# Characterization, quantification, and *in vivo* effects of vitamin $B_6$ antagonists from flaxseed on amino acid metabolism in a rodent model of moderate vitamin $B_6$ deficiency

By

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# ABSTRACT

Vitamin $B_6$, or more specifically the active form pyridoxal 5′-phosphate (PLP), plays a crucial role as a cofactor for numerous enzymes linked to carbohydrate, fatty acid, and amino acid metabolism. There is a high prevalence of moderate vitamin $B_6$ deficiency in the population that may be further exacerbated through the ingestion of vitamin $B_6$ antagonists/anti-pyridoxine factors present in the food supply. For example, flaxseed (*Linum usitatissimum L.)* contains the anti-pyridoxine factor 1-amino D-proline (1ADP) in the form of a dipeptide called linatine (1-[(n-γ-L-glutamyl)-amino]-D-proline). Due to its content of nutrients linked to health (omega-3 fatty acids and phytoestrogens), flaxseed ingestion has increased. As such, a portion of the population, particularly those presenting with moderate vitamin $B_6$ deficiency, may be at risk of further deterioration of $B_6$ status upon ingestion of these anti-pyridoxine factors. In order to address these issues, the current study was designed to: 1) characterize and quantify the total amount of anti-pyridoxine factors present in flaxseed through the use of UPLC/ESI-MS analysis, 2) investigate the *in vivo* effects of synthetic and flaxseed-derived 1ADP on amino acid metabolism using a rat model of moderate $B_6$ deficiency, and 3) identify novel biomarkers of vitamin $B_6$ inadequacy using a HPLC-Qtof-MS based non-targeted metabolomics approach. The total anti-pyridoxine content, measured as1ADP equivalents, in the flaxseed extract was found to be 177-437 μg/g of whole flaxseed, depending on the variety tested. Plasma biochemical analyses revealed that $B_6$ vitamers, particularly PLP concentrations were reduced ($P \leq 0.001$), due to 1ADP ingestion (10 mg/kg diet) irrespective of the sources. The reduction was also found to be dose dependent ($r = -0.669$, $P \leq 0.001$) when moderate $B_6$-deficient rats were fed with

synthetic 1ADP. Oral ingestion of flaxseed-derived 1ADP in marginally vitamin $B_6$-deficient rats increased plasma cystathionine ($P \leq 0.001$), and decreased plasma α-aminobutyric acid ($P \leq 0.001$) and glutamic acid ($P = 0.017$) concentrations compared to the controls. However, the ingestion of synthetic 1ADP elicited greater perturbations in amino acid profile compared to the flaxseed-derived 1ADP, which was predominantly in the form of the dipeptide linatine. Additionally, oral ingestion of the synthetic as well as the flaxseed-derived 1ADP significantly ($P \leq 0.05$) inhibited the activities of hepatic PLP-dependent enzymes involved in transsulphuration reactions of methionine metabolism, namely cystathionine β-synthase and cystathionine γ-lyase. The use of a non-targetted metabolomics analytical approach identified ten potential lipophilic markers of vitamin $B_6$-insufficiency: glycocholic acid, glycoursodeoxycholic acid, murocholic acid, N-docosahexaenoyl GABA, N-arachidonoyl GABA, lumula, nandrolone, orthothymotinic acid, cystamine and 3-methyleneoxindole. These data serve to highlight potential deleterious effects of nutritional co-insults of the ingestion of anti-pyridoxine factors linked to flaxseed in a population at risk for moderate vitamin $B_6$ deficiency.

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could not have been possible without the constant encouragement and endless love from my wife, Shanta.

# DEDICATION

I dedicate this thesis to my family

To my wife Shanta and my lovely daughter Leina

To my parents Nodiachand and Nungshitombi

I would not have been able to do this without you.

# FOREWORD

This thesis was prepared following a manuscript format, and it is composed of four manuscripts. The thesis begins with a general introduction (chapter 1) and literature review (chapter 2) followed by hypotheses and objectives (chapter 3). Manuscript I (chapter 4), II (chapter 5), and IV (chapter 7) were published in Journal of Agricultural and Food Chemistry, Journal of Nutritional Biochemistry, and European Journal of Nutrition respectively. While, manuscript III (chapter 6) has been submitted to Journal of Nutrition. The thesis is concluded with an overall summary and discussion (chapter 8).

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# ABBREVIATIONS

| | |
|---|---|
| 1ADP | 1-Amino D-proline |
| 4-PA | 4-Pyridoxic acid |
| ASBT | Apical sodium-dependent bile acid transporter |
| Ar-EA | N-Arachidonoyl ethanolamide |
| CBS | Cystathionine β-synthase |
| CGL | Cystahtionine γ-lyase |
| CTH | Cystathionine |
| CYP | Cytochrome P450 |
| Cys | Cysteine |
| DRI | Dietary reference intake |
| FAAH | Fatty acid amide hydrolase |
| FE | Flaxseed extract |
| FXR | Farnosoid X receptor |
| GTH | Glutathione |
| Hcy | Homocysteine |
| H&E | Hematoxylin and eosin |
| HPLC | High performance liquid chromatography |
| METH | Methionine |
| NAGABA | N-Arachedonoyl γ-aminobutyric acid |
| NDGABA | N-Docosohexaenoyl γ-aminobutyric acid |
| NTCP | Sodium taurocholate co-transporting polypeptide |

| | |
|---|---|
| OATP | Organic anion transporting polypeptide |
| OST | Solute transporters |
| PL | Pyridoxal |
| PLK | Pirdoxal kinase |
| PLP | Pyridoxal 5′-phosphate |
| PN·HCl | Pyridoxine·Hydrochloride |
| PNPOx | Pyridoxine phosphate oxidase |
| Q-tof | Quadrupole time of flight |
| RM | Remethylation |
| RSM | Response surface methodology |
| SAH | S-adenosylhomocysteine |
| SAM | S-adenosylmethionine |
| SHMT | Serine hydroxymethyl transferase |
| SULT | Sulphotransferase |
| THF | Tetrahydrofolate |
| TM | Transmethylation |
| TS | Transsulphuration |
| UGT | UDP-glucuronosyltransferase |
| UPLC-MS | Ultrahigh-performance liquid chromatography-mass spectrometry |

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1. Introduction

Vitamin $B_6$ serves as a cofactor for many enzymes in amino acid, sugar, and fat metabolism [1,2]. Most mammalian cells rely on the nutritional supply of vitamin $B_6$ as source of pyridoxal 5′-phosphate (PLP), the active form of vitamin $B_6$. PLP plays a vital role as a cofactor for enzymes involved in sulphur amino acid metabolism, notably those involved in the transsulphuration and remethylation pathways of the methionine cycle [3-5]. Vitamin $B_{6,}$ as PLP, also plays an active role in connecting protein with carbohydrate metabolism via the Krebs cycle by transferring the amino group from amino acids to α-keto acids during the transamination reaction [6]. Additionally, PLP-dependent enzymes play crucial roles in the metabolism of neurotransmitters, one carbon units, tetrapyrolic compounds, and amino sugars. It is also involved in the modulation of steroid receptor-mediated gene expression and regulation of immune function [7]. Therefore, the *in vivo* availability of PLP is critical to the maintenance of metabolic homeostasis.

Vitamin $B_6$ status is affected by several factors including nutrition, physiology, drugs, genetic, and naturally occurring vitamin $B_6$ antagonist/anti-pyridoxine components. In addition to the pyridoxine from dietary sources, natural $B_6$ antagonists may be present in common foods. For example, flaxseed which is known for its bioactive compounds linked to reduction in diabetes, cancer, and cardiovascular diseases [8], has also been shown to contain linatine [9]. When hydrolyzed using hydrocholic acid, this dipeptide releases a vitamin $B_6$ antagonist called 1-amino D-proline (1ADP). $B_6$ vitamers, particularly PLP, forms a hydrazone complex with 1ADP, thus leading to hydrazone poisoning [10]. As a result, vitamin $B_6$ requirement is increased with the administration of $B_6$ antagonists [11,12].

Vitamin $B_6$ deficiency causes extensive impairments in metabolic processes because of its multifaceted functions associated with different enzymes. Thus, marginal to severe vitamin $B_6$ deficiency in humans has been found to associate with inflammation, cardiovascular diseases [13], and impaired immune functions [1]. Moreover, several investigators have reported inverse association between serum PLP levels (and vitamin $B_6$ intake) with different types of cancer [1,14,15]. Therefore, dietary supply of vitamin $B_6$ as well as $B_6$ antagonists play crucial roles in maintaining the homeostasis of the *in vivo* PLP pool in order to prevent development of vitamin $B_6$-linked diseases.

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# CHAPTER 2

# LITERATURE REVIEW

## 2.1. Vitamin $B_6$: An overview

### 2.1.1. General description of vitamin $B_6$

Vitamin $B_6$ is a generic term assigned to pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL) and their related phosphorylated forms, which differ in the identity of the chemical groups present at the 4′ position (Figure 2.1). Out of the six forms of vitamin $B_6$, pyridoxal 5′-phosphate (PLP) is biologically active and functions as a cofactor in more than 140 different enzymatic reactions [1]. When considering the distribution of vitamin $B_6$ in the body, muscles represent the major storage site, accounting for approximately 80% of the total $B_6$ vitamers [2] while the blood plasma contains < 0.1% of the total body vitamin $B_6$ [3]. PLP represents more than 90% of the vitamin in plasma bound to albumin and it is considered as the most commonly used indicator for vitamin $B_6$ status [3-5].

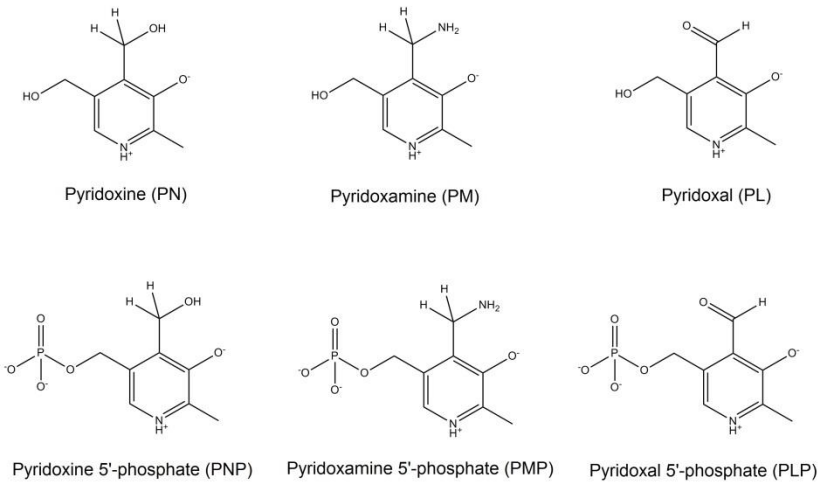



Figure 2.1. Structure of $B_6$ vitamers

2.1.2. Biology of vitamin $B_6$

*2.1.2.1. Digestion and absorption*

The naturally occurring forms of vitamin $B_6$ are PLP, PMP, and PN. $B_6$ vitamers can only pass through the intestinal lumen in the form of PL, PM, and PN; and therefore, phosphorylated vitamers must be dephosphorylated before absorption in the small intestine [6]. The major enzymes responsible for dephosphorylation are alkaline phosphatase or other intestinal phosphatases. They hydrolyze the phosphate group to release PL, PM, and PN. They are absorbed primarily in the jejunum through passive diffusion in the animals [7]. However, a protein known as Tpn1p which was identified in *S. cerevisiae* facilitates the uptake of vitamin $B_6$ across the plasma membrane through active transport system [8]. It was also found that there is evidence of existence of specialized carrier-mediated process of pyridoxine uptake in intestinal epithelial Caco-2 cells which is Na+-independent but highly dependent on the change of pH of the buffer [9]. A similar carrier-mediated uptake of vitamin $B_6$ was also demonstrated in young adult mouse colonic epithelial cells and purified human colonic apical membrane vesicles. It was further confirmed when unlabeled pyridoxine inhibited the rate of uptake of [$^3$H] pyridoxine; while no effect was seen on the uptake of unrelated compounds such as isoniazid, pennicillamine, theophylline, and homocysteine [10].

*2.1.2.2. Metabolism of vitamin $B_6$*

The liver is the major organ responsible for vitamin $B_6$ metabolism. In the cytoplasm of hepatocytes, ATP kinase (i.e., pyridoxal kinase, PLK) phosphorylates the unphosphorylated $B_6$ vitamers before they undergo subsequent metabolism. In healthy humans, the homeostasis of $B_6$ vitamers, especially PLP, is maintained by PLK and

pyridoxine 5′-phosphate oxidase (PNPOx) enzymes and their related genes [1,11]. $B_6$ vitamers, particularly PL, are catabolized by aldehyde oxidase or by NAD-dependent dehydrogenase to yield 4-pyridoxic acid and 5-pyridoxic acid, and these are subsequently excreted through urine [3]. Aldehyde oxidase is mostly concentrated in the liver, while dehydrogenase activity is found in mitochondria, cytosol, and microsomes of many tissues. Therefore, dehydrogenase enzymes are more responsible for the formation of pyridoxic acid than aldehyde oxidase [6]. These enzymes also help in maintaining the free *in vivo* PLP concentrations in the body by regulating the catalytic conversion of pyridoxal to 4-pyridoxic acid [1].

*2.1.2.3. Storage of vitamin $B_6$*

Out of the different $B_6$ vitamers, PLP and PL represent the predominant circulating forms of vitamin $B_6$ [12]. Vitamin $B_6$, particularly PLP, is stored in different body pools including muscle, liver, plasma and erythrocytes. PLP in the muscle pool represents around 75-80% of the total vitamin $B_6$ in the body and atleast 60% of that serves as coenzymes for glycogen phosphorylase [13]. In tissue, vitamin $B_6$ is concentrated in mitochondria and cytoplasm and retained there due to phosphorylation reactions [14].

*2.1.2.4. Excretion of vitamin $B_6$*

As mentioned earlier, the major excretory product of vitamin $B_6$ is pyridoxic acid, and the quantity excreted is dependent on the amount of $B_6$ intake. Shibata *et al* (2013) found that excretion of 4-pyridoxic acid was almost zero in the urine after feeding of a pyridoxine free-diet for 2 days in rats [15]. Additionally, the correlation ($r \geq 0.30$) of vitamin $B_6$ intake and 4-pyridoxic acid excretion suggests a dose dependent association

between vitamin $B_6$ excretion as pyridoxic acid, and vitamin $B_6$ intake in humans [16]. At least 40-60% of dietary vitamin $B_6$ is excreted as pyridoxic acid. Therefore, this urinary marker is a useful short term indicator of vitamin $B_6$ status [17].

2.1.3. Measurement of vitamin $B_6$ status

The measurement of vitamin $B_6$ concentrations has been well established. With the advancement of technology and methodologies, the measurement of the concentrations of $B_6$ vitamers can be accomplished with higher specificity and sensitivity, thus furthering the understanding of the role of vitamin $B_6$ in nutrition. There are several markers for the estimation of vitamin $B_6$ status, and these can be broadly categorized as either direct or indirect methods (Table 2.1.)

*2.1.3.1. Direct method for vitamin $B_6$ measurement*

For many years, plasma PLP has been the most frequently used direct measure or marker for vitamin $B_6$ status [17,18]. Plasma PLP is the primary form of circulating $B_6$ vitamers, comprising 70-90% of total vitamin $B_6$ in the blood [17]. It has been recognized to be an appropriate index for vitamin $B_6$ status as its concentration directly reflects tissue $B_6$ levels. Generally, plasma concentrations lower than 20 nmoles/L are considered indicative of a vitamin $B_6$ deficiency in humans [5]. Plasma PL has also been suggested to be an additional marker for $B_6$ status. PL is the primary precursor for the major forms of $B_6$ vitamers, and it crosses cell membranes prior to conversion to PLP. Plasma PL contributes around 8-30% of total plasma $B_6$ vitamers [17]. Additionally, measuring the total vitamin $B_6$ concentration, including all forms of $B_6$ vitamers, might provide more detailed data on the estimation of vitamin $B_6$ content. Besides plasma biomarkers, urinary 4-PA, the major metabolic product of all forms of $B_6$ vitamers, may be an effective

marker of status, as it corresponds to 50% of total $B_6$ intake. Urinary 4-PA excretion responds almost immediately to changes in dietary vitamin $B_6$ supply. Therefore, it is also considered as another good index for vitamin $B_6$ status. According to previous work, the excretion of more than 3 μmoles/day of 4-PA is considered to be associated with adequate $B_6$ status in humans [5].

The direct measurement of total vitamin $B_6$, which includes all of its possible $B_6$ vitamers, can be performed by using either a microbiological technique [17] or via high-performance liquid chromatography (HPLC) with an appropriate detection system [19,20]. Quantification of only plasma PLP has been commonly performed with an enzymatic method using tyrosine apodecarboxylase [5]. On the other hand, HPLC methods have also been extensively used due to their ability to separate and quantify the major $B_6$ vitamers with higher sensitivity. Plasma PLP and PL (as semicarbazide derivatives), and urinary 4-PA have been accurately measured using a HPLC and fluorescence detection [20].

*2.1.3.2. Indirect method for vitamin $B_6$ measurement*

*(a) Erythocyte transaminase test*

One of the most commonly used indirect methods of $B_6$ status is the measurement of α-erythrocyte aspartate aminotransferase (α-EAST) and α-alanine aminotransferase (α-EALT) activity or/and stimulation. Because of the nature of the life span of erythrocytes, α-EAST and α-EALT are considered long-term indices of vitamin $B_6$ status [17]. When the activation coefficient of α-EAST is greater than 1.6 and of α-EALT is greater than 1.25, this indicates that a deficiency of vitamin $B_6$ is likely. As the α-EALT activity is

more responsive to vitamin $B_6$ intake, it is considered to be a better indicator than α-EAST [5,17].

*(b) Tryptophan load test*

One of the oldest methods for the determination of vitamin $B_6$ status is the measurement of urinary xanthurenic acid, a primary metabolite of tryptophan metabolism. This metabolic pathway proceeds via a PLP-dependent enzyme kynureninase. During vitamin $B_6$ deficiency, a substantial increase in the excretion of xanthurenic acid is observed. When the deficiency is worsened, 3-hydroxykynurenine and kynurenine are also excreted in urine [5]. The urinary excretion of 30-40 μmoles/day of xanthurenic acid after a 2 g tryptophan load is considered to be normal. These tryptophan metabolites can be separated and quantified using existing HPLC methodology [17]. However, this has been a challenging method given the fact that there is ambiguity to level of xanthurenic acid excretion that is representative of adverse $B_6$ status [5].

*(c) Methionine load test*

The methionine load test, in addition to the tryptophan load test, has also been used as an indirect method for vitamin $B_6$ status. This test is based on the acitivity of three PLP-dependent enzymes i.e., cystathionine β-synthase, cystathionine γ-lyase, and cysteine sulphinic acid decarboxylase [21]. A vitamin $B_6$ deficiency caused a dramatic increase in the excretion of the metabolites of methionine catabolism, particularly cystathionine and homocysteine. A methionine load test of 3g resulted in the excessive excretion of cystathionine, homocysteine, cysteine, and sulphinic acid in adults of $B_6$ deficiency [5].

Table 2.1. Parameters for the assessment of vitamin $B_6$ status and their corresponding reference values for adult humans [5,17].

| **Parameters** | **Markers** | **Reference value for adequate status** |
|---|---|---|
| Direct method | Plasma PLP | > 30 nmoles/L |
| | Plasma total vitamin $B_6$ | > 40 nmoles/L |
| | Urinary 4-PA excretion | > 3μmoles/day |
| Indirect method | α-Erythrocyte aspartic transaminase activity | < 1.8 (< 80%) |
| | α-Erythrocyte alanine transaminase activity | < 1.25 (< 25%) |
| | Urinary xanthurenic excretion | < 65 μmoles/day |
| | Urinay cystathionine excretion | < 350 μmoles/day |
| Diet intake | Vitamin $B_6$ intake | > 1.2-1.5 g/day |

2.1.4. Major functions of vitamin $B_6$

Vitamin $B_6$ acts as a cofactor for a number of PLP-dependent enzymes which represent a class of the most versatile organic catalysts in biological systems. It acts as a co-enzyme during the transamination and transsulphuration reactions for the synthesis and catabolism of proteinogenic and non-proteinogenic amino acids, including homocysteine [22,23]. Vitamin $B_6$ is required for the bioconversion and synthesis of bioactive amines or/and neurotransmitters from amino acids. For instance, it is required for the decarboxylase enzymes to convert histidine to histamine, tryptophan to serotonin, 3,4 –dihydroxyphenyl alanine to dopamine, and glutamate to γ-aminobutyric acid [5,24].

In the case of glucose metabolism, the coenzyme PLP acts as an indispensable structural determinant to activate glycogen phosphorylase enzyme during glycogenolysis. It also functions as a proton donor or acceptor through the 5′-phosphate group of PLP

during those glycogenolytic reactions [25]. Vitamin $B_6$ as PLP helps in the synthesis and functioning of hemoglobin. It first assists in the functioning of δ-aminolevulinic acid synthase, an enzyme involved in heme synthesis. Later, it binds to two sides of hemoglobin to modulate the affinity of oxygen [26]. Vitamin $B_6$ is also required for the activity of both the serine C-palmitoyltransferase and sphingosine 1-phosphatase enzymes which act as entry and exit gates in the metabolism of sphingolipids [27]. Thus, PLP is involved in catalyzing numerous diverse reactions including transamination, decarboxylation, transsulphuration, deamination, cleavage, racemization and replacement reactions [28].

Vitamin $B_6$ has also been found to be involved in the modulation of gene expression. High levels of $B_6$ vitamers lead to a reduction in the transcription of progesterone, androgen, oestrogens, and certain glucocorticoid hormones [3,29]. Vitamin $B_6$, as PLP, has the ability to react with lysine residues of the steroid hormone receptor protein, thereby preventing or interrupting hormone-binding processes [11]. This inhibitory effect of PLP on the binding of progesterone receptor to ATP-sepharose was concentration-dependent [30]. Moreover, vitamin $B_6$ also modulates the glycoprotein IIb gene expression by interacting with tissue specific transcription factors to regulate platelet agregration [3].

In addition to the function of PLP as a coenzyme, vitamin $B_6$ also exhibits anti-oxidative capacity when assayed *in vitro* similar to the activities observed with ascorbic acid and tocopherols [31,32]. An *in vivo* study also showed that $B_6$ vitamers act as anti-oxidative modulators of many reactive oxygen species including superoxide radicals. For

instance, PL at a concentration of 1mM was equivalent to one unit of superoxide dismutase (SOD) in terms of its quenching ability to superoxide radicals [33].

### 2.2. Sulphur amino acid metabolism and role of vitamin $B_6$

Sulphur-containing amino acids play a substantial role in the methylation, control of oxidative status, and the maintenance of cellular integrity, in addition to their well-defined roles as substrates for protein synthesis [34]. The primary sulphur-containing amino acids in mammals are methionine and cysteine. Methionine is an indispensable amino acid, while cysteine is a conditionally indispensable amino acid obtained as a metabolite during methionine metabolism [35]. Defects in sulphur amino acid metabolism lead to thiol imbalance, which has been associated with a variety of diseases including cardiovascular disease, alzheimer's, cancers, and HIV [36].The metabolism of methionine involves three pathways: Transmethylation (TM), Transsulphuration (TS), and Remethylation (RM) (Figure 2.2).

#### 2.2.1. Transmethylation pathway

The first step in methionine metabolism is the formation of high-energy sulphonium compound, S-adenosylmethionine (SAM), an important methyl donor. Methionine adenosyl transferase (MAT) enzyme catalyzes the formation of SAM transferring an adenosyl moiety of ATP to the sulphur atom of methionine [37]. In the next step, SAM condenses with glycine to form S-adenosylhomocysteine (SAH) catalyzed by cytosolic enzyme glycine N-methyltransferase (GNMT). As a result, a methyl group and sarcosine are released. Subsequently, a homocysteine molecule is formed by releasing the adenosyl group with the help of the reversible enzyme S-adenosylhomocysteine hydrolase (SAH-H) [23,35,38].

2.2.2. Transsulphuration pathway

Homocysteine is an essential nonproteinogenic sulphur-containing amino acid that plays a significant role in one-carbon metabolism and methylation reactions [36]. In order to maintain normal homocysteine levels of 5-12 μmol/L in plasma [1], vitamin $B_6$ plays a crucial role as a co-factor for three enzymes directly linked to two pathways of the methionine cycle - namely TS and RM. In the TS pathway, homocysteine is condensed with serine to form cystathionine with the help of a PLP-dependent enzyme cystathionine β-synthase (CBS). It is subsequently broken down into cysteine and α-ketobutyrate through the action of another PLP-dependent enzyme cystathionine γ- lyase (CGL). Cysteine subsequently acts as a substrate for protein anabolism and as a precursor for the synthesis of the sulphur-containing compounds, including Co-enzyme A, taurine and glutathione [35]. The latter compound plays an important role in immune modulation, xenobiotic detoxification, and also acts as an antioxidant to equilibrate redox status [39,40].

2.2.3. Remethylation pathway

Homocysteine is also necessary for its role in accepting a methyl group from betaine or 5-methyl tetrahydrofolate in the step that reforms methionine [41]. The remethylation reaction of homocysteine to form methionine (Figure 2.2) is catalyzed by betaine-homocysteine methyltransferase (BHMT) or/and methionine synthase (MS). Betaine (trimethylglycine) acts as a methyl donor to convert homocysteine to methionine by BHMT, and dimethylglycine is released as a byproduct [36]. MS, a vitamin $B_{12}$-dependent enzyme, is however responsible for *de novo* synthesis of the methionine methyl group by utilizing 5′-methyl tetrahydrofolate as a methyl donor in the

tetrahydrofolate (THF) coenzyme system [37]. In order to complete the folate cycle, one methyl group from serine is transferred to tetrahydrofolate (THF) to yield 5,10-methylene THF, and it is regulated by a PLP-dependent enzyme serine hydroxymethyltransferase (SHMT) [23,41,42].

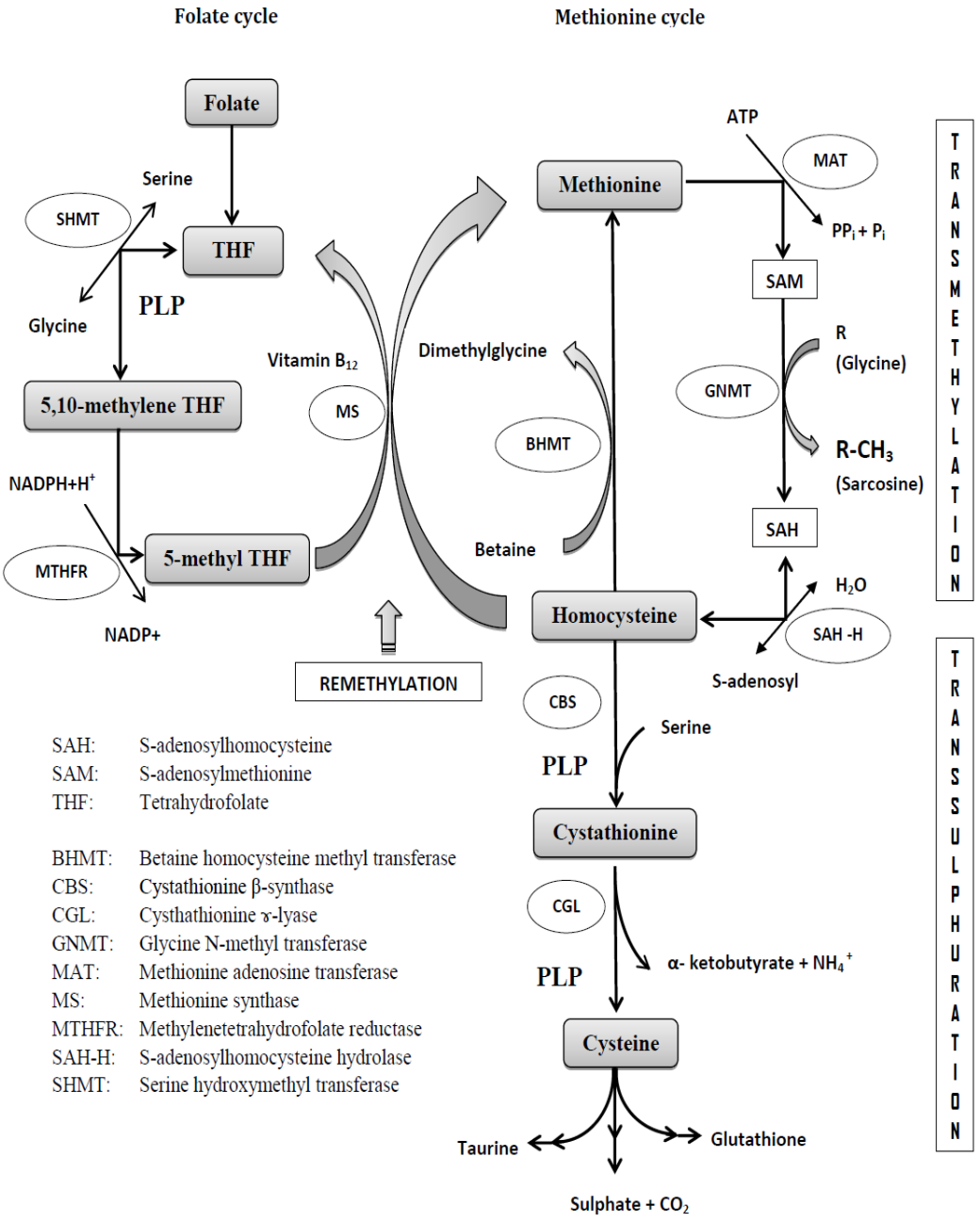



Figure 2.2. Role of PLP in sulphur amino acid metabolism

## 2.3. Epidemiology and factors influencing vitamin $B_6$ status

### 2.3.1. Epidemiology of vitamin $B_6$ status

Status of vitamin $B_6$ in individuals varies with country to country due to the differences in the intake of vitamin $B_6$ from dietary sources. Epidemiological studies on vitamin $B_6$ status have been based on either nutrient intake from the food supply or plasma PLP concentration. As indicated earlier, plasma PLP is the most frequently used measure for determing vitamin $B_6$ status [17,18]. Categorization of vitamin $B_6$ status/insufficiency is not very clear; however, researchers have been trying to group the individuals based on plasma PLP concentrations. Individuals are considered as deficient and marginally deficient in vitamin $B_6$ when their plasma PLP cocnentrations are less than 20 nmoles/L and 20-30 nmoles/L respectively [4,18]. According to the data from the Canadian Community Health Survey in 2004, the percentage of the population consuming vitamin $B_6$ lower than the estimated average requirement (EAR) was higher in older people, estimated to be 32% for females over 70 years of age. However, it was less than 10% in those less than 30 years of age (Table 2.2). Additionally, the National Health and Nutrition Examination Survey (2004) reported that 16% of the male and 32% of the female US population has plasma PLP concentrations below 20 nmoles/L (Table 2.3)[43]. These epidemiological studies suggested that overt $B_6$ deficiency is rare in developed countries, while marginal/moderate deficiency may be highly prevalent.

Table 2.2. Vitamin $B_6$ intake of the Canadian household population from foods (adapted from Canadian Community Health Survey, 2004)

| Age (years) | Vitamin $B_6$ intake from food (mg/d) | | | | | |
|---|---|---|---|---|---|---|
| | Male | | | Female | | |
| | n | Mean | % < EAR | n | Mean | % < EAR |
| 9-13 | 2080 | 1.83 | < 3 | 1980 | 1.54 | F |
| 14-18 | 2288 | 2.22 | < 3 | 2256 | 1.51 | 11.1 |
| 19-30 | 1804 | 2.29 | F | 1854 | 1.59 | 9.6 |
| 31-50 | 2596 | 2.23 | F | 2686 | 1.65 | 15.9 |
| 51-70 | 2550 | 2.08 | 10.9 | 3200 | 1.66 | 19.4 |
| > 70 | 1520 | 1.83 | 23.1 | 2610 | 1.55 | 32.5 |

Table 2.3. Vitamin $B_6$ status of US population (adapted from National Health and Nutrition Survey 2004 [43])

| Age (years) | Plasma PLP | | | |
|---|---|---|---|---|
| | Nonusers of supplemental $B_6$ | | Users of supplemental $B_6$ | |
| | n | % < 20 nmoles/L | n | % < 20 nmoles/L |
| < 13 | 903 | 25 | 275 | 11 |
| 13-20 | 1317 | 19 | 196 | 10 |
| 21-34 | 556 | 27 | 197 | 16 |
| 35-44 | 410 | 26 | 163 | 15 |
| 45-64 | 632 | 23 | 428 | 7 |
| ≥ 65 | 581 | 24 | 501 | 6 |
| Sex | | | | |
| Male | 2351 | 16 | 834 | 2 |
| Female | 2048 | 32 | 926 | 15 |

2.3.2. Factors influencing vitamin $B_6$ status

The major factors influencing vitamin $B_6$ status thus predisposing an individual to a vitamin $B_6$ deficiency include genetic aberrations, drug interactions, and physiological and nutritional factors. In addition to these, vitamin $B_6$ requirement is elevated due to special conditions, including chronic renal failure, and certain poisoning conditions [5].

2.3.2.1. Genetic factors

The salvage pathways of vitamin $B_6$ biosynthesis maintain the homeostasis of endogenous PLP in animals. Two ubiquitous enzymes–an ATP-dependent pyridoxal kinase (PLK) and a FMN-dependent pyridoxine 5′-phosphate oxidase (PNPOx)–are actively involved in this pathway. A pdxK gene located on chromosome 21q22.3 of humans and its homolog pdxY in bacteria code for the PLK enzyme. The PLK enzyme from the pdxK gene is active for all three unphosphorylated $B_6$ vitamers, while the PLK from pdxY gene is responsible for the phosphorylation of only PL to PLP [44]. PNPOx, which catalyzes the 4′-hydroxyl group of PNP or the 4′-amino group of PMP into the aldehyde group of PLP, is coded by a pdxH gene in humans, with a location on chromosome- 17q21.2 [1]. As such, any type of genetic aberrations/polymorphism affects the status of the related enzymes. Similarly, the ALPL gene regulates the expression of alkaline phosphatase enzyme, and mutations in the ALPL gene may impair the catabolism of vitamin $B_6$ [45]. Moreover, any polymorphism in methylenetetrahydrofolate reductase gene, for example MTHFR C667T, that regulates 1-carbon methylation may influence vitamin $B_6$ requirement [46,47].

2.3.2.2. Interactions with drugs

Certain drugs/compounds may interact with freely available PLP to cause a vitamin $B_6$ deficiency, and these interactions can typically be grouped into three categories. The first group includes those drugs that directly inhibit the PLK enzyme but do not form covalent complexes with $B_6$ vitamers, including theophylline (a drug used to treat respiratory diseases) and caffeine (a natural bioactive compound present in coffee). The second group of drugs reacts with plasma PLP to form hydrazone complexes, and these complexes inhibit the activity of the PLK enzyme. Antituberculosis drugs, including isonicotinic acid hydrazide, dopamine and cycloserine, come under this group. The third group of drugs form complexes with freely available PLP but do not inhibit the PLK enzyme. This class of drugs, including penicillamine and levodopa reduce freely available endogenous PLP [48]. Additionally, compounds that are used in oral contraceptive pills like ethinylestradiol and mestranol also reduce plasma PLP concentration in the body [5].

2.3.2.3. Physiological factors

Physiological factors influencing vitamin $B_6$ status include the age of the person, disorders in absorption, physical activity level, pregnancy, smoking and drinking [5]. The concentration of plasma PLP in elderly persons was significantly lower as compared to younger individuals [43,49]. Some of the possibilities to explain the age-related PLP decrease may be the reduction of vitamin $B_6$ absorption and regulation of 4-pyridoxic acid production in the liver [50]. Heavy exercise increases protein and glycogen breakdown, which subsequently may cause an increased $B_6$ requirement [51]. During pregnancy, low plasma PLP is observed due to an increased PLP dephosphorylation in

the maternal circulation in order to provide sufficient pyridoxal for placental transport to the developing fetus [52]. Additionally, a low vitamin $B_6$ status has also been associated with chronic alcoholism and heavy smokers, likely due to the increased susceptibility of PLP to the alkaline phosphatase enzyme. This enzyme hydrolyzes PLP to PL in the erythrocytes, resulting in plasma PLP concentrations that are significantly reduced [53,54].

2.3.2.4. Nutritional factors

The plasma PLP concentration is directly related to the dietary supply of vitamin $B_6$. The recommended dietary allowances (RDA) values for vitamin $B_6$ are 1.8mg/day and 1.3 mg/day for Canadian men and women, respectively; and it is the only water soluble vitamin whose requirement increases with aging [2]. An insufficient dietary supply of vitamin $B_6$ leads to a low endogenous PLP level [11]. Bioavailability is also an important factor which affects the body's vitamin $B_6$ status. In most commonly consumed foods, vitamin $B_6$ bioavailability ranges from 70 -80%. Increased protein intake appears to decrease freely available $B_6$ vitamers even though the actual $B_6$ content is not changed. This might be due to increased tissue retention of PLP after a high protein diet as well as increased transaminase activity, the enzymes involved in amino acid catabolism [2]. Therefore, there is still a consideration of the protein intake when evaluating the $B_6$ requirement [55]. On the other hand, low carbohydrate diets lead towards increases in gluconeogenesis and glycogenolysis in order to supply required glucose units for obligate glucose-utilizing tissues, and these processes require PLP as a cofactor. Low carbohydrate diets, therefore, can mimic metabolic conditions (ie: protein and fat utilization) that arise with high protein diets. Therefore, vitamin $B_6$ requirement may

increase with a decrease in carbohydrate and an increase in the protein content of the diet [56], however this remains to be fully elucidated.

