AMERICAN UNIVERSITY OF BEIRUT

ACUTE EFFECT OF PHOSPHORUS INGESTION ON POSTPRANDIAL PROTEIN SYNTHESIS IN RATS

by
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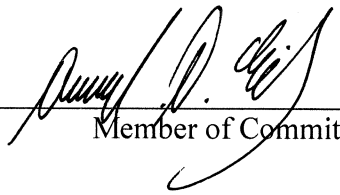

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AN ABSTRACT OF THE THESIS OF

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Title: Acute Effect of Phosphorus Ingestion on Postprandial Protein Synthesis in Rats

Phosphorus is vital to energy production as it provides the phosphate in adenosine triphosphate (ATP). Protein synthesis is a high energy demanding process. Therefore, phosphorus is important for protein synthesis required for the growth, maintenance, and repair of all tissues and cells. The objective of this study was to investigate the acute effect of phosphorus in meal on *in vivo* postprandial protein synthesis in rats. Forty male Sprague-Dawley rats weighing around 230-270 grams each were randomly divided into five groups as follows: a control group (fasting), two phosphorus-free groups (one with rats sacrificed 2 hours after food ingestion and the other 4 hours after) and two phosphorus adequate groups (one with rats sacrificed 2 hours after food ingestion and the other 4 hours after). *In vivo* fractional protein synthesis in plasma and liver was measured by an intraperitoneal injection of a flooding dose of L-phenylalanine containing L-[ring-$^{2}H_5$] Phenylalanine as a stable isotope tracer (1.5 mmol/kg body weight). The protein synthesis rates in the different groups did not differ significantly at p-value < 0.05, although the rats, fed a phosphorus containing diet 2 hours before their sacrifice, had an overall higher mean protein synthesis rates as compared to rats fed a phosphorus free diet, but the latter failed to reach statistical significance.

Keywords: ATP, protein synthesis, phosphorus, L-phenylalanine.

CONTENTS

LIST OF ABBREVIATIONS

25(OH)D 1α-hydroxylase	25-Hydroxyvitamin D 3alpha-hydroxylase
2,3-DPG	2,3-diphosphoglycerate
X	Multiply
%	Per Cent
°C	Degree Celsius
&	And
/	Per
=	Equal To
ATP	Adenosine Triphosphate
AUB	American University of Beirut
BW	Body Weight
CHO	Carbohydrates
CKD	Chronic Kidney Failure
d	Day
dl	Deciliter
ECF	Extracellular Fluid
EDTA	Ethylenediaminetetraacetic Acid
EAA	Essential Amino Acid
FGF-23	Fibroblastic Growth Factor-23
FGF-7	Fibroblastic Growth Factor-7
FSR	Fractional Protein Synthesis Rate
g	Gram
GCMS	Gas Chromatography-Mass Spectrometry
HCl	Hydrogen Chloride
H_2PO_4	Dihydrogen Phosphate
HPO_4	Mono-hydrogen Phosphate
Hr	Hour

i.p.	Intra Peritoneal
Kcal	Kilocalorie
Kg	Kilogram
L	Liter
m	Meter
MEPE	Matrix Extracellular Phosphoglycoprotein
ml	Milliliter
mm	Millimeter
Mol%	Percent Molar Enrichment
mmol	Millimole
NaCl	Sodium Chloride
NaPi	Sodium Phosphorus
NaOH	Sodium Hydroxide
NH_3	Ammonia
PTH	Parathyroid Hormone
RDA	Recommended Dietary Allowance
RT	Retention Time
SD	Standard Deviation
sFRP-4	Secreted Frizzled Related Protein-4
TCA	Trichloroacetic Acid
tRNA	Transfer Ribonucleic acid

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CHAPTER ONE
INTRODUCTION

Nowadays, phosphorus has gained a lot of interest due to its number of properties. It is the second most abundant mineral in the body, after calcium. Its recommended daily allowance (RDA) is set at 700mg/d. Phosphorus is found in milk products, meats, and legumes. However, phosphorus in meat product is much more bioavailable as compared to legumes, which contain the mineral primarily in the form of phytate that is not easily hydrolyzed in the body. In fact, the enzyme phytase required for the breakdown of phytate is absent in humans. Phosphorus is required for many biological processes such as bone remodeling, cell structure, and energy metabolism. It is the main component of the energy carrying molecule, adenosine triphosphate (ATP) . The latter is involved in the metabolism of fats, carbohydrates, and proteins as they are high energy demanding processes. Among them, protein metabolism seems to have the highest demand of ATP [1]. Consequently, factors that contribute to the reduction in ATP formation have shown to negatively impact protein synthesis [2-4]. In addition, phosphate, being a major component of ATP, plays a crucial role in the formation of ATP. As a matter of fact, hypophosphatemia has been linked with impaired ATP synthesis [5]. Consequently, reductions in phosphorus levels in the body can result in impaired protein synthesis by inhibiting ATP formation. Furthermore, increase in phosphorus ingestion has been linked with improvements in insulin sensitivity [6, 7]. Insulin is known to stimulate protein synthesis. Thus, another way

that phosphorus might affect protein synthesis rates is through its capacity to increase insulin sensitivity [8, 9].

Therefore, the objective of this study is to investigate the acute effect of phosphorus in meal on *in vivo* postprandial protein synthesis in rats.

CHAPTER TWO
LITERATURE REVIEW

A. Phosphorus Overview

1. Distribution

Phosphorus is the second most abundant mineral in the body, after calcium, making up about 1% of total body weight. This means that the total phosphorus in a healthy adult weighing 60kg should be around 600-700g. About 85% of the body's phosphorus is in the skeleton in hydroxyapatite phase, 14% in soft tissues, and the remaining 1% in blood and body fluids. Phosphorus is found, in majority, in the intracellular fluid with an intracellular to extracellular ratio equivalent to 100:1. It is present in every cell [10]. Phosphorus is primarily present in the body in the form of organic phosphate as a complex with carbohydrates, lipids, and proteins. In the serum, about 85% of the phosphate is in the free form and 15% protein bound. The two major forms of inorganic phosphate in the serum are dihydrogen phosphate (H_2PO_4) and mono-hydrogen phosphate (HPO_4). The ratio of HPO_4 to H_2PO_4 is 4:1 and plays a role in the maintenance of proper acid-base balance. The average serum phosphate concentration in adults ranges from 2.5 to 4.5 mg/dl. However, children, who require more phosphorus for bone remodeling, have the highest value ranging from 4.5 to 8.3 mg/dl. Serum phosphorus concentration fluctuates throughout the day and reaches its lowest point early in the morning between 8 and 11:00am due to diurnal variations [11-13].

2. Phosphorus Requirements, Intake, and Absorption

The RDA of phosphorus is 700 mg per day. Current daily intake of phosphorus ranges from 1.0g/d to 1.5g/d [11]. Based on the RDA, this value is high. As a matter of fact, the current RDA is based on the lower end of the normal serum phosphorus concentration range. However, if it were based on the middle value, it would have been 2.1g per day; and thus, the current daily intake would have been considered low. Phosphorus is present in a variety of food such as meat, legumes, and dairy products. Proteins are rich in phosphorus. In fact, one gram of protein contains about 13 to 15 mg of phosphorus. However, the bioavailability of phosphorus varies in the different sources. Plant proteins are not readily bioavailable as compared to animal proteins. The phosphorus in plants is in the form of phytate that requires phytase enzymes to break it down and release the phosphorus. Humans lack the enzyme phytase which explains the lower absorption. Absorption from meat is more than 70%. About 90% of the inorganic phosphorus in additives and preservatives is absorbed in the gastrointestinal tract. In addition, the presence of some dietary minerals, such as aluminum and calcium, are known to form complexes with phosphorus leading to their excretion through the feces. As such, an increase in these minerals in the diet would result in a decrease in phosphorus absorption. In fact, a calcium:phosphorus ratio that exceeds three reduces the absorption of phosphorus and can subsequently lead to a deficiency [13, 14].

3. Functions

Maintenance of phosphorus homeostasis is critical as phosphorus is involved in a number of biological processes among which bone mineralization, cell structure, nucleic acid metabolism, and energy production. In fact, phosphorus is vital for energy production as it provides the phosphate in adenosine triphosphate (ATP), energy-carrying molecule found in the cells of all living things. Consequently, phosphorus plays an important role in the utilization of carbohydrates and fats for energy production as well as in protein synthesis, being highly energy dependent processes. Protein synthesis is required for the growth, maintenance, and repair of all tissues and cells [12]. In addition, phosphate is required for the bone as part of the calcium phosphate hydroxyapatite. The bone mass ratio of calcium:phosphorus is 2.2 in the bone of adults. Any significant deviation from this ratio could negatively affect the bone health [15, 16]. Phosphorus is also required for the formation of 2,3-diphosphoglycerate (2,3-DPG) involved in the release of oxygen from hemoglobin. An increase of this molecule reduces the affinity of oxygen for hemoglobin allowing the oxygen to be delivered to the tissues [5, 17]. Phosphorus is involved in the activation of proteins by phosphorylation. It also contributes to the maintenance of acid-base balance by maintaining a proper ratio of HPO_4 to H_2PO_4 as mentioned earlier [11].

B. Phosphorus Homeostasis

1. Role of Organs

The major organs involved in phosphorus homeostasis are the intestine, bone, and kidney. Phosphorus absorption occurs all throughout the gastrointestinal tract, however, the jejunum is the major site of absorption. It occurs through two mechanisms: a saturable carrier-mediated active transport system dependent on sodium that is stimulated by the active form of Vitamin D_3, and a passive load-dependent diffusion. The intestinal absorption of phosphorus typically ranges between 55% to 80% and does not vary much across a wide range of intakes. For this reason, there seems to be no adaptive mechanisms of absorption under low intakes [5, 12]. The primary regulator of plasma phosphorus is the kidney at the level of the proximal tubules. The latter is the principal route of excretion of the mineral. The kidney regulates phosphorus levels mainly through the stimulation of hormones that will be discussed later. The role of the skeleton is also very important. In fact, the rate at which the bone is remodeled determines the concentration of phosphorus in the plasma. An increase in bone resorption would subsequently lead to an increase in serum phosphorus levels. This is due to the fact that about 85% of body's phosphorus is stored in the skeleton. Current daily phosphorus intake ranges between 1000mg to 1500mg/day which represents about 20mg/kg/d of phosphorus. Under normal conditions, about 16mg/kg/d is absorbed in the intestines. About 3mg/kg/d is excreted through the bile and intestinal secretions leading to a net excretion of 7mg/kg/d through the feces. Once in the extracellular fluid, about 3mg/kg/d of phosphorus moves in and out of the bone. And finally, the kidney, being the major site of excretion and reabsorption, will excrete about

13mg/kg/d via the proximal tubules. However, in the case of deficiency of this mineral, there will be a reduction in the amount excreted which is a good indicator of serum phosphorus levels. *Figure 1* summarizes the movement of phosphorus in the different organs [12, 18].

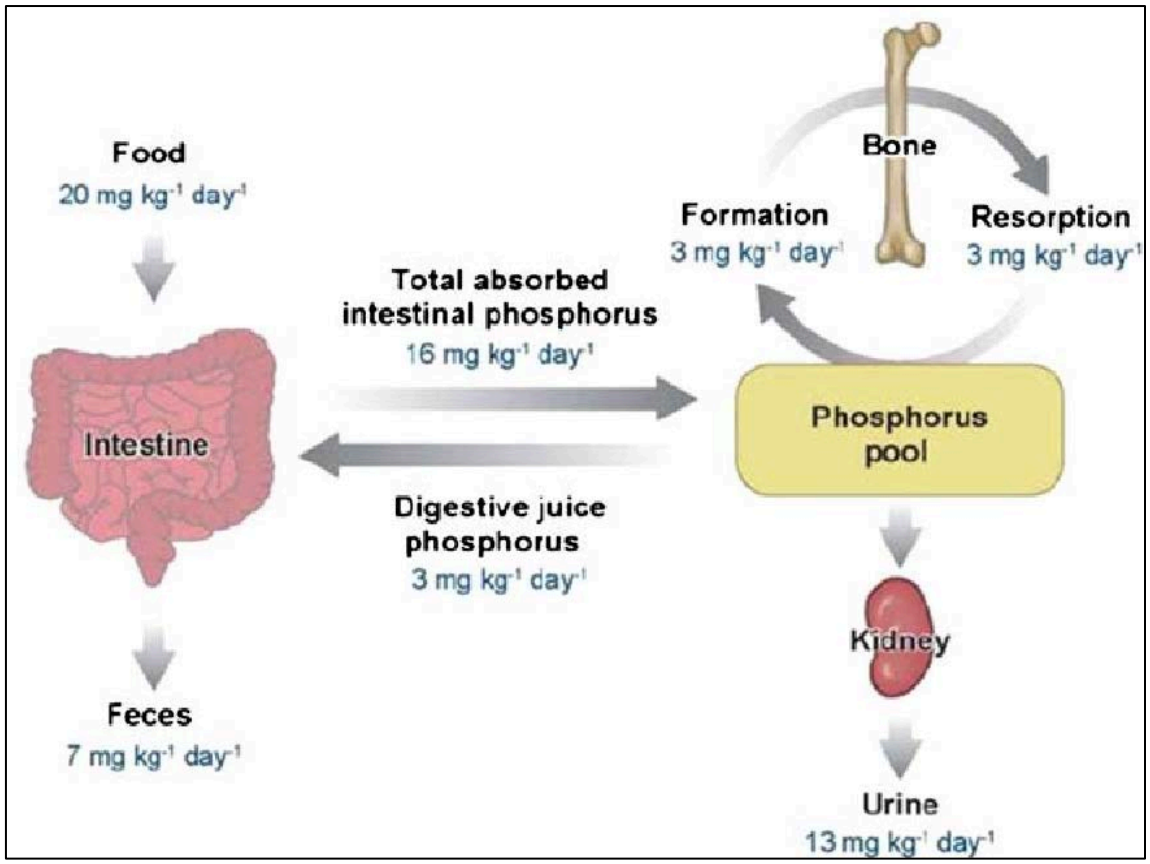

Figure 1: Phosphorus homeostasis

*[18] Shaikh, A., Berndt, T., and Kumar, R., Regulation of phosphate homeostasis by the phosphatonins and other novel mediators. Pediatric Nephrology, 2008. **23**(8): p. 1203–1210.*

