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Master's Thesis
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OPTIMIZATION OF SOLVENT EXTRACTION METHOD AND DETERMINATION OF UNBOUND ENNIATIN CONCENTRATION IN BOVINE SERUM ALBUMIN USING RAPID EQUILIBRIUM DIALYSIS (RED)

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difference was observed between the two groups for each enniatin using two-way ANOVA; $p<0.0021$ (**), $p<0.0002$ (***), $p<0.0001$ (****).

Figure 10:Recovery of ENNs from BSA containing test samples and plastic binding samples after the incubation time of 6 hours. Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation. Statistically significant difference was observed between the two groups for each enniatin using two-way ANOVA; $p<0.0001$ (****).

Figure 11:Recovery of ENNs from BSA containing test samples and plastic binding samples after the incubation time of 6 hours. Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation. Statistically significant difference was observed between the two groups for each enniatin using two-way ANOVA; $p<0.0001$ (****).

Figure 12: Bound fraction % of ENNs from test samples BSA after 6 hours and 24 hours of incubation. Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation. Statistically significant difference was observed between the two groups for each enniatin using paired t-test, $p<0.005$ (**).

List of Abbreviations

ABS	Absolute
ACN	Acetonitrile
BEA	Beauvericin
BSA	Bovine Serum Albumin
Cmax	Maximum (peak) concentration
C(time)	Maximum concentration at a specific time point after dose administration
CONTAM	Panel on Contaminants in the Food Chain
CYP450	Cytochrome P450 Enzymes
DMSO	Dimethyl sulfoxide EFSA
EFSA	European Food Safety Authority
ENN	Enniatin
ESI	Electrospray ionization
EURL	European Union Reference Laboratory
FBS	Fetal Bovine Serum
HBSS	Hanks' Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazine-1-ethane-sulfonic acid
HSA	Human Serum Albumin
IV	Intravenous
LC/MS	Liquid Chromatography Mass Spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LPO	Lipid Peroxidation

MEM	Minimum Essential Medium
MRM	Multiple reaction monitoring
MRP 2	Multidrug resistance-associated protein 2
NOAEL	No Observed Adverse Effect Level
PPB	Plasma Protein Binding
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PBTK	Physiologically based toxicokinetic modelling
P-gp	P-glycoprotein
PO	Per os (Oral)
RED	Rapid Equilibrium Dialysis
TK	Toxicokinetics
Vd	Volume of distribution
UC	Ultracentrifugation
UF	Ultrafiltration
(f_{ub})	Unbound fraction
ZEN	Zearalenone

Abstract

Enniatins (ENNs) are *Fusarium* mycotoxins commonly present in contaminated food and feed. The most prevalent types of ENNs are ENNA, ENNA1, ENNB, and ENNB1. Possessing ionophoric properties and lipophilic in nature, these toxins raise concerns for potential health risks in humans and animals. Understanding the toxicokinetics of ENNs is essential for comprehending their impact on the body. This study initially focuses on optimizing the extraction of all four enniatins from various matrices to achieve optimal recovery rates. Unlike previous investigations limited to ENNA's binding affinity to human and rat plasma samples, our study delved into the plasma protein binding (PPB) of all enniatins to bovine serum proteins. By employing a rapid equilibrium dialysis assay with incubation periods of 6 and 24 hours, we determined PPB and unbound concentrations of enniatins. Notably, ENNB exhibited the highest unbound fraction (71%) after 24 hours, implying a potential for increased toxicity compared to the other three enniatins. This finding underscores the importance of assessing binding characteristics across different species to better understand the toxicological implications of ENNs.

Keywords: *Enniatins; Extraction; Plasma protein binding; Rapid Equilibrium Dialysis; LC/MS.*

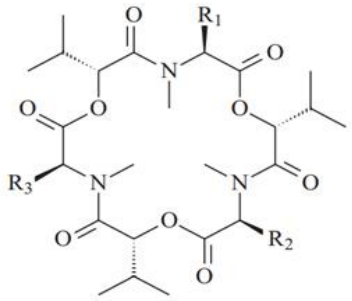
Chapter 1: Introduction

Food contaminants pose a major concern to food safety and public health. The food items can be contaminated during their processing and production. Environmental pollutants, process contaminants and natural toxins are the three primary types of contaminants in food and feed (EFSA, 2023). Mycotoxins belong to natural toxins which are produced by fungi under certain environmental conditions as secondary metabolites and are known to be food and feed contaminants (Escrivá et al., 2016). Over the past few years, more attention has been given to mycotoxins referred to as "mycotoxins of emerging concern", which despite their increasing prevalence are not yet regulated (Gruber-Dorninger et al., 2017; Jestoi, 2008; Malachova et al., 2011).

Enniatins (ENNs), also referred to as fusaria toxins are emerging mycotoxins produced by *Fusarium* species (Juan et al., 2014). Several studies have shown the significant presence of ENNs in food and feed commodities. ENNs have been identified as contaminants in cereals such as barley, oats, wheat and rye (5.3 to 284.2 mg/kg) and cereal-based products like pasta (1 to 1100 mg/kg) (Juan, Mañes, et al., 2013; Juan, Ritieni, et al., 2013). The commonly found ENNs in contaminated food and feed items are ENNA, ENNA1, ENNB and ENNB1. An investigation was carried out to determine the concentration of the four enniatins in Finnish grain samples. The results revealed the highest concentrations obtained for each enniatin from different grains, with ENNB being the most dominant toxin with the highest concentration in wheat samples (18300 µg/kg). In addition to this, the highest concentrations obtained in Finnish barley samples for ENNB1, ENNA1 and ENNA were 5720 µg/kg, 2000 µg/kg and 950 µg/kg respectively (Jestoi, et al., 2004). Another comprehensive analysis of multiple mycotoxins in maize silage conducted in northwest Spain discovered that ENNB was the most frequently detected mycotoxin in 51% of the samples, with an average concentration of 157 µg/kg (Dagnac et al., 2016). Pasta and baby food products of multi-cereal origin sold in Italian supermarkets were assessed to check for the presence of enniatins in them. It was found that elevated levels of ENN B at concentrations of 1,100 µg/kg and 106µg/kg, followed by ENNB1 at 110µg/kg and 4µg/kg, ENNA1 at 92µg/kg and 9µg/kg and ENNA at 27µg/kg and 8µg/kg were detected in baby food and pasta samples respectively (Juan, Mañes, et al., 2013).

ENNs are cyclohexadepsipeptides consisting of alternating segments composed of three N-methyl amino acids (valine, leucine, isoleucine) and three hydroxy acids, predominantly hydroxyisovaleric acid (Ivanova et al., 2012) as illustrated in Figure 1. ALOGPS 2.1 was used

to predict pKa and logP values (Rozentale et al., 2018; Tetko et al., 2005) which is given in Table 1. ENNA exhibits the highest lipophilicity among the four ENNs, with ENNA1, ENNB1, and ENNB following in decreasing order of lipophilicity (logP) and molecular weights.



ENN	R1	R2	R3
A	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$
A_1	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)_2$
B	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)_2$
B_1	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$

Figure 1: Chemical structure of ENNs (with different side chains) (Prosperini et al., 2017).

Table 1: Physiochemical properties of ENNs.

Enniatin	Molecular Formula	Molecular weight (g/mol)	pKa	LogP
ENNA	C36H63N3O9	681.9	18.8	4.79
ENNA1	C35H61N3O9	667.9	18.8	4.39
ENNB	C33H57N3O9	639.8	18.8	3.81
ENNB1	C34H59N3O9	653.8	18.8	4.06

1.1 Toxicity and Toxicokinetic studies

ENNs toxicity is due to their ionophoric nature as it allows them to form complexes with Ca^{2+} and K^+ which are mono and divalent cations. These complexes facilitate the integration of ENNs in cell membranes. Furthermore, in the membrane ENNs can form cation pores resulting in disturbance of these ion equilibrium leading to disruption in osmolarity. This has an impact on the overall cellular homeostasis and causes cytotoxic effects (Fraeyman et al., 2017; Gruber-

Dorninger et al., 2017; Kamyar et al., 2004). Toxicity tests are performed to assess the adverse effects of a compound, determine the No Observed Adverse Effect Level (NOAEL) dose and identify certain endpoints (Parasuraman, 2011). Toxicokinetics (TK) refers to the absorption, distribution, metabolism and excretion of toxic substances in animal and human models (Chen et al., 2023). The 3Rs of animal use (refinement, replacement and reduction) are the guiding principles ensuring the ethical use of animals for research and scientific testing (Naderi et al., 2012). Toxicokinetics data is crucial for comprehending toxicity tests performed, identifying interspecies variations and validating the 3Rs (Creton et al., 2009). Additionally, TK data can also be obtained from absorption and distribution assessed via *in vitro* testing thus eliminating *in vivo* testing of toxic substances in animals. This is in accordance with the revised OECD Test Guideline 417. Additionally, for *in vitro* to *in vivo* extrapolation, TK analysis is crucial and can be used as input parameters of Physiologically Based Toxicokinetic Modelling (PBTK) for holistic risk assessment (Coecke et al., 2013).

1.1.1 *In vivo* studies

Several *in vivo* studies to determine the toxicity and toxicokinetics of enniatins have been conducted on various animal models, like chickens, pigs and rodents. For toxicological investigations, among these animal models, rodents are the most selected models. (Parasuraman, 2011). The several reasons for the selection of rodents for toxicity studies are short life cycles, genetic similarities to humans, small size and shorter gestation periods (Bryda, 2013). Prolonged exposure to mycotoxins can lead to adverse effects such as reduced immunity, feed refusal and reduced reproductive abilities which was seen in tested animal (Council for Agricultural Science and Technology, 2003). One of the first *in vivo* studies was conducted on immuno-compromised (HIV-infected) mice by administering a mixture of ENNs (A1, B, B1) into them. The mice received a dose of ENNs mixture of 10 to 40 mg/kg per day per body weight via intraperitoneal injection every 8 hours for 6 days. Acute toxicity was observed which ultimately resulted in the mortality of mice (McKee et al., 1997).

In another study, ENNA (465 mg/kg of food) was fed to Wistar rats, for 28 days and its immunotoxicity results revealed variations in peripheral blood lymphocytes (PBL) as there was a reduction in cytotoxic T cells (CD8+) and an increase in helper T cells (CD4+). The authors concluded that ENNA downregulated the expression of surface antigens of PBL and lowered immunity in Wistar rats (Juan et al., 2014). However, in another study when ENNA was

administered at a lower dose of 20.9 mg/kg body weight per day for 28 days in Wistar rats, no adverse effects were reported (Manyes et al., 2014). When 5 mg/kg body weight of ENN B was intraperitoneally administered in mice for two consecutive days to examine the distribution of ENNB in tissues, the results showed the highest accumulation of ENNB (2.9 ± 0.6 µg/kg) in the liver and the lowest in the kidney (0.1± 0.01 µg/kg). However, no acute tissue damage was observed. Accumulation in the liver suggested that it acts as the main organ for detoxification (Rodríguez-Carrasco et al., 2016).

Furthermore, the oral administration of a mixture of enniatins containing A (1.19 mg/kg body weight), A1 (2.16 mg/kg body weight), B (1.03 mg/kg body weight) and B1 (1.41 mg/kg body weight) to Wistar rats resulted in no serious effects after 8 hours (Escrivá et al., 2015). In a different study, the oral and intravenous administration of ENNB and ENNB1 (0.2 mg/kg body weight) was carried out in broiler chicken and *in vivo* toxicokinetics analysis was performed. Low absolute oral bioavailability values (F) were observed for both enniatins (ENNB: 0.11, ENNB1: 0.05). The half-lives and volume of distribution of ENNB and ENNB1 in broiler chickens are given in Table 2 below.

Table 2: Half-life (T1/2, h) and Volume of Distribution (Vd, L/kg) of ENNB and ENNB1 in Broiler chickens after intravenous (IV) and oral (PO) administration.

TK Parameters	ENNB		ENNB1	
	IV	**PO**	**IV**	**PO**
T1/2 (h)	3.32	2.87	2.63	1.49
Vd (L/kg)	33.91	29.39	25.09	14.36

The high half-lives ($T_{1/2}$) were observed for both routes of administration due to the high volume of distribution (Vd). This indicated that both the ENNs (ENNB, ENNB1) were rapidly distributed in broiler tissues (Fraeyman et al., 2016). However, the toxicokinetic studies of ENNB1 (0.05 mg/kg body weight oral/IV) in pigs revealed contrasting results compared to the previous study as high absolute oral bioavailability (0.91), lower half-life (0.24 hours) and lower volume of distribution were observed for ENNB1 in pigs highlighting high inter-species variability and the importance of species-specific studies (Devreese et al., 2014). The oral administration of ENNA (5mg/kg body weight) in rats resulted in oral bioavailability of 46%

and slow absorption, as it took 4 hours for ENNA to reach its peak concentration of 116.3 ng/mL (Bhateria et al., 2022).

1.1.2 *In vitro* studies

In vitro studies were carried out to investigate the cytotoxic effects of ENNs (A, A1, B, B1) in Caco-2 cells. The cytotoxic effects tested involved determining whether reactive oxygen species production is responsible for effects like cell damage, disruption of cell cycle, apoptosis and necrosis. The results revealed that when Caco-2 cells were exposed to 1.5μM-3μM of the four enniatins individually, excessive production of ROS was observed, causing membrane lipid peroxidation (LPO) (Ferrer et al., 2009). LPO affects membrane integrity resulting in cell dysfunction and damage. After incubating cells with ENNA, A1 and B1 for 24 hours, cell cycle arrest at the G2/M phase was detected. The cell cycle was arrested at the S phase after 72 hours of exposure to all four enniatins. Early apoptotic cells and necrotic cells were increased after 24 hours to 48 hours and 72 hours of exposure respectively (Prosperini et al., 2013).

Elevated absorption was observed when ENNB1 (4.8 μM) was transported across the Caco-2 cell layer in the basolateral to apical direction suggesting the involvement of efflux transporters like P-glycoprotein (Pgp) and multidrug resistance-associated protein 2 (MRP 2). However, a decrease in the permeability of ENNB1 across the Caco-2 cell layer (apical to basolateral direction) was observed as these transporters located on the apical side involved are efflux pumps responsible for pumping ENNB1 out of the cell leading to reduced absorption (Ivanova et al., 2010).

Various studies have been conducted to determine the metabolic profile of the ENNs. Cytochrome P450 enzymes are involved in phase I metabolism. CYP reaction phenotyping experiments can be carried out to determine which CYP isoforms are required for the metabolism of the compound. ENNA underwent 74% of *in vitro* metabolism via CYP3A4 enzyme in rat and human microsomes, followed by CYP1A2, CYP2C9 and CYP2E1 (Bhateria et al., 2022). Isoforms CYP3A4, CYP2C19, and CYP1A2 in human liver microsomes mediated the metabolism of ENNB (Fæste et al., 2011). ENNB1 was also metabolised in vitro by CYP3A4 isoform in human hepatic microsomes (Ivanova et al., 2019). Furthermore, the phase I metabolism of ENNB was studied in human, dog and rat hepatic microsomes. In total, twelve metabolites were identified, among which, five were found to be monohydroxylated, two were demethylated and four were carboxylated. The majority of N-demethylated and

monooxygenated metabolites were produced in rat hepatic microsomes after incubation with ENNB, in human microsomes it was an accumulation of all the types of metabolites, while in dog microsomes it was predominantly carboxyl metabolites. Three common metabolites formed in all the liver microsomes tested were oxygenated metabolites. Four common metabolites formed in human and rat microsomes were oxygenated and desmethyl metabolites. Five common metabolites formed only in human and dog microsomes were a result of multiple oxidations. These results indicated that the type of metabolites formed were species-specific (Ivanova et al., 2011).

The toxic effects of enniatins on animals can be assessed through the investigations of their toxicokinetics characteristics. The risk assessment carried out by the European Food Safety Authority (EFSA) identified that the presence of enniatins in high concentrations in food and feed items, along with evidence of toxicity, indicate potential harmful effects on human and animal health. Furthermore, the EFSA Panel on Contaminants in the Food Chain (CONTAM) concluded that short-term exposure to enniatins is not of any concern, but chronic exposure might pose a potential risk (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2014). Given the insufficiency of data on these toxins, EFSA highlighted the need to perform additional *in vivo* toxicity studies and link them to vitro toxicity studies. Furthermore, toxicokinetic investigations of enniatins in animal models like rodents can be extrapolated to humans through physiologically based toxicokinetic modelling (PBTK), thereby aiding the human risk assessment studies of enniatins (Bhateria et al., 2022).