While the diet contributes vitamin $B_6$, it can also be a source of naturally-occurring vitamin $B_6$ antagonists that are present in common foods and known to affect the bioavailability and metabolism of $B_6$ vitamers. Flaxseed contains linatine (1-[(n-γ-L-glutamyl)-amino]-D-proline), a naturally occurring dipeptide that yields a toxic compound called 1-amino D-proline (1ADP) after hydrolysis [57]. Certain wild mushrooms including *Gyromita esculenta L.*and *Agaricus biporis L.* also contain Gyromitrin, methylhydrazine and agaritine. These active compounds formed complexes with PLP to cause hydrazine poisoning. Similarly, most of the leguminous plants, especially *Canavalia sp.*, contain considerable amount of canavaline and canaline that can also form complexes and oximes with $B_6$ vitamers. In addition to these, a neurotoxin from *Vicia* called β-cyanoalanine also acts as a vitamin $B_6$ antagonist [58]. As a result, these anti-nutritive compounds reduce the endogenous PLP pool or/and cause toxicity within cells.

**2.4. Flaxseed: A nutrient overview**

2.4.1. The nutritive and anti-nutritive components of flaxseed

Flax (*Linum usitatissimum L.*) is known for its content of omega-3 fatty acids, fibre and lignans. About 42-45% of the flaxseed is oil and the polyunsaturated fatty acids account for more than 70% of the oil's composition. The traditional flaxseed contains a high amount of α-linolenic acid (ALA), representing 57% of total fatty acids [59]. Flaxseed also contains soluble fibres which help in lowering blood cholesterol levels, and insoluble fibres which increase bowel movement in the colon [60]. Moreover, flaxseed is

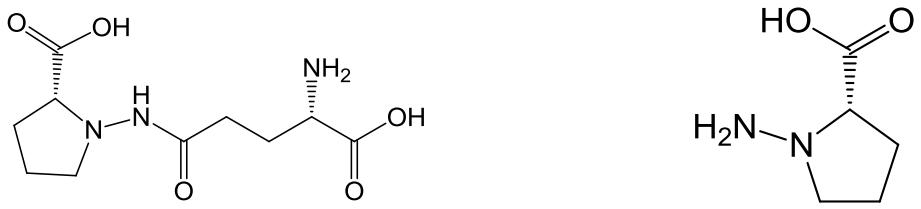
also a rich source of lignans which have the potential to protect against certain chronic diseases including, cancers, cardiovascular diseases, diabetes, and kidney diseases [61].

In addition to the positive attributres mentioned above, flaxseed also contains several anti-nutritive compounds, including phytic acid, cyanogenic glucosides and linatine. A very small amount of trypsin inhibitor and oxalates are also found in flaxseed. Phytic acid is a large compound and a very strong chelator of mineral cations such as potassium, magnesium, iron and zinc, and has also been implicated in the binding to proteins and starches. The amount of phytic acid in flaxseed varies from 0.8-1.5% on a dry weight basis [62]. The cyanogenic glucosides present in significant amounts in flaxseed are linustatin and neolinustatin. However, linamarin and lotraustralin, the corresponding monoglycosides, are also present in immature seeds but diminish to trace levels in mature seeds [63]. These compounds have the ability to release hydrogen cyanide upon acid or enzymatic hydrolysis which can cause cyanide toxicity [64]. Linustatin is the main cyanogenic compound present in flaxseed at a concentration of 213-352 mg/100 g of seed accounting for 54-76% of the total cyanogenic glucosides, while the content of neolinustatin ranges from 91-203 mg/100 g of seed [65]. Linatine, the third anti-nutrient found in flaxseed acts as anti-pyridoxine factor. The amount of linatine present in the flaxseed ranges from 80-100 μg/g [57,58,66].

2.4.2. The anti-vitamin $B_6$ components in flaxseed

Linatine (1-[(*n*-*γ*-L-glutamyl)-amino]-D-proline), as mentioned above, is a dipeptide present in flaxseed (Figure 2.3A). Although, it was first isolated and characterized in the seed of the flax plant, linatine has been found to occur in all parts of the immature flax plant [58]. It is a polar compound, thus very soluble in water and

insoluble in anhydrous organic solvents. Kratzer (1946) first identified this vitamin $B_6$ antagonist when he observed the establishment of a vitamin $B_6$ deficiency in chicks fed a diet containing linseed meal [66]. Elemental analysis showed that the empirical formula of linatine is $C_{10}H_{17}N_3O_5$ with an observed molecular weight of 259 g/mol. It is formed by 1-amino D-proline (1ADP, Figure 2.3B), a toxic compound, bound to a glutamic acid by a peptide bond through the γ-carbonyl of the glutamic acid. Upon hydrolysis, linatine releases 1ADP and glutamic acid, while hydrogenolysis yields proline and glutamine [57].




(A) Linatine (B) 1-amino D-proline

Figure 2.3. Structure of anti-pyridoxines present in flaxseed: (A) Linatine (B) 1-amino D-proline

2.4.3. Extraction and quantification of linatine from flaxseed

After the extraction of oil from flaxseed, linatine is retained in the meal. This dipeptide is a polar compound, and can be easily extracted using water or aqueous alcohol as a solvent. Klosterman and colleagues extracted linatine from flaxseed cotyledons by using 70% aqueous ethanol. They also found that this compound was

dialyzable and thus a small molecule [67]. Afterwards, linatine was successfully extracted from linseed, at pilot scale, using 70% isopropyl alcohol as a solvent. The extraction procedure included mechanical stirring extraction for 1h followed by filtration using a rotary vacuum filter. The clear filtrate obtained was passed through an ion exchange column, and the eluate containing linatine was concentrated under reduced pressure to get considerably pure linatine [68].

Linatine present in flaxseed extracts can be quantified by either direct or indirect methods. The direct method of quantification involves separation of linatine present in the sample using a chromatography column such as thin layer chromatography, gas and liquid chromatography, along with a suitable detector. The indirect method is based on the hydrolysis of linatine present in the sample to release 1ADP followed by the quantification of free 1ADP either using a colorimetric determination or chromatographic method with a suitable detector. Klosterman and his colleagues (1967) hydrolyzed linatine by incubating the sample in 1M HCl for 4h at 100°C. The hydrolysate was separated by using a resin column followed by thin layer chromatography. Quantification was done using a colorimetric method where 1ADP reacts with PLP at pH 6 to form a hydrazone complex, which can be detected at 374 nm [57].

## 2.5. Biological and toxicological properties of 1-amino D-proline

### 2.5.1. *In vitro* effect of 1-amino D-proline

*In vitro* analysis revealed potential anti-vitamin $B_6$ as well as bactericidal properties of 1ADP [58,69] . This vitamin $B_6$ antagonist inhibited a variety of PLP-requiring enzymes from *E. coli*, including glutamic aminotransferase, tryptophanase, tyrosine decarboxylase, and glutamic decarboxylase. The inhibiton of the acitivities of

these enzymes was due to the toxicity induced by hydrazone complexes—confirmed by spectrophotometric evidence—formed in the cell when 1ADP was added to the respective holoenzymes [57,58]. Addition of PLP in the above enzyme complexes reversed the inhibition of the decarboxylases and tyrosinase, but not the aminotransferase. Interestingly, linatine did not show inhibition of these enzymes *in vitro* because the reactive hydrazino function is masked by the γ -glutamyl group [58]. In terms of bactericidal properties, 1ADP was more potent than its optical isomer, 1-amino L-proline (1ALP) and several other hydrazino acids against 20 microorganisms [58,69]. Besides, linseed extract at a concentration of 0.015g-equivalent also inhibited at least 55 mm diameter zone of *Azotobacter vinelandii O.* growth in a seeded agar plates when assayed by using microbiological tests [57].

2.5.2. *In vivo* effect of 1-amino D-proline

*In vivo* effect of 1ADP was first noticed when Kratzer (1946) showed development of vitamin $B_6$ deficiency symptoms in chicks when fed with diets containing linseed meal. He also found that an addition of pyridoxine in the diet can counteract the observed deficiency symptoms [66]. The active toxic component of linatine, 1ADP, acts as an asymmetrically substituted secondary hydrazine responsible for the *in vivo* hydrazone toxicity and vitamin $B_6$ deficiency of the linseed diets [57,58,70]. The extract from the linseed showed an $LD_{50}$ of 50 g-equivalent/chick, and an application of 1 mg pyridoxine injection or 40 ppm of pyridoxine to the diet can counteract the effect of the antagonist to prevent the development of deficiency symptoms [57]. However, the toxic effects had not been observed in mature poultry or mammals [58].

The intraperitoneal injection of 1-aminoproline (mixture of 1-amino D-proline and 1-amino L-proline) impaired amino acid metabolism in young rats. These rats showed significant increases in the concentrations of α-aminoadipic acid, citrulline, and cystathionine in liver, kidney, pancreas, plasma, and urine due to exposure to 1-aminoproline application. Moreover, rats with vitamin $B_6$ deficiency were found to be more sensitive to 1-aminoproline toxicity than the normal rats [71].

Tsuji and his colleagues' (1977) studied the kinetics of 1-aminoproline metabolism using radiolabelled substrates. After an intraperitoneal injection of radioactive 1-aminoproline in rats, radioactivity was distributed to most of the tissues. The kidney had the highest specific activity 1 hour following the injection, which decreased by 60% 7 hours post-injection. It was also observed that 80% of the radioactivity administered was detected in the urine. Among the several radioactive compounds in the urine, the 1-aminoprolyl hydrazone of pyridoxal, a dephosphorylated product of 1-aminoprolyl hydrazone of pyridoxal 5-phosphate, was identified [72].

In another experiment, injection of 1-aminoproline resulted in the accumulation of (R)-β-aminoisobutyric in the kidney and liver of rats [73]. It also greatly disturbed methionine metabolism, resulting in the accumulation of L-cystathionine and its derivatives. Other unknown compunds, which were later, identified as S-[L-2-(acetylamino)-2-carboxyethyl]-L-homocysteine and diastereoisomers of L-cystathionine sulfoxide were also excreted in the urine of those rats [74]. Moreover, application of 1ADP depressed exogenous carnitine production by as much as 60-80% in the profused rat liver, which can be reversed by the addition of adequate pyridoxine in the perfusing

medium. This result indicated potential inhibitory effect of 1ADP on carnitine and its related metabolism [75].

Interestingly, 1-amino-L-proline was more toxic than its optical isomer 1-amino D-proline in rats [71], while it was other way in chicks and some bacteria [57,69]. The latter results provided evidence of the sterospecificity of 1-aminoproline toxicity amongst organisms. Although, 1ADP acts as an anti-pyridoxine factor in chicks and other lower mammals, flaxseed has not been associated with vitamin $B_6$ deficiency in healthy humans [60].

### 2.6. Major clinical impairments due to vitamin $B_6$ deficiency

Because of the diverse and versatile function of vitamin $B_6$ in the body, its deficiency causes extensive impairments. Primarily, vitamin $B_6$ deficiency causes microcytic anemia due to decreased haemoglobin synthesis [5]. Immune function is normally impaired by $B_6$ deficiency, especially in the elderly because of the reduced production of lymphocytes and interleukin (IL)-2 [76].

Vitamin $B_6$ deficiency reduces the activity of the PLP-dependent enzyme SHMT, resulting in a lack of methylene groups for the synthesis of 5,10 methylene -THF. As a result, methylation processes will be perturbed resulting in outcomes such as DNA hypomethylation and impaired DNA excision repair [77]. Eventually, altered DNA methylation may lead to tumor development [78]. Apart from this, a deficiency of PLP also reduces CBS and CGL activities which may hamper the TS pathway. As a result, the generation of cysteine, an important component of glutathione is disturbed. Therefore, the production of a well-known detoxifying agent of several carcinogenic compounds by glutathione S- transferases and glutathione peroxidase from glutathione is hampered,

potentially leading to an increase risk of cancer [5]. Epidemiological studies also disclosed a significant inverse correlation between serum PLP (and vitamin $B_6$ intake) with different types of cancer. Individuals having plasma PLP < 30 nmoles/L were found to have a higher risk of pancreatic and lung cancers [5].

Marginal to severe vitamin $B_6$ deficiency in humans has been associated with inflammation and several cardiovascular diseases [79]. Impairments in PLP-dependent enzymes, including CBS, CGL and SHMT, can elevate *in vivo* homocysteine levels and subsequently increase SAH concentrations via the reversible SAH-H reaction [80]. Perturbation in the SAM/SAH ratio can adversely affect overall metabolic homeostasis. Elevated SAH levels lead to impaired TM reactions [81], causing a detrimental effect on the methylation of macromolecules and xenobiotic toxicants [34,82]. Elevated levels of homocysteine along with reduced methylation capacity due to a decrease in the SAM/SAH ratio seems to be linked to the increased risk of a number of pathological conditions including diabetes and cardiovascular diseases [35,83,84] . Additionally, impairments of enzymes involved in TS pathway may inhibit the *in vivo* synthesis of $H_2S$, an important modulatory gaseous molecule, as well as other sulfur-containing compounds, including glutathione [38,85] . Therefore, vitamin $B_6$ deficiency can predispose to perturbed hepatic function via the promotion of oxidative stress, fibrosis, and steatosis [86-88]. Recently, a meta-analysis concluded that supplementation of folate with vitamin $B_6$ and $B_{12}$ reduces the risk of stroke [89].

Early findings have also demonstrated the role of vitamin $B_6$ in the prevention of several neurocognitive defects including peripheral neuropathy and seizures [35].

Peripheral neuropathy caused by vitamin $B_6$ deficiency also has been reported to be associated with chronic peritoneal dialysis [90].

**2.7. Metabolomics: an approach to the study of modern nutrition**

Modern nutritional research targets to improve our understanding of the effects of nutrients/ food ingredidents on human health. The search for new biomarkers and pathways has been a promising step in the field of nutitrional biochemistry. Existing biomarkers allow us a glimpse into the effect of $B_6$ deficiency on metabolic processes. In order to gain more insight about the potential pathways affected by $B_6$ deficiency, new techniques and approaches may be required. Metabolomics offers one such opportunity.

2.7.1. What is metabolomics?

Metabolomics is a technology that has been applied to profile the entirety of metabolites in a complex biological sample [91]. The Metabolomics Society defines metabolomics as a newly emerging field of omics research whose primary objective is the extensive characterization of low molecular-weight metabolites present in the biological system. It also provides an overview of the global biochemical events in the cellular level [92]. It can be considered as an ‘imaging approach’ for the biochemical processes occurring inside the body [93]. The study of metabolites reveals useful biological information of molecules associated with biological end-points and can be implicated in the development of numerous human diseases [94]. It is also an advanced tool for the identification of important biomarkers of diseases/diagnoses which can lead to the discovery of novel pathways linked to disease development [95]. Using a metabolomics approach, several hundreds and/or thousands of metabolites can be monitored in biological samples based on their accurate mass.

2.7.2. Methodology in metabolomics.

Experimental strategies in metabolomics analysis are varied. However, in general, it involves the following steps: (a) sample preparation, (b) data acquisition, (c) statistical analysis, and (d) pathway mapping. Metabolomics can be performed on a range of different sample types including tissue, cells, blood, urine and saliva [94]. Additionally, targeted and non-targeted approaches have proved fruitful in deciphering a variety of biochemical pathways of human metabolism. Targeted metabolomics is used to quantify several hundred known metabolites present in complex biological samples, by using gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS)-based equipment with a multiple-reaction monitoring (MRM) approach. On the other hand, non-targeted metabolomics provides a broader technique of identifying unknown metabolites. The latter is performed by collecting all the possible ions (*m/z*) through scanning the mass profile. The most recent and advanced equipment of conducting untargeted metabolomics approach is using mass spectrometry-equipped with quadrupole time-of-flight (Qtof) which can give a mass accuracy of four points after the decimal. The mass spectral data collected are analyzed, and ions which are statistically different are sorted out. Identification and characterization of those potential markers are performed by searching existing databases or via NMR [91,96]. Therefore, the combination of targeted and non-targeted approaches towards metabolomics may lead to a better understanding of complex physiological pathways.

2.7.3. Metabolomics in the study of modern nutrition and vitamin $B_6$

Modern nutrition has shifted the focus regarding the impact of nutrients on human health and diseases to the molecular level. Recently, metabolomics has been projected as

a powerful tool to understand the impact of complex dietary molecules on human health and diseases [92]. Nutrimetabolomics or nutritional metabolomics has also been proposed as the beginning of personalized nutrition [97]. Targeted metabolomics approaches have been recently used to study the metabolic sequelae of low vitamin $B_6$ intake in humans [97,98]. Metabolomics analysis of marginal vitamin $B_6$ deficiency revealed a widespread perturbation in amino acid, sugar, and organic acid metabolism in healthy humans. It also illustrated the usefulness of metabolomics in studying the complex effects due to altered vitamin $B_6$ intake [98,98]. Plasma PLP, homocysteine and cystathionine are well-recognized biomarkers for vitamin $B_6$ deficiency [18,77,98]. However, additional opportunity exists to address alternative lipophilic biomarkers associated with disruptions in vitamin $B_6$ status, either through inadequate dietary supply and/or the ingestion of an antivitamin $B_6$ factor, namely 1ADP.

**2.8. Summary**

Vitamin $B_6$ is a collective term used for PL, PN, PM, and their phosphorylated forms. The liver is the major organ for vitamin $B_6$ metabolism, and the final product, pyridoxic acid is excreted through the urine. PLP, the active form of vitamin $B_6$ plays critical roles as a cofactor in numerous enzymes particularly associated with amino acid, carbohydrate, and fat metabolism. Its role in TS and RM pathways of methionine metabolism is highly crucial. Impairment in these pathways leads to imbalances in methylation process, production of homocysteine and xenobiotic detoxificants. Additionally, vitamin $B_6$ helps in maintaining the immune responses and preventing neurogenerative diseases.

There is a high prevalence of marginal vitamin $B_6$ deficiency in the general population, particularly in developed countries including North America. $B_6$ deficiency is mostly developed due to the insufficient intake of pyridoxine from the food. In addition to this, vitamin $B_6$ status is affected by different factors including genetic aberrations, interactions with drugs, physiological and nutritional factors. Many common foods including flaxseed contains vitamin $B_6$ antagonist. The anti-pyridoxine factor present in flaxseed is lintaine, a dipepetide of glutamic acid and 1ADP. Intraperitoneal injection of 1ADP had exhibited *in vivo* toxic effects of vitamin $B_6$ antagonist due to hydrozone toxicity in rats.

Severe vitamin $B_6$ deficiency is not common in humans, but its deficiency can cause diverse impairments in humans ranging from microcystic anemia, reduce immune function and cognitive ability, and defects in amino acid metabolism. The study of $B_6$ deficiency in the context of modern nutrition uses different advanced tools. Metabolomics is one of the modern tools that results in the study of hundreds to thousands of metabolites simultaneously in order to better understand pathways and to discover new markers linked to a defect in metabolism. Recently, targeted and non-targeted metabolomics have been used to understand more about vitamin $B_6$ and its metabolism as well as to discover novel biomarkers of $B_6$ deficiency.

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# CHAPTER 3
# HYPOTHESES AND OBJECTIVES

### 3.1. Hypotheses

(i) Optimization of extraction procedures using response surface methodology (RSM) will increase the efficiency of extraction of vitamin $B_6$ antagonists from flaxseed.

(ii) Vitamin $B_6$ antagonist contained in flaxseed will impair PLP-dependent enzymes and perturb amino acid metabolism in moderately vitamin $B_6$-deficient rats.

(iii) A metabolomics approach will reveal potential pathways affected due to vitamin $B_6$ deficiency induced by the ingestion of 1ADP during low B6 status.

### 3.2. Objectives

The main purpose of this research was to investigate the impact of a vitamin $B_6$ antagonist linked to flaxseed on amino acid metabolism, particularly the TS pathway of methionine cycle. The current research will also study the potential deleterious effect that the $B_6$ antagonist has on metabolic homeostasis. Therefore, the above hypotheses will be tested through the following objectives:

(i) Identification, characterization, and quantification of total anti-pyridoxines present in flaxseed.

(ii) Development of a rodent model of moderate vitamin $B_6$ deficiency.

(iii) *In vivo* investigation of the oral exposure of synthetic 1ADP in moderate vitamin $B_6$-deficient rats.

(iv) *In vivo* investigation of the oral exposure of flaxseed-derived 1ADP in moderate vitamin $B_6$-deficient rats.

(v) Metabolomics investigation for novel lipophilic biomarkers of vitamin $B_6$ inadequacy, induced by the application of an anti-pyridoxine, using a rodent model.

# CHAPTER 4

# MANUSCRIPT- I

## Identification, characterization and quantification of an anti-pyridoxine factor from flaxseed using ultra high performance liquid chromatography-mass spectrometry

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## 4.1. Abstract

In the present study, the anti-pyridoxine compounds linatine (1-[(n-γ-L-glutamyl)-amino]-D-proline) and 1-amino-D-proline (1ADP) were quantified following extraction from defatted flaxseed using aqueous isopropanol as a solvent, with extraction variables including time, temperature, and the solid-solvent ratio. Both linatine and 1ADP were identified, characterized and quantified via UPLC/ESI-MS using authentic standards. In order to optimize the extraction conditions for these anti-pyridoxine compounds, a respond surface methodology was applied using a second-order polynomial to describe the experimental data. The predicted model for the optimal extraction was significant ($P < 0.05$) with a $R^2$ of 0.82. A varietal analysis showed that amount of anti-pyridoxine present in flaxseed was ranged from 177-437 μg 1ADPE /g of whole seed. The current study establishes the content of specific anti-pyridoxine factors in flaxseed and positions the data for use in subsequent risk assessment modeling.

**Key words:** anti-pyridoxine, linatine, 1-amino D-proline, flaxseed

## 4.2. Introduction

The seeds of the flax plant (*Linum usitatissimum L.)* contain several nutritionally-relevant compounds. Flaxseeds contain 35-45% oil by weight, of which 52% is the α-linolenic acid (ALA), an omega-3 fatty acid [1]. Dietary ALA supplementation lowers the production of inflammatory biomolecules such as interleukin-6 (IL-6) and C-reactive protein (CRP) [2,3]. Flaxseed consumption suppressed the development of atherosclerosis by reducing plasma total cholesterol (TC), low density lipoprotein (LDL) and triglycerides (TGs) in a rat model [4]. For humans, the Dietary Reference Intake (DRI) process led to the establishment of an adequate intake for ALA in the amount of 1.1 and 1.6 g/d for women and men, respectively [5] . Given its enriched status, flaxseed has been positioned as a good source of ALA for the human diet. Additionally, flaxseed also represents a good source of cyclolinopeptides [6,7], fibre, and contains significant levels of lignans especially isolariciresinol, pinoresinol and secoisolariciresinol diglucoside (SDG) [8,9]. The latter phytoestrogenic compounds possess putative anti-tumorigenic [10,11], anti-oxidant [12] and hypotensive properties [13]. Given the increased interest in the potential health-promoting aspects of whole flaxseed, it's utilization in the commercial food supply has expanded. While the positive attributes of flax consumption have been the focus of attention in recent years, it is important to recognize the potential anti-nutritive factors present in whole seeds.

Flaxseed contains several anti-nutritive compounds, including phytic acid [14], cyanogenic glycosides [5,15,16] and the dipeptide linatine [17]. Linatine (1-[(n-γ-L-glutamyl)-amino]-D-proline), is a polar compound first identified by Kratzer [18] to possess anti-pyridoxine activity. Chicks fed with a diet containing linseed meal

established signs of vitamin $B_6$ deficiency [19]. Subsequent studies confirmed the structure of the compound as $C_{10}H_{17}N_3O_5$ with an observed molecular weight of 259.26. It is a dipeptide formed by 1-amino-D-proline (1ADP) bound to a glutamic acid by a peptide bond through the γ-carbonyl of the glutamic acid [17]. The potent anti-pyridoxine component of linatine is purported to be 1ADP, which has a molecular weight of 130.15. The latter compound acts as an asymmetrically substituted secondary hydrazine that is thus responsible for the *in vivo* hydrazone toxicity of the active pyridoxyl-5-phosphate (PLP), ultimately leading to the vitamin $B_6$ deficiency symptoms [19]. Numerous PLP-dependent enzymes, including glutamic aminotransferase, tryptophanase, tyrosine decarboxylase, and glutamic decarboxylase, demonstrated marked activity inhibition when treated, *in vitro*, with 1ADP [20]. Additionally, the intraperitoneal injection of 1ADP greatly disturbed methionine metabolism resulting in the accumulation of L-cystathionine and its derivatives [21]. Given the recognized potential for the presence of an anti-pyridoxine factor in the food supply, the establishment of an analytical technique for the measurement of linatine and 1ADP in flaxseed is required prior to any risk assessment analysis.

Previous research has relied upon the use of first generation chromatography (paper, thin layer) techniques coupled with ninhydrin-based, spectrophotometric analysis for linatine and 1ADP analysis [17,22-24]. The present study was designed to develop an Ultrahigh-performance liquid chromatography-mass spectrometry (UPLC-MS) technique for the measurement of linatine and 1ADP. Additionally, the objectives included the optimization of extraction conditions using response surface methodology (RSM) with four factors (solvent concentration, time, and temperature and the liquid-solid ratio), each

tested at three distinct levels. The final objective of this study was the determination of the content of the anti-pyridoxine components in selected varieties of commercial flaxseed.

**4.3. Materials and methods**

4.3.1. Materials

Flaxseed samples [a harvest composite sample made of flaxseed samples (N. 150) harvested within one year in Western Canada and nine different varieties] were provided by the Canadian International Grain Commission, Winnipeg, Canada. Isotopically-labeled D-proline (2,5,5-$d_3$) was obtained from C/D/N Isotopes (QC, Canada) while 1-amino D-proline was purchased from Santa Cruz Biotech (Dallas, TX, USA). All solvents (analytical and HPLC grades) were purchased from Sigma-Aldrich (Oakville, ON, Canada). A bulk of composite flaxseed was defatted through the use of a Soxhlet apparatus, using hexane as solvent. After drying, it was re-ground using a commercial seed/bean grinder, and passed through 50 mesh sieves to obtain a fine powder (particle size 300 μm) and it was used for optimization experiment.

4.3.2. Ultrasound-assisted extraction of anti-pyridoxine factors from flaxseed

Ultrasound-assisted extraction was performed in a VWR 250 HT ultrasonic cleaning bath (VWR International, AB, Canada). The flaxseed samples (500 mg) were accurately weighed in a 50 mL centrifuge tube, the required amount of extracting solvent was added, and the contents homogenized at 13,500 rpm for 1min using a Ultra-Turrax T25 homogenizer (IKA-Labortechnik, Wilmington, NC, USA). The mixture was transferred into a 100 mL volumetric flask and sonicated for the designed times and at designed temperatures. Temperatures were controlled through the use of a circulating

thermostatic water bath. The resultant flaxseed extracts were centrifuged at 5000 x g for 20 min at 4°C, and the supernatants collected. The extracts were concentrated using a rotary evaporator set to 35°C. The concentrated extracts were diluted to a constant volume (10 mL) followed by centrifugation at 12,000 x g for 20 min and filtered through 0.2 μm filter before injection onto the UPLC-MS system. All extracted samples were freshly analyzed.

4.3.3. UPLC-MS Conditions

An Acquity UPLC® system coupled with a Quattro Micro Atmospheric pressure ionization (API) tandem mass spectrometry (Waters Corporation, Milford, MA, USA) was used for the analysis. De-ionized water (18.2 MΩ) as solvent A and acetonitrile as solvent B, both containing 0.1% formic acid were used as mobile phases and the column was a Hypersil Gold C18 column; 150 x 3.0 mm, 3μm (Thermo Scientific Inc., Waltham, MA, USA). The gradient elution was programmed at 0, 2, 3, 3.4, 3.5 and 8 min with 0, 20, 50, 50, 0 and 0% of solvent B respectively at a flow rate of 0.16mL/min. The column was maintained at 35°C while the auto-*sampler* was maintained at a temperature of 4°C. The tandem mass spectrometry was operated in positive electro spray ionization (ESI+) mode, with the conditions tuned based on 1ADP. The main parameters of MS were as follows: capillary voltage was 3kV; cone voltage was 20V; source temperature was 120°C; desolvation temperature was 500°C; desolvation gas ($N_2$) flow was 600L/h and cone gas flow was 50L/h. Multiple reaction monitoring (MRM) mode was set to monitor the mass transition of precursor ion to the product ion. The mass transition was monitored at 118.9 > 72.9 for d3-D-proline (internal standard), 131.0 > 85 for 1ADP and 259.9 > 85

for linatine. Data acquisition and processing was done using MassLynx V4.1 (Waters Corporation, Milford, MA, USA).

4.3.4. Purity analysis and validation of the presence of linatine in flaxseed extract

The flaxseed extract was concentrated in rotary evaporator to form a crude linatine extract. It was fractionated using a Varian 920LC High Performance Liquid Chromatography (HPLC) system, equipped with a UV-detector and fraction collection capabilities (Varian Inc, Palo Alto, California, USA). Fractions of each 15 sec were collected using a reverse phase Luna C18 $_{(2)}$ column; 250 x 4.6 mm; 5µm (Phenomenex, Torrance, CA, USA) and solvent conditions as described above. The identification of linatine in the fraction was confirmed on the basis of the theoretical mass spectrum, using UPLC-MS. The final linatine fraction collected was lyophilized to obtain a white amorphous solid. The amorphous solid was dissolved in de-ionized water and scanned at 200 – 400 nm to identify $\lambda_{max}$ using a DU 8000 spectrophotometer (Beckman Coulter Inc, Brea, CA, USA). The purity of the linatine in the solution was determined by normalizing the peak areas detected by UV-detector of HPLC at $\lambda_{max}$ using same column and method mentioned above.

The amorphous linatine fraction at a concentration of 50 µg/mL was analyzed by an Agilent 6538 Q-TOF mass spectrometry coupled with dual ESI source (Agilent Technologies, Santa Clara, CA, USA) in positive mode using a Poroshell C18 column; 2.1×50 mm, 2.7 µm (Agilent Technologies, Santa Clara, CA, USA) with a flow rate of 0.6 mL/min using the same solvent and gradient system mentioned above for UPLC-MS method. For the mass spectrometry and MS/MS analyses, data was acquired for the fragments range of 50-1200 *m/z*. ESI capillary voltage was set a fast switch polarity mode

ESI+/- with 4000V (+/-) ion mode with a fragmentor voltage of 60V. A drying gas ($N_2$) at a temperature of 300°C was supplied at a flow rate of 11L/min. The reference masses used were 121.050873, 922.00978 and119.03632 m/z (Agilent technologies, Mississauga, ON, Canada). Find by auto and targeted MS/MS algorithms options within the Agilent MassHunter Qualitative Analysis B.05.00 were used to verify the presence linatine in the sample using the Metlin database and library

Furthermore, to confirm the presence of linatine in the amorphous solid, 1mg of the sample was hydrolyzed in 1M HCl for 2 h at 100°C. The hydrolyzed solution was passed through a $H^+$-form resin column (Bio-Rad, Hercules, CA, USA). It was then washed with de-ionized water and the sample was eluted with 3M $NH_4OH$ solution. The eluate was dried under $N_2$ at 35°C and reconstituted with de-ionized water to 5mL. Verification was done by comparing the retention time and daughter ion mass spectrum as described above in UPLC-MS/MS method.

4.3.5. Preparation of calibration curve and quantification of anti-pyridoxines

A calibration curve was prepared according to FDA guidelines [25]. Fifty mL of the sample extract was incubated at 70°C in a water bath for 48 h until all the 1ADP and linatine had been degraded. A calibration curve was prepared by spiking the resulting extract with known concentrations of the analyte, either 1ADP or linatine, into the same sample matrix. The concentrations of standards were chosen on the basis of the concentration range of the sample. A blank sample matrix was also run concurrently with the calibration curve. The concentrations of anti-pyridoxine factors in the samples were determined based on the peak area response of 1ADP and linatine with respect to the internal standard (d3-D-proline) at a concentration of 200ng/mL. The total anti-

pyridoxine content was expressed as 1-amino D-proline equivalents (1ADPE) using the equation (1) given below. The limit of detection (LOD) and the lower limit of quantification (LLOQ) were also determined.

$$\text{Total anti-pyridoxine (1ADPE } \mu g/g) = \frac{1}{2}\text{ (Linatine) + 1ADP ----- (1)}$$

4.3.6. Experimental design of extraction procedures

An isopropanol-water mixture was used as the extraction solvent [17]. Four factors, isopropanol concentration (A, %v/v), time (B, minutes), temperature (C,°C) and liquid-solid ratio (D, mL/g) were selected as the primary influential parameters for extraction yield. The anti-pyridoxine compounds (1ADP and linatine) from flaxseed were first extracted based on a single-factor experiment to determine the three levels of each factor. During the entire extraction process, the chosen temperature of the sonication bath was kept steady (within ±1.5°C).

After examining the test results obtained from single-factor extraction, a design based on a central composite model with three levels and four variables was used to find out the best extraction parameter combination to optimize the recovery of anti-pyridoxines from flaxseed. The variables were generated according to the equation [26]:

$$x_i = (X_i - X_0)/\Delta X \text{ ----- (2)}$$

Where, $x_i$ was the value of the independent variable $X_i$, $X_0$ was the value of $X_i$ at the centre point and ΔX was the step change value.

Table 4.1 shows the four independent variables with their respective levels. The central composite design matrix and experimental extraction values are listed in table 4.2. Out of the three responses (1ADP, linatine and total 1ADPE), extraction of total 1ADPE

was considered as the response for the regression analysis. The model was fitted on the second-order polynomial function as follows [27]:

$$Y = bo + \sum_{i=1}^{4} A_i X_i + \sum_{i=1}^{4} A_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} A_{ij} X_i X_j \text{ ----- (3)}$$

Where, Y was the predicted variable function; $A_i$, $A_{ii}$ and $A_{ij}$ were the regression coefficients of linear, quadratic and interaction terms of the independent variables, $X_i$ and $X_j$.

The experimental design and calculation of the predicted data were carried out using Design Expert 8.0.7.1 software to estimate the best response. The predicted model was verified by conducting three additional experiments. The final response was also compared with the samples extracted with either ethanol or methanol.

Table 4.1. Factors and levels of response surface methodology for the extraction of anti-pyridoxine factors from flaxseed

| Independent Variables | Levels | | |
|---|---|---|---|
| | -1 | 0 | 1 |
| Isopropanol concentration (A, %) | 30 | 40 | 50 |
| Extraction time (B, min) | 20 | 30 | 40 |
| Extraction temperature (C,°C) | 25 | 35 | 45 |
| Liquid/solid ratio (D, mL/g) | 30 | 40 | 50 |

4.3.7. Varietal analysis and stability of anti-pyridoxine on storage

Nine flaxseed varieties, grown in different locations were defatted with SER 148 Solvent extractor (VELP Scientifica, Via stazione, MB, Italy) using hexane as solvent according to the AOAC official method guidelines [28] to avoid the interference of oil during extraction procedures. Anti-pyridoxine content of those samples were determined

using the optimized extraction parameters. To check the storage stability of anti-pyridoxines, 1ADP and linatine in solution or in the sample extract were incubated at 3°C and 37°C for 48 h. Every 6 h, the samples were injected in UPLC-MS and the changes in peak area ratio were noted.

4.3.8. Statistical Analysis

The design of the experiment for the optimization of extraction using RSM was performed using Design Expert 8.0.7.1 software (Trial Version, State-Ease Inc., Minneapolis, MN, USA). Statistical analysis was performed using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA). All data were analyzed using one-way ANOVA with Bonferroni's multiple comparison and $P < 0.05$ was set for all analyses to reflect statistically significant differences.

