Food and Chemical Toxicology 161 (2022) 112827

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Acute and subchronic 90-days toxicity assessment of propyl-propane-thiosulfinate (PTS) in rats

Antonio Cascajosa-Lira [a], Silvia Pichardo [a,*], Alberto Baños [b], Enrique Guillamón [b], Verónica Molina-Hernández [c], Rosario Moyano [c], Ángeles Jos [a], Ana M Cameán [a]

[a] Área de Toxicología, Facultad de Farmacia, Universidad de Sevilla, Profesor García González n 2, 41012, Seville, Spain
[b] DMC Research Center, Camino de Jayena, 82, 1862, Alhendín, Spain
[c] Departamento de Anatomía y Anatomía Patológica Comparadas y Toxicología, Facultad de Veterinaria, Universidad de Córdoba, Campus de Rabanales, 14014, Córdoba, Spain

ARTICLE INFO

Handling Editor: Dr. Jose Luis Domingo

Keywords:
Subchronic toxicity
90-Days
Organosulfur compounds
Allium
Propyl-propane-thiosulfinate

ABSTRACT

The organosulfur compounds (OSC) extracted from *Allium spp*. exhibit antibacterial, antifungal, and antioxidant properties. The agri-food industry is taking advantage of these properties by using them as natural feed and food additives. In the present work, an acute and a subchronic 90-days toxicity studies have been conducted for the first time to assess the safety of the OSC propyl-propane-thiosulfinate (PTS). Both studies were carried out following the Organization for Economic Co-operation and Development test guidelines (425 and 408, respectively). The acute study provided a maximum tolerated dose (MTD) of 175 mg/kg and the subchronic study established the Non Observed Adverse Effect Level (NOAEL) $\geq$ 55 mg/kg body weight (b.w.)/day in both sexes. In addition, the subchronic study performed on rats exposed to 14, 28 and 55 mg/kg b.w./day PTS, revealed no changes in any of the hematological parameters measured as well as no differences in body weight and water/food consumption. However, biochemical parameters were altered in some groups, although they were not biologically significant (Ca^{2+} in female rats, and the thyroids hormones T3 and T4 in rat males). Furthermore, the histopathological assessment evidenced no abnormality on the gastrointestinal, respiratory, lymphoid, urinary, circulatory, nervous, musculoskeletal, and reproductive systems.

1. Introduction

Organic sulfur compounds (OSCs) are phytochemical molecules present in many species of the *Allium* genus. This genus includes more than 600 species, among them, the edibles highlight: garlic (*A. sativum*) onion (*A. cepa*), leek (*A. porrum*), chive (*A. schoenoprasum*), shallot (*A. ascalonicum*), and giant flowering onion (*A. giganteum*). The OSCs are well-known for their biological properties, among which antibacterial, antifungal, antioxidants, antiviral, antiprotozoal, anti-inflammatory properties stand out (Farhat et al., 2021; Putnik et al., 2019; Vezza et al., 2019). In onion, the most common OSCs are isoalliin (S-propenyl-L-cysteine sulfoxide) that changes into methiin (S-methyl-L-cysteine sulfoxide), also present in garlic; and propiin (S-propyl-L-cysteine sulfoxide) that, due to the action of alliinase, leads to propyl-propane-thiosulfinate (PTS) ($C_6H_{14}O_1S_2$) (Guillamón et al., 2021). Their properties are of great interest to the agri-food industry, in which there has recently been a high boom in the demand for additives of natural origin (Cascajosa-Lira et al., 2020a). Additionally, in the last years the number of research and patents of OSCs have increased: the use of dialkyl thiosulfonate or thiosulfinate to reduce the number of apicomplexa in animals (Bravo and Lillehoj, 2013) or the use of PTS and propyl propane thiosulfonate (PTSO) for the prevention and reduction of parasites in aquatic animals (Baños Arjona et al., 2016). PTS has demonstrated antifungal activity against *Verticillium dahliae* in olive trees suggested that this compound could be used as natural tool for Crop Pest Management (Falcón-Piñeiro et al., 2021). Moreover, PTS has shown beneficial effects on goats as an inhibitor of methanogenesis (Martínez-Fernández et al., 2013, 2015). Another use for PTS is as sensory additive in animal nutrition, improving the palatability of feed and being an alternative to the use of antibiotics in farm animals. It has been suggested to be used as an alternative of antibiotics (Peinado et al., 2012, 2013) supporting the decrease in bacteria resistance generated by their excessive application in farm animals. The regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22

* Corresponding author. Área de Toxicología, Facultad de Farmacia, Universidad de Sevilla, Profesor García González n 2, 41012, Seville, Spain.
E-mail address: spichardo@us.es (S. Pichardo).

https://doi.org/10.1016/j.fct.2022.112827
Received 22 October 2021; Received in revised form 7 December 2021; Accepted 17 January 2022
Available online 22 January 2022

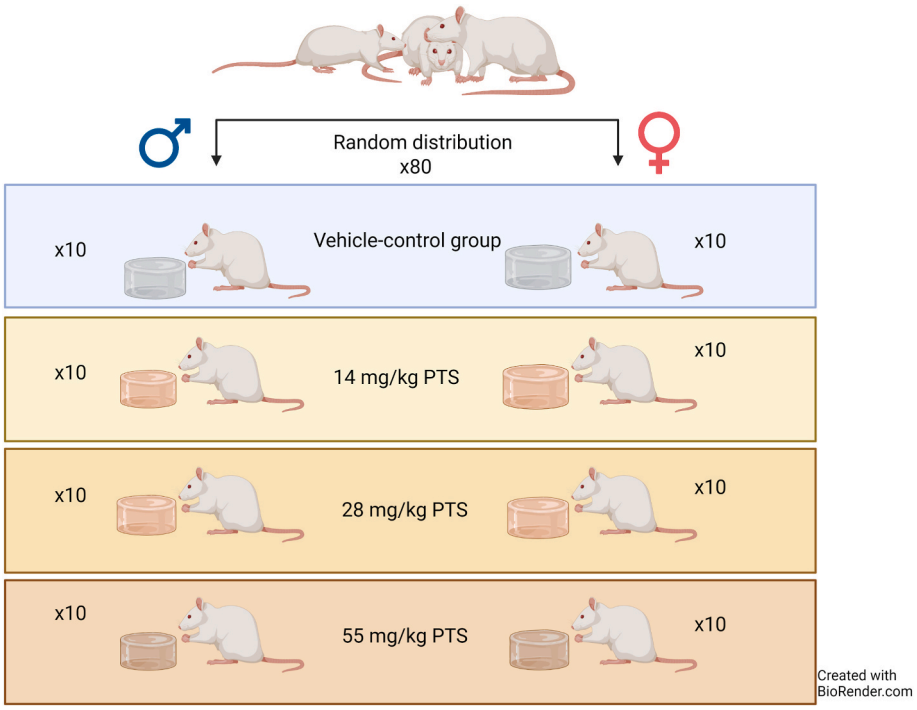

Fig. 1. Flowchart of the subchronic toxicity study.

September 2003 sets the parameters of handling of the additives used in animal nutrition. With respect to this legislation, the use of antibiotics as growth promoters is forbidden; the development of alternative products to replace those antibiotics is expected. In addition, OSCs or essential oils are being employed as additives in food and feed (Cascajosa-Lira et al., 2021a), either because of their flavoring character or their antioxidant or antimicrobial properties (Llana-Ruiz-Cabello et al., 2015; Maisanaba et al., 2017). In this sense, they can be a good alternative for additives of chemical origin, such as sulfites, used in meats, wines or juices that cause allergic reactions in some consumers (EFSA, 2016a) and also could degrade molecules with nutritional value such as thiamine or vitamin B1 (Singh et al., 2005). For this reason, there is an increased demand of new products that guarantee safety and similar production levels, without generating unwanted effects, such as human resistances or side effects, being additives of natural origin a good alternative.

In this regard, it is necessary to have a comprehensive knowledge of its toxicological profile to guarantee its safety. Regarding the toxicological profile of PTS, at the present time there are available cytotoxicity assays in mammalian cells as well as mutagenicity and genotoxicity assessment *in vitro* (Mellado-García et al., 2017) and an *in vivo* genotoxicity study in rats (Cascajosa-Lira et al., 2021). The European Food Safety Authority (EFSA) required a 90-days oral toxicity study among the toxicological assays for the approval of food and feed additives (EFSA 2012; 2016b, 2017) following the guideline 408 of the Organization for Economic Cooperation and Development (OECD).

Recently, several subchronic studies in rats treated with OSC such as PTSO (Cascajosa-Lira et al., 2020b) and *Allium* extract (Mellado-García et al., 2016) have been published. Moreover, different essential oils have shown antioxidant and antimicrobial characteristics that are as well of interest in the agri-food industry (Lira et al., 2020; Llana-Ruiz-Cabello et al., 2017; Mellado-García et al., 2016; Mishra et al., 2018; Preece et al., 2021). However, the acute and subchronic studies of PTS itself has not been conducted so far. Therefore, the objectives of this work were to carry out, for the first time, an acute toxicity study (OECD 425, 2008) and a 90-day subchronic toxicity study by oral route in rats (OECD 408, 2018) in order to study the possible adverse effects in long terms of PTS by establishing the NOAEL (no observed adverse effect level).

2. Materials and methods

2.1. Dose preparation and acute toxicity study design

PTS was supplied by DMC Research Center (Granada, Spain) with a 76% of purity (cod. 19.10.2020). The results of the PTS analysis by UPLC-MS-MS were reported in Cascajosa-Lira et al. (2021a).

In order to determine the maximum tolerable dose (MTD), the acute oral toxicity study (Up and Down procedure, OECD 425, 2008) was carried out. Initially, one rat was administered by gastric tube with a single dose of 175 mg/kg body weight (b.w.) PTS. Then, the single doses to the consecutive rats were administered in increasing/decreasing order: 550 and 2000 mg/kg b.w. PTS depending on the morbidity or mortality of the animals. Mortality and clinical sign were observed for two weeks before sacrifice. If animals showed moribund signs or obvious severe pain, then they were humanely sacrificed. Three animals were dosed with 175 mg/kg b.w., 3 were exposed to 550 mg/kg b.w., and only 1 animal was treated with 2000 mg/kg b.w.

2.2. Subchronic study design

A total of 80 rats (40 males and 40 females with similar body weight mean in a range of ±20% as mentioned in the guideline) were randomly distributed into eight groups (10 rats/sex/group of doses). The distribution of rats in this study is described in Fig. 1. The highest dose was set at 55 mg/kg b.w. accordingly with the MTD and the palatability of the product, and descending doses were selected using a factor of root of ten according to the guideline (OECD 408, 2018) recommendations: 14, 28, 55 mg/kg b.w. Therefore, 3 groups of rats from each sex were exposed to 14, 28, 55 mg/kg b.w./day PTS. The commercial powder neutral gelatin from pork protein was employed as the vehicle for the test substance in all groups including the control. The dose was mixed in 3 mL of liquid gelatin. PTS was included in the gelatin that solidified at 4 °C overnight following Cascajosa-Lira et al. (2020b). In addition, the vehicle-control group were fed with gelatin without PTS.

2.3. Animal conditions and husbandry services

All animals received human care in accordance with the guidelines for the protection of animals used for the science purposes (Directive, 2010/63/EU, Decision, 2020/569/UE, and RD 1386/2018). All procedures have been approved by the Ethical Animal Experimentation Committee of the University of Córdoba and by the Junta de Andalucía (project nº 22/12/2020/154).

