A preliminary study in Wistar rats with enniatin A contaminated feed.

EN A test in vivo

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Abstract

A 28-day repeated dose preliminary assay, using enniatin A naturally contaminated feed through microbial fermentation by a *Fusarium tricinctum* strain, was carried out employing two months-old female Wistar rats as *in vivo* experimental model. In order to simulate a physiological test of a toxic compound naturally produced by fungi, five treated animals were fed during twenty-eight days with fermented feed. As control group, five rats were fed with standard feed. At the 28th day, blood samples were collected for biochemical analysis and the gastrointestinal tract, liver and kidneys were removed from each rat for enniatin A detection and quantitation. Digesta were collected from stomach, duodenum, jejunum, ileum and colon. Enniatin A present in organs and in biological fluids was analyzed by liquid chromatography-diode array detector (LC-DAD) and confirmed by LC-mass spectrometry linear ion trap (MS-LIT); also several serum biochemical parameters and a histological analysis of the duodenal tract were performed. No adverse effect was found in any treated rat at the EN A concentration (20.91 mg/kg bw/day) tested during the 28-day experiment. EN A quantitation in biological fluids ranged from 1.50 to 9.00 mg/kg, whereas in the gastrointestinal organs the EN A concentration ranged from 2.50 to 23.00 mg/kg. The high EN A concentration found in jejunum liquid and tissue points to them as an absorption area. Finally, two EN A degradation products were identified in duodenum, jejunum and colon content, probably produced by gut microflora.

Keywords: Enniatin A, *Fusarium tricinctum*, *in vivo* study, LC-DAD, LC-MS-LIT,

1. Introduction

Enniatins (ENs) are secondary fungal metabolites that have been known for several decades (Ivanova et al., 2006). Chemically there are six-membered cyclic depsipeptides, which are commonly composed of three D-α-hydroxyisovaleric acid (Hiv) residues linked alternatively to three L-configured N-methyl amino acid residues to give an 18-membered cyclic skeleton (Zhukhlistova et al., 1999). ENs are produced by strains of several species of fungal genera as *Alternaria, Fusarium, Halosarpheia and Verticillium* (Supothina et al., 2004). ENs produced by *Fusarium subglutinans*, *Fusarium proliferatum and Fusarium tricinctum* are cereals contaminants, especially maize and its derivatives. ENs have been found as worldwide natural contaminants of several food and feed products (Jestoi, 2008).A few years ago, Meca et al. (2010a) have reported ENs contamination of cereals available in the Spanish market and their levels ranged from 0.51 to 11.78 mg/kg.

ENs possess a wide range of biological activities: these substances are known as ionophores, phytotoxins, anthelmintic and antibiotics compounds (Jestoi, 2008). ENs antibiotic effects have been used in a pharmaceutical commodity with anti-inflammatory properties called fusafungine (Akbas et al., 2004). There are applications for ENs in respiratory tract infections treatment and it has been reported a positive effect on wound healing after tonsillectomy (Akbas et al., 2004). Several studies have indicated that ENs change the monovalent ion transport across membranes and disrupt the ionic selectivity of cell walls. This effect is particularly debilitating in mitochondrial membranes, resulting in the uncoupling of oxidative phosphorylation (Tonshin et al., 2010).

Several studies have evaluated the ENs cytotoxic activity *in vitro* using as experimental model rodent, monkey, porcine, insect and human cell lines (Fornelli et al., 2004; Vongvilai

et al., 2004; Ivanova et al., 2006; Jestoi, 2008; Lee et al., 2008; Behm et al., 2009; Dornetshuber et al., 2009; Hyun et al., 2009; Watjen et al., 2009; Meca et al., 2010b, 2011). In the scientific literature, only few studies related to the ENs toxicity *in vivo* are available. In particular, Bosch et al. (1989) studied the toxicity of ENs, among other mycotoxins, in Fusarium contaminated feed on twenty day old white female Spargue Dawley rats, evidencing no toxic signs. To be sure about which mycotoxins where responsible of the effects, they administrated a mixture of ENNs in single oral dose (0.05 mg/g body weight (bw)). McKee et al. (1997) studied a hypothetic ENs property to reduce the human immunodeficiency virus (HIV) growth using the hollow fiber assay and employing mice as biological model. They used an ENs A1, B and B1 purified mixture (from 1.25 to 40 mg/kg) injected intraperitoneally every 8h during 6 days. Any anti-HIV properties were not found but 40, 20 and 10 mg/kg doses were lethal.

Considering the lack of information in physiological conditions related to the ENs toxicity *in vivo*, the aims of this research were: a) to study the EN A *in vivo* potential toxicity trough a repeated dose assay using standard rat feed contaminated by a microbial fermentation of *Fusarium tricintum* strain; b) to evaluate the EN A presence in several rat organs after a 28-day continuous ingest and c) to identify possible EN A degradation products from gut microflora.

2. Materials and methods

2.1 Chemicals

Acetonitrile, methanol, and ethyl acetate were purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm^{-1} resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water

were degassed for 20 min using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., CT, USA). Potato dextrose broth (PDB) was obtained from Insulab (Valencia, Spain). Phosphate Buffered Saline (PBS), glycerol and ENs A standard solution stock (purity: 99% molecular weight 682.92 g/mol) were purchased from Sigma Aldrich (Madrid, Spain).

2.2. Strain and culture conditions for the ENs production on rat feed

A solid medium represented by the rat feed (Autoclaved Harlan lab blocks, Castellar del Vallés, Spain) was utilized in this study. The medium was prepared weighting 5 kg in two 2.5 L Erlenmeyer flasks and autoclaved at 121ºC during 20 min. Each one was inoculated with 25 ml of a conidia suspension (10^6 conidia/ml sterile water) of *Fusarium tricinctum* CECT 20232 in PDB. Conidial concentration was measured by optical density at 600 nm in sterile water and adjusted to 10^6 conidia/ml PDB as reported Kelly et al. (2006).

F. tricinctum CECT 20232 strain was obtained from the Spanish Type Culture (CECT Valencia, Spain), in sterile 18% glycerol. Fermentations were carried out at 25ºC on an orbital shaker (IKA Ks 260 basic, Stanfen, Germany) in batch culture for 30 days. At the end of fermentations, the solid culture was autoclaved at 121ºC during 20 min to promote fungi inactivation, and after drying and milling, ENs analysis was done.

2.3 ENs extraction from rat feed

A modified method based on Chelkowski et al. (2007) for mycotoxins extraction was performed. Briefly, ENs contained in fifteen grams of dried contaminated feed were extracted with 100 ml methanol–water mixture (75:25) using an Ika T18 basic Ultraturrax (Staufen, Germany) for 5 min. Samples were then filtered through Phenomenex No. 4 filter paper (Torrance, CA, USA) and thereafter the solvent was removed under reduced

pressure. Each extract was dissolved in 5 ml of methanol and filtered through a 0.22 µm filter Phenomenex before toxin identification and quantitation by liquid chromatography (LC)-DAD as reported by Meca et al. (2010a) (see 2.8).

2.4 *In vivo* study design

Ten female Wistar rats (average body weight: 250 g) were acquired from Pharmacy animal facility (Universitat de València, Spain). The Institutional Animal Care and Use Committee of the University of Valencia approved all animal procedures (protocol nº A1338818442265). Animals were divided in two groups: 5 rats in the control group and 5 in the treated one. Each group was housed in one cage in a windowless room with a 12h light-dark cycle. The study rooms were maintained under controlled conditions appropriate for the species (temperature 22ºC, relative humidity 45-65%). After 7 days of adaptation, the control group was fed with the Harlan autoclaved lab box feed, while the test group was fed with EN A contaminated feed (see 2.2). Treatment was maintained for 28 days in order to simulate a preliminary subchronic study to be able to analyze EN A distribution. The body weight of each rat was controlled weekly using a weighing scale. Rats were sacrificed by isoflurane gas asphyxiation and blood samples were collected via cardiac puncture. Blood samples were allowed to clot for 30 min and then the serum layer was separated by centrifugation at 1000 rpm for 30 min at 4ºC. Serum was kept at -20ºC until analysis. The gastrointestinal tract (from stomach to rectum) was removed from all rats and digesta were collected from stomach, duodenum, jejunum, ileum and colon. Digesta collection of the intestinal compartments was carried out flushing the tissues with 1 ml PBS twice. Also the liver, kidneys, heart, thymus and spleen of each animal were recollected after terminal

sacrifice (Figure 1). Each organ collected was weighted for further comparison between the treated and the control animals.