Table 4.2. Central Composite Design matrix and experimental extraction values of anti-pyridoxines

| Std | Run | A | B | C | D | 1ADP (μg/g) | Linatine (μg/g) | Total 1ADPE (μg/g)* |
|---|---|---|---|---|---|---|---|---|
| 9 | 1 | 30 | 20 | 25 | 50 | 47.5 | 995.5 | 545.2 |
| 24 | 2 | 40 | 30 | 35 | 50 | 5.8 | 620.3 | 315.9 |
| 23 | 3 | 40 | 30 | 35 | 30 | 43.1 | 784.6 | 435.4 |
| 8 | 4 | 50 | 40 | 45 | 30 | 17.8 | 828.6 | 432.1 |
| 28 | 5 | 40 | 30 | 35 | 40 | 33.7 | 854.1 | 460.7 |
| 4 | 6 | 50 | 40 | 25 | 30 | 16.7 | 641.9 | 337.7 |
| 14 | 7 | 50 | 20 | 45 | 50 | 10.8 | 948.3 | 485.0 |
| 1 | 8 | 30 | 20 | 25 | 30 | 51.0 | 637.0 | 369.4 |
| 18 | 9 | 50 | 30 | 35 | 40 | 22.6 | 839.7 | 442.4 |
| 21 | 10 | 40 | 30 | 25 | 40 | 29.2 | 902.7 | 480.6 |
| 16 | 11 | 50 | 40 | 45 | 50 | 19.7 | 945.8 | 492.7 |
| 20 | 12 | 40 | 40 | 35 | 40 | 37.4 | 898.1 | 486.5 |
| 6 | 13 | 50 | 20 | 45 | 30 | 12.5 | 801.8 | 413.4 |
| 30 | 14 | 40 | 30 | 35 | 40 | 39.3 | 850.5 | 464.5 |
| 12 | 15 | 50 | 40 | 25 | 50 | 23.5 | 858.9 | 452.9 |
| 13 | 16 | 30 | 20 | 45 | 50 | 29.3 | 843.7 | 451.2 |
| 10 | 17 | 50 | 20 | 25 | 50 | 23.7 | 728.8 | 388.1 |
| 19 | 18 | 40 | 20 | 35 | 40 | 31.6 | 897.7 | 480.5 |
| 29 | 19 | 40 | 30 | 35 | 40 | 31.0 | 861.9 | 462.0 |
| 22 | 20 | 40 | 30 | 45 | 40 | 14.5 | 713.4 | 371.3 |
| 26 | 21 | 40 | 30 | 35 | 40 | 37.7 | 943.5 | 509.4 |
| 11 | 22 | 30 | 40 | 25 | 50 | 18.7 | 907.9 | 472.7 |
| 7 | 23 | 30 | 40 | 45 | 30 | 33.8 | 659.8 | 363.7 |
| 25 | 24 | 40 | 30 | 35 | 40 | 40.6 | 874.8 | 478.0 |
| 5 | 25 | 30 | 20 | 45 | 30 | 31.4 | 532.6 | 297.7 |
| 2 | 26 | 50 | 20 | 25 | 30 | 21.2 | 766.5 | 404.4 |
| 3 | 27 | 30 | 40 | 25 | 30 | 17.4 | 449.9 | 242.4 |
| 27 | 28 | 40 | 30 | 35 | 40 | 41.5 | 894.7 | 488.8 |
| 15 | 29 | 30 | 40 | 45 | 50 | 34.3 | 835.0 | 451.8 |
| 17 | 30 | 30 | 30 | 35 | 40 | 29.2 | 797.5 | 427.9 |

*Total 1ADPE = $\frac{1}{2}$ (Linatine) + 1ADP

Note: A, B, C, and D represents isopropanol concentration (%), extraction time (min), extraction temperature (˚C), and liquid/solid ratio (mL/g) respectively.

## 4.4. Results and Discussion

### 4.4.1. Identification and characterization of anti-pyridoxines in flaxseed

The anti-pyridoxines were detected using an ESI+ mode in MRM mass chromatograms. These compounds were identified by comparing the retention times and daughter ion mass spectrums of the authentic standards. The 1ADP was eluted at 5.48 min while linatine was eluted at 7.33 min (Figure 4.1). There was an increase in the peak area of 1ADP when the sample was spiked with synthetic standard. This confirmed the presence of free 1 ADP in flaxseed (Figure S1, Appendix). It was verified by comparing the mass spectra of the authentic standard parent ion at *m/z* 131 and base ion at *m/z* 85 (Figure 4.2). This was the first report indicating the presence of free 1ADP in flaxseed. Earlier, it was suggested that free 1ADP was not detected in the freshly prepared flaxseed extract when quantified using either by pyridoxal phosphate assay or amino acid analyzer [17]. The latter results may be due to the lack of sensitivity of the methods and the instruments used at the time. In the current study, the limit of detection (S:N = 5) and lower limit of quantification (S:N = 10) for 1ADP were 10 ng/mL and 15 ng/mL respectively, showing the higher sensitivity of the new method.

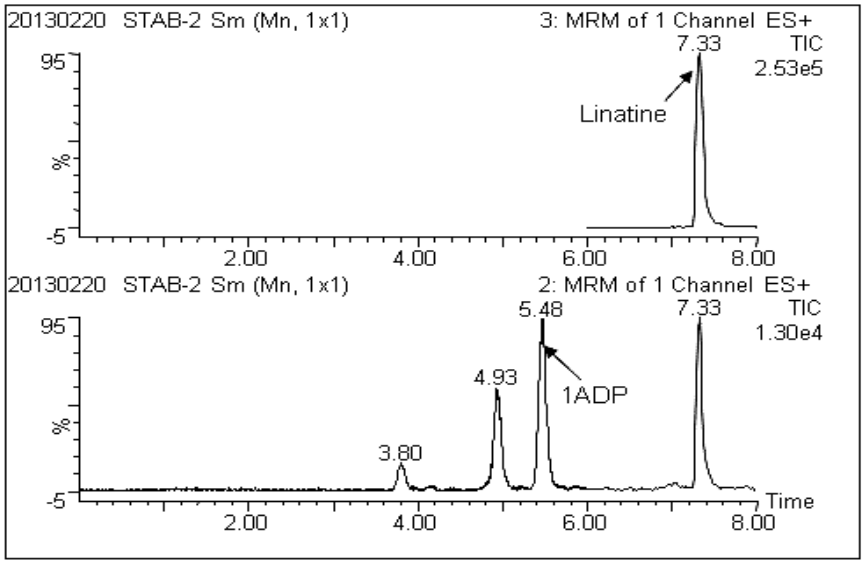


Figure 4.1. Chromatogram of linatine and 1-amino D-proline in flaxseed extract

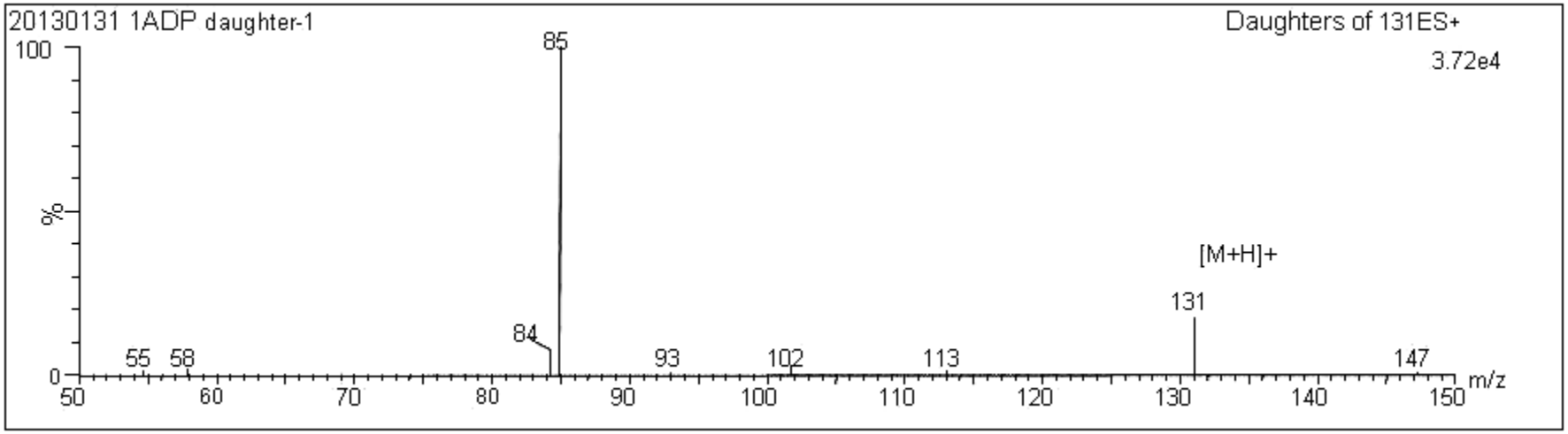


Figure 4.2. UPLC-mass spectrum of 1ADP: Parent ion at m/z 131, [M+H]+ and base ion m/z 85

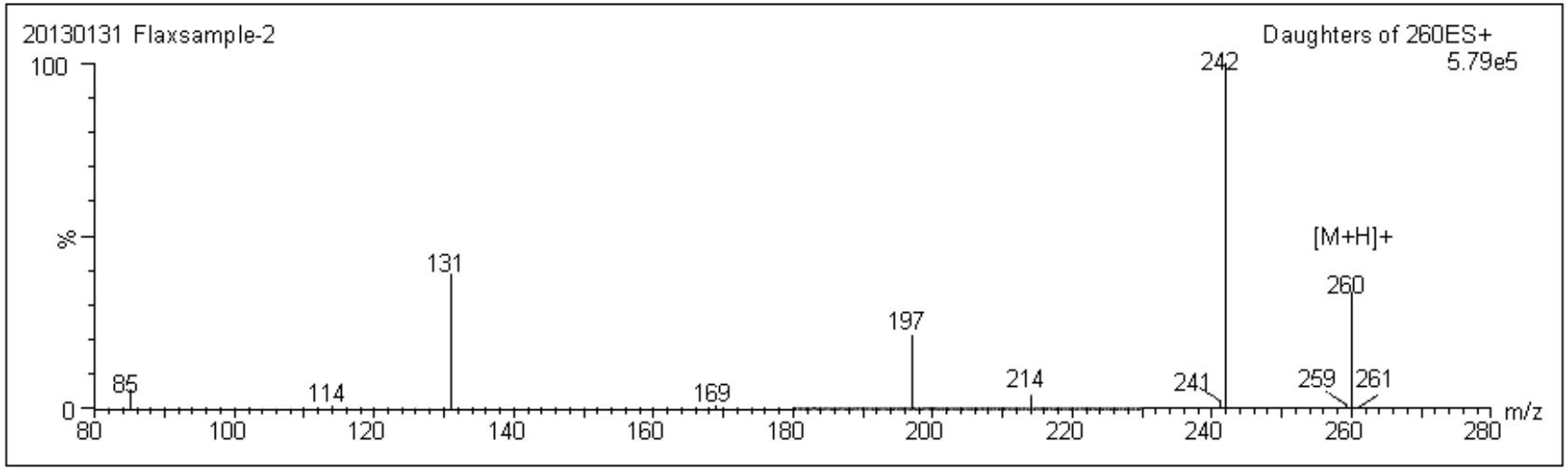


Figure 4.3. UPLC-mass spectrum of linatine in flaxseed extract: Parent ion at m/z 260, [M+H]+

Linatine was identified and characterized based on its supposed mass spectral profile. We could not obtain a synthetic linatine standard as it was not commercially available. The mass spectrum of the chromatogram, obtained by scanning the flaxseed extract at *m/z* 260 $[M+H]^+$, was analyzed to validate the authenticity of linatine (Figure 4.3). Besides the parent ion, there were two major base peaks at *m/z* 242 $[M+H-18]^+$ and *m/z* 131 $[M+H-129]^+$. The first base peak was formed by losing a $H_2O$ molecule (-18u) while the second base peak was formed by losing the glutamyl group (-129u) as $[M+H-C_5H_7NO_3]^+$ to give 1ADP spectrum. Apart from those two major base peaks, the minor peak obtained with m/z 197 $[M+H-63]^+$ was due to the loss of a $H_2O$ (-18u) plus a COOH group (-63u) as $[M+H-H_2O-COOH]^+$. The occurrence of common peaks at *m/z* 131 and *m/z* 85 in both 1ADP and sample extract provided an additional evidence supporting the presence of linatine in flaxseed.

4.4.2. Purity analysis and validation of the presence of linatine

The $\lambda_{max}$ of a linatine solution prepared by dissolving the amorphous solid, obtained after the fractionation of crude flaxseed extract, into de-ionized water was found at 268 nm. The purity of linatine in the solution calculated after normalizing the peak area of three consecutive HPLC injections detected at $\lambda_{268nm}$ was 78% . The mass spectrum from Q-TOF (Figure S2, Appendix) showed a similar fragmentation of linatine as mentioned above in the case of UPLC-MS with a *m/z* value of 260.1217, $[M+H]^+$. Targeted MS/MS molecular feature analysis had provided a compatibility score of 99.15 of linatine compared to the database available in online MassHunter software. This indicated a very high chance of presence of linatine in the sample. Moreover, linatine was hydrolyzed into 1ADP which was not found in the un-hydrolyzed sample (Figure S3,

Appendix). The appearance of 1ADP peak in the chromatogram of hydrolyzed sample also confirmed the presence of linatine in the amorphous solid fractionated from the crude extract. This amorphous solid was used as an authentic linatine standard for quantification.

4.4.3. Extraction parameters

Ultrasound-assisted extraction of bioactive compounds from plant sources have been documented to be affected by extraction solvent, time, temperature and ratio of liquid-solid [22-24].The effects of the specific test parameters (isopropanol concentration, sonication time, temperature and liquid-solid ratio) on the anti-pyridoxines extraction are displayed in Figure 4.4(A-B).

An isopropanol concentration of 30% resulted in the highest amount of 1ADP and linatine extraction. These results indicated that the efficiency of extraction was higher when the proportion of organic solvent was the lowest. Opportunities to use solvent concentrations lower than 30% were hampered by the increased extraction of water-soluble flax-derived gums and mucilages [29-31], which created challenges during sample preparation (gelation). Therefore, the range for optimum isopropanol concentration was considered to be 30-50%. A similar trend was also observed in liquid-solid ratio, as the extraction efficiency was highest when the ratio was 50:1, indicating that higher water contents enhanced the extraction efficiency. The range for the liquid-solid ratio for extraction was selected as 30-50:1 (v:w). Similarly, range of sonication time and temperature were selected as 20-40 min and 25-45 °C, respectively. The above selected parameters were used for the RSM design.

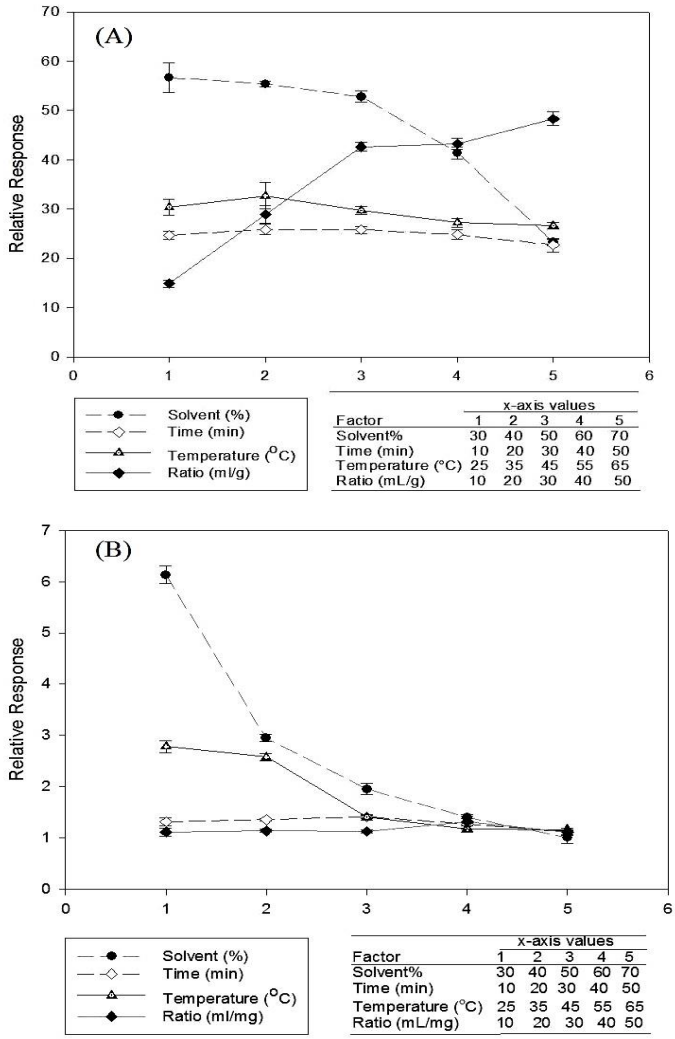


Figure 4.4A-B. Effect of single-factor test on linatine (A) and 1-amino D-proline (B) extractions from flaxseed. Values on x-axis represent the five levels of each single factor.

4.4.4. Optimization of extraction conditions

Based on the values obtained from the single-factor experiments, the levels of independent variables chosen for the Central Composite design i.e., isopropanol concentration (A, %v/v), sonication time (B, min), sonication temperature (C,°C) and liquid-solid ratio (D, ml/g), were selected as design variables in the RSM (Table 4.1). Out

of the three anti-pyridoxine responses i.e., 1ADP, linatine and total 1ADPE (Table 4.2); extraction of total 1ADPE was employed as the response value with four factors and three levels for the optimization using RSM design. Analysis of variance (ANOVA) for response surface of quadratic model analyses was chosen to test the significance and adequacy of the design. The value of 'prob > F' of model was significant with $P = 0.0082$ and the Lack of Fit was not significant ($P= 0.052$). This suggested that the model terms were precise and applicable (Table 4.3). The coefficient of determination ($R^2$) of the model was 0.82 and the regression equation after replacing experimental points was:

$$Y= -307.92 + 26.54A – 11.28B + 17.48C + 0.63D + 0.02AB +0.27AC -0.18AD +0.1BC - 0.01BD -0.003CD – 0.36 A^2 + 0.12B^2 -0.45C^2 +0 .15 D^2 \quad \text{------ (4)}$$

Each 3D plot (Figure 4.5) represents the number of combinations of the two test variables. After carrying out optimization of all the parameters on the basis of mathematical model, the points of prediction for maximum extraction of total anti-pyridoxine were: isopropanol concentration 35.97% (v/v), time 24.89 min, temperature 31.54°C, liquid-solid ratio 44.68:1(mL/g). At these points the predicted values for linatine, 1ADP and total anti-pyridoxine (as 1ADPE) were 916.5µg/g, 41.3µg/g and 499.5 µg/g respectively.

Table 4.3. Analysis of variance for the fitted quadratic polynomial model on total 1ADPE

| Source | SS | DF | MS | F-Value | Prob > F | |
|---|---|---|---|---|---|---|
| Model | 68404.14 | 14 | 4886.01 | 4.04 | 0.0082 | significant |
| Residual | 15717.47 | 13 | 1209.04 | | | |
| Lack of Fit | 13879.53 | 8 | 1734.94 | 4.72 | 0.0521 | not significant |
| Pure Error | 1837.94 | 5 | 367.59 | | | |
| Cor Total | 84121.61 | 27 | | | | |

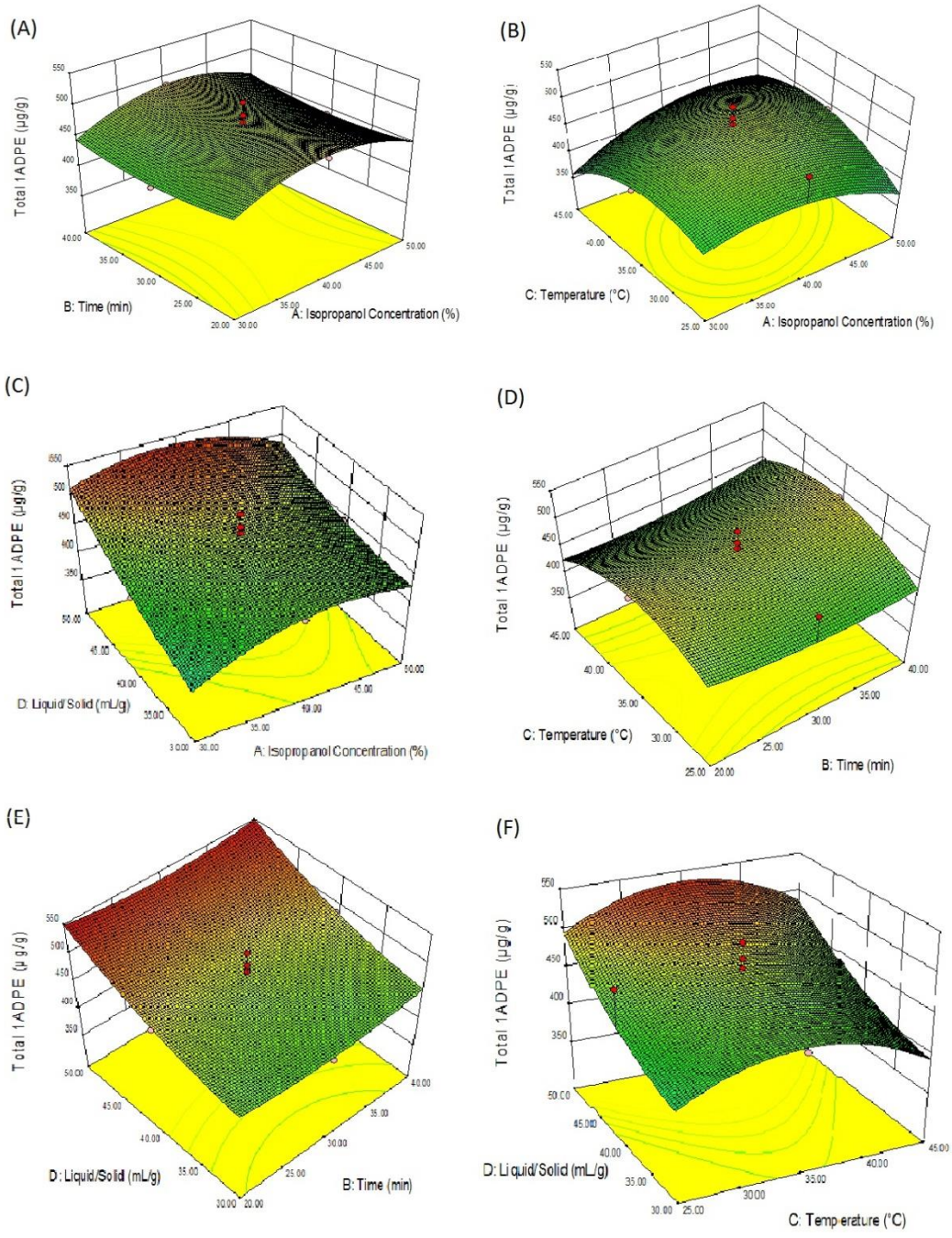


Figure 4.5. A 3D plot showing the correlative effects of different parameters on total anti-pyridoxine extraction.

4.4.5. Verification of the predicted model

In order to validate the adequacy of the model equation, a verification experiment was carried out under the optimal condions. Extraction conditions were adjusted to the following: isopropanol concentration, 36%; sonication time, 25 minutes; temperature, 32°C; and liquid-solid ratio, 45:1 (mL/g). The results showed that the anti-pyridoxine content values were: linatine, 1030 ± 21.7 μg/g; 1ADP, 42 ± 1.0 μg/g; and total 1ADPE, 557.9 ± 10.8 μg/g (of defatted flaxseed, n = 3). These results provide evidence that the experimental values were not only consistent with the predicted values, but also better than any single-factor experiment. Therefore, the extraction conditions obtained by response surface methodology were considered optimized and repeatable.

Efficiency of anti-pyridoxine extraction using different solvents at optimized conditions were also evaluated (Table 4.4). Isopropanol have significantly ($P < 0.001$) higher capacity to extract both linatine and 1ADP compared to ethanol and methanol. Ethanol was able to extract more 1ADP than methanol, however, linatine extraction efficiency were not significantly different for both the solvents.

Table 4.4. Comparison of 1ADP and linatine extraction from flaxseed using different solvents

| Solvent type | Concentration (μg/g of defatted flaxseed) * | |
|---|---|---|
| | 1ADP | Linatine |
| Ethanol | $32 \pm 0.7^{b}$ | $919.3 \pm 15.4^{b}$ |
| Methanol | $15.2 \pm 0.9^{c}$ | $850.9 \pm 26.4^{b}$ |
| Isopropanol | $42 \pm 1.0^{a}$ | $1030 \pm 21.7^{a}$ |

*Data represents means ± SE, n = 3, values with different superscript are significantly different ($P < 0.05$).

4.4.6. Varietal analysis and stability of anti-pyridoxines on storage

The oil removed from the nine varieties of flaxseed ranged from 32-43%. The amount of anti-pyridoxine compounds present in the nine varieties was significantly different (Table 4.5). The total amount of anti-pyridoxine content (1ADPE) in the tested varieties ranged from 177-437 μg/g for whole seed. Of the nine varieties, Normandy had the highest amount of anti-pyridoxine (437 ± 10.8 μg/g) while Taurus had the lowest (177.3 ± 5 μg/g). These results may provide evidence of genotypic variation in the 1ADPE content of flaxseed, but this remains to be rigorously tested.

The stability of 1ADP and linatine during storage is shown in Figure S4 (Appendix). Standard 1ADP solution was stable for 48 h at 3°C but, it was readily degraded in 3 h at 37°C. The amount of 1ADP content in sample increased during storage at both temperatures up to 12 h incubation. The slight degradation of linatine is likely resulting in the release of 1ADP in the sample solution. Linatine in the sample also

degraded at both temperatures; however, the rate of degradation was faster at 37°C as compared to 3°C.

**4.5. Conclusion**

In conclusion, this study establishes a new method for assessing the anti-pyridoxine content of flaxseed in advance of risk assessment modeling for specific populations. Our method provided a better extraction of 1ADP and linatine from flaxseed by optimizing the extraction parameters using a RSM design. The UPLC-MS/MS method, which has higher specificity and sensitivity for those analytes revealed that the amount of anti-pyridoxine content in flaxseed was up to 437μg/g of whole seed, much higher than previously reported by Klosterman et al [17]. Furthermore, we have documented the presence of free 1ADP in flaxseed, which has never been reported before. While varietal differences in 1ADP equivalents were apparent, further work is required to ascertain the impact of genotype by environment effects on the anti-pyridoxine content of flaxseed. Future work will assess the oral toxicity of anti-pyridoxines, especially 1ADP and linatine as it will be important to assess the risk factors associated with the consumption of flaxseed and its related products.

Table 4.5. Varietal and environmental effects on the content of anti-pyridoxine compounds in flaxseed

| SL | Variety | Place grown | Anti-pyridoxine content in defatted flaxseed (µg/g)* | | | Fat removed% | Anti-pyridoxine content in whole flaxseed (µg/g)* | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Linatine | 1ADP | Total 1ADPE | | Linatine | 1ADP | Total 1ADPE |
| 1 | Taurus | MB, Canada | 530.9±15.8 | 11.6±0.7 | 277.0±7.8$^{e}$ | 36.0 | 339.8±10.1 | 7.4±0.5 | 177.3±5.0$^{f}$ |
| 2 | CDC-Sorrel | MB, Canada | 919.7±12.1 | 3.5±0.2 | 463.4±6.1$^{d}$ | 39.8 | 554.6±7.3 | 2.1±0.1 | 279.4±3.6$^{e}$ |
| 3 | CDC-Bethune | MB, Canada | 794.0±29.2 | 56.1±3.2 | 453.1±13.0$^{d}$ | 32.8 | 533.5±19.6 | 37.7±2.2 | 304.5±8.7$^{de}$ |
| 4 | CDC-Arras | SK, Canada | 1138.6±13.0 | 6.5±0.4 | 575.7±6.6$^{c}$ | 40.9 | 672.9±7.7 | 3.8±0.2 | 340.3±3.9$^{c}$ |
| 5 | Lightening | MB, Canada | 1038.0±17.0 | 32.6±1.9 | 551.6±7.0$^{c}$ | 43.5 | 586.5±9.6 | 18.4±1.1 | 311.7±3.9$^{de}$ |
| 6 | Normandy | MB, Canada | 1331.9±33.9 | 47.8±2.2 | 713.7±17.6$^{a}$ | 38.8 | 816.4±20.8 | 29.3±1.4 | 437.5±10.8$^{a}$ |
| 7 | Hanley | MB, Canada | 1083.7±13.6 | 24.8±1.6 | 566.6±7.7$^{c}$ | 34.6 | 708.7±8.9 | 16.2±1.0 | 370.6±5.1$^{b}$ |
| 8 | Vimy | SK, Canada | 1255.0±35.8 | 6.7±0.2 | 634.2±18.1$^{b}$ | 38.1 | 776.8±22.1 | 4.1±0.1 | 392.6±11.2$^{b}$ |
| 9 | Jantarol | Poland | 868.6±17.4 | 20.0±0.9 | 454.3±8.3$^{d}$ | 32.5 | 586.3±11.7 | 13.5±0.6 | 306.7±5.6$^{de}$ |

*Data represents means ± SE, n = 3, values with different superscript are significantly different ($P < 0.05$)

Total 1ADPE = $\frac{1}{2}$ (Linatine) + 1ADP

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# CHAPTER 5

## MANUSCRIPT-II

## Oral exposure to the anti-pyridoxine compound 1-amino D-proline further perturbs homocysteine metabolism through the transsulphuration pathway in moderately vitamin $B_6$-deficient rats

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## 5.1. Abstract

Pyridoxal 5′-phosphate (PLP) serves as an important cofactor in a myriad of metabolic reactions, including the transsulfuration pathway, which converts homocysteine (Hcy) to cysteine (Cys). While overt vitamin $B_6$ deficiency is rare, moderate deficiency is common, and may be exacerbated by anti-pyridoxine factors in the food supply. To this end, we developed a model of moderate $B_6$ deficiency and a study was conducted to examine the *in vivo* effect of 1-amino D-proline (1ADP), an anti-pyridoxine factor found in flaxseed, on indices of Hcy metabolism through the transsulphuration pathway in moderately $B_6$-deficient rats. Male weaning rats received a semi-purified diet containing either 7 (control; CD) or 0.7 mg/kg diet of PN·HCl (moderately deficient; MD), each with one of 4 levels of 1ADP, *viz.* 0, 0.1,1 and 10 mg/kg diet for 5 weeks. The changes in vitamin $B_6$ biomarkers were more pronounced only in rats of MD group rather than the CD group. Plasma PLP was significantly reduced, while plasma Hcy (8-fold) and cystathionine (11-fold) were increased in rats consuming the highest amount of 1ADP in the MD group. The activities of hepatic CBS and CGL enzymes were significantly reduced in rats consuming the highest 1ADP compared to the lowest, for both levels of PN·HCl. Liver histopathological examination also revealed that rats receiving 10 mg/kg diet of 1ADP in the MD group developed dilations of central veins and sinusoids, and mild steatosis supported by data showing significantly higher hepatic total triglyceride concentrations, in comparison to other treatment groups. The current data provide evidence that the consumption of an anti-pyridoxine factor linked to flaxseed may pose a risk for subjects who are moderate/severe vitamin $B_6$-deficient.

**Key words:** 1-amino D-proline, linatine, anti-pyridoxine factor, homocysteine, pyridoxal 5′-phosphate, moderate vitamin $B_6$ deficiency, nutritional co-insults.

## 5.2. Introduction

Pyridoxal 5′-phosphate (PLP) serves as a cofactor for many enzymes in amino acid, carbohydrate, and fatty acid metabolism. Most mammalian cells rely on the nutritional supply of vitamin $B_6$ as a source of PLP [1,2]. Nutritional supply plays a vital role in regulating PLP-dependent enzymes, including those involved in homocysteine (Hcy) metabolism of the methionine cycle [3]. In the transmethylation (TM) pathway of methionine metabolism, the enzyme methionine adenosyl transferase (MAT) adds an ATP to methionine to generate S-adenosylmethionine (SAM), an important methyl donor. After donating a methyl group, SAM is subsequently converted to S-adenosylhomocysteine (SAH) and Hcy by the reversible enzyme SAH hydrolase [4]. Perturbation of an optimal SAM/SAH ratio by factors including an elevated Hcy concentration, can adversely affect metabolic homeostasis, including hepatic lipid biosynthesis [5,6]. Additionally, elevated levels of Hcy have been associated with a number of pathological conditions including diabetes and cardiovascular diseases [7]. In order to maintain normal Hcy level, vitamin $B_6$ plays a crucial role as a co-factor for three enzymes directly linked to two major pathways of Hcy metabolism, - namely transsulphuration (TS) and remethylation (RM) in the methionine cycle. In the TS pathway, Hcy condenses with serine to form cystathionine (CTH) with the help of a PLP-dependent enzyme cystathionine β-synthase (CBS). It is subsequently broken down into cysteine (Cys) and α-ketobutyrate through the action of another PLP-dependent enzyme cystathionine γ-lyase (CGL). In the RM pathway, in order to complete the folate cycle, one methyl group from serine is transferred to tetrahydrofolate (THF) to yield 5,10-methylene THF that is regulated by a PLP-dependent enzyme serine hydroxymethyltransferase (SHMT) [1,3]. Therefore, vitamin $B_6$ status influences Hcy flux and overall sulphur amino acid metabolism.

Apart from the role of PLP as a coenzyme, vitamin $B_6$ has putative anti-oxidative [8] and anti-inflammatory properties [9,10]. Additionally, it functions as a proton donor or acceptor through the 5-phosphate group of PLP to activate glycogen phosphorylase [11]. Vitamin $B_6$ has also been shown to be important for fatty acid metabolism [12], with deficiency states linked to impaired cholesterol metabolism [13] and hepatic steatosis with glucose deprivation or high-protein diets [14]. Vitamin $B_6$ deficiency has been linked to chronic hepatic diseases as well, including jaundice, hepatitis, necrosis and biliary cirrhosis [15]. The administration of the $B_6$ antagonists acetyl hydrazine and isoniazide induced hepatic necrosis and steatosis, and a concurrent $B_6$-deficiency increased susceptibility to these antagonists [16,17]. Therefore, the metabolic availability of PLP is critical to the maintenance of hepatic metabolic homeostasis.

Several factors such as nutrition, physiology, drugs, genetics and naturally occurring vitamin $B_6$ antagonists modulate the status of $B_6$ in the body. In addition to the pyridoxine from dietary sources, natural $B_6$ antagonists may be present in common foods. For example, gyromitrin and methylhydrazine from wild mushroom (*Gyromita esculenta L.),* agaritine from *Agaricus biporis,* canavaline and canaline from several leguminous plants, and mimosine from *Mimosa sp*. all form hydrazone complexes with PLP, thus leading to hydrazone poisoning [18]. Additionally, flaxseed, known for bioactive compounds including alpa-linolenic acid and enterolignans which may be linked to a reduction in chronic diseases [19], has also been shown to contain 1-amino D-proline (1ADP), an anti-pyridoxine factor, in the form of linatine [20]. $B_6$ vitamers also form a hydrazone complex with 1ADP, thus leading to hydrazone poisoning [21]. This hydrazino acid inhibits a variety of PLP-dependent enzymes including glutamic acid aminotransferase, tryptophanase, tyrosine decarboxylase

and glutamic decarboxylase [18]. Intraperitoneal injection of 1ADP in young rats led to increases in the concentrations of α-aminoadipic acid, citrulline and CTH in liver, kidney, pancreas, plasma and urine [22]. Methionine metabolism was also greatly disturbed by the injection of 1ADP resulting in the accumulation of L-CTH and its derivatives [23]. Additionally, vitamin $B_6$-deficient rats were shown to be more sensitive to 1ADP than $B_6$-adequate rats [22].

With respect to the risk of exposure to 1ADP, its effect may be more pronounced in cases of moderate vitamin $B_6$ deficiency, a condition more likely to be experienced in the population rather than overt deficiency [24,25]. Subjects replete with $B_6$ may be more resistant to the effect of 1ADP, however this remains to be determined. Given the limited toxicity data related to the oral exposure of 1ADP, as well as the lack of information on the interaction of anti-pyridoxine exposure with $B_6$ status, we developed a rodent model of moderate vitamin $B_6$ deficiency and investigated the *in vivo* effects of 1ADP on Hcy metabolism in said model.

## 5.3. Methods and materials

### 5.3.1. Rats and diets

#### *5.3.1.1. Development of a rodent model of moderate vitamin $B_6$ deficiency: A pilot study*

In order to establish a level of dietary vitamin $B_6$ for subsequent studies examining moderate deficiency, the latter condition was defined at the outset as one that would lead to depressed plasma PLP concentrations but with no impact on growth or feed efficiency. For the pilot study, the experimental groups consisted of 12 Sprague-Dawley male rats (3-4 weeks old; 70±5 g), purchased from the University of Manitoba Biological Resource Unit. They were individually housed in polypropylene cages in a room maintained at a temperature

of 20 ± 2ºC with 12 h light/dark rhythm at 50-70% relative humidity. After acclimatization for a week, they were randomly divided into three groups (n = 4) and fed with a semi-purified diet (AIN-93G, based on vitamin-free casein) with three levels of vitamin $B_6$ viz, 7 (control diet; CD), 0.7 (moderately limited $B_6$ content diet; MD) and 0.07 mg/kg diet of PN·HCl (severely limited $B_6$ content diet; SD) *ad libitum* for five weeks. Food intake was monitored daily while body weight was taken every 3 days. They were sacrificed after 12 h of fasting to collect blood and tissue samples for the analysis.

*5.3.1.2. Effect of 1ADP on the metabolism of moderately vitamin $B_6$ deficient model: Main study*

Forty-eight male Sprague-Dawley rats weighing 80 ± 7g were purchased from the same source and housed individually at polypropylene cages maintaining same conditions mentioned above. After acclimatization for a week, rats were randomly divided into eight groups (n = 6) and fed with AIN-93G (based on vitamin free casein) diet containing either 7 (CD) or 0.7 (MD) mg/kg diet of PN·HCl. Each group of rats was received one of the four levels of 1ADP (Santa Cruz Biotechnology Inc, Dallas, TX, USA) viz, 0, 0.1, 1 and 10 mg/kg diet *ad libitum* for five weeks. Food intake was monitored daily and body weight was measured every week. At the end of the experiments, urine, blood and tissue samples were collected as described above.