The rats, Sprague-Dawley strain, were 7 weeks old. Initially, all of them become stable for an acclimatization phase of 7 days. Animals were individually housed in type 3H cages with aspen wood bedding and food completely available using standard dry pellet diet for rodents. They were kept in a room with controlled conditions of hygiene behind a barrier system, a range of temperature of 21±2 °C with a 10–15 air changes per hour and a relative humidity between 30 and 70% under 12 h light/dark cycle.

2.4. Clinical observation and ophthalmic examination

Examination included observations of skin and fur, eye and mucous membranes, respiratory and circulatory effects, automatic effects as salivation, central nervous system, motor activity, including tremors and convulsions, changes in motor activity, gait and posture, reactivity to handling or sensory stimuli, and normally or strange behavior. For the ophthalmic examination, before and after treatment, the following organs were also observed: cornea, crystalline lens, conjunctivae, sclera, iris, and fundus of all animals.

2.5. Body weight, food, and water consumption

These three parameters were weekly recorded in order to avoid the stress of the animals and the means per groups and sex were calculated. The total food consumed per cage was recorded and weekly mean intake per rat was calculated, required to determinate feed ratio conversion efficiency (FCE) according to Cascajosa-Lira et al. (2020b) by the ratio of food intake (g)/weight gained (g).

2.6. Haematology, biochemistry and, endocrinology

As recommended OECD 408 (2018), once the animals were sacrificed under anesthesia effects, blood samples were extracted from the heart by an intracardiac injection. Then, the hematological parameters were determined on an automatic hematological analyzer Cell-Dyn 3700 (Abbot, GMI, MI, USA): red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hematoglobin (MCH), mean corpuscular hematoglobin concentration (MCHC), blood platelet count (PLT), red cell volume distribution (RDW), prothrombin time (PT), cefaline time (CT), white blood cell count (WBC), neutrophils (NE), lymphocytes (LY), monocytes (MO), eosinophils (EO) and basophils (BA).

The standard serum biochemistry parameters were analyzed with an automatic chemistry analyzer Cobas 6000 (Roche Diagnostics, IN, USA), to determinate these biochemistry parameters: glucose (GLUC), blood urea nitrogen (UREA), creatine (CREAT), bile acids (BILI-T), total cholesterol (CHOL), high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides (TRIGL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), albumin (ALB), total protein (TOT PROT), sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) ions.

The hormones: triiodothyronine (T3) and thyroxine (T4) were also measured using an analyzer Cobas 6000 (Roche Diagnostics, IN, USA). Thyroid stimulating hormone (TSH) was measured by a commercial ELISA kit from Demeditec (DEV9977 TSH rat ELISA, Demeditec Diagnostics GmbH, Germany).

2.7. Necropsy, organ weight and histopathology

All animals survived the complete treatment. Then, rats were fasted overnight (for 18 h before the sacrifice) and deeply anaesthetized with isofluorane. Later, they were euthanized with CO_2 and exsanguinated by intracardiac injection according to directive 2010/63 UE. All rats received a complete pathology examination through the necropsy. The following tissues and organs were collected from animals and weighed wet *in situ* after dissection: brain, liver, heart, spleen, kidneys, thymus, adrenal glands, uterus with cervix and ovaries (females) and testes, epididymis and seminal gland (males).

For the histopathological assay, tissues from the gastrointestinal system (liver, pancreas, glandular and nonglandular stomach, and small and large intestines), from the respiratory, lymphoid, urinary, circulatory, musculoskeletal, and nervous systems (lung, spleen, kidney, heart, skeletal muscle, cerebellum, and cerebrum), and the reproductive system (ovary and testes) were taken from the control and the PTS administered rats (14, 28 and 55 mg/kg b.w./day). All the tissue samples were fixed in 10% buffered formalin for 24h and routinely processed and embedded in paraffin wax. Tissue sections (4 μm thick) were stained with haematoxylin and eosin (H&E).

2.8. Statistical analysis

Statistical analysis was carried out using Graph-Pad Prism 9 software (GraphPadSoftware Inc., La Jolla, USA). Continuous variables were summarized using mean and standard deviation (SD) and were reported by sex and dosage. One-way analysis of variance (ANOVA) was carried out to test differences in continuous variables. Normality assumption was tested using Kolmogorov-Sminorv's test. If non-normality was observed, comparison was performed with Kruskal-Wallis test. If those tests were statistically significant, multiple comparisons were performed using Tukey-Kramer/Dunn's Multiple Comparisons Tests.

3. Results

3.1. Acute study. LD50 and MTD

Following the guidelines of the OECD 425, the first dose assayed was 175 mg/kg b.w. given by gavage. Considering the results obtained in each step the dose increased to 550 and 2000 mg/kg b.w. The results obtained in each dose were the following. None of the rats orally exposed to 175 mg/kg b.w. died or experienced any clinical injury. Different responses were recorded in rats exposed to 550 mg/kg b.w.; one rat was sacrificed for human reasons; another rat survived with severe irreversible signs of toxicity; and the other rat survived showing light signs of toxicity. In addition, two out of the three rats exposed to 550 mg/kg b.w. underwent a decrease in weight gain above 20%. The rat dosed with 2000 mg/kg b.w. of PTS died immediately after exposure. Consequently, the LD50 of PTS was stablished at 550 mg/kg b.w., and MTD at 175 mg/kg b.w. However, due to palatability acceptance of the product, the dose for the subchronic study was lowered at 55 mg PTS/kg b.w. as previously explained in section 2.2.

3.2. Clinical and ophthalmological observation

No changes in gait, posture, or handling response, abnormal, clonic, or tonic movements, and stereotypes or bizarre behavior was recorded in any group. The ophthalmological examination performed at the end of the study showed no abnormalities in all doses and sex groups during the study period. Some contact injuries were observed in seven rats in the 28 and 55 mg/kg b.w./day dose groups on back, face and hands. No other clinical observations were noted.

Table 1
Effect of 90 days oral exposure to PTS on body weight and food consumption in Sprague Dawley. Values are mean ± SD for 10 rats/sex/group. F: Statistics ANOVA test; K.W: Kruskal-Wallis Statistic; N.S.: Not Significant.

	MALE				FEMALE			
PARAMETERS	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
	(0 mg/kg/day)	**(14 mg/kg/day)**	**(28 mg/kg/day)**	**(55 mg/kg/day)**	**(0 mg/kg/day)**	**(14 mg/kg/day)**	**(28 mg/kg/day)**	**(55 mg/kg/day)**
	N = 10	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**
Inicial body weight (g)	360.5 ± 12.18	313.00 ± 31.34	309.80 ± 13.35	317.10 ± 14.12	223.40 ± 12.22	222.00 ± 13.03	229.20 ± 12.15	220.40 ± 13.83
	F(36.3) = 0.5309 p = 0.6640; N.S.				**F(36.3) = 0.8938 p = 0.4537; N.S.**			
Final body weight (g)	647.40 ± 48.14	623.00 ± 63.24	645.00 ± 37.96	658.50 ± 35.15	334.70 ± 23.35	345.00 ± 19.29	351.50 ± 27.50	334.80 ± 17.59
	F(36.3) = 0.9836 p = 0.4118; N.S.				**KW = 2.697 p = 0.4408; N.S.**			
Body weight gain (g)	340.90 ± 43.83	310.00 ± 64.22	335.20 ± 29.85	341.40 ± 30.93	121.30 ± 17.60	123.00 ± 9.78	122.30 ± 18.00	114.40 ± 12.08
	F(36.3) = 1.118 p = 0.3546; N.S.				**F(36.3) = 0.7174 p = 0.5481; N.S.**			
Total feed intake (g)	3241.10 ± 314.04	3165.60 ± 293.63	3304.60 ± 249.09	3485.40 ± 263.36	2127.80 ± 215.17	2171.80 ± 219.48	2210.20 ± 291.03	2049.40 ± 208.61
	F(36.3) = 2.358 p = 0.0879; N.S.				**F(36.3) = 0.8560 p = 0.4727; N.S.**			
Feed conversion ratio	9.56 ± 0.71	10.55 ± 1.93	9.90 ± 0.77	10.25 ± 0.86	17.79 ± 2.54	17.72 ± 1.99	18.32 ± 3.20	18.00 ± 1.73
	F(36.3) = 1.315 p = 0.2845; N.S.				**F(36.3) = 0.1258 p = 0.9442; N.S.**			

Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA-Tukey test (F values). N.S. not significant.

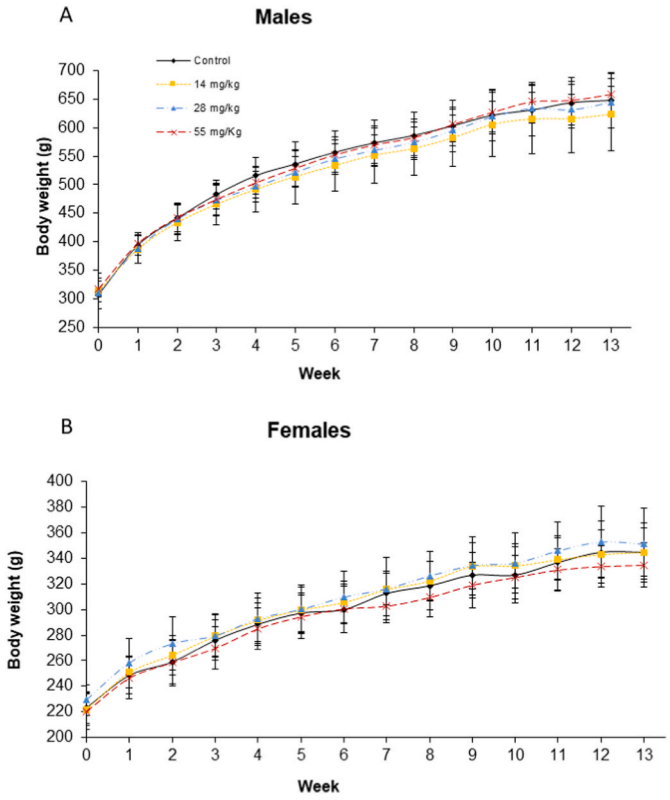

Fig. 2. Mean body weights (g) of A) male and B) female rats orally exposed to 14, 28 and 55 mg/kg b.w./day PTS and control rats for 90 days. Values are mean ± standard deviation (SD) for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by ANOVA-Tukey tests.

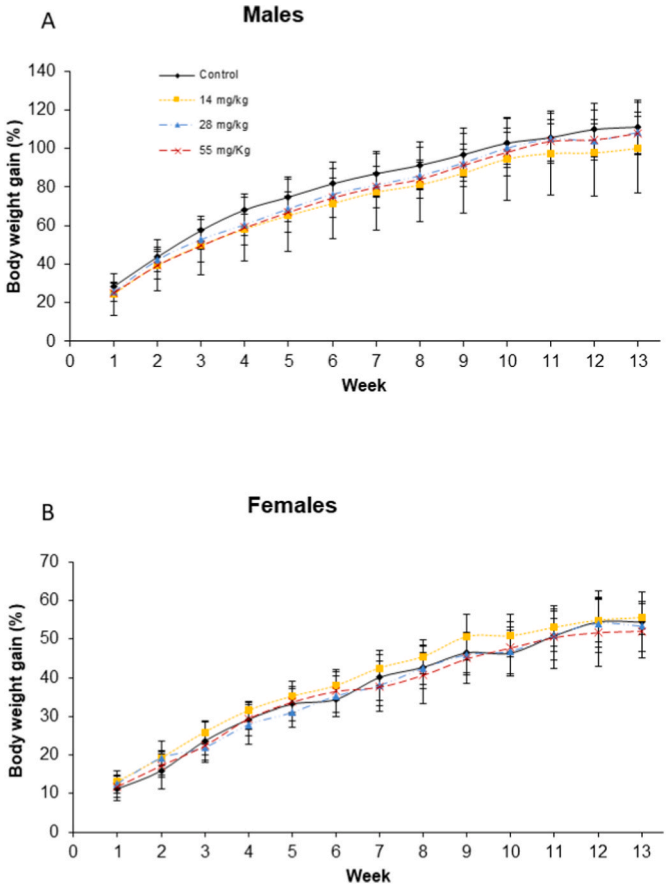

Fig. 3. Mean body weight gain (%) of A) male and B) female rats orally exposed to 14, 28 and 55 mg/kg b.w./day PTS and control rats for 90-day. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test or ANOVA-Tukey tests.