1.2 Plasma Protein Binding

The degree of compound binding to plasma proteins has a significant impact on its absorption, distribution and accumulation in the target tissues. Toxicity is determined by the unbound/free fraction (f_{ub}) of toxin which can be transported across cell membranes and reach their target organs (Smith et al., 2010). Additionally, the degree of protein binding can also affect clearance as only the free fraction of the compound can be eliminated (Levy, 1980). Key proteins in blood plasma include lipoproteins, α1-acid glycoprotein and the most prevalent serum albumin. Generally, acidic and neutral compounds show greater binding affinity towards albumin, while basic compounds bind to α1-acid glycoprotein and lipoproteins (Lobell & Sivarajah, 2003). Several techniques are used to measure plasma protein binding including ultrafiltration, ultracentrifugation and equilibrium dialysis (ED). Among these methods, equilibrium dialysis

is preferred over the other techniques as it requires lower plasma volumes and limits the extent of unspecific binding. (Kariv et al., 2001; Pacifici & Viani, 1992). However, long equilibration times and low sample processing capacity are the major drawbacks associated with ED. Rapid equilibrium dialysis is utilized instead to determine the concentrations of bound and free fractions of the compound. It consists of a Teflon base plate and disposable dialysis cells/inserts with a greater surface area-to-volume ratio compared to traditional ED methods. This results in elevated sample throughput and shorter equilibration periods (Waters et al., 2008). F_{ub} acts as an important input parameter in physiologically based toxicokinetic models (PBTK) crucial for performing risk assessment studies (Dimitrijevic et al., 2023). The plasma protein binding in the RED device is illustrated in Figure 2.

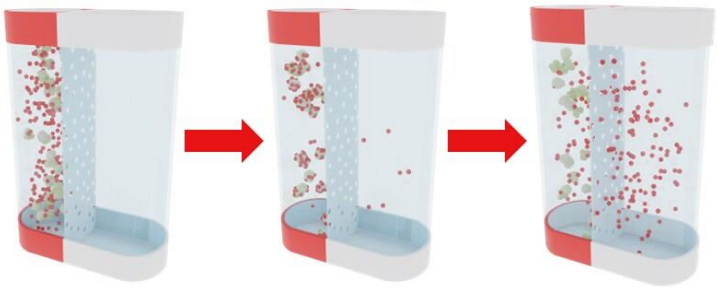


Figure 2: Plasma protein binding in RED Device.

Due to their physicochemical properties, enniatins are expected to be highly bound to plasma proteins. From one of the studies it was found that ENNA showed high binding affinity to plasma proteins (99.43% ± 0.05% in rat plasma and 98.94% ± 0.01% in human plasma). A single-use Rapid Equilibrium Dialysis (RED) device was used to determine the extent of binding of ENNA to the plasma proteins (Bhateria et al., 2022). However, the assessment of plasma protein binding of other prevalent enniatins in different species remains unexplored, resulting in a significant knowledge deficit.

The correct quantification of enniatins is crucial for performing the RED assay. A set of preliminary solvent extraction studies were carried out to determine the amount of enniatins using Liquid chromatography/Mass spectrometry (LC/MS). These experiments were focused on evaluating the efficiency of the organic solvent in the extraction of enniatins from serum-free and serum-containing samples. For most of the extraction experiments, acetonitrile (ACN) is a commonly used solvent. Following this, the recovery rates were calculated for the enniatins. Recovery was considered acceptable in the range of 70 % – 120 % (Serrano et al.,

2015). The optimization of these extraction tests is important in determining the recovery rates and binding affinity of ENNs to plasma proteins.

The aim of this project was to first develop and optimize the solvent extraction method for the four enniatins (A, A1, B, B1) using LC/MS to obtain optimal recovery rates. Furthermore, to evaluate the plasma protein binding of the enniatins the RED device was utilized and the unbound fractions were calculated.

Chapter 2: Materials and Methods

2.1 Chemicals and solvents

Enniatin A (m. wt: 681.9 g/mol), Enniatin A1 (m.wt: 667.9 g/mol) Enniatin B (m.wt: 639.8 g/mol) and Enniatin B1 (m.wt:653.8 g/mol) were obtained as powders from PARC and zearalenone (318.36 g/mol) was obtained from Sigma Aldrich. Dimethyl sulfoxide (DMSO) ACS grade was obtained from Merck (Merck Germany 102952). Hank's Balanced Salt Solution (HBSS) and Minimum Essential Medium (MEM) without phenol red were purchased from Gibco (Gibco/14025-050), acetonitrile LC grade (ACN-001204102BS), formic acid (99-100%) was procured from VWR chemicals (VWR chemicals/20318.297P). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and Bovine Serum Albumin (BSA) were obtained from Sigma Aldrich (A2153-100G). FBS (Fetal Bovine Serum) was obtained from Capricorn Scientific (cat no: FBS-12A). Phosphate buffered saline 1X (PBS) was obtained from Invitrogen (cat no: 10010015). The Pierce Rapid Equilibrium Dialysis (RED) device reusable plate with 48 inserts of 8K MWCO was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Master stock solutions of ENNs (A, A1, B and B1) were prepared in DMSO to obtain the concentration of 10mM. Appropriate working solutions were obtained when the master stock solutions were diluted with DMSO. The aliquots from each working solution of ENNs were mixed to obtain the composite dosing mixture solution. All the prepared solutions were stored in amber glass vials at -20ºC.

2.2 Analytical Chemistry

2.2.1 Extraction experiments

(i) Pilot extraction study (Individual ENNs)

The first pilot extraction study was carried out for ENNB1 from 25% FBS solution in amber glass vials, glass tubes and eppendorf tubes. In the second pilot study extraction of all four ENNs (A, A1, B, B1) was carried out from different matrices (MEM, HBSS, FBS (2 %, 50 %, 100 %)). Additionally, the abiotic degradation under incubation time periods of 1 to 2 hours was also tested. Extraction solvent acidified ACN (0.1% formic acid) was added to the ENNB1 and ENNs sample mixture in 3/1 (v/v) ratio to achieve the final concentration of 1 µM of ENNB1 and individual ENNs in the first and second pilot study respectively. Both the pilot experiments were performed in triplicates and the samples underwent vortexing for 10 seconds for homogenization (Eppendorf Thermomixer F1.5) and centrifuged (Salmenkipp) at 3,700 xg

for 15 minutes at 4ºC. The supernatants obtained were collected in amber autosampler vials and subjected to LC/MS analysis.

(ii) Extraction studies (Mixture of ENNs)

The extraction tests of four ENNs (A, A1, B, B1) in a mixture were carried out from two types of exposure media consisting of HBSS. The BSA free-exposure media with the ENNs mixture and BSA (4g/L) containing exposure media with the ENNs mixture were the positive controls and test samples respectively. Similar to the pilot studies acidified ACN (900 µL) was added to the 300 µL positive controls and test samples in 3/1 (v/v) ratio to obtain the final concentration of 0.25 µM of individual ENNs.The extractions were performed in triplicates and the test samples were incubated on an orbital plate shaker (Heidolph Titramax 1000) for 30 minutes in the dark and vortexed for 5 minutes. Finally, they were centrifuged for 15 minutes at 8000 xg at room temperature. The supernatants obtained were injected into the LC/MS system. The % STD for positive control samples to nominal concentration and the recovery % for test samples to positive control samples were calculated as:

$$\%\ \boldsymbol{STD\ to\ nominal\ concentration} = \boldsymbol{ABS}\left(\frac{\boldsymbol{Actual\ conc\ of\ positive\ controls - nominal\ conc}}{\boldsymbol{nominal\ conc}}\right) * \mathbf{100} \qquad (\boldsymbol{Eq.1})$$

$$\boldsymbol{Recovery}\ \%\ \boldsymbol{to\ positive\ control} = \left(\frac{\boldsymbol{Actual\ concentration\ of\ test\ sample}}{\boldsymbol{mean\ of\ positive\ control}}\right) * \mathbf{100} \qquad (\boldsymbol{Eq.2})$$

(iii) Optimization of the extraction studies

The optimization tests were performed to re-confirm if the acidity of acetonitrile impacts its efficiency in improving the extraction of ENNs when added to different sample mixtures in 3/1 (v/v) ratio. In the first optimization test, the extraction of four ENNs (A, A1, B, B1) in a mixture were carried out from BSA free-exposure media containing HBSS. In the second optimization test, the extraction of four ENNs in a mixture was carried out from BSA free-exposure media containing HBSS or MilliQ water and exposure media consisting of HBSS and 4g/L BSA. Acidified ACN /ACN (300 µL) was added to the 100 µL BSA free sample mixtures in a 3/1 (v/v) ratio and acidified ACN /ACN (100 µL) was added to the 100 µL BSA free sample mixtures in 1/1 (v/v) ratio as well, to achieve the final concentration of 0.25 µM or 0.5 µM of individual ENNs respectively. For the extraction with BSA, 600 µL acidified ACN /ACN was added to 200 µL sample mixtures to achieve a final concentration of 0.25 µM of individual

ENNs. Both the optimization experiments were performed in triplicates and the test samples with BSA were subjected to the same centrifugation (Eppendorf Centrifuge 5430) parameters as mentioned in section (ii). Additionally, these BSA samples also underwent overnight freezing and were centrifugated after 24 hours. The supernatants were collected and analyzed with LC/MS. The recovery rates for the first optimization test were calculated from equation 1 and for the second optimization test the % STD for positive control samples to nominal concentration and the recovery % for test samples to positive control samples were calculated from equations 1 and 2 respectively.

(iv) Extraction studies using larger sample volumes and determination of unspecific binding

The final extraction tests were performed using larger volumes of sample mixtures in glass tubes to determine whether the extraction efficiency was influenced by the increased sample volumes. Zearalenone was used as the internal standard. The dosing mixture solution with ENNs (10 µL) was spiked into five experimental conditions which were acidified ACN with PBS (positive control samples (7990 µL); Condition A), PBS (PBS controls (1990 µL); Condition B), PBS (Abiotic (1990 µL); Condition C), PBS with 4g/L BSA solution ((1990 µL); Condition D) and PBS with 3T3-L1 fibroblasts (PBS/cells (1990 µL); Condition E). All the conditions were performed in triplicates. For the sampling of tubes for conditions B, D and E 6 mL acidified ACN (with 2µM ZEN) was added and the test conditions D and E underwent 30 minutes of incubation on the orbital shaker. This was followed by an additional 30 minutes of incubation at -20^{o}C and centrifugation for 30 minutes at 8000 xg. The PBS samples tested for abiotic conditions (Condition C) were left uncovered and incubated in the dark at room temperature for four hours. After the incubation period 6 mL acidified ACN (2µM ZEN) was added to the sample mixture and the solutions were vortexed for 30 seconds for homogenization. Furthermore, the unspecific binding of enniatins to the 12 well plates was assessed by spiking 10 µL dosing mixture to 1990 µL of PBS in wells. The plates were incubated for 4 hours in the dark at room temperature. After incubation, for the well plating sampling the contents from each well were taken out into separate glass tubes and 6 mL acidified ACN (2µM ZEN) was added to it and vortexed for 30 seconds. Furthermore, acidified ACN (2µM ZEN) was added to the wells and then collected in separate glass tubes containing PBS and centrifuged for 30 seconds. Similar to the previous experiments acidified ACN (2µM ZEN) was added to the sample mixtures in a 3/1 (v/v) to achieve the final concentration of 0.5 µM of individual ENNs. The resulting aliquots were collected from all the conditions and analyzed using LCMS. The % STD for positive control samples to nominal concentration and

the recovery % for test samples to positive control samples were calculated from equations 1 and 2 respectively.

2.2.2 LC/MS Instrumentation and Optimization

LC/MS analysis of enniatins was performed using Shimadzu LCMS 8045 (Shimadzu, Japan). A Kinetic C18 (1.7 µm x 100 Å x 150 mm x 2.1 mm) Phenomenex column equipped with a guard column holder (Phenomenex AJ0-9000) and guard column (Phenomenex AJ0-8782) was utilized. The LC column temperature was set at 40ºC, the injection volume was 1 µL with sampling speed of 0.5 µL/min and a constant flow rate of 0.3mL/min was maintained. The mobile phases were composed of (A) MilliQ water with 0.1 % formic acid and (B) acetonitrile with 0.1% formic acid. The LC gradient elution started with 100% A (0-2 minutes), then 40% A and 60% B (2-9 minutes), 2% A and 98% B (9-12 minutes) and finally back to 100% A (12-18 minutes). The flow rate and total run time for each sample analysis were 0.3 mL/min and 18 minutes respectively. Mass Spectrometry was performed for ion detection using an electrospray ionization (ESI) source in positive ion mode with multiple reaction monitoring (MRM) mode.

The MS parameters set for the quantification of the enniatins were: nebulizing gas flow rate (3 L/min), heating gas flow rate (10 L/min), interface temperature (300ºC), desolvation temperature (526ºC), DL temperature (250ºC), heat block temperature (400ºC) and drying gas flow rate (10 L/min). Table 3 provides the retention times, precursor and product ions and collision energies of the tested enniatins. Data collection and analysis was done using LabSolutions CS software. For the optimized extraction studies a smaller LC column (Kinetic C18 1.7 µM x 100 Å x 50 mm x 2.1 mm Phenomenex column) was utilized.

Table 3: Retention times, precursor and product ions, collision energies of enniatins.

Enniatin	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)
ENNA	11.2	682.60	210.2	-30
ENNA1	10.8	668.60	210.2	-25
ENNB	9.8	640.45	196.2	-21
ENNB1	10.3	654.45	196.2	-32

2.2.3 Method performance

The validation of LC/MS method performance was performed by evaluating parameters like linearity, LOQ and LOD. The linearity of the method is proved when the obtained responses are directly proportional to the tested sample concentrations. Determination of linearity involves analyzing the calibration curve and the coefficient of determination (R^2) of >0.9 indicates linearity. The standard calibration curves were prepared for different concentration ranges by serial dilutions for each extraction experiment. For the first extraction studies with mixture of ENNs (section ii), two sets of calibration curves at five concentration levels (62.5, 125, 250, 500, 1000 nM) were prepared and measured at the start and end of the LC/MS run. For the first and second optimization tests (section iii), two and four sets of calibration curves were prepared respectively at ten concentration levels (0.0244, 0.0976, 0.3906, 1.5625, 6.25, 25, 100, 400, 1600 and 8000nM) and measured at the beginning and end of the LC/MS run. Finally, for extraction studies with larger volumes (section iv) calibration curves at ten concentration levels (0.0244, 0.0976, 0.3906, 1.5625, 6.25, 25, 100, 400, 1600 and 6400nM) were prepared and measured at the beginning and end of the LC/MS run. These calibration curves were generated using both Lab Solutions CS software and Microsoft Excel where peak areas (mV* min) were plotted against the nominal concentrations (nM) of the calibration standards. When the internal standard (zearalenone) was included in the experiment, the ratio of peak area (analyte/IS) was considered and plotted against the nominal concentrations of the calibration standards. The curves were plotted on a logarithmic scale and were used for the quantification of enniatins. LOD (Limit of Detection) is the "smallest concentration of analyte that can be detected but not quantified and clearly distinguished from zero" (Thompson et al., 2002) and LOQ (Limit of Quantification) is the "smallest concentration of analyte which can be detected and quantified with acceptable trueness and precision" (EURACHEM, 1998). The European Union Reference Laboratory (EURL) for mycotoxins in feed and food set guidelines for the estimation of the limit of detection (LOD) and limit of quantification (LOQ) of mycotoxins (European Commission. Joint Research Centre., 2016). The LOD and LOQ were calculated from the blank signals as:

$$\boldsymbol{xLOD = 3.9 * \frac{s_{y,b}}{b}} \qquad (\boldsymbol{Eq.3})$$

$$\boldsymbol{xLOQ = 3.3 * xLOD} \qquad (\boldsymbol{Eq.4})$$

Where :

***xLOD*:** Limit of detection

***$S_{y,b}$*:** Standard deviation of blank signals

***b*:** Slope of the calibration curve

***xLOQ*:** Limit of quantification

2.3 Assessment of plasma protein binding of enniatins using RED

A preliminary Rapid Equilibrium Dialysis assay was performed to evaluate the plasma protein binding of enniatins and its non-specific binding to the RED device. The dosing mixture solution with ENNs (10 µL and 15 µL) was spiked to 1990 µL and 2985 µL acidified ACN with PBS to obtain the appropriate sample and buffer RED controls respectively. The RED sample controls with BSA were obtained after 10 µL dosing mixture solution was added to 490 µL PBS with BSA solution followed by the 1500 µL addition of acidified ACN (2µM ZEN). The RED test samples without BSA were prepared in triplicates by adding 490 µL and 735 µL of PBS to the sample and buffer compartments of the RED insert respectively. The dosing mixture solution with ENNs of 10 µL and 15 µL was then spiked in these sample and buffer chambers respectively. The RED test samples with BSA were also analyzed in triplicates by adding the test sample (490 µL) and PBS dialysis buffer (750 µL) to separate sample and buffer chambers respectively. The dosing mixture solution with ENNs of 10 µL was then spiked in these sample chambers. After 6 hours of incubation (test samples without BSA) and incubation time points of 6 hours and 24 hours (test samples with BSA) at 37°C, the contents were collected from the sample and buffer chambers and transferred to separate amber glass vials. To these vials containing sample and buffer contents, 1500 µL and 2250µL acidified ACN (2µM ZEN) were added respectively to achieve 3/1 (v/v) ratio and the final concentration of 0.5 µM of individual ENNs. Additionally, to evaluate potential unspecific binding to the RED apparatus, 1500µL and 2250 µL of acidified ACN (2µM ZEN) were added to the sample and buffer chambers and then collected in the vials already containing 500 µL and 750µL PBS respectively. The test samples with BSA were subjected to the same centrifugation conditions as in conditions D and E (Section 2.2.1, iv). The resulting aliquots were collected and analyzed using LCMS.