2. Role of Hormones

Phosphorus homeostasis is regulated by a number of hormones such as the parathyroid hormone (PTH), the active form of vitamin D (also called calcitriol or 1,25 dihydrovitamin D_3), and the phosphatonins. Calcitriol increases the absorption of phosphorus in the intestine and reabsorption in the kidney. PTH reduces the reabsorption of phosphorus by the kidney, promotes bone resorption, and the activation of the inactive form of vitamin D in the kidney by stimulating the enzyme 25-Hydroxyvitamin D 3 alpha-hydroxylase. This enzyme is responsible for the conversion of the inactive form of Vitamin D_3 to its active form. PTH stimulates the secretion of phosphorus through the kidney by reducing the number of NaPi cotransporters in the proximal tubules that are responsible of the reabsorption of the mineral. As such, any increase of phosphorus would activate PTH. PTH, in turn, would stimulate the excretion of phosphorus from the kidney in order to maintain normal serum phosphorus concentration. *Figure 2* summarizes the role of PTH and 1,25 dihydrovitamin D_3 in phosphorus homeostasis.

Phosphatonins such as fibroblastic growth factor-23 (FGF-23), fibroblastic growth factor-7 (FGF-7), secreted frizzled related protein-4 (sFRP-4), and matrix extracellular phosphoglycoprotein (MEPE) in the kidney were discovered more recently and have been found to also be involved in the regulation of phosphorus in the body. As a matter of fact, they inhibit the reabsorption of phosphorus. In addition, some phosphatonins, most specifically FGF-23 and sFRP-4 might also suppress the activation of vitamin D_3 and thus lead to the reduction in phosphorus absorption in the intestine [14].

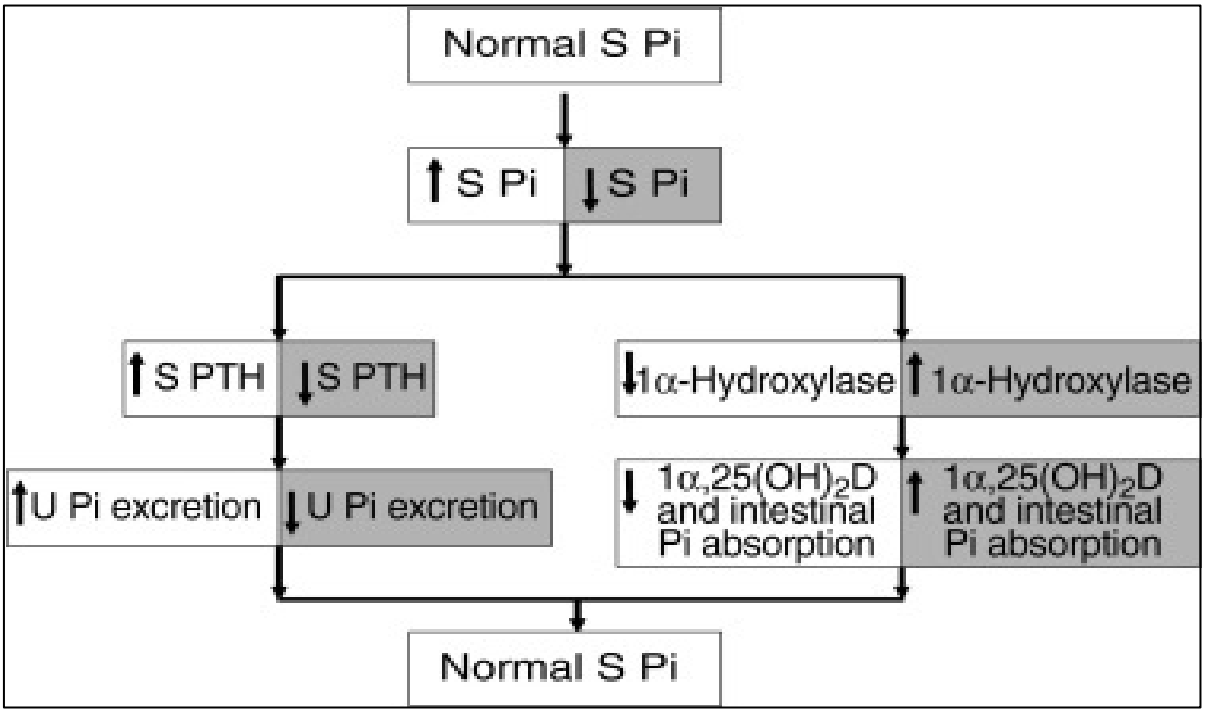

Figure 2: Hormonal regulation of phosphorus homeostasis

[19] Schiavi, S. and Kumar, R., The phosphatonin pathway: New insights in phosphate homeostasis. Kidney International, 2004. ***65****(1): p. 1-14.*

Figure 3 illustrates the role of the phosphatonins on phosphorus homeostasis: the current model being the previously known mechanism, and the proposed model being the most recently discovered role of phosphatonins.

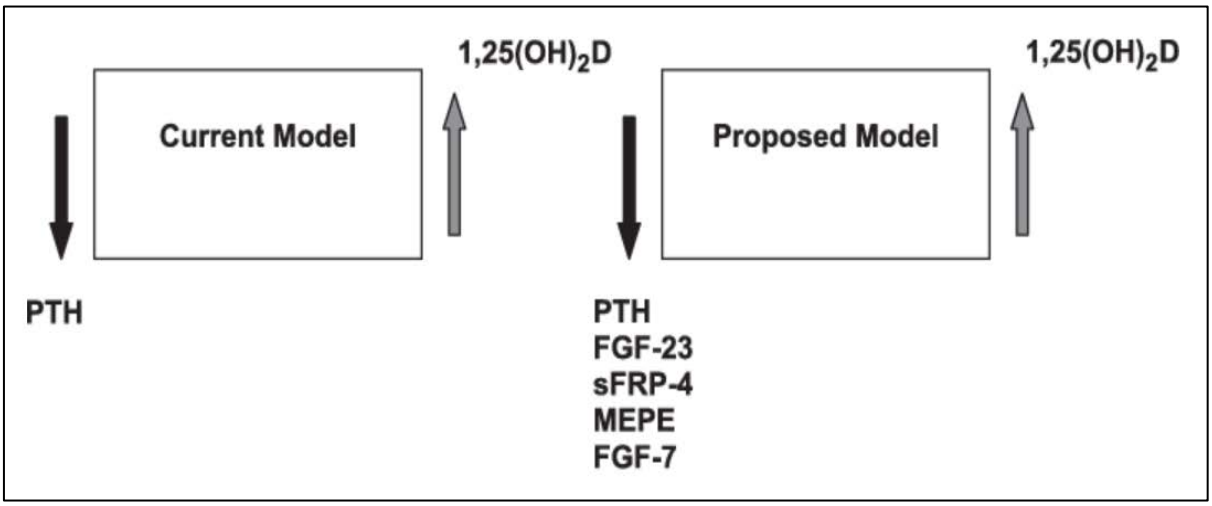

Figure 3: Factors regulating renal phosphorus reabsorption

[20] Berndt, T., Schiavi, S., and Kumar, R.,"Phosphatonins" and the regulation of phosphorus homeostasis. American journal of physiology. Renal physiology, 2005. ***289****: p. 1170–1182.*

C. Health Implications of Altered Phosphorus Homeostasis

1. Hypophosphatemia

Phosphorus homeostasis is critical for the maintenance of a number of biological processes in the body. Any variation of serum phosphorus concentration beyond the accepted range can result in altered health status. Hypophosphatemia is characterized by a plasma phosphorus concentration less than 2.5mg/dl. It can be caused by either rapid shifts of extracellular phosphorus into intracellular compartment, reduced intestinal absorption or increased loss through the feces, or increased excretion through the kidneys. Diet induced hypophosphatemia is uncommon since phosphorus is present in many foods. Many medical interventions such as hemodialysis, anti-acid treatments, and use of diuretics can lead to an imbalance in phosphorus homeostasis. One of the major causes of hypophosphatemia is alcoholism which is characterized with increased intestinal loss of the mineral through the feces. Hypophosphatemia has many consequences among which nervous system

complications, cardiac failure, osteomalacia, and poor postsurgical outcomes depending on the severity of the deficiency, but that can also be recovered with phosphorus supplementation [21, 22].

2. Hyperphosphatemia

Hyperphosphatemia is a condition characterized with a very high serum phosphorus levels, above 5mg/dl. The three major causes of hyperphosphatemia are either rapid shifts of intracellular phosphorus into extracellular compartment, increased intestinal absorption, or decreased excretion through the kidneys. Hyperphosphatemia is one of the major characteristics of patients with chronic kidney failure (CKD). CKD is due to a defective capacity of the kidney to excrete phosphorus. It is usually associated with high calcium levels. These two factors highly contribute to the development of cardiovascular diseases primarily due to vascular calcification. The latter results from a "*high calcium x phosphorus*" product that lead to the calcification of the soft tissues [23]. Dietary phosphate restriction, coupled sometimes with phosphate binders, is used as a treatment of this condition. In case of no response to this treatment, hemodialysis is required to "clean" the blood [24]. Hyperphosphatemia can also result from many other diseases such as tumor lysis syndrome which is a group of metabolic disturbances that can appear after the treatment of cancer. Hypothyroidism, excessive phosphorus intake, vitamin D toxicity can also cause a rise in phosphate levels in the serum. For this reason, hyperphosphatemia is best managed by treating the underlying condition [25].

D. Protein Metabolism

1. Protein Digestion

Protein digestion starts in the stomach where large peptides are broken down into smaller peptides by the actions of hydrochloric acid (HCl) and pepsin by creating an acidic environment. The smaller polypeptides travel then into the small intestine. Their arrival generates the secretion of digestive hormones, cholecystokinin (CCK) and secretin involved in the degradation of the proteins. These hormones will stimulate the pancreas to release proteolytic enzymes which will subsequently allow the breakdown of the polypeptides into individual amino acids [26]. These amino acids are then absorbed by the blood stream and transported to the liver and cells for protein synthesis. As the body does not have any protein storage, the excess amino acids will be decomposed or used to produce glucose, ketones, or fatty acids. The decomposition of amino acids results in the production of ammonia that if not eliminated is very toxic. The urea cycle is a cycle that uses the ammonia generated to produce urea and thus, allow the excretion of ammonia via the urine [27].

2. Protein Synthesis and Degradation

Protein metabolism is the process characterized by the balance between the synthesis and degradation of proteins [28].

The body in normal conditions strives to maintain a neutral protein balance where protein synthesis is equal to protein breakdown. Protein turnover is the net result of synthesis and

degradation of proteins for optimal protein functions. In certain cases, protein balance can be positive, meaning that protein synthesis is greater than breakdown in the body. This state usually happens during pregnancy, growth, or recovery after protein loss from trauma or malnutrition. A negative protein balance is when the body degrades more protein than what it produces leading to a net loss of protein. This typically happens after a trauma, starvation, or major surgery where the body starts losing lean body mass [29, 30].

Protein synthesis is vital for the growth, maintenance, and repair of all tissues and cells. For this reason, it is critical to insure proper protein synthesis rates to avoid loss of tissues in the body. The building blocks of proteins are amino acids that are obtained by the ingestion of proteins in the intestine and the breakdown of other proteins within the cell. Dietary protein plays an important role in the maintenance of a stable protein balance. First, because as the body continuously breaks down amino acids, if protein is not supplied in the diet to counteract the loss of protein, this would lead to a reduction in body protein mass [31]. Second, some amino acids, called essential amino acid (EAA) cannot be synthesized by the body and thus only diet can supply them. There are nine EAA which are phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine. If one of these amino acids is not adequate in the diet, protein synthesis is impaired [32]. The rest of the amino acids are categorized into two major groups: the conditionally essential amino acids and the non-essential amino acids. The conditionally essential amino acids are the amino acids that depend on the adequate supply of the essential amino acids to be produced. The non-essential amino acids are the ones that can be produced in the body [33, 34].