3.3. Body weight, body weight gain, food consumption, feed conversion efficiency, and water consumption

There are no significant differences between groups of doses in the final body weight, body weight gain, total food intake or feed conversion efficiency (Table 1). The mean body weight per week of male and female rats exposed to PTS (14, 28 and 55 mg/kg b.w./day) was not significantly changed through the complete experiment in males (Fig. 2A) and females (Fig. 2B). Similarly, no significant changes were observed in % body weight gain in males (Fig. 3A) and females (Fig. 3B). There were significant differences in food consumption in control and 14 mg/kg b.w./day groups in comparison to 55 mg/kg b.w./day in males for three weeks as were shown in Fig. 4A. In contrast, there were no significant

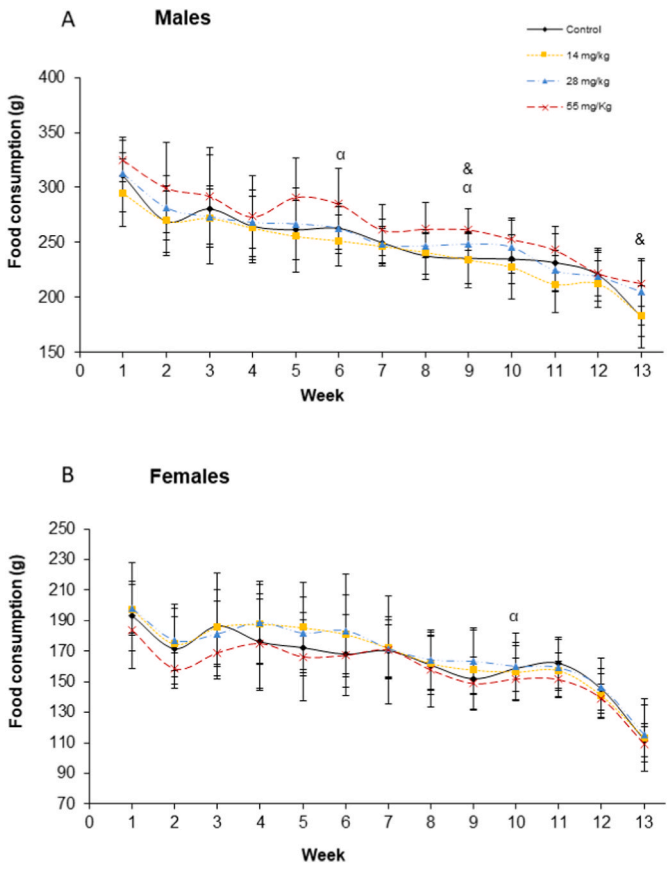

Fig. 4. Mean Food consumption (g) of A) male and B) female rats orally exposed to 14, 28 and 55 mg/kg b.w./day of PTS and control rats for 90-day. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test or ANOVA-Tukey tests. The significance levels observed are α $p < 0,05$ when 14 mg/kg b.w./day, and 55 mg/kg b.w/day. groups were compared. & $p < 0,05$ when control group and 55 mg/kg were compared.

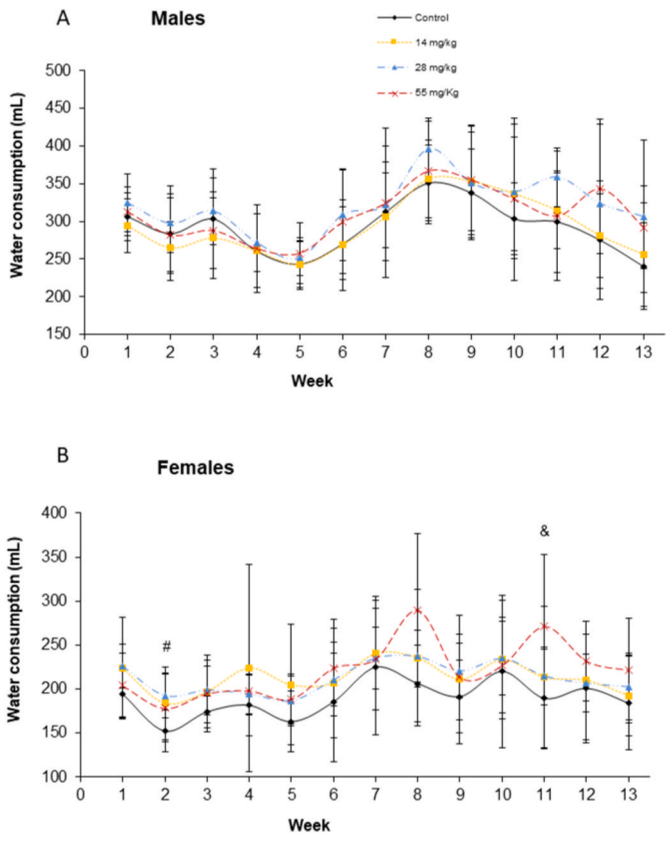

Fig. 5. Mean water consumption (mL) of A) male and B) female rats orally exposed to 14, 28 and 55 mg/kg b.w./day of PTS and control rats for 90-day. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test or ANOVA-Tukey tests. The significance levels observed are #$p < 0,05$ when control group and 28 mg/kg b.w./day were compared & $p < 0,05$ when control group and 55 mg/kg b.w./day were compared.

changes in female groups (Fig. 4B). The water consumption in male rats did not show any significant differences (Fig. 5A). But in the other hand, female rats exposed to 28 and 55 mg/kg b.w./day showed significant differences in comparison with control group in the second week and in the eleventh week respectively (Fig. 5B).

3.4. Biochemistry and haematology

Haematology parameters are shown in Table 2. There were no significant differences in any parameter measured for male and female rats. In addition, there were no significant differences in total and differential leukocyte counts for rats fed with PTS for both sexes (Table 3).

Clinical biochemistry parameters of rats exposed to all levels of PTS doses (14, 28, 55 mg/kg b.w.) are shown in Table 4. These results revealed significant differences in Ca^{2+} in female rats exposed to 14 mg/kg b.w./day in comparison with control group. In addition, thyroids hormones showed significant differences in male rats. T3 levels revealed significant increase in rats exposed to 14 mg/kg b.w./day Besides, T4 levels are also increased in the same group of dosage and additionally in 55 mg/kg b.w./day in comparison with control group in male rats.

3.5. Necropsy and organ weights

The macroscopy examination of the organs and tissue did not show any remarkable damage in any dosage level assayed of PTS. The organ weight (brain, liver, heart, spleen, kidney, thymus, adrenals, testes, epididymis, seminal gland, uterus, and ovaries) of male and female rats of control and treated groups are shown in Table 5 as well as the ratio of the weight of the organ with respect to the total weight in Table 6 and with respect to the brain weight in Table 7. Subchronic administration of PTS did not revealed any significant difference in the organ weight or ratios.

3.6. Histopathology

The histopathology of the organs from the gastrointestinal, respiratory, lymphoid, urinary, circulatory, nervous, musculoskeletal, and reproductive systems were firstly assessed in the control and 55 mg/kg b.w./day PTS groups including male and female rats. The results obtained from the histopathological study revealed no observable pathological features in the rats administered with 55 mg/kg b.w./day PTS dose compared with the controls in any studied organs. Moreover, the comparative study between male and female rats no displayed histopathological differences neither.

The representative histological images of the gastrointestinal system are included in Fig. 6. Thus, control and 55 mg/kg b.w./day PTS male and female rats presented a histologically normal liver with hepatocytes extending from the portal spaces to central veins without pathological features. In addition, pancreases showed both endocrine and exocrine components with normal appearance no displaying any pathological change in the samples from the control and 55 mg/kg b.w./day PTS male and female rats. Both glandular and non-glandular (squamous) stomachs did not display pathological changes in any group studied showing an

Table 2
Haematology parameters of Sprague Dawley male and female rats fed with different doses of PTS in the diet for 90-day. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values). N.S. not significant.

HAEMATOLOGY DATA SUMMARY									
PARAMETERS		MALE				FEMALE			
		Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 1**	**Group 2**	**Group 3**	**Group 4**
		(0 mg/kg/day)	**(14 mg/kg/day)**	**(28 mg/kg/day)**	**(55 mg/kg/day)**	**(0 mg/kg/day)**	**(14 mg/kg/day)**	**(28 mg/kg/day)**	**(55 mg/kg/day)**
		N = 10	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**
RBC 10^6/µl	**MEAN**	9.20	9.23	9.14	9.25	8.40	8.21	8.47	8.78
	ST. DEV.	0.41	0.54	0.79	0.53	0.32	0.66	0.61	1.74
		F(36.3) = 0.0731 p = 0.9740; N.S.				**KW = 1.730 p = 0.6303; N.S.**			
HGB g/dL	**MEAN**	15.09	15.50	15.10	15.21	14.60	14.27	14.60	14.27
	ST. DEV.	0.45	0.94	1.12	0.80	0.55	1.22	0.55	1.22
		KW = 4.772 p = 0.1893; N.S.				**KW = 3.321 p = 0.3447; N.S.**			
HCT %	**MEAN**	53.93	54.61	53.97	53.78	52.80	54.84	53.57	52.19
	ST. DEV.	1.32	3.22	4.03	2.99	3.35	13.31	2.19	4.68
		F(36.3) = 0.1449 p = 0.9323; N.S.				**KW = 0.2993 p = 0.9602; N.S.**			
MCV fL	**MEAN**	58.66	59.22	59.13	58.20	63.83	63.60	62.42	62.76
	ST. DEV.	1.89	0.83	1.97	1.85	1.42	1.88	1.85	2.00
		KW = 0.9939 p = 0.8027; N.S.				**F(36.3) = 1.511 p = 0.2288; N.S.**			
MCH pg	**MEAN**	16.42	16.50	16.57	16.45	17.39	17.39	17.08	16.36
	ST. DEV.	0.49	0.40	0.48	0.36	0.48	0.48	0.42	3.96
		KW = 0.9297 p = 0.8183; N.S.				**KW = 3.515 p = 0.3188; N.S.**			
MCHC g/dL	**MEAN**	27.80	27.89	28.00	28.29	27.26	27.36	27.34	26.01
	ST. DEV.	0.73	0.52	0.41	0.35	0.43	0.20	0.41	5.80
		KW = 4.881 p = 0.1807; N.S.				**KW = 1.320 p = 0.7244; N.S.**			
PLT 10^3/µl	**MEAN**	939.20	873.72	912.80	999.40	861.20	786.20	871.55	784.93
	ST. DEV.	142.21	348.62	76.25	97.41	151.21	407.11	288.75	694.09
		F(36.3) = 0.7095 p = 0.5527; N.S.				**KW = 2.746 p = 0.4324; N.S.**			
RDW %	**MEAN**	17.99	17.73	17.89	18.56	15.77	15.49	15.66	16.98
	ST. DEV.	1.34	1.32	1.19	0.89	0.89	1.19	0.67	3.96
		F(36.3) = 0.9070 p = 0.4470; N.S.				**KW = 5.236 p = 0.1553; N.S.**			
T PRO seg	**MEAN**	20.63	23.57	21.50	21.39	24.01	23.28	24.72	23.53
	ST. DEV.	0.49	2.03	0.97	0.67	1.07	1.64	0.95	0.78
		KW = 6.188 p = 0.1028; N.S.				**KW = 3.514 p = 0.3190; N.S.**			
T CEF seg	**MEAN**	24.55	23.57	21.87	21.79	26.38	21.83	22.42	24.75
	ST. DEV.	1.99	2.03	0.70	2.16	5.15	1.83	2.69	0.07
		KW = 9.811 p = 0.0202; N.S.				**KW = 5.903 p = 0.1164; N.S.**			

RBC: Erythrocyte count; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet (thrombocyte) count; RDW: red blood cell distribution width; T PRO: prothrombin time; T CEF: cephalin time.
Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA-Tukey test (F values). N.S. not significant.

intact mucosa. The small and large intestines studied from the male and female rats of the control and PTS-exposed group presented the normal histology of these organs with enterocytes together with mucous cells lining an intact epithelium.