(i) Calculations of recovery rates and unbound fractions of ENNs

The recoveries and free fraction of enniatins were determined by considering the concentrations of enniatins in the sample chamber, buffer chamber and control concentration. For the test samples with BSA, enniatins bind to the plasma proteins in the sample chamber and the unbound/free fraction of enniatins crosses the semipermeable membrane between the sample and the buffer chamber to reach the buffer chamber. The system is set to reach equilibrium when the concentration of the free fraction of ENNs in the sample chamber is equal to the concentration of the free fraction of enniatins in the buffer chamber. The concentration in the sample chamber represents the total concentration (bound fraction+free fraction) and the concentration in the buffer chamber represents the free concentration (free fraction) of enniatins.

The free/unbound fraction was calculated as:

$$\%\ \boldsymbol{free\ fraction} = \left(\frac{\boldsymbol{conc(\ buffer\ chamber)}}{\boldsymbol{conc\ (sample\ chamber)}}\right) * \mathbf{100} \qquad (\boldsymbol{Eq.5})$$

The bound fraction was calculated as:

$$\%\ \boldsymbol{bound\ fraction} = \mathbf{100} - \%\ \boldsymbol{free\ fraction} \qquad (\boldsymbol{Eq.6})$$

The recovery of each sample from the RED assay was calculated as:

$$\boldsymbol{Recovery}\ \% = \left(\frac{(\boldsymbol{conc\ (buffer\ chamber)} * \mathbf{750\mu L} + \boldsymbol{conc\ (sample\ chamber)} * \mathbf{500\mu L})}{\boldsymbol{conc\ (sample\ control)} * \mathbf{500\mu L}}\right) * \mathbf{100} \qquad (\boldsymbol{Eq.7})$$

2.4 Statistical Analysis

The experiments were conducted in technical triplicates, and the data obtained was presented as mean values with standard deviations indicated by error bars. The differences between groups were evaluated using suitable statistical analysis using various methods, including two-way ANOVA with multiple comparisons using Dunnett, Tukey and Sidak tests, multiple t-tests with the Holm-Sidak method and paired t-tests. Statistical significance was determined at $p<0.0332$ (*), $p<0.0021$(**), $p<0.0002$(***) and $p<0.0001$(****). The data analysis was performed using GraphPad Prism 8.0.2 software (GraphPad Software, San Diego, CA, USA) and Microsoft Excel.

Chapter 3: Results and Discussion

3.1 Analytical Chemistry

3.1.1 Extraction experiments

(i) Pilot Extraction Studies (Individual ENNs)

The first pilot study showed that amber glass vials (65 % -77 %) gave recovery rates of ENNB1 greater than glass tubes (34 % - 41 %) and eppendorf tubes (24 %) with the recovery rates being the lowest from eppendorf tubes as shown in Table 4. The recoveries for all the conditions tested in triplicates were not satisfactory suggesting low efficiency of the extraction solvent acidified ACN to extract ENNB1. This can be attributed to two primary factors. Firstly, ENNB1 exhibited a certain level of binding with the eppendorf tubes made of plastic, leading to the lowest recovery rates compared to glass containers, which demonstrated relatively higher recovery rates. Secondly, inadvertent exposure of ENNB1 to light, given its light sensitivity, likely contributed to the diminished recovery rates. Due to these considerations, subsequent sets of experiments were repeated and exclusively utilized amber glass vials for the extraction process.

Table 4: Recovery rates of ENNB1 from 25% FBS solution under various conditions.

Type of container	Conditions	Recovery (%)	Mean recovery (%)	Standard deviation
Amber glass vials	**Open, 1 hour**	99.60 67.41 65.67	77.56	19.10
Amber glass vials	**Closed, 2 hours**	0.39 106.91 89.14	65.48	57.06
Eppendorf tubes	**Closed, 2 hours**	74.87 0.39 0.40	25.22	42.99
Glass tubes	**Open, 1 hour**	29.21 45.13 40.52	38.29	8.19
Glass tubes	**Closed, 2 hours**	46.05 44.34 32.96	41.12	7.11

The second pilot study focussing on the extraction of all four ENNs from different matrices showed high efficiency of acidified ACN in extracting ENNs from HBSS, MEM and 2 % FBS by obtaining high recovery rates > 80 %. These tests were also performed in triplicates and the high extraction may be due to the negligible interference of HBSS, MEM and lower

concentrations of FBS solution in the extraction process of ENNs. However, the extraction of ENNs from 50 % and 100 % FBS gave some varying results. High recovery rates of > 80 % were observed for ENNA, ENNA1 and ENNB from 50 % FBS but ENNB1 displayed lower recovery rates. Extraction from 100% FBS yielded different results showing ENNA having the lowest recovery followed by ENNA1 and ENNB1 with all three enniatins having recovery below 80 %, while ENNB showed the highest recovery rate exceeding 80 %. The results for ENNA, ENNA1, ENNB and ENNB1 are presented in Table 5, Table 6, Table 7 and Table 8 respectively. This discrepancy suggests that the lipophilicity of ENNA, ENNA1, ENNB1, and ENNB plays a role. The highest Log P value (4.79) and lipophilicity of ENNA among the ENNs potentially caused greater binding to plasma proteins. Conversely, ENNB has the lowest Log P value of 3.81 among the ENNs and showed the highest recoveries. This demonstrated the need to enhance the extraction efficacy of acidified ACN for all ENNs across various matrices. The effect of abiotic degradation on ENNs recovery was minimal as no significant difference was observed in the recoveries between the open and closed conditions in both pilot studies. Additionally, some degree of variability was observed among the triplicates of each condition resulting in very high standard deviations of samples in both the studies. The R^2 value of 0.9805 (ENNB1) was obtained from the calibration curve from the first pilot study. Subsequently, high R^2 values of 0.9987, 0.9997, 0.9993, and 0.9997 were obtained for ENNA, ENNA1, ENNB and ENNB1 respectively from the resulting calibration curves from the second pilot study indicating linearity.

Table 5: Recovery rates of ENNA from different matrices under various conditions.

Conditions	Recovery (%)	Mean Recovery (%)	Standard Deviation
ENNA+HBSS(Open, 1 hour)	98.37 97.43 99.14	98.31	0.85
ENNA+HBSS(Closed, 2 hours)	97.52 96.29 97.25	97.02	0.64
ENNA+MEM (Closed, 2 hours)	98.78 97.61 96.05	97.48	1.36
ENNA+ 2% FBS (Closed, 2 hours)	78.28 97.35 95.79	90.47	10.58
ENNA + 50% FBS (Closed, 2 hours)	93.88 78.03 92.39	88.10	8.75

ENNA +100% FBS (Closed, 2 hours)	29.30 54.17 95.35	59.60	33.36

Table 6: Recovery rates of ENNA1 from different matrices under various conditions.

Conditions	Recovery (%)	Mean Recovery (%)	Standard Deviation
ENNA1+HBSS(Open, 1 hour)	99.28 80.84 96.68	92.27	9.98
ENNA1+HBSS(Closed, 2 hours)	94.47 94.34 94.20	94.34	0.1
ENNA1+MEM (Closed, 2 hours)	94.23 95.18 91.96	93.15	1.65
ENNA1+ 2% FBS (Closed, 2 hours)	95.28 97.29 86.89	93.15	5.51
ENNA1 + 50% FBS (Closed, 2 hours)	79.56 99.84 96.83	92.08	10.94
ENNA1 +100% FBS (Closed, 2 hours)	99.24 61.41 43.87	68.17	28.29

Table 7: Recovery rates of ENNB from different matrices under various conditions.

Conditions	Recovery (%)	Mean Recovery (%)	Standard Deviation
ENNB+HBSS(Open, 1 hour)	97.71 99.96 99.12	98.93	1.13
ENNB+HBSS(Closed, 2 hours)	99.65 99.78 51.16	83.53	28.03
ENNB+MEM (Closed, 2 hours)	97.91 72.11 98.00	89.34	14.92
ENNB+ 2% FBS (Closed, 2 hours)	99.39 82.88 99.91	94.06	9.68
ENNB + 50% FBS (Closed, 2 hours)	98.34 97.55 96.40	97.43	0.97
ENNB +100% FBS (Closed, 2 hours)	99.97 96.15 91.66	95.93	4.16

Table 8: Recovery rates of ENNB1 from different matrices under various conditions.

Conditions	Recovery (%)	Mean Recovery (%)	Standard Deviation
ENNB1+HBSS(Open, 1 hour)	46.95 99.62 99.70	82.09	30.42
ENNB1+HBSS(Closed, 2 hours)	99.85 99.80 76.22	91.96	13.62
ENNB1+MEM (Closed, 2 hours)	75.99 66.44 95.79	79.41	14.97
ENNB1+ 2% FBS (Closed, 2 hours)	99.83 99.96 99.93	99.91	0.06
ENNB1 + 50% FBS (Closed, 2 hours)	99.92 4.42 56.34	53.56	47.81
ENNB1 +100% FBS (Closed, 2 hours)	99.97 5.94 99.93	68.58	54.24

(ii) Extraction studies (Mixture of ENNs)

The recovery of ENNA, ENNA1, ENNB, and ENNB1 from exposure media without BSA (positive controls) was determined to be 92.34 %, 92.63 %, 89.50 %, and 99.13 %, respectively. The recovery of 123.33 %, 129.54 %, 130.53 %, and 126. 15% for ENNA, A1, B, and B1, respectively were obtained from exposure media with BSA (test samples). These results are presented in Table 9. Statistically significant difference was observed between the positive control and the test samples ($p < 0.05$). However, high standard deviation values were observed, indicating greater variability among triplicates for the samples tested. The recovery percentages for all the enniatins were above the desired threshold of >80 %, highlighting that acidified ACN was efficient in the extraction of enniatins irrespective of the matrix used. Among the enniatins, ENNB exhibited the highest recovery percentage from exposure media containing BSA, followed by ENNA1, ENNB1, and ENNA. This outcome aligned with the findings of the second pilot experiment, confirming the success of acidified ACN in extracting enniatins from exposure media, both with and without BSA. The recovery of the four enniatins is illustrated in Figure 3. R^2 values of 0.9454, 0.9127, 0.9568 and 0.9708 were obtained for ENNA, ENNA1, ENNB and ENNB1 respectively from the resulting calibration curves indicating linearity.

Table 9: Recovery of ENNs from exposure media without BSA (positive controls) and from exposure media with BSA (test samples).

Enniatin	Samples	Recovery (%)	Mean Recovery (%)	Standard Deviation
ENNA	**Positive controls**	86.40 95.38 95.23	92.34	5.14
	Test samples	137.84 174.89 57.25	123.33	60.14
ENNA1	**Positive controls**	87.02 95.54 95.34	92.63	4.86
	Test samples	138.93 191.71 57.98	129.54	67.35
ENNB	**Positive controls**	83.22 92.88 92.41	89.50	5.44
	Test samples	134.68 197.69 59.21	130.53	69.33
ENNB1	**Positive controls**	98.49 9.45 99.43	99.13	0.54
	Test samples	133.14 185.09 60.21	126.15	62.73

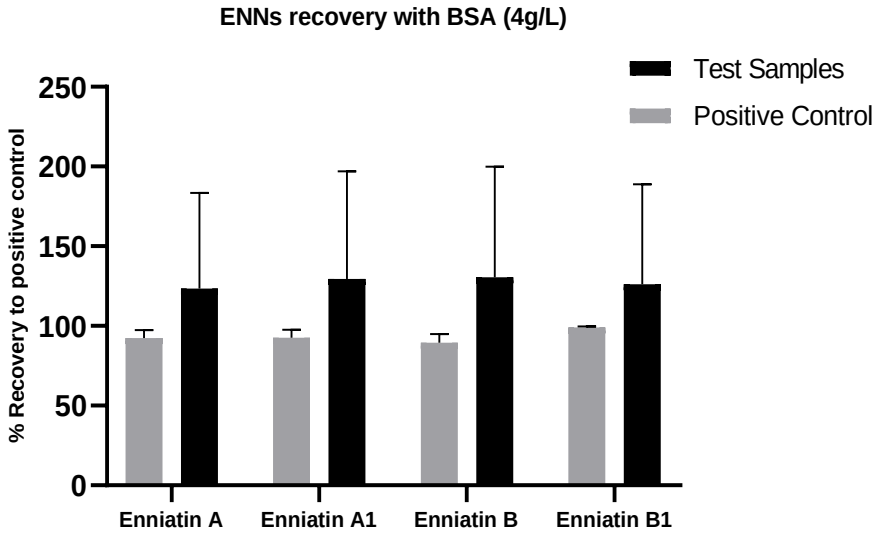


Figure 3: Recovery of ENNs from exposure media without BSA (positive controls) and from exposure media with BSA (test samples). Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation. Statistically significant difference was observed between positive control and test samples using paired t-test; $p<0.05$ (**).

(iii) Optimization of the extraction studies

In the first optimization test, the recovery of ENNA, ENNA1, ENNB, and ENNB1 from samples with the ENNs mixture prepared in BSA-free HBSS and acidified ACN at a 3/1 (v/v) ratio were determined to be 152.95 %, 145.44 %, 113.79 %, and 110.16 %, respectively. Conversely, recovery lower than the threshold of > 80 % was obtained from samples with ENNs mixture prepared in BSA-free HBSS and only acetonitrile at the same ratio i.e. ENNA (53.07 %), ENNA1(43.60 %), ENNB (53.96 %) and ENNB1(49.43 %). These results are shown in Table 10. This indicated that the extraction efficiency and recovery with acidified ACN is greater compared to acetonitrile alone. Statistical analysis revealed a significant difference between the conditions with acetonitrile and those with acidified ACN ($p< 0.0032$ (*), $p< 0.0021$(**)). This enhancement in recovery could be attributed to the acidification of acetonitrile, which potentially increases the solubility of ENNs and induces ionization, thereby improving extraction efficiency. Additionally, the introduction of acidified ACN into the ENNs mixture, prepared in HBSS at 3/1 (v/v) ratio, resulted in enhanced recovery rates compared to an equivalent addition (1/1 (v/v) ratio) of acidified ACN/acetonitrile to the ENNs mixture in HBSS. This suggested that a greater volume of extraction solvent results in increased extraction efficiency. Notably, the obtained recovery rates for 3/1 (v/v) ratio for ENNA and ENNA1 surpassed the recommended range of 70 % - 120 %, as specified (Serrano et al., 2015), indicating a likelihood of contamination/carryover during the extraction process. Overall, the recovery for all ENNs remained above 80 %, when acidified ACN was added at a 3:1 ratio into the ENNs mixture prepared in HBSS highlighting the efficiency of the extraction of enniatins. Similar to previous experiments, high standard deviation values were noted, indicating greater variability among triplicates for the conditions tested. The recovery of the four enniatins based on the acidity of the extraction solvent is illustrated in Figure 4. R^2 values of 0.9742, 0.9717, 0.9850 and 0.9781 were obtained for ENNA, ENNA1, ENNB and ENNB1 respectively from the resulting calibration curves indicating linearity.