2.5 Histological and biochemical analysis

Histological analyses of duodenum tissue from treated and control rats, focused on enterocytes atrophy determination and on the presence of cells liquid in the gut tissue, were carried out by Echevarne laboratory (Barcelona, Spain). Duodenum tissue samples were fixed in formaldehyde (40% v/v in water), embedded in paraffin, sectioned at 4 µm and stained with haematoxylin and eosin before analysis. Biochemical parameters analyzed in serum were: bile salts, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase, total bilirubin, cholesterol, alkaline phosphatase, gamma-glutamyl transpeptidase and urea through ELISA kit analysis (Echevarne laboratory, Barcelona, Spain).

2.6 EN A surrogate recovery

Each tissue (0.5 g) and digest (0.5 ml) was placed in a 15 ml plastic test tube and fortified with 5 µl of EN A at 1000 ppm. 30 min after spiking, each sample was extracted with 1 ml of ethyl acetate using a vortex VWR international (Barcelona, Spain) for 3 min. Then, mixtures were centrifuged at 4000 rpm and at 4ºC during 15 min (Centrifuge 5810R, Eppendorf, Germany). Organic phases were collected into new tubes. Ethyl acetate addition, vortex, centrifugation and collection steps were repeated three times. The extracts were then evaporated dryness under nitrogen flow at 30ºC and reduced pressure (5 psi), in order to accelerate organic phase evaporation by decreasing the partial vapor pressure of the solvent just above the liquid surface (Turbovap LV, Zymark, Runcorn, UK). Dried samples were resuspended in 1 ml of methanol and filtered with a 0.22 µm filter (Phenomenex, Madrid, Spain) prior to their LC analysis.

2.7 EN A extraction from intestinal fluids

EN A contained in the stomach and intestinal fluids were extracted according to Meca et al. (2012). One milliliter of each intestinal fluid was placed in a 15 ml test tube, and extracted with 2 ml of ethyl acetate using a vortex VWR international (Barcelona, Spain) for 3 min. Following steps were performed as described in section 2.6.

2.8 EN A extraction from tissues

EN A contained in the tissues collected from control and treated rats was extracted as follows: 0.5 g of each tissue was introduced in a 15 ml plastic tube and 2 ml of PBS (1X, pH 7.5) was added. Sample were completely grounded using an Ultraturrax T8 IKA (Staufen, Germany) during 3 min. EN A was extracted from the PBS solution using 4 ml of ethyl acetate employing a vortex (VWR international, Barcelona, Spain) during 3 min. Following steps were performed as described in section 2.6.

2.9 LC-DAD analysis

LC analyses of EN A (Meca et al., 2010a) were performed using LC-10AD pumps and a diode array detector (DAD) (Shimadzu, Japan). A Gemini (150 x 4.6 mm, 5 µm) Phenomenex column was used. LC conditions were set up using a constant flow at 1.0 ml/min of acetonitrile–water (70:30 v/v) as starting eluent system. The starting ratio was kept constant for 5 min and then it was linearly modified to 90% acetonitrile in 10 min. After 1 min the mobile phase was set to the initial conditions in 4 min. All samples were filtered through a 0.22 µm syringe filter Phenomenex prior to injection (20 µL) into the column. EN A was detected at 205 nm. Mycotoxin identification was performed by comparing retention times and UV spectra of samples with those of pure standards. A further confirmation action was performed by co-injecting pure standards together with

each sample. Mycotoxin quantitation was determined by comparing tested samples peak areas with a calibration curve performed with standards (n=4).

2.10 LC-MS-Linear Ion Trap (LIT) confirmation

An applied Biosystems/AB SCIEX QTRAP® linear ion traps mass spectrometer (Concord, Ontario, Canada), coupled to a Turbo Ion Spray source was used. This instrument is based on a triple-quadrupole path (QqQ) in which the third quadrupole can also be operated as a linear ion trap (QqLIT) with improved performance. In the QqLIT configuration the Q TRAP can also operate in enhanced resolution scan (ER) and in enhanced product ion scan (EPI) modes. Applied Biosystem/MDS SCIEX Analyst software version 1.3.2 was used for data acquisition and processing.

A Gemini (150 x 2.0 mm, 5 μm) Phenomenex column was used. LC was set using a constant flow of 0.2 ml/min of acetonitrile/water (70:30 v/v) with 0.1 % of HCOOH isocratically. The instrument was operated in the positive ion electrospray mode using the following parameters: cone voltage 40 V, capillary voltage 3.80 kV, source temperature 350ºC, desolvation temperature 270ºC and collision gas energy 5 eV. EN A identification and quantitation was performed using the modality of ER, utilizing the mass range from 700 to 900 Da. The utilization of the mass spectrometry associated to a linear ion trap permitted to obtain an enhanced characterization of the isolated compounds.

2.11 Mass spectrometry characterization of the EN A degradation products

Characterization of the newly formed compounds was performed as explained in 2.10 using the LC coupled to LIT in ER mode.

2.12 Calculations

Recoveries of fortified tissues and biological fluid samples were calculated as the percentage of the EN A detected amount related to the total EN A spiked in each of them. Recovery studies were performed in triplicate and the spiking levels were 1.0, 5.0 and 10 µg/g.

For the treated rats liquid contents and tissues, the absolute amount of mycotoxins (mg) was calculated by multiplying the measured sample volume or weight by the EN A concentration found.

3. Results and discussion

3.1. Method performance

Mean recovery of fortified tissues and biological fluid samples (n = 3) at 3 levels of EN A (1.0, 5.0 and 10 µg/g.), was of 97.8% (range= 70-156%) with a relative standard deviations of 3.5% (range= 1.5-5.5%). Intra-day (n= 5) and inter-day (5 different days) precision were 2.4% and 9.0%, respectively. These values were below ±10% which is the maximum variation for certification exercises for several mycotoxins (2002/657/EC). The limit of detection (LOD) and the limit of quantitation (LOQ) calculated as signal to noise ratio, S/N = 3 and S/N = 10, were 0.2 and 0.6 µg/g respectively (Table 1).

3.2 EN A quantitation of contaminated feed

Feed contamination by fungi strain was carried out in order to reproduce experimentally the natural mycotoxin presence in a food matrix. *Fusarium tricinctum* strain CECT 2032, through microbial fermentation, produced the mycotoxin in rat feed. In figure 2a is shown the LC-MS-LIT chromatogram of the EN A detected at 465 mg/kg in the contaminated feed. Moreover, in figure 2b is evidenced the MS-LIT spectrum of the bioactive compound

EN A, with three characteristic signals that identify the structure of this bioactive compound as the molecular weight (MW=682.92 g/mol), the sodium and the potassium adduct. The EN A identification was also confirmed by the comparison of the retention time (RT=27.61 min) of the EN A standard solution with the peak of the EN A present in the sample.

3.3 EN A distribution in rat tissues and biological fluids

This study was designed as a 28-day repeated dose assay in rats using the bioactive compound EN A. Ten 2 months-old female Wistar rats were divided in two groups (treated and control), five in each cage. During 28 days, the treated group was fed *ad libitum* with the EN A contaminated feed whereas the control group was fed simultaneously with standard feed.

Rats were observed and weighted weekly (Table 2a). First weight measure was taken on day 0 and, the last measure was obtained, the sacrifice day. As none of them showed significant weight gain or loss, it was assumable that all of them ate a similar amount of feed during the assay. Considering that each animal consumed daily approximately 11.82 g of contaminated feed, the EN A daily intake was of 5.50 mg/per rat. Finally, considering that the mean weight of the treated animals was 263.48 g (Table 2a), the EN A daily intake was 20.91 mg/kg bw/day.