Fig. 7 showed the histological images of the respiratory, lymphoid, urinary, circulatory, nervous, and musculoskeletal systems. Lungs from the control and 55 mg/kg b.w./day PTS male and female rats presented the normal histological architecture with empty alveoli, bronchioles, and bronchi. Spleens from control and PTS administered rats did not display pathological features and they were constituted by the red pulp showing a physiological small amount of intracytoplasmic pigment within the splenic macrophages, and the white pulp with arterioles surrounded by lymphoid tissue. Kidneys from both control and PTS administered groups showed a normal histology, with glomerulus displaying a Bowman's space between the Bowman's capsule and the nest of capillaries and proximal and distal tubules presented their normal appearance with a brush border in the proximal tubules appearing as a full lumen and the distal tubules without brush border appearing as an empty lumen. Hearts did not show any pathology neither the control nor 55 mg/kg b.w./day PTS groups displaying a myocardium with normal cardiomyocytes with one or two central nuclei that sometimes are enlarged in normal conditions. The skeletal muscle was also histologically normal presenting long and multi-nucleated myofibers. The evaluation of the nervous system sampling such as cerebrum and cerebellum from the control and 55 mg/kg b.w./day PTS male and female rats did not show any visible pathological feature displaying the normal architecture of the tissue with neurons with normal morphology.

The representative images of the reproductive system are shown in Fig. 8. Ovaries from control and 55 mg/kg b.w./day PTS female rats presented ovarian follicles in different stages that indicate a normal functionality of the organ. Testes from control and 55 mg/kg b.w./day PTS male rats displayed no pathology and histologically showed tightly

Table 3
Differential white blood cells count data of Sprague Dawley male and female rats fed with different doses of PTS in the diet for 90-day. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values). N.S. not significant.

DIFFERENTIAL WHITE BLOOD CELLS COUNT DATA SUMMARY									
		MALE				FEMALE			
		Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
		(0 mg/kg/day)	(14 mg/kg/day)	(28 mg/kg/day)	(55 mg/kg/day)	(0 mg/kg/day)	(14 mg/kg/day)	(28 mg/kg/day)	(55 mg/kg/day)
		N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10	N = 10
WBC 10^3/μL	**MEAN**	14.74	13.27	14.18	14.16	8.49	7.54	7.46	6.90
	ST. DEV.	2.05	4.09	2.96	4.04	1.69	2.66	2.95	3.01
		KW = 0.6582 p = 0.8830; N.S.				**F(34.3) = 0.6459 p = 0.5908; N.S.**			
NE %	**MEAN**	18.70	17.51	13.91	15.33	17.41	12.74	13.81	18.99
	ST. DEV.	7.51	5.89	4.71	6.42	11.70	7.10	3.70	9.22
		F(36.3) = 1.189 p = 0.3270; N.S.				**F(34.3) = 1.112 p = 0.3573; N.S.**			
LY %	**MEAN**	74.80	74.64	78.51	77.49	70.89	79.22	80.95	70.64
	ST. DEV.	8.06	7.70	4.98	8.93	16.23	10.08	3.11	17.92
		KW = 0.6574 p = 0.5836; N.S.				**F(34.3) = 1.928 p = 0.1430; N.S.**			
MO %	**MEAN**	1.38	1.55	2.91	1.74	1.85	1.80	0.62	2.02
	ST. DEV.	1.70	2.04	3.54	2.18	1.69	2.41	0.35	1.80
		KW = 2.321 p = 0.5086; N.S.				**KW = 6.572 p = 0.0869; N.S.**			
EO %	**MEAN**	4.01	6.24	3.75	4.88	9.28	5.74	4.14	8.05
	ST. DEV.	2.63	3.82	1.75	3.82	9.43	4.13	1.64	8.96
		F(36.3) = 1.285 p = 0.2944; N.S.				**KW = 1.347 p = 0.7179; N.S.**			
BA %	**MEAN**	0.37	0.46	0.65	0.56	0.55	0.50	0.49	0.29
	ST. DEV.	0.37	0.29	0.50	0.55	0.57	0.36	0.33	0.40
		F(36.3) = 0.7941 p = 0.5052; N.S.				**F(34.3) = 0.7351 p = 0.5381; N.S.**			

WBC: total leukocyte count; NE: neuthrophil; LY: lymphocyte; MO:monocyte; EO:eosinophil; BA:basophil.
Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA-Tukey test (F values). N.S. not significant.

packed seminiferous tubules with the Sertoli cells and the germinal epithelium with the spermatozoids in the lumen indicating the functional spermatogenesis and presenting the interstitial Leydig cells surrounding the seminiferous tubules.

4. Discussion

The increasing use of PTS in the food industry, as well as phytochemical preparations including this compound, has led to the need of assuring its safety. The guidance for submission of food additive evaluations published by EFSA (EFSA, 2012) describes a tiered approach, which consists of three tiers. Within the first tier, a subchronic toxicity study should be conducted for a period of at least 90 days (OECD 408, 2018) in rodents. In the present work, we performed for the first time for PTS a repeated dose 90-days oral toxicity study in rats, and according to the lack of toxicity observed in all parameters measured, the NOAEL was estimated to be ≥ 55 mg/kg b.w./day. Previous subchronic toxicity studies of similar compounds to PTS and natural extract containing it have been previously conducted. A 90-day study of an *Allium*-based commercial extract (Proallium AP®) has reported no toxicity, establishing the NOAEL at 400 mg/kg b.w./day in the (Mellado-García et al., 2016). Likewise, no sign of toxicity was reported in the subchronic study performed using the main compound of Proallium AP®, PTSO, which is an analogue of PTS. In the latter study, the NOAEL was set at ≥ 55 mg/kg b.w./day (Cascajosa-Lira et al., 2020b). This finding agrees with the ones obtained in the present study, both compounds showed the same value of NOAEL. It is worth noting that a higher dose could not be assayed due to palatability problems. Therefore, a higher PTS dose could have been probably established for NOAEL.

No significant change was observed in most of the parameters measured in the present work (body weight, food and water consumption, FCE, and biochemical and blood parameters). The only value showing a significant decrease was Ca^{2+} in female rats exposed to the lowest dose of PTS (14 mg/kg b.w./day). However, although this value indicated a significant change from a statistical point of view, this is not a remarkable change from a biological perspective since it can be considered as a normal value in Sprague-Dawley rats. The value obtained was 9.81 ± 1.1 mg/dL Ca^{2+}. Similar results have been reported in control female rats, 9.9 ± 0.7 mg/dL Ca^{2+} (Matsuzawa et al., 1986) and 9.6 ± 0.29 mg/dL Ca^{2+} (An et al., 2019).

Moreover, our study measured not only total cholesterol but also high density lipoprotein (HDL) and low density lipoprotein (LDL) as recommended in the OECD guideline 408 (2018). These parameters were measured taking into account that the vast majority of OSCs that have biological activity affect cholesterol levels (Ghyasi et al., 2019; Liu and Yeh, 2000, 2001; Sun et al., 2018). In this sense, mice fed with a high-fat diet and orally treated with different doses of PTSO (0.1, 0.5 and 1 mg/kg b.w./day) for 5 weeks showed beneficial effects of PTSO such as decrease of body weight and reduction of the total and LDL cholesterol without modifying the HDL cholesterol (Vezza et al., 2021). The values of total cholesterol, HDL and LDL found in the present study were similar to those obtained in another subchonic study in rats fed with mango extract (Tajiri et al., 2021). Although the total cholesterol was higher in our study (93.2 ± 11.94 in male rats and 100.3 ± 8.11 in female rats) compared to the mango extract study (66 ± 13 male and 101 ± 12 female), the values obtained for HDL were lower in the present study (41.6 ± 13.33 male and 51.90 ± 5.13) in comparison to the other study (47.0 ± 9.9 in male and 64.0 ± 8.4).

All the 90-day toxicity study previously mentioned followed the guidelines proposed by EFSA, OECD 408 (2018). However, none

Table 4
Clinical biochemistry of Sprague Dawley male and female rats fed with different doses of PTS in the diet for 90-day. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values). The significance levels observed are *p < 0.05 and **p < 0.01 in comparison to control group values.