5.3.2. Plasma biochemistry

*5.3.2.1. Plasma vitamin $B_6$*

Plasma vitamin $B_6$ vitamers, including PLP, pyridoxal (PL) and 4-pyridoxic acid (4-PA) were measured as their semicarbazide derivatives. Samples (500 µL) were mixed with 40 µL of derivatizing agent containing 250 mg/mL of semicarbazide and glycine (Sigma-

Aldrich, Oakville, ON, Canada) in amber microcentrifuge tubes. They were then incubated at room temperature in the dark for 30 minutes. The samples were deproteinized by adding 40 µL of 70% perchloric acid and supernatants were collected after centrifuging at 10,000 x g for 10 min. The pH of each supernatant was stabilized at 3-5 by adding 30 µL of 25% NaOH before injection onto a reverse phase-HPLC (Varian Inc, Palo Alto, CA, USA). The isocratic mobile phase for chromatography was 60 nmol/L disodium hydrogen phosphate containing 9.5% methanol (V/V) and 400 mg/L EDTA disodium salt, adjusted to pH 6.5 with concentrated phosphoric acid. A 5-µm reverse phase column (Luna $C_{18(2)}$, 4.6x250 mm, Phenomenex, Torrance, CA, USA) protected with 3x4 mm guard column at a rate of 1.5 mL/min was used. The derivatized PLP and PL were detected at $\lambda_{Ex}$ = 380 nm, emission $\lambda_{Em}$ = 450 nm; 4-PA was detected at $\lambda_{Ex}$ = 320 nm, emission $\lambda_{Em}$ = 420 nm [26].

*5.3.2.2. Plasma Homocysteine and Cysteine*

Twenty µLof 10% tris (2-carboxylethyl) phosphine (TCEP) (Pierce chemical Co, Rockford, IL, USA) were added to 150 µL of sample and incubated at room temperature for 30 min. After incubation, 125 µL of 0.6 M perchloric acid was added to deproteinize the sample. After centrifuging at 10,000 x g for 10 min, 50 µL of the supernatant was added to a tube containing 100 µl of potassium borate buffer (pH 10.5), which contained 5 mmol/L ethylenediaminetetraacetic acid (EDTA) and 50 µL of ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F)(Wako Chemicals, Richmond, VA, USA) in the borate buffer at a concentration of 1 mg/mL. The solution was incubated for 1 h at 60ºC and allowed to cool down at 4ºC before injection onto a reverse phase-HPLC. A 20 µL of the sample was injected onto a $C18_{(2)}$ column to run at a flow rate of 1.0 mL/min. Plasma total Hcy and Cys concentrations were analyzed according to a reverse phase-HPLC (Shimadzu Inc, Nakagyo-

ku, Kyoto, Japan) method with fluorescence detection ($\lambda_{Ex}$ = 385 nm, $\lambda_{Em}$ = 515 nm), as previously described [27].

*5.3.2.3. Plasma cystathionine*

Plasma samples were mixed with 2M acetic acid containing an internal standard, isotope labeled 3,3,4,4-$d_4$ DL-cystathionine (CDN Isotopes Inc, Pointe-Claire, QC, Canada) in 1:2 ratio to precipitate proteins. After centrifugation (10,000 x g for 10 min), supernatants were loaded onto a cation-exchange column of AG50W-8 resin (Bio-rad, Hercules, CA, USA). Columns were washed with de-ionized water (5mL x 4) followed by elution with 3 mL of 3M $NH_4OH$. Samples were completely dried under a stream of $N_2$ gas at 35ºC. Amino acids were esterified by reacting with 500 µL of a 5:1 mixture of 1-propanol-acetyl chloride for 40 min at 110ºC. The esterified samples were dried again under $N_2$, and derivatized with 100µL of a 4:1 mixture of heptafluorobutyric anhydride and ethyl acetate at 60ºC for 40 min. The derivatized samples were re-dried under $N_2$ and reconstituted with 200 µL of ethyl acetate before injection onto a gas chromatography/mass spectrometry (GC/MS) for CTH analysis [28,29].

5.3.3. Hepatic enzyme activity

*5.3.3.1. Cystahionine- γ- lyase activity*

Liver samples were homogenized with 20% potassium phosphate buffer (100 mM), pH 7.5 and centrifuge at 10,000 x g to collect the supernatant sample. CGL enzyme activity was determined by incubating 250 µL of potassium phosphate buffer, 4.0 mM L-CTH, 0.32 mM NADH, 1.5 units of lactate dehydrogenase and 25 µL of supernatant sample in micro-plate reader at 37ºC. The decrease in optical density at 340 nm was monitored with a spectrophotometer and recorded. A blank reaction was also performed in the same manner by

omitting L-CTH from the reaction mixture. Maximum velocities were calculated from the linear portion of the graph. CGL activity was expressed in terms of protein content in the sample [30].

*5.3.3.2. Cystathionine - β-synthase activity*

The activity of CBS enzyme in crude liver homogenate was measured using a non-radioactive method. Frozen liver samples were homogenized in 10 volume of 0.05 M potassium phosphate buffer (pH 7.2). Each homogenate was then centrifuged at 14000 x g for 10 min and the supernatant stored on ice until assayed. The supernatant (20 µL) was mixed with 20 µL of a reaction mixture (0.1 M serine, 5 mM EDTA and 2.5 mM propargylglycine in 1 M Tris buffer, pH 8.4), 20µL of 2.5 mM pyridoxal 5′-phosphate, 100 µL of water and 20 µL of Hcy reagent for 5 min. The Hcy reagent was made by dissolving 0.0 154 g of Hcy thiolactone in 400 µL of 2.5 M KOH. The solution was then neutralized by adding 600 µL of a mixture of 2.57 mL of 4.5 M HCI and 4.43 mL of 1 M Tris buffer, pH 8.4. It was then incubated for 1 h at 37 °C and the reaction stopped by adding 100 µL of 40 % (w/v) trichloroacetic acid. Each sample was thoroughly vortex-mixed and centrifuged at 10000 x g for 5 min. The newly formed CTH in the sample was derivatized using the method mentioned above and analyzed via GC/MS [31].

5.3.4. Hepatopathological analysis

*5.3.4.1. Hepatic histology*

Liver samples were quickly removed and fixed in neutral buffer formalin. After fixation, specimens were dehydrated and embedded in wax. The sections of approximately 5 µm thickness were stained with hematoxylin and eosin (HE) following a standard protocol. Stained slides were observed under Nikon Eclipse E100 light microscope (Nikon

Corporation, Tokyo) at different magnifications. Liver sections were examined for histopathological symptoms of early liver injury such as dilation of central veins (CV) and sinusoids, macrovesicular steatosis, inflammation, and necrosis. Each slide was analyzed in a blinded manner and graded on a scale ranging from 0-3 (0 is none; 1 is up to 33%; 2 is 33-66%; 3 is > 66%) based on the symptoms observed around a random representative CV [32]. The average of three individual grading scores was presented as the final score. Photographs were taken by Sony DSP 3CCD color digital camera (Sony Corporation, Tokyo, Japan) using Northern Eclipse version 8.0 imaging software (Empix Imaging Inc., New York, USA).

*5.3.4.2. Hepatic total triglycerides*

Liver sample (400 mg) was homogenized with 5 mL of sodium phosphate buffer (pH 6.8). The homogenate was centrifuge at 15,000 g for 30 min at 4ºC and the supernatant was collected. Total triglyceride content was analyzed using a commercially available kit (Cayman Chemical Company, Michigan, USA). In this colorimetric assay, triglycerides were hydrolyzed by a lipase enzyme to produce glycerol and free fatty acids. The glycerol released formed a brilliant purple color compound after a coupled enzymatic reaction and the absorbance was measured at 540 nm.

5.3.5. Statistical analysis

All the data are presented as means ± SE. Data were tested for normality using the Shapiro-Wilk statistic and normalized by log transformation if necessary, and outliers (values beyond 2.5 x σ) were omitted from the analysis by examination of the residuals. A general linear model (GLM) with Bonferroni's post hoc test was performed for treatment effects and multiple comparisons. Correlations between the treatments and respective biomarkers were

determined using Pearson's correlation coefficient. Statistical significance was set at $P < 0.05$ and data were analyzed using SPSS 16.0 (SPSS Inc, Chicago, IL, USA).

## 5.4. Results

### 5.4.1. Development of a rodent model of moderate vitamin $B_6$ deficiency: A pilot study

#### *5.4.1.1. Performance data*

Food consumption declined significantly ($P<0.005$) by the second week of the study in the SD group compared to the CD and MD group. At the end of the fifth week, both food intake and final body weights were significantly reduced (~1.4 fold reduction, $P<0.001$) in SD rats compared to CD, while there was no significant difference between the rats in the CD and MD groups. The pattern of growth exhibited by all three groups is depicted in Figure 5.1. Relative liver weights (RLW), expressed per 100 g body weight, showed no significant differences between treatment groups. The feed efficiency ratio (FER) was significantly lower in SD compared to CD and MD rats (Table 5.1).

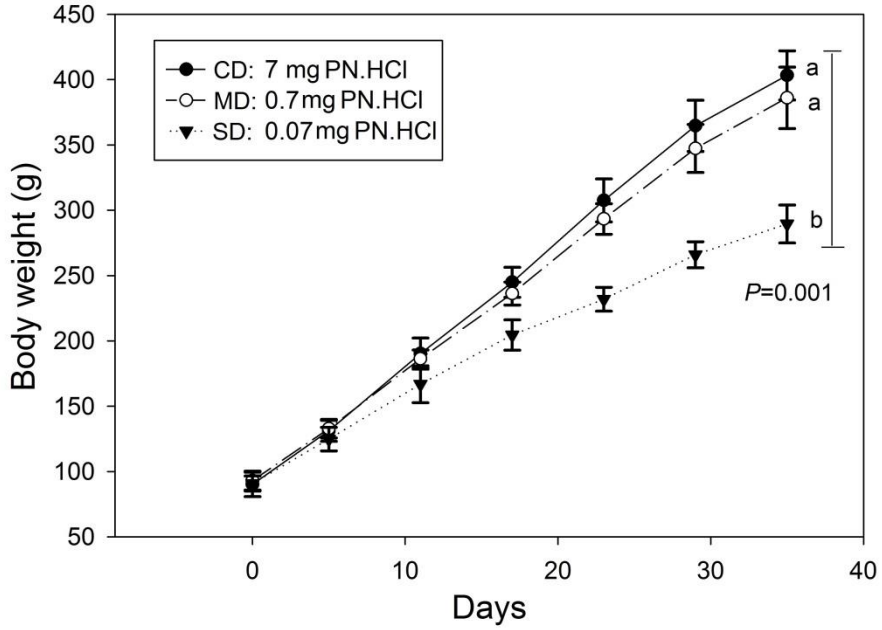


Figure 5.1. Growth curve of rats during a pilot study design to develop a rodent model of moderate vitamin $B_6$ deficiency. CD,Control diet (7 mg/kg diet of PN·HCl); MD, Moderately $B_6$ deficient (0.7 mg/kg diet of PN·HCl); SD, Severely $B_6$ deficient (0.07 mg/kg diet of PN·HCl). Means ± SE with different superscripts are significantly different (n = 4).

Table 5.1. Performance and weight of tissues derived from rats that had consumed diets differing in vitamin $B_6$ levels (pilot study).

| PN·HCl (mg/kg diet) | Initial body weight (g) | Final body weight (g) | Weight gain (g) | Total feed intake (g) | Feed Efficiency Ratio (FER) | Relative liver weight (RLW) |
|---|---|---|---|---|---|---|
| CD :7 | 90.1 | 403.3[a] | 313.1[a] | 837.4[a] | 0.37[a] | 4.5 |
| MD: 0.7 | 93.0 | 386.1[a] | 293.0[a] | 780.1[a] | 0.38[a] | 4.7 |
| SD: 0.07 | 90.8 | 289.5[b] | 198.7[b] | 591.9[b] | 0.33[b] | 4.5 |
| SE | 3.8 | 9.6 | 11.1 | 19.1 | 0.01 | 0.2 |
| *P* value | 0.86 | 0.001 | 0.001 | 0.001 | 0.007 | 1.05 |

Means within a column with unlike superscript differs ($P < 0.05$, n = 4). FER= Body weight gain/Feed consumed, RLW = liver weight x 100/Body weight. CD: control diet, MD: moderately deficient diet, SD: severely deficient diet, SE: standard error

*5.4.1.2. Plasma vitamin $B_6$*

The values obtained for plasma PLP, PL and 4-PA determined at the end of the five week experimental period are shown in Table 5.2. The plasma PLP of CD rats was significantly ($P < 0.001$) higher compared to the rats from the MD or SD groups. A similar trend was also observed for both plasma PL and 4-PA ($P < 0.001$). The significant ($P < 0.001$) correlation between dietary PN·HCl level and plasma PLP (r = 0.97), PL (r = 0.975) and 4-PA (r = 0.797) provided evidence of a dose-dependent association between $B_6$ intake and markers of $B_6$ status.

*5.4.1.3. Plasma thiols and hepatic enzyme activity*

Plasma thiol concentrations are provided in Table 5.2. Plasma Hcy concentrations of the SD rats were significantly ($P < 0.001$) higher than those of CD and MD group; however,

there was no significant ($P$ = 0.881) difference between the CD and MD rats. Plasma Hcy was increased more than 7-fold in SD rats compared to CD and MD group. On the other hand, plasma Cys was not significantly different between treatments. There was a significant ($P$ < 0.001) increase in plasma CTH as $B_6$ intake decreased from the CD treatment to the SD treatment. Hepatic CGL enzyme activity of MD and SD rats were reduced to 26 and 55%, respectively, of CD activity levels. In the case of hepatic CBS enzyme activity, there was no significant ($P$ = 0.122) difference between those rats from the CD and MD groups; however, SD rats exhibited significantly ($P$ < 0.001) lower activities (by more than half) when compared to the CD rats (Table 5.2).

Table 5.2. Plasma and hepatic vitamin $B_6$ biomarkers of rats that had consumed diets differing in vitamin $B_6$ content (pilot study).

| PN·HCl (mg/kg diet) | $B_6$ vitamers (nmoles/L) | | | Plasma thiols (μmoles/L) | | | Hepatic enzyme activity (nmoles/min/mg protein) | |
|---|---|---|---|---|---|---|---|---|
| | PLP | PL | 4-PA | CTH | Hcy | Cys | CGL | CBS |
| CD :7 | 525.1 [a] | 607.1 [a] | 54.0 [a] | 1.1[c] | 8.9[b] | 175.9 | 5.8[a] | 8.8[a] |
| MD 0.7 | 35.5 [b] | 24.7 [b] | 1.7 [b] | 2.4[b] | 8.1[b] | 167.0 | 3.2[b] | 7.2[a] |
| SD: 0.07 | 13.6 [c] | 8.5 [c] | 2.0 [b] | 8.8[a] | 39.1[a] | 164.2 | 1.5[c] | 3.2[b] |
| SE | 33.7 | 35.6 | 14.4 | 0.9 | 2.4 | 10.1 | 0.3 | 0.5 |
| $P$ value | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 |

Means with different superscripts within the column are significantly different ($P$ < 0.05, n = 4). As required, data were log-transformed to homogeneity of variance before analysis. CD: control diet, MD: moderately deficient diet, SD: severely deficient diet, SE: standard error.

On the basis of the observed changes in growth rates, feed efficiency, and plasma vitamers as well as thiol concentrations, a dietary inclusion rate of 0.7 mg/kg diet of PN·HCl was chosen to elicit a "moderate" deficiency. Rats consuming this diet had similar weight gains as those consuming the CD diet, but exhibited depressed plasma PLP concentrations (35.5 nmoles/L). However, the depression in PLP status was not sufficient to cause an increase in plasma Hcy concentration (8.1 µmoles/L).

5.4.2. Effect of 1ADP on the Hcy metabolism of moderately vitamin $B_6$ deficient model: Main study

*5.4.2.1. Performance data*

There was no significant effect of 1ADP ($P = 0.112$) on the final body weight of rats in the CD group. However, rats in the MD group, which consumed 10 mg/kg diet of 1ADP were significantly lighter (~1.4-fold reduction in body weight, $P < 0.001$) compared to other rats (Figure 5.2). The FER was also significantly ($P < 0.001$) reduced in rats consuming the 10 mg/kg diet of 1ADP in the MD group but not in the CD group (Table 5.3).

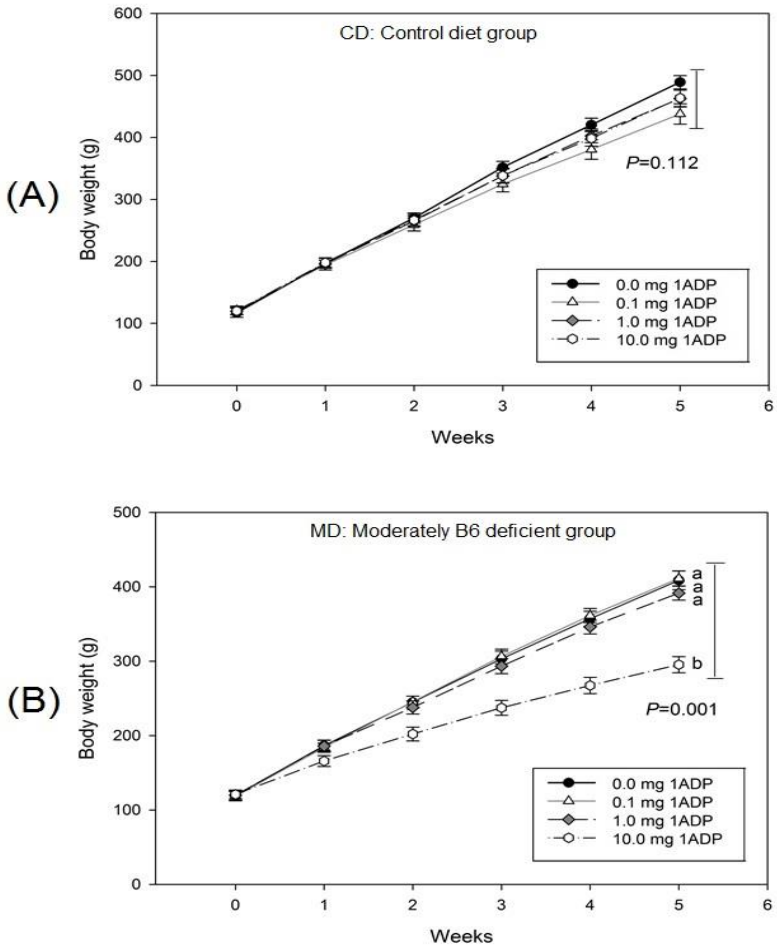


Figure 5.2. Effect of 1ADP exposure and vitamin $B_6$ status on growth in a rat model of moderately vitamin $B_6$ deficiency. (A) CD, Control diet group; (B) MD, Moderate $B_6$ deficient group. Means ± SE with different superscripts are significantly different (n = 6).All the 1ADP concentrations are expressed in mg/kg diet

*5.4.2.2. Plasma vitamin $B_6$*

Oral intake of 1ADP did not affect ($P$ = 0.645) the concentration of plasma PLP in rats on the CD treatment. However, in the MD group, plasma PLP was reduced ($P$ = 0.004) to half in rats consuming10 mg/kg diet of 1ADP compared to those which consumed no 1ADP. Plasma PL did not differ as a result of 1ADP consumption in either the CD or MD treatments. Plasma 4-PA, on the other hand, was significantly (P < 0.001) reduced in rats

consuming 10 mg/kg diet of 1ADP in the CD group, as compared to the other levels of 1ADP in the CD group (Table 5.4), but rats in the MD treatment group had reached a nadir in plasma PA concentrations. Plasma PLP was significantly and negatively correlated with 1ADP dietary levels in the MD group (r = - 0.669, $P < 0.001$) but not the CD group (r = - 0.237, $P = 0.264$).

*5.4.2.3. Plasma thiols*

Data for plasma thiol concentrations are given in Table 5.4. Plasma CTH concentrations were significantly *(*$P < 0.001$) higher in rats consuming 1ADP in both the CD and MD groups in dose dependent manners. The increase in plasma CTH in rats fed with 10 mg/kg diet of 1ADP, relative to the 1ADP-free diets, were 4.3 and 11.4 fold higher in the CD and MD groups, respectively. A similar trend was also observed for plasma Hcy concentrations, with pronounced increases at the highest 1ADP level in the MD group. Plasma Cys concentrations were not significantly affected by $B_6$ or 1ADP level. The correlations between 1ADP and plasma CTH were significant in both MD (r = 0.916, $P < 0.001$) and CD (r = 0.879, $P < 0.001$) treatment groups. Similar relationships were evident for plasma Hcy and 1ADP levels (MD: r = 0.906, $P < 0.001$; CD: r = 0.676, $P < 0.001$).

Table 5.3. Performance data of rats fed with two levels of vitamin $B_6$ and four levels of 1ADP (main study).

| 1ADP (mg/kg diet) | Initial body weight (g) | | Final body weight (g) | | Weight gain (g) | | Total feed intake (g) | | Feed efficiency ratio (FER) | | Relative liver weight (RLW) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CD | MD | CD | MD | CD | MD | CD | MD | CD | MD | CD | MD |
| 0 | 117.2 | 121 | 489.1 | 408.6$^{a}$ | 371.9$^{a}$ | 287.6$^{a}$ | 992.9$^{a}$ | 796.5$^{a}$ | 0.38 | 0.37$^{a}$ | 4.4 | 4.3 |
| 0.1 | 121.1 | 122.1 | 437.8 | 411.3$^{a}$ | 316.8$^{b}$ | 288.6$^{a}$ | 885.2$^{b}$ | 806.4$^{a}$ | 0.36 | 0.36$^{a}$ | 4.3 | 4.5 |
| 1 | 119.6 | 121.6 | 462.6 | 391.3$^{a}$ | 343.3$^{ab}$ | 269.9$^{a}$ | 946.7$^{ab}$ | 792.7$^{a}$ | 0.36 | 0.34$^{a}$ | 4.6 | 4.4 |
| 10 | 120.1 | 124.1 | 463.6 | 295.3$^{b}$ | 343.5$^{ab}$ | 171.2$^{b}$ | 930.1$^{ab}$ | 591.6$^{b}$ | 0.37 | 0.30$^{b}$ | 4.5 | 4.3 |
| SE | 6.5 | 3.8 | 13.9 | 10.7 | 10.6 | 10.8 | 22.9 | 19.7 | 0.01 | 0.01 | 0.14 | 0.15 |
| *P* value | 0.98 | 0.947 | 0.112 | 0.001 | 0.014 | 0.001 | 0.027 | 0.001 | 0.284 | 0.001 | 0.422 | 0.118 |

Means with different superscripts within the group are significantly different ($P < 0.05$, n = 6). As required, data were log-transformed to homogeneity of variance before analysis. CD and MD represent vitamin $B_6$ levels of 7 and 0.7 mg/kg diet of PN·HCl, respectively; FER= Body weight gain/Feed consumed, RLW = liver weight x 100/Body weight, SE, standard error.

Table 5.4. Plasma vitamin $B_6$ biomarkers of rats fed with two levels of vitamin $B_6$ and four levels of 1ADP (main study).

| 1ADP (mg/kg diet) | PLP (nmoles/L) | | PL (nmoles/L) | | 4-PA (nmoles/L) | | CTH (μmoles/L) | | Hcy (μmoles/L) | | Cys (μmoles/L) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CD | MD | CD | MD | CD | MD | CD | MD | CD | MD | CD | MD |
| 0 | 910.1 | 45.6$^{a}$ | 568.6 | 16.0 | 33.6$^{a}$ | 0.2 | 1.0$^{b}$ | 2.7$^{b}$ | 8.9$^{b}$ | 9.7$^{bc}$ | 246.3 | 265.1 |
| 0.1 | 854.8 | 41.1$^{a}$ | 389.9 | 17.7 | 33.4$^{a}$ | 0.3 | 1.1$^{b}$ | 2.6$^{b}$ | 8.2$^{b}$ | 8.6$^{c}$ | 251.1 | 258.2 |
| 1 | 790.5 | 34.7$^{ab}$ | 585.6 | 12.2 | 27.5$^{a}$ | 0.4 | 1.3$^{b}$ | 4.6$^{b}$ | 9.3$^{b}$ | 17.2$^{b}$ | 252.0 | 262.7 |
| 10 | 737.3 | 23.3$^{b}$ | 317.9 | 12.1 | 10.7$^{b}$ | 0.4 | 4.2$^{a}$ | 31.1$^{a}$ | 12.4$^{a}$ | *79.3$^{a}$ | 265.0 | 245.7 |
| SE | 92.3 | 4.3 | 84.7 | 1.8 | 4.1 | 0.1 | 0.36 | 3.1 | 0.7 | 6.5 | 8.7 | 8.6 |
| *P* value | 0.645 | 0.004 | 0.082 | 0.126 | 0.001 | 0.333 | 0.001 | 0.001 | 0.003 | 0.001 | 0.488 | 0.419 |

Means with different superscripts within the group are significantly different ($P < 0.05$, n = 6). As required, data were log-transformed to homogeneity of variance before analysis. CD and MD represent vitamin $B_6$ levels of 7 and 0.7 mg/kg diet of PN·HCl, respectively. The asterisks (*) indicated that one data point was omitted as outlier during the statistical analysis; SE, standard error.

*5.4.2.4. Hepatic enzyme activity*

Hepatic CGL activity was significantly ($P < 0.001$) reduced due to oral exposure to 1ADP at levels ≥ 1mg/kg diet, with the effects being more pronounced in the MD group compared to CD group (Figure 5.3). Hepatic CBS activity showed a similar trend (Figure 5.4).

*5.4.2.5. Hepatic histology*

Rats consuming the MD diet with 10 mg/kg diet of 1ADP exhibited symptoms of early liver injury compared to those rats consuming the CD diet without 1ADP (Figure 5.5), with evidence of significant dilation of central veins and sinusoids ($P \leq 0.001$). Macrovesicular steatosis was also significantly developed in those rats consuming the highest amount of 1ADP in the MD group. However, liver injury did not reach the level of inflammation or necrosis (Table 5.5).

*5.4.2.6. Hepatic total triglycerides*

To further strengthen the data of liver injury, in particular hepatic steatosis, the total triglyceride content of the liver was analyzed. The total hepatic triglyceride contents of rats in the CD group did not differ significantly in response to graded levels of 1ADP. However, rats consuming the low vitamin $B_6$ diet (MD group) did exhibit significantly higher hepatic triglycerides ($P < 0.001$) in response to 1ADP inclusion at a level of 10 mg/kg diet, in comparison to lower 1ADP inclusion levels (Figure 5.6).

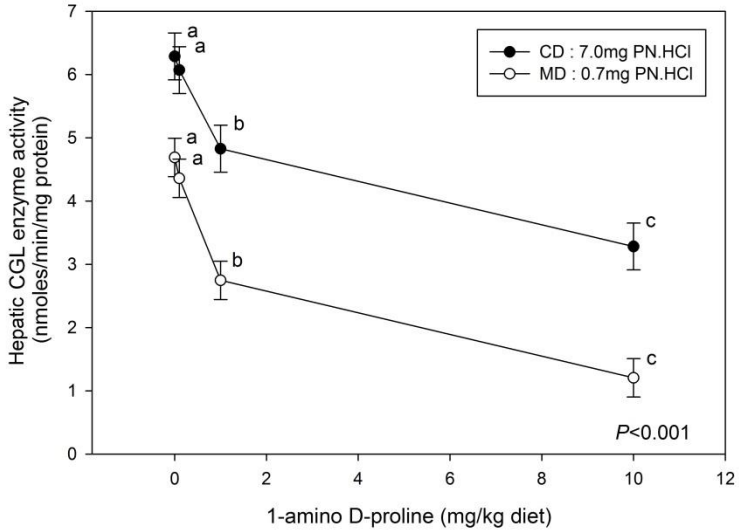


Figure 5.3. Effect of 1ADP exposure and vitamin $B_6$ status on hepatic CGL activity in rat model of moderate vitamin $B_6$ deficiency. CD, Control diet (7 mg/kg diet of PN·HCl); MD, Moderately $B_6$ deficient (0.7 mg/kg diet of PN·HCl). Means ± SE with different superscripts within the group are significantly different (n = 6).

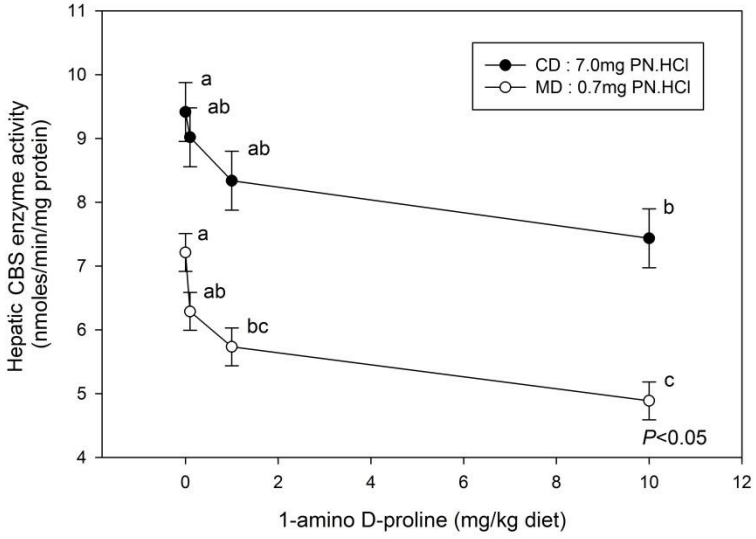


Figure 5.4. Effect of 1ADP exposure and vitamin $B_6$ status on hepatic CBS activity in rat model of moderate vitamin $B_6$ deficiency. CD, Control diet (7 mg/kg diet of PN·HCl); MD, Moderately $B_6$ deficient (0.7 mg/kg diet of PN·HCl). Means ± SE with different superscripts within the group are significantly different (n = 6).

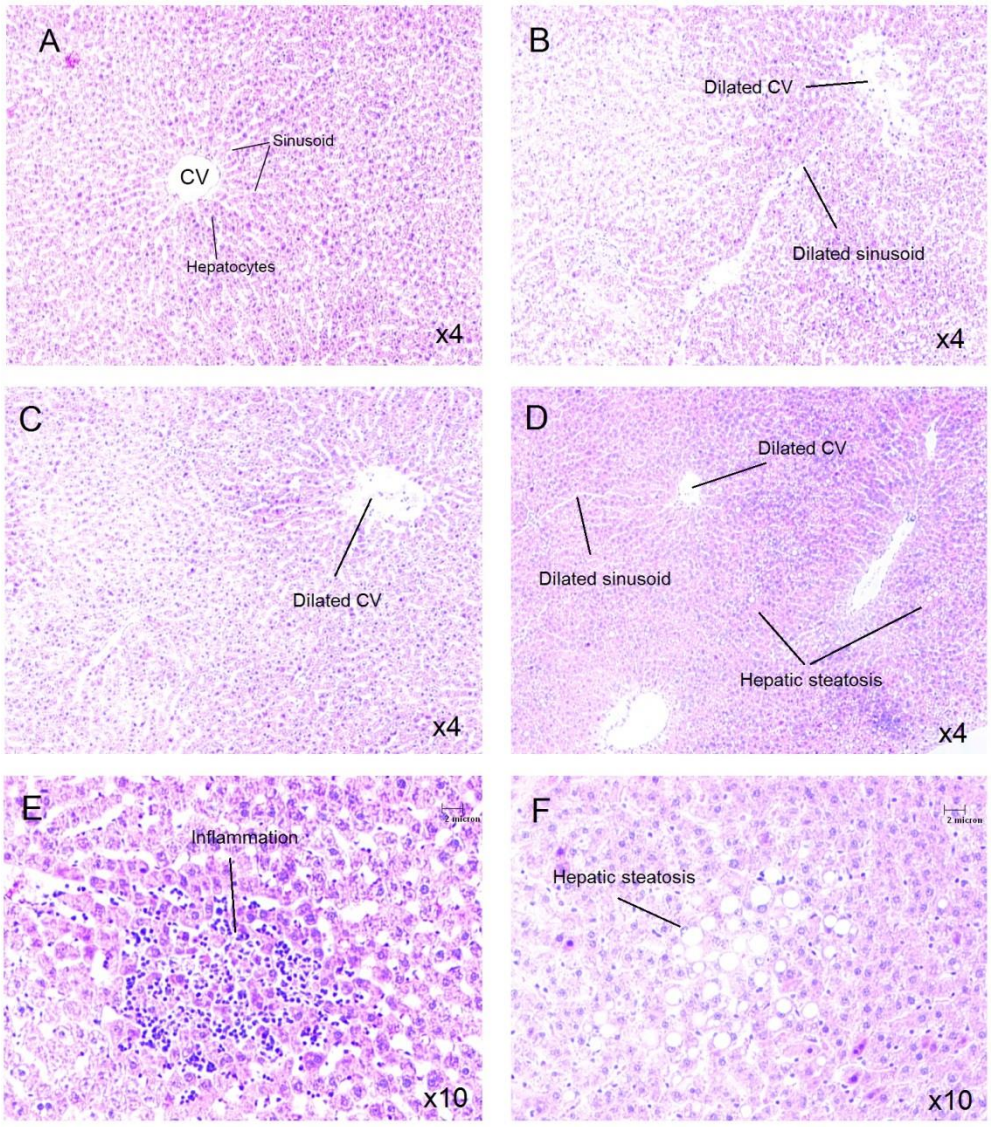


Figure 5.5. Effect of 1ADP exposure and vitamin $B_6$ status on the histology of representative samples of selected treatments in a rat model of moderate vitamin $B_6$ deficiency. (A) 7 mg/kg diet of PN·HCl (CD) , 0 mg/kg diet of 1ADP; (B) 7 mg/kg diet of PN·HCl (CD), 10 mg/kg diet of 1ADP; (C) 0.7 mg/kg diet of PN·HCl (MD), 0 mg/kg diet of 1ADP; (D) 0.7 mg/kg diet of PN·HCl (MD), 10 mg/kg diet of 1ADP; (E) a representative slide showing inflammation; (F) A representative slide showing hepatic steatosis.

Table 5.5. Numerical grading of histopathological symptoms for the lowest and highest levels of 1ADP exposure in rats that were both adequate or marginally deficient in vitamin $B_6$ (main study).

| Histopathology | CD | | MD | | SE | *P* value |
|---|---|---|---|---|---|---|
| | 1ADP:0 | 1ADP:10 | 1ADP:0 | 1ADP:10 | | |
| Central vein dilation | $0.28^{b}$ | $0.83^{b}$ | $0.85^{b}$ | $1.82^{a}$ | 0.24 | 0.002 |
| Sinusoidal dilation | $0.67^{b}$ | $1.17^{b}$ | $1.40^{b}$ | $2.37^{a}$ | 0.19 | <0.001 |
| Macrovesicular steatosis | $0.28^{ab}$ | $0.33^{ab}$ | $0.05^{b}$ | $1.10^{a}$ | 0.21 | 0.012 |
| Inflammation | 0.33 | 0.38 | 0.40 | 0.67 | 0.20 | 0.653 |
| Necrosis | 0 | 0 | 0.17 | 0.17 | 0.12 | 0.582 |

Means with different superscripts are significantly different (n = 6). Grading score: 0 is none, 1 is up to 33%, 2 is 33-66% and 3 is >66%. CD and MD represent vitamin $B_6$ levels of 7 and 0.7 mg/kg diet of PN·HCl, respectively; SE, standard error.

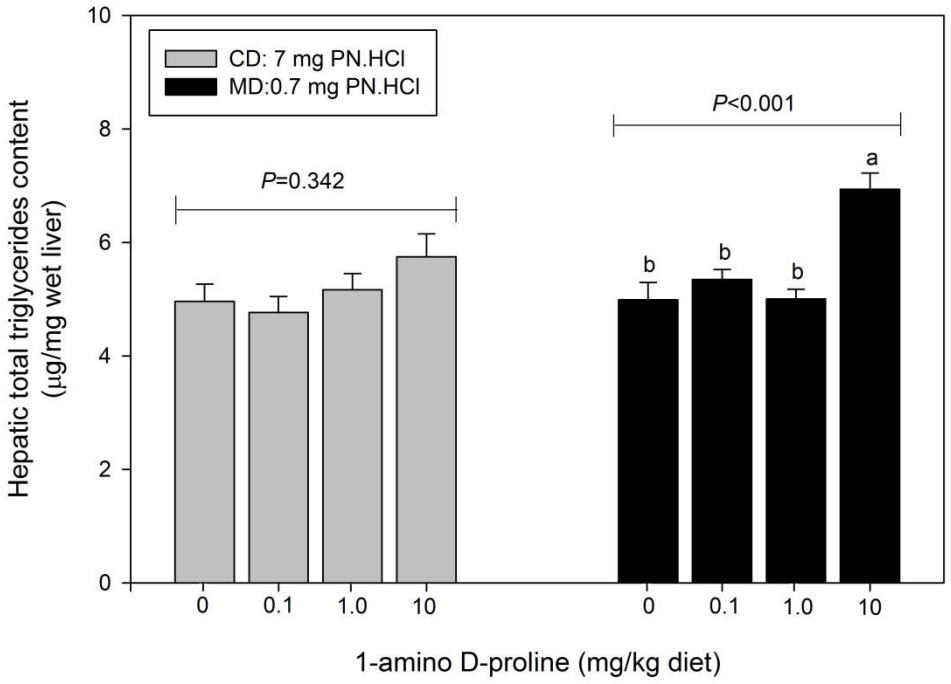


Figure 5.6. Effect of 1ADP exposure and vitamin $B_6$ status on the hepatic triglyceride content in a rat model of moderate vitamin $B_6$ deficiency. CD, Control diet (7 mg/kg diet of PN·HCl); MD, Moderately $B_6$ deficient (0.7 mg/kg diet of PN·HCl). Means ± SE with different superscripts within the group are significantly different (n = 6).