After the animals terminal sacrifice, they were examined and neither visible weight nor morphological tissue or organ changes were observed (Table 2b). The histological analysis of the duodenum tissue was focused on enterocytes atrophy and cellular infiltration determination in the analyzed tissue. No differences were found between treated and

control animals. Biochemical blood parameters analyzed in treated and control animals serum did not show any significant differences between them (Table 3). Bile salts, GTP and GOT showed lower values in treated than in control rats, but there were no statistically significant differences between both animal groups. All biochemical parameters analyzed were within the standard healthy range. This result supports the data reported in the scientific literature describing that ENs inhibit the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) (Tomoda et al., 1992).

No adverse effect was observed in treated rats at the mycotoxin concentration used during the 28 day treatment. The lack of toxic effects produced by ENs on the animal model studied is in agreement with the data published by Bosch et al., (1989). The authors tested the toxic effect of deoxynivalenol (DON), zearalanenone (ZEA), moniliformin (MON), fusaraneone-X (FX), 3-15 Acetyl-DON and ENs A, A_1, B, B_1 naturally present in contaminated corn on twenty day-old white virgin female Sprague Dawley rats. During five days, treated animals were fed with a 1:1 mixture of fermented *Fusarium* rice culture and complete rat diet, whereas control rats received only complete rat diet. Surviving rats were sacrificed by cervical dislocation and examined for gross pathological changes in the tissues. To be certain of the ENs effects, they administered orally 2 mg of ENs mixture to rats weighting 40 g approximately each. The observation lasted 5 days and no toxic signs were found. This result is comparable with the data observed in our study.

Very scarce scientific literature related with *in vivo* toxic effects of ENs is available. McKee et al. (1997) administrated intraperitoneally to mice ENs in a concentration range from 1.25 to 40 mg/kg bw/8h during six days. The top three doses of the ENs mixture (40, 20, 10 mg/kg bw) tested in the hollow-fiber assay were toxic to all mice in the tested groups. With the highest dose, most deaths occurred between days 2 and 3, while for the 20

and 10 mg/kg bw dose groups, deaths occurred between days 4 and 5. For all surviving groups, there was a dose-dependent weight loss. These toxic effects indicated that a maximum-tolerated dose for the ENs was achieved within the tested dose range. Unfortunately, a comparison between the results reported by McKee et al. (1997) and those reported in this study was not possible due to the different species assayed as well as the different route of the toxin administration chosen. The use of solvents to dissolve compounds to test is not the best approach to study any molecule toxicity *in vivo* due to the response that the animals can have to the solvent. EN A oral administration of a naturally contaminated rat feed was chosen for our approach in order to simulate a natural intake of the compound studied. Usually, the bioactive compound administration in animal experiments through alternative methodologies to the oral intake as intraperitoneal injection, promotes the reaching of observed adverse effect levels due to the bypass of the gastrointestinal digestion reaction that can influence the structure of the compound studied (Jestoi, 2008). The last important point is the interaction between the compound studied and the matrix effects generated by the other feed components. This phenomenon is absent in the experiments carried out with standard solutions of toxic compounds intraperitoneally injected as proven by McKee et al. (1997).

The EN A concentration was determined in several organs and biological fluids. Digesta from stomach, duodenum, jejunum, ileum and colon were evaluated. The gastrointestinal tract and the kidneys were also analyzed. The EN A chromatogram present in the liver sample of a treated animal with the contaminated feed compared with the control rat is shown in figure 3.

As exposed in figure 4a, the lowest EN A concentration was detected in colon and duodenum with 2.2±0.7 mg/kg and 2.9±0.6 mg/kg respectively, probably because of a weak absorption of the bioactive compound in those gastrointestinal tract parts. The highest EN A concentration was observed in liver with 22.7±1.0 mg/kg and it may be related to its detoxification function of bioactive compounds transported from the intestine through the portal vein and others present in the human body. The molecules transported are normally accumulated into the hepatocytes where they are metabolized by the enzymes present in the bile that can modify their chemical structure. Liver and kidneys are particularly susceptible to organ toxicity as they are the sites of toxin filtration and toxin metabolic breakdown. The secondary products produced by toxic compound metabolism can also be accumulated in the liver and may be potentially toxic for the animal body (Kerns and Di, 2008). However, no EN A was detected neither in kidneys, stomach nor ileum.

Regarding the intraintestinal liquids, the highest EN A data was observed in the jejunum content with 9.6±1.1 mg/kg, whereas the lowest in the duodenal liquid with 1.3±0.2 mg/kg. Significant EN A concentrations were measured in the colon content with 7.3±0.7 mg/kg, whereas the EN A data found in the gastric content and in serum were of 4.6±0.2 and 5.0±0.5 mg/kg respectively (Figure 4b). No EN A was observed in ileum content.

3.4 LC-MS-LIT determination of ENs degradation products

The gastrointestinal content extracts were also injected in the LC-MS-LIT to identify possible degradation products produced through the gastrointestinal fermentation by gut microflora. Two degradation products were detected in the duodenal compartment represented by the EN A with the loss of an isoleucine (Ile) group, an aminoacid

characteristic of the ENs structure, and by the EN A with the loss of a hydroxivaleric acid unit (HyLv). The concentration in duodenum digesta of these two degradation products was of 89.7±3.2 and of 123.55±4.1 mg/L respectively. The presence of these newly formed compounds was confirmed employing the technique of the LC-MS coupled to the LIT. As explained in table 4 the structure of the degradation compound ENA-Ile was confirmed by the fragment with *m/z* = 577.1 that represents the molecular weight (MW) of the compound formed. By fragmentation of this signal, two diagnostic signals were obtained in MS^2 with *m/z* of 547.3 represented by the EN A-Ile with the loss of a carbonyl group and *m/z* of 292.4, the EN A with the loss of two Ile group. The last confirmation of the structure of this degradation product was obtained by the MS^3 spectra, where are evidenced the characteristic fragments of the two principal ENs components as the Ile and HyLv. The MS^1 fragment of the degradation product composed by the EN A with the HyLv group loss presents a *m/z* of 637.4. The structure of this product formed was confirmed by the fragments in MS^2 with *m/z* of 537.1 and MS^3 with *m/z* of 533.4. They represent the EN A with the loss of four molecules of water and by the EN A with the loss of two HyLv units. Definitive confirmation fragments in MS^3 were the signals with *m/z* of 84 represented by one HyLv unit and 168.0, two HyLv units. The presence of this important EN A structural component in MS^3 confirmed the structure of the degradation product formed.

The adducts formed between the EN A and the macronutrients present in rat feed detected and characterized are described in table 5. In the duodenum and jejunum compartments was detected the adduct formed with the EN A and two molecules of glucose. As shown in table 5, this newly formed compound presents a *m/z* of 1022.0. The structure of the bioactive compound formed was confirmed by the fragments obtained in MS^2 and represented by the

signals with a *m/z* 937.3 and 916.3 that confirmed the loss by the structure of the adduct of an EN A component as the HyLv. In MS^3 spectra was observed an important diagnostic signal with *m/z* of 181.16 represented by the MW of a glucose unit. The concentrations calculated for this newly formed compound in the duodenum and jejunum compartments were 196.74±6.3 and 149.39±4.9 mg/L respectively.

Another important adduct detected in the duodenal compartment was the reaction product originated by the reaction between the EN A and the glucuronic acid. Uridine diphosphate glucuronosyltransferase (UDP-GT or UGT) is a family of inducible microsomal isoenzymes associated with the liver, intestine, lung and olfactory epithelium. These isozymes catalyze glucuronidation, the transfer of glucuronic acid from the high-energy nucleotide UDP-glucuronic acid (UDP-GA) to an electronegative group on a wide variety of endogenous and xenobiotic substrates (Hayes, W.A., 2007). The concentration found of this adduct in the duodenal compartment was 121.98±6.8 mg/L. The confirmation of the adduct formed was carried out using the LC-MS coupled with the LIT operating in MS^1, MS^2 and MS^3. The ER spectra in MS^1 evidenced a diagnostic fragment with a *m/z* 112.3 that represents the EN A coupled with two units of glucuronic acid. The presence of that compound was confirmed in the MS^2 spectra with a fragment with a *m/z* of 914.4. The final confirmation of the coupling adduct obtained with the reaction between the EN A and the glucuronic acid was evidenced in the MS^3 spectra whit the fragment corresponding to the MW of the glucuronic acid with a *m/z* of 195.1.