CLINICAL BIOCHEMISTRY DATA SUMMARY									
PARAMETERS		MALE				FEMALE			
		Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
		(0 mg/kg/ day)	**(14 mg/kg/ day)**	**(28 mg/kg/ day)**	**(55 mg/kg/ day)**	**(0 mg/kg/ day)**	**(14 mg/kg/ day)**	**(28 mg/ kg/day)**	**(55 mg/kg/ day)**
		N = 10	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**	**N = 10**
GLUC	MEAN	119.90	117.90	133.80	122.20	131.40	132.70	132.90	143.70
mg/dL	ST. DEV.	17.87	19.32	46.21	35.76	12.28	23.97	21.69	23.42
		KW = 1.051 p = 0.7889; NS				**F(36.3) = 0.7508; p = 0.5291; NS**			
UREA	MEAN	35.22	35.32	35.87	34.56	37.84	34.88	34.12	37.4
mg/dl	ST. DEV.	6.26	4.56	3.97	3.81	8.95	7.98	9.39	4.88
		KW = 0.9968 p = 0.8020; N.S.				**F(36.3) = 0.5271; p = 0.6665; N.S.**			
CREAT	MEAN	0.29	0.27	0.27	0.28	0.325	0.29	0.294	0.317
mg/dL	ST. DEV.	0.11	0.03	0.07	0.04	0.04	0.06	0.05	0.02
		KW = 5.580 p = 0.1339; N.S.				**KW = 6.697; p = 0.0822; N.S.**			
BILI-T	MEAN	0.07	0.09	0.10	0.10	0.12	0.11	0.09	0.11
mg/dL	ST. DEV.	0.03	0.02	0.03	0.02	0.04	0.02	0.02	0.03
		KW = 7.146 p = 0.0674; N.S.				**F(36.3) = 2.035 p = 0.1263; N.S.**			
CHOL	MEAN	93.20	95.10	89.00	98.80	100.3	97.5	99.5	100.3
mg/dL	ST. DEV.	11.94	14.44	10.52	11.98	8.11	13.77	8.66	8.72
		KW = 2.862 p = 0.4133; N.S.				**F(36.3) = 0.1719; p = 0.9147; N.S.**			
HDL	MEAN	41.60	40.80	37.70	42.70	51.90	49.70	51.50	51.90
mg/dL	ST. DEV.	12.33	8.04	5.23	7.59	5.13	8.04	5.34	4.33
		KW = 1.817 p = 0.6113; N.S.				**F(36.3) = 0.3192 p = 0.8114; N.S.**			
LDL	MEAN	30.48	28.88	26.82	28.84	30.42	32.66	33.75	33.90
mg/dL	ST. DEV.	16.43	5.37	8.59	6.83	3.95	8.18	4.83	4.16
		KW = 0.7147 p = 0.8697; N.S.				**KW = 1.339 p = 0.7199; N.S.**			
TRIGL	MEAN	89.9	75.7	71.4	77.5	89.90	75.70	71.40	77.50
						11.97	21.87	11.02	16.56
mg/dL	ST. DEV.	11.97	21.87	11.02	16.56				
		F(36.3) = 0.5760; p = 0.6345; N.S.				**F(36.3) = 2.480; p = 0.0767; N.S.**			
AST	MEAN	206.35	200.83	197.08	192.64	216.33	241.18	203.15	221.947
U/L	ST. DEV.	5.51	81.36	46.59	29.85	36.33	117.93	74.25	57.80
		KW = 2.109 p = 0.5500; N.S.				**KW = 2.724; p = 0.4362; N.S.**			
ALT	MEAN	38.46	40.50	53.75	36.39	51.15	49.66	53.64	47.668
U/L	ST. DEV.	5.51	6.94	43.71	6.54	28.71	23.65	40.31	22.12
		KW = 2.059 p = 0.5604; N.S.				**KW = 0.3293; p = 0.9544; N.S.**			
ALKP	MEAN	110.40	92.60	103.80	106.30	80	71.2	79.9	64.1
U/L	ST. DEV.	32.02	21.39	25.99	25.57	17.39	29.45	23.88	17.67
		KW = 2.931 p = 0.4024; N.S.				**KW = 5.532; p = 0.1367; N.S.**			
ALB	MEAN	4.27	4.36	4.30	4.42	4.28	4.45	4.49	4.70
g/dl	ST. DEV.	0.16	0.19	0.22	0.06	0.99	0.46	0.24	0.31
		F(36.3) = 1.117 p = 0.3566; N.S.				**KW = 3.196 p = 0.3624; N.S.**			
TOT PROT	MEAN	5.91	6.23	6.02	6.00	6.07	6.02	6.05	6.15
g/dl	ST. DEV.	0.37	0.45	0.42	0.39	0.34	0.53	0.33	0.37
		KW = 3.742 p = 0.2907; N.S.				**F(36.3) = 0.1923 p = 0.9009; N.S.**			
Na^+	MEAN	138.20	136.70	137.20	138.40	137.80	136.60	136.40	137.10
mmol/L	ST. DEV.	3.61	5.19	3.43	3.72	3.43	3.10	2.55	4.53
		KW = 0.9696 p = 0.8086; N.S.				**KW = 0.3218 p = 0.8095; N.S.**			
K^+	MEAN	8.33	11.37	10.59	8.78	9.01	10.02	9.82	9.94
mmol/L	ST. DEV.	2.65	1.87	2.34	2.72	2.91	1.81	1.56	2.55
		KW = 6.246 p = 0.1002; N.S.				**KW = 1.339 p = 0.7199; N.S.**			
Ca^{++}	MEAN	11.01	10.43	11.57	11.74	11.5	9.81 *	10.23	10.57
mg/dL	ST. DEV.	1.31	0.68	2.02	1.19	1.83	1.10	1.34	1.15
		KW = 6.676 p = 0.0830; N.S.				**F(36.3) = 2.689; p = 0.0608**			
T3	MEAN	90.50	111.18**	94.90	99.83	91.74	102.98	103.63	103.84
mcg/dL	ST. DEV.	8.25	24.11	6.33	9.42	11.48	8.51	7.58	11.56
		KW = 13.15 p = 0.0043				**F = 3.500 p = 0.0254; N.S.**			
T4	MEAN	5.68	7.09**	6.48	6.84*	4.20	4.82	5.17	4.34
mcg/dL	ST. DEV.	1.00	0.89	0.96	0.77	0.99	0.86	0.75	0.74
		F(36.3) = 4.598 p = 0.0080;				**KW = 7.272 p = 0.0637;N.S.**			
TSH	MEAN	2.39	1.56	1.35	1.79	1.22	1.35	1.00	1.33
mcg/mL	ST. DEV.	1.21	0.80	0.76	1.24	0.76	0.66	0.57	0.42
		F(36.3) = 0.1916 p = 0.1445; N.S				**F(36.3) = 0.6496 p = 0.5885; N.S**			

GLUC: glucose; CREAT: creatinine; Bili-T: Bilirubin. total; CHOL: cholesterol total; HDL: high-density lipoprotein LDL: low-density lipoprotein; TRIGL: triglycerides; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALKP: alkaline phosphatase; ALB: albumin; TOT PROT: protein. total; Na^+: sodium; K^+: potassium; Ca++: calcium; T3: Triiodothyronine; T4: thyroxine, TSH: thyroid stimulating hormone.
Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA-Tukey test (F values). The significance levels observed are *p < 0.05 and **p < 0.01 in comparison to control group values. N.S. not significant.

Table 5
Organ weight of Sprague Dawley male and female rats fed with different doses of PTS for 90-day. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values). N.S. Not significant.

ORGAN WEIGHT DATA SUMMARY										
MALE						FEMALE				
PARAMETERS		Group 1 (0 mg/kg/day) N = 10	Group 2 (14 mg/kg/day) N = 10	Group 3 (28 mg/kg/day) N = 10	Group 4 (55 mg/kg/day) N = 10	PARAMETERS		Group 1 (0 mg/kg/day) N = 10	Group 2 (14 mg/kg/day) N = 10	Group 3 (28 mg/kg/day) N = 10	Group 4 (55 mg/kg/day) N = 10
BODY W. (g)	**MEAN**	629.80	596.90	620.80	640.20	**BODY W. (g)**	**MEAN**	331.40	437.55	433.02	323.80
	ST. DEV.	48.97	57.65	35.70	31.39		**ST. DEV.**	23.11	65.26	63.76	17.09
		F(36.3) = 1.710 p = 0.1823; N.S.						**F(36.3) = 1.026 p = 0.3926; N.S.**			
BRAIN (g)	**MEAN**	2.34	2.27	2.27	2.29	**BRAIN (g)**	**MEAN**	2.08	2.05	2.09	2.05
	ST. DEV.	0.18	0.10	0.15	0.12		**ST. DEV.**	0.08	0.12	0.07	0.09
		F(36.3) = 0.5894 p = 0.6259; N.S.						**F(36.3) = 0.5587 p = 0.6457; N.S.**			
LIVER (g)	**MEAN**	16.87	15.44	16.67	17.42	**LIVER (g)**	**MEAN**	8.45	8.93	9.05	8.63
	ST. DEV.	1.96	2.28	1.67	1.51		**ST. DEV.**	0.78	1.33	1.36	1.08
		F(36.3) = 1.977 p = 0.1349; N.S.						**F(36.3) = 0.5676 p = 0.6399; N.S.**			
HEART (g)	**MEAN**	1.93	1.78	2.01	1.95	**HEART (g)**	**MEAN**	1.14	1.11	1.15	1.13
	ST. DEV.	0.23	0.23	0.26	0.17		**ST. DEV.**	0.13	0.11	0.11	0.07
		F(36.3) = 1.930 p = 0.1421; N.S.						**F(36.3) = 0.3177 p = 0.8125; N.S.**			
SPLEEN (g)	**MEAN**	1.25	1.12	1.26	1.21	**SPLEEN (g)**	**MEAN**	0.77	0.80	0.77	0.80
	ST. DEV.	0.13	0.11	0.16	0.14		**ST. DEV.**	0.10	0.09	0.07	0.11
		F(36.3) = 2.089 p = 0.1188; N.S.						**F(36.3) = 0.3384 p = 0.7976; N.S.**			
KIDNEYS (g)	**MEAN**	3.79	3.74	3.97	4.05	**KIDNEYS (g)**	**MEAN**	2.03	2.15	2.13	2.06
	ST. DEV.	0.36	0.48	0.43	0.29		**ST. DEV.**	0.20	0.21	0.26	0.13
		F(36.3) = 1.514 p = 0.2285; N.S.						**F(36.3) = 0.7733 p = 0.5390; N.S.**			
THYMUS (g)	**MEAN**	0.94	0.90	0.86	1.02	**THYMUS (g)**	**MEAN**	0.76	0.62	0.78	0.68
	ST. DEV.	0.26	0.21	0.25	0.37		**ST. DEV.**	0.17	0.09	0.08	0.17
		KW = 1.209 p = 0.7509; N.S.						**F(36.3) = 3.077 p = 0.0397; N.S.**			
TESTES (g)	**MEAN**	3.97	3.84	3.97	4.10	**UTE./CERV. (g)**	**MEAN**	0.79	0.76	0.80	0.68
	ST. DEV.	0.30	0.24	0.34	0.36		**ST. DEV.**	0.21	0.26	0.31	0.12
		F(36.3) = 1.147 p = 0.3432; N.S.						**KW = 1.253 p = 0.7404; N.S.**			
EPIDIDIMS (g)	**MEAN**	2.16	2.21	2.16	2.19	**OVARIES (g)**	**MEAN**	0.23	0.23	0.24	0.21
	ST. DEV.	0.43	0.38	0.38	0.39		**ST. DEV.**	0.04	0.05	0.07	0.05
		F(36.3) = 0.0378 p = 0.9900; N.S.						**F(36.3) = 0.8214 p = 0.4906; N.S**			
ADRENALS (g)	**MEAN**	0.09	0.08	0.09	0.09	**ADRENALS (g)**	**MEAN**	0.09	0.11	0.12	0.09
	ST. DEV.	0.03	0.02	0.03	0.03		**ST. DEV.**	0.02	0.03	0.04	0.02
		KW = 0.9271 p = 0.8189; N.S.						**F(36.3) = 1.450 p = 0.2445; N.S.**			
SEMINAL GLANDS (g)	**MEAN**	3.76	3.36	3.73	3.70						
	ST. DEV.	0.34	0.56	0.37	0.52						
		F(36.3) = 1.646 p = 0.1959; N.S.									

Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA-Tukey test (F values). N.S. not significant.

included the latest additional parameters needed in this kind of studies. In the present study, the modified 90-day study has been conducted, which includes the measurement of thyroid hormones (T3, T4 and TSH), as indicators of endocrine-related effects (EFSA, 2012). In our study, the hormone T3 significantly increased in the group of male rats exposed to 14 mg/kg b.w./day This increase from 90.50 ± 8.25 ng/dl in the control group to 111.18 ± 24.11 ng/dl in the treated group had no biological significance since the values for the hormone T3 in control Sprague-Dawley rats ranges from 80 to 100 ng/dl (Walker et al., 1980) and even higher up to 131 ng/dl (Waner and Nyska, 1988). Likewise, the statistical differences observed in the hormone T4 are not considered toxicologically relevant since differences were only found in one sex and it did not follow a dose-dependent pattern.

In the histopathological study, no discernible alteration was observed in any of the organs studied including the gastrointestinal, respiratory, lymphoid, urinary, circulatory, nervous, musculoskeletal, and reproductive systems. Similarly, Cascajosa-Lira et al. (2020b) did not report histopathological damage in rats exposed to 55 mg/kg b.w/day of PTSO for 90 days. However, in the acute study carry out in the present work, damage was observed in two of the rats exposed to 550 mg/kg b.w. of PTS, alteration was visible in stomach, cecum, and spleen (data not shown). No clinical signs evidencing damage were observed in rats exposed to 175 mg/kg b.w PTS. Hence, this dose was established as the MTD. Similarly, rats orally exposed to 175 mg/kg b.w., of PTSO

Table 6
Relative organ weight/body weight of Sprague Dawley male and female rats fed with different doses of PTS in the diet for 90-day. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values). N.S. Not significant.