**5.5. Discussion**

The crucial role of vitamin $B_6$ as a cofactor has been studied extensively, with overt deficiency leading to impairments in the activity of a wide-range of PLP-dependent enzymes [33]. Epidemiological studies have shown that, while overt deficiency is rare, the prevalence of moderate vitamin $B_6$ deficiency in the general population is more pronounced, ranging from approximately 18% in North-America and reaching up to 50% of elderly population in Europe [25,34]. Marginal $B_6$ deficiency has been linked to the development of chronic inflammation in rheumatoid arthritis, colorectal cancer and cardiovascular diseases [35,36]. Additionally, several factors including the consumption of $B_6$ antagonists may further perturb

the biochemical availability of endogenous-PLP [11]. In view of these, the present study was established to investigate the metabolic implications of a moderate vitamin $B_6$ deficiency in conjunction with the exposure to a dietary anti-pyridoxine agent linked to a commonly consumed commodity. As such, these studies were designed to mirror conditions relevant to those experienced in the general human population.

The classic deficiency symptoms that appear with inadequate $B_6$ status include reductions in feed intake, weight gain and feed efficiency ratio (FER) [37], with the latter likely due to perturbations in *in vivo* protein turnover in growing animals [38]. The anorexia induced by $B_6$-deficiency may be a function of reduced feed palatability [37]. For the current study, these classic symptoms developed only in those rats consuming the most restricted $B_6$ diet (pilot study) and in those rats consuming MD diet with concurrent exposure to the anti-pyridoxine agent 1ADP. Given that the inclusion of 1ADP in the CD diet had no impact on performance of the rats is strong evidence that the toxicological effects of 1ADP were due to its impact on $B_6$ status. Furthermore, prior studies have demonstrated that reductions in weight gain and FER elicited by anti-pyridoxine compounds were not solely the effect of reduced intake, but rather due to the metabolic insults on amino acid, carbohydrate and lipid metabolism [37,39]. Clearly, those rats consuming adequate vitamin $B_6$ are generally protected from the oral exposure to 1ADP, at least as it pertains to growth and feed intake.

Common vitamin $B_6$ antagonists, including hydrazines, cycloserine, penicillamine and ethanol, react with $B_6$ vitamers to lower the circulating and storage forms of PLP in the body [11]. In the current study, 1ADP was shown to elicit similar effects, including a significant ($P = 0.004$) reduction in plasma PLP in those rats consuming the MD diet as the level of 1ADP increased, however these effects were not observed in rats on the CD diet.

These anti-pyridoxines, particularly hydrazines or its derivatives, can condense with the carbonyl group of PLP or PL to form corresponding hydrazones [18], resulting in distinctly reduced total endogenous $B_6$-vitamer pools. Subnormal plasma PLP concentrations have been shown to impair one-carbon and methionine metabolism leading to the disturbance in nucleic acid synthesis because of the absence of methylene groups for 5,10-methylene-THF production [40] . As mentioned earlier, vitamin $B_6$ also plays a crucial role in Hcy metabolism, with deficiency leading to hyperhomocysteinemia, particularly in the fed-state [27] and hypercystathionemia [28]. Plasma Cys concentrations were not significantly affected by either $B_6$ intake or exposure to 1ADP. In a previous study in pigs, plasma Cys were similarly unaffected by low $B_6$ intake, and only decreased with prolonged $B_6$ deficiency [4]. Perhaps the most intriguing finding of this study was the observed increases in plasma Hcy and CTH concentrations in rats fed with 10 mg/kg diet of 1ADP, irrespective of the level of dietary $B_6$ intake, indicative of a severely compromised transsulphuration pathway. An elevated level of plasma Hcy has been considered to be an independent risk factor for certain chronic diseases, including hypertension and cardiovascular diseases [41,42]. Therefore, the current data provide evidence that the oral exposure to 1ADP can negatively impact $B_6$ and homocysteine metabolism even if subjects are $B_6$ replete, but consequences are more severe in moderately deficient animals.

The transsulphuration of Hcy to Cys in eukaryotic cells requires two key PLP-dependent enzymes CBS and CGL. As mentioned earlier, human CBS is a unique heme-containing enzyme which catalyzes the condensation of serine with Hcy by β-replacement reaction to produce CTH [43]. The latter is further catabolized by CGL to produce Cys and α-ketobutyric acid in an α,γ-elimination reaction [44]. In the current study, the activities of

hepatic CBS and CGL enzymes were significantly reduced in rats from the MD group in comparison to those from the CD group. Within each group, rats receiving the higher doses of 1ADP exhibited greater reductions in enzyme activities compared to control rats. Previous data from pigs has provided evidence that the mRNA expression of both CBS and CGL is not suppressed during moderate $B_6$ deficiency [27] . Given the importance of PLP for the activity of both CBS and CGL enzymes [43,45], the reduction of these enzyme activities might be due to the subnormal $B_6$ levels developed either by feeding low pyridoxine, 1ADP exposure, or a combination of both. While previous studies have noted that the activity of CGL is more sensitive to $B_6$ deficiency than the activity of CBS [46], the current findings showing impacts on both enzymes is consistent with other published works [43,47,48]. Hence, the current study confirms that the impairment of CBS and CGL enzymes leads to hyperhomocysteinemia [27,49] and hypercystathioninemia [28]. However, the novelty of the current work lies in the observed effects of dietary $B_6$ inadequacy concurrent with oral 1ADP exposure. The fact that the hepatic activities of both CGL and CBS from rats consuming adequate $B_6$ levels (CD group) were decreased highlight the potent inhibitory effects of 1ADP or its hydrazone on PLP-dependent enzyme activities. Apparently, even low levels of oral exposure to 1ADP in subjects who are well-nourished with respect to $B_6$ can cause measured reductions in PLP-dependent enzyme activities, however the reductions are not sufficient to lead to measured perturbations in associated metabolite levels until much higher rates of oral exposure are realized.

As a secondary assessment of the potential negative interaction between marginal $B_6$ status and the concurrent exposure to a dietary $B_6$ antagonist, histological examinations of liver sections were conducted. Vitamin $B_6$ deficiency, as well as the administration of anti-

pyridoxine agents, has been linked to certain hepatic diseases, including necrosis [15,16] and steatosis [17]. While precise mechanisms remain to be elucidated, impairments in PLP-dependent enzymes, including CBS and CGL, can give lead to elevated Hcy and subsequent increases in SAH concentrations via the reversible SAH hydrolase reaction [50,51]. Elevated SAH levels are linked to impaired transmethylation reactions [52], with subsequent detrimental effects on the methylation of macromolecules, including DNA, and xenobiotic toxicants [53,54] . Impairments in the enzymes of the TS pathway may also inhibit the biosynthesis of $H_2S$, an important modulatory gaseous molecule, as well as other sulfur-containing compounds, including glutathione [55,56] . Impairments in the above-mentioned processes can predispose to perturbed hepatic function via the promotion of oxidative stress, fibrosis, and steatosis [5,6,57]. In the current study, the development of early stage liver injury in rats receiving 10 mg/kg diet of 1ADP in the MD group was evident, with the occurrence of dilated CV and sinusoids. In order to provide additional support to the histological evidence, which may be prone to the artifactual presentation of pathological indices due to sample processing, total hepatic triglyceride concentrations were also measured. The latter data support the histological evidence of an initial stage of macrovessicular steatosis, as total hepatic triglyceride content in the MD group were significantly higher in those rats exposed to the highest level of dietary 1ADP (Fig.7). Defects in cholesterol and bile acid metabolism have also been reported in $B_6$-deficient individuals [13,58], thus highlighting the importance or this water-soluble vitamin in lipid homeostasis. However, the ingestion of the anti-pyridoxine 1ADP did not induce hepatic inflammation and necrosis significantly in this model. Further research is required to

determine if a longer term of exposure to these nutritional co-insults may predispose to greater liver injury.

The current study provides the first evidence of metabolic perturbations arising from the oral ingestion of the $B_6$ antagonist, 1ADP, concurrent with the existence of a marginal $B_6$ status. As such, the data serve to highlight the potential risks associated with nutritional co-insults that are likely to be experienced by the population. While marginal $B_6$ status alone caused minor perturbations in performance and metabolic indices, the concurrent exposure to 1ADP further impacted markers of $B_6$ status, including the reduction of the activity of the transsulphuration pathway, leading to increases in both Hcy and CTH. Examination of the risks associated with the intake of 1ADP is timely given its occurrence in whole flaxseed. The incorporation of flaxseed into processed food products has increased, due to the recognition of flaxseed as a rich source of alpha-linolenic acid and lignans [59,60]. A recent study found that flaxseed contained 170-400 µg/g of 1ADP equivalents, depending on the variety tested [20]. The consumption of 25-30 g/day of flaxseed, a serving size recommended by some [61-63], equates to an oral exposure to approximately 10 mg/kg diet of 1ADP per day, or 0.15 mg/kg body weight per day (assume 65 kg body weight). In the current study, rats consuming diets containing 1 mg/kg diet of 1ADP consumed approximately 0.05 mg/kg body weight per day, therefore the observed effects are relevant in relation to risk assessment exercises.

**5.6. Conclusion**

In conclusion, the current data provide, to our knowledge, the first data to support the oral toxicity of 1ADP when evaluated in both adequate and moderately deficient vitamin $B_6$ states. The importance of this study lies in the identification of potential risks to segments of

the population who present with compromised $B_6$ status when they are exposed to foods containing 1ADP and related-compounds. Future studies will further elucidate this risk, including the identification of other pathways impacted by sub-optimal $B_6$ status as assessed through the use of a metabolomics approach [64]. Finally, the data serve to draw attention to the importance of assessing the metabolic implications of concurrent nutritional insults that may be experienced by the human population, particularly as it pertains to the introduction of foods that have may not have had a long history of human usage.

## 5.7. References

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# CHAPTER 6

# MANUSCRIPT-III

## Effect of an anti-pyridoxine factor from flaxseed on the plasma amino acid profile of moderately vitamin $B_6$-deficient rats

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## 6.1. Abstract

Pyridoxal 5-phosphate (PLP) plays a crucial role as a cofactor in amino acid metabolism. There is a high prevalence of moderate vitamin $B_6$ deficiency in the population that may be exacerbated through the ingestion of 1-amino D-proline (1ADP), a $B_6$ antagonist found in flaxseed. Given prior evidence of the impact of synthetic 1ADP on indices of pyridoxine metabolism, the current study was designed to investigate the effects of 1ADP, in either synthetic form or derived from flaxseed on amino acid metabolism in moderately vitamin $B_6$-deficient rats. Male weanling rats (n =8/treatment) consumed a semi-purified diet containing either 7 (optimum $B_6$) or 0.7 mg/kg diet (moderate $B_6$) of PN·HCl, each with 0 or 10mg/kg diet of 1ADP, in either synthetic form or as a flaxseed extract, for 5 weeks. Plasma biochemical analyses revealed a significant reduction ($P \leq 0.001$) in $B_6$ vitamers due to 1ADP ingestion, irrespective of source, in moderately $B_6$-deficient rats. Moderate $B_6$-deficient rats consuming 1ADP as a flaxseed extract exhibited, relative to controls, a significant increase in plasma cystathionine ($P < 0.001$), and decreases in plasma α-aminobutyric acid ($P < 0.001$) and glutamic acid ($P = 0.017$) concentrations. However, the synthetic 1ADP elicited greater perturbations in plasma amino acids, compared to the 1ADP extracted from flaxseed. Both synthetic 1ADP and the flaxseed extract inhibited activities of PLP-dependent enzymes including cystathionine β-synthase and cystathionine γ-lyase ($P < 0.001$). The metabolic perturbations of 1ADP exposure were not evident in rats consuming adequate $B_6$. The current data provide evidence that an anti-pyridoxine agent now prevalent in the human food supply may pose challenges to those individuals presenting with moderate $B_6$ status.

**Key words:** vitamin $B_6$, anti-pyridoxine,1-amino-D-proline, flaxseed, cystathionine, moderate $B_6$ deficiency, amino acid

## 6.2. Introduction

Vitamin $B_6$ is known for its biological role as a cofactor in a myriad of metabolic reactions, due to its ability to link various carbon and nitrogen enzymatic reactions and its involvement in the biosynthesis of biogenic amines and one-carbon units [1,2]. Usually, pyridoxal 5′-phosphate (PLP), the active form of vitamin $B_6$, binds to the ε-amino acid of active lysine residues contained in vitamin $B_6$-dependent enzymes, thus forming a Schiff base/external aldimine. The latter species then acts as a common central intermediate for all PLP-catalyzed reactions, including decarboxylation, racemization, transamination, β-elimination and aldol cleavage, and thus serves to perform diverse functions in amino acid, sugar and lipid metabolism [2,3]. In certain amino acid metabolic pathways, including those of the transsulphuration and remethylation pathways of methionine metabolism, vitamin $B_6$ is required as a cofactor [4-6]. In the transsulphuration pathway, PLP acts as a coenzyme for cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL) to convert homocysteine (Hcy) to cysteine (Cys), releasing α-aminobutyric acid as a byproduct. In the remethylation pathway, Hcy is converted back to methionine (METH) by coupling several enzymes including serine hydroxymethyltransferase (SHMT). This PLP-dependent enzyme is involved in the reversible reaction to transfer a -$CH_2$- group from serine to tetrahydrofolate (THF) to form 5,10-methylenetetrahydrofolate (5,10-MTHF) along with glycine [5,7,8].

The body's vitamin $B_6$ status is primarily modulated by its dietary supply, and may be perturbed by the ingestion of anti-pyridoxines [9]. Several drugs (isoniazide, cycloserine, penicillamine and mestranol), as well as natural substances (gyromitrin, canavanine, agaritin, thyophylline, caffeine and linatine) act as vitamin $B_6$ antagonists in the body either by reducing free PLP and/or inhibiting the activity of pyridoxal kinase (PLK) and pyridoxine

phosphate oxidase (PNPOx) enzymes [9-11]. Linatine, a naturally occurring dipeptide of glutamic acid and 1-amino D-proline (1ADP), is a vitamin $B_6$ antagonist present in flaxseed. Chemically, it is 1-[(n-γ-L-glutamyl)-amino]-D-proline. After hydrolysis using hydrochloric acid, linatine releases1ADP, the active form of this anti-pyridoxine [12]. Previous studies have documented that the total anti-pyridoxine content, expressed in 1ADP equivalents (1ADPE), was 177 - 437 µg/g of whole flaxseed depending on the variety sampled [12,13]. 1ADP binds with PLP to form hydrazone complexes, and these complexes can reduce the free *in vivo* PLP pool or cause hydrazone toxicity in the body [12,14]. We have previously shown that the toxicity of synthetic 1ADP was much more evident in animals who were moderately vitamin $B_6$ deficient, as compared to those who were replete. The oral ingestion of synthetic 1ADP at a concentration of 10 mg/kg diet caused a severe impairment in Hcy metabolism in moderately vitamin $B_6$-deficient rats [15].

Consumption of flaxseed has been increasing because of its purported health benefits, including the reduction of risk factors associated with diabetes, cancer and cardiovascular diseases [16-18]. Concurrent ingestion of anti-nutrients including the vitamin $B_6$ antagonist 1ADP present in flaxseed by the general population, particularly those who have moderate $B_6$ deficiency, may be a matter of concern. As overt vitamin $B_6$ deficiency is rare and moderate deficiency is prevalent [19,20], the current study was designed to evaluate the effect of anti-pyridoxine from flaxseed on amino acid metabolism in a rodent model of moderate vitamin $B_6$ deficiency.

## 6.3. Materials and methods

### 6.3.1. Preparation of flaxseed extract

Flaxseed was obtained from a commercial market and was defatted by using a Soxhlet apparatus, using hexane as a solvent. The defatted flaxseed was reground to obtain a fine powder. Anti-pyridoxines from the ground, defatted flaxseed were extracted through the use of an ultrasonification method, previously described with minor modifications including the use of 40% isopropanol at 25°C with 10:1 solvent/solid ratio for 30 min [13]. The extract was concentrated under reduced pressure and freeze dried. Then, it was finely ground and the amount of total anti-pyridoxine content (1ADP equivalents) was quantified using ultra high-performance liquid chromatography/mass-spectrometry (UPLC/MS) using synthetic 1ADP (Santa Cruz Biotechnology Inc, Dallas, TX, USA) and in-house linatine as standards [13]. The flaxseed extract containing the concentrated anti-pyridoxine was stored at -20°C until use.

6.3.2. Animals and diets

Forty-eight male Sprague-Dawley rats weighing 112 ± 9g were purchased from the University of Manitoba Central Animal Care. They were individually housed in polypropylene cages in a room maintained at a temperature of 20 ± 2°C with 12h light/dark rhythm at 50-70% relative humidity. After acclimatization for one week, rats were randomly divided into six groups (n = 8) and fed a semi-purified diet (AIN-93G, based on vitamin-free casein) containing pyridoxine·hydrocloride (PN·HCl) at 7 mg/kg diet (optimum $B_6$-diet) or 0.7 mg/kg diet (moderate $B_6$-deficient diet). Each group of rats were also fed *ab libitum* with 10 mg/kg diet of anti-pyridoxine either in the form of synthetic 1ADP (Santa Cruz Biotechnology Inc, Dallas, TX, USA) or flaxseed extract or none (control) for five weeks. Food intake was monitored daily and body weight was measured every week. At the end of

the experiment, plasma and tissue samples were collected after 12 h of fasting for biochemical analyses.

6.3.3. Plasma biochemical analyses

Plasma $B_6$ vitamers including PLP, pyridoxal (PL), 4-pyridoxic acid (4-PA) were measured as their semicarbazide derivatives using high-performance liquid chromatography (HPLC) and fluorescence detection as previously described [15]. Plasma total thiols, including Hcy, Cys and glutathione were quantified using ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate by HPLC with fluorescence detection [15,21]. Plasma amino acid profile analysis was performed using a commercially available kit (EZ-faast kit, Phenomenex, Torrance, CA, USA) through the use of a Varian 450-GC gas chromatography couple with Varian 240-MS IT mass spectrometer. The injection temperature was 250°C, and column temperature was programmed at 110°C, 160°C and 320°C corresponding with 0, 2.5 and 9.8 min of run-time. After electronic ionization, masses ranging from 50-350 m/z were forwarded for detection. The identification and quantification of amino acids present in the samples was subsequently performed using authentic standards.

6.3.4. Hepatic PLP-dependent enzyme activity

Hepatic CBS and CGL enzyme activities were determined using previously described methods [15,22] with some modifications. Liver samples (0.5g) were homogenized with ice-cold 5mL of 50 mM potassium phosphate buffer (pH 6.8) and centrifuged at 15,000 x g at 4°C to collect the supernatant [15]. For the evaluation of CBS enzyme activity, the supernatant sample was incubated in a reaction mixture containing a radioactive material, 25 mM L-[U-$^{14}$C]serine (~78 000 dpm/μmol) (PerkinElmer,Boston, MA, USA) along with 0.15 mM L-cystathionine, 41.67 mM DL-homocysteine, 0.32 mM S-adenosylmethionine, 2.08

mM DL-propargylglycine, 125 mM Tris, 2.08 mM EDTA. After incubation at 37°C, the newly formed $^{14}$C-cystathionine was separated using a cation-exchange column, and radioactivity was counted to determine the CBS enzyme activity [22]. The hepatic activity of CGL was determined using a spectrophotometric method. The sample was incubated in a reaction mixture containing 4.0 mM L-cystathionine, 0.32 mM NADH, 1.5 units of lactate dehydrogenase at 37°C. The CGL enzyme activity was evaluated based on the decrease in absorbance at 340nm [15].

6.3.5. Statistical analysis

Statistical analysis was done using SPSS 16.0 (SPSS Inc, Chicago, IL, USA). Data were tested for normality before analysis using the Shapiro-Wilk statistic and normalized by $\log_{10}$ transformation if necessary. A general linear model (GLM) with full factorial design was used for the analysis by setting the statistical significance at $P < 0.05$. Feed intake was used as a covariate in the analysis of all plasma biochemical analytes. Treatment effects within the same vitamin $B_6$ levels were compared using Bonferroni's test.

**6.4. Results**

6.4.1. Preparation of flaxseed extract

Upon quantification by UPLC-MS, the amount of anti-pyridoxines in freeze dried flaxseed extract was found to be 305.3µg of linatine and 16.6µg of 1ADP per 50 mg of sample. According to the molar calculation as mentioned in our previous study [13], the total amount of anti-pyridoxines, expressed as 1ADP equivalents (1ADPE), present in 50 mg sample was 169.2µg (152.6µ + 16.6µg), and this value was used in the preparation of the flaxseed extract-based diets that contained 10 mg/kg diet of 1ADPE.

6.4.2. Performance data

Rats in the optimal $B_6$ group who were exposed to either synthetic 1ADP or 1ADP from a flaxseed extract for five weeks, did not show any significant differences in feed intake, final body weight, feed efficiency ratio (FER) or total weight gain (Fig. 6.1 and Table 6.1). However, in the case of the moderate $B_6$-deficient group, rats receiving synthetic 1ADP had a significant reduction ($P < 0.001$) in feed intake, FER and total body weight gain. As a result, final body weight was also significantly reduced ($P < 0.001$). However, the rats fed with flaxseed extract including those of moderate $B_6$-deficient group were not different from the control rats in terms of growth performance parameters. Additionally, feeding of 1ADP, irrespective of source, did not change the relative liver weight (RLW) of rats consuming diets with either optimal or moderately deficient levels of PN·HCl (Table 1). One mortality was observed over the course of the study (rat consuming 1ADP in the optimal $B_6$ group). Death was sudden and the subject did not exhibit anorexia or reduced weight gain, however post-mortem examination could not reveal the cause of death.

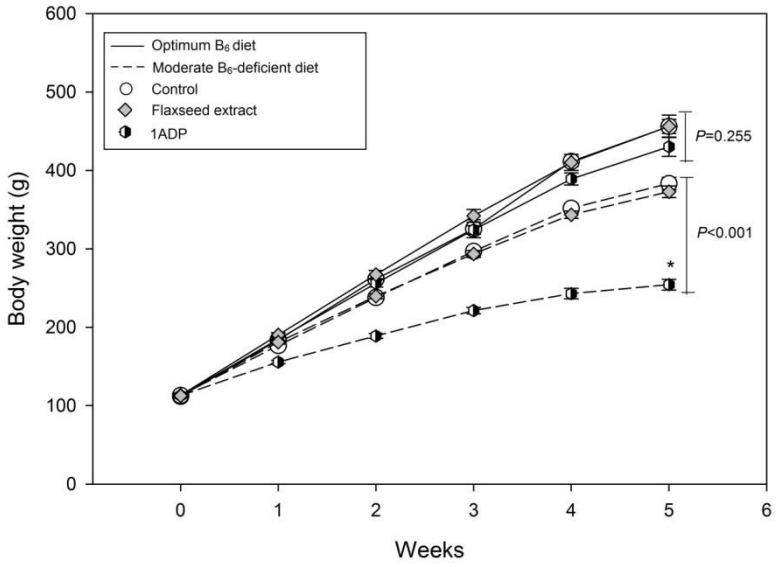


Figure 6.1. Effect of vitamin $B_6$ and anti-pyridoxine factors on growth in a rat model of moderate vitamin $B_6$ deficiency. Data represents mean ± SE (n = 8); optimum $B_6$ diet means PN·HCl: 7 mg/kg diet; moderate $B_6$-deficient diet means PN·HCl: 0.7 mg/kg diet. The asterisks (*) denotes significant different within the diet group.

Table 6.1. Effect of flaxseed extract and synthetic 1ADP on body weight (g), feed intake (g), feed efficiency ratio in a rat model of moderate vitamin $B_6$ deficiency.

| Vitamin group (PN·HCl) | Treatment | Initial body weight | Final body weight | Total weight gain | Relative liver weight (RLW) | Feed intake | Feed efficiency ratio (FER) |
|---|---|---|---|---|---|---|---|
| Optimum $B_6$ (7) | Control | 112±6 | 456±9 | 344±6 | 3.49±0.13 | 929.9±15.3 | 0.37±0.01 |
| | FE | 113±3 | 456±14 | 343±16 | 3.49±0.06 | 917.8±24.7 | 0.39±0.03 |
| | 1ADP[T] | 114±3 | 430±12 | 316±12 | 3.57±0.07 | 868.8±24.8 | 0.37±0.01 |
| Moderate $B_6$-deficient (0.7) | Control | 113±3 | 383±8[a] | 270±10[a] | 3.44±0.08 | 779.9±15.8[a] | 0.35±0.01[a] |
| | FE | 112±2 | 373±7[a] | 260±7[a] | 3.35±0.09 | 770.2±18.8[a] | 0.34±0.01[a] |
| | 1ADP | 114±2 | 255±8[b] | 141±8[b] | 3.54±0.14 | 514.3±19.0[b] | 0.28±0.02[b] |

Data represents mean ± SE (n = 8), [T] n = 7; different superscriptions in the same group were significantly different; RLW= liver weight x 100/body weight; FER= total weight gain/total feed intake; FE, flaxseed extract; 1ADP, 1-amino D-proline; PN·HCl, pyridoxine·hydrochloride (mg/kg diet).

### 6.4.3. Plasma vitamin $B_6$ vitamers

As expected, we found a significant difference in plasma $B_6$ vitamer concentrations, with levels being dependent upon the amount of dietary PN·HCl. Feeding of an anti-pyridoxine, either in the form of synthetic 1ADP or as a flaxseed extract, did not affect the vitamin $B_6$ profile of rats in the optimum $B_6$ group. However, for rats consuming the 0.7 mg PN·HCl/kg diet, the ingestion of synthetic 1ADP caused a significant reduction in both plasma PLP and PL, whereas the ingestion of 1ADP from a flaxseed extract elicited a significant reduction in plasma PLP (Table 6.2). Despite of lowered plasma PN concentrations in rats fed with antipyridoxines compared to the control rats, there was no significant differences between the treatments ($P = 0.152$) due to the compounding effect of food intake. On the other hand, plasma 4-PA, the by-product of vitamin $B_6$ catabolism was

not affected ($P = 0.617$) when rats were fed with either flaxseed extract or 1ADP, and stratified according to dietary $B_6$ supply.

6.4.4. Plasma thiols

Besides the $B_6$ vitamers, the plasma thiols, particularly Hcy and CTH, are important biomarkers of vitamin $B_6$ deficiency [21,23]. In the current study, we found increases in plasma Hcy and CTH concentrations due to both vitamin $B_6$ insufficiency and 1ADP exposure ($P < 0.001$). As given in Table 6.3, plasma Hcy concentrations were elevated by 8 fold in those rats on the moderately deficiency $B_6$ diets who consumed the synthetic 1ADP, but no changes were observed due to the 1ADP derived from the flaxseed extract. However, Hcy concentrations were not significantly affected by 1ADP exposure (synthetic or from flaxseed) in the optimum vitamin $B_6$ group. Plasma CTH concentrations were significantly increased due to 1ADP exposure, in rats consuming both adequate and marginally deficient $B_6$ diets, with the greatest effects observed with the synthetic 1ADP in the marginally deficient treatment group (Table 3). Somewhat unexpectedly, a significant reduction in plasma Cys concentration was observed in rats receiving synthetic 1ADP in the optimum $B_6$ group ($P = 0.005$), but not the marginally deficiency group. There were no significant effects due to either $B_6$ level or 1ADP exposure on plasma METH concentrations (Table 6.3).

6.4.5. Plasma amino acid profile

Plasma amino acid profile of the current study is depicted in Table 6.4. Plasma α-aminobutyric acid ($P < 0.001$) and glutamic acid ($P = 0.017$) were significantly reduced by the oral ingestion of 1ADP irrespective of sources (either synthetic or flaxseed extract) in moderately vitamin $B_6$-deficient rats. Additionally, plasma glycine ($P = 0.02$) concentration was elevated, and plasma serine ($P = 0.013$) and asparagine ($P = 0.016$) concentrations were

reduced in rats of the moderately $B_6$-deficient group when exposed to the synthetic 1ADP. Plasma amino acid concentrations were not significantly impacted due to the exposure to 1ADP in rats consuming the optimum vitamin $B_6$ diet, with the exception that plasma α-aminobutyric acid concentrations were significantly reduced to the exposure to synthetic 1ADP ($P \leq 0.013$), irrespective of vitamin $B_6$ levels. Other plasma amino acids given in Table 6.4 were not affected by the oral ingestion of either the flaxseed extract containing 1ADP or the synthetic 1ADP.

Table 6.2. Effect of flaxseed extract and synthetic 1ADP on plasma vitamin $B_6$ vitamers in a rat model of moderate vitamin $B_6$ deficiency.

| Vitamin group (PN·HCl) | Treatment | $B_6$ vitamers (nmoles/L) | | | |
|---|---|---|---|---|---|
| | | PN | PLP | 4-PA | PL |
| Optimum $B_6$ (7) | Control | 319.6±41.4 | 731.1±62.2 | 29.5±4.3 | 571.1±64.9 |
| | FE | 278.1±38.4 | 721.3±50.8 | 29.9±4.2 | 566.1±58.9 |
| | 1ADP[Ŧ] | 266.0±47.4 | 662.8±26.5 | 25.1±2.6 | 397.2±42.6 |
| Moderate $B_6$-deficient (0.7) | Control | 164.1±21.4[a] | 63.4±3.0[a] | 2.6±0.9 | 39.3±3.2[a] |
| | FE | 112.3±6.6[b] | 47.1±3.0[b] | 2.4±0.5 | 29.7±2.3[b] |
| | 1ADP | 72.3±9.4[b] | 19.8±2.0[c] | 3.6±1.3 | 9.8±1.0[c] |

Data represents mean ± SE (n = 8), [Ŧ]n = 7; different superscriptions in the same group were significantly different, ($P < 0.05$); PN, pyridoxine; PLP,pyridoxal 5-phosphate; 4-PA, 4-pyridoxic acid; PL, pyridoxal; FE, flaxseed extract; 1ADP, 1-amino D-proline; PN·HCl, pyridoxine·hydrochloride (mg/kg diet).

Table 6.3. Effect of flaxseed extract and synthetic 1ADP on plasma thiol concentrations in a rat model of moderate vitamin $B_6$ deficiency.

| Vitamin group (PN·HCl) | Treatment | Plasma thiols (μmole/L) | | | | |
|---|---|---|---|---|---|---|
| | | METH | Cys | Hcy | GTH | CTH |
| Optimum $B_6$ (7) | Control | 49.9±1.3 | 281.7±6.0$^{a}$ | 10.2±0.7 | 29.8±1.6 | 1.7±0.0$^{c}$ |
| | FE | 49.9±1.8 | 283.9±6.9$^{a}$ | 11.4±0.3 | 36.2±2.5 | 2.2±0.1$^{b}$ |
| | 1ADP $^{Ŧ}$ | 46.3±1.2 | 255.8±5.0$^{b}$ | 12.0±0.5 | 32.0±2.8 | 3.4±0.2$^{a}$ |
| Moderate $B_6$-deficient (0.7) | Control | 47.6±0.8 | 280.1±6.2$^{a}$ | 8.5±0.5$^{b}$ | 30.2±1.9 | 2.3±0.1$^{c}$ |
| | FE | 48.4±1.0 | 263.4±6.1$^{ab}$ | 9.5±0.6$^{b}$ | 33.8±2.9 | 4.6±0.9$^{b}$ |
| | 1ADP | 48.0±1.9 | 248.6±4.5$^{b}$ | 71.7±15.7$^{a}$ | 32.7±2.4 | 32.2±5.9$^{a}$ |

Data represents mean ± SE (n = 8), $^{Ŧ}$ n = 7; different superscriptions in the same group were significantly different ($P < 0.05$); METH, methionine; Cys, Cysteine; Hcy, homocysteine; GTH, glutathione; CTH, cystathionine; FE, flaxseed extract; 1ADP, 1-amino d-proline; PN·HCl, pyridoxine·hydrochloride (mg /kg diet).

Table 6.4. Effect of flaxseed extract and synthetic 1ADP on major plasma free amino acids (μmoles/L) in a rat model of moderate vitamin $B_6$ deficiency.

| Plasma amino acid | Optimum $B_6$ (PN·HCl:7) | | | Moderate $B_6$-deficient (PN·HCl:0.7) | | |
|---|---|---|---|---|---|---|
| | Control | FE | 1ADP[Ŧ] | Control | FE | 1ADP |
| Alanine | 618.7±27.1 | 705.5±25.2 | 618.6±39.5 | 564.9±16.2 | 542.1±29.8 | 494.3±30.5 |
| Sarcosine | 10.8±0.1 | 10.9±0.1 | 10.9±0.1 | 10.8±0.1 | 10.9±0.1 | 11.1±0.1 |
| Glycine | 284.5±22.2 | 297.1±19.4 | 289.0±15.5 | 442.4±24.2$^{b}$ | 510.8±38.5$^{b}$ | 898.6±74.4$^{a}$ |
| α-Aminobutyric acid | 56.1±4.4$^{a}$ | 44.3±4.4$^{ab}$ | 33.0±3.3$^{b}$ | 45.9±4.0$^{a}$ | 19.0±1.9$^{b}$ | 13.8±1.1$^{b}$ |
| Valine | 183.7±9.4 | 197.8±8.8 | 177.0±6.0 | 188.1±8.3$^{a}$ | 189.5±7.1$^{a}$ | 151.5±8.7$^{b}$ |
| Leucine | 147.7±7.8 | 158.3±8.2 | 142.6±6.2 | 146.6±6.5 | 147.3±6.4 | 135.5±9.8 |
| Isoleucine | 92.7±5.2 | 96.0±5.2 | 86.1±4.5 | 90.6±4.2 | 92.4±4.7 | 82.9±6.0 |
| Threonine | 682.1±71.7$^{a}$ | 590.0±39.9$^{ab}$ | 443.9±38.2$^{b}$ | 766.3±90.6$^{a}$ | 648.2±64.9$^{ab}$ | 485.7±43.2$^{b}$ |
| Serine | 602.5±60.1$^{a}$ | 471.6±38.9$^{b}$ | 496.5±45.1$^{b}$ | 584.6±49.9$^{a}$ | 469.2±23.5$^{ab}$ | 352.7±32.4$^{b}$ |
| Proline | 171.0±7.1 | 168.2±4.4 | 162.0±6.6 | 183.1±8.5 | 187.7±4.2 | 185.5±10.6 |
| Asparagine | 96.1±5.6 | 94.8±3.1 | 97.0±4.3 | 99.3±4.7$^{a}$ | 87.3±5.8$^{ab}$ | 79.1±4.4$^{b}$ |
| 4-Hydroxyproline | 64.9±6.6 | 55.7±4.6 | 59.7±6.3 | 65.1±6.2 | 59.9±6.3 | 51.4±5.6 |
| Glutamic acid | 205.6±10.6 | 213.2±4.9 | 200.9±20.2 | 197.2±14.0$^{a}$ | 151.8±8.6$^{b}$ | 129.4±6.0$^{b}$ |
| Phenylalanine | 64.1±1.8 | 69.2±1.4 | 65.0±1.7 | 62.4±1.0 | 61.6±2.2 | 57.7±1.4 |
| α-Aminoadipic acid | 2.0±0.2 | 2.3±0.2 | 2.3±0.2 | 2.5±0.1 | 2.9±0.3 | 2.5±0.2 |
| Glutamine | 955.7±97.2 | 778.2±93.4 | 867.0±114.2 | 961.7±118.5 | 796.5±92.8 | 758.6±81.8 |
| Ornithine | 60.5±2.6 | 66.1±4.2 | 62.4±2.6 | 86.5±7.4 | 91.6±8.2 | 87.1±5.9 |
| Lysine | 302.8±13.7 | 271.2±7.9 | 273.0±12.9 | 271.5±16.1$^{a}$ | 257.4±13.2$^{ab}$ | 209.7±13.3$^{b}$ |
| Histidine | 61.8±5.1 | 59.4±5.9 | 60.3±4.5 | 62.7±5.3 | 65.7±6.5 | 84.0±8.6 |
| Tyrosine | 58.7±1.9 | 56.5±2.1 | 52.6±1.9 | 51.3±1.5 | 52.0±1.9 | 49.1±2.3 |
| Proline-hydroxyproline | 23.1±1.3 | 20.3±0.1 | 20.5±0.3 | 21.1±0.3 | 20.3±0.2 | 20.5±0.3 |
| Tryptophan | 82.3±7.2 | 67.7±6.2 | 78.4±8.2 | 84.4±6.9 | 71.1±5.0 | 72.3±6.6 |

Data represents mean ± SE (n = 8), [Ŧ] n = 7; different superscriptions in the same group were significantly different ($P < 0.05$); FE, flaxseed extract; 1ADP, 1-amino D-proline; PN·HCl, pyridoxine·hydrochloride (mg/kg diet).