Table 10: Recovery of ENNs from solvent combinations with acetonitrile and acidified ACN.

Enniatin	Samples	Recovery (%)	Mean Recovery (%)	Standard Deviation
ENNA	**Acetonitrile (3:1)**	36.29 61.93 61.00	53.07	14.54
	Acidified ACN (3:1)	160.99 170.03 127.83	152.95	22.21

ENNA1	**Acetonitrile (3:1)**	30.13 48.42 52.24	43.60	11.82
	Acidified ACN (3:1)	160.62 142.17 133.54	145.44	13.83
ENNB	**Acetonitrile (3:1)**	37.72 62.07 62.09	53.96	14.06
	Acidified ACN (3:1)	126.15 112.73 102.50	113.79	11.86
ENNB1	**Acetonitrile (3:1)**	40.52 52.43 55.36	49.44	7.86
	Acidified ACN (3:1)	126.42 108.38 95.69	110.16	15.44

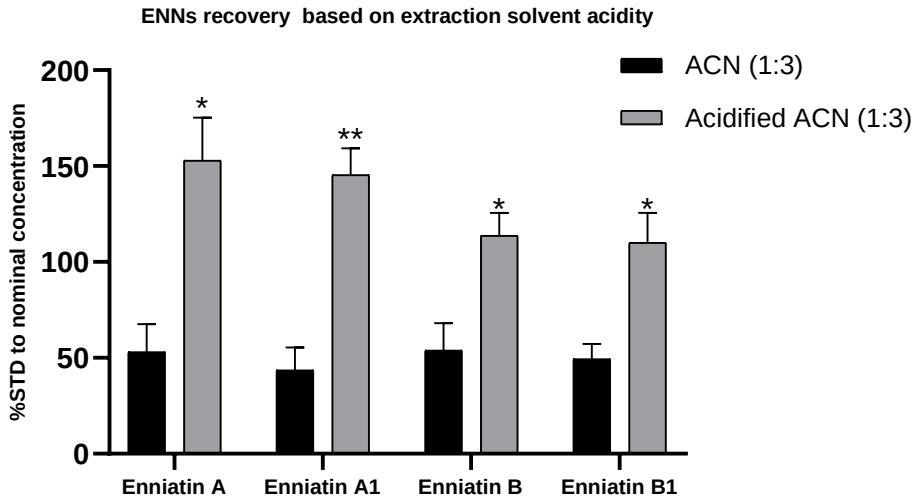


Figure 4: Recovery of ENNs from solvent combinations with acetonitrile and acidified ACN.Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation. Statistically significant difference was observed between the two groups for each enniatin using multiple t-tests; p<0.0032 (*), p<0.0021 (**).

In the second optimization test, the recovery for each enniatin utilizing acidified ACN and acetonitrile with MilliQ water ranged from 57.90 % to 66.56 % and 59.71 % to 67.76 %, respectively. Similarly, for HBSS, the recovery rates with acidified ACN and acetonitrile varied between 52.86 % to 59.77 % and 53.48 % to 60.20 %, respectively. These findings are shown in Table 11 and Figure 5 . These findings indicated that the recoveries of enniatins from both

MilliQ and HBSS (without BSA) fell below the desired threshold of >80 %, signifying inefficient recovery. HBSS showed slightly lower recovery compared to MilliQ possibly due to the HBSS containing salts and buffers that might interfere with extraction efficiency. This contradicted earlier results where recovery rates exceeding 80% were achieved for solvent combinations with ENNs mixture (with HBSS) and acidified ACN in 3/1 (v/v) ratio. However, statistically significant difference was noted between the groups using HBSS and those using MilliQ for each enniatin ($p<0.0001$ (****)). Lower standard deviation values were obtained as well, indicating reduced variability among triplicates for the tested conditions. Notably, there was no observed phase separation in any of the tested solvent combinations.

Table 11: Recovery of ENNs from solvent combinations (exposure media without BSA) with acetonitrile and acidified ACN.

Enniatin	Samples	Recovery (%)	Mean Recovery (%)	Standard Deviation
ENNA	**Acidified ACN/MilliQ (3:1)**	58.25 57.35 58.10	57.90	0.48
	Acetonitrile/MilliQ (3:1)	57.58 60.86 60.70	59.71	1.84
	Acidified ACN/HBSS (3:1)	52.29 53.86 52.45	52.86	0.86
	Acetonitrile/HBSS (3:1)	52.78 53.60 54.05	53.48	0.64
ENNA1	**Acidified ACN/MilliQ (3:1)**	53.77 53.68 54.82	54.09	0.63
	Acetonitrile/MilliQ (3:1)	53.54 57.04 56.58	55.72	1.90
	Acidified ACN/HBSS (3:1)	47.64 48.87 49.20	48.57	0.82
	Acetonitrile/HBSS (3:1)	48.47 48.69 48.59	48.58	0.11
ENNB	**Acidified ACN/MilliQ (3:1)**	66.61 66.26 66.81	66.56	0.28
	Acetonitrile/MilliQ (3:1)	66.96 67.66 68.66	67.76	0.85
	Acidified ACN/HBSS (3:1)	59.66 60.36 59.29	59.77	0.54

	Acetonitrile/HBSS (3:1)	59.38 60.48 60.76	60.20	0.73
ENNB1	**Acidified ACN/MilliQ (3:1)**	61.48 60.63 60.70	60.94	0.47
	Acetonitrile/MilliQ (3:1)	61.27 62.75 63.19	62.40	1.00
	Acidified ACN/HBSS (3:1)	53.94 53.71 54.71	54.12	0.52
	Acetonitrile/HBSS (3:1)	54.42 55.39 54.68	54.83	0.50

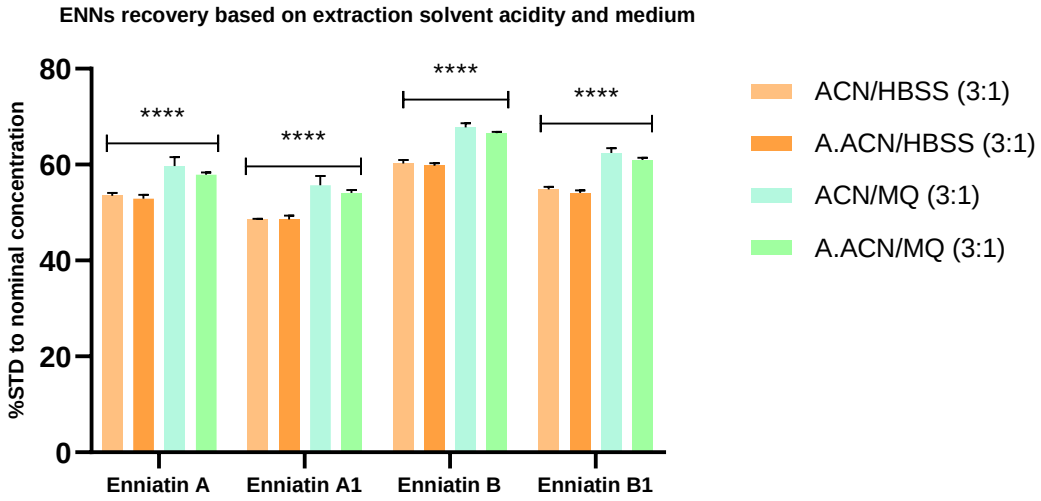


Figure 5: Recovery of ENNs from solvent combinations (exposure media without BSA) with acetonitrile and acidified ACN. Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation. Statistically significant difference was observed between the HBSS group and MIlliQ group for each enniatin using two-way ANOVA test; p<0.0001 (****).

Recovery for each enniatin from frozen samples containing BSA with acidified ACN and acetonitrile ranged from 70.35 % to 79.28 % and 72.62 % to 83.83 %, respectively. Conversely, recovery from unfrozen samples containing BSA with acidified ACN and acetonitrile was lower, ranging between 28.11 % to 33.89 % and 19.67 % to 23.62 %, respectively. These findings are shown in Table 12 and Figure 6. These findings suggested that the recoveries of enniatins from frozen samples with BSA approached >70%, indicating that lower temperatures might enhance recovery efficiency. Additionally, very high standard deviation values were

obtained hinting at considerable variability among triplicates for the tested conditions. Surprisingly, no statistical significance was observed between the frozen and unfrozen samples following a two-way ANOVA test. This visual impression of a difference might be due to high variability and type II error (false positive). Moreover, there was no statistically significant difference in the extraction efficiency between acidified ACN and only acetonitrile, regardless of the solvent combinations and temperature conditions. The potential reason for the discrepancy in recovery rates obtained with acidified ACN or ACN between the first and second optimization studies could be attributed to the preparation of two and four sets of calibration curves respectively. This variation in the number of calibration curve sets might have influenced the calculated concentrations and therefore differences in the recovery rates between the two studies were observed. R^2 values of 0.9742, 0.9982, 0.9963 and 0.9982 were obtained for ENNA, ENNA1, ENNB and ENNB1 respectively from the resulting calibration curves indicating linearity.

Table 12: Recovery of ENNs from solvent combinations (HBSS with BSA) with acetonitrile and acidified ACN from before and after freezing the samples.

Enniatin	Samples	Recovery (%)	Mean Recovery (%)	Standard Deviation
ENNA	**BSA /Acidified ACN/HBSS (3:1)**	3.55 86.10 6.22	31.96	46.91
	BSA/Acetonitrile/HBSS (3:1)	3.74 3.88 62.66	23.43	33.98
	BSA /Acidified ACN/HBSS (3:1) (After freezing)	79.78 85.57 72.42	79.26	6.60
	BSA /Acetonitrile /HBSS (3:1) (After freezing)	81.96 96.90 72.64	83.83	12.24
ENNA1	**BSA /Acidified ACN/HBSS (3:1)**	4.14 90.64 6.88	33.89	49.17
	BSA/Acetonitrile/HBSS (3:1)	4.38 4.50 61.99	23.63	33.23
	BSA /Acidified ACN/HBSS (3:1) (After freezing)	77.73 81.70 69.06	76.17	6.46
	BSA /Acetonitrile /HBSS (3:1) (After freezing)	77.20 90.35 71.06	79.54	9.85

ENNB	**BSA /Acidified ACN/HBSS (3:1)**	2.43 77.28 4.64	28.12	42.59
	BSA/Acetonitrile/HBSS (3:1)	2.62 2.68 53.74	19.68	29.50
	BSA /Acidified ACN/HBSS (3:1) (After freezing)	73.32 74.23 63.85	70.47	5.75
	BSA /Acetonitrile /HBSS (3:1) (After freezing)	72.09 83.98 61.79	72.62	11.10
ENNB1	**BSA /Acidified ACN/HBSS (3:1)**	3.14 79.47 5.36	29.33	43.44
	BSA/Acetonitrile/HBSS (3:1)	3.26 3.28 56.94	21.16	30.99
	BSA /Acidified ACN/HBSS (3:1) (After freezing)	70.86 76.03 64.17	70.36	5.95
	BSA /Acetonitrile /HBSS (3:1) (After freezing)	73.20 84.77 64.98	74.32	9.94

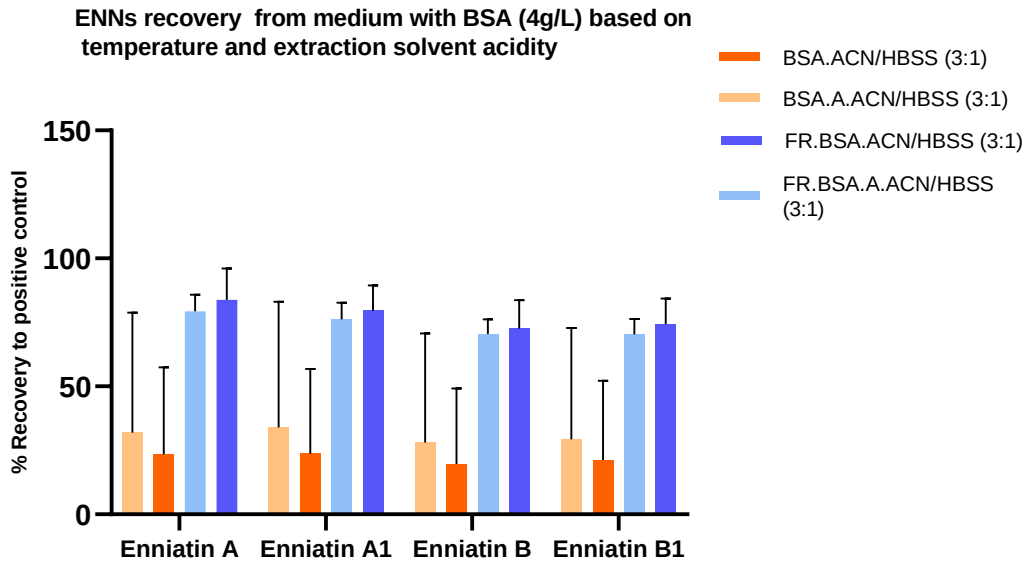


Figure 6: Recovery of ENNs from solvent combinations (HBSS with BSA) with acetonitrile and acidified ACN from before and after freezing the samples. Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation.

(iv) Extraction studies using larger sample volumes and determination of unspecific binding

The last optimization experiment aimed at enhancing extraction efficiency by utilizing larger sample volumes in comparison to previous tests. The recovery rates for positive controls of all ENNs exceeded 99 %. For PBS controls and PBS controls under abiotic conditions, recovery rates ranged from 99.01 % to 112.46 % and 106.31 % to 113.83 %, respectively. For PBS with BSA and PBS with 3T3-L1 fibroblasts, remarkably high recovery rates ranging from 119.46% to 140.47 % and 125.86 % to 142.37 %, respectively were achieved. These results are presented by Table 13 and Figure 7. No statistically significant difference was observed between the recovery rates of PBS controls, PBS controls under abiotic conditions, and the positive controls. However, elevated standard deviation values indicated increased variability among triplicates for conditions B (PBS controls) and C (PBS controls under abiotic conditions) No statistically significant difference was observed between the recovery rates of PBS with BSA and PBS with 3T3-L1 fibroblasts. Overall, a high recovery of > 80 % was observed for ENNs sample mixtures of higher volumes, for all enniatins.

Table 13: Recovery of ENNs from various conditions tested in glass tubes.