E. Phosphorus & Protein Synthesis

1. Phosphorus and Insulin

The literature suggests a role of insulin on the stimulation of protein synthesis. In fact, several studies have shown that phosphorus might improves insulin sensitivity. In a human experiment, co-ingestion of phosphorus with glucose induced improvements in insulin sensitivity index by reducing postprandial insulin and glucose levels. The availability of phosphorus allowed glucose to be trapped in the cells through phosphorylation. This consequently reduced the release of insulin as the latter depends on the amount of glucose present in the plasma. Both the reduction in glucose and insulin levels might have contributed to the improvements in insulin sensitivity [6]. In addition, a study comparing micronutrient rich and not rich bread revealed lower postprandial glucose levels with the micronutrient rich bread [7]. In another study, human subjects under normoglycemic conditions had an improved insulin sensitivity after the ingestion of phosphorus [35]. Moreover, the role of insulin in the stimulation of protein synthesis has been mentioned many times in the literature [8, 9]. In a human study, the local administration of a high dose of insulin to the muscle increased protein synthesis rates as compared to the low dose where no change occurred [8]. Consequently, phosphorus by improving insulin sensitivity could potentially increase protein synthesis rates.

2. Phosphorus and ATP Synthesis

Adequate intake of phosphorus is a requirement for proper ATP synthesis. Any deficiency of the micronutrient has been negatively associated with ATP production [36]. After food ingestion, the body relies, first, on the available phosphorus stored within the cells. However, as the storage of free phosphorus in the intracellular milieu is very limited, the body soon after necessitates the phosphorus present in the extracellular fluid (ECF). Low availability of phosphorus in the ECF would thus negatively impact ATP production. For this reason, the phosphorus in food becomes of primary importance for the regulation of phosphorus in the circulation. In addition, there exist no adaptive mechanism that improves phosphorus absorption at low intakes. Consequently, low intakes of phosphorus, by reducing ECF phosphorus levels, are expected to negatively affect ATP synthesis [5]. In a recent study, mice were found to have both a reduction in serum inorganic phosphorus concentration and muscle ATP synthesis rates after a diet induced hypophosphatemia. The reintroduction of phosphorus in the diet contributed to the normalization of serum phosphorus levels as well as muscle ATP synthesis rates [2]. Dietary lifestyle is an important contributor to the availability of phosphorus for ATP synthesis. In fact, nowadays, the consumption of a diet high in carbohydrates (CHO) and low in phosphorus is more common. This type of diet is typically characterized by a sharp rise in insulin secretion which promotes both the uptake of phosphorus by the peripheral cells and the phosphorylation of macronutrients (such as CHO) present in the food ingested. As such, the increased requirements of phosphorus for the phosphorylation of the compounds does not leave enough phosphorus for ATP production [5].

3. ATP Synthesis and Protein Synthesis

Protein synthesis is a highly energy dependent process. The energy cost for the formation of one peptide bond is equivalent to 4 ATP, or in other terms 0.67 Kcal per one gram of protein synthesized. Two ATPs are required for the attachment of the amino acid to tRNA, 1 ATP for the binding of tRNA to the ribosome, and 1 ATP for the movement of the growing peptide [1]. For this reason, when ATP synthesis is slowed down or inhibited, protein synthesis is negatively affected. In fact, Jewett et al. studied the effect of ATP synthesis on protein synthesis in cell-free system Escherichia coli bacteria. A cell-free system provides the opportunity to remove or add components freely, which allows the observation of the direct effect of a compound on another compound. As such, protein synthesis rates were calculated at different ATP concentration. The end results suggested a direct implication of ATP concentration on protein synthesis rates in the bacteria extracts [3]. Furthermore, hypophosphatemia has been linked with muscle weakness and heart failure in humans and mice [2, 4]. The muscle weakness might be explained by the reduction in ATP synthesis resulted by the diet-induced hypophosphatemia [2]. In addition, a human study compared the cardiac profile of newly admitted patients to the hospital with low serum phosphorus levels with those within the normal range. The results revealed that patients with hypophosphatemia were more prone to respiratory muscle weakness as compared to those with normophosphatemia who had no symptoms at all [37].

CHAPTER THREE
MATERIALS AND METHODS

A. Animals

This study was approved by the Institutional Animal Care and Use Committee of the American University of Beirut. Forty, six weeks old, male Sprague–Dawley rats weighing around 230-270 grams (American University of Beirut, Lebanon) were maintained on a control diet (**Table 1**) for one week with the purpose of adaption to their new environment. The adaptation period was followed by an additional week, where the rats were fed the same diet, as part of the experiment. During these two weeks, both water and food were served *ad libitum*.

B. Experimental Design

At the end of two weeks, the forty male Sprague-Dawley rats were fasted overnight and then divided equally into five groups for the day of the sacrifice (n=8). After the overnight fast, all the rats, except the rats in group 1, were provided with 1g of food dissolved in 3ml of water by gavage, followed by 1 mL of water to wash the feeding tube of any food residues remaining. Then, they were injected, into the peritoneum (i.p. injection), with 1 ml of a flooding dose of L-Phenylalanine (1.50 mmol/kg BW; Sigma) containing L-[ring-$^{2}H_{5}$]Phenylalanine at 40 % molar enrichment (0.60 mmol/kg; Cambridge isotope Laboratories). The diet received on the day of the sacrifice was different than the one during the two weeks. **Table 2** summarizes the composition of the diet for the

different groups. The rats in the control group (group 1) were not provided food, only 3 ml of water. Then, they received the i.p. injection in the fasted state and were sacrificed an hour later. The second and fourth groups of fasted rats received 1g of phosphorus free experimental diet dissolved in 3ml water, to consume within a period of 10mins, before being injected the 1 mL of L-[ring-$^{2}H_{5}$]Phenylalanine 40 % molar enrichment. The third and fifth groups of fasted rats received the same treatment as the second and fourth groups except that the experimental diet included 0.3% phosphorus. The rats of group 2 and 3 were sacrificed 2 hours after food ingestion; while, the rats of group 4 and 5 were sacrificed 4 hours after. All the animals were sacrificed by decapitation. In all the five groups, blood samples were collected in EDTA tubes and stored on ice. The organs of the animals were first washed with ice-cold saline (154 mmol NaCl/L at pH 7.4) and snap-frozen in liquid nitrogen before being stored at -80°C until analysis. The collected blood samples were centrifuged at 5000*rpm* for 15min at 3°C and stored in the freezer at -80°C.

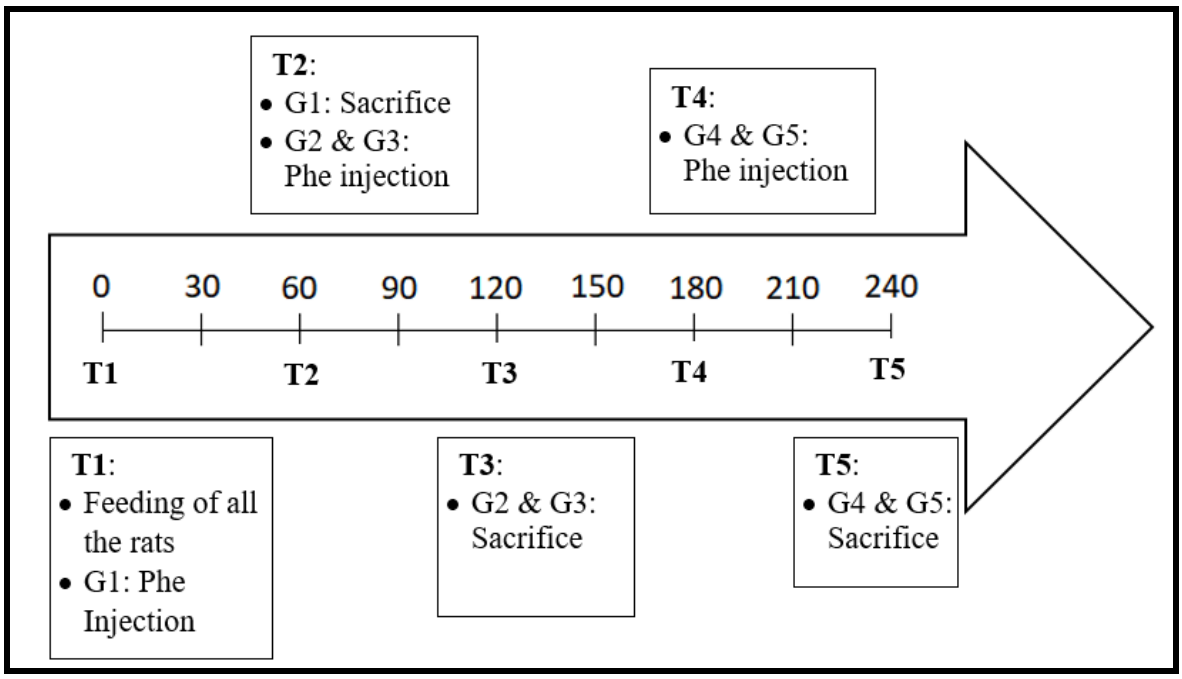

Figure 4: Experimental time frame.

T represents the time in minutes. Control group (G1) was given water only before being sacrificed. G2 received a phosphorus free gavage and was sacrificed 2 hours later; G3 was given a phosphorus containing gavage and sacrificed two hours later; G4 was given a phosphorus free gavage and sacrificed 4 hours later; G5 was given a phosphorus containing gavage and sacrificed 4 hours later.

C. Sample Preparation and GC-MS

Plasma (0.75mL) and tissue samples (0.5 g) were homogenized in 10% trichloroacetic acid (TCA) and the homogenate was centrifuged at 3000*rpm* for 20 min at 4 °C. The supernatant of the plasma was collected to measure the enrichment of free phenylalanine in plasma. The protein pellet was washed three times with 5% TCA and centrifuged at 3000*rpm* for 20 min at 4 °C before being hydrolyzed in 5mL of 6N HCl at 100°C for 18 h. The supernatant and pellet were applied to a cation-exchange column (Dowex AG 50W - 8H resin; 100-200 mesh; Bio-Rad). Before loading the sample, the column was washed successively with 6mL of 1M HCl, 6mL of deionized water, 6mL of

1M NaOH, 6mL of deionized water, 6mL of 1M HCl, 6mL of deionized water. Finally, the free amino acids were eluted with 6mL of 10% NH_3 aqueous solution and dried. Dried samples and blood were reacted with a 100ul of *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA): acetonitrile mixture (1:1 ratio) at 100°C for an hour that was later on centrifuged at 3000*rpm* for 15 min at room temperature. The isotopic enrichment in L-[ring-$^2H_5$] Phenylalanine in the tissues and the plasma free and bound pools was determined by gas chromatography-mass spectrometry (GCMS). A 100-µl volume of the solution mixture was transferred into vials to be analyzed by the GCMS. GCMS was used to monitor 234 and 239 m/z in the electron impact mode which were then converted to percent molar enrichment (mol%) using calibration curves prepared with L-[ring-$^2H_5$] Phenylalanine standards. A 1-µl volume was injected in a capillary column, 30 m x 0.25mm x 0.25 m film thickness (Agilent 5973N-MSD and DB-17MS), using a temperature program from 90°C for 5min, which was increased to 130°C at 5°C/min, and then increased up to 240°C at 40°C/min. The 240°C was hold for 5min. The flow rate of helium was at 1ml/min. The transfer line, injector, and ion source temperatures were 250°C with a 10:1 split ratio [38, 39]. *Figure 4 and 5* summarize the steps of the experiment.

A great amount of time was spent for the development and validation of this experiment. One of the major challenges encountered throughout the development of the method was with the GCMS machine. In fact, the large number of users within the same week, in addition to the repeated technical issues in the machine, considerably affected the results obtained. Perhaps, the use of high-performance liquid chromatography instead of GCMS would have been a better option based on the situation of the laboratory.