6.4.6. Hepatic enzyme activity

In the current study, we found a significant reduction ($P < 0.001$) in hepatic CBS (Fig. 6.2) and CGL (Fig. 6.3) enzyme activities due to either flaxseed extract or synthetic 1ADP treatment compared to the respective controls. Hepatic CGL enzyme activity of rats fed with either flaxseed extract or synthetic1ADP was reduced to half of that observed in the moderate $B_6$-deficient group consuming no 1ADP. However, for the optimum $B_6$ group, the reductions in enzyme activities were less pronounced in rats exposed to the flaxseed extract as compared to the synthetic 1ADP. Nevertheless, the reduction of hepatic CBS enzyme activity was more response in rats fed with synthetic 1ADP compared to those fed with the flaxseed extract in moderate $B_6$-deficient group. But, the reductions were of a similar magnitude in the optimum $B_6$ group.

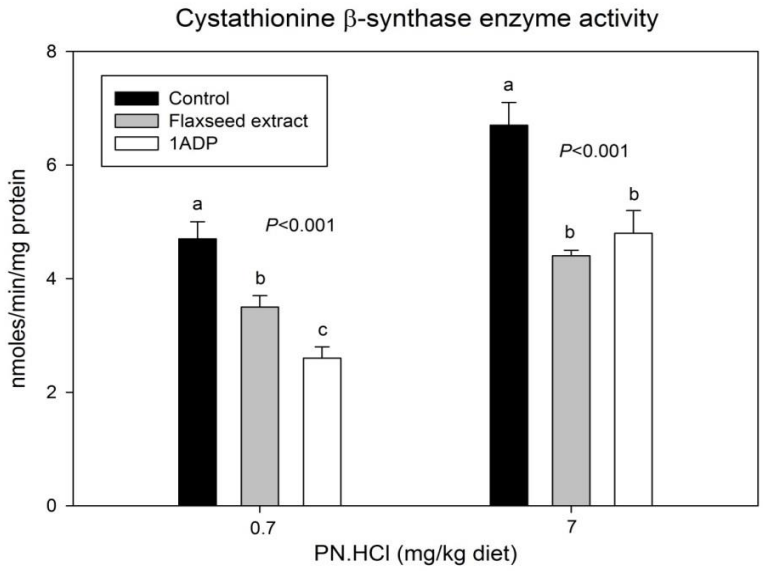


Figure 6.2. Effect of flaxseed extract and synthetic 1ADP on hepatic CBSenzyme activity in a rat model of moderate vitamin $B_6$ deficiency. Data represents mean ± SE (n = 8), different superscriptions in the same group were significantly different, ($P < 0.05$); 1ADP, 1-amino D-proline; PN·HCl, pyridoxine·hydrochloride (mg/kg diet).

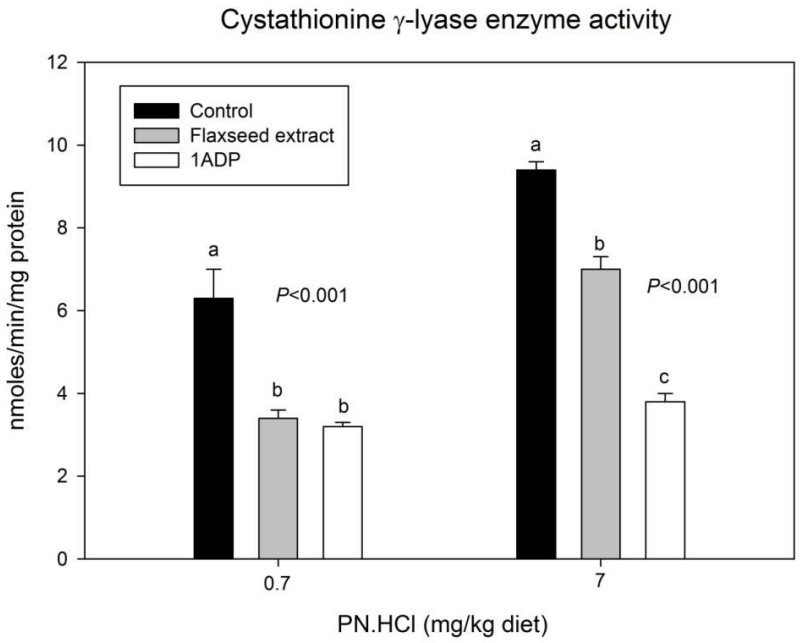


Figure 6.3. Effect of flaxseed and synthetic 1ADP on hepatic CGLenzyme activity in a rat model of moderate vitamin $B_6$ deficiency. Data represents mean ± SE (n = 8), different superscriptions in the same group were significantly different, ($P < 0.05$); 1ADP, 1-amino D-proline; PN·HCl, pyridoxine·hydrochloride (mg/kg diet).

## 6.5. Discussion

Dietary vitamin $B_6$ has a substantial impact on the concentration of plasma PLP [9,24]. Marginal vitamin $B_6$ deficiency can be defined by low circulating PLP, but with variable impacts on PLP-dependent enzymes. In humans, plasma PLP concentration of 20-30 nmol/L or a vitamin $B_6$ intake of 0.7 mg/day indicates marginal $B_6$ status [4,25]. Within the general population, marginal $B_6$ deficiency is prevalent in 24% of the American population and 21% of the Canadian elderly (> 50 years) population [19,26]. Additionally, women have a higher occurrence of marginal $B_6$ status (32%) compared to their male counterparts (16%) and its prevalence in women consuming oral contraceptive pills is as high as 76% of the female population [19]. Inadequate $B_6$ status has also been implicated in the development of many

common diseases including chronic inflammation, colorectal cancer, and cardiovascular diseases [27,28]. The consumption of compounds possessing anti-pyridoxine activity concurrent with marginal $B_6$ deficiency may therefore increase the risk for the development of metabolic defects/disease associated with low $B_6$ status [14,29]. As such, the current study was designed to investigate the potential responses in biomarkers elicited by nutritional co-insults due to the oral consumption of a flaxseed-derived component in a model of moderate $B_6$ deficiency.

Vitamin $B_6$ deficiency normally causes reductions in food intake, weight gain, and FER in growing animals [15,30]. Animals deficient in $B_6$ have been documented to derive less metabolizable energy per unit of gross energy, signifying overt defects in intermediary metabolism as well as nitrogen retention due to sub-optimal vitamin $B_6$ status [31]. In the present study, rats in the moderate $B_6$ group receiving synthetic 1ADP exhibited a significant reduction in FER as well as weight gain compared to other groups. These data support previous findings from a study that examined the impact of feeding an anti-pyridoxine, 4-deoxypyridoxine, which led to significantly lower body weights in rats of low vitamin $B_6$ status compared to their pair-fed counterparts [32]. Wofson *et al* (1987) also suggested that the reduction in growth performance of vitamin $B_6$-deficient rats was not solely due to a reduction in feed intake, but also to the associated metabolic insults on amino acid, protein, and lipid metabolism [30]. However, in the current study, rats receiving the anti-pyridoxine 1ADP in the form of a flaxseed extract showed neither reduction in FER or in weight gain. As the anti-pyridoxine treatments were matched on the basis of 1ADP equivalents (molar sum of 1ADP and the dipeptide linatine), these results may be a reflection of the

bioavailability or biopotency of the naturally occurring dipeptide linatine relative to synthetic 1ADP in a rodent model (see discussion below).

Tissue concentrations of the classical vitamin $B_6$ markers, including PLP, PL, PN and 4-PA, primarily reflect the nutritional supply of pyridoxine, but can be affected by the ingestion of vitamin $B_6$ inhibitors [9,10,33]. In the current study, the significant alteration of major vitamin $B_6$ markers—developed as a result of anti-pyridoxine administration—is in line with the findings of previous studies [15,29,34]. Our data showed that the flaxseed extract reduced plasma PLP concentrations, with significant evidence in moderately $B_6$-deficient rats, indicating a potential binding ability of naturally occurring 1ADP to endogenous $B_6$ vitamers. Even though, there was a lower value of plasma PLP concentrations in the rats receiving synthetic 1ADP than that of flaxseed extract, when compared, they were not statistically significant because of the compounding effect of food intake. However, the synthetic 1ADP indicated a stronger and wider affinity to $B_6$ vitamers over naturally occurring ones, as it had significantly reduced both plasma PLP and PL concentrations. Therefore, the current study provided evidence that oral ingestion of an anti-pyridoxine, either in the form of synthetic 1ADP or as a flaxseed extract, reduced the overall $B_6$ vitamer pool in the body. One of the main reasons for lowering *in vivo* $B_6$ vitamers in the current study might be the result of the formation of hydrazone complexes of 1ADP with freely available endogenous PLP and PL. These findings, also suggested by others, were more pronounced during low $B_6$ status [10,14]. Other potential mechanisms include an anti-pyridoxine induced inactivation of vitamin $B_6$ salvage enzymes such as PLK and PNOx [11] and increased urinary excretion of $B_6$ vitamers during anti-pyridoxine exposure [35]. It is well known that the PLK and PNOx enzymes play a crucial role in maintaining the

homeostasis *in vivo* of $B_6$ vitamers through their phosphorylation and inter-conversion [11,36]. Interestingly, their activities have been shown to be inhibited by several $B_6$ antagonists such as isoniazid, cycloserine, and dopamine, which have a similar mode of action as 1ADP causing *in vivo* PLP deficiency [11]. However, further work is needed to characterize the effect of this anti-pyridoxine on *in vivo* vitamin $B_6$ homeostasis and its catabolism.

A deficiency of vitamin $B_6$ alters the plasma amino acid profile because of the involvement of this co-factor in numerous PLP-dependent enzymes particularly those involved in transamination, transsulphuration, and decarboxylation reactions [6]. For instance, administration of anti-pyridoxine agents DL-penicillamine, thiosemicarbazide, and semicarbazide-HCl significantly reduced γ-aminobutyric acid content in the mouse brain due to the inhibition of the activity of a PLP-dependent glutamic acid decarboxylase enzyme [37]. Additionally, an intraperitoneal injection of 1ADP (50 mg/kg body weight) in male weanling rats led to a significant increase in plasma α-aminoadipic acid, citrulline, and CTH concentrations, signifying major perturbations in amino acid metabolism [14]. In another experiment, the perfusion of 1ADP into an isolated rat liver significantly reduced the synthesis of carnitine from lysine residues [38]. Marginal to severe vitamin $B_6$ restriction is well documented to impair methionine metabolism [4,15,23]. Several studies have demonstrated significantly higher plasma Hcy and CTH concentrations in both animal models and humans when subjected to a vitamin $B_6$-restricted diet [15,21,23,24]. In the current study, the significant increase in plasma Hcy that was observed in response to oral 1ADP exposure was likely due to the inhibition of hepatic CBS activity in the transsulphuration pathway, a condition that was more pronounced under low vitamin $B_6$

status. The latter mechanism is supported by the evidence that plasma Hcy concentration is higher (40-fold) in mice lacking CBS activity in comparison to wild-type mice [39]. Additionally, hepatic CGL enzyme activity was also inhibited due to the feeding of anti-pyridoxine either from flaxseed or synthetic 1ADP, leading to increases in plasma CTH and a significant reduction in plasma α-aminobutyric acid concentrations. A similar pattern of inhibition of CBS and CGL enzyme activities had already been reported in the liver of vitamin $B_6$-deficient animals [21,24,40]. The current findings support the contention that moderate vitamin $B_6$ deficiency as well as oral exposure to an anti-pyridoxine were able to independently inhibit activities of PLP-dependent enzymes including hepatic CBS and CGL in rats.

Glycine decarboxylation through the glycine cleavage system and the conversion of glycine to serine by SHMT in one carbon metabolism also require PLP. Several *in vitro*, animal, and human studies have demonstrated that vitamin $B_6$ deficiency reduces the activities of these PLP-dependent enzymes [21,23,24,41,42]. An inhibition of SHMT and/or the glycine cleavage system have been speculated to be the mechanisms explaining the shifts in plasma glycine/serine ratios during low vitamin $B_6$ status [23,24,43]. Therefore, one of the reasons for the elevation of plasma glycine and reduction of plasma serine concentrations in the current study might be due to the impairments of these two enzymes which led to reduced transformation of *in vivo* glycine to serine. Additionally, reductions in plasma asparagine and glutamic acid concentrations as a result of 1ADP feeding during low vitamin $B_6$ status might be related to the impairment of other PLP-dependent enzymes involved in transamination and inter-conversion of amino acids. Our data is supported by the previous study of Swendseid *et al* (1964), who found higher concentrations of CTH and glycine, and lower

concentrations of serine and total glutamine and asparagine in the plasma of vitamin $B_6$-deficient rats [43].

Responses induced by dietary vitamin $B_6$ deficiency or the exposure to anti-pyridoxine agents on amino acid profile can exhibit variability, likely related to the duration or severity of the imposed deficiency [4,14,44]. Gregory *et al* (2013) recently showed that marginal $B_6$ deficiency alone can cause minor perturbations in metabolic indices including *in vivo* amino acid profiles [4], and a concurrent exposure to synthetic anti-pyridoxine further exacerbated vitamin $B_6$ status [15]. Interestingly, the current data provided evidence of a perturbation of certain $B_6$ markers due to the co-insult of a flaxseed-derived anti-pyridoxine factor during moderate $B_6$ deficiency, when provided at a dose of 0.5 mg 1ADPE/kg body weight per day. Consumption of 25-30 g/day of flaxseed, a serving size recommended by some [45-47], will result in the intake of 0.15 mg 1ADPE/kg body weight per day (assuming a body weight of 65 kg), a concentration within the same order of magnitude as that provided in the current study. As such, our data serve to highlight potential deleterious effects of the oral exposure of an anti-pyridoxine factor linked to flaxseed on a population presenting with moderate $B_6$ deficiency. However, the bioefficacy of naturally occurring bioactive compounds largely depends on their bioavailability [48,49]. For example, synthetic drugs tend to have better liberation, absorption, distribution and metabolism when compared to naturally occurring bioactive components [48]. The major anti-pyridoxine factor present in flaxseed is the dipeptide linatine, which must liberate free 1ADP via the hydrolysis of the γ-glutamyl bond attached to glutamic acid in the gastrointestinal tract, in order to induce *in vivo* anti-pyridoxine toxicity. Incomplete hydrolysis or/and reduced absorption and metabolism of linatine contained in flaxseed extract might be responsible for the lowered

potency of flaxseed-derived 1ADP compared to the synthetic 1ADP. Additionally, the percentage of total anti-pyridoxine present in the flaxseed extract was 0.64%. As such, there exists the possibility for the presence of other components in the extract that may have negated the anti-pyridoxine toxicity. Nevertheless, flaxseed-derived 1ADP did demonstrate an ability to perturb markers of amino acid and vitamin $B_6$ metabolism in the current study.

### 6.6. Conclusion

Vitamin $B_6$ is required as a cofactor for a myriad of enzymes particularly linked to transsulphuration and transamination reactions, key components in intermediary metabolism. The current study provided evidence that the oral ingestion of 1ADP, either from flaxseed or as a synthetic source, lowered free *in vivo* $B_6$ vitamers potentially due to hydrazone formation, thus impairing amino acid metabolism. The most intriguing finding of this study was the ability of flaxseed-derived 1ADP to inhibit the activities of PLP-dependent enzymes during moderate vitamin $B_6$-deficiency, which may be alleviated by the consumption of adequate $B_6$. However, further investigations are necessary to better understand the mechanism of action of 1ADP and the protective role of vitamin $B_6$ during anti-pyridoxine toxicity. Additional opportunities also exist to investigate a long term effect of this naturally occurring anti-pyridoxine linked to flaxseed on other metabolic pathways.

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# CHAPTER 7

# MANUSCRIPT-IV

## Investigation of vitamin $B_6$ inadequacy, induced by exposure to the anti-$B_6$ factor 1-amino D-proline, on plasma lipophilic metabolites of rats: a metabolomics approach

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## 7.1. Abstract

Vitamin $B_6$ status in the body is affected by several factors including dietary supply of the antivitamin $B_6$ factor, 1-amino D-proline (1ADP) which is present in flaxseed. Owing to the prevalence of moderate $B_6$ deficiency in the general population, a co-insult of 1ADP exposure may lead to a further deterioration of $B_6$ status. To this end, we applied a non-targeted metabolomics approach to identify potential plasma lipophilic biomarkers of 1ADP injury in moderately vitamin $B_6$-deficient rats. Male weanling rats (n = 6/treatment) received a semi-purified diet containing PN·HCl at either 7 (optimal $B_6$) or 0.7mg/kg diet (moderate $B_6$), each with 0 or 10mg/kg diet of synthetic 1ADP for 5 weeks. Plasma lipophilic metabolites were extracted in acetonitrile for analysis via LC-QTOF/MS. Ten potential lipophilic biomarkers: glycocholic acid, glycoursodeoxycholic acid, murocholic acid , N-docosahexaenoyl GABA, N-arachidonoyl GABA, lumula, nandrolone, orthothymotinic acid , cystamine, and 3-methyleneoxindole were identified out of >2500 detected entities. Changes in these metabolites revealed potential defects in the biosynthesis and metabolism of bile acid components, N-acyl amino acids, analgenic androgens, anti-inflammatory and neuroprotective molecules. These data provide new insights into the impact of $B_6$ inadequacy, induced by the application of 1ADP, on pathways linked to vitamin $B_6$ metabolism.

**Key words:** Metabolomics, biomarkers, vitamin $B_6$, antivitamin $B_6$ and 1-amino D-proline

## 7.2. Introduction

Pyridoxal 5′-phosphate (PLP), the active form of vitamin $B_{6,}$ serves as a co-factor for a myriad of enzymes associated with amino acid, sugar and fat metabolism [1-3]. It is required as a co-factor in both the transsulphuration and remethylation pathways of the methionine cycle [4,5]. Vitamin $B_6$ is also necessary in the activation of hydrolases and decarboxylases used in the synthesis of neurotransmitters, such as γ-aminobutyric acid (GABA), dopamine and serotonin [2]. In addition, vitamin $B_6$ has been shown to influence the functions of key hormones including glucocorticoids [6,7], androgen [8], and progesterone [9]. Other roles of vitamin $B_6$ include its putative antioxidant activity to protect against induced neurotoxicity [10]. Low plasma PLP, a marker of diminished vitamin $B_6$ status, adversely affects polyunsaturated fatty acid metabolism, leading to defects in arachidonic acid and hepatic cholesterol biosynthesis [11-13].

The dietary provision of vitamin $B_6$ represents the primary source of pyridoxine in the body. The diet can also be a source of antivitamin $B_6$ factors: Natural $B_6$ antagonists such as gyromitrin, agaritine and canavaline may be present in some common foods, including mushrooms and certain leguminous plants [14]. Flaxseed, a good source of the omega-3 fatty acid α-linolenic acid, lignans and dietary fibre [15] also contains the $B_6$ antagonist 1-amino D-proline (1ADP) and its precursor linatine [14,16,17]. We recently quantified the total amount of antivitamin $B_6$ components present in the flaxseed, and it was found to be 170-400 μg/g of whole flaxseed based on different varieties [17]. When consumed, free 1ADP complexes with PLP to form a hydrazone complex [16], that leads to the inhibition of a variety of PLP-dependent enzymes including aminotransferases, tryptophan indole-lyase, glutamate and tyrosine decarboxylase [14,16].

The intraperitoneal administration of 1ADP (26 mg/kg body weight) to Wistar rats perturbed amino acid metabolism as evident through elevations in α-aminoadipic acid, citrulline and cystathionine concentrations in liver, kidney, pancreas, plasma and urine, and vitamin $B_6$-deficient rats were more sensitive to 1ADP than adequately nourished rats [18]. As moderate vitamin $B_6$ deficiency is prevalent within the North American population [19,20], the possible deleterious effects of 1ADP exposure through the consumption of flaxseed deserves attention.

Metabolic profiling is considered an advanced tool for the identification of important biomarkers of diseases/diagnoses which can lead to the development of biologically-relevant, mechanistic pathways [21]. Several hundreds and/or thousands of metabolites may be monitored in biological fluids based on their accurate mass through the use of a metabolomics methodological approach. Targeted metabolomic approaches have been recently used to study the metabolic sequelae of low vitamin $B_6$ intake in humans [22]. Plasma PLP, homocysteine and cystathionine are well-recognized biomarkers for vitamin $B_6$ deficiency [5,23]. However, additional opportunity exists to address alternative biomarkers associated with disruptions in vitamin $B_6$ status, either through inadequate dietary supply and/or the ingestion of an antivitamin $B_6$ factor, namely 1ADP. In the current study, we identified the potential lipophilic biomarkers of vitamin $B_6$ deficiency as well as 1ADP-injury, through the use of a non-targeted metabolomics approach, by high performance liquid chromatography/quadrupole time-of flight mass spectrometry (HPLC-Qtof-MS) using a rodent model of moderate vitamin $B_6$ deficiency with or without co-exposure to an antivitamin $B_6$ agent.

## 7.3. Materials and methods

### 7.3.1. Materials

Acetonitrile and formic acid were purchased from Fisher Scientific (ON, Canada) while synthetic 1ADP and lumula were obtained from Santa Cruz Biotechnology, Inc (TX, USA). N-arachidonoyl γ-aminobutyric acid was purchased from Cayman Chemical Company (MI, USA). All the reference solutions for HPLC-QTOF-MS were obtained from Agilent Technologies (CA.USA). De-ionized water was prepared in-house using Milli Q20 System from Millipore Corporation (MA, USA).

### 7.3.2. Animals and diets

Twenty-four male Sprague-Dawley rats, weighing 80 ± 7g, were purchased from the University of Manitoba Central Animal Care Facility, and housed individually in polypropylene cages maintained at a temperature of 20 ± 2°C with a 12 h light/dark cycle at 50-70% relative humidity. Following a one week acclimatization period during which animals were maintained on commercial lab chow, rats were randomly divided into four groups (n=6). They were assigned to receive one of 4 treatment diets, based on the AIN-93G formulation, using vitamin-free casein: a) 0.7 mg/kg pyridoxine.HCl (PN·HCl) of diet, 0 mg/kg 1ADP [L]; b) 0.7 mg/kg PN·HCl, 10 mg/kg 1ADP [L+1ADP]; c) 7.0 mg/kg PN·HCl; 0 mg/kg 1ADP [N]; and d) 7 mg/kg PN·HCl, 10 mg/kg 1ADP [N+1ADP]. Food intake was monitored daily and body weight was measured weekly over a 35 day study period. Full details on the model and the confirmation of moderate $B_6$ deficiency have been presented elsewhere [24]. In brief, feeding of 0.7 mg/kg diet of PN.HCl decreased plasma PLP to a level of 30-40 nmoles/L without any significant changes in food consumption, growth and plasma homocysteine (<12 μmoles/L) compared to the optimum $B_6$ supply (7 mg/kg diet of

PN.HCl). This condition was considered to be a moderate vitamin $B_6$ deficiency in a rodent model; and hence, a dietary supply of 0.7 mg/kg diet of PN.HCl was chosen to mimic a moderate vitamin $B_6$ deficiency in humans for this study. Additionally, we chose 10 mg/kg diet of 1ADP in the current study as this antivitamin $B_6$ at this concentration concentration impaired growth performance as well as profiles of several $B_6$ markers including plasma PLP content in the rats of a model previously describe [24]. At the end of the experiment, rats were fasted for 12h and euthanized using isoflurane. Blood was collected through cardiac puncture, and then immediately transferred in a vacutainer heparin tube to centrifuge at 5,000 x g for 10 min at 4°C. The plasma was harvested and stored at -80°C prior to analysis.

7.3.3. Sample preparation

Plasma samples (100µL) were mixed with acetonitrile (200 µL) in 1.5 mL eppendorf tubes, vortexed vigorously (30 sec) and the mixture was centrifuged for 10 min (10,000 x g at 4°C). The supernatant was transferred into a new tube and completely dried under a gentle stream of $N_2$. Prior to HPLC-Qtof-MS analysis, the dried samples were reconstituted with 100 µL of 80% acetonitrile prepared in deionized water [25].

7.3.4. HPLC-Qtof-MS conditions

Non-targeted metabolomics analysis was performed on an 1290 Infinity Agilent HPLC system coupled to a 6538 UHD Accurate LC-Qtof-MS from Agilent Technologies (CA, USA) equipped with a dual electrospray ionization (ESI) source. A 3x50 mm, 2.7µ Agilent Poroshell column (Agilent Technologies) was used to separate metabolites while the column temperature was maintained at 60°C. The mobile phases A and B were water and acetonitrile, with 0.1% formic acid. A sample size of 2 µL was injected by maintaining the HPLC flow rate at 0.7 mL/min with a gradient program of: 0, 0.5, 16, 17 and 22 min with 30,

30 100, 100 and 30% of solvent B, respectively. A post-run time of 2 min was buffered before injecting the next sample. The auto-sampler was maintained at a temperature of 15°C. The mass detection was operated using dual electrospray with reference ions of *m/z* 121.050873 and 922.009798 for positive mode; and *m/z* 119.03632 and 980.016375 for negative mode. The main parameters for MS were as followed: gas temperature, 300°C; drying $N_2$ gas flow rate, 11L/min; Nebulizer pressure, 50 psig; fragmentor voltage, 175V; skimmer voltage 50V and OCTRF Vpp voltage, 750V. Targeted MS/MS mode was used to identify the potential biomarkers. As part of the MassHunter Software, the collision energy was applied by setting an appropriate equation having a slope value of 5 and offset value of 2.5. A full range mass scan from 50-3000 *m/z* with an extended dynamic range of 2 GHz standardized at 3200 was applied. Data acquisition rate was maintained at the rate of 3spectra/s at a time frame of 333.3 ms/spectra with a transient/spectrum ratio of 1932.

7.3.5. Data processing and statistical analyses

The workflow utilized for data processing comprised several algorithms used by Agilent Mass Hunter Qualitative (MHQ, B.05) and by Mass Profiler Professional (MPP, 12.6). The raw data files were first acquired and stored as "*.d" files using an Agilent MassHunter Acquisition software (B.05) ready to be processed in MHQ. The Molecular Feature Extraction (MFE) considered a naïf extraction procedure was the first algorithm applied to the total ion chromatograms (TIC) files. The MFE parameters were set to allow the extraction of detected features satisfying an absolute abundances >4,000 counts. The MFE parameters were set to provide information regarding $[M+H]^+$, isotopes and their corresponding $Na^+$ adducts. The resulting extracted ions were treated as single features for which potential formula were generated. The collected information summarizing retention

time (RT), exact masses and ion abundances were converted into compound exchange format files ("*.cef") and were exported to MPP for further subsequent comparative and statistical analyses. Using alignment and normalization procedures, individual "*.cef" files were binned and combined to generate new "*.cef" files. These new files were reopened in MHQ for further data mining procedure using a 'Find by ion' algorithm. This targeted feature algorithm helped with minimizing the false positive and negative features found by MFE procedure. Second series of individual "*.cef" files were created from original individual "*.d" files and exported into MPP software for statistical and differential analysis. A frequency filtration was used to only accept features that were detected in at least one of the four conditions (treatments). This filtration step was employed to ensure an elimination of the potential feature extraction artifacts. Other MPP filtering procedures such as number of detected ions (set to '2') and charge states (set to 'all charge states permitted') were also applied. The RT compound alignment parameters were set to 0.15 min with a mass tolerance of 2.0 mDa. The data were normalized using a percentile shift algorithm set to 75 and were adjusted to the baseline values of the median of all samples. The final entity list with > 2000 entities and the 4 treatments (non-averaged) were used for sample classification and statistical analysis.

The partial least square discrimination (PLSD) was used as the class prediction algorithm to build a prediction model with the following validation parameters; number of components (4), validation type (N-fold), number of folds (3) and number of repeats (10). The prediction results were generated to examine the predicted label and confidence value for each individual sample (n = 24). In addition, Lorenz Curves were displayed for each of the 4 class fraction (4 treatments) to visualize the ordering of this measure for each class.

One way ANOVA (P<0.05) followed by a Tukey's honestly significant difference (HSD) were performed by MPP software (version 12.6).

All statistical analysis including the partial least square discrimination (PLSD) and one way ANOVA (P<0.05) followed by a Tukey's honestly significant difference (HSD) were performed by MPP software (version 12.6).

7.3.6. Identification of plasma biomarkers

Metabolites or potential markers with at least two-fold significant changes ($P$<0.05) in any of the different groups of rats were first annotated on the basis of their exact mass data corresponding to *m/z* peaks by searching them against Metlin (> 64,000 metabolites) and SimLipids (> 39,000 lipids) database. The identities of the selected metabolites were further confirmed using their mass spectra and chromatographic retention times against commercially pure standards when available. Mass fragmentation experiments were conducted on both standards and plasma samples using the same chromatographic standards described above. Identification and confirmation of those compounds for which pure standards were not available were achieved either by comparing the ion fragmentation (MS/M spectra) from the previously published studies or percent confidence score based on exact mass. A mass error of less than 5 ppm was set to formulate the elemental compositions.

**7.4. Results**

The raw data generated from the HPLC-QTOF-MS analyses were detected and normalized automatically using Mass Hunter software to generate a multivariate data matrix, which was then converted to "*cef" files for subsequent processing and analysis in MPP software. The class prediction built using the PLSD algorithm applied to the final filtered entity list with > 2000 entities was successful with a clear separation between the groups

(Figure 7.1). The Lorenz Curves generated for each treatment showed a clear linear slope from 0 to 1 on Y axis with all 6 samples within each treatment followed by all other items in 3 other treatments resulting in a flat curve thereafter (Supplemental Fig. S6). The rats of [L+1ADP] group showed a tight clustering of entities compare to the remaining groups: [N], [N+1ADP], and [L]. A total of 237 entities out of > 2500 detected were significantly different after a one way ANOVA ($P \leq 0.05$). Among those, 23 metabolites were annotated based on exact mass and MS information available in Metlin and Lipidomics database. Thereafter, 10 metabolites out of 23 were identified by comparing the fragmentation patterns of their ion either with commercial standards or with MS/MS spectra available in our libraries.

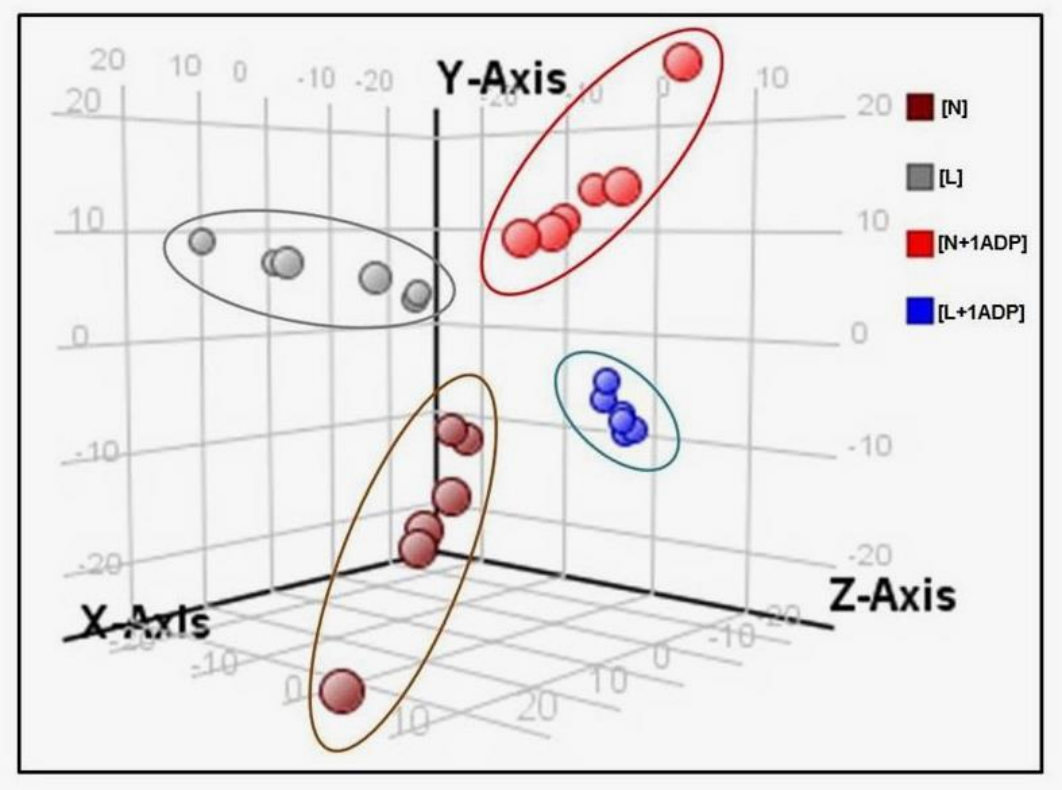


Figure 7.1. An overview of the partial least square discrimination (PLSD) analyses with plasma metabolites to investigate the effect of different treatments (n = 6). 1ADP: 1-amino D-proline, L: low vitamin $B_6$, N: normal vitamin $B_6$.

Here, we present glycocholic acid (*m/z* 466.3164) eluted at 2.53 min as an example to elucidate biomarkers identification. In the ESI+ mode, $C_{26}H_{43}NO_6$ was generated as the most likely molecular formula of the metabolite and MS/MS information was used to generate its molecular structure. After considering the elemental composition and fragmentation patterns, Metlin database predicted this metabolite having a parent ion of *m/z* 466.3164 $[M+H]^+$ to be glycocholic acid- calculated mass was 465.3087 (Figure 7.2A-B). The two major daughter ion fragments were *m/z* 412.2850: [M+H-76.0128]+ and *m/z* 337.2521: [M+H-151.0457]+. The first base peak might have been formed by losing three OH groups (-51u) and two H atoms (-2u): [M+H-OH-OH-OH-$H_2$]+. The second base peak is suggested to be formed from the additional breakdown of two $CH_3$ groups (-30u) and one COOH group (-45u): [M+H-OH-OH-OH-$H_2$-$CH_3$-$CH_3$-COOH]+ (Figure 7.2C). By comparing the ion fragmentation patterns and MS/MS spectra of library with that of the samples, the presence of glycocholic acid was confirmed. Similarly, we identified the remaining potential metabolites for the *in vivo* effect of perturbations in $B_6$ status (Figure S7-15).

We also evaluated the possible deleterious effect of 1ADP on those identified metabolites on each treatment. Of the 10 metabolites, two of them were down-regulated whereas the remaining eight were up-regulated (Table 7.1). Glycocholic acid, a common bile acid component, was detected in the plasma of all rats. However, its concentration was significantly lower in rats fed with optimum $B_6$ as compared to those receiving low vitamin $B_6$. The nutritional co-insult experienced with the concurrent feeding of 1ADP in rats consuming low vitamin $B_6$ [L+1ADP] increased plasma glycocholic acid concentration by 2.5-fold compared to those rats receiving only low vitamin $B_6$ [L] even though such change was not observed in rats of optimum $B_6$ group. Alternatively, N-acyl-amino acids namely N-

arachidonoyl GABA (NAGABA) and N-docosahexaenoyl GABA (NDGABA) were detected only in rats from the low vitamin $B_6$ group and their concentrations in plasma were elevated significantly ($P \leq 0.001$) by the addition of 1ADP to the diet. Plasma glycoursodeoxycholic acid, murocholic acid, lumula and nandrolone were present exclusively in rats consuming 1ADP in the low vitamin $B_6$ group [L+1ADP]. Although, orthothymotinic acid was not detected in the plasma of control rats [N], its concentration was significantly elevated in rats fed with 1ADP in the low $B_6$ group [L+1ADP]. Conversely, cystamine and 3-Methyleneoxindole concentrations in plasma were significantly ($P \leq 0.001$) lower in rats consuming the low vitamin $B_6$ diet [L] compared to the control rats [N]. Plasma concentrations of these two markers were also reduced significantly by the ingestion of 1ADP even in rats fed with optimum $B_6$ level [N+1ADP], and they were not detected in rats fed with 1ADP in the low $B_6$ group [L+1ADP]. The overall change in abundance of potential biomarkers of antivitamin $B_6$ injury with respect to different treatments is also shown in table 7.1 and figure 7.3. Our data indicated that glycocholic acid, orthothymotinic acid, NAGABA, NDGABA, cystamine, and 3-methyleneoxindole were significantly affected as a result of lower $B_6$ intake [L], while glycoursodeoxycholic acid, murocholic acid, lumula, and nandrolone were impacted due to $B_6$ deficiency induced by the exposure to 1ADP [L+1ADP]. Therefore, the earlier group of metabolites is potentially more sensitive markers of $B_6$ deficiency compare to the latter.