Enniatin	Samples	Recoveries (%)	Mean Recovery (%)	Standard Deviation
ENNA	**Positive control**	99.41 99.29 99.35	99.35	0.062
	PBS control	150.77 150.23 41.07	114.03	63.18
	PBS control (Abiotic)	67.87 134.61 129.21	110.56	37.08
	PBS/BSA	141.41 140.99 139.00	140.47	1.28
	PBS/3T3-L1 fibroblasts	143.98 143.07 140.05	142.37	2.05
ENNA1	**Positive control**	99.52 99.41 99.47	99.47	0.05
	PBS control	138.38 139.95 44.65	107.65	54.57
	PBS control (Abiotic)	67.85 126.72 124.37	106.31	33.33

	PBS/BSA	137.41 137.03 130.56	135.00	3.85
	PBS/3T3-L1 fibroblasts	133.95 135.73 128.67	132.78	3.67
ENNB	**Positive control**	99.72 99.73 99.70	99.72	0.01
	PBS control	120.37 118.73 57.92	99.01	35.59
	PBS control (Abiotic)	80.62 113.94 119.21	104.59	20.92
	PBS/BSA	119.36 115.58 123.42	119.46	3.92
	PBS/3T3-L1 fibroblasts	137.78 125.58 114.22	125.86	11.78
ENNB1	**Positive control**	99.91 99.90 99.91	99.90	0.005
	PBS control	141.71 142.70 52.96	112.46	51.53
	PBS control (Abiotic)	78.57 132.39 130.54	113.83	30.55
	PBS/BSA	131.01 133.31 130.53	131.61	1.48
	PBS/3T3-L1 fibroblasts	136.42 128.62 142.67	135.90	7.04

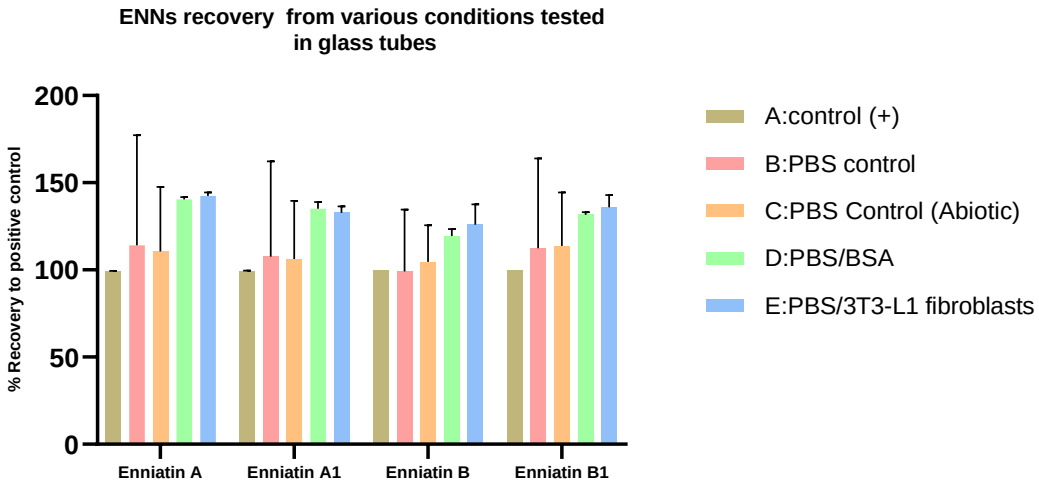


Figure 7: Recovery of ENNs from various conditions tested in glass tubes. Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation.

Recoveries of ENNs from the well medium exceeded the targeted threshold of >80 % except for ENNA. as illustrated in Figure 8. Among the enniatins, the highest recovery rates from well medium were observed for ENNB and ENNB1 at 103.24 % and 103.30 % respectively, followed by ENNA1 at 85.29 %, and ENNA at 75.88 % as shown in Table 14. The likely explanation for this trend is the lower lipophilicity of ENNB and ENNB1. The analysis of the unspecific binding of each enniatin to the plastic wells revealed overall lower recovery rates compared to those from the medium for all enniatins as the enniatins were already extracted. Additionally, due to the non-specific binding of ENNA to wells, it might be present in significant amounts and therefore exhibited the highest recovery, followed by ENNA1, ENNB1, and ENNB. This disparity may be attributed to the preconditioning of pipette tips with acidified ACN (2µM ZEN) to minimize any non-specific binding. Statistical significance was noted between positive controls and unspecific medium samples, as well as unspecific plastic binding samples for ENNA and ENNA1, and between positive controls and unspecific plastic binding samples for ENNB and ENNB1 ($p<0.0001$ (****)). R^2 values of 0.9969, 0.9969, 0.9989, and 0.9971 were obtained for ENNA, ENNA1, ENNB and ENNB1 respectively from the resulting calibration curves indicating linearity.

Table 14: Recovery of ENNs from medium and wells (Unspecific binding).

Enniatin	Samples	Recovery (%)	Mean Recovery (%)	Standard Deviation
ENNA	**Well unspecific (medium)**	80.59 71.81 75.24	75.88	4.42
	Well unspecific (plastic)	50.35 40.06 46.47	45.63	5.19
ENNA1	**Well unspecific (medium)**	85.85 83.09 86.92	85.29	1.97
	Well unspecific (plastic)	33.75 25.99 29.40	29.71	3.88
ENNB	**Well unspecific (medium)**	105.98 101.08 102.66	103.24	2.50
	Well unspecific (plastic)	17.00 11.32 14.21	14.18	2.84
ENNB1	**Well unspecific (medium)**	103.56 101.07 105.26	103.30	2.11
	Well unspecific (plastic)	25.96 20.33 23.09	23.12	2.81

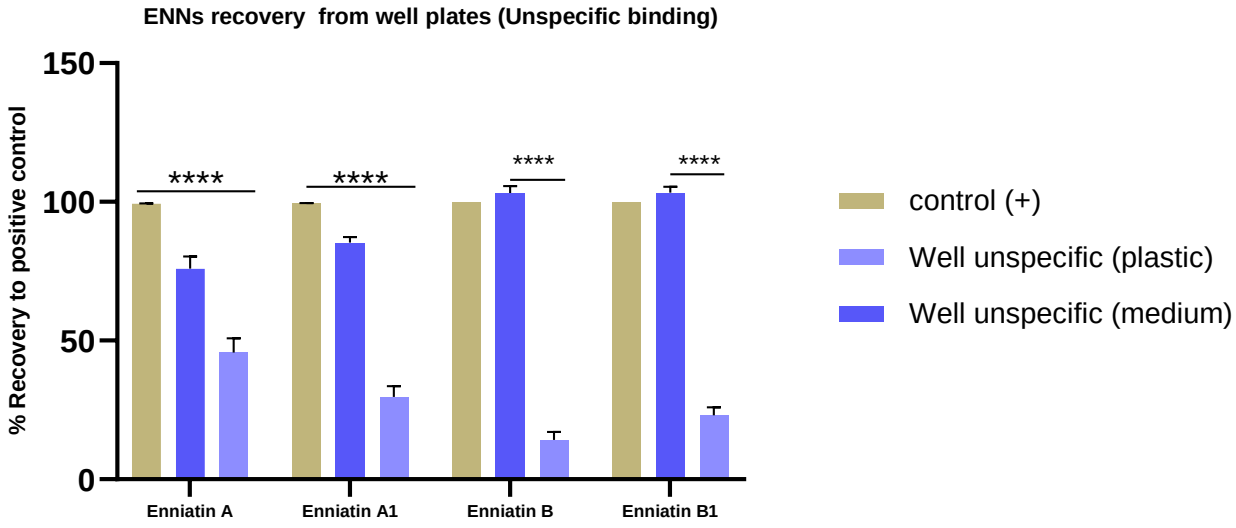


Figure 8: Recovery of ENNs from medium and wells (Unspecific binding). Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation. Statistically significant difference was observed between the two or three groups for each enniatin using a two-way ANOVA test;. $p<0.0001$ (****).

3.1.2 Method performance

The calibration curves prepared for all the extraction experiments gave high R^2 values and confirmed the linearity of the method. The LOD and LOQ were calculated for the extraction studies with mixture of ENNs (section ii) and the optimization tests (section iii) using equations 3 and 4. The highest LOD and LOQ values obtained from all the calibration curves were determined to be the method's LOD and LOQ which are compiled in Table 15. The LODs and LOQs values of the method for each enniatin were as follows: ENNA (LOD = 1, LOQ = 3.30 nM), ENNA1 (LOD = 0.65, LOQ = 2.15 nM), ENNB (LOD = 2.16, LOQ = 7.13 nM) and ENNB1 (LOD = 1.62, LOQ = 5.35 nM).

Table 15: LOD and LOQ values of ENNs.

Enniatin	Extraction studies with mixture of ENNs		First Optimization test		Second Optimization test	
	LOD (nM)	LOQ (nM)	LOD (nM)	LOQ (nM)	LOD (nM)	LOQ (nM)
ENNA	0.05	0.17	0.34	1.14	1.00	3.30
ENNA1	0.01	0.04	0.45	1.48	0.65	2.15
ENNB	0.01	0.05	0.31	1.03	2.16	7.13
ENNB1	0.03	0.12	1.00	3.30	1.62	5.35

3.2 Assessment of plasma protein binding of enniatins using RED

Recovery rates and unbound fractions of enniatins from the RED device were determined from the BSA free test samples and unspecific plastic binding samples, following a 6-hour incubation period. These results indicated that the highest recovery was observed for ENNB (199.25 %) followed by ENNB1 (190.56 %), ENNA1 (184.15 %) and ENNA (163.68 %). Similarly, the unbound fraction of ENNB (111.12 %) was highest followed by ENNB1 (107.33 %), ENNA1 (100.62 %) and ENNA (95.31 %). The recovery rates obtained were over the expected range indicating a likelihood of contamination/carryover during the extraction process. In the evaluation of unspecific plastic binding to the RED inserts, ENNA exhibited the highest recovery, followed by ENNA1, ENNB1, and ENNB, with hardly any bound fraction observed. A statistically significant difference was noted between the BSA-free test samples and unspecific plastic binding samples for all enniatins ($p < 0.0021$ (**), $p < 0.0002$ (***), $p < 0.0001$ (****)) as illustrated in Figure 9. The analysis of the plastic binding of each enniatin revealed overall lower recovery rates compared to those from the unspecific binding for all enniatins as the enniatins were already extracted from BSA-free test samples. Additionally,

due to the plastic binding of ENNA to RED inserts, it might be present in significant amounts and therefore exhibited the highest recovery, followed by ENNA1, ENNB1, and ENNB.

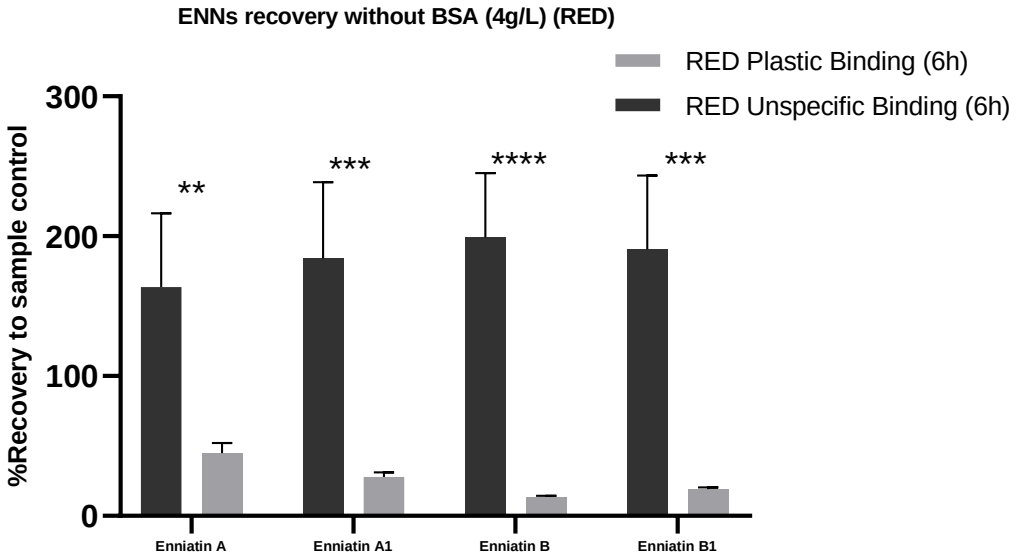


Figure 9: Recovery of ENNs from BSA-free test samples and unspecific plastic binding samples after the incubation time of 6 hours. Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation. Statistically significant difference was observed between the two groups for each enniatin using two-way ANOVA; $p<0.0021$ (**), $p<0.0002$ (***), $p<0.0001$ (****).

Recovery rates and unbound fractions of enniatins from the RED device were determined from BSA containing test samples and plastic binding samples following a 6-hour and 24-hour incubation period. At the 6-hour incubation period results indicated that the highest recovery was observed for ENNB (90.24 %) followed by ENNB1 (85.14 %), ENNA1 (75.87 %) and ENNA (71.84 %). Similarly, the unbound fraction of ENNB (43.68 %) was highest followed by ENNB1 (36.27 %), ENNA1 (25.06 %) and ENNA (19.02 %). At the 24-hour incubation period, results indicated that the highest recovery was again observed for ENNB (71.67 %) followed by ENNB1 (68.14 %), ENNA1 (58.43 %) and ENNA (54.15 %). Similarly, the unbound fraction of ENNB (51.87 %) was highest followed by ENNB1 (44.77 %), ENNA1 (32.01 %) and ENNA (22.91 %). The analysis of the plastic binding of each enniatin again revealed overall lower recovery rates compared to those from the binding for all enniatins as the enniatins were already extracted from BSA-containing test samples. Additionally, due to the plastic binding of ENNA to RED inserts, it might be present in significant amounts and therefore exhibited the highest recovery and unbound fraction, followed by ENNA1, ENNB1, and ENNB. The results for 6-hour and 24-hour incubation periods are presented in Table 16

and Table 17 respectively. A statistically significant difference was noted between the test sample and plastic binding samples with BSA for all enniatins after 6 hours and 24 hours of incubation ($p<0.0001$ (****)) illustrated by Figure 10 and Figure 11 respectively. The bound fraction of enniatins at 6-hour and 24-hour incubation periods are illustrated in Figure 12.

Table 16: Recovery and unbound fraction of enniatins from BSA containing test samples and plastic binding samples after 6 hours of incubation.

Enniatin	**(RED) test samples with BSA (6h)**		**(RED) sample with BSA (plastic binding) (6h)**	
	Recovery (%)	**Unbound fraction (%)**	**Recovery (%)**	**Unbound fraction (%)**
ENNA	71.84	19.02	12.17	86.79
ENNA1	75.87	25.06	9.00	83.96
ENNB	90.24	43.63	6.30	65.06
ENNB1	85.14	36.27	8.44	74.83

Table 17: Recovery and unbound fraction of enniatins from BSA containing test samples and plastic binding samples after 24 hours of incubation.

Enniatin	**RED test sample with BSA (24h)**		**RED sample with BSA(plastic binding) (24h)**	
	Recovery (%)	**Unbound fraction (%)**	**Recovery (%)**	**Unbound fraction (%)**
ENNA	54.15	22.91	18.70	154.215
ENNA1	58.43	32.01	15.20	143.52
ENNB	71.67	51.87	13.30	135.00
ENNB1	68.14	44.77	14.50	123.28

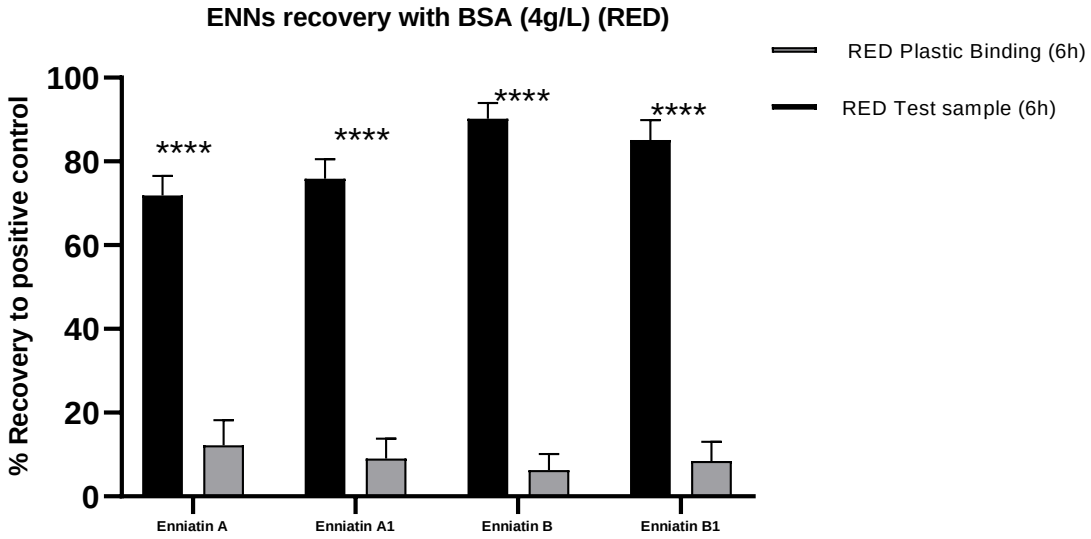


Figure 10:Recovery of ENNs from BSA containing test samples and plastic binding samples after the incubation time of 6 hours. Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation. Statistically significant difference was observed between the two groups for each enniatin using two-way ANOVA; p<0.0001 (****).