Related to the adducts formed with EN A and macronutrients, in the colonic compartment was detected the product of the reaction between the EN A and four glucose units. This product was detected at the concentration of 42.02±8.2 mg/L and presents in MS^1 spectra a

MW of 1517.8 g/mol. The presence of the glucose in the adduct structure was confirmed by the localization of several fragments in the MS^2 spectra and, in particular, one fragment present in the MS^3 spectra with a *m/z* of 724.6, represented by four glucose units.

Among the adducts detected in several intestinal compartments, only the reaction product between the EN A and two glucose units was found in serum. This compound can be considered the only adduct detected in this study that was absorbed by the intestinal epithelium and was detected in the rat blood (66.11±7.1 mg/L). The reasons for the presence of this compound in the systemic circulation possibly because of the high concentrations detected in the duodenum and jejunum compartment that favored the absorption of the adduct formed. The structure of this adduct observed in serum was confirmed by several diagnostic fragments as the ion detected in MS^1 spectra with a *m/z* of 1065.1 that represents the MW of the adduct, the signal detected in the MS^2 spectrum with a *m/z* of 1013 represented by the adduct with the loss of an EN A structural component as the Ile and, definitely, the presence of the ion with a *m/z* of 181.6 in the MS^3 spectrum, confirmed the glucose presence in the structure of the newly formed compound.

To sum up, EN A intestinal degradation products and adducts are described for the first time. Further investigation is needed in order to fill the gap of the metabolic routes affecting EN A.

Conclusion

The results obtained in this study confirmed that the EN A concentration of 20.91 mg/kg bw/day present in rat feed through a microbial fermentation by a strain of *Fusarium tricinctum*, used during 28 days on Wistar rats simulating a preliminar subchronic toxicity

study, does not provoke any observed adverse effect on the animals. No statistical differences on the biochemical blood parameters or on the histological analysis carried out on the duodenum tissue were found when comparing controls with treated animals. Thus, we can confirm that 20.91 mg/ kg bw/day of EN A is a non-toxic level for young adult rats during medium term ingestion.

EN A was detected in several organs and contents of the gastrointestinal tract, but also in serum confirming its intestinal absorption. EN A degradation products and adducts, probably produced by gut microbial fermentation, were identified and characterized.

It is presented for the first time experimental data of interest that give information about toxicokinetic processes and potential effects after oral administration *in vivo* of emerging mycotoxins that may be of interest to international institutions when conducting risk evaluation assessment.

Further investigation may be focused on the calculation of the lowest-observed-adverse-effect-level (LOAEL) for the ENs in order to establish a dose-response relationship, a fundamental step to assess the risk related to the intake of these mycotoxins.

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Declaration of interest

The authors report no declarations of interest.

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Legend of figures

Figure 1. Schematic representation of the *in vivo* study carried out on EN A toxicity.

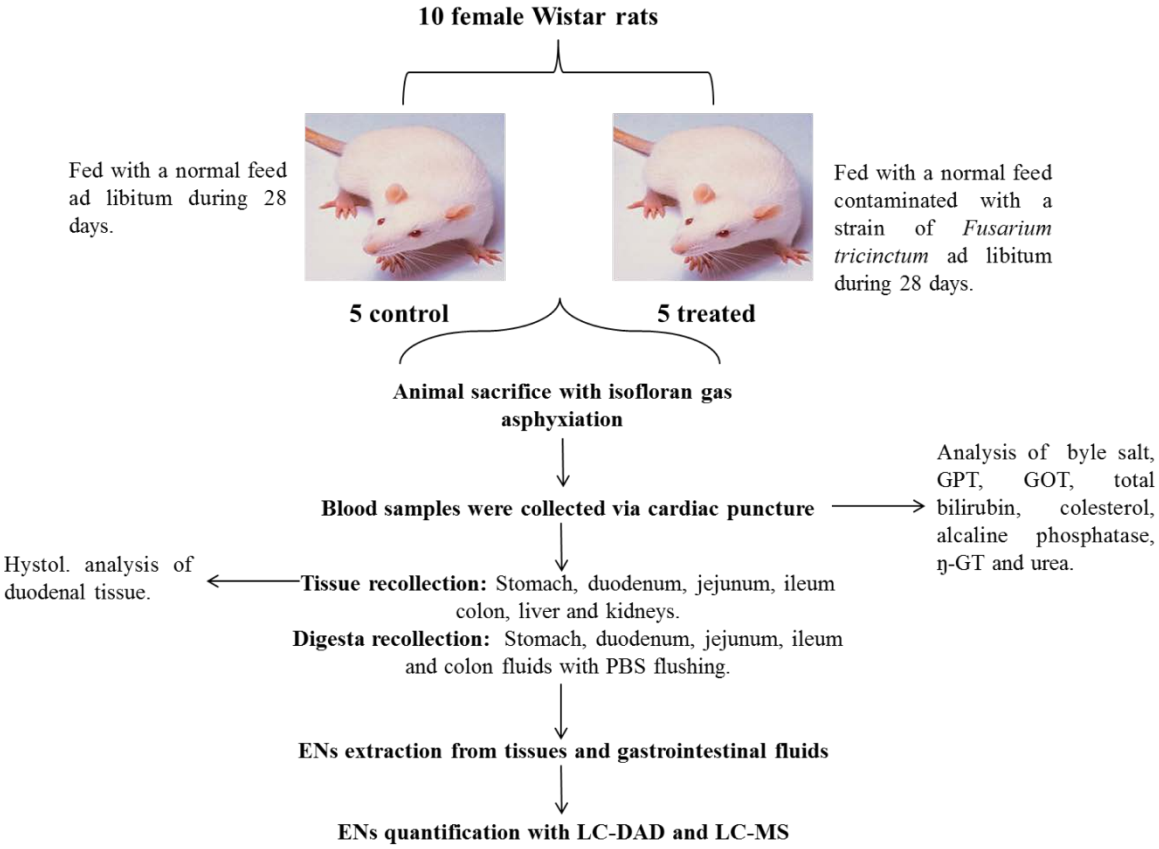
Figure 2. a) LC-MS-LIT chromatogram of the EN A present in the feed contaminated with the strain of *Fusarium tricinctum* CECT 20232 and b) mass spectrum in linear ion trap (MS-LIT) of the EN A.

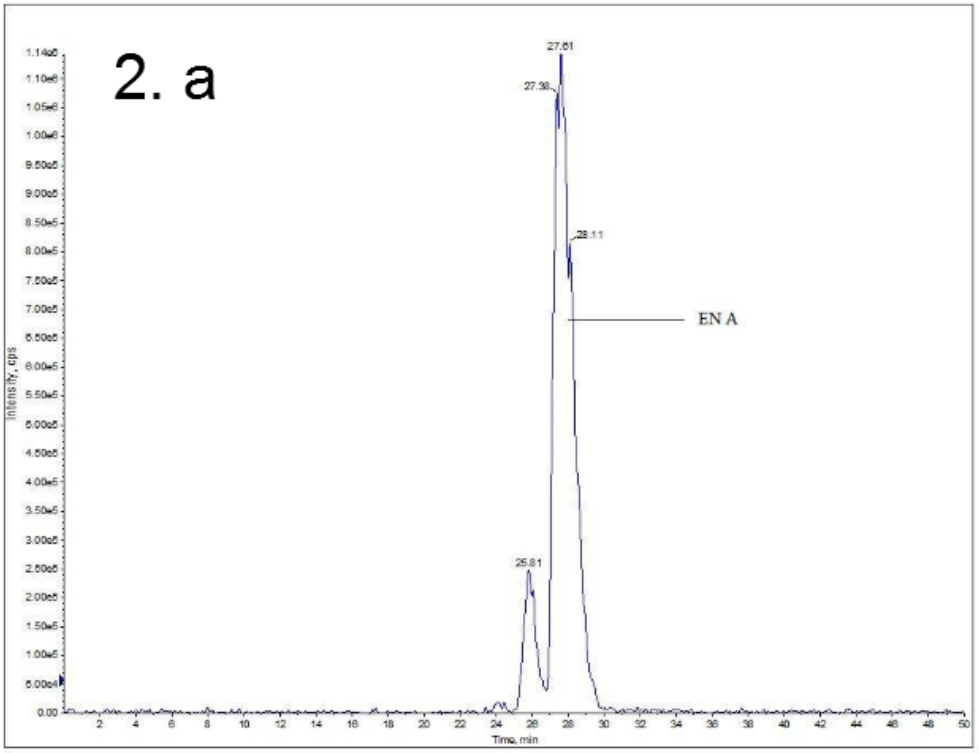
Figure 3. a) LC-DAD chromatogram of the ENA present in the liver of the rat treated with the feed contaminated with the EN A, compared with b) the liver of the control animals.