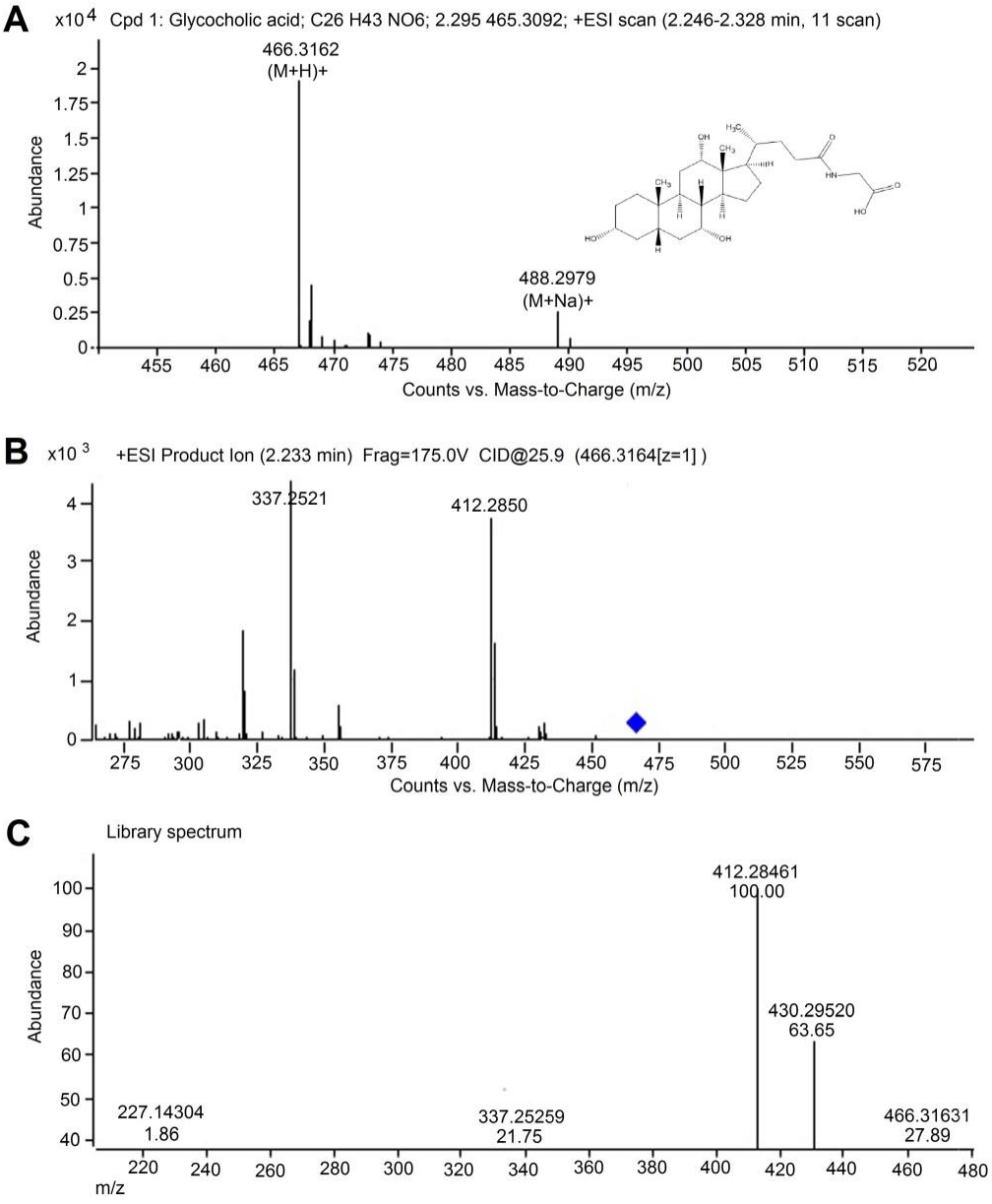


Figure 7.2. Mass spectra of glycocholic acid : (a) MS spectra of sample (b) MS/MS spectra of sample, '♦' precursor ion of glycocholic acid, *m/z* 466.3164; and its major fragment ions, *m/z* 412.2850 and 337.2521 (c) MS/MS spectra from Metlin Library (adapted from Metlin library).

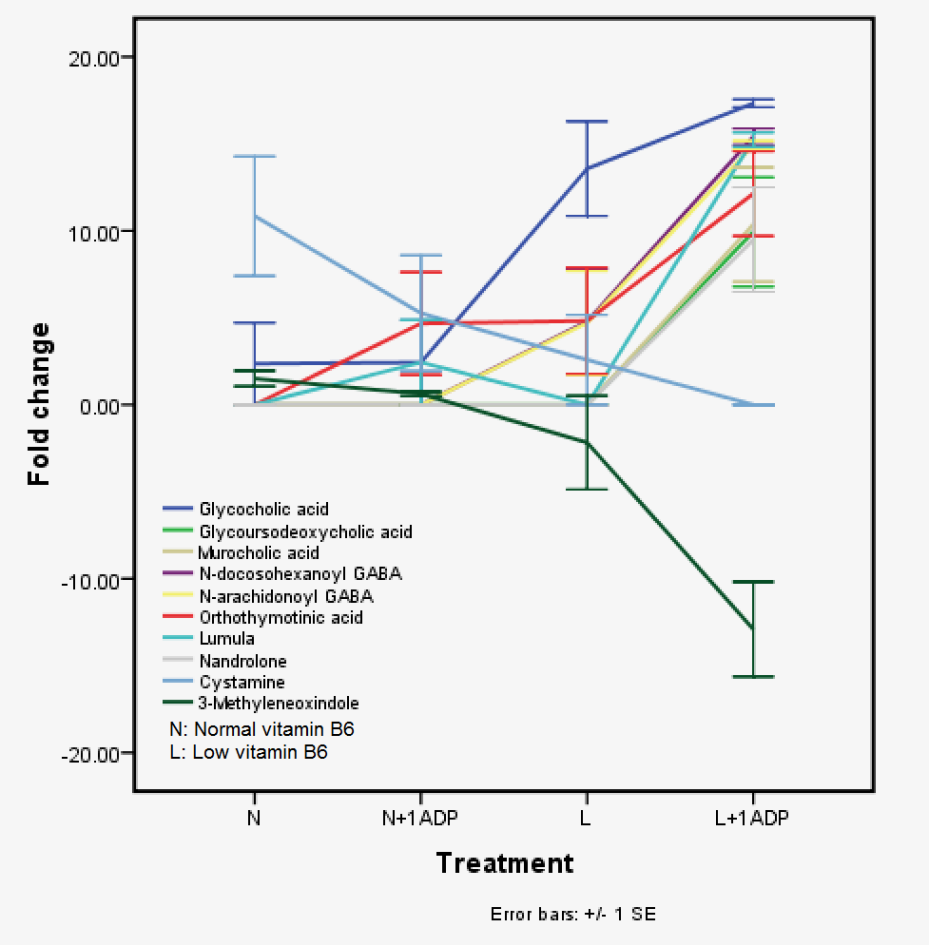


Figure 7.3. Effect of vitamin $B_6$ deficiency and 1ADP-injury on selected plasma lipophilic metabolites in rats (showing their fold changes).

**7.5. Discussion**

The significant role of vitamin $B_6$ in amino acid metabolism had been studied extensively, and the impairment of sulphur amino acid metabolism is found in marginal vitamin $B_6$ deficiency [26], which represents 16% of male and 32% of American population [20]. Vitamin $B_6$ deficiency is expected to disturb a wide-range of metabolic pathways owing to the importance of PLP as a cofactor for numerous enzymes. However, classical biomarkers of vitamin $B_6$ such as PLP, provide limited information about other pathways which could have been affected indirectly due to $B_6$ deficiency. Therefore, potential effects of $B_6$ inadequacy on other metabolic pathways including neurotransmitter biosynthesis, organic acids and lipid metabolism needs to be further investigated. Additionally, we also had demonstrated that a concurrent feeding of antivitamin $B_6$ during low vitamin $B_6$ status further perturbed homocysteine metabolism and initiated hepatic steatosis due to increase accumulation of liver triglycerides [24]. In view of these, the current study was designed to develop novel lipophilic biomarkers of $B_6$-inadequacy induced by oral exposure to anti-$B_6$ factor 1ADP, in order to understand better about the roles of this water soluble vitamin on other biochemical pathways particularly linked to lipid metabolism

A clear treatment separation in the PLSD plot indicated the presence of distinct biomarkers for the treatment combinations. Low vitamin $B_6$ intake tended to impair the biosynthesis and catabolism of the identified metabolites; however the impairments were more significant for those rats receiving low vitamin $B_6$ along with the vitamin $B_6$ antagonist. As discussed earlier, the intraperitoneal administration of 1ADP disturbed methionine metabolism by forming hydrazone complexes with PLP, and the impacts were more prevalent during vitamin $B_6$ deficiency [18]. A recent metabolomics analysis conducted by

using a combination of $^{1}$H-NMR and targeted mass spectral approach revealed that vitamin $B_6$ restriction not only impaired amino acid but also carbohydrate, neurotransmitters, and organic acid profiles. They found a significant increase in plasma cystathionine, proline, asparagine, glutamine, myo-inositol, 2-oxoglutarate, pyruvate, and trimethylene-N-oxide concentrations and significant decrease in plasma glutamate concentration in healthy individuals due to $B_6$-restriction [22]. Interestingly, in the current study, we also found changes in plasma concentrations of certain organic acids (bile acids), and metabolites potentially related to neurotransmitters biosynthesis such as N-acyl amino acids.

The classically recognized function of bile acids, a family of steroidal molecules, is their involvement in the absorption of dietary lipids. However, they have been identified as ligands of the Farnosoid X receptor (FXR), a nuclear bile acid receptor. This receptor plays a crucial role in the regulation of bile acid and cholesterol metabolism [27]. Moreover, hepatic cholesterol synthesis has been shown to be significantly elevated in pyridoxine-deficient animals, contributing to increased cholesterol turnover and conversion to bile acids [13]. Similar observations were also noted in the current study where certain plasma bile acid components were significantly increased as a result of either low vitamin $B_6$ status (glycocholic acid), the effect of oral 1ADP exposure during low vitamin $B_6$ status (glycoursodeoxycholic acid and murocholic acid) or their interaction (glycocholic acid). Besides low circulatory PLP, 1ADP/hydrazone as well as potential synergistic effects, might have led to the deactivation of the FXR receptor, subsequently leading to an increase in hepatic bile acid biosynthesis from cholesterol. The latter may result from an up-regulation of expression of cholesterol-7 α hydrolase or cytochrome P450 7A1 (CYP7A1), a rate-limiting enzyme of bile acid synthesis and CYP8B1[28]. Under normal conditions, bile acid

homeostasis is maintained by the effective reabsorption of bile acid components via the enterohepatic cycle. However, when FXR is deactivated by its antagonist, the normal functions of this nuclear receptor are affected [28], leading to an up-regulation of the apical sodium-dependent bile acid transporter (ASBT) and a down-regulation of the solute transporters (OSTα and OSTβ) in enterocytes. Additionally, the deactivation of FXR increased the expression of the organic anion transporting polypeptide (OATP) and sodium taurocholate co-transporting polypeptide (NTCP) and reduced the expression of bile acid modification enzymes including sulphotransferase 2A1 (SULT2A1), UDP-glucuronosyltransferase 2B4 (UGT2B4) and CYP3A4 in hepatocytes (Figure 7.4). As a result, the entire enterohepatic cycle and detoxification processes may have been impaired. Previous study suggested that such conditions caused injury to hepatocytes, and ultimately bile acids are leaked into the blood [29]. Our histological data also provided evidence of development of early stage of liver injury in rats fed with 1ADP during low $B_6$ status with the occurrence of dilated sinusoids and central veins [24].

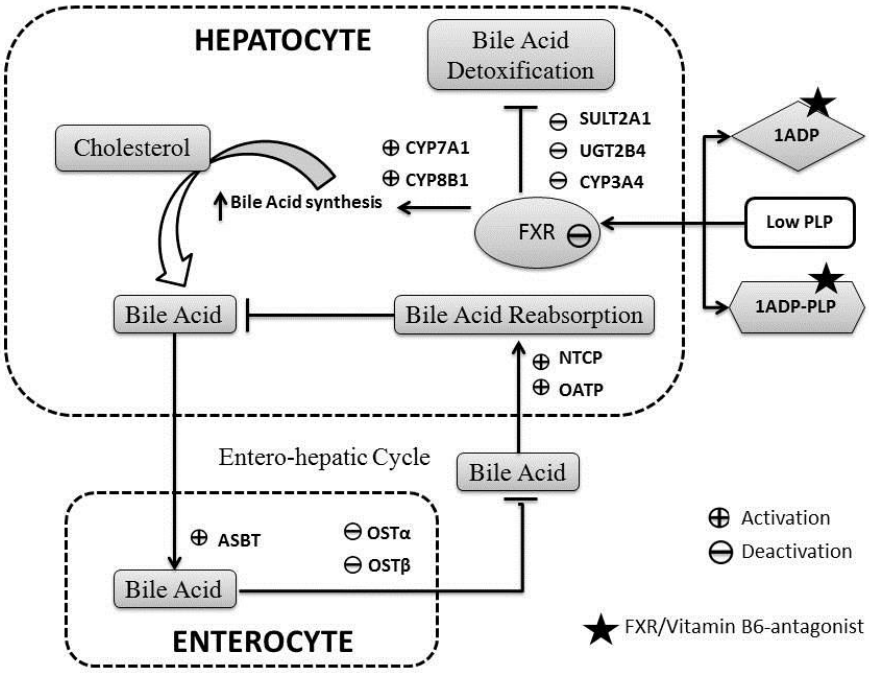


Figure 7.4. Hypothetical mechanism of antivitamin $B_6$, 1ADP action during low vitamin $B_6$ status on the biosynthesis of bile acids. Low PLP status and antivitamin $B_6$ compounds including 1ADP and its hydrazones might have deactivated Farnesoid X receptor (FXR), a nuclear bile acid receptor, which increased bile acid biosynthesis from cholesterol by up-regulating CYP7A1 and CYP8B1 in the hepatocytes. Deactivation of this nuclear receptor also up-regulates ASBT (enterocytes), NTCP (hepatocytes) and OATP (hepatocytes); and down-regulates OST (enterocytes) which ultimately impair enterohepatic cycle. Additionally, deactivated FXR might have reduced bile acid detoxification process by down-regulating SULT2A1, UGT2B4 and CYP3A4 in hepatocytes. Such conditions are potentially linked to liver injury due to increased accumulation of bile acids.

N-acyl amino acids have been shown to have anti-inflammatory, anti-nociceptive, vasoprotective, angiogenic and neuroprotective effects [30] and their biosynthesis and catabolism have not been fully elucidated [31]. The increased plasma concentrations of N-acyl amides particularly NAGABA and NDGABA in this study might be due to their increased biosynthesis, reduced catabolism, or a combination of the two. The possible biosynthetic pathway of NAGABA is from the hydrolysis of N-arachidonoyl ethanolamide (Ara-EA), by fatty acid amide hydrolase (FAAH) enzyme [31]. Elevated levels of plasma N-acyl amino acids might be due to the increased sensitivities of certain steroid and glucocorticoid hormones including progesterone to FAAH enzyme by either low vitamin $B_6$ or the application of 1ADP during low vitamin $B_6$ status, which in turn may increase the catabolism of Ara-EA [9, 32].

One of the sulphur containing target compounds in the field of neuroprotective research is cystamine, an oxidized disulfide form of cysteamine. Cystamine degrades to cysteamine and they exist in redox-equilibrium in cells which is subsequently converted to hypotaurine and taurine. Cystamine/cysteamine is considered to be a potential treatment of neurodegenerative diseases, including Parkinson's and Hungtington's diseases, due to its protective role against the neuron degradation process [33]. Given the crucial role that vitamin $B_6$ plays in neurotransmitter synthesis [34], plus the fact that 1ADP exposure leads to the development of seizure in rats [18], *in vivo* cystamine/cysteamine might have been extensively utilized to protect against neuronal degradation in low pyridoxine status. Additionally, degradation of the neuroprotective components might have been enhanced by the feeding of 1ADP in the current study.

Prostaglandins, lipophilic hormones derived from arachidonic acid, play a key role in generating inflammatory responses to injury and infection [35], and the compound lumula, unoprostone N-ethyl amide, is an example. The concentration and profile of prostaglandins changes drastically during an inflammatory response. In general, they are elevated immediately in an acute inflammation before the recruitment of leukocytes and are therefore considered to be important biomarkers for inflammation and oxidative stress conditions [36]. Interestingly, vitamin $B_6$ deficiency has been shown to modulate the biosynthesis of prostaglandins in the body, with the direction of changes dependent on the specific prostaglandin under study [37, 38]. Therefore, elevated plasma levels of lumula might have a link with inflammation and oxidative stress concurrent with the development of moderate $B_6$ deficiency.

Nandrolone is an anabolic androgenic steroid hormone. The biosynthesis and metabolic pathway of this hormone, particularly in relation to the involvement of cofactors including vitamins, has not been fully described. However, it has been reported that its function and metabolism are similar to testosterone [39] and it has been linked to reward pathways[40]. Higher concentrations of nandrolone in the body caused a significant reduction in certain reward-motivated neurotransmitters, including dopamine, 5-hydroxytryptamine and noradrenaline [41]. A similar inverse relationship has also been observed in case of vitamin $B_6$ deficiency, where the production of such endoneurotransmitters responsible for shaping mood and behavior were reduced in response to low circulatory PLP in the body [2,34]. Therefore, the elevated levels of plasma nandrolone observed in the current study might have been related to the increased expression of certain steroid hormones under vitamin $B_6$ deficient conditions.

The current metabolomics study provided evidences of potential metabolic perturbations linked to lipophilic metabolites arising from vitamin $B_6$ inadequacy induced by oral ingestion of the anti-$B_6$ factor 1ADP. Interestingly, the change in potential plasma lipophilic biomarkers were found to be alleviated in those rats supplemented with adequate vitamin $B_6$ level, and thus indicated a crucial role of pyridoxine in normalizing those impaired metabolic pathways. Altogether, the current data indicated that $B_6$ inadequacy, either due to low intake or induced by oral exposure to anti-$B_6$ factor, potentially impaired plasma concentrations of bile acids, N-acyl amino acids, analgenic androgens, anti-inflammatory and neuroprotective molecules. Therefore, this data not only broadens biomarker selection for $B_6$-inadequacy but also provides information to better understand the effects of this water soluble vitamin on other biochemical pathways, particularly linked to lipophilic metabolites.

**7.6. Conclusion**

Metabolomic investigations on the effect of low vitamin $B_6$, especially a low status and toxicity induced by antivitamin $B_6$ ingestion, are limited. However, targeted metabolomics of vitamin $B_6$ restriction in cultured HepG2 cells and healthy humans revealed fundamental changes in the metabolism of amino acids, carbohydrates, organic acid, neurotransmitters, and metabolites of the one-carbon pathway [22, 42]. This metabolomics data on oral exposure of antivitamin $B_6$ in a rodent model identified novel plasma lipophilic biomarkers and their associated metabolic pathways of vitamin $B_6$ deficiency and antivitamin $B_6$ effect. Knowing the fact that flaxseed contains 1ADP, further investigations on $B_6$-deficiency induced by 1ADP intake might be necessary, and therefore, the newly reported potential lipophilic biomarkers should be assessed in relation to classic biomarkers of

vitamin $B_6$ deficiency. Additionally, more detailed understanding of these mechanisms underlying the effect of $B_6$ antagonist during low/moderate $B_6$ deficiency needs to be explored.

Table 7.1. Selected plasma lipophilic metabolites for low vitamin $B_6$ and/or 1ADP-injury in rats showing their abundance

| Compound name | ESI mode | RT (min) | *m/z* | Formula | Calculated mass | Associated Pathway | Direction of change | Abundance (x$10^3$)* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | [N] | [N+1ADP] | [L] | [L+1ADP] | SE |
| Glycocholic acid | + | 2.334 | 466.3164 | $C_{26}H_{43}NO_6$ | 465.3087 | Bile acid metabolism [13, 29] | Up | 3.0$^{c}$ | 4.1$^{c}$ | 68.4$^{b}$ | 176.9$^{a}$ | 16.7 |
| Glycoursodeoxycholic acid | + | 3.889 | 450.3214 | $C_{26}H_{43}NO_5$ | 449.3139 | Bile acid metabolism [13, 29] | Up | ND$^{b}$ | ND$^{b}$ | ND$^{b}$ | 20.9$^{a}$ | 1.6 |
| N-docosahexaenoyl GABA | + | 2.516 | 414.2999 | $C_{26}H_{39}NO_3$ | 413.2927 | N-acyl amino acid metabolism [30] | Up | ND$^{c}$ | ND$^{c}$ | 7.1$^{b}$ | 54.7$^{a}$ | 7.3 |
| N-arachidonoyl GABA | + | 2.237 | 412.2845 | $C_{24}H_{39}NO_3$ | 389.2929 | N-acyl amino acid metabolism [30] | Up | ND$^{c}$ | ND$^{c}$ | 6.0$^{b}$ | 33.5$^{a}$ | 3.4 |
| Lumula | + | 5.194 | 410.3260 | $C_{24}H_{43}NO_4$ | 409.3187 | Inflammation/oxidative stress [35, 36] | Up | ND$^{b}$ | ND$^{b}$ | ND$^{b}$ | 50.1$^{a}$ | 10.0 |
| Murocholic acid | - | 3.844 | 391.2854 | $C_{24}H_{40}O_4$ | 392.2923 | Bile acid metabolism [13, 29] | Up | ND$^{b}$ | ND$^{b}$ | ND$^{b}$ | 35.7$^{a}$ | 4.5 |
| Nandrolone | + | 3.618 | 275.2006 | $C_{18}H_{26}O_2$ | 274.1932 | Anabolic androgens/Reward pathway [39, 40] | Up | ND$^{b}$ | ND$^{b}$ | ND$^{b}$ | 13.6$^{a}$ | 1.5 |
| Orthothymotinic acid | - | 1.248 | 193.0870 | $C_{11}H_{14}O_3$ | 194.0939 | Analgesic effect [43] | Up | ND$^{c}$ | 5.6$^{b}$ | 7.7$^{b}$ | 20.5$^{a}$ | 2.2 |
| Cystamine | + | 0.499 | 175.0338 | $C_4H_{12}N_2S_2$ | 152.0445 | Neuroprotective effect [33, 34] | Down | 55.8$^{a}$ | 18.9$^{b}$ | 7.7$^{bc}$ | ND$^{d}$ | 7.6 |
| 3-Methyleneoxindole | + | 0.430 | 146.0596 | $C_9H_7NO$ | 145.0523 | Indole acetic acid metabolism [44] | Down | 181.1$^{a}$ | 80.3$^{ab}$ | 62.8$^{b}$ | ND$^{c}$ | 25.9 |

Values and notations with different superscript in the same raw were significantly different at $P < 0.05$ between the treatments (n=6). 1ADP: 1-amino D-proline, L: low vitamin $B_6$, N: normal vitamin $B_6$, ND: not detected or abundance of the mass was below the cut-off threshold level.

*Abundance is the area obtained for the detected ions.

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# CHAPTER 8

# OVERALL DISCUSSION AND SUMMARY

## 8.1. General discussion and summary

The roles of vitamin $B_6$ in human metabolism have been discussed in the previous chapters. Overt vitamin $B_6$ deficiency leads to impairments in the activities of numerous PLP-dependent enzymes including cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL) of the transsulphuration pathway of sulphur amino acid metabolism [1-3]. Epidemiological studies have indicated that severe vitamin $B_6$ deficiency is rare but marginal/moderate deficiency does exist in the general population of North American and European countries [4,5]. The prevalence of vitamin $B_6$ deficiency might be due to a poor diet or exposure to smoking, excessive alcoholism, and certain drugs such as oral contraceptive pills. In the case of elderly people, reduced absorption and increased catabolism of vitamin $B_6$ is one of the primary reasons of low plasma PLP concentrations. Therefore, Morris and his colleague have suggested an increase in the amount of vitamin $B_6$ supplementation for those individuals who are prone to $B_6$ deficiency [4]. Additionally, *in vivo* $B_6$ status is further exacerbated by several factors including exposure to anti-pyridoxine factors present in common foods. For instance, linatine and 1ADP contained in flaxseed had been shown to act as vitamin $B_6$ antagonists in chicks and rats [6-9].

The discovery of linatine, an anti-pyridoxine factor, led to the identification of yet another anti-nutritive component present in flaxseed [10]. The presence of a vitamin $B_6$ antagonist in flaxseed was confirmed when Klosterman and his colleagues (1967) isolated and characterized linatine and its active form 1ADP [11,12]. His research team estimated the amount of linatine present in flaxseed as 100 μg/g . At that time, they used chemical analyses and an amino acid analyzer for its characterization and quantification [11]. We have re-examined the methodology with the intent of devising an improved procedure for the

extraction, identification, and quantification of vitamin $B_6$ antagonists from flaxseed. The use of ultrasound-assisted solvent extraction improved the release of matrix-bound bioactive molecules, leading to better extraction efficiency, as has been shown for other compounds [13,14]. Response surface methodology yielded an algorithm for the optimization of extraction parameters with respect to maximum yield of 1ADP. Additionally, ultra-high performance liquid chromatography/mass spectrometry (UPLC/MS) led to the measurement of samples with higher sensitivity when characterizing and quantifying the anti-pyridoxine factors extracted from flaxseed. To the best of our knowledge, flax is the only source of naturally occurring 1ADP, and our results led to the first demonstration of the presence of free 1ADP in flaxseed, where Klosterman and his colleagues could not detect this compound using the methods available at the time. A varietal analysis revealed that the total anti-pyridoxine (1ADPE) found in flaxseed was 177-437μg/g of whole seed, which is much higher than the value mentioned during previous work in the 1960's. However, the establishment of such improved methods may help in the more efficient extraction, isolation, characterization, and accurate quantification of the vitamin $B_6$ antagonists, particularly linatine and 1ADP, in other food/plant samples.

The *in vivo* studies established to investigate the metabolic insults of anti-pyridoxines linked to flaxseed were conducted using a rodent model of moderate vitamin $B_6$ deficiency. We first developed the rodent model to simulate conditions relevant to those individuals having moderate $B_6$ status in the general population. Moderate vitamin $B_6$ deficiency, which we define by low circulating $B_6$ vitamers yet sufficient status to maintain basic biological and physiochemical functions, was achieved when rats were fed with 0.7 mg/kg diet of pyridoxine.hydrochloride. In human clinical settings, blood PLP concentrations of 20-40

nmoles/L are also considered reflective of a moderate/marginal vitamin $B_6$ deficiency (status) [15,16].

One of the key biomarkers, which is affected directly by inadequate vitamin $B_6$ intake as well as anti-pyridoxine toxicity, is endogenous PLP [7,17-19]. Low intake of pyridoxine directly reflects the concentration of endogenous $B_6$ vitamers [4]. In addition to this, we have shown that the oral consumption of anti-pyridoxine factors, particularly 1ADP, lowered free *in vivo* $B_6$ vitamers as a result of potential hydrazone toxicity to the cells. The reduction of freely available vitamin $B_6$ might be due to the utilization of $B_6$ vitamers to form hydrazone complexes. The latter may result due to the trapping of the carbonyl group of $B_6$ vitamers (PL and PLP) by the hydrazide group of 1ADP [20]. Additionally, the possibility exists regarding the inhibition of the activities of PLK and PNOx, the enzymes that are responsible for the synthesis and inter-conversion of PLP *in vivo,* due to anti-pyridoxines or the associated hydrazone complexes. Inhibition of these enzymes might have further reduced the *in vivo* PLP pool to cause $B_6$ deficiency.

Furthermore, the toxicity developed to the cells after the administration of anti-pyridoxine agents may also be due to the inhibition of the activities of several PLP-dependent enzymes linked to carbohydrate, amino acid, and fatty acid metabolism. For instance, the intraperitoneal injection of 1-aminoproline (mixture of 1-amino D-proline and 1-amino L-proline), at a dose of 15-50 mg/kg body weight, in vitamin $B_6$ deficient-rats significantly disturbed *in vivo* amino acid profiles, and led to convulsions [7]. In a similar study, the activity of glutamate decarboxylase was significantly reduced, and severe convulsions were prevalent when Massieu and his colleagues (1994) injected 80 mg/kg of hydrazone of pyridoxal phosphate intraperitoneally [21]. This indicates that hydrazones of $B_6$

vitamers are likely responsible for the inhibition of the activities of these enzymes. Our data also exhibited a similar inhibition ($P < 0.001$) of activities of PLP-depended enzymes, including CBS and CGL, after the oral exposure to 1ADP, irrespective of the source (either synthetic or natural). Impairments in the functions of these enzymes led to the defects in the biosynthesis of certain key amino acids linked to methionine metabolism including Hcy, CTH, and α-ketobutyric acid during moderate $B_6$ deficiency status. This finding is supported by the results of earlier study which stated an accumulation of large quantity of CTH during intraperitoneal injection of 1-aminoproline in rats [6]. Surprisingly, our data on plasma Cys concentrations were not consistent between the two *in vivo* studies. We found that feeding of 10 mg/kg diet in the second *in vivo* study (chapter 6) significantly reduced plasma Cys concentrations compared to the control; whereas, there was no effect in our earlier study (chapter 5). The reason for this discrepancy is not entirely clear. We did see an increase in homogeneity due to a decrease in the standard error (SE) for the second study (n = 8) compared to the previous one (n = 6), which may have provided more power for the detection of significant differences. Increased levels of Hcy and CTH are also associated with a defect in the functioning of the SHMT enzyme of the remethylation pathway of methionine cycle [22,23]. In this study, plasma glycine concentration was increased and serine concentration was decreased as a result of a potential reduction in the activity of SHMT enzyme as well as impairments in glycine-serine cleavage system due to 1ADP exposure. Subsequently, production of 1-carbon unit ($-CH_2-$) might have been reduced, thus hampering the biosynthesis and metabolism of macromolecules, including nucleic acids and xenobiotic compounds [24]. Additionally, an elevated level of Hcy causes an imbalance in the SAH/SAM ratio which ultimately impairs the transmethylation pathway of methionine

cycle [25]. These biochemical insults led to the perturbation of various hepatic functions via promoting oxidative stress, hypomethylation of nucleic acids, fibrosis and steatosis [26,27]. A significant accumulation of fatty acids, as well as the presence of dilated central veins and sinusoids were observed in the livers of rats consuming synthetic 1ADP (10 mg/kg diet) along with low $B_6$ diet in the current study. The latter is found to be consistent with acute hepatic injury.

Responses to vitamin $B_6$ markers and/or toxicity to the cells as a result of exposure to a diet containing low vitamin $B_6$ or anti-pyridoxine agents can exhibit variability, likely due to the severity and duration of exposure [7,28]. Interestingly, as indicated above, we found evidence that flaxseed-derived 1ADP impaired certain $B_6$ markers in moderate $B_6$-deficient rats, when feeding at a dose of 0.5 mg 1ADPE/kg body weight per day. Besides, some researchers suggested a serving size of 25-30 g/day flaxseed to prevent the development of many metabolic diseases, including diabetes, hypertension ,and other cardiovascular diseases [29-31], and that will result in the intake of 0.15 mg 1ADPE/kg body weight per day (assuming a body weight of 65 kg), a concentration comparable with that of the current study. Therefore, our data serve to highlight potential detrimental effects which might be experienced by a population presenting moderate $B_6$ deficiency as a result of nutritional co-insult of vitamin $B_6$ antagonist present in flaxseed.

The biochemical insults created due to the administration of anti-pyridoxine agents led to the development of classic vitamin $B_6$ deficiency symptoms, including reductions in feed intake and growth performance in rats [32,33]. Similar observations were also found in rats receiving synthetic 1ADP at a dose of 10 mg/kg diet in the moderate $B_6$ deficiency group of the current study. However, the rats receiving optimum $B_6$ were able to withstand 1ADP

toxicity. This might be due to the presence of surplus PLP to neutralize 1ADP toxicity by forming hydrazone complexes in rats of optimum $B_6$ group, which is not the case for rats deficient in $B_6$. This theory is also supported by the evidence that pyridoxine application prior to the injection of 1ADP prevented 1ADP-induced seizures and convulsions in rats [7]. The severity of impairments on biochemical and metabolic insults on rats due to flaxseed extract was less pronounced compared to that of synthetic 1ADP. The difference in the potency of naturally occurring 1ADP (linatine) and synthetic 1ADP might be due to relative differences in their bioavailability and metabolism. Naturally occurring bioactive compounds normally have low liberation, absorption, transportation and metabolism compared to synthetic drugs, leading ultimately to a reduction in their bioavailability [34]. The *in vivo* toxicity of the dipeptide linatine is directly related to the release free 1ADP, the potent and active anti-pyridoxine component, in gastrointestinal tract. Incomplete metabolism of the same might also be responsible for the lowered toxicity of linatine present in flaxseed extract compared to the synthetic 1ADP. Nevertheless, flaxseed-derived 1ADP did demonstrate an ability to perturb markers of amino acid and vitamin $B_6$ metabolism in the current study.

To understand better the pathways affected by 1ADP, we further investigated the plasma samples of the selected treatments of the *in vivo* study using a non-targeted metabolomics approach. Metabolomics capitalizes on the identification of novel biomarkers and elucidation of biochemical pathways to improve diagnosis, progression and therapy. An ideal biomarker assists early identification of disease development with higher sensitivity and specificity [35]. The goal of nutritional metabolomics, on other hand, is to understand what happens to the entire metabolome with changes in diet [36]. In the current study, we separated and detected lipophilic plasma metabolites by using liquid chromatography-

quadrupole time of flight/mass spectrometry (LC-Qtof/MS). Out of more than 2500 detected entities; 237 entities were significantly different as a result of oral ingestion of synthetic 1ADP compared to the control. We had identified 10 metabolites of interest based on their specific mass spectrum against a database of authentic standards. The metabolites identified included glycocholic acid, glycoursodeoxycholic acid, murocholic acid, N-docosahexaenoyl GABA, N-arachidonoyl GABA, lumula, nandrolone and orthothymotinic acid. These compounds are linked with the biosynthesis and metabolism of bile acid components, N-acyl amino acids, analgenic androgens, anti-inflammatory and neuroprotective molecules. These results obtained through the global screening of lipophilic novel metabolites indicated a potential defect in fatty acid metabolism due to the feeding of 1ADP concurrent with vitamin $B_6$ deficiency. Non-targeted metabolomics screens potential metabolites and pathways affected by a treatment or a disease in a much wider range, which tend to be overlooked with targeted approaches. The current study also provided the first *in vivo* metabolomics data of 1ADP toxicity. It also provided more data on the involvement of this water soluble anti-pyridoxine linked to flaxseed in hampering fatty acid metabolism.

**8.2. Strengths of the current research project**

In the current research project, we utilized advanced methods for extraction, optimization, characterization, and quantification of anti-pyridoxine agents present in flaxseed. For instance, the anti-pyridoxines present in the samples were extracted more efficiently through the use of an ultrasound-assisted extraction method, after optimizing the extraction parameters by using response surface methodology (RSM). Quantification of total anti-pyridoxine content using a UPLC/MS in multiple reaction monitoring (MRM) mode contributed to strong confidence in the method we developed. Additionally, varietal

screening of total anti-pyridoxine content helped us to gather more data on effect of genetic and environment on the biosynthesis of vitamin $B_6$ antagonist, particularly linatine in flax plant.

Moderate vitamin $B_6$ deficiency is reasonably well defined in humans; however, it is not the case in animals. To this end, the development of a rat model of moderate $B_6$ deficiency, to mimic a subset of the human population of interest, provided an advantage while performing *in vivo* studies. The amount of anti-pyridoxine added in each treatment in the current study was calculated based on the potential amount of vitamin $B_6$ antagonist that may present in a serving size of flaxseed, suggested by some [29-31]. To our knowledge, this is the only study which has investigated the effect of this anti-pyridoxine on hepatic CBS and CGL enzyme activities. Therefore, our data can be used for dose comparison and risk assessment exercise for future studies. Furthermore, metabolomics approach widens the area of a research project. In the current study, it provided evidence of the effects of vitamin $B_6$ inadequacy as well as anti-pyridoxine on other potential pathways besides the classical ones, including amino acid metabolism. The use of LC-Qtof/MS, one of the most advanced equipment for identifying low molecular weight metabolites, coupled with Metlin (> 64,000 metabolites) and SimLipids (> 39,000 lipids) database, also provided a strong confidence over identification of novel biomarkers.

**8.3. Limitations and future directions**

As the general population consumes whole flaxseed rather than flaxseed extracts, the use of a flaxseed extract and synthetic 1ADP might not truly represent the effects of normal flaxseed consumption. Whole flaxseed has a more complex matrix than flaxseed extract and synthetic 1ADP; and thus, their potency differs. Therefore, consumption of flaxseed as a

whole might have a different story in terms of 1ADP toxicity compared to feeding of synthetic 1ADP or flaxseed extract.

Vitamin $B_6$ antagonist caused toxicity to the cell most probably due to hydrazone formation, but, our *in vivo* studies could not demonstrate the exact mechanism of action. We also could not provide a long term effect of this anti-pyridoxine linked to flaxseed. Moreover, we used a rat model to represent moderate $B_6$ deficiency in humans; thus, the potential toxic effects of this anti-pyridoxine need to be evaluated using human clinical trials.

Nevertheless, the current short term research experiments provided data on potential impairments of PLP-dependent enzymes due to consumption of 1ADP derived from synthetic as well as flaxseed extract. Therefore, a long term study using the current model might be required to strengthen our data before moving to clinical trials. *In vitro* studies might also be conducted to better understand the mechanisms of action of 1ADP related to human metabolism. Further investigations on potential impact of this anti-pyridoxine on fatty acids and one-carbon metabolism as well as neurotransmitter biosynthesis might be promising steps to see its effect on other metabolic pathways. Ultimately, a human clinical trial will provide crucial data on the *in vivo* toxicity effect of this anti-pyridoxine factor linked to flaxseed.