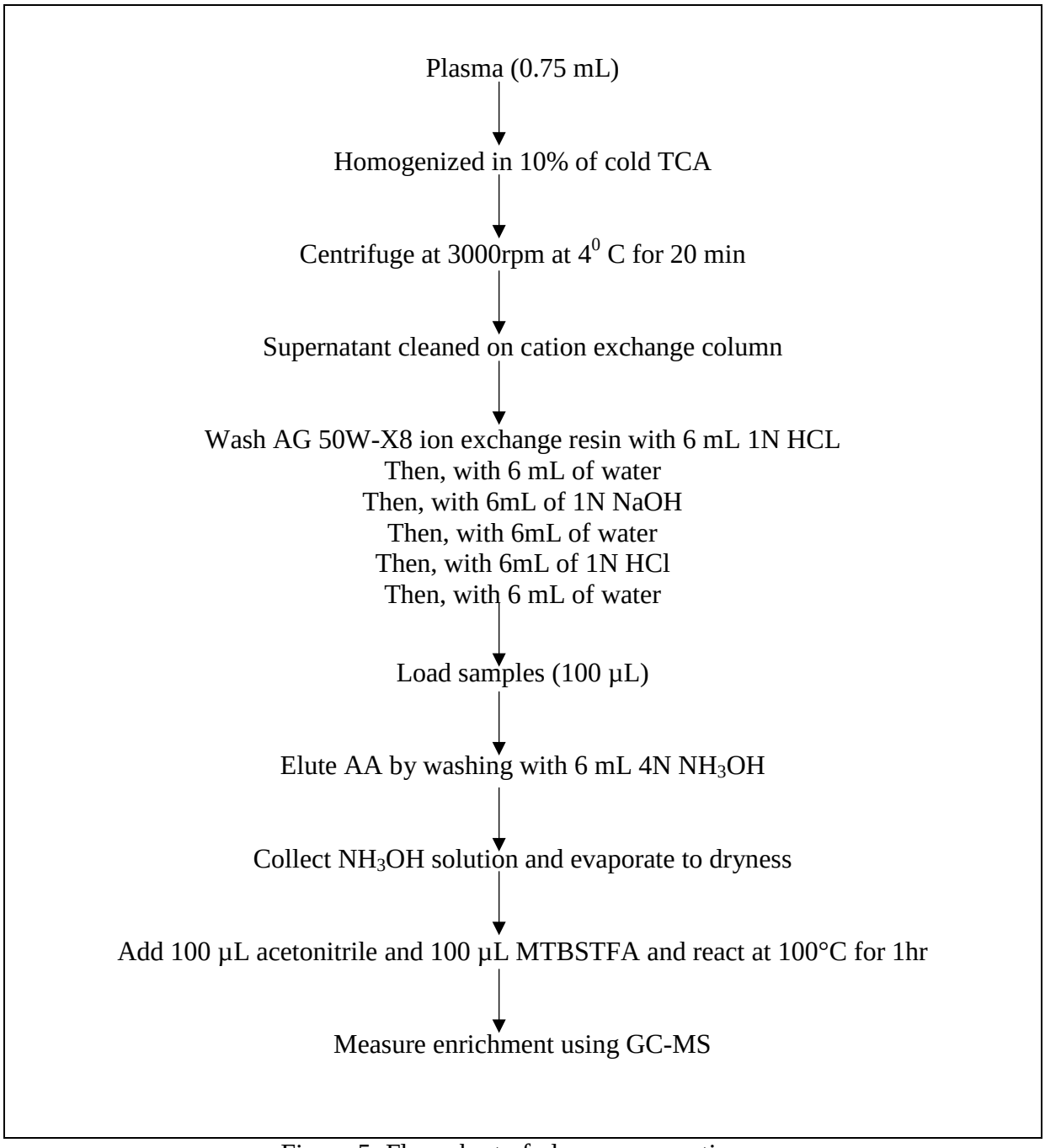

Figure 5: Flow chart of plasma preparation

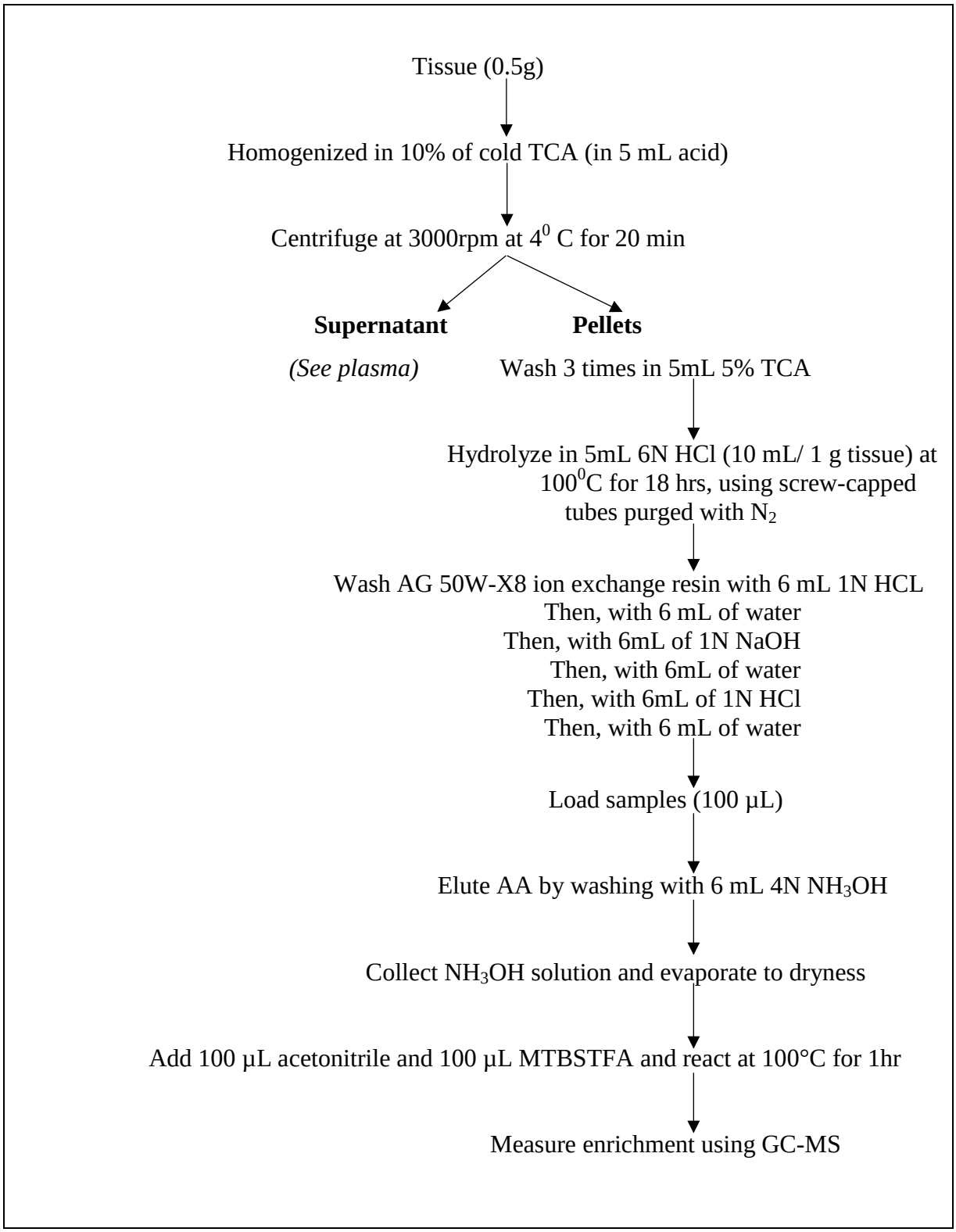

Figure 6: Flow chart of tissue preparation

Table 1: Composition of the control diet

Ingredients	Diet % (Kcal)
Protein (egg white)*	20 (80)
Fat (corn oil)	10 (90)
Starch (CHO)	30 (120)
Sucrose (CHO)	30 (120)
Cellulose	5.5
Mineral mix**	3.5
Vitamin mix	1
KH2PO4 (P added)***	0.27
Total calories (Kcal)/ 100g	410

* Egg white is used as it is low in phosphorus but contains all the essential amino acids [40]
**Phosphorous Free Mineral Mix used to make the low phosphorus diet.
***MW KH2PO4 = 136 g of which P = 31 g.

D. Preparation of Standard Curves

A standard curve was prepared using different mol% as follows: 0, 0.5, 1, 2, 5, 10, 20, 30, 40% of L-[ring-$^{2}H_{5}$] Phenylalanine molar enrichments. A10-µl of each enrichment was dried and then reacted with 50-µl acetonitrile and 50-µl MTBSTFA at 100°C for an hour. The standards were then analyzed using the same procedure by the GCMS.

E. Calculations

The equation used for the calculation of the fractional protein synthesis rate (FSR) is the following:

$$\mathbf{FSR= (E_{Bound--t}\ x1440min/d\ x100\%)/\ (E_{Free--t}\ x\ t)} \quad [39]$$

where FSR is fractional protein synthesis rate (%/d), $E_{Bound—t}$ and $E_{Free\ t}$ are the isotopic enrichment of L-[ring-$^{2}H_{5}$] Phenylalanine in the bound and free tissue pools at time t respectively, and t is the time interval of phenylalanine injection and tissue sampling (60min).

All analyses were performed using IBM SPSS Statistics 23. All the values were expressed in mean ±SD. *P-values* of < 0.05 were considered significant.

Table 2: Experimental Diet

Ingredients	GROUP 2 & 4	GROUP 3 &5
	Diet %	Diet %
Protein (egg white)*	20	20
Fat (corn oil)	10	10
Starch (CHO)	30	30
Sucrose (CHO)	30	30
Cellulose	5.5	5.5
Mineral mix**	3.5	3.5
Vitamin mix	1	1
KH2PO4 (P added)	-	0.27
Total P	-	0.3
Total calories (Kcal) / 100g	410	410

*of which 0.03% phosphorus
**Phosphorous Free Mineral Mix used to make the low phosphorus diet.

CHAPTER FOUR
RESULTS

A. Validation of the Method

A great amount of time was spent for the development and validation of the method. All the results were calculated based on the values obtained from the standard curve. As mentioned earlier, different mol% of L-[ring-$^{2}H_{5}$] Phenylalanine solutions were prepared for the development of the standard curve. For each %mol, the corresponding 239/234 ratio was calculated through the observed values in the mass spectrum. The calculated ratios allowed the development of the standard curve. Similarly, in Buraczenka et al., 239 and 234 m/z were monitored in the electron impact mode which is one of the reasons why they were monitored in the current study: 239 m/z representing the labelled phenylalanine and 234 m/z representing the non-labelled phenylalanine [41]. In addition, the higher the mol% of L-[ring-$^{2}H_{5}$] Phenylalanine in the prepared standard solutions, the higher the intensity at 239 m/z as compared to the intensity at 234 m/z. *Figure 7* and *figure 9* are the chromatogram generated by the GCMS of the prepared standards of 10 and 30 mol% in L-[ring-$^{2}H_{5}$] Phenylalanine respectively. *Figure 8* and *figure 10* are respectively the mass spectrum of these standards. The mass spectrums indicate a clear increase in intensity at 239 m/z observed in *figure 10* with 30 mol% of L-[ring-$^{2}H_{5}$] Phenylalanine compared to 10 mol% of L-[ring-$^{2}H_{5}$] Phenylalanine in *figure 8*. The peaks observed at 239 and 234 m/z, for the 10 mol% in labelled phenylalanine standard solution, have an absolute value equivalent to 2.46×10^{5} and 4.24×10^{7} respectively with a calculated 239/234 ratio of

0.0058 *(figure 8).* As for the 30 mol% in labelled phenylalanine standard solution, the intensity of the peak at 239 m/z is 5.52 x 10^6 and at 234 m/z is 4.87 x 10^7 respectively with a 239/234 ration equivalent to 0.1133 *(figure 10).*These observation were a further confirmation that the intensity at 239 m/z represented the intensity of L-[ring-$^2H_5$] Phenylalanine and at 234 m/z, the non-labelled phenylalanine. Consequently, the GCMS was used to monitor 234 and 239 m/z in the electron impact mode for the tissue and plasma samples which were then converted to mol% using the calibration curves prepared with L-[ring-$^2H_5$] Phenylalanine standards.

The standard solutions also allowed the determination of the retention time (RT) of phenylalanine. The RT, which is the required time for a solute to pass through the chromatography, of phenylalanine was about 18.35min. In fact, both *figure 6 and 8* show the apparition of the peak representing phenylalanine at 18.34min RT.

Based on these results, two standard curves were developed. *Figure 11* is the calibration curve for the high mol% in L-[ring-$^2H_5$] Phenylalanine standards ranging from 10 to 40% with 0% as the origin that were used to analyze the results of the plasma samples. *Figure 12* is the standard curve for the low mol% in L-[ring-$^2H_5$] Phenylalanine standards ranging from 0 to 2% that were used to analyze the results of the liver samples.

Figure 13 is the chromatogram obtained for one of the liver samples. The RT of phenylalanine is at 18.35min. *Figure 14* is the mass spectrum of the same sample. The peaks observed at 239 and 234 m/z have an absolute value equivalent to 5.81 x 10^5 and 8.51 x 10^7 respectively. The calculated 239/234 ratio is thus *0.0068*. Based on the standard curve (*figure 11*), this is equivalent to a 0.78 mol% of L-[ring-$^2H_5$] Phenylalanine.

Figure 15 is the chromatogram obtained for one of the plasma samples. The RT, however, is 17.35min. The change in the RT is one of the other problems faced with the GCMS. In fact, the RT of phenylalanine should not change from one sample to another as the method used for analysis did not vary between the different samples. *Figure 16* is the mass spectrum of same plasma sample. The calculated 239/234 ratio was 0.13, thus a mol% enrichment equivalent to 33.73.