ORGAN WEIGHT/BODY WEIGHT RATIO DATA SUMMARY											
MALE						FEMALE					
PARAMETERS		**Group 1**	**Group 2**	**Group 3**	**Group 4**	**PARAMETERS**		**Group 1**	**Group 2**	**Group 3**	**Group 4**
		(0 mg/kg/day)	**(14 mg/kg/day)**	**(28 mg/kg/day)**	**(55 mg/kg/day)**			**(0 mg/kg/day)**	**(14 mg/kg/day)**	**(28 mg/kg/day)**	**(55 mg/kg/day)**
		N = 10	**N = 10**	**N = 10**	**N = 10**			**N = 10**	**N = 10**	**N = 10**	**N = 10**
BRAIN (%)	**MEAN**	0.37	0.39	0.37	0.36	**BRAIN (%)**	**MEAN**	0.63	0.62	0.062	0.63
	ST. DEV.	0.02	0.06	0.04	0.01		**ST. DEV.**	0.05	0.05	0.05	0.03
		F(36.3) = 1.038 p = 0.3875; N.S.						**F(36.3) = 0.3136 p = 0.8154; N.S.**			
LIVER (%)	**MEAN**	2.68	2.58	2.68	2.73	**LIVER (%)**	**MEAN**	2.55	2.69	2.66	2.67
	ST. DEV.	0.23	0.25	0.17	0.28		**ST. DEV.**	0.20	0.41	0.35	0.31
		F(36.3) = 0.6541 p = 0.5856; N.S.						**KW = 0.9906 p = 0.8035; N.S.**			
HEART (%)	**MEAN**	0.31	0.30	0.32	0.30	**HEART (%)**	**MEAN**	0.34	0.33	0.34	0.35
	ST. DEV.	0.04	0.02	0.04	0.03		**ST. DEV.**	0.03	0.04	0.03	0.02
		F(36.3) = 1.042 p = 0.3856; N.S.						**F(36.3) = 0.5525 p = 0.6498; N.S.**			
SPLEEN (%)	**MEAN**	0.20	0.19	0.20	0.019	**SPLEEN (%)**	**MEAN**	0.23	0.24	0.23	0.25
	ST. DEV.	0.01	0.01	0.03	0.02		**ST. DEV.**	0.02	0.02	0.02	0.03
		F(36.3) = 1.458 p = 0.2424; N.S.						**F(36.3) = 1.372 p = 0.2669; N.S.**			
KIDNEYS (%)	**MEAN**	0.60	0.61	0.64	0.63	**KIDNEYS (%)**	**MEAN**	0.61	0.65	0.63	0.64
	ST. DEV.	0.06	0.06	0.05	0.05		**ST. DEV.**	0.06	0.06	0.06	0.05
		F(36.3) = 0.9699 p = 0.4183; N.S.						**F(36.3) = 0.6669 p = 0.5779; N.S.**			
THYMUS (%)	**MEAN**	0.15	0.15	0.14	0.16	**THYMUS (%)**	**MEAN**	0.23	0.19	0.23	0.21
	ST. DEV.	0.03	0.03	0.04	0.06		**ST. DEV.**	0.05	0.03	0.03	0.05
		F(36.3) = 0.5200 p = 0.5712; N.S.						**F(36.3) = 2.765 p = 0.0559; N.S.**			
TESTES (%)	**MEAN**	0.63	0.65	0.64	0.64	**UTE./CERV. (%)**	**MEAN**	0.24	0.23	0.24	0.21
	ST. DEV.	0.06	0.06	0.05	0.03		**ST. DEV.**	0.07	0.07	0.10	0.04
		F(36.3) = 0.1471 p = 0.9309; N.S.						**KW = 0.6537 p = 0.8840; N.S.**			
EPIDIDIMS (%)	**MEAN**	0.34	0.37	0.35	0.34	**OVARIES (%)**	**MEAN**	0.07	0.07	0.07	0.06
	ST. DEV.	0.07	0.06	0.06	0.06		**ST. DEV.**	0.01	0.01	0.02	0.02
		F(36.3) = 0.4606 p = 0.7115; N.S.						**F(36.3) = 0.4182 p = 0.7410; N.S.**			
ADRENALS (%)	**MEAN**	0.01	0.01	0.01	0.01	**ADRENALS (%)**	**MEAN**	0.03	0.03	0.03	0.03
	ST. DEV.	0.00	0.00	0.00	0.00		**ST. DEV.**	0.01	0.01	0.01	0.01
		KW = 0.4327 p = 0.9334; N.S.						**F(36.3) = 0.9642 p = 0.4202; N.S.**			
SEMINAL GLANDS (%)	**MEAN**	0.60	0.56	0.60	0.58						
	ST. DEV.	0.04	0.07	0.06	0.07						
		F(36.3) = 0.9367 p = 0.4330; N.S.									

Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA-Tukey test (F values). N.S. not significant.

damage in the hepatic and intestinal mucosal epithelium was observed (Cascajosa-Lira et al., 2020b).

As previously mentioned, the guidance for submission of food additive evaluations published by EFSA (EFSA, 2012) describes a tiered approach, within the first tier includes a subchronic toxicity study. In addition to this, an *in vitro* genotoxicity study is also required. Previous *in vitro* studies carried out in our laboratory have evidenced no mutagenicity of PTS measured by Ames test and mouse lymphoma assay (MLA), although genotoxic effects were recorded in the micronucleus test (MN) at 17.25 μM without S9 and from 20 μM in presence of S9. Moreover, the comet assay detected DNA breaks damage in Caco-2 cells induced by PTS at 280 μM (Mellado-García et al., 2017). These contradictory *in vitro* results justified the need to perform a combined *in vivo* assay of comet assay and MN for PTS, and we have demonstrated that PTS was not genotoxic at oral doses assayed up to 55 mg/kg b.w. (Cascajosa-Lira et al., 2021b).

In vivo research on OSCs is very scarce, and in the case of PTS very few studies are available, making it difficult to compare the toxicity results obtained. Recently, an acute toxicity study of mice intraperitoneally exposed to a single dose of diallyl sulfide (DAS) reported that a dose of 1280 mg/kg had no toxic effect (Dutta et al., 2021). Shipkowski et al. (2021) stablished a NOAEL of 300 mg/kg for male and female guinea pigs administered sulfolane for 28 days. Both experiments obtained higher toxicity values than the given in the present study. However, it should be noted that they are different OSCs and experimental animals; moreover, lower exposure time were used (1 day and 28 days). Martínez-Fernandez et al. (2013, 2015) exposed goats to α-cyclodextrin-PTS complex in order to assess its potential use as antimethanogenic additive in feed. Positive but limited antimethanogenic effect was described, and additionally, no adverse effect was observed in

Table 7
Relative organ weight/brain weight of Sprague Dawley male and female rats fed with different doses of PTS in the diet for 90-day. Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA test (F values). N. S. Not significant.

ORGAN WEIGHT/BRAIN WEIGHT RATIO DATA SUMMARY											
MALE						FEMALE					
PARAMETERS		**Group 1**	**Group 2**	**Group 3**	**Group 4**	**PARAMETERS**		**Group 1**	**Group 2**	**Group 3**	**Group 4**
		(0 mg/kg/day)	**(14 mg/kg/day)**	**(28 mg/kg/day)**	**(55 mg/kg/day)**			**(0 mg/kg/day)**	**(14 mg/kg/day)**	**(28 mg/kg/day)**	**(55 mg/kg/day)**
		N = 10	**N = 10**	**N = 10**	**N = 10**			**N = 10**	**N = 10**	**N = 10**	**N = 10**
LIVER (%)	**MEAN**	722.90	684.98	740.26	764.88	**LIVER (%)**	**MEAN**	408.45	437.55	433.02	421.74
	ST. DEV.	97.13	128.98	125.93	82.98		**ST. DEV.**	47.46	65.62	63.76	55.61
		KW = 3.691 p = 0.2969; N.S.						**KW = 0.9906 p = 0.8035; N.S.**			
HEART (%)	**MEAN**	82.66	78.55	88.35	85.41	**HEART (%)**	**MEAN**	55.09	55.35	54.99	54.24
	ST. DEV.	12.06	11.78	8.79	8.53		**ST. DEV.**	5.11	4.10	6.34	5.99
		F(36.3) = 1.602 p = 0.2059; N.S.						**F(36.3) = 0.5525 p = 0.6498; N.S.**			
SPLEEN (%)	**MEAN**	53.08	49.41	55.78	53.05	**SPLEEN (%)**	**MEAN**	37.29	39.34	36.89	39.08
	ST. DEV.	3.37	6.78	11.37	7.32		**ST. DEV.**	5.16	4.57	3.08	5.43
		F(36.3) = 1.166 p = 0.3361; N.S.						**F(36.3) = 1.372 p = 0.2669; N.S.**			
KIDNEYS (%)	**MEAN**	162.37	167.41	175.56	177.37	**KIDNEYS (%)**	**MEAN**	98.09	105.41	101.94	100.73
	ST. DEV.	19.94	20.05	25.55	14.34		**ST. DEV.**	10.74	12.96	13.28	8.74
		F(36.3) = 3.404 p = 0.3334; N.S.						**F(36.3) = 0.6669 p = 0.5779; N.S.**			
THYMUS (%)	**MEAN**	40.03	40.00	38.53	45.16	**THYMUS (%)**	**MEAN**	36.88	30.46	37.33	32.97
	ST. DEV.	9.93	9.60	13.34	17.12		**ST. DEV.**	8.80	4.59	3.96	7.42
		KW = 0.7873 p = 0.8525; N.S.						**F(36.3) = 2.765 p = 0.0559; N.S.**			
TESTES (%)	**MEAN**	170.21	169.64	175.61	179.48	**UTE./CERV. (%)**	**MEAN**	38.20	37.57	38.36	33.43
	ST. DEV.	20.37	15.00	21.28	13.20		**ST. DEV.**	9.99	12.95	14.46	5.90
		F(36.3) = 0.6920 p = 0.5629; N.S.						**KW = 0.6537 p = 0.8840; N.S.**			
EPIDIDIMS (%)	**MEAN**	92.03	97.15	95.02	95.44	**OVARIES (%)**	**MEAN**	11.71	10.11	10.99	11.37
	ST. DEV.	16.62	15.38	15.28	14.88		**ST. DEV.**	3.36	2.78	2.09	2.72
		F(36.3) = 0.1873 p = 0.9044; N.S.						**F(36.3) = 0.4182 p = 0.7410; N.S.**			
ADRENALS (%)	**MEAN**	3.85	3.67	3.85	3.89	**ADRENALS (%)**	**MEAN**	5.54	4.53	4.52	5.21
	ST. DEV.	0.90	1.15	1.17	1.28		**ST. DEV.**	1.97	0.88	0.84	1.46
		KW = 0.5298 p = 0.9123; N.S.						**KW = 0.9642 p = 0.4202; N.S.**			
SEMINAL GLANDS (%)	**MEAN**	160.88	148.65	165.09	161.96						
	ST. DEV.	12.76	26.18	23.55	22.18						
		F(36.3) = 1.102 p = 0.3609; N.S.									

Values are mean ± SD for 10 rats/sex/group. The differences between control and treated groups for male and female rats were evaluated by Kruskal-Wallis test (K.W.) or by ANOVA-Tukey test (F values). N.S. not significant.

any of the animals treated with PTS. Also, the use of an *Allium* extract (Garlicon®, DOMCA, Spain) containing PTS and PTSO as phytobiotics supplemented in diet of laying hens showed improvements such as increases in egg production and size, enhancing also the beneficial bacterial content in intestine (Rabelo-Ruiz et al., 2021a). Similarly, supplementation with the same *Allium* extract (Garlicon®) in the diet of weaning piglets, improved productive parameters such as body weight, average daily gain, or feed conversion ratio levels with respect to control diet by modulating gut microbiota (Rabelo-Ruiz et al., 2021b). However, none of these works carried out *in vivo* toxicity studies. Other OSC, DAS, has evidenced effects such as hepatotoxicity in rats induced by 200 mg of DAS (Davenport and Wargovich, 2005). However, most of the studies based on OSCs reported beneficial effects such as antioxidant activity (Wu et al., 2019) and antimutagenicity (Guyonnet et al., 2000). In addition to the scarcity of toxicity studies of OSCs, it is very challenging to establish a clear relationship between the structure and bioactivity of OSCs because of their diverse and complex structure. Furthermore, these compounds are prone to enzymatic modifications and therefore they may undergo degradation processes (Putnik et al., 2019).