**8.4. Final conclusion**

Vitamin $B_6$, as PLP, plays a crucial role as a cofactor in numerous enzymes linked to carbohydrate, lipids, and amino acid metabolism. Inadequate vitamin $B_6$ intake causes impairments in the proper functioning of those PLP-depended enzymes, and this condition is associated with the development of many common diseases such as diabetes, cardiovascular and other metabolic defects [17]. There is a high prevalence of moderate vitamin $B_6$

deficiency in the general population, particularly in developed countries [4]and endogenous free PLP pool is further exacerbated by the application of anti-pyridoxine components present in common foods including linatine and 1ADP from flaxseed. Feeding of anti-pyridoxine factor linked to flaxseed had shown to impair PLP-dependent enzymes, and the deleterious effects were found to be more sensitive to low vitamin $B_6$ status. One of the major pathways which were impaired by oral ingestion of anti-pyridoxines was transsulphuration reactions of methionine metabolism. Defects in this pathway cause imbalances in endogenous thiol concentrations. As a result, the production of xenobiotic detoxicants and one carbon metabolism were hampered, subsequently leading to liver injury. A non-targeted metabolomics study also provided evidences to support a potential deleterious effect of 1ADP on fatty acid metabolism. Interestingly, these deleterious effects of 1ADP might be prevented by the application of adequate vitamin $B_6$. Further studies are required to confirm the potential toxicity effect of this anti-pyridoxine linked to flaxseed on humans.

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# CHAPTER 9

# APPENDIX

## 9.1. Supplementary materials

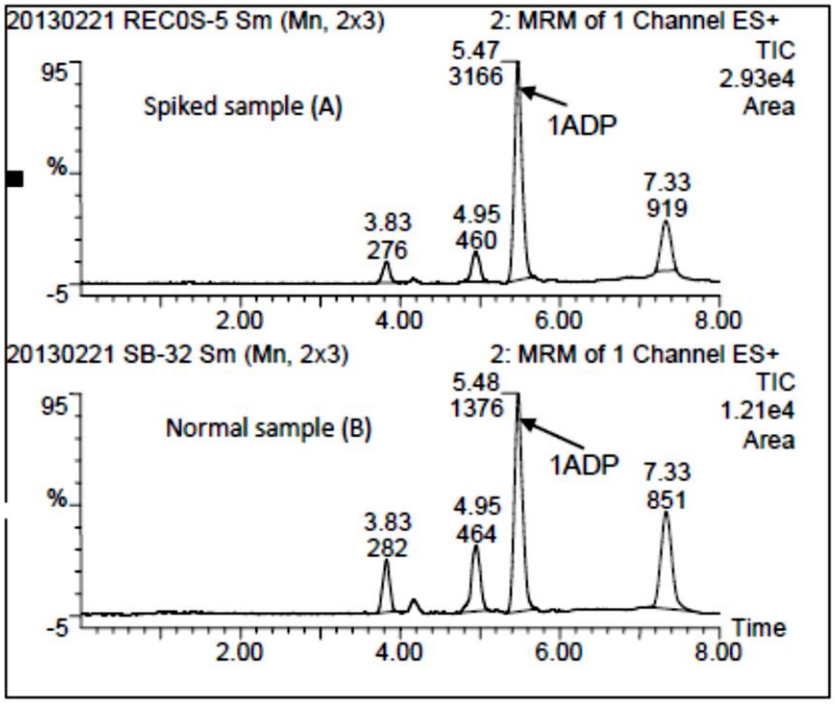


Figure S1. Chromatogram of 1ADP in spiked sample (A) and normal sample (B).

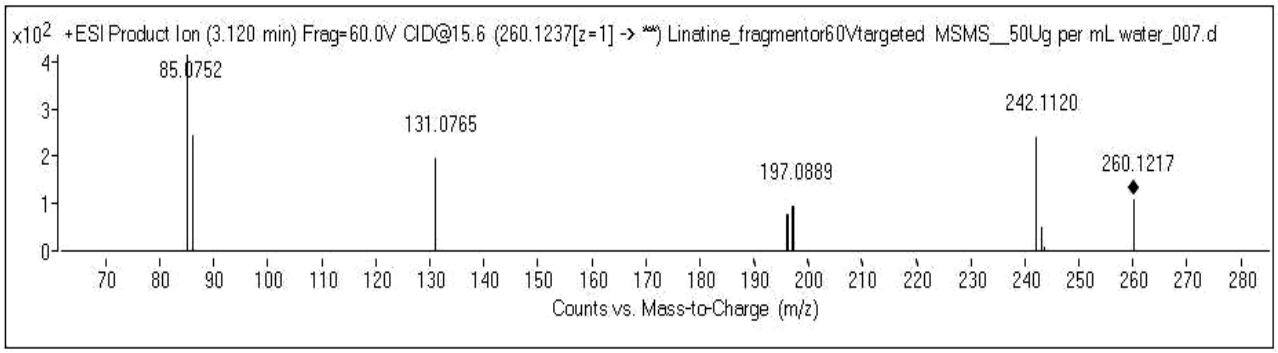


Figure S2. Q-TOF mass spectrum of linatine (Purified fraction): Parent ion at m/z 260.1217, $[M+H]^+$

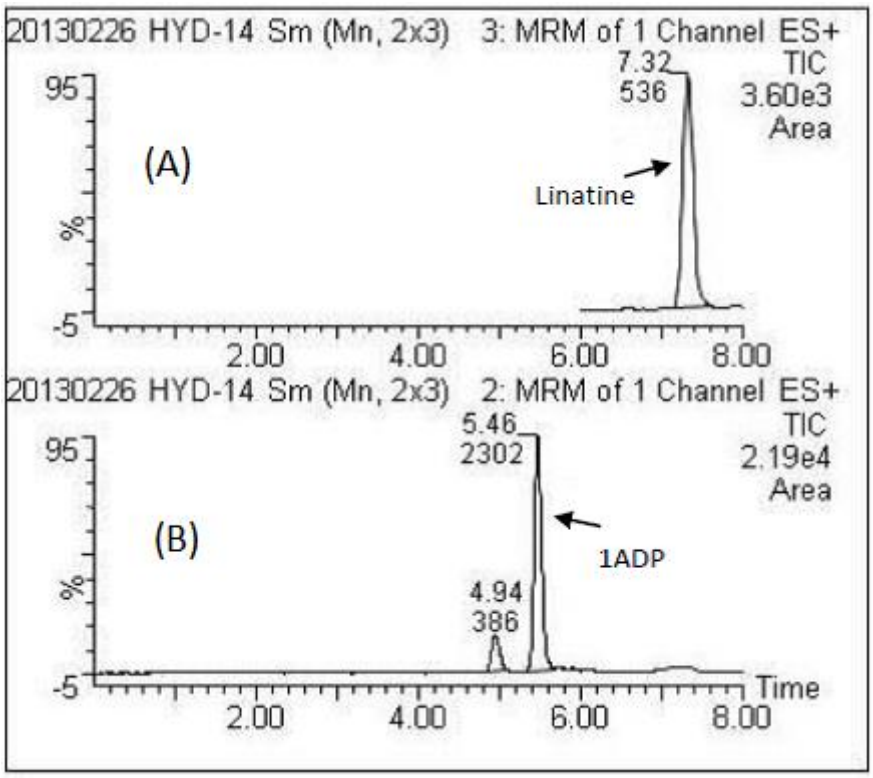


Figure S3. Chromatogram of linatine fraction before (A) and after (B) hydrolysis.

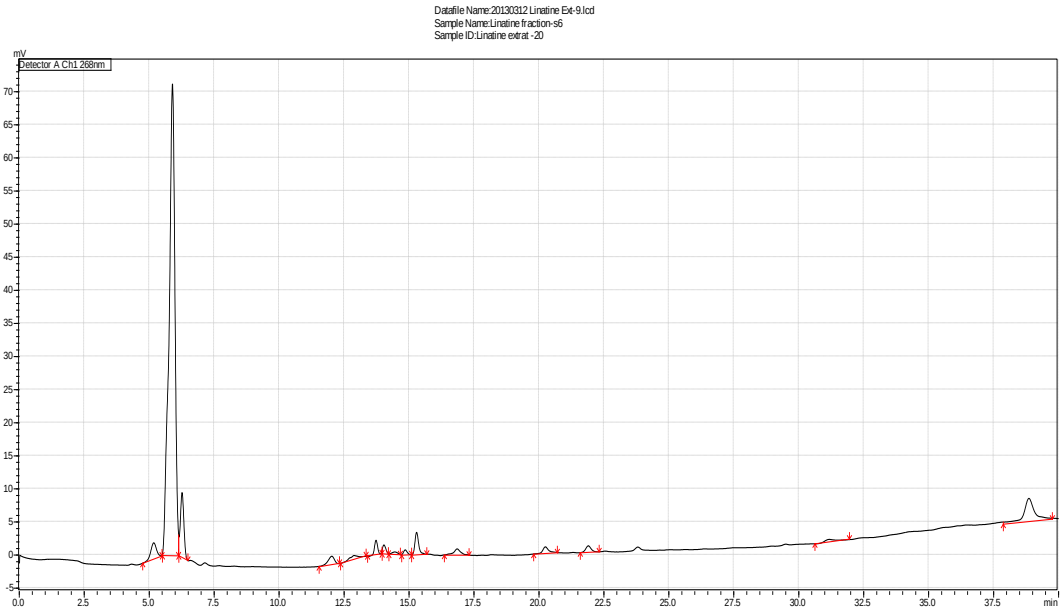


Figure S4. Chromatogram showing purity of linatine extract using HPLC/UV detector

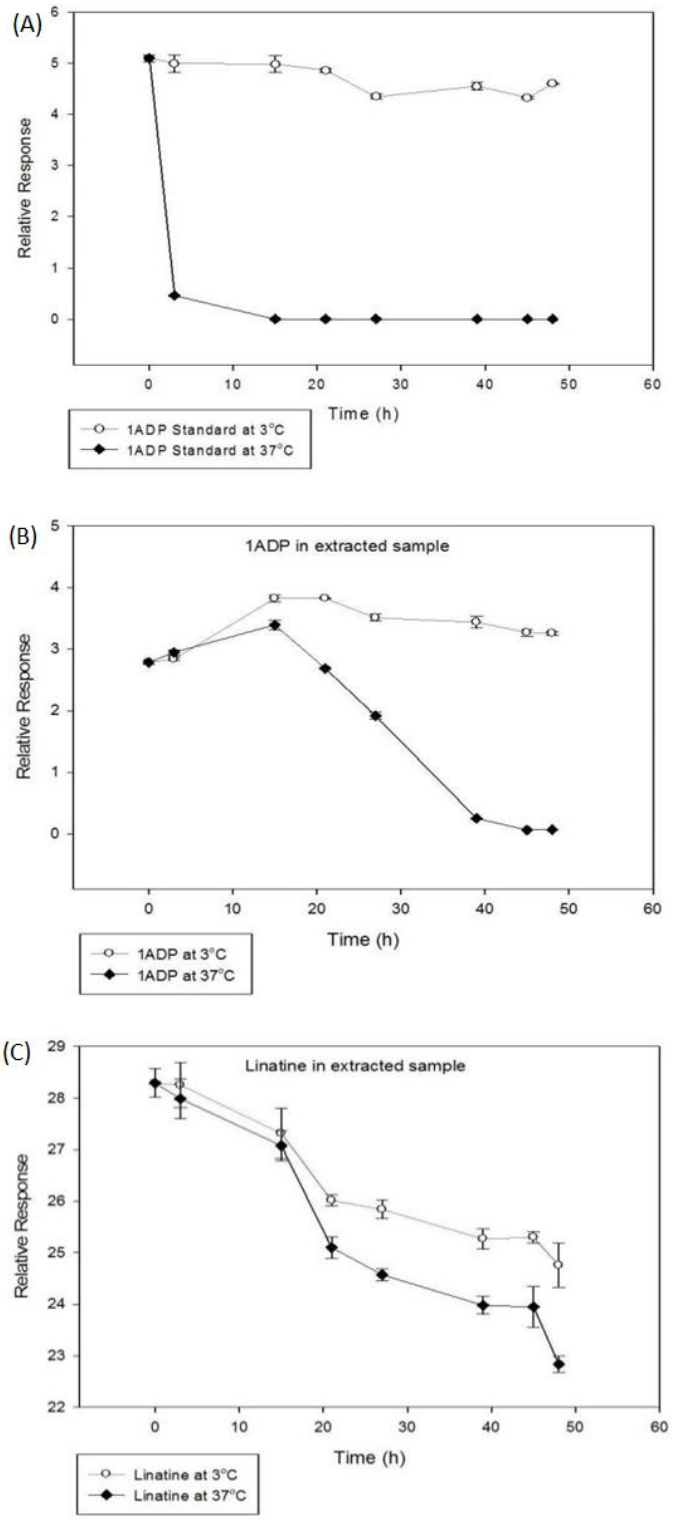


Figure S5. Stability of 1ADP and linatine during storage at 3 and 37°C.

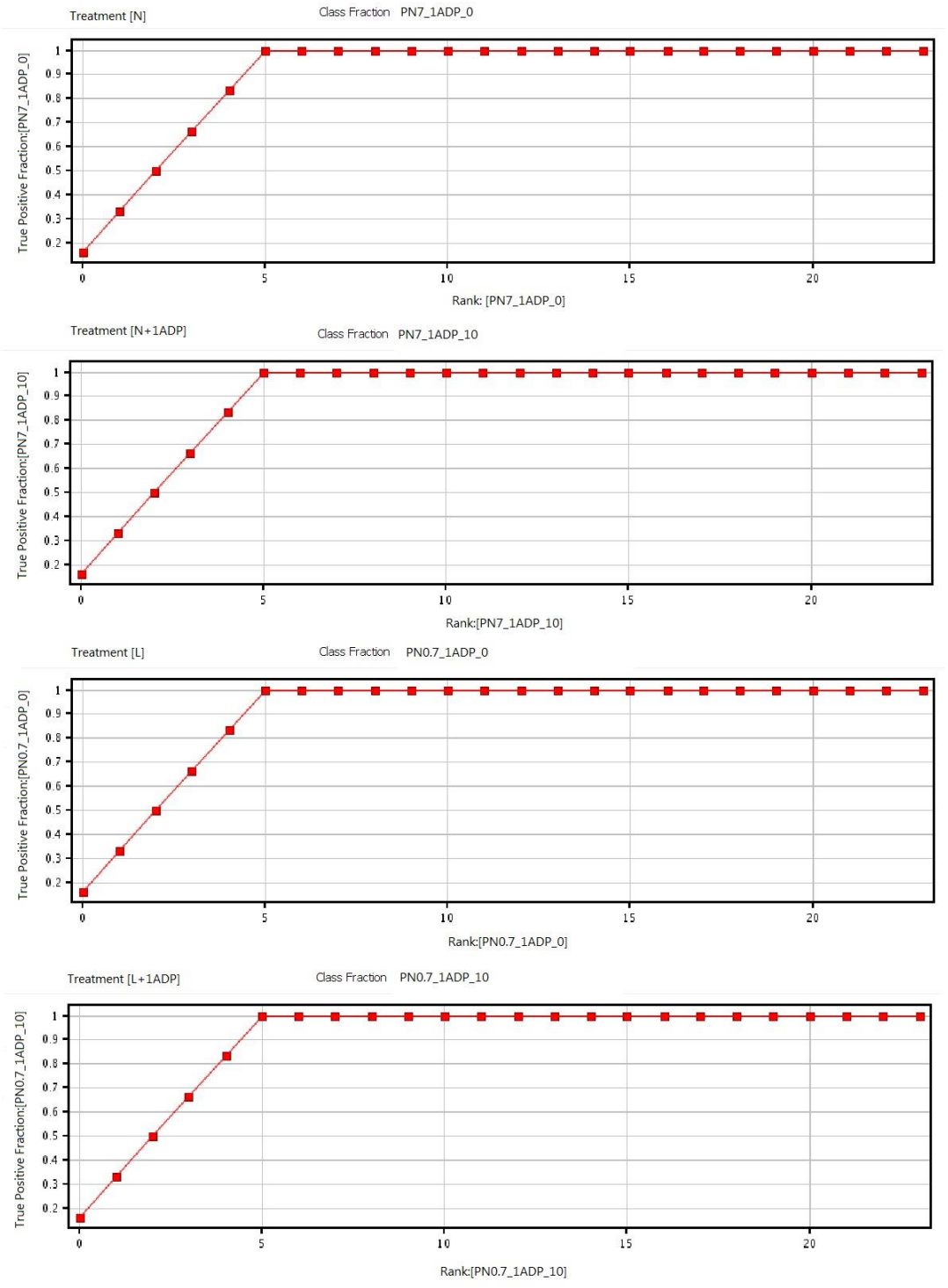


Figure S6. Lorenz curves of each treatment

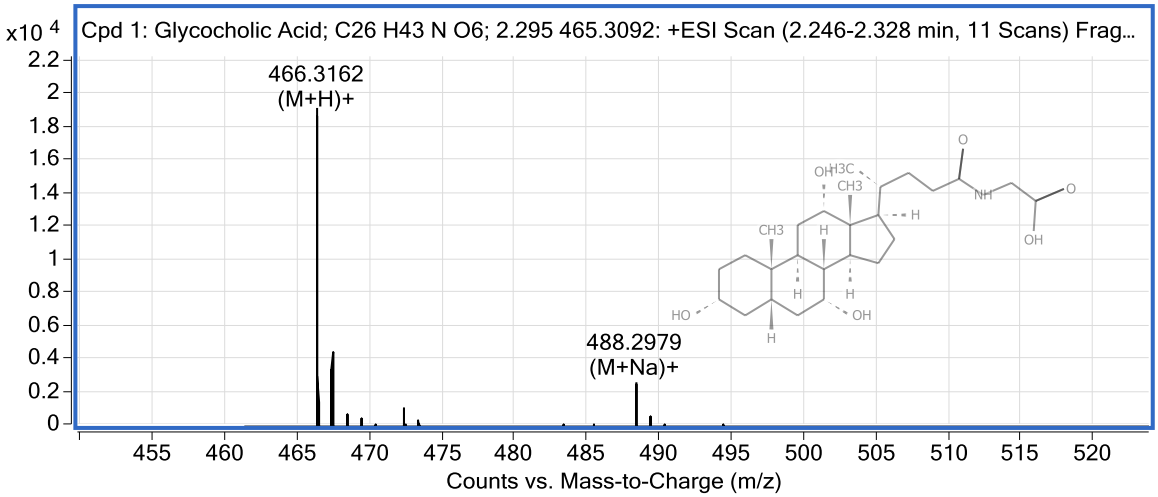


Figure S7. Mass spectrum of Glycocholic acid (*m/z* 466.3162)

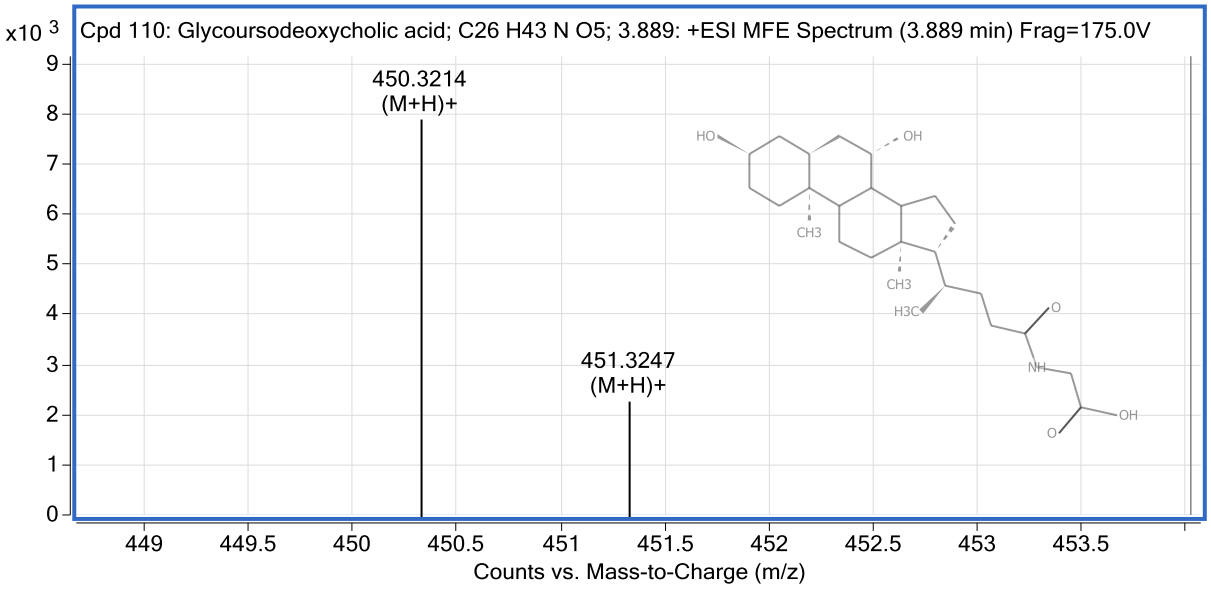


Figure S8. Mass spectrum of Glycoursodeoxycholic acid (*m/z* 450.3214)

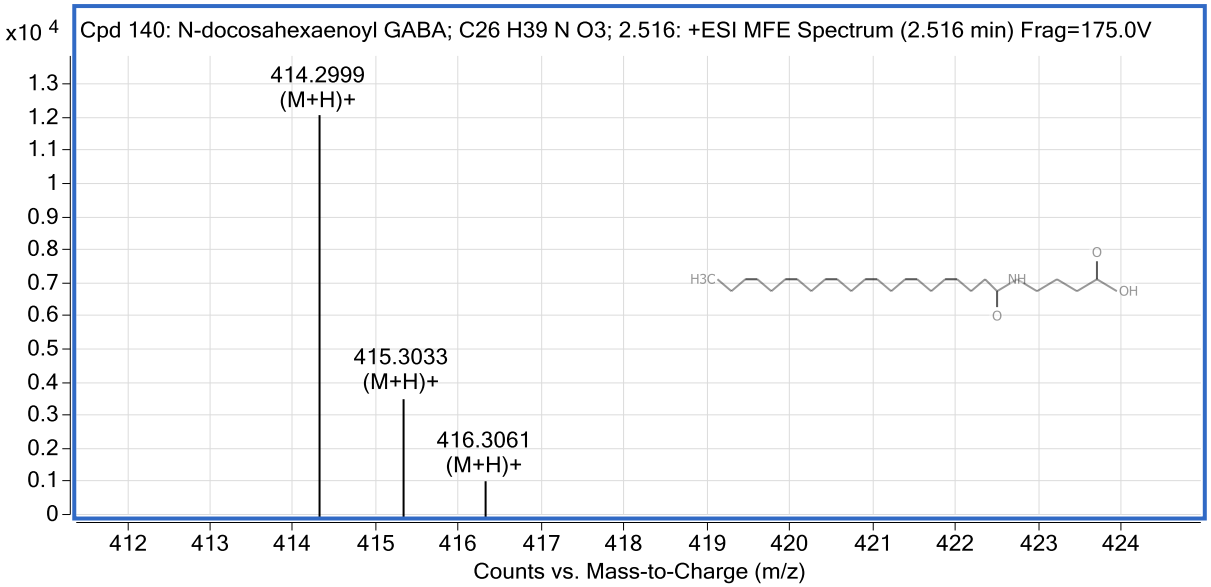


Figure S9. Mass spectrum of N-docosahexaenoyl GABA (*m/z* 414.2999)

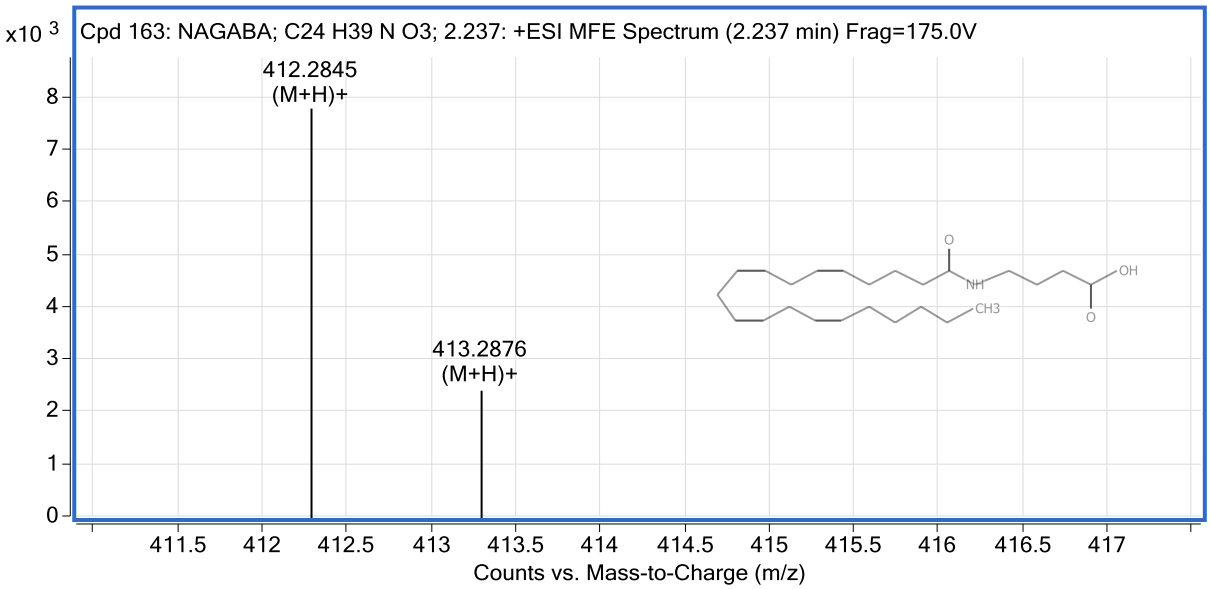


Figure S10. Mass spectrum of N-arachidonoyl GABA (NAGABA)

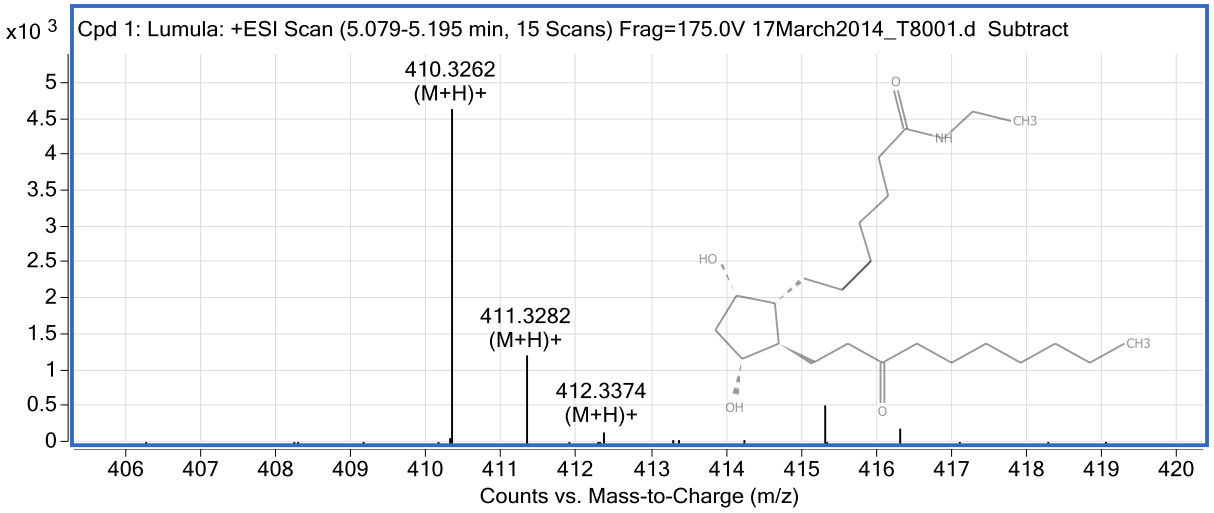


Figure S11. Mass spectrum of Lumula (*m/z* 410.3262)

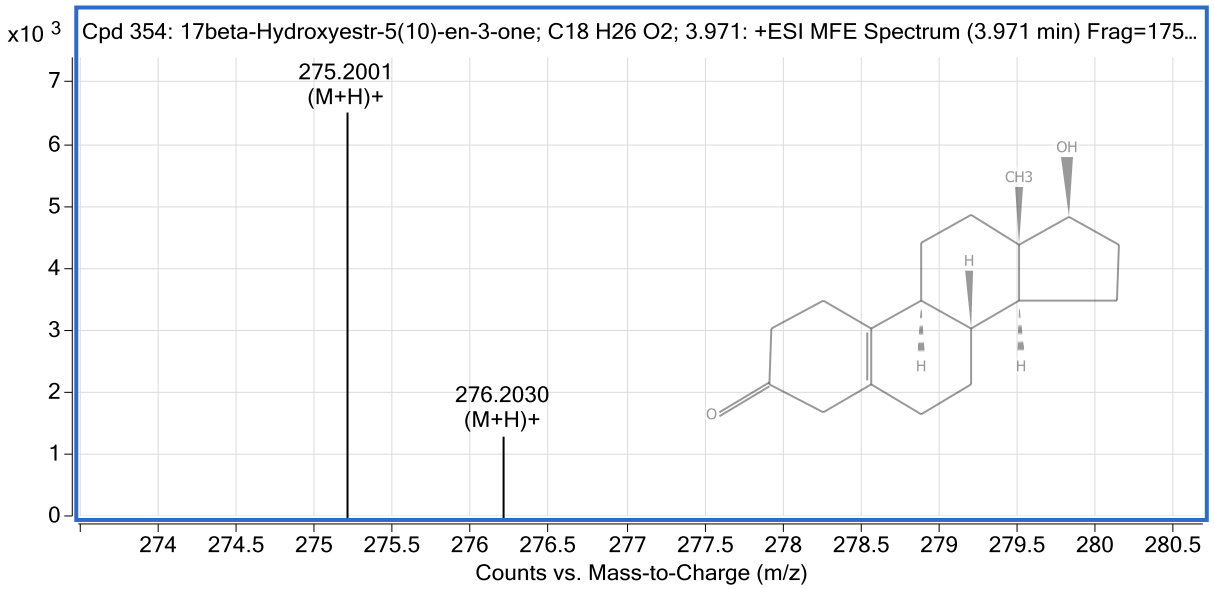


Figure S12. Mass spectrum of Nandrolone (*m/z* 275.2001)

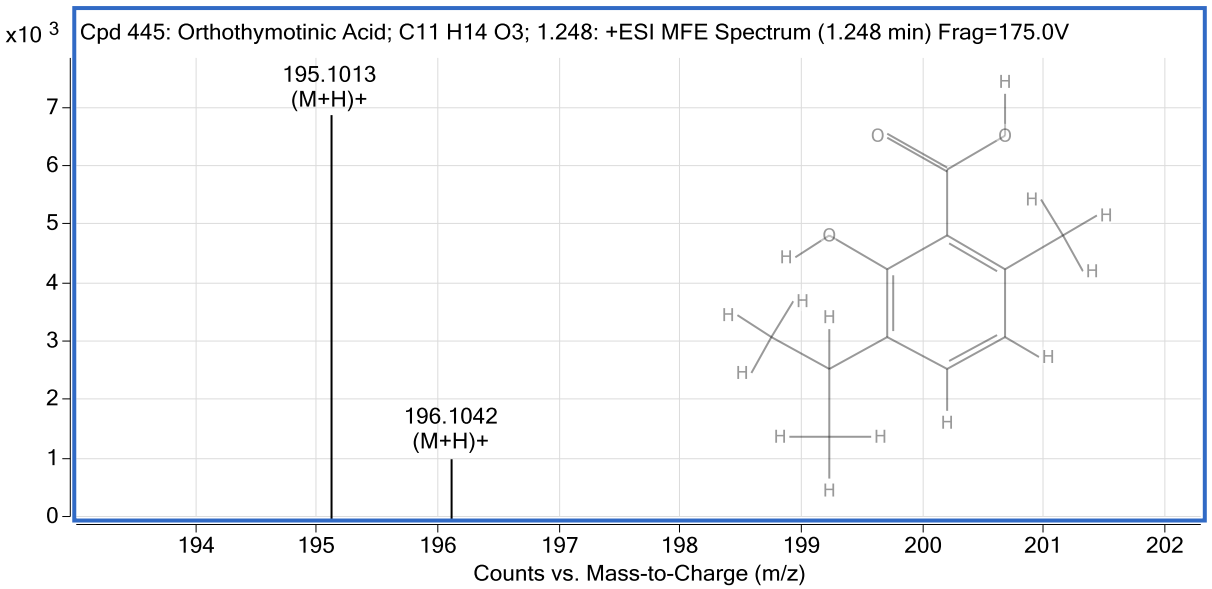


Figure S13. Mass spectrum of Orthothymotinic acid (*m/z* 195.1013)

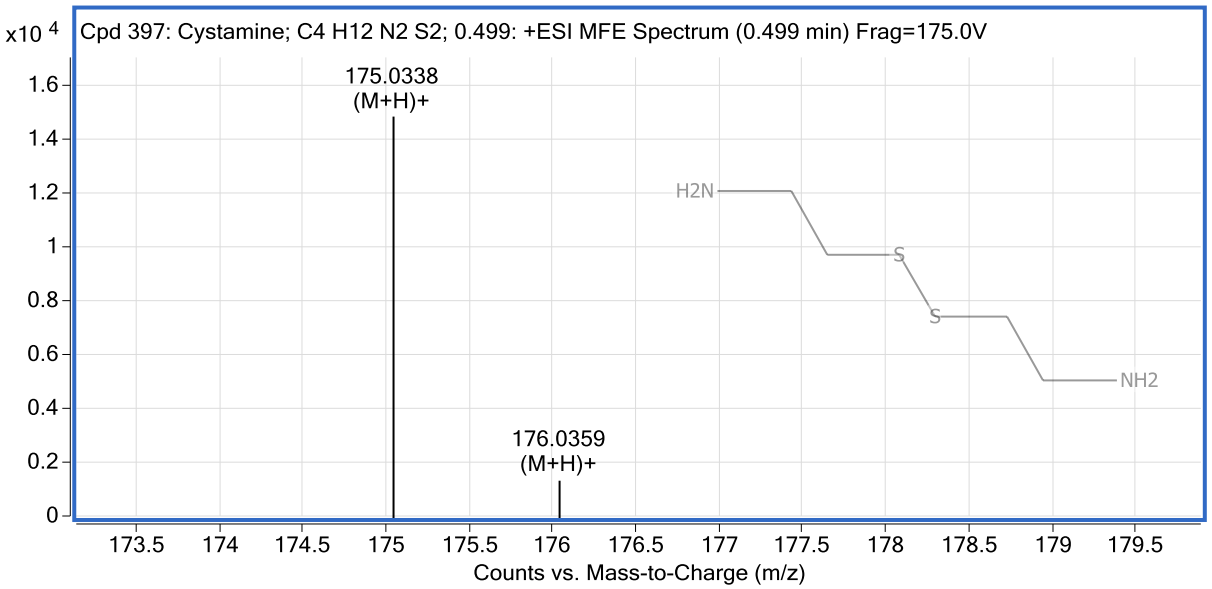


Figure S14. Mass spectrum of Cystamine (*m/z* 175.0338)

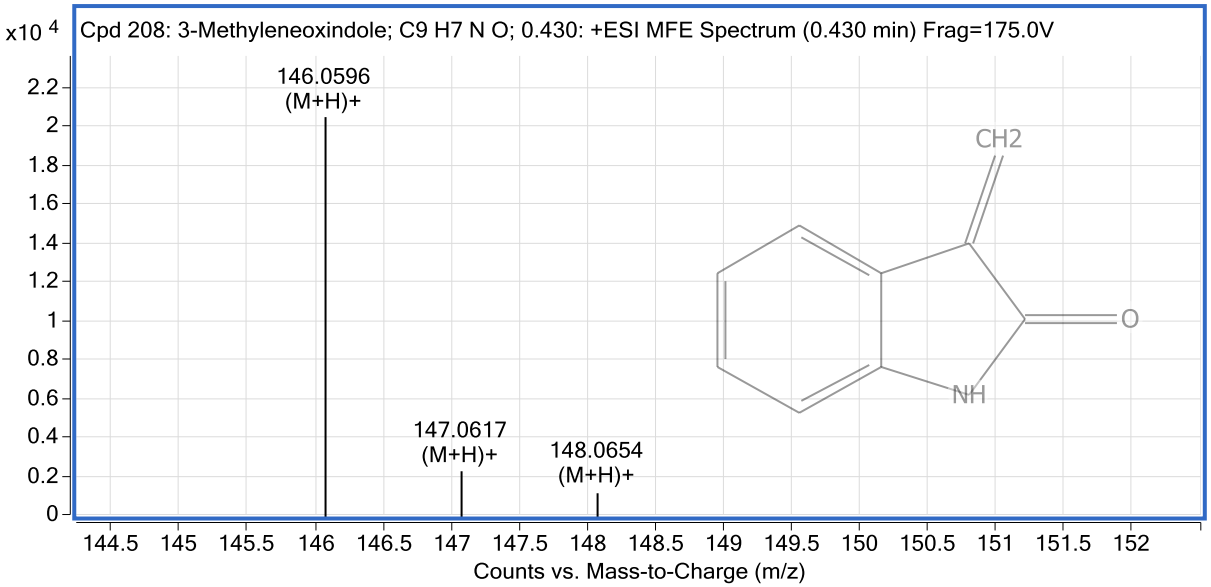


Figure S15. Mass spectrum of 3-Methyleneoxindole (*m/z* 146.0596)