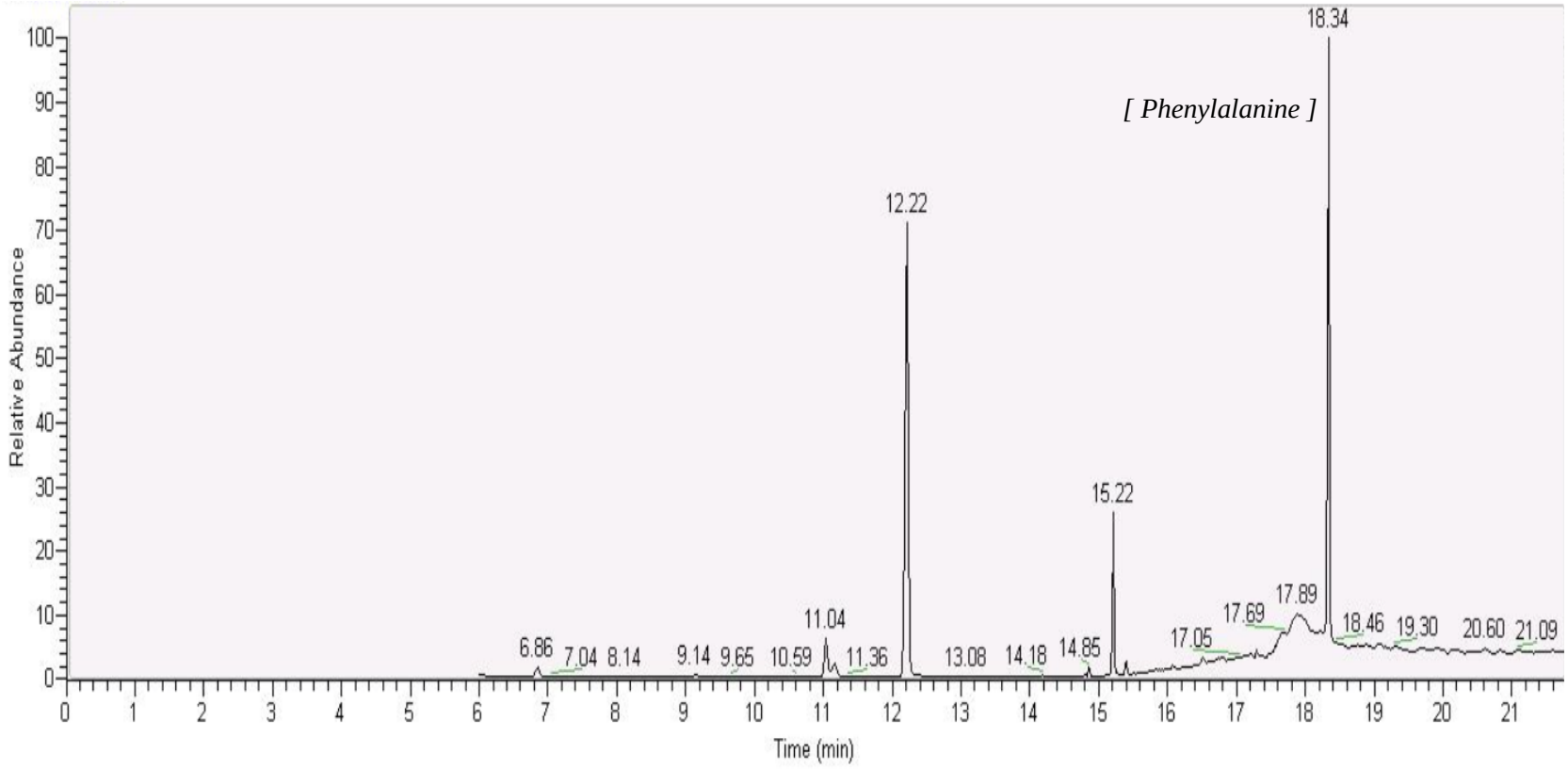

Figure 7: Sample chromatogram reading of phenylalanine in a standard solution of 10 mol% of L-[ring-$^{2}H_{5}$]Phenylalanine

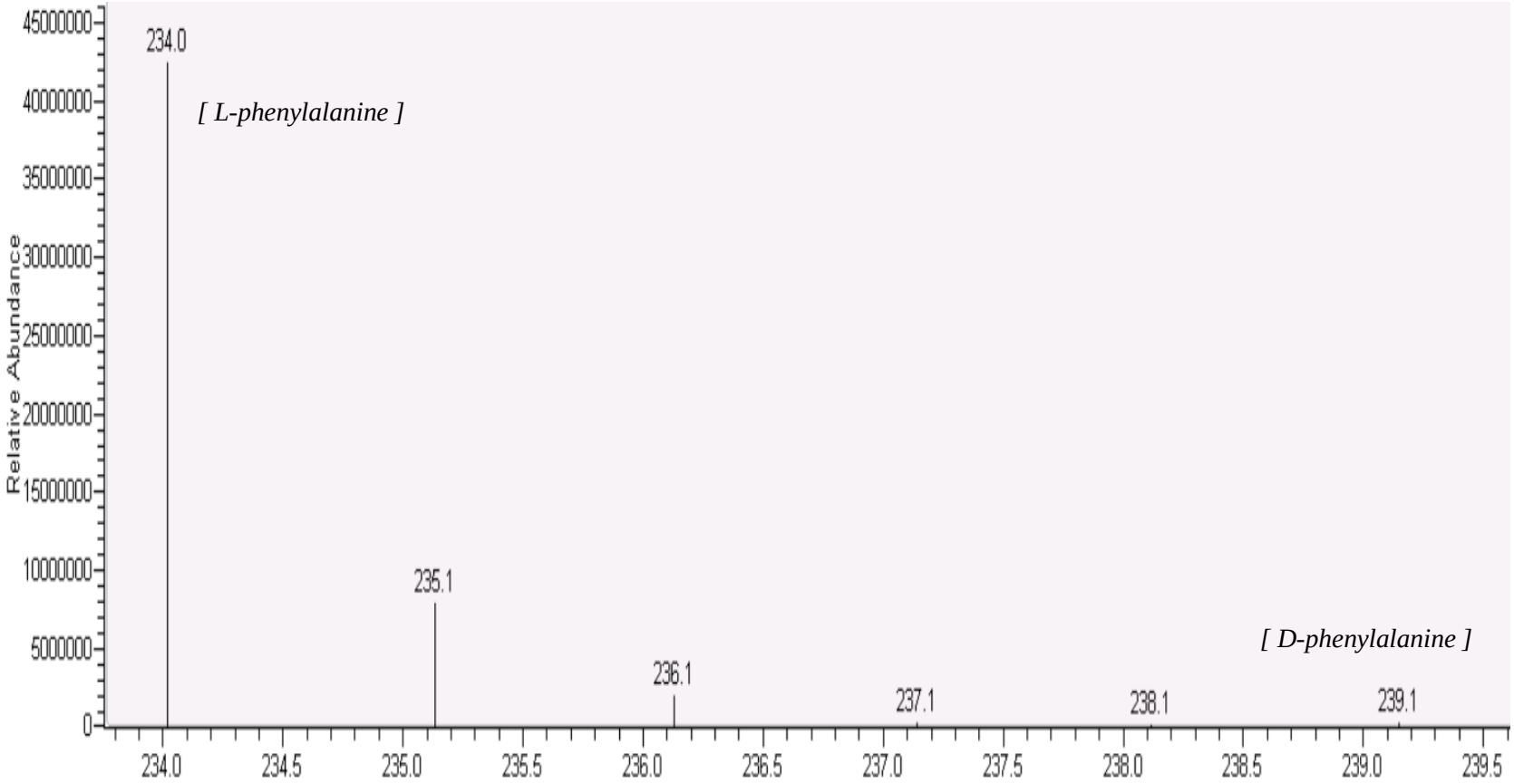

Figure 8: Sample mass spectrum reading of phenylalanine in a standard solution of 10 mol% of L-[ring-$^{2}H_{5}$]Phenylalanine.
L-phenylalanine being the natural form of phenylalanine and D-phenylalanine being the labelled form.

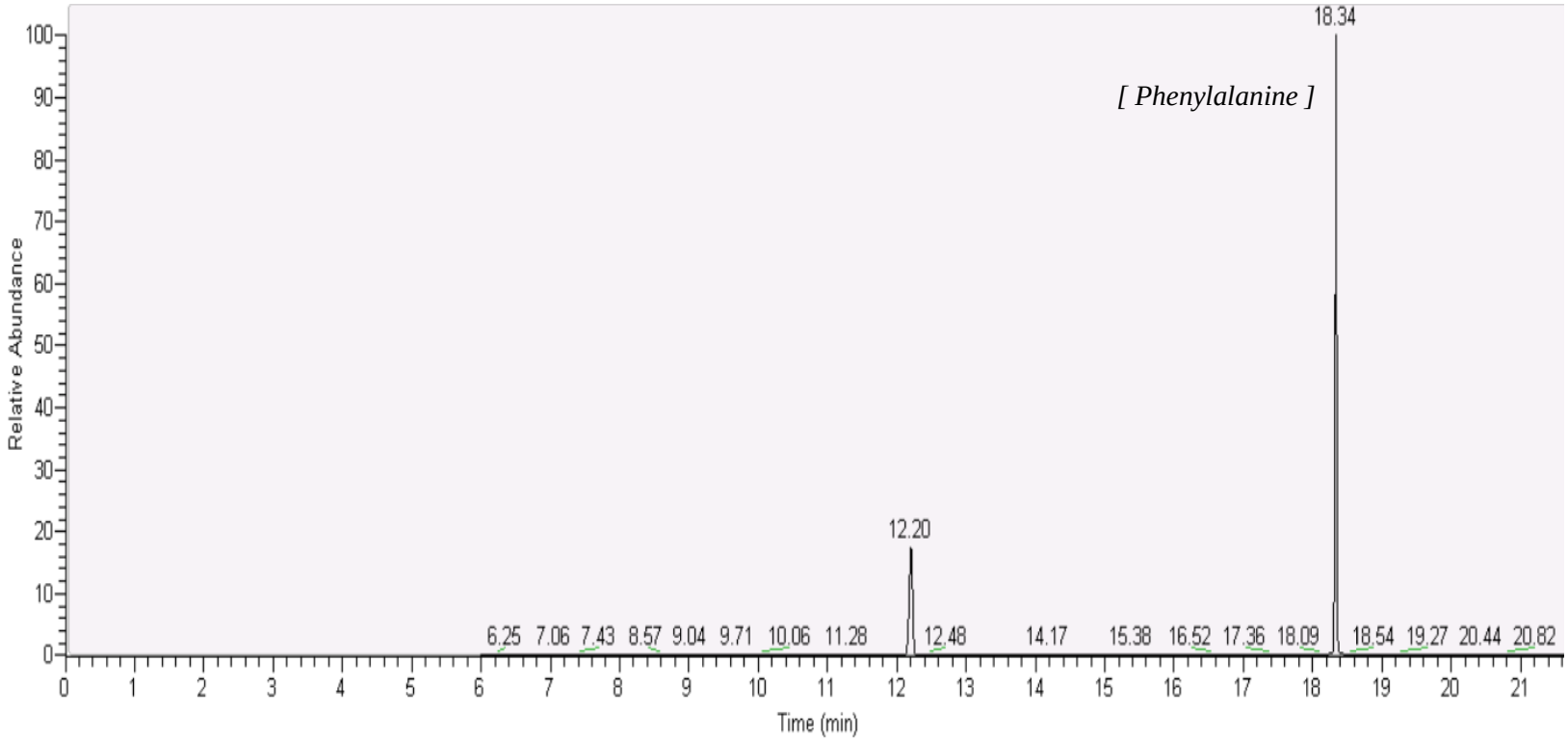

Figure 9: Sample chromatogram reading of phenylalanine in a standard solution of 30 mol% of L-[ring-$^{2}H_{5}$]Phenylalanine