In order to perform an accurate risk assessment based on the NOAEL obtained in the present study, the use of PTS as well as the content in the food should be considered. The most promising use of PTS is as additive in food and feed (Guillamón et al., 2021). In the case of its use as feed additive, no residue of PTS has been found in meat of animal consuming feed containing PTS. However, in the use as food additive, for instance as dressings such as garlic sauce, the content of PTS could reach 30 mg/kg. In the inquiry into Spanish consumers (AEC, 2021), the percentile 95 consumes 88.88 g/day of dressing. In this case, a person weighting 70 kg would ingests 0.038 mg/kg b.w./day PTS, this is around 1450 times lower that the NOAEL (55 mg/kg b.w./day). Therefore, even in the worst scenario of high consumers, the use of PTS as additive in food is expected to be safe regarding the present subchronic study.

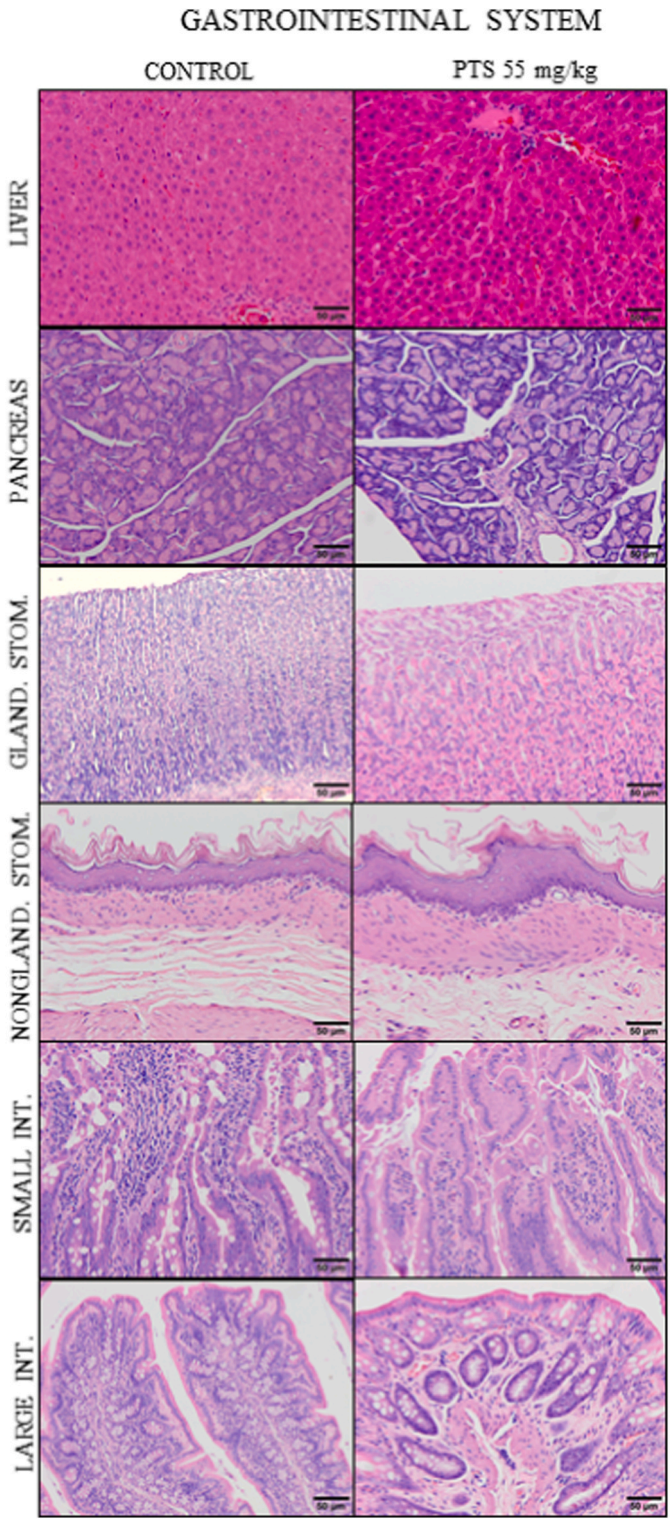

Fig. 6. Representative histological images of the gastrointestinal organs comparing both control and 55 mg/kg b.w./day PTS rats for 90 days. H&E stain.

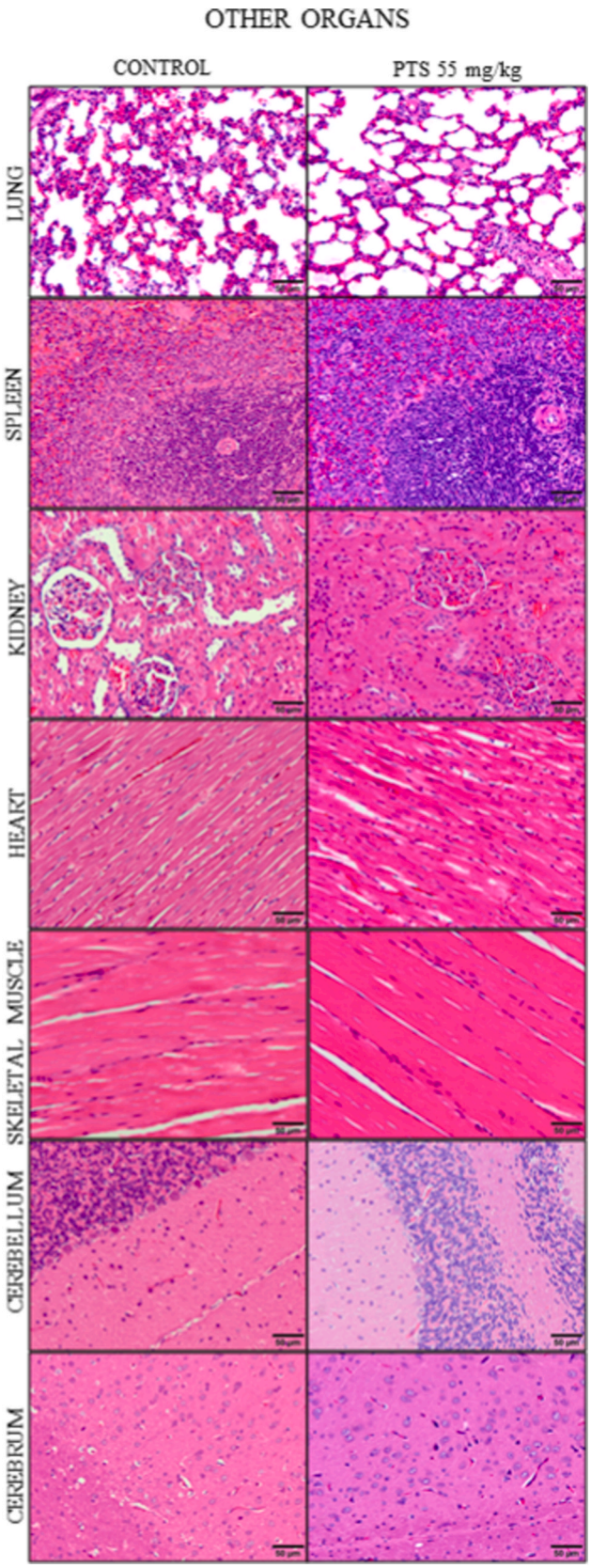

Fig. 7. Representative histological images of organs from respiratory, lymphoid, renal, circulatory, musculoskeletal, and nervous systems comparing both control and 55 mg/kg b.w./day PTS rats. H&E stain.

5. Conclusions

Administration of PTS to male and female rats in the diet for 90-days, following the OECD 408 guideline, caused no significant changes in any hematological and biochemical parameters measured. Moreover, no differences in body weight and water/food consumption, as well as none histopathological abnormalities were recorded. Therefore, the NOAEL

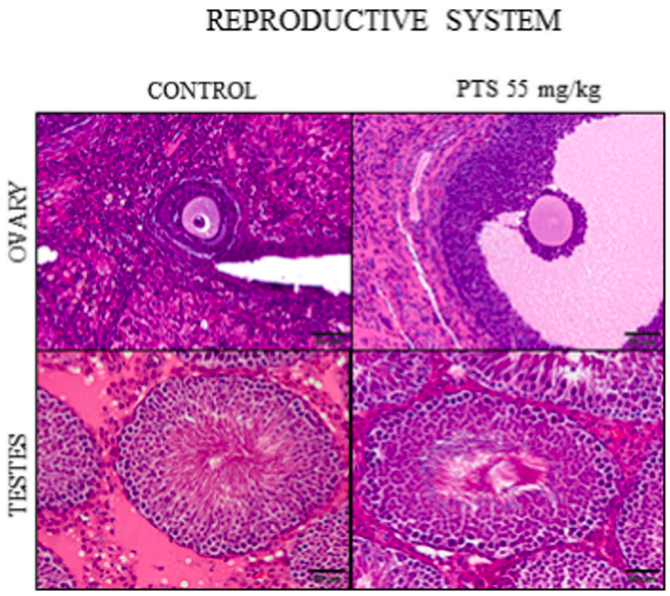

Fig. 8. Representative histological images of reproductive system comparing both control and 55 mg/kg b.w./day PTS rats. H&E stain.

was set≥55 mg/kg b.w./day PTS in both sexes.

CRediT authorship contribution statement

Antonio Cascajosa-Lira: Investigation, Formal analysis, Writing – original draft. **Silvia Pichardo:** Writing – original draft, Supervision, Project administration. **Alberto Baños:** Resources, Writing – review & editing. **Enrique Guillamón:** Resources. **Verónica Molina-Hernández:** Formal analysis, Writing – original draft. **Rosario Moyano:** Supervision. **Ángeles Jos:** Writing – review & editing. **Ana M Cameán:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Acknowledgements

The authors would like to thank the Spanish Ministerio de Ciencia e Innovación (Project RTC-2017-6199-2), and Junta de Andalucía (Projects AT 2017–5323 and P18-TP-2147) for its financial support. Antonio Cascajosa Lira thanks the Spanish Ministerio de Universidades for the funding FPU grant (FPU2019-01247).

References

EFSA, 2016. ANS panel (EFSA panel on food additives and nutrient sources added to foods). Scientific opinion on the Re-evaluation of sulfur dioxide (E 220), sodium sulfite (E 221), sodium bisulfite (E 222), sodium metabisulfite (E 223), potassium metabisulfite (E 224), calcium sulfite (E 226), calcium bisulfite (E 227) and potassium bisulfite. EFSA J. 14.

AEC, 2021. Agencia Española de Consumo, Segruidad Alimentaria y Nutrición. Encuesta ENALIA 2. Encuesta Nacional de Alimentación en población adulta, mayores y embarazadas. AECOSAN [web]. Online available: https://www.aesan.gob.es/AECOSAN/web/seguridad_alimentaria/subdetalle/enalia.htm.

An, M.J., Lee, J.S., Park, Y.C., Park, C.J., Kim, H.J., 2019. 90-Day repeated oral toxicity test of D-allulose produced from Microbacterium foliorum. Regul. Toxicol. Pharmacol. 109, 104485. https://doi.org/10.1016/j.yrtph.2019.104485.

