. <https://doi.org/10.1016/j.crfs.2021.05.001>.
- Solorzano-Puerto, A., Albertuz-Crespo, M., Lopez-Machado, I., Ariza-Romero, J.J., Baños-Arjona, A., Exposito-Ruiz, M., Gutiérrez-Fernández, J., 2018. *In vitro* antibacterial activity of propyl-propane-thiosulfinate and propyl-propane-thiosulfonate derived from *Allium* spp. against gram-negative and gram-positive multidrug-resistant bacteria isolated from human samples. *BioMed Res. Int.* 1–11. <https://doi.org/10.1155/2018/7861207>, 2018.
- Talhaoui, N., Taamalli, A., Gómez-Caravaca, A.M., Fernández-Gutiérrez, A., Segura-Carretero, A., 2015. Phenolic compounds in olive leaves: analytical determination, biotic and abiotic influence, and health benefits. *Food Res. Int.* 77, 92–108. <https://doi.org/10.1016/j.foodres.2015.09.011>.
- Vera, P., Canellas, E., Nerin, C., 2018. Identification of non volatile migrant compounds and NIAS in polypropylene films used as food packaging characterized by UPLC-MS/QTOF. *Talanta* 188, 750–762. <https://doi.org/10.1016/j.talanta.2018.06.022>.
- Wang, R., Dong, S., Wang, P., Li, T., Huang, Y., Zhao, L., Su, X., 2021. Development and validation of an ultra performance liquid chromatography-tandem mass spectrometry method for twelve bisphenol compounds in animal feed. *J. Chromatogr. B* 1178, 122613. <https://doi.org/10.1016/j.jchromb.2021.122613>.
- Yoo, M., Kim, S., Lee, S., Shin, D., 2014. Validated HPLC method and temperature stabilities for oil-soluble organosulfur compounds in garlic macerated oil. *J. Chromatogr. Sci.* 52, 1165–1172. <https://doi.org/10.1093/chromsci/bmt166>.