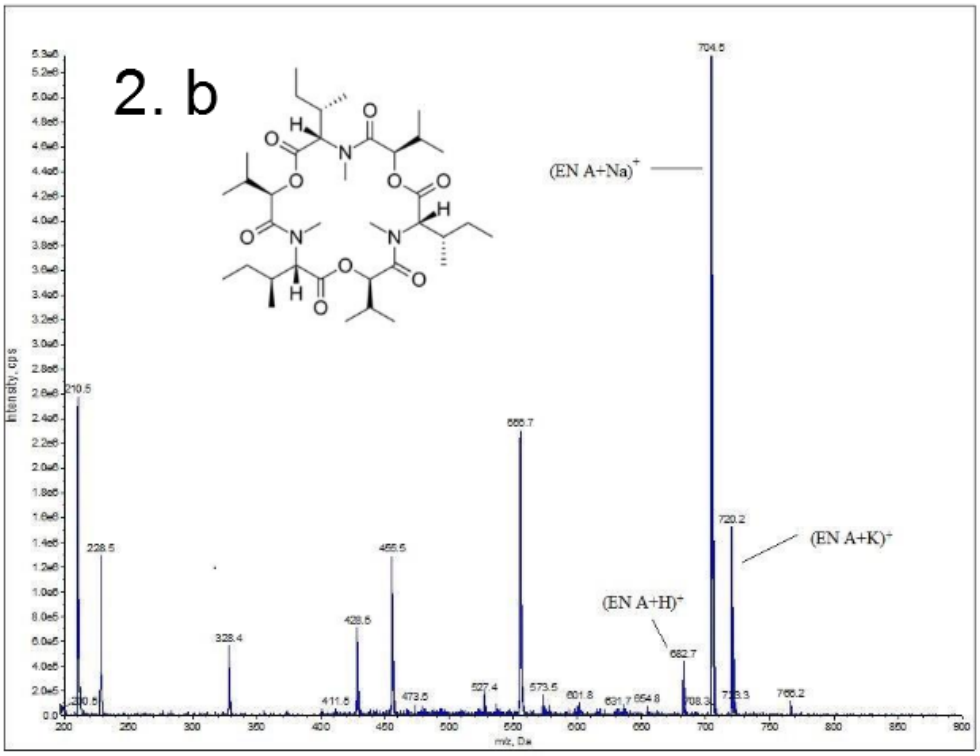
Figure 4. EN A concentration detected by LC-DAD in organs, gastrointestinal liquids and serum of treated female rats (n=5). Their diet consisted in EN A contaminated feed (465 mg/kg) *ad libitum* during 28 days. Control rats (n=5) ate standard feed and no trace of EN A was detected during the whole analysis in any sample. a) Different organs from treated rats. b) Mycotoxin concentration present in the gastrointestinal liquids and serum of treated animals.

Figure 5. Quantification of the a) EN A degradation products originated by the microbial fermentation of the ENA present in the rat feed by the intestinal microflora and b) adducts of formation of the EN A with the glucose in the liquid of several intestinal compartments. Ile: isoleucine. HyLv: hydroxivaleric acid. Duod: duodenum. Gluc. Ac.: glucuronic acid. Glu: glucose. Col: colon.

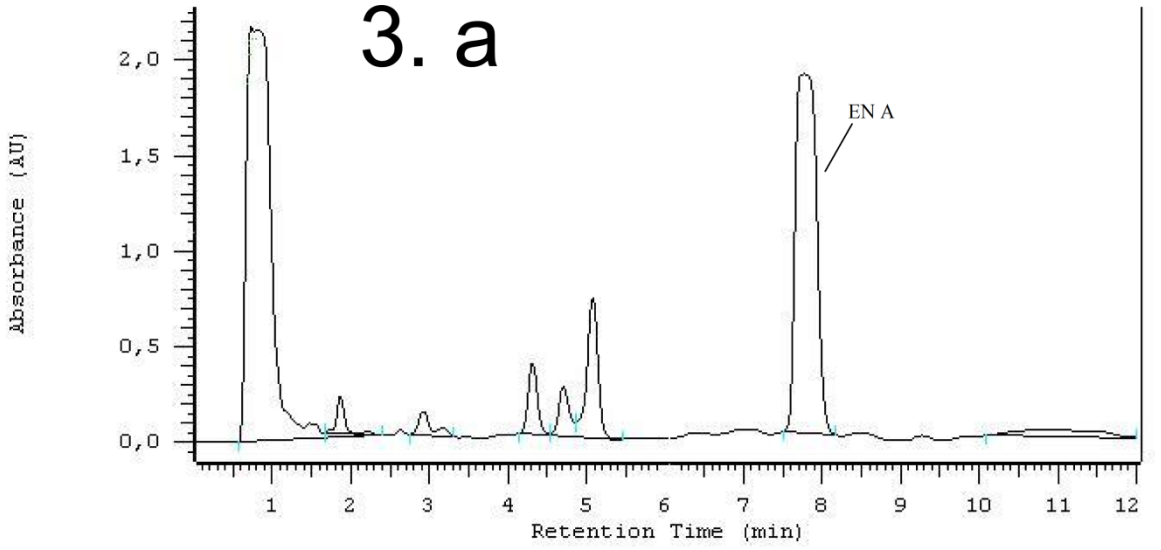
Figure 6. LC-MS-LIT chromatogram of the EN A and of the adduct of formation between the minor *Fusarium* mycotoxin and the glucose evidenced in the serum of treated animals. Glu: glucose.

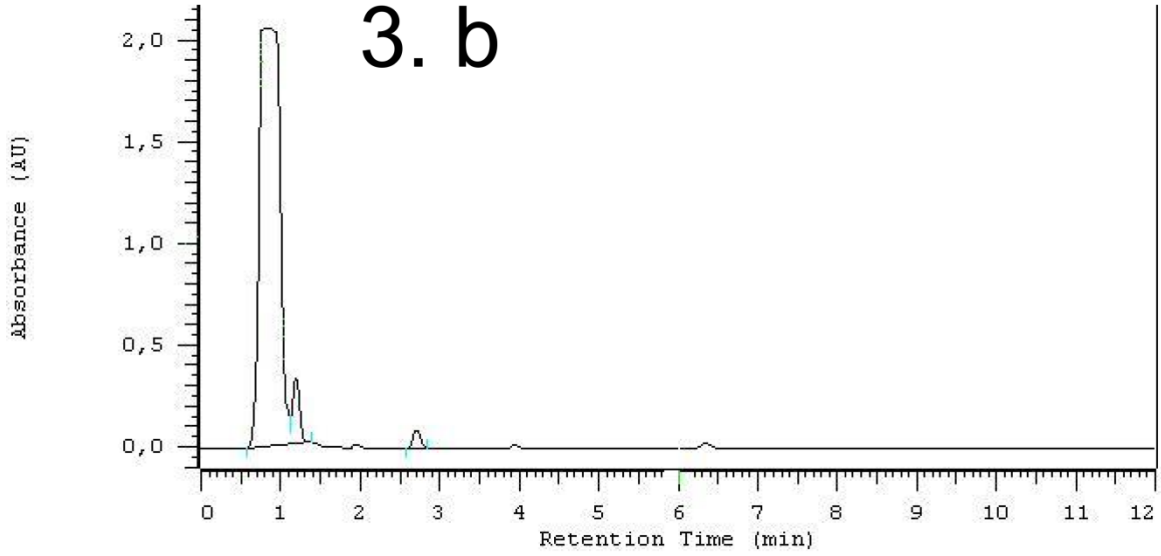

10 female Wistar rats
Fed with a normal feed ad libitum during 28 days.
Fed with a normal feed contaminated with a strain of Fusarium tricinctum ad libitum during 28 days.
5 control
5 treated
Animal sacrifice with isofloran gas asphyxiation
Blood samples were collected via cardiac puncture
Analysis of byle salt, GPT, GOT, total bilirubin, colesterol, alcaline phosphatase, ŋ-GT and urea.
Hystol. analysis of duodenal tissue.
Tissue recollection: Stomach, duodenum, jejunum, ileum colon, liver and kidneys.
Digesta recollection: Stomach, duodenum, jejunum, ileum and colon fluids with PBS flushing.
ENs extraction from tissues and gastrointestinal fluids
ENs quantification with LC-DAD and LC-MS