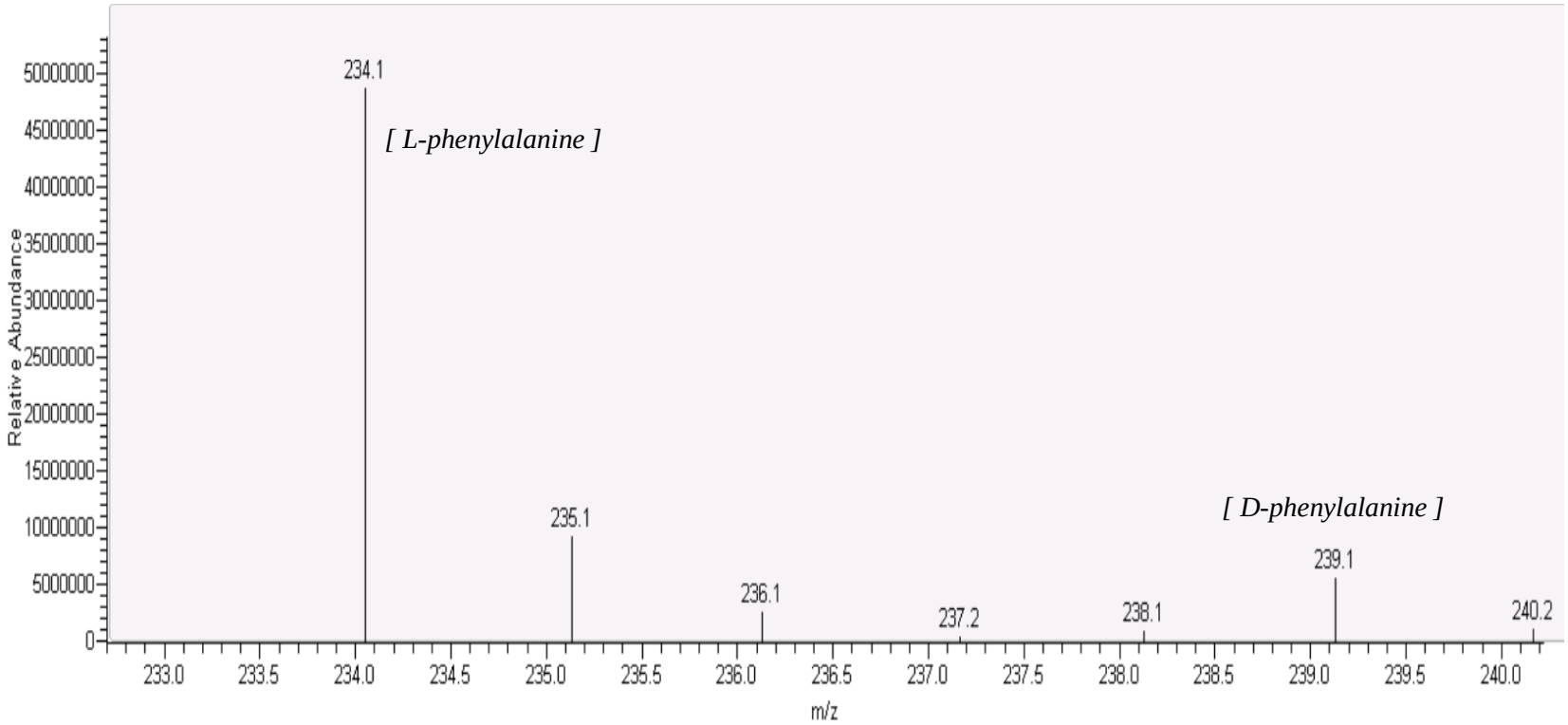

Figure 10: Sample mass spectrum reading of phenylalanine in a standard solution of 30 mol% of L-[ring-$^{2}H_{5}$]Phenylalanine. *L-phenylalanine being the natural form of phenylalanine and D-phenylalanine being the labelled form.*

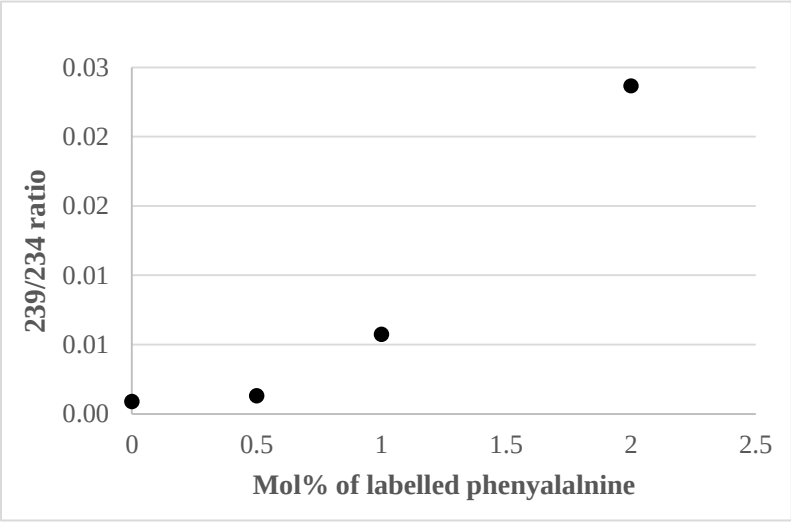

Figure 11: Standard curve for low mol% in labelled Phenylalanine

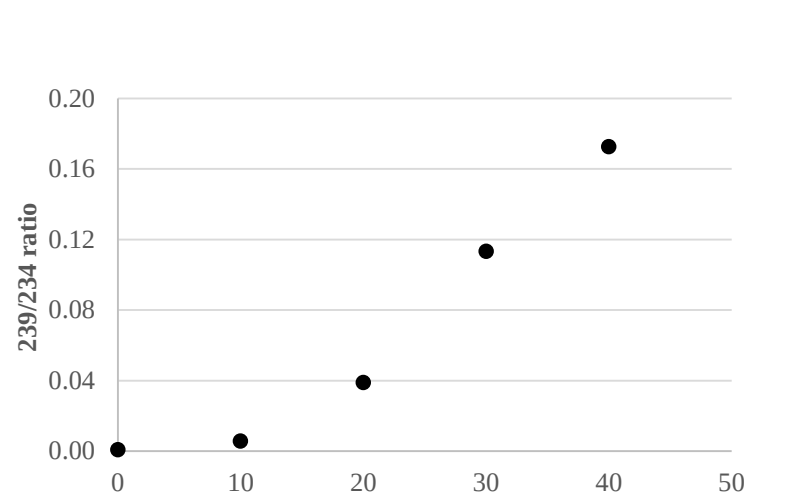

Figure 12: Standard curve for high mol% in labelled Phenylalanine

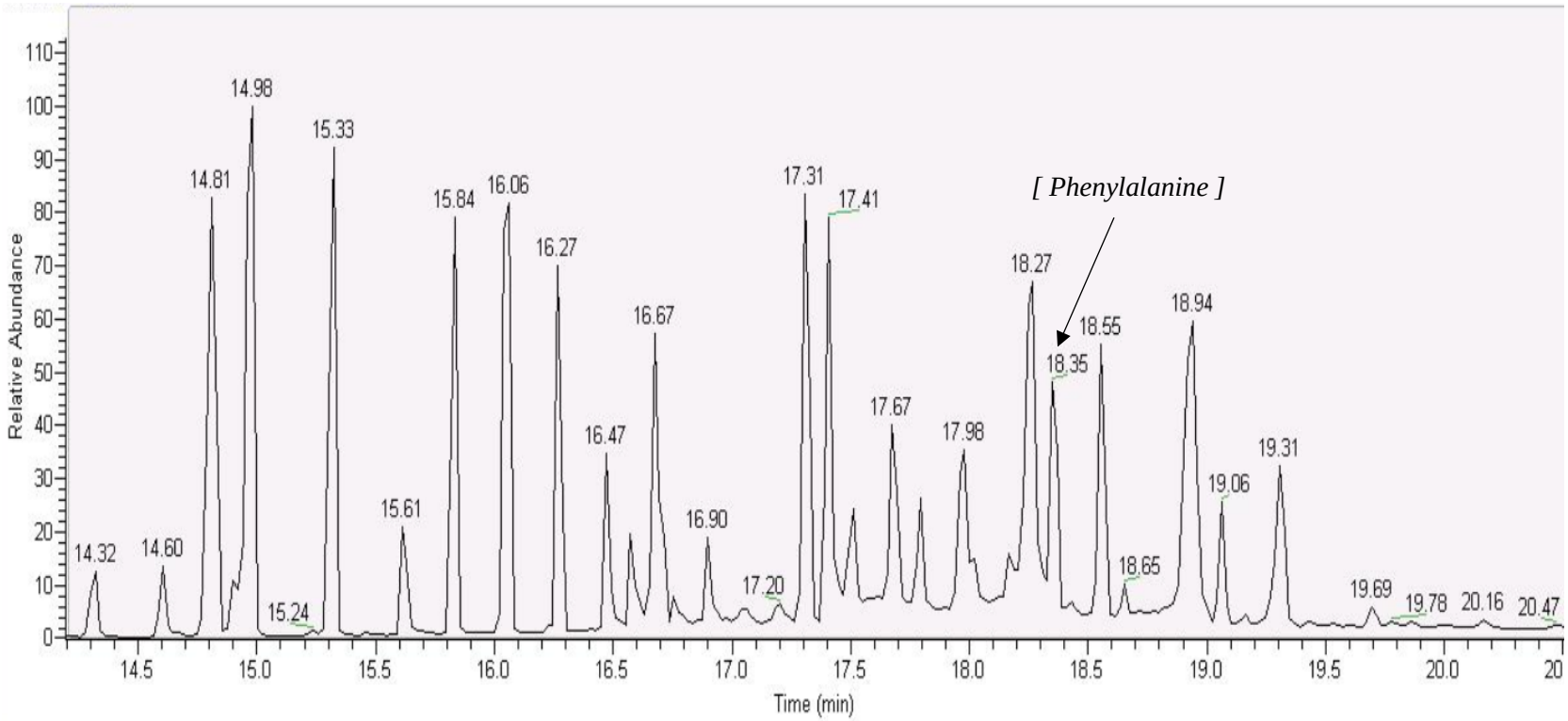

Figure 13: Sample chromatogram reading of phenylalanine in a liver sample

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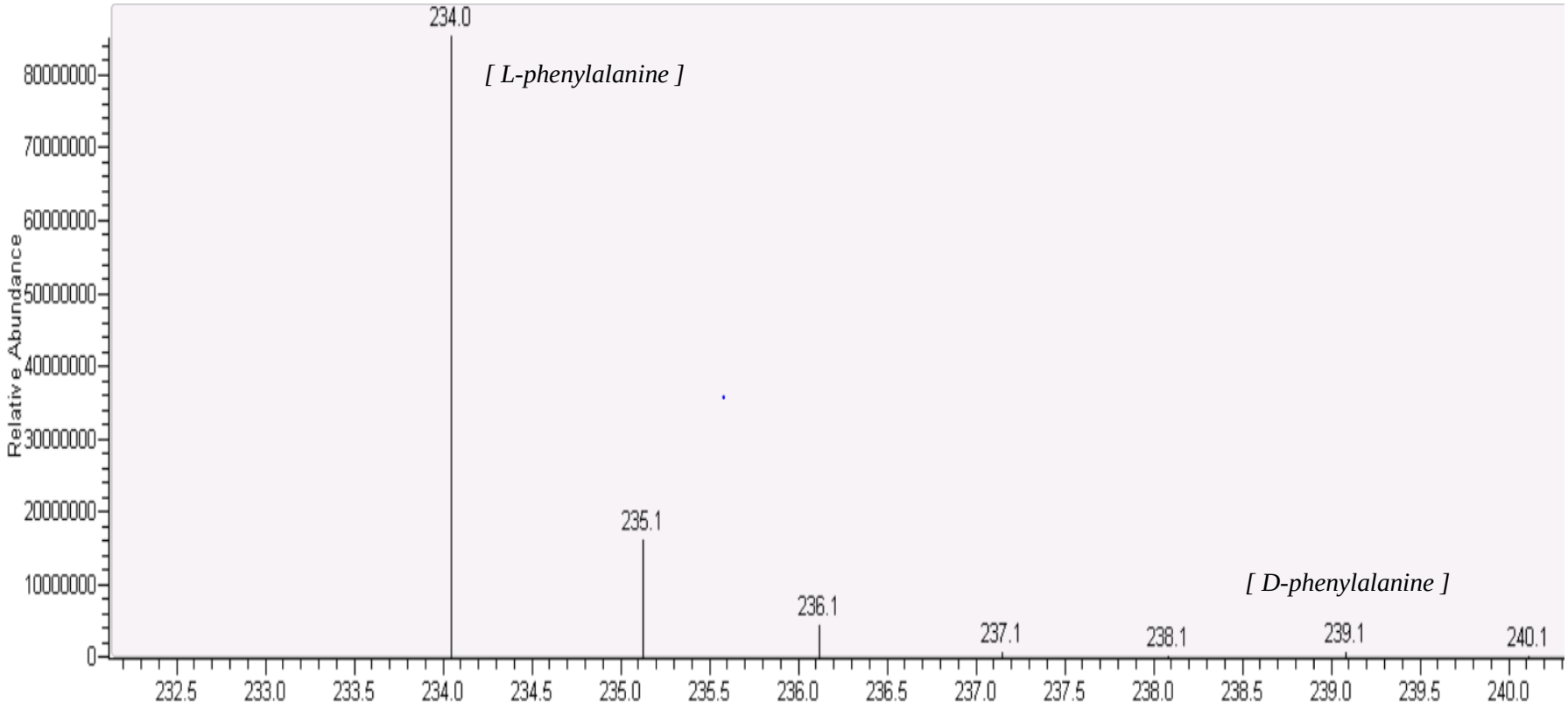

Figure 14: Sample mass spectrum reading of phenylalanine in a liver sample.
L-phenylalanine being the natural form of phenylalanine and D-phenylalanine being the labelled form.