Baños Arjona, A., Sanz, A., Brotman, K.A., 2016. Use of propyl propanethosulfinate and propyl propanethosulfonate for the prevention and reduction of parasites in aquatic animals. United States Patent. US 9.271,947 B2 2.

Bravo, D., Lillehoj, H., 2013. Use of at Least One Dialkyl Thosulfonate or Thosulfinate for Reducing the Number of Apcomplexan an Animal. United States. Patent Application Publication. US 2013/00794.02 A1 1.

Cascajosa-Lira, A., Prieto, A.I., Puerto, M., Baños, A., Valdivia, E., Jos, A., 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., Puerto, M., 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, 111612. https://doi.org/10.1016/j.fct.2020.111612.

Cascajosa-Lira, A., Prieto Ortega, A.I., Guzmán-Guillén, R., Cătunescu, G.M., de la Torre, J.M., Guillamón, E., , Jos, Á., Cameán Fernández, A.M., 2021a. Simultaneous determination of Allium compounds (Propyl propane thiosulfonate and thiosulfinate) in animal feed using UPLC-MS/MS. Food Chem. Toxicol. 157, 112619. https://doi.org/10.1016/j.fct.2021.112619.

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., 2021b. Genotoxicity evaluation of propyl-propane-thiosulfinate (Pts) from allium genus essential oils by a combination of micronucleus and comet assays in rats. Foods 10. https://doi.org/10.3390/foods10050989.

Davenport, D.M., Wargovich, M.J., 2005. Modulation of cytochrome P450 enzymes by organosulfur compounds from garlic. Food Chem. Toxicol. 43, 1753–1762. https://doi.org/10.1016/j.fct.2005.05.018.

Dutta, A., Dahiya, A., Prakash, A., Agrawala, P.K., 2021. Acute toxicity of diallyl sulfide derived from Allium sativum (garlic) in mice and its possible mechanisms. Phytomedicine 1 (3), 100084. https://doi.org/10.1016/j.phyplu.2021.100084.

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2016. Scientific opinion on recent developments in the risk assessment of chemicals in food and their potential impact on the safety assessment of substances used in food contact materials, 2016b. EFSA J. 14 (1), 4357, 28 pp, https://doi.org/10.2903/j.efsa.2016.4357.

EFSA Scientific Committee, 2012. Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data. EFSA J. 10 (3), 2579. https://doi.org/10.2903/j.efsa.2012.2579 [32 pp.].

Falcón-Piñeiro, A., Remesal, E., Noguera, M., Ariza, J.J., Guillamón, E., Baños, A., Navas-Cortes, J.A., 2021. Antifungal activity of propyl-propane-thiosulfinate (PTS) and propyl-propane-thiosulfonate (PTSO) from allium cepa against verticillium dahliae: in vitro and in planta assays. J. Fungi 7. https://doi.org/10.3390/jof7090736.

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.

Ghyasi, R., Mohaddes, G., Naderi, R., 2019. Combination effect of voluntary exercise and garlic (Allium sativum) on oxidative stress, cholesterol level and histopathology of heart tissue in type 1 diabetic rats. J. Cardiovasc. Thorac. Res. 11, 61–67. https://doi.org/10.15171/jcvtr.2019.10.

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. https://doi.org/10.3390/foods10081680.

Guyonnet, D., Belloir, C., Suschetet, M., Siess, M.H., Le Bon, A.M., 2000. Liver subcellular fractions from rats treated by organosulfur compounds from Allium modulate mutagen activation. Mutat. Res. Genet. Toxicol. Environ. Mutagen 466, 17–26. https://doi.org/10.1016/S1383-5718(99)00234-X.

Liu, L., Yeh, Y.-Y., 2000. Inhibition of cholesterol biosynthesis by garlic compounds. Lipids 35, 197–203.

Liu, L., Yeh, Y.Y., 2001. Water-soluble organosulfur compounds of garlic inhibit fatty acid and triglyceride syntheses in cultured rat hepatocytes. Lipids 36, 395–400. https://doi.org/10.1007/s11745-001-0734-4.

Llana-Ruiz-Cabello, M., Pichardo, S., Maisanaba, S., Puerto, M., Prieto, A.I., Gutiérrez-Praena, D., Jos, A., Cameán, A.M., 2015. 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., Maisanaba, S., Puerto, M., Pichardo, S., Jos, A., Moyano, R., Cameán, A.M., 2017. A subchronic 90-day oral toxicity study of Origanum vulgare essential oil in rats. Food Chem. Toxicol. 101, 36–47. https://doi.org/10.1016/j.fct.2017.01.001.

Maisanaba, S., Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Pichardo, S., Puerto, 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.

Martínez-Fernández, G., Abecia, 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.

Martínez-Fernández, G., Abecia, 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 (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 91, 1–11. https://doi.org/10.1093/femsec/fiv079.

Matsuzawa, T., Nomura, M., Unno, T., 1986. Clinical pathology reference ranges of laboratory animals. Chem. Pharm. Bull. 34, 430–433.

Mellado-García, P., Puerto, M., Pichardo, S., Llana-Ruiz-Cabello, M., Moyano, R., Blanco, A., Jos, A., Cameán, A.M., 2016. Toxicological evaluation of an Allium-based

commercial product in a 90-day feeding study in Sprague-Dawley rats. Food Chem. Toxicol. 90, 18–29. https://doi.org/10.1016/j.fct.2016.01.019.
Mellado-García, P., Maisanaba, S., Puerto, M., Prieto, A.I., Marcos, R., Pichardo, S., Cameán, A.M., 2017. In vitro toxicological assessment of an organosulfur compound from Allium extract: cytotoxicity, mutagenicity and genotoxicity studies. Food Chem. Toxicol. 99, 231–240. https://doi.org/10.1016/j.fct.2016.12.007.
Mishra, A.K., Mishra, A., Pragya, Chattopadhyay, P., 2018. Screening of acute and sub-chronic dermal toxicity of Calendula officinalis L essential oil. Regul. Toxicol. Pharmacol. 98, 184–189. https://doi.org/10.1016/j.yrtph.2018.07.027.
OECD, 2008. Test No. 425: Acute Oral Toxicity: Up-And-Down Procedure, OECD Guidelines for the Testing of Chemicals, Section, vol. 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264071049-en.
OECD, 2018. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents, OECD Guidelines for the Testing of Chemicals, Section, vol. 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264070707-en.
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.
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.
Preece, K., Glávits, R., Foster, J.R., Murbach, T., Endres, J.R., Hirka, G., Vértesi, A., Béres, E., Szakonyiné, I.P., 2021. A toxicological evaluation of geranylgeraniol. Regul. Toxicol. Pharmacol. 124, 104975. https://doi.org/10.1016/j.yrtph.2021.104975.
Putnik, P., Gabrić, D., Roohinejad, S., Barba, F.J., Granato, D., Mallikarjunan, K., Lorenzo, J.M., Bursać 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., 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., 2021a. Allium-based phytobiotic enhances egg production in laying hens through microbial composition changes in ileum and cecum. Animals 11, 1–15. https://doi.org/10.3390/ani11020448.
Rabelo-Ruiz, M., Teso-Pérez, C., Peralta-Sánchez, J.M., Ariza, J.J., Martín-Platero, A.M., , Casabuena-Rincón, Ó., Vázquez-Chas, P., Guillamón, E., Aguinaga-Casañas, M.A., Maqueda, M., Valdivia, E., Baños, A., Martínez-Bueno, M., 2021b. Allium extract implements weaned piglet's productive parameters by modulating distal gut microbiota. Antibiotics 10, 1–15. https://doi.org/10.3390/antibiotics10030269.
EFSA FEEDAP Panel (EFSA Panel on Products or Substances used in Animal Feed), Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M.L., Bories, G., Chesson, A., Cocconcelli, P.S., Flachowsky, G., Gropp, J., Kolar, B., Kouba, M., López-Alonso, M., López Puente, S., Mantovani, A., Mayo, B., Ramos, F., Saarela, M., Villa, R.E., Wallace, R.J., Wester, P., Anguita, M., Dujardin, B., Galobart, J., Innocenti, M.L., 2017. Guidance on the assessment of the safety of feed additives for the consumer. EFSA J. 15 (10), 5022, 17.
Shipkowski, K.A., Cora, M.C., Cesta, M.F., Robinson, V.G., Waidyanatha, S., Witt, K.L., Vallant, M.K., Fallacara, D.M., Hejtmancik, M.R., Masten, S.A., Cooper, S.D., Fernando, R.A., Blystone, C.R., 2021. Comparison of sulfolane effects in Sprague Dawley rats, B6C3F1/N mice, and Hartley Guinea pigs after 28 days of exposure via oral gavage. Toxicol. Rep. 8, 581–591. https://doi.org/10.1016/j.toxrep.2021.02.004Singh, M. Thompson, M., Sullivan, N., Child, G., 2005. Thiamine deficiency in dogs due to the feeding of sulphite preserved meat. Australian Veterinary Journal 83, 412–417. https://doi.org/10.1111/j.1751-0813.2005.tb13078.x.
Sun, Y.E., Wang, W., Qin, J., 2018. In: Anti-hyperlipidemia of Garlic by Reducing the Level of Total Cholesterol and Low-Density Lipoprotein, vol. 97. Medicine, United States. https://doi.org/10.1097/MD.0000000000010255.
Tajiri, H., Tanaka, W., Takashima, M., Matsuyama, H., Sugita, T., Hidaka, K., Sakakibara, H., 2021. Subchronic safety evaluation of hot-water extract from thinned immature mangos (Mangifera indica 'Irwin'): 90-days oral toxicity study in rats. Toxicol. Rep. 8, 1046–1053. https://doi.org/10.1016/j.toxrep.2021.05.005.
Vezza, T., Algieri, F., Garrido-Mesa, J., Utrilla, M.P., Rodríguez-Cabezas, M.E., Baños, A., Guillamón, E., García, F., Rodríguez-Nogales, A., Gálvez, J., 2019. The immunomodulatory properties of propyl-propane thiosulfonate contribute to its intestinal anti-inflammatory effect in experimental colitis. Mol. Nutr. Food Res. 63, 1–31. https://doi.org/10.1002/mnfr.201800653.
Vezza, T., Garrido-Mesa, J., Diez-Echave, P., Hidalgo-García, L., Ruiz-Malagón, A.J., García, F., Sánchez, M., Toral, M., Romero, M., Duarte, J., Guillamón, E., Baños, A., Morón, R., Gálvez, J., Rodríguez-Nogales, A., Rodríguez-Cabezas, M.E., 2021. Allium-derived compound propyl propane thiosulfonate (Ptso) attenuates metabolic alterations in mice fed a high-fat diet through its anti-inflammatory and prebiotic properties. Nutrients 13. https://doi.org/10.3390/nu13082595.
Walker, P., Dubois, J.D., Dussault, J.H., 1980. Free thyroid hormone concentrations during postnatal development in the rat. Pediatr. Res. 14, 247–249. https://doi.org/10.1203/00006450-198003000-00014.
Waner, T., Nyska, A., 1988. Thyroxine (T4) and triiodothyronine (T3) levels in the Fischer 344 inbred rat. Lab. Anim 22, 276–280. https://doi.org/10.1258/002367788780746458.
Wu, Y., Tang, X., Yang, W., Fan, J., Tang, L., Wang, C., Yu, Z., Jia, X.D., Fan, B., 2019. Subchronic toxicity of cerium nitrate by 90-day oral exposure in wistar rats. Regul. Toxicol. Pharmacol. 108 https://doi.org/10.1016/j.yrtph.2019.104474.