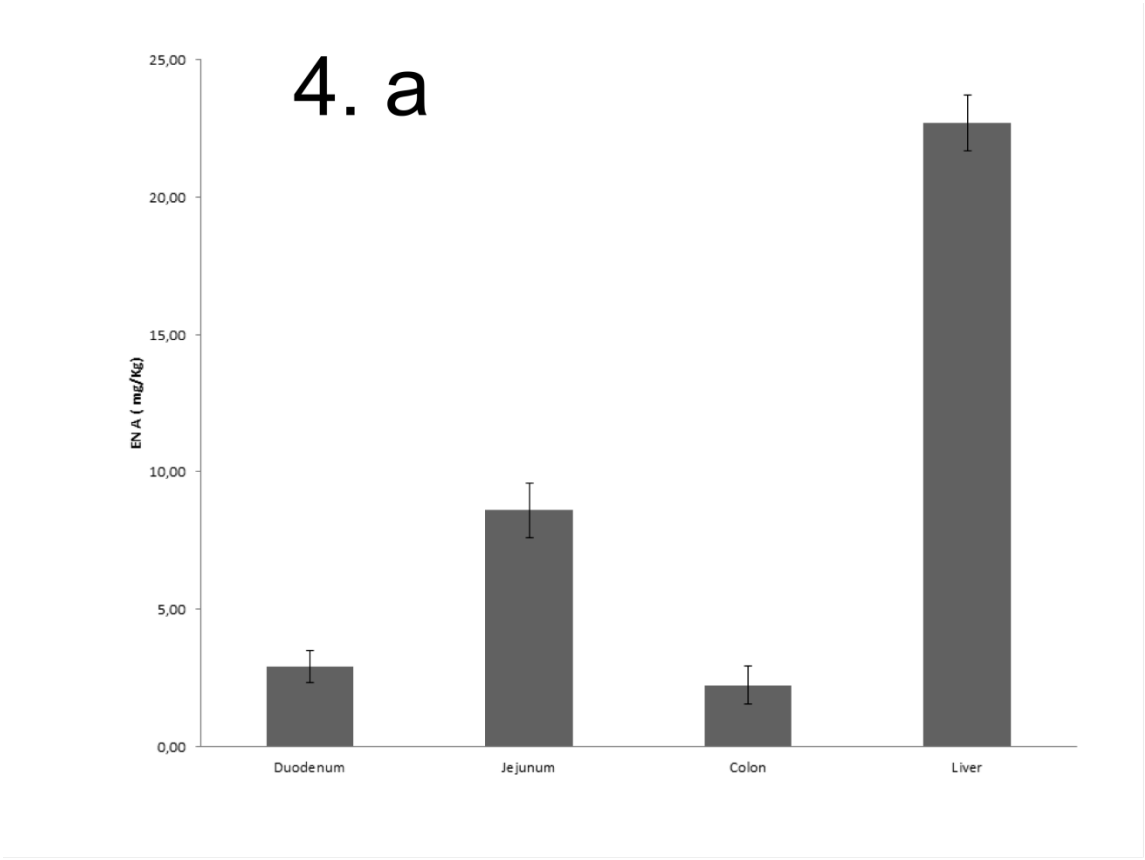

2. a
27.61
27.38
28.11
EN A
25.81
Intensity, cps
Time, min

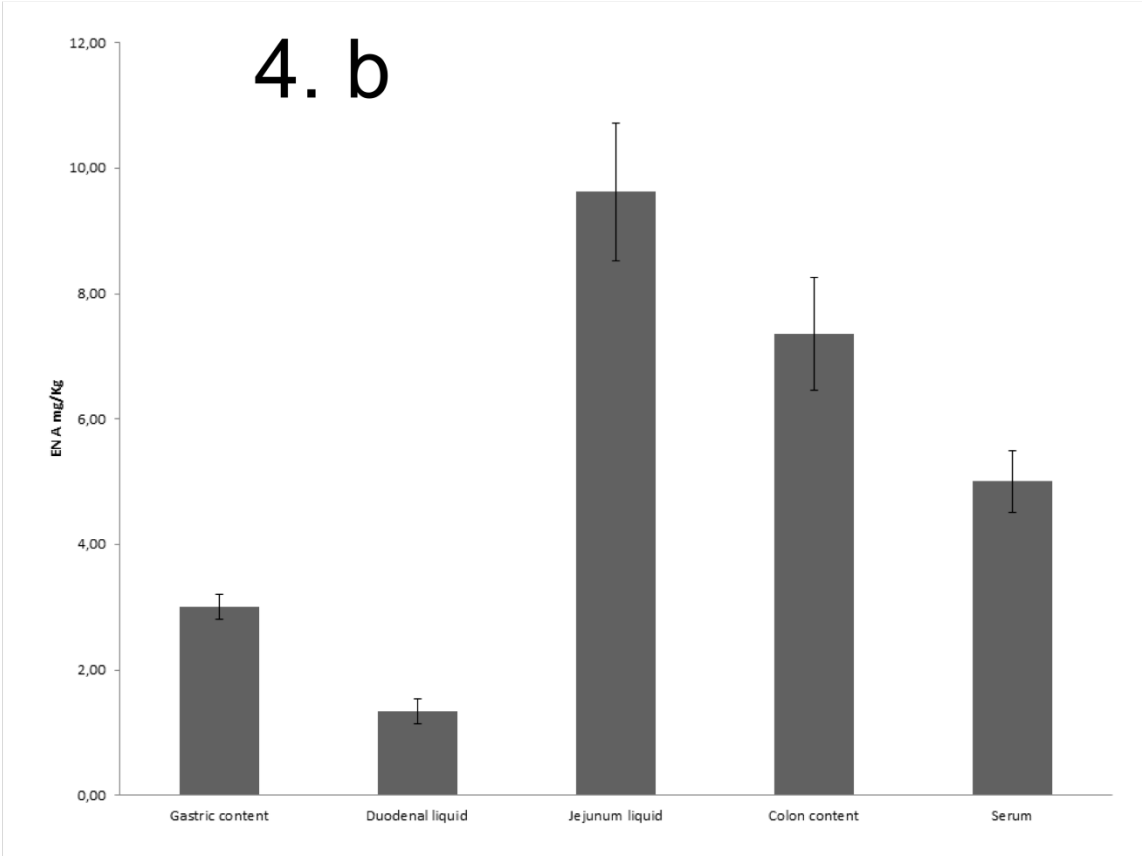

2. b
704.5
(EN A+Na)+
720.2
(EN A+K)+
(EN A+H)+
682.7
210.5
228.5
328.4
428.5
455.5
555.7
Intensity, cps
m/z, Da

1


3. a
Absorbance (AU)
2,0
1,5
1,0
0,5
0,0
EN A
1
2
3
4
5
6
7
8
9
10
11
12
Retention Time (min)