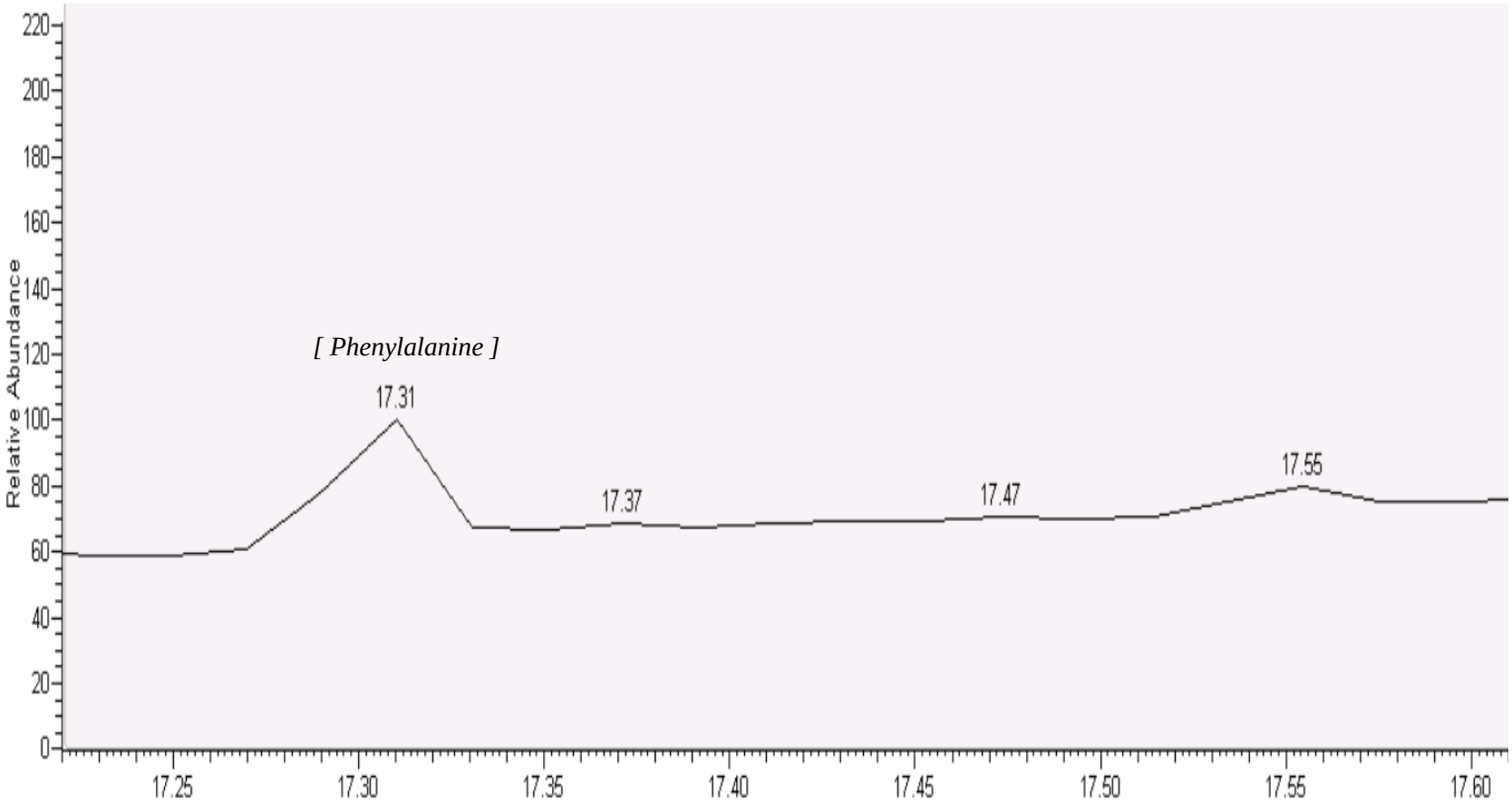

Figure 15: Sample chromatogram reading of phenylalanine in a plasma sample

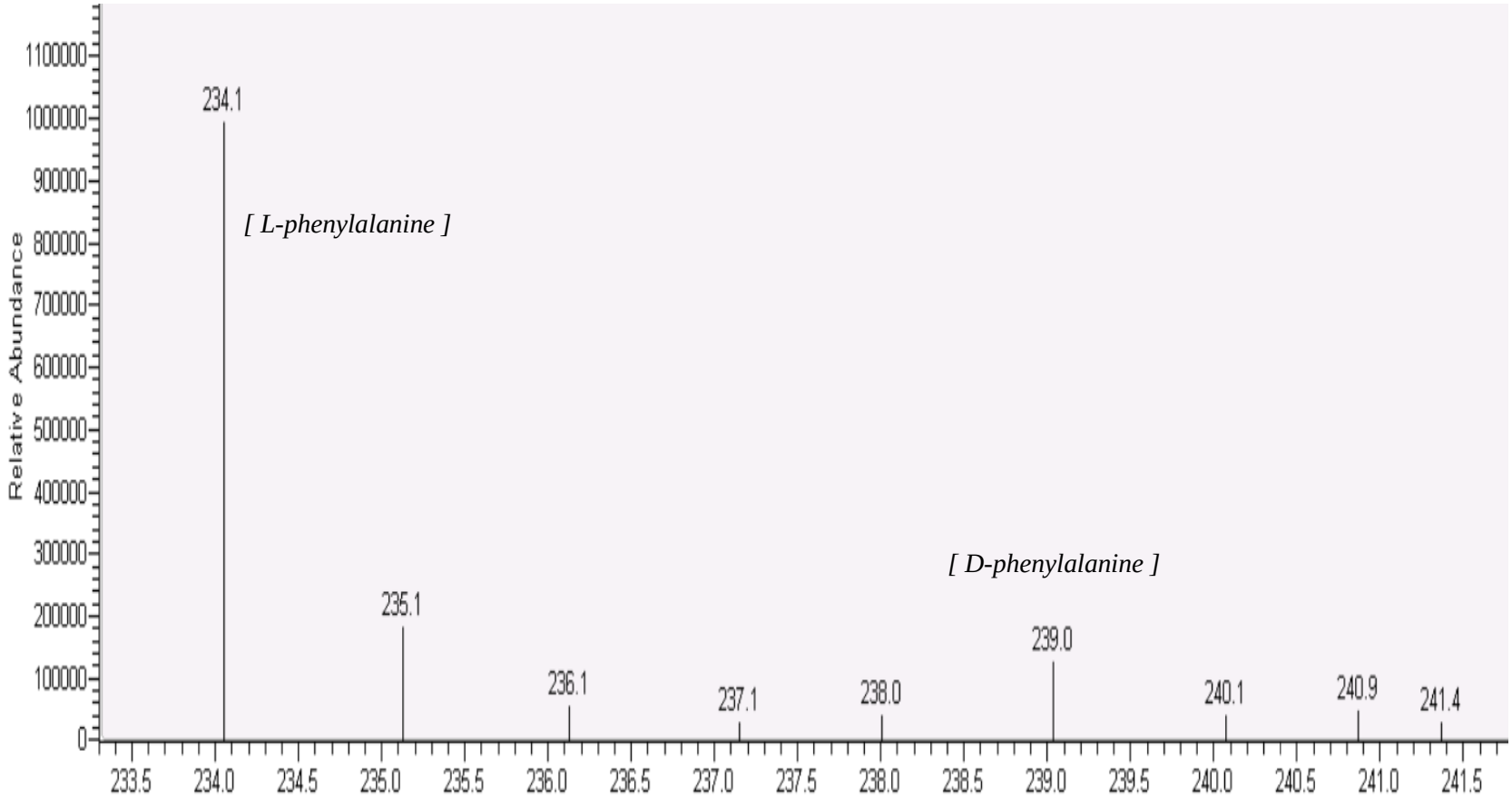

Figure 16: Sample mass spectrum reading of phenylalanine in a plasma sample.
L-phenylalanine being the natural form of phenylalanine and D-phenylalanine being the labelled form.

B. Initial and Final Weights in the Different Groups

All values are expressed as mean ±SD.

The mean BW of the rats at the beginning of the experiment was 249.42g (±36.09) and did not differ significantly between the groups, with the lowest mean weight in group 4 of 239.09g (±26.1050) and highest mean weight in group 2 of 265.68g (±45.9544). At the end of the experiment, all the rats increased in weight. The final mean BW of the rats was 339.00g (±30.47) with no significant difference of BW in the different groups.

Table 3: Mean initial and final weights of the different groups

	Control (n=8)	**2Hrs – P (n=8)**	**2Hrs + P (n=8)**	**4Hrs – P (n=8)**	**4Hrs + P (n=8)**
Initial Weight (g)	254.66 ± 48.69	265.68 ± 45.95	245.46 ± 25.96	239.09 ± 26.11	242.19 ± 29.79
Final Weight (g)	339.40 ± 37.59	339.51 ± 36.36	339.56 ± 31.95	338.66 ± 26.86	339.04 ± 26.37

Control group was given water only before being sacrificed. Group 2Hrs-P received a phosphorus free gavage and was sacrificed 2 hours later; Group 2Hrs+P was given a phosphorus containing gavage and sacrificed two hours later; Group 4Hrs-P was given a phosphorus free gavage and sacrificed 4 hours later; Group 4Hrs+P was given a phosphorus containing gavage and sacrificed 4 hours later. All the values are expressed in mean ±SD.

C. Labeled Phenylalanine Enrichment and Protein Synthesis Rates

It seemed more appropriate to compare the protein synthesis rates of group 2 with group 3, as well as group 4 with group 5 separately, based on the time of sacrifice of the rats. As such, the mean phenylalanine enrichment in the bound pool of liver in group 3 was not significantly different than the mean in group 2. It is 1.119% enrichment in group 3, fed with phosphorus as compared to 0.79% in group 2. Similarly, there was no significant difference between the other groups. *Figure 4* and *Table 4* summarize the observed results.

Table 4: Labelled phenylalanine enrichment in liver (mol%)

	Control (n=8)	**2Hrs – P (n=8)**	**2Hrs + P (n=8)**	**4Hrs – P (n=8)**	**4Hrs + P (n=8)**
Mol% (SD)	0.61 (± 0.20)	0.79 (± 0.28)	1.12 (± 0.79)	0.71 (± 0.40)	0.72 (± 0.30)

Control group was given water only before being sacrificed. Group 2Hrs-P received a phosphorus free gavage and was sacrificed 2 hours later; Group 2Hrs+P was given a phosphorus containing gavage and sacrificed two hours later; Group 4Hrs-P was given a phosphorus free gavage and sacrificed 4 hours later; Group 4Hrs+P was given a phosphorus containing gavage and sacrificed 4 hours later. All the values are expressed in mean ±SD.

The FSR in the liver of the control group was the lowest of all the groups (48.99 %/d (±15.79)). Although the rats in group 3 (89.48 %/d (± 63.41)) seemed to have a higher mean FSR as compared to group 2 (62.78 %/d (± 22.65)), however, they do not differ significantly. Similarly, the FSR in the liver of group 4 and 5 was not significantly different, 56.78 %/d (± 32.39) and 57.77 %/d (± 23.74) respectively. The FSR calculated was based on an average plasma phenylalanine enrichment of 30.00% for all the groups.

Table 5: Fractional protein synthesis in the liver

	Control (n=8)	2Hrs – P (n=8)	2Hrs + P (n=8)	4Hrs – P (n=8)	4Hrs + P (n=8)
FSR liver %/d (SD)	48.99 (±15.79)	62.78 (±22.65)	89.48 (±63.41)	56.78 (±32.39)	57.77 (±23.74)

Control group was given water only before being sacrificed. Group 2Hrs-P received a phosphorus free gavage and was sacrificed 2 hours later; Group 2Hrs+P was given a phosphorus containing gavage and sacrificed two hours later; Group 4Hrs-P was given a phosphorus free gavage and sacrificed 4 hours later; Group 4Hrs+P was given a phosphorus containing gavage and sacrificed 4 hours later. All the values are expressed in mean ±SD.