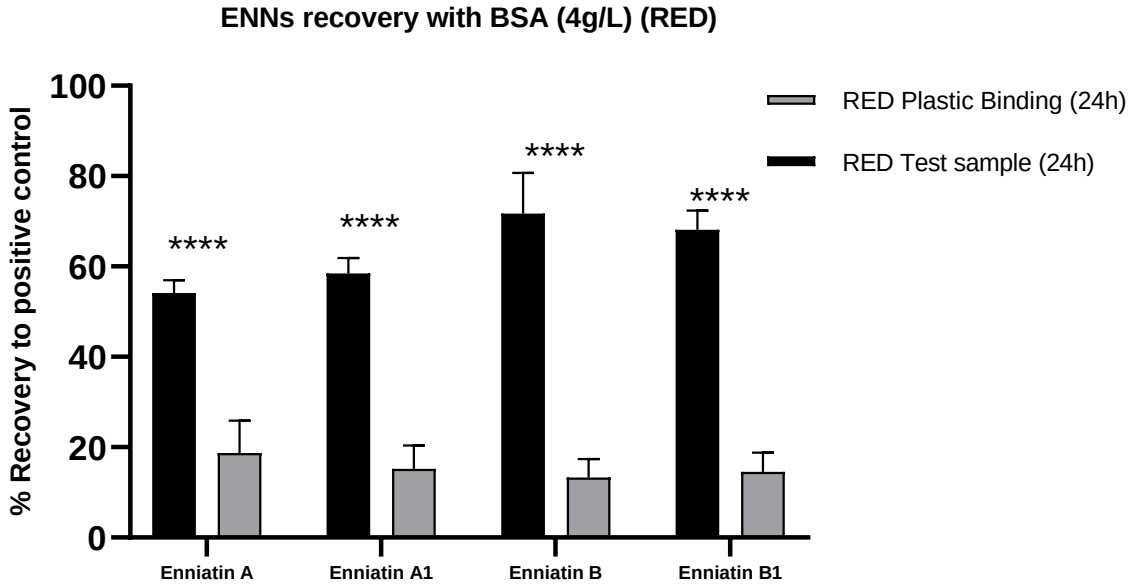


Figure 11:Recovery of ENNs from BSA containing test samples and plastic binding samples after the incubation time of 6 hours. Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation. Statistically significant difference was observed between the two groups for each enniatin using two-way ANOVA; p<0.0001 (****).

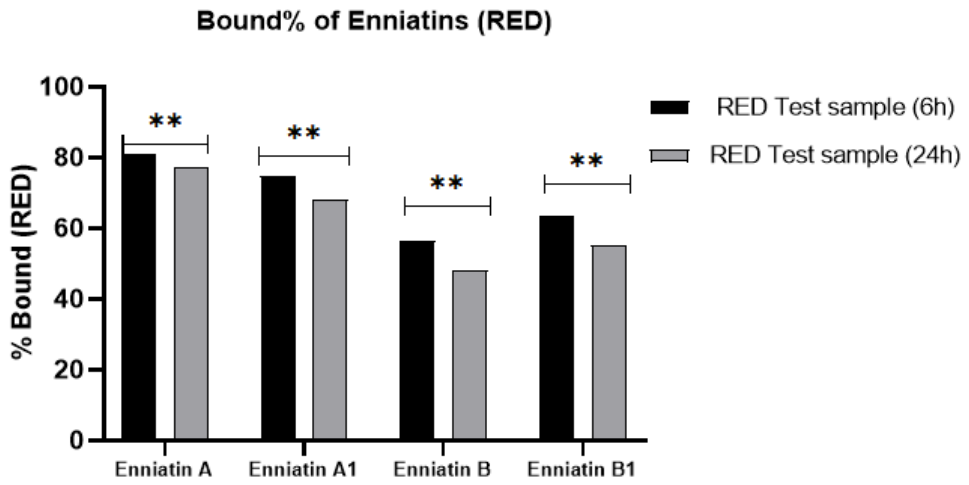


Figure 12: Bound fraction % of ENNs from test samples BSA after 6 hours and 24 hours of incubation. Results are presented as the mean of triplicates of each condition and error bars represent the standard deviation. Statistically significant difference was observed between the two groups for each enniatin using paired t-test, $p<0.005$ (**).

The recovery and unbound fraction of ENNB from test samples (-/+ BSA 4g/L) for both the incubation time points of 6 hours and 24 hours was the highest followed by ENNB1, ENNA1 and ENNA. The unbound fraction of enniatins from the test samples with BSA increased from 6 hours to 24 hours and a decrease in the recovery was also observed. This trend can be attributed to the lipophilicity order of ENNs: ENNA > ENNA1 > ENNB1 > ENNB. Additionally, the recovery of enniatins from plastic binding was notably low, and the unbound fractions (fu) were high. This outcome is a result of the pipette tips being pre-conditioned with acidified ACN (2µM ZEN) to alleviate any non-specific binding. The single-use Rapid Equilibrium Dialysis (RED) plate was used to find that ENNA showed high binding affinity to plasma proteins (99.43% ± 0.05%) in rat plasma and (98.94% ± 0.01%) in human plasma after 4 hours of incubation period (Bhateria et al., 2022). Our investigation was focused on evaluating the binding extent of ENNA and other ENNs to plasma proteins in bovine serum over 6-hour and 24-hour incubation periods. The binding affinity of ENNA was found to be 80.98% and 77.08% for 6 hours and 24 hours, respectively, slightly lower than the values obtained for rat and human serum after 4 hours. There are several possible reasons for this observed difference in different species. Serum albumins consist of three helical domains and two subdomains. Sudlow sites I and II present in the subdomains IIA and IIB respectively are the primary binding sites (Sudlow et al., 1975). Among the serum proteins, HSA (Human

Serum Albumin) and BSA share 76% sequence similarity (Huang et al., 2004). Apart from homologous similarities, there are also several differences between HSA and BSA which might influence their binding affinity. BSA and HSA have 585 and 583 amino acids respectively and differences in their sequences at the linker loop between subdomain IIA and IIB and subdomain IB have been observed (Majorek et al., 2012). From molecular dynamic and structural studies, it was observed that BSA is more rigid than HSA and the characteristics of the albumins are determined by the movement of domains I and III (Ketrat et al., 2020). Additionally, HSA exhibits greater hydrophobicity and thermal stability than BSA. This might increase HSA's ability to attract amphiphilic and hydrophobic compounds compared to BSA (Akdogan et al., 2012; Michnik et al., 2006). These variations along with differences in binding pockets, charge distributions and surface structures are also seen between HSA and BSA which might influence their binding affinity for certain compounds (Bujacz, 2012). The mycotoxin zearalenone (ZEN) which was used as the internal standard is a cereal contaminant that is also produced by *Fusarium* species similar to enniatins. The interaction studies between ZEN and HSA showed a stable complex formation and ZEN showed high binding affinity to HSA between Sudlow sites I and II (Poór et al., 2017). Our study suggested that prolonged exposure to enniatins decreases their bound fraction, as we observed an increase in the free fraction of ENNs after 24 hours which is more biologically active and capable of interacting with target tissues, eliciting toxic effects. Additionally, the free fraction (fu) of enniatin, influences its distribution, metabolism, and elimination within the body. According to EFSA's 2014 assessment, ENNs pose a low risk of acute toxicity, but the potential risk to human health after chronic exposure to lower levels remains unclear. A RED validation study was performed to determine plasma protein binding of certain drugs obtained recoveries in the range of 70 % - 125%. The dialysis was carried out at 3 incubation time points and it was observed that the equilibrium for all the tested compounds was achieved after 4 hours (Waters et al., 2008). When the plasma protein binding of ENNA was assessed using RED, the recovery of >90% was achieved after 4 hours of incubation(Bhateria et al., 2022). In contrast, the evaluation of the plasma protein binding of ennitains in our study gave the recovery rates within the accepted range after 6 hours and lower than the range after 24 hours of incubation. This indicates that ENNs fall in the applicability domain of the RED assay if the incubation period is between 4 hours to 6 hours as the recovery rates obtained until 6 hours fall within the accepted range. Overall, this suggests that the RED assay has to be validated for all the ENNs for accurate determination of plasma protein binding. An equilibrium dialysis study was performed at the time interval of 4-6 hours

and it was found that after the equilibrium was reached the recovery of 100% and the unbound fraction of 25% was obtained in the absence of unspecific binding. Whereas in the presence of unspecific binding, the recovery decreased to 40% and the unbound fraction stayed unchanged (25%). This indicated that the unspecific binding leading to low recovery had no effect on the unbound fraction. This study also strongly suggested that having recovery as the main criterion for the evaluation of assay quality may not be feasible (Di et al., 2012). As mentioned earlier apart from rapid equilibrium dialysis (RED), other methods such as ultrafiltration (UF) and ultracentrifugation (UC) are also used to measure unbound fractions. One such study was performed to compare these techniques and determine the unbound fraction of compounds with varying degrees of lipophilicity in human plasma samples. It was found that UF and UC were more suitable for assessing PPB for polar and hydrophilic compounds with Log $P_{OW} < 2$ and high f_u of >70%. RED was suitable for all the compounds regardless of their lipophilicity (Dimitrijevic et al., 2023). Among the enniatins, ENNA, with its high bound fraction and lipophilicity, can be considered less toxic compared to ENNA1, ENNB, and ENNB1. Conversely, ENNB, with the highest unbound fraction and lowest lipophilicity, appears to be the most toxic among ENNA, ENNA1, and ENNB1. The lipophilicity is a crucial parameter which influences the toxicokinetics i.e. absorption, metabolism, distribution and excretion and also the bioavailability of a compound (Morak-Młodawska et al., 2023). However other factors like Cmax and C(time) can also be studied to evaluate toxicokinetic assessment (S 3 A Toxicokinetics: A Guidance for Assessing Systemic Exposure in Toxicology Studies, 2006). According to one study, ENNB exhibits higher cytotoxic potential among enniatins, surpassing several other mycotoxins like patulin, ochratoxin A, zearalenone, and citrinin in V79-4 fibroblasts supplemented with 10% Fetal Calf Serum (FCS) (Föllmann et al., 2009). ENNB and ENNB1 demonstrated absorption rates exceeding 65% after 4 hours of exposure to Caco-2 cells (Meca et al., 2012). Notably, ENNB is also known to display the highest permeability((kp,v = 9.44 × 10−6 cm/h) through an ex vivo human skin barrier model (Taevernier et al., 2016). Beauvericin (BEA), a cyclic hexadepeptide is also an emerging mycotoxin similar to enniatins with ionophoric properties. Plasma protein binding of Beauvericin (BEA) was studied in humans and rats using HT Dialysis and showed high recovery rates of > 70% and high bound fractions of 99.88 ± 3.53% and 99.93 ± 2.90% was obtained from human and rat plasma proteins respectively (Yuan et al., 2022).

3.8 Conclusion

Emerging mycotoxins such as Enniatins (ENNs), have gained attention due to their significant presence in food and feed commodities. These toxins are known for their lipophilic nature and ionophoric properties, which disrupt cellular functions, leading to cytotoxicity. Several *in vivo* and *in vitro* toxicity studies have highlighted their potential adverse effects even at low concentrations. Toxicokinetics factors play a crucial role in understanding how ENNs affect the body. This study was aimed at understanding the toxicological implications of four prevalent enniatins namely ENNA, ENNA1, ENNB and ENNB1. The solvent extraction and analytical measurement experiments were optimized for all four enniatins to obtain the best recovery rates from different matrices. The extraction efficiency was higher when acidified acetonitrile was used and the ratio between the ENNs sample volume to acidified acetonitrile volume was set to 1:3. Additionally, with these parameters, better recovery rates were also obtained at lower temperatures and with larger sample volumes. However, the LC/MS method still needs to be completely validated for the accurate measurement of the concentration of the enniatins. This is in terms of the matrix effects, accuracy, specificity, precision and stability (Sveshnikova et al., 2019). Given their physicochemical properties, ENNs were expected to bind strongly to plasma proteins. For this study, we used fetal bovine serum and bovine serum albumin (4g/L). Plasma protein binding affects the distribution and elimination of ENNs within the body, therefore we investigated the extent of plasma protein binding of enniatins using Rapid Equilibrium Dialysis (RED). The assay was performed at 2 incubation time points of 6 hours and 24 hours and also unspecific binding to the RED device was also analyzed. It was found that among all the enniatins ENNB had the highest unbound fraction and recovery rate after 24 hours. This suggested that ENNB might elicit some toxic effects, considering its significant presence in several food commodities as seen in several studies. Therefore, the enniatin can be further studied along with other enniatins by performing in vitro to *in vivo* extrapolation (IVIVE) using physiologically based toxicokinetic models (PBTK).

3.9 Recommendations for future research

PPB values derived from the RED assay can be utilized as input parameters for constructing and validating physiologically based (PBK) models of ENNs. This will facilitate the prediction of variations in blood and tissue concentrations in bovine samples for the purpose of toxicological risk assessment. The solvent extraction of all enniatins from diverse concentrations of human and rat plasma samples can be performed and subsequently the recovery rates can be calculated. This approach will be crucial in understanding the inter-species toxicity and toxicokinetic differences. The LC/MS analytical method should be validated for the accurate measurement of the concentration of the analytes. The scope of extraction experiments can be expanded by employing larger volumes of enniatin sample mixtures with Human Serum Albumin and Rat Serum. The extraction process of all enniatins from other plasma proteins, such as α1-acid glycoprotein can also be investigated. The RED assay can be performed to determine the unbound fraction of enniatin in human and rat plasma samples and compare these results with existing data on unbound fractions of enniatins obtained from bovine serum albumin. Finally, the PPB values obtained from the RED assay with human and rat plasma samples can be used for PBK modelling procedures.

Acknowledgements

First and foremost, I want to sincerely thank my examiner, Dr Nynke Kramer for introducing me to my supervisor and this project. I am grateful for this opportunity to do this master's thesis titled – ‘ Optimization of solvent extraction method and determination of unbound enniatin concentration in bovine serum albumin using rapid equilibrium dialysis (RED).’

I want to express my heartfelt gratitude to my supervisor, Chrysanthi Pachoulide, for her constant encouragement, guidance and genuine support throughout this thesis. Under her mentorship, I have not only honed my research skills but also seen significant academic growth.

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I wish to express my deepest gratitude to my family and friends for their constant support, encouragement and unconditional love.

Finally, I acknowledge all sources of help and have quoted and consulted others published work for which the references have been cited accordingly. Apart from such quotations, this thesis is entirely my work.

Appendix

A. Calibration Curves

A.1 Pilot Extraction Studies (Individual ENNs)

A.1.1 Pilot Study 1 (ENNB1)

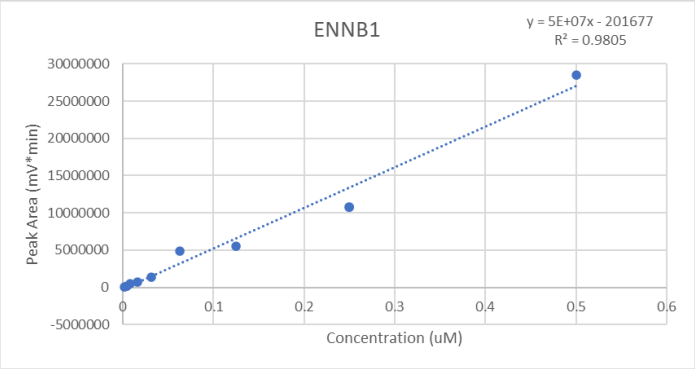

Figure 13: Calibration curve of ENNB1.

A.1.2 Pilot Study 2 (ENNA,ENNA1,ENNB, ENNB1)

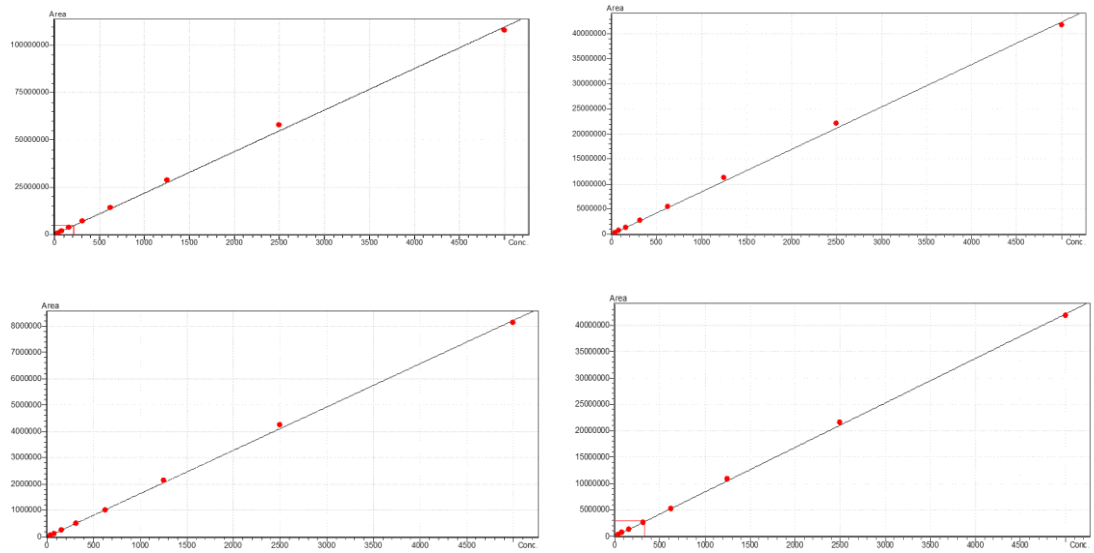

Figure 14: Calibration curves (Top; left to right - ENNA, ENNA1, Bottom ; left to right- ENNB, ENNB1.