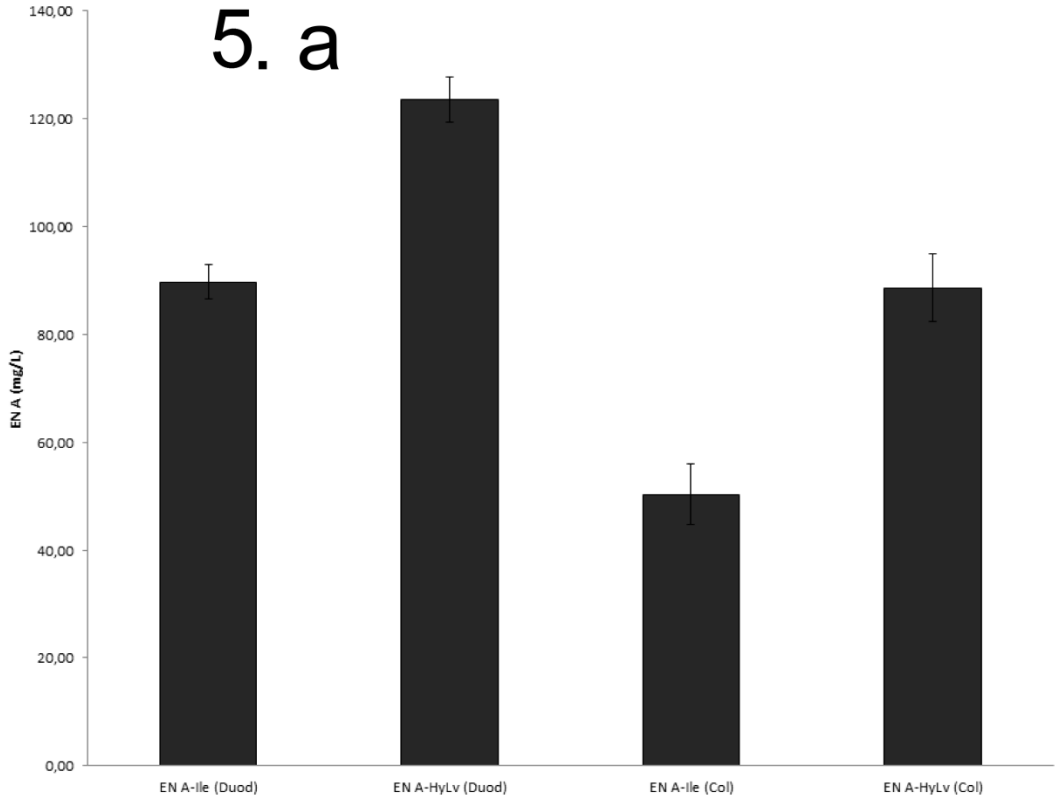

3. b
Absorbance (AU)
2,0
1,5
1,0
0,5
0,0
0
1
2
3
4
5
6
7
8
9
10
11
12
Retention Time (min)


4. a
25,00
20,00
15,00
10,00
5,00
0,00
EN A (mg/Kg)
Duodenum
Jejunum
Colon
Liver

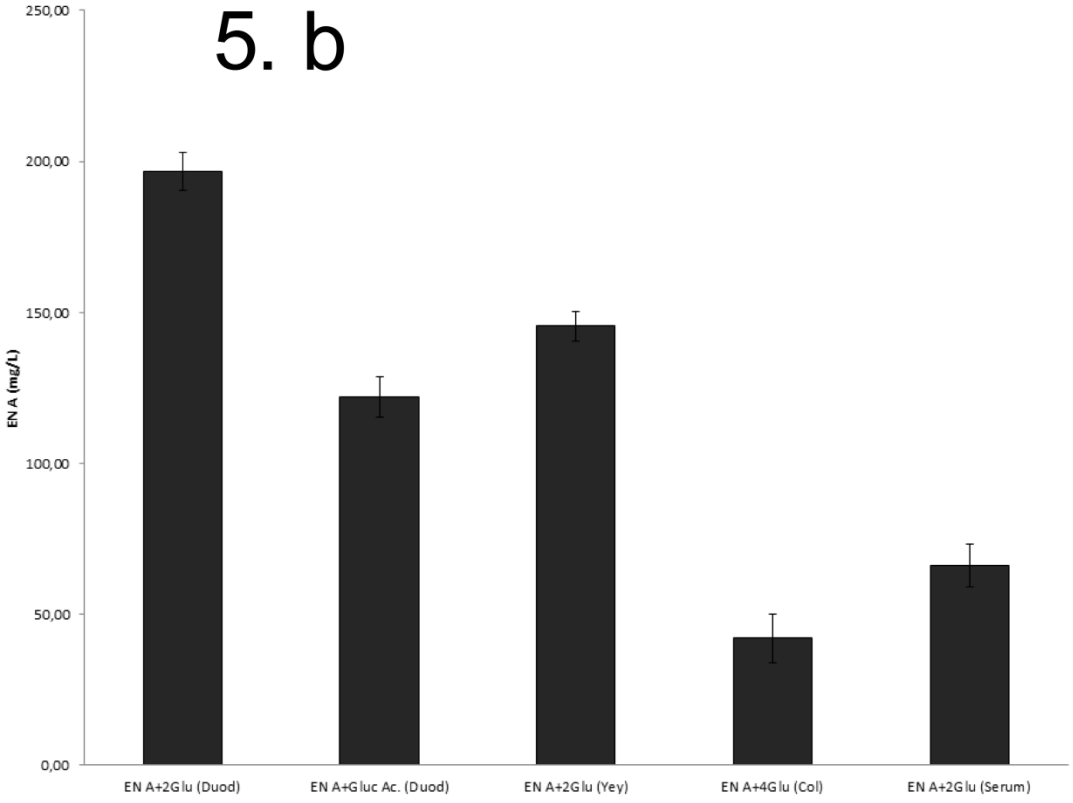

4. b
12,00
10,00
8,00
6,00
4,00
2,00
0,00
EN A mg/Kg
Gastric content
Duodenal liquid
Jejunum liquid
Colon content
Serum

1

5. a

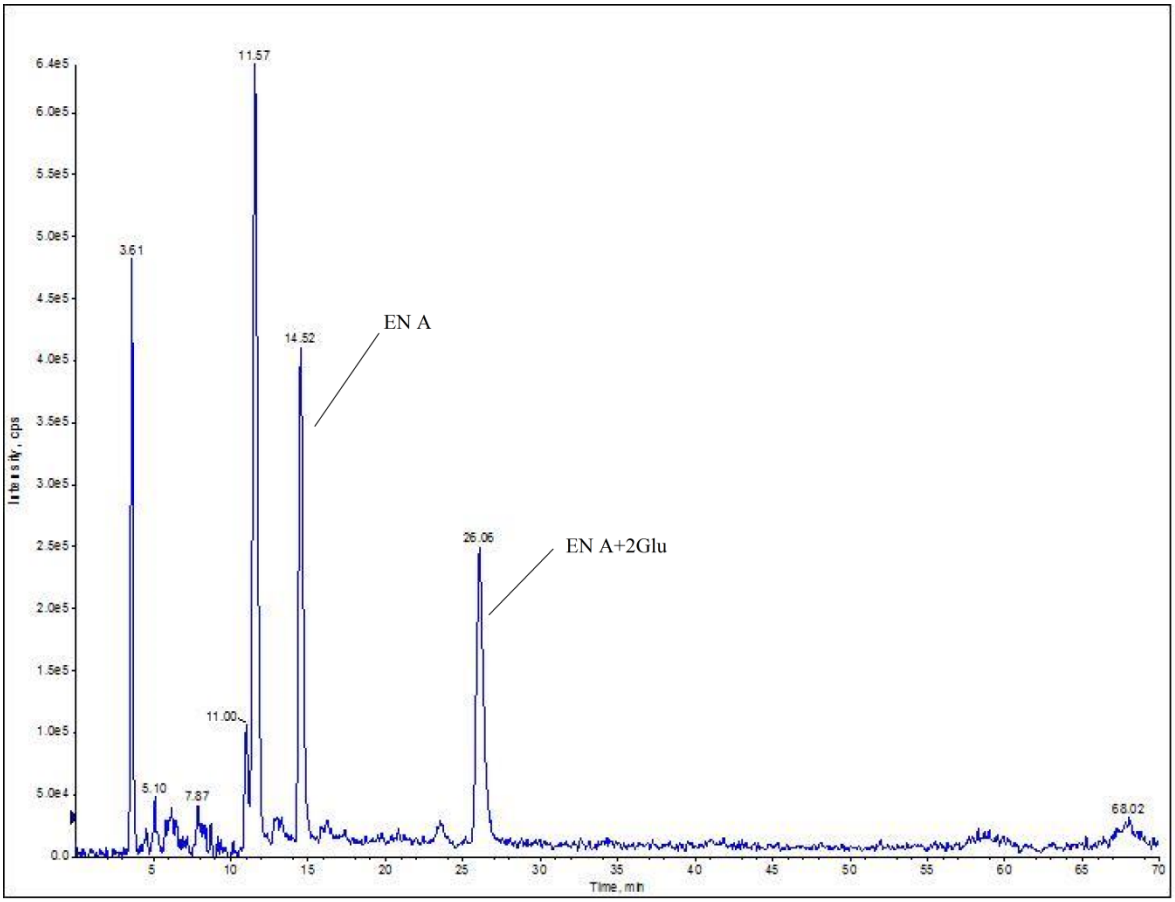

140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
EN A (mg/L)
EN A-Ile (Duod)
EN A-HyLv (Duod)
EN A-Ile (Col)
EN A-HyLv (Col)

5. b


250,00
200,00
150,00
100,00
50,00
0,00
EN A (mg/L)
EN A+2Glu (Duod)
EN A+Gluc Ac. (Duod)
EN A+2Glu (Yey)
EN A+4Glu (Col)
EN A+2Glu (Serum)

1

1


Intensity, cps
Time, min
3.61
5.10
7.87
11.00
11.57
14.52
EN A
26.06
EN A+2Glu
68.02

2

Table 1. EN A mean recoveries, inter-day and intra-day variations, limit of detection (LOD) and limit of quantitation (LOQ) of the analytical method applied to the different matrices analyzed in this study in which EN A has been detected.

Sample	*Mean recovery (%)*	*Inter-day variation (%)*	*Intra-day variation (%)*	*LOD (mg/Kg)*	*LOQ (mg/Kg)*
Gastric content	*156.3±1.5*	*2.0*	*8.7*	*0.2*	*0.6*
Duodenum content	*97.2±3.4*	*2.2*	*9.6*	*0.2*	*0.6*
Duodenum	*97.1±2.6*	*2.5*	*9.2*	*0.2*	*0.6*
Jejunum content	*98.2±3.4*	*2.5*	*7.5*	*0.2*	*0.6*
Jejunum	*97.5±2.0*	*3.1*	*10.2*	*0.2*	*0.6*
Colon content	*70.2±2.2*	*2.4*	*8.8*	*0.2*	*0.6*
Colon	*71.4±3.1*	*2.0*	*9.6*	*0.2*	*0.6*
Liver	*93.1±2.0*	*2.6*	*9.1*	*0.2*	*0.6*
Serum	*100.0±2.8*	*2.4*	*8.6*	*0.2*	*0.6*

Table 2. a) Weekly body weight measurements during the study of the rats used. At week 0, first day of the 28 day-study b) Comparison of organs weight of these rats measured at terminal sacrifice.

a)

	BODY WEIGHT (g)									
	CONTROL RATS					TREATED RATS				
	1	2	3	4	5	1	2	3	4	5
Week 0	242.0	269.8	289.2	229.0	236.4	268.8	261.4	252.6	236.6	255.4
Week 1	244.6	273.2	295.2	228.4	244.6	262.4	255.8	256.2	233.0	248.2
Week 2	252.0	274.0	296.0	229.0	250.0	272.2	269.8	253.8	234.8	259.2
Week 3	253.4	290.0	270.8	227.6	252.2	279.6	274.6	265.2	230.2	267.8
Week 4	255.4	285.3	262.6	230.3	257.1	284.4	270.2	268.1	222.3	274.3

b)

	ORGAN WEIGHT (g)									
	CONTROL RATS					TREATED RATS				
	1	2	3	4	5	1	2	3	4	5
Liver	8.40	9.31	9.82	8.07	7.61	7.54	8.12	7.43	7.15	7.58
Kidneys	1.61	1.85	1.59	1.41	1.36	1.53	1.66	1.46	1.59	1.63
Heart	0.84	0.97	1.03	0.72	0.92	0.92	0.89	0.97	0.93	0.78
Thymus	0.53	0.53	1.02	0.43	0.53	0.62	0.55	0.56	0.51	0.79
Spleen	0.42	0.51	0.50	0.38	0.46	0.57	0.54	0.55	0.50	0.63

Table 3. Biochemical parameters analyzed in control (n=5) and treated (n=5) rat serum. GPT=glutamic-pyruvic transaminase, GOT=glutamic-oxaloacetic transaminase, ɣ-GT=gamma-glutamyl transpeptidase.

Samples	***Bile salts***	***GPT***	***GOT***	***Total bilirubin***	***Cholesterol***	***Alkaline phosphatase***	***ɣ -GT***	***Urea***
	µmol/L	*U/L*	*U/L*	*mg/dL*	*mg/dL*	*U/dL*	*U/L*	*mg/dL*
Controls rats	*47.6±3.1*	*41.2±3.9*	*98.2±9.7*	*0.1±0.05*	*92.6±9.6*	*71.5±7.1*	*<5*	*42.6±3.5*
Treated rats	*28.5±3.2*	*28.4±3.9*	*84.8±9.8*	*0.1±0.03*	*85.6±8.7*	*68.8±8.6*	*<5*	*47.2±5.4*

Table 4. EN A intestinal degradation products. Two degradation products from ENN A found in the intestinal compartment of the treated rats, in which compartment where they were discovered and their MS^1, MS^2 and MS^3 fragments.

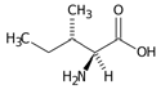
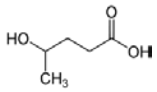
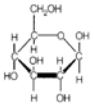
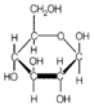
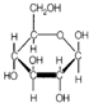
Degradation product	Biological liquid	$[M+H]^+$ m/z	Fragment	Structure	MS^2 fragments		MS^3 fragments	
					m/z	Fragment	m/z	Fragment
$(EN\ A+K-Ile)^+$	Duodenum	577.1	Ile		547.3	$(EN\ A+K-Ile-C=O)^+$	85.0	$(HyLv)^+$
	Colon				292.4	$(EN\ A+K-2Ile)^+$	144.2	$(Ile)^+$
$(EN\ A+K-HyLv)^+$	Duodenum	637.4	HyLv		537.1	$(EN\ A+K-4H2O)^+$	84.0	$(HyLv)^+$
	Colon				533.4	$(EN\ A+K-2HyLv)^+$	168.0	$2(HyLv)^+$

Table 5. ENN A intestinal adducts. Four adducts formed with ENN A and macronutrients present in the rats feed, in which compartment where they were discovered and their MS^1, MS^2 and MS^3 fragments.

Adducts	Biological liquid	$[M+H]^+$ m/z	Adduct Fragment	Structure	MS^2 fragments		MS^3 fragments	
					m/z	Fragment	m/z	Fragment
$(EN\ A+2Glu-H_2O)^+$	Duodenum	1022.0	Glu		937.3	$(EN\ A+2Glu-H_2O-HyLv)^+$	181.16	$(Glu)^+$
	Jejunum				916.3	$(EN\ A+2Glu-2H_2O-HyLv)^+$	84.0	$(HyLv)^+$
$(EN\ A+K+4Glu)^+$	Colon	1517.8	Glu		1442.4	$(EN\ A+K+4Glu-HyLv)^+$	84.0	$(HyLv)^+$
					1373.8	$(EN\ A+K+4Glu-HyLv-Ile)^+$	144.2	$(Ile)^+$
					1042.4	$(EN\ A+K+4Glu-HyLv-3Ile-2H_2O)^+$	724.6	$4(Glu)^+$
$(EN\ A+2Gluc\ Ac.)^+$	Duodenum	1112.3	Gluc Ac.		914.4	$(EN\ A+Gluc\ Ac.)^+$	195.1	$(Gluc\ Ac.)^+$
							390.2	$2(Gluc\ Ac.)^+$
$(EN\ A+Na+2Glu)^+$	Serum	1065.1	Glu		903.9	$(EN\ A+Na+2Glu-Ile-H_2O)^+$	181.16	$(Glu)^+$