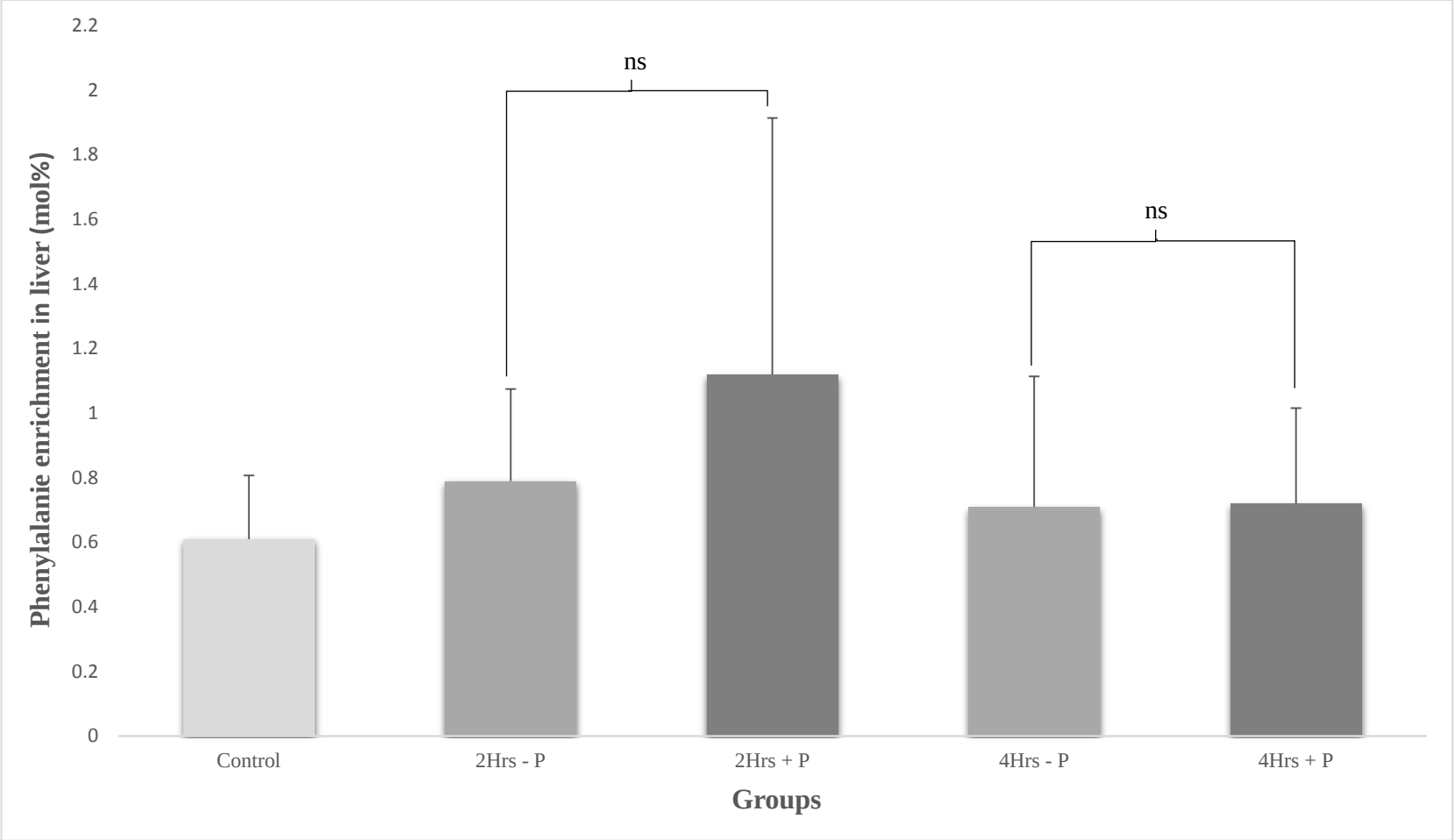

Figure 17: Bound pool in liver of tracer phenylalanine enrichment.

Control group was given water only before being sacrificed. Group 2Hrs-P received a phosphorus free gavage and was sacrificed 2 hours later; Group 2Hrs+P was given a phosphorus containing gavage and sacrificed two hours later; Group 4Hrs-P was given a phosphorus free gavage and sacrificed 4 hours later; Group 4Hrs+P was given a phosphorus containing gavage and sacrificed 4 hours later. All the values are expressed in mean ±SD.

CHAPTER FIVE
DISCUSSION

The objective of this study was to compare the differences in FSR in the liver after the ingestion of a meal with and without phosphorus. A higher FSR was expected for the groups that received a diet with phosphorus than the groups without phosphorus. The mean FSR obtained were comparable to the values of previous studies [39, 42]. However, based on the results, the presence of the phosphorus in the meal did not significantly affect protein synthesis in the liver whether after two or four hours of food ingestion. Although, the group 3 seemed to have a higher mean FSR of 89.48 %/d (± 63.41), as compared to the group 2 of 62.78 %/d (± 22.65), suggesting that there might be a relation between dietary phosphorus and FSR. The lack of significance in the results is mainly due to the large within group variations which suggest the need to conduct further studies to get more accurate results.

ATP synthesis is a process that requires proper amount of phosphate. In fact, each molecule of ATP necessitates three phosphorus. In addition, ATP synthesis primarily relies on the phosphorus present in the ECF as intracellular phosphorus levels are quickly depleted after about 60-90min of food ingestion. Consequently, the lack of phosphorus in food, implicated in the amount of ECF phosphorus, should negatively affect ATP synthesis. This has been observed in several animal studies where diet induced hypophosphatemia resulted in reduced ATP formation [2, 5]. Studies have also confirmed the role of ATP on protein synthesis which relies on high ATP dependent processes. The energy cost for the formation

of one peptide bond is equivalent to 4 ATP. Any condition that affects ATP formation could thus alter protein synthesis rates. This been said, the inadequate supply of phosphorus could negatively affect ATP formation and have thus consequences on protein synthesis rates [1]. In addition, the ingestion of egg white was associated with low urea production in rats, as compared to the ingestion of cottage cheese. Egg white is low in phosphorus, unlike cottage cheese that is high in phosphorus. Urea production is an indicator of protein metabolism. The low urea production suggests a slower removal rate and decreased protein synthesis [43]. Furthermore, after a diet-induced hypophosphatemia, rats were found to have reduced muscle tension that was explained by the reduction in ATP synthesis. This was reversed with the reintroduction of phosphorus [44]. All these studies suggest a link between phosphorus intake and protein synthesis. Although the results of our study were not statistically significant, the high mean FSR after 2 hours of food ingestion of the rats that received a diet with phosphorus as compared to the ones with a phosphorus free diet stresses the need to conduct further studies.

This study was conducted, as much as possible, in a controlled environment. However, as in any experiment, this study could have been subject to limitations related to the physiological response of the animals at the time of sacrifice as well as their adaptation to the experimental environment.

CHAPTER SIX

CONCLUSION

In conclusion, our results did not show any statistical difference in FSR in the liver of rats fed a phosphorus containing diet against those fed a phosphorus free diet. However, the lack of significance was due to the high within group variation, although the observed mean FSR of the rats fed a phosphorus containing diet two hours before their sacrifice seemed to be higher compared to the phosphorus free diet. This been said, improved working conditions, related specifically to the accessibility as well as the general state of the GCMS, are required for a better validation of the method used. Therefore, all these conditions suggest the need to conduct further studies for a better understanding of the relationship of phosphorus intake with protein synthesis rates.

BIBLIOGRAPHY

1. Bender, D., *Introduction to Nutrition and Metabolism*. Fourth Edition ed. 2012, London: CRC Press.

2. Pesta, D., et al., *Hypophosphatemia promotes lower rates of muscle ATP synthesis.* The FASEB jounal, 2016. **30**(10): p. 3378–3387.

3. Jewett, M., et al., *Continued Protein Synthesis at Low [ATP] and [GTP] Enables Cell Adaptation during Energy Limitation.* Journal of bacterology, 2009. **191**(9): p. 1083-1091.

4. Geerse, D., et al., *Treatment of hypophosphatemia in the intensive care unit: a review*. Critical care, 2010. **14**(4).

5. Obeid, O., *Low phosphorus status might contribute to the onsetof obesity.* Etiology and Pathophysiology 2013. **14**(8): p. 659-664.

6. Khattab, M., et al., *Phosphorus ingestion improves oral glucose tolerance of healthy male subjects: a crossover experiment.* Nutrition Journal, 2015. **14**(112).

7. Su-Que, L., et al., *Effect of consumption of micronutrient enriched wheat steamed bread on postprandial plasma glucose in healthy and type 2 diabetic subjects.* Nutrition Journal, 2013. **12**(64).

8. Fujita, S., et al., *Effect of insulin on human skeletal muscle protein synthesis is modulated by insulin-induced changes in muscle blood flow and amino acid availability.* American Journal of Physiology-Endocrinology and Metabolism, 2010. **291**(4): p. 745-754.

9. Abdulla, H., et al., *Role of insulin in the regulation of human skeletal muscle protein synthesis and breakdown: a systematic review and meta-analysis.* Diabetologia, 2016. **59**(1): p. 44-55.

10. Chang, A. and C. Anderson, *Dietary Phosphorus Intake and the Kidney.* Annual Review of Nutrition, 2017(37): p. 321-346.

11. Vinod, B., *Serum Inorganic Phosphorus*. 3rd ed. Clinical Methods: The History, Physical, and Laboratory Examinations. , ed. H.W. Walker HK, Hurst JW. Vol. Chapter 198. 1990, Boston: Butterworths.

12. Maria, P. and A. Uri, *Phosphate homeostasis and its role in bone health.* Pediatric Nephrology, 2012: p. 2039–2048.

13. Gaasbeek, A. and A. Meinders, *Hypophosphatemia: an update on its etiology and treatment.* The American journal of medicine, 2005: p. 1094-1101.

14. González-Parra, E., et al., *Phosphorus and Nutrition in Chronic Kidney Disease.* International Journal of Nephrology, 2012. **2012**: p. 5 pages.

15. Sapir-koren, R. and G. Livshits, *Bone mineralization and regulation of phosphate homeostasis.* IBMS BoneKEy, 2011. **8**: p. 286-300.

16. Bonjour, J.-P., *Calcium and Phosphate: A Duet of Ions Playing for Bone Health.* Journal of the American College of Nutrition, 2011: p. 438-448.

17. Kraft, M., *Phosphorus and Calcium: A Review for the Adult Nutrition Support Clinician.* Nutrition in Clinical Practice, 2015. **20**: p. 21–33.

18. Shaikh, A., T. Berndt, and R. Kumar, *Regulation of phosphate homeostasis by the phosphatonins and other novel mediators.* Pediatric Nephrology, 2008. **23**(8): p. 1203–1210.

19. Schiavi, S. and R. Kumar, *The phosphatonin pathway: New insights in phosphate homeostasis.* Kidney International, 2004. **65**(1): p. 1-14.

20. Berndt, T., S. Schiavi, and R. Kumar, *"Phosphatonins" and the regulation of phosphorus homeostasis.* American journal of physiology. Renal physiology, 2005. **289**: p. 1170–1182.

21. Brunelli, S. and S. Goldfarb, *Hypophosphatemia: Clinical Consequences and Management.* Journal of the American society of nephrology, 2007. **18**(7): p. 1999-2003.

22. Håglin, L., *Using phosphate supplementation to reverse hypophosphatemia and phosphate depletion in neurological disease and disturbance.* Nuttitional nueroscience, 2016. **19**(5): p. 213-223.

23. Askar, A., *Hyperphosphatemia.* Saudi medical journal, 2015. **36**(1).

24. Shamana, A. and S. Kowalski, *Hyperphosphatemia Management in Patients with Chronic Kidney Disease.* Saudi pharmaceutical journal, 2016. **24**(4): p. 494-505.

25. Howard, S., D. Jones, and C. Pui, *The Tumor Lysis Syndrome.* The New England journal of medicine, 2012. **364**(19): p. 1844-1854.

26. Freeman, H., Y. Kim, and M. Sleisenger, *Protein digestion and absorption in man.* The American journal of medicine, 1979. **67**(6): p. 1030–1036.

27. Dimski, D., *Ammonia Metabolism and the Urea Cycle: Function and Clinical Implications* Journal of veterinary internal medicine, 1994. **8**: p. 73-78.

28. Rothman, S., *How is the balance between protein synthesis and degradation achieved?* Theoretical biology and medical modelling, 2010. **7**(25).

29. Poortmans, J., et al., *Protein turnover, amino acid requirements and recommendations for athletes and active populations.* Brazilian journal of medical and biological research, 2012. **45**(10): p. 875–890.

30. Kondrup, J., *Basic concepts in nutrition: Energy and protein balance.* The European e-Journal of Clinical Nutrition and Metabolism, 2008. **3**: p. 117-120.

31. Arnal, M., et al., *Dietary control of protein turnover.* Diabete & metabolisme, 1987. **13**(6): p. 630-642.

32. Mitch, W., C. Jurkovitz, and B. England, *Mechanisms that cause protein and amino acid catabolism in uremia.* American journal of kidney diseases: the official journal of the National Kifnay Foundation, 1993. **21**(1): p. 91-95.

33. Reeds, P., *Dispensable and Indispensable Amino Acids for Humans.* The Journal of Nutrition, 2000. **130**(7): p. 1835–1840.

34. Fürst, P., *Basics in clinical nutrition: Proteins and amino acids.* Clinical nutrition, 2009. **4**(2): p. 62-65.

35. Nowicki, M., et al., *Changes in plasma phosphate levels influence insulin sensitivity under euglycemic conditions.* the Journal of clinical endocrinology and metabolism, 1