A.2 Extraction studies (Mixture of ENNs)

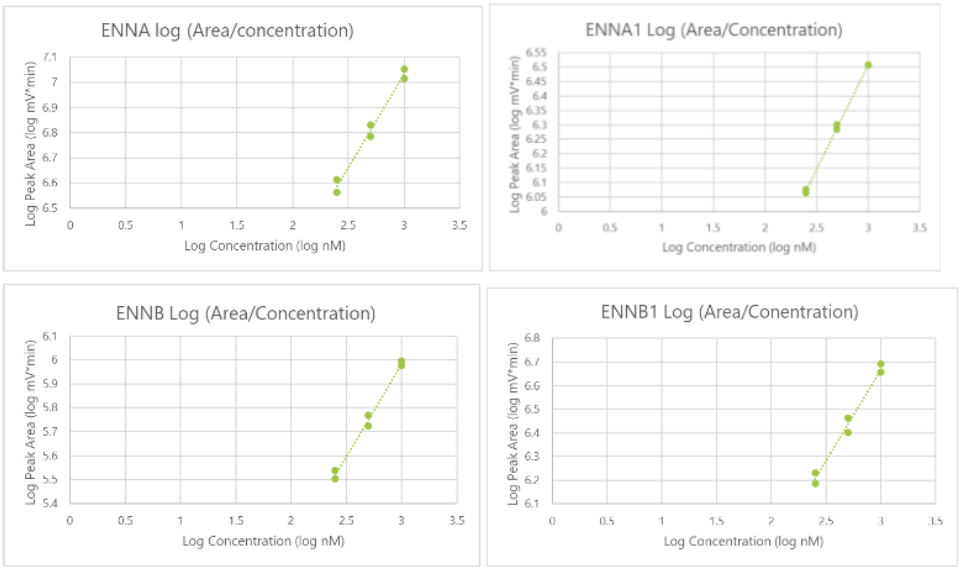

Figure 15: Calibration curves of ENNA,ENNA1,ENNB and ENNB1.

A.3 Optimization of the extraction studies

A.3.1 First optimization study

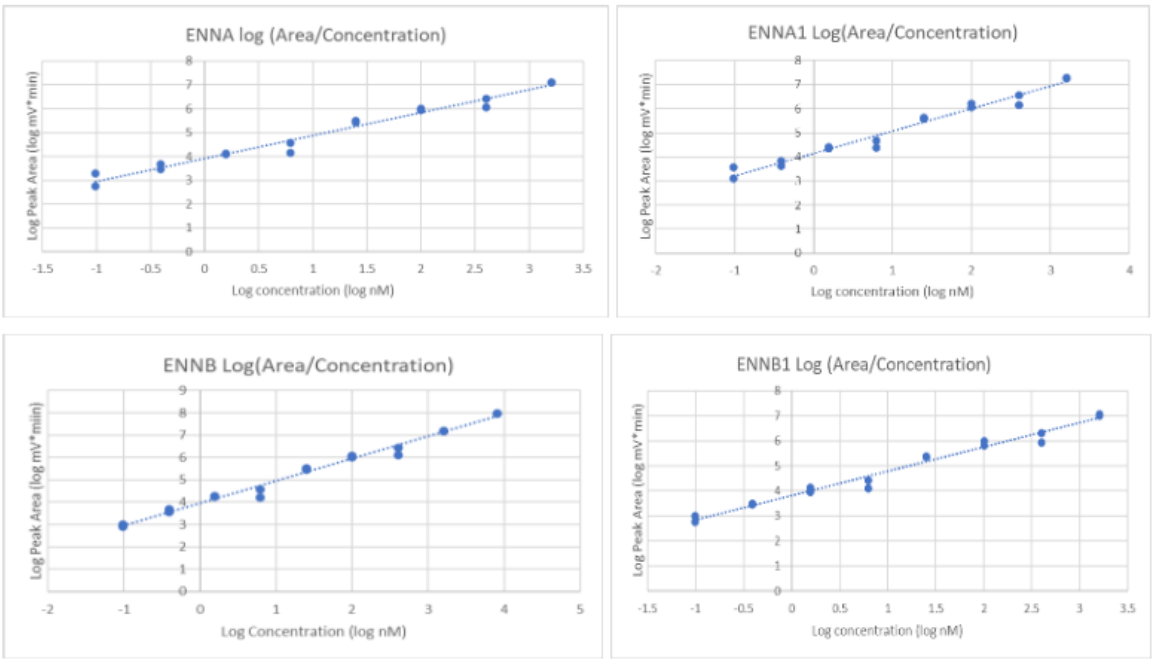

Figure 16: Calibration curves of ENNA, ENNA1, ENNB and ENNB1.

A.3.2 Second optimization study

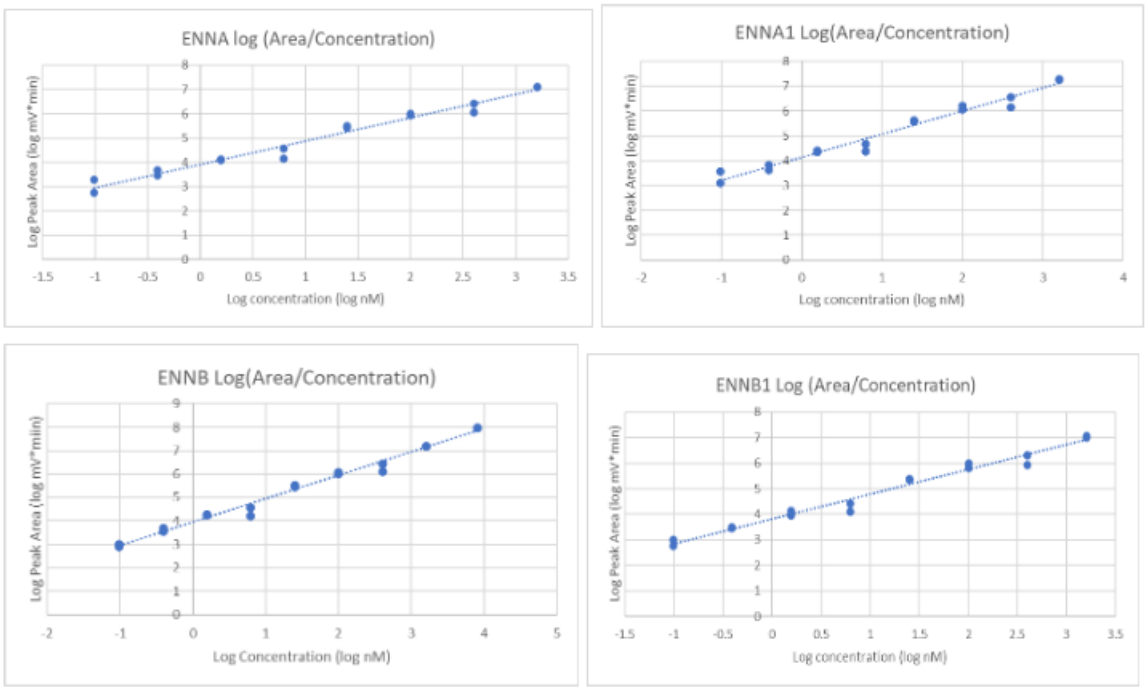

Figure 17: Calibration curves of ENNA, ENNA1, ENNB and ENNB1.

A.4 Extraction studies using larger sample volumes and determination of unspecific binding

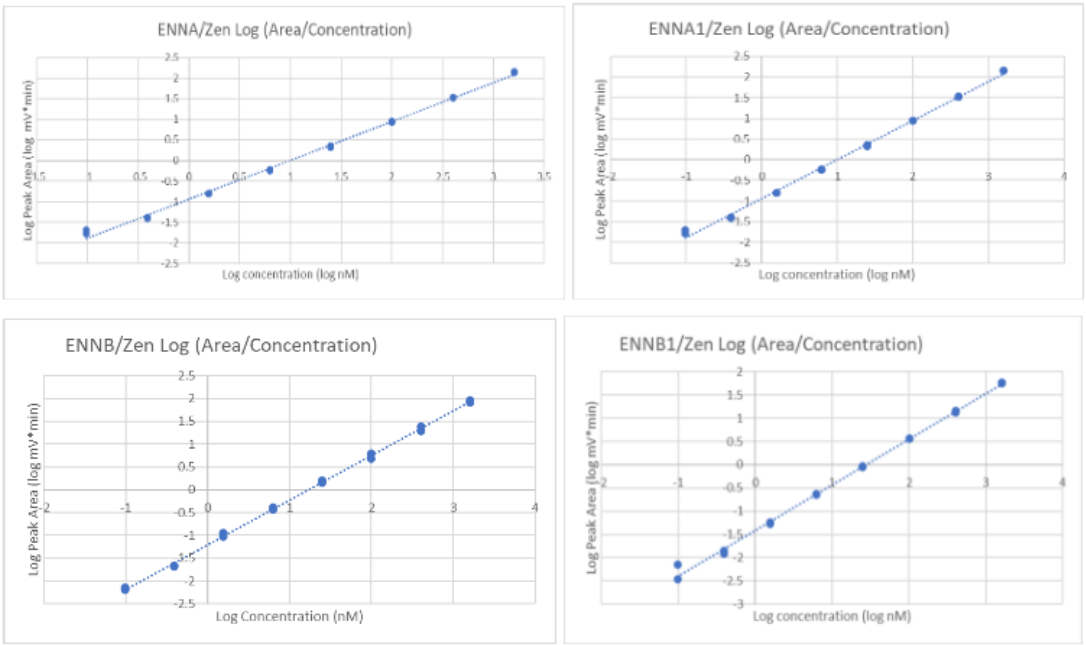

Figure 18: Calibration curves of ENNA, ENNA1, ENNB and ENNB1.

B. Preparation of solutions

B.1 2% FBS solution: 2.5 mL FBS was added to 7.5 mL HBSS to obtain 10 mL of 25% FBS solution.

B.2 25% FBS solution: 0.1 mL FBS was added to 4.9 mL HBSS to obtain 5 mL of 25% FBS solution.

B.3 50% FBS solution: 2.5 mL FBS was added to 2.5 mL HBSS to obtain 5 mL of 50% FBS solution.

B.4 Acidified acetonitrile (0.1%): Acetonitrile was acidified by adding 500 µL of formic acid to 499.5 mL of acetonitrile.

B.5 Acidified acetonitrile (2µM ZEN): ZEN stock (10mM) solution of 80 µL was added to acidified ACN.

B.6 Phosphate Buffered Saline with BSA (4g/L): A total of 48 mg of BSA was added to 12 mL of PBS to obtain the desired solution.

B.7 Acidified ACN with PBS (3/1 (v/v)): The solution was prepared by combining 60 mL of the pre-prepared acidified ACN (2µM ZEN) with 20 mL of PBS, maintaining a ratio of 3:1 between acidified ACN and PBS.

B.8 PBS with 3T3-L1 fibroblasts: A PBS volume of 8mL, containing a maximum quantity of 3T3-L1 fibroblasts, was prepared. The cells underwent trypsinization with 2 mL of trypsin and were subsequently re-suspended in 6 mL of PBS. After centrifugation, the supernatant was discarded, and the cells were re-suspended in 8 mL of PBS. Cell counting resulted in a concentration of $7.10*10^5$ cells/mL, equating to a total of 5,680,000 cells available. Therefore, in each triplicate (2 mL of the cell/PBS suspension), there were a total of 1,420,000 cells.

References

European Food Safety Authority Chemical contaminants in food and feed. *European Food Safety Authority*. 2023https://www.efsa.europa.eu/en/topics/topic/chemical-contaminants-food-feed

Akdogan, Y., Reichenwallner, J., & Hinderberger, D. (2012). Evidence for Water-Tuned Structural Differences in Proteins: An Approach Emphasizing Variations in Local Hydrophilicity. *PLoS ONE*, *7*(9), e45681. https://doi.org/10.1371/journal.pone.0045681

Bhateria, M., Karsauliya, K., Sonker, A. K., Yahavi, C., & Singh, S. P. (2022). Cytochrome P450 isoforms contribution, plasma protein binding, toxicokinetics of enniatin A in rats and in vivo clearance prediction in humans. *Food and Chemical Toxicology*, *164*, 112988. https://doi.org/10.1016/j.fct.2022.112988

Bryda, E. C. (2013). The Mighty Mouse: The impact of rodents on advances in biomedical research. *Missouri Medicine*, *110*(3), 207–211.

Bujacz, A. (2012). Structures of bovine, equine and leporine serum albumin. *Acta Crystallographica Section D Biological Crystallography*, *68*(10), 1278–1289. https://doi.org/10.1107/S0907444912027047

Chen, M., Du, R., Zhang, T., Li, C., Bao, W., Xin, F., Hou, S., Yang, Q., Chen, L., Wang, Q., & Zhu, A. (2023). The Application of a Physiologically Based Toxicokinetic Model in Health Risk Assessment. *Toxics*, *11*(10), 874. https://doi.org/10.3390/toxics11100874

Coecke, S., Pelkonen, O., Leite, S. B., Bernauer, U., Bessems, J. G., Bois, F. Y., Gundert-Remy, U., Loizou, G., Testai, E., & Zaldívar, J.-M. (2013). Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches. *Toxicology in Vitro*, *27*(5), 1570–1577. https://doi.org/10.1016/j.tiv.2012.06.012

Council for Agricultural Science and Technology (Ed.). (2003). *Mycotoxins: Risks in plant, animal, and human systems*. Council for Agricultural Science and Technology.

Creton, S., Billington, R., Davies, W., Dent, M. P., Hawksworth, G. M., Parry, S., & Travis, K. Z. (2009). Application of toxicokinetics to improve chemical risk assessment: Implications for the use of animals. *Regulatory Toxicology and Pharmacology*, *55*(3), 291–299. https://doi.org/10.1016/j.yrtph.2009.08.001

Dagnac, T., Latorre, A., Fernández Lorenzo, B., & Llompart, M. (2016). Validation and application of a liquid chromatography-tandem mass spectrometry based method for the assessment of the co-occurrence of mycotoxins in maize silages from dairy farms in NW Spain. *Food Additives & Contaminants: Part A*, *33*(12), 1850–1863. https://doi.org/10.1080/19440049.2016.1243806

Devreese, M., Broekaert, N., De Mil, T., Fraeyman, S., De Backer, P., & Croubels, S. (2014). Pilot toxicokinetic study and absolute oral bioavailability of the Fusarium mycotoxin enniatin B1 in pigs. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *63*, 161–165. https://doi.org/10.1016/j.fct.2013.11.005

Di, L., Umland, J. P., Trapa, P. E., & Maurer, T. S. (2012). Impact of Recovery on Fraction Unbound Using Equilibrium Dialysis. *Journal of Pharmaceutical Sciences*, *101*(3), 1327–1335. https://doi.org/10.1002/jps.23013

Dimitrijevic, D., Fabian, E., Funk-Weyer, D., & Landsiedel, R. (2023). Rapid equilibrium dialysis, ultrafiltration or ultracentrifugation? Evaluation of methods to quantify the unbound fraction of substances in plasma. *Biochemical and Biophysical Research Communications*, *651*, 114–120. https://doi.org/10.1016/j.bbrc.2023.02.021

EFSA Panel on Contaminants in the Food Chain (CONTAM). (2014). Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. *EFSA Journal*, *12*(8). https://doi.org/10.2903/j.efsa.2014.3802

Escrivá, L., Font, G., Berrada, H., & Manyes, L. (2016). Mycotoxin contamination in laboratory rat feeds and their implications in animal research. *Toxicology Mechanisms and Methods*, *26*(7), 529–537. https://doi.org/10.1080/15376516.2016.1206163

Escrivá, L., Font, G., & Manyes, L. (2015). Quantitation of enniatins in biological samples of Wistar rats after oral administration by LC-MS/MS. *Toxicology Mechanisms and Methods*, *25*(7), 552–558.

EURACHEM (Ed.). (1998). *The fitness for purpose of analytical methods: A laboratory guide to method validation and related topics* (Engl. ed. 1.0). LGC.

European Commission. Joint Research Centre. (2016). *Guidance document on the estimation of LOD and LOQ for measurements in the field of contaminants in feed and food.* Publications Office. https://data.europa.eu/doi/10.2787/8931

Fæste, C. K., Ivanova, L., & Uhlig, S. (2011). In Vitro Metabolism of the Mycotoxin Enniatin B in Different Species and Cytochrome P450 Enzyme Phenotyping by Chemical Inhibitors. *Drug Metabolism and Disposition*, *39*(9), 1768–1776. https://doi.org/10.1124/dmd.111.039529

Ferrer, E., Juan-García, A., Font, G., & Ruiz, M. J. (2009). Reactive oxygen species induced by beauvericin, patulin and zearalenone in CHO-K1 cells. *Toxicology in Vitro*, *23*(8), 1504–1509. https://doi.org/10.1016/j.tiv.2009.07.009

Föllmann, W., Behm, C., & Degen, G. H. (2009). The emerging Fusarium toxin enniatin B: In-vitro studies on its genotoxic potential and cytotoxicity in V79 cells in relation to other mycotoxins. *Mycotoxin Research*, *25*(1), 11–19. https://doi.org/10.1007/s12550-008-0002-y

Fraeyman, S., Croubels, S., Devreese, M., & Antonissen, G. (2017). Emerging Fusarium and Alternaria Mycotoxins: Occurrence, Toxicity and Toxicokinetics. *Toxins*, *9*(7), 228. https://doi.org/10.3390/toxins9070228

Fraeyman, S., Devreese, M., Antonissen, G., De Baere, S., Rychlik, M., & Croubels, S. (2016). Comparative Oral Bioavailability, Toxicokinetics, and Biotransformation of Enniatin B1 and Enniatin B in Broiler Chickens. *Journal of Agricultural and Food Chemistry*, *64*(38), 7259–7264. https://doi.org/10.1021/acs.jafc.6b02913

Gruber-Dorninger, C., Novak, B., Nagl, V., & Berthiller, F. (2017). Emerging Mycotoxins: Beyond Traditionally Determined Food Contaminants. *Journal of Agricultural and Food Chemistry*, *65*(33), 7052–7070. https://doi.org/10.1021/acs.jafc.6b03413

Huang, B. X., Kim, H.-Y., & Dass, C. (2004). Probing three-dimensional structure of bovine serum albumin by chemical cross-linking and mass spectrometry. *Journal of the American Society for Mass Spectrometry*, *15*(8), 1237–1247. https://doi.org/10.1016/j.jasms.2004.05.004

Ivanova, L., Denisov, I. G., Grinkova, Y. V., Sligar, S. G., & Fæste, C. K. (2019). Biotransformation of the Mycotoxin Enniatin B1 by CYP P450 3A4 and Potential for Drug-Drug Interactions. *Metabolites*, *9*(8), 158. https://doi.org/10.3390/metabo9080158

Ivanova, L., Egge-Jacobsen, W. M., Solhaug, A., Thoen, E., & Fæste, C. K. (2012). Lysosomes as a Possible Target of Enniatin B-Induced Toxicity in Caco-2 Cells. *Chemical Research in Toxicology*, *25*(8), 1662–1674. https://doi.org/10.1021/tx300114x

Ivanova, L., Fæste, C. K., & Uhlig, S. (2011). In vitro phase I metabolism of the depsipeptide enniatin B. *Analytical and Bioanalytical Chemistry*, *400*(9), 2889–2901. https://doi.org/10.1007/s00216-011-4964-9

Ivanova, L., Uhlig, S., Eriksen, G., & Johannessen, L. (2010). Enniatin B1 is a substrate of intestinal P-glycoprotein, multidrug resistance-associated protein 2 and breast cancer resistance protein. *World Mycotoxin Journal*, *3*(3), 271–281. https://doi.org/10.3920/WMJ2010.1225

Jestoi *, M., Rokka, M., Yli-Mattila, T., Parikka, P., Rizzo, A., & Peltonen, K. (2004). Presence and concentrations of the *Fusarium*- related mycotoxins beauvericin, enniatins and moniliformin in finnish grain samples. *Food Additives and Contaminants*, *21*(8), 794–802. https://doi.org/10.1080/02652030410001713906

Jestoi, M. (2008). Emerging ***Fusarium*** -Mycotoxins Fusaproliferin, Beauvericin, Enniatins, And Moniliformin—A Review. *Critical Reviews in Food Science and Nutrition*, *48*(1), 21–49. https://doi.org/10.1080/10408390601062021

Juan, C., Mañes, J., Raiola, A., & Ritieni, A. (2013). Evaluation of beauvericin and enniatins in Italian cereal products and multicereal food by liquid chromatography coupled to triple quadrupole mass spectrometry. *Food Chemistry*, *140*(4), 755–762. https://doi.org/10.1016/j.foodchem.2012.08.021

Juan, C., Manyes, L., Font, G., & Juan-García, A. (2014). Evaluation of immunologic effect of Enniatin A and quantitative determination in feces, urine and serum on treated Wistar rats. *Toxicon*, *87*, 45–53. https://doi.org/10.1016/j.toxicon.2014.05.005

Juan, C., Ritieni, A., & Mañes, J. (2013). Occurrence of Fusarium mycotoxins in Italian cereal and cereal products from organic farming. *Food Chemistry*, *141*(3), 1747–1755. https://doi.org/10.1016/j.foodchem.2013.04.061

Kamyar, M., Rawnduzi, P., Studenik, C. R., Kouri, K., & Lemmens-Gruber, R. (2004). Investigation of the electrophysiological properties of enniatins. *Archives of Biochemistry and Biophysics*, *429*(2), 215–223. https://doi.org/10.1016/j.abb.2004.06.013

Kariv, I., Cao, H., & Oldenburg, K. R. (2001). Development of a high throughput equilibrium dialysis method. *Journal of Pharmaceutical Sciences*, *90*(5), 580–587. https://doi.org/10.1002/1520-6017(200105)90:5<580::aid-jps1014>3.0.co;2-4

Ketrat, S., Japrung, D., & Pongprayoon, P. (2020). Exploring how structural and dynamic properties of bovine and canine serum albumins differ from human serum albumin. *Journal of Molecular Graphics & Modelling*, *98*, 107601. https://doi.org/10.1016/j.jmgm.2020.107601

Levy, G. (1980). Effect of Plasma Protein Binding on Renal Clearance of Drugs. *Journal of Pharmaceutical Sciences*, *69*(4), 482–483. https://doi.org/10.1002/jps.2600690437

Lobell, M., & Sivarajah, V. (2003). In silico prediction of aqueous solubility, human plasma protein binding and volume of distribution of compounds from calculated pK_a and AlogP98 values. *Molecular Diversity*, *7*(1), 69–87. https://doi.org/10.1023/B:MODI.0000006562.93049.36

Majorek, K. A., Porebski, P. J., Dayal, A., Zimmerman, M. D., Jablonska, K., Stewart, A. J., Chruszcz, M., & Minor, W. (2012). Structural and immunologic characterization of bovine, horse, and rabbit

serum albumins. *Molecular Immunology*, *52*(3–4), 174–182. https://doi.org/10.1016/j.molimm.2012.05.011

Malachova, A., Dzuman, Z., Veprikova, Z., Vaclavikova, M., Zachariasova, M., & Hajslova, J. (2011). Deoxynivalenol, Deoxynivalenol-3-glucoside, and Enniatins: The Major Mycotoxins Found in Cereal-Based Products on the Czech Market. *Journal of Agricultural and Food Chemistry*, *59*(24), 12990–12997. https://doi.org/10.1021/jf203391x

Manyes, L., Escrivá, L., Serrano, A. B., Rodríguez-Carrasco, Y., Tolosa, J., Meca, G., & Font, G. (2014). A preliminary study in Wistar rats with enniatin A contaminated feed. *Toxicology Mechanisms and Methods*, *24*(3), 179–190. https://doi.org/10.3109/15376516.2013.876135

McKee, T. C., Bokesch, H. R., McCormick, J. L., Rashid, M. A., Spielvogel, D., Gustafson, K. R., Alavanja, M. M., Cardelline, J. H., & Boyd, M. R. (1997). Isolation and characterization of new anti-HIV and cytotoxic leads from plants, marine, and microbial organisms. *Journal of Natural Products*, *60*(5), 431–438. https://doi.org/10.1021/np970031g

Meca, G., Mañes, J., Font, G., & Ruiz, M. J. (2012). Study of the potential toxicity of enniatins A, A1, B, B1 by evaluation of duodenal and colonic bioavailability applying an in vitro method by Caco-2 cells. *Toxicon*, *59*(1), 1–11. https://doi.org/10.1016/j.toxicon.2011.10.004

Michnik, A., Michalik, K., Kluczewska, A., & Drzazga, Z. (2006). Comparative DSC study of human and bovine serum albumin. *Journal of Thermal Analysis and Calorimetry*, *84*(1), 113–117. https://doi.org/10.1007/s10973-005-7170-1

Morak-Młodawska, B., Jeleń, M., Martula, E., & Korlacki, R. (2023). Study of Lipophilicity and ADME Properties of 1,9-Diazaphenothiazines with Anticancer Action. *International Journal of Molecular Sciences*, *24*(8), 6970. https://doi.org/10.3390/ijms24086970

Naderi, M. M., Sarvari, A., Milanifar, A., Boroujeni, S. B., & Akhondi, M. M. (2012). Regulations and ethical considerations in animal experiments: International laws and islamic perspectives. *Avicenna Journal of Medical Biotechnology*, *4*(3), 114–120.

Pacifici, G. M., & Viani, A. (1992). Methods of Determining Plasma and Tissue Binding of Drugs: Pharmacokinetic Consequences. *Clinical Pharmacokinetics*, *23*(6), 449–468. https://doi.org/10.2165/00003088-199223060-00005

Parasuraman, S. (2011). Toxicological screening. *Journal of Pharmacology & Pharmacotherapeutics*, *2*(2), 74–79. https://doi.org/10.4103/0976-500X.81895

Poór, M., Kunsági-Máté, S., Bálint, M., Hetényi, C., Gerner, Z., & Lemli, B. (2017). Interaction of mycotoxin zearalenone with human serum albumin. *Journal of Photochemistry and Photobiology B: Biology*, *170*, 16–24. https://doi.org/10.1016/j.jphotobiol.2017.03.016

Prosperini, A., Berrada, H., Ruiz, M. J., Caloni, F., Coccini, T., Spicer, L. J., Perego, M. C., & Lafranconi, A. (2017). A Review of the Mycotoxin Enniatin B. *Frontiers in Public Health*, *5*, 304. https://doi.org/10.3389/fpubh.2017.00304

Prosperini, A., Juan-García, A., Font, G., & Ruiz, M. J. (2013). Reactive oxygen species involvement in apoptosis and mitochondrial damage in Caco-2 cells induced by enniatins A, A1, B and B1. *Toxicology Letters*, *222*(1), 36–44. https://doi.org/10.1016/j.toxlet.2013.07.009

Rodríguez-Carrasco, Y., Heilos, D., Richter, L., Süssmuth, R. D., Heffeter, P., Sulyok, M., Kenner, L., Berger, W., & Dornetshuber-Fleiss, R. (2016). Mouse tissue distribution and persistence of the food-born fusariotoxins Enniatin B and Beauvericin. *Toxicology Letters*, *247*, 35–44. https://doi.org/10.1016/j.toxlet.2016.02.008

Rozentale, I., Bogdanova, E., & Bartkevics, V. (2018). A rapid and sensitive method for the control of selected regulated and emerging mycotoxins in beer. *World Mycotoxin Journal*, *11*(4), 503–517. https://doi.org/10.3920/WMJ2017.2298

S 3 A Toxicokinetics: A Guidance for Assessing Systemic Exposure in Toxicology Studies. (2006).

Serrano, A., Capriotti, A., Cavaliere, C., Piovesana, S., Samperi, R., Ventura, S., & Laganà, A. (2015). Development of a Rapid LC-MS/MS Method for the Determination of Emerging Fusarium mycotoxins Enniatins and Beauvericin in Human Biological Fluids. *Toxins*, *7*(9), 3554–3571. https://doi.org/10.3390/toxins7093554

Smith, D. A., Di, L., & Kerns, E. H. (2010). The effect of plasma protein binding on in vivo efficacy: Misconceptions in drug discovery. *Nature Reviews. Drug Discovery*, *9*(12), 929–939. https://doi.org/10.1038/nrd3287

Sudlow, G., Birkett, D. J., & Wade, D. N. (1975). The characterization of two specific drug binding sites on human serum albumin. *Molecular Pharmacology*, *11*(6), 824–832.

Sveshnikova, N., Yuan, T., Warren, J. M., & Piercey-Normore, M. D. (2019). Development and validation of a reliable LC–MS/MS method for quantitative analysis of usnic acid in Cladonia uncialis. *BMC Research Notes*, *12*(1), 550. https://doi.org/10.1186/s13104-019-4580-x

Taevernier, L., Veryser, L., Roche, N., Peremans, K., Burvenich, C., Delesalle, C., & De Spiegeleer, B. (2016). Human skin permeation of emerging mycotoxins (beauvericin and enniatins). *Journal of Exposure Science & Environmental Epidemiology*, *26*(3), 277–287. https://doi.org/10.1038/jes.2015.10

Tetko, I. V., Gasteiger, J., Todeschini, R., Mauri, A., Livingstone, D., Ertl, P., Palyulin, V. A., Radchenko, E. V., Zefirov, N. S., Makarenko, A. S., Tanchuk, V. Yu., & Prokopenko, V. V. (2005). Virtual Computational Chemistry Laboratory – Design and Description. *Journal of Computer-Aided Molecular Design*, *19*(6), 453–463. https://doi.org/10.1007/s10822-005-8694-y

Thompson, M., Ellison, S. L. R., & Wood, R. (2002). Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). *Pure and Applied Chemistry*, *74*(5), 835–855. https://doi.org/10.1351/pac200274050835

Waters, N. J., Jones, R., Williams, G., & Sohal, B. (2008). Validation of a Rapid Equilibrium Dialysis Approach for the Measurement of Plasma Protein Binding. *Journal of Pharmaceutical Sciences*, *97*(10), 4586–4595. https://doi.org/10.1002/jps.21317

Yuan, Y., Meng, G., Li, Y., & Wu, C. (2022). Study on In Vitro Metabolism and In Vivo Pharmacokinetics of Beauvericin. *Toxins*, *14*(7), 477. https://doi.org/10.3390/toxins14070477

Rapid Equilibrium dialysis (RED) inserts and plates. (n.d.). https://www.thermofisher.com/order/catalog/product/89809?gclid=CjwKCAjw17qvBhBrEiw

A1rU9w1AcdMvTF8zJrxTbXqgAlc9CSwTDuwgVVWMXSYSHzzm4R-E6Hh5pQhoC-cgQAvD_BwE&ef_id=CjwKCAjw17qvBhBrEiwA1rU9w1AcdMvTF8zJrxTbXqgAlc9CSwTDuwgVVWMXSYSHzzm4R-E6Hh5pQhoC-cgQAvD_BwE:G:s&s_kwcid=AL!3652!3!657327261489!e!!g!!red%20device!13368767886!157267306028&cid=bid_pca_ppf_r01_co_cp1359_pjt0000_bid00000_0se_gaw_nt_pur_con&gad_source=1

OpenAI, "ChatGPT" https://www.openai.com/chatgpt Query: "Rephrase the above paragraph and make it grammatically accurate"

Open AI, "ChatGPT" https://www.openai.com/chatgpt Query: From 2 w2-wayOVA analysis, no statistically significant difference was obtained, but in the visual representation of data, there was a difference observed. What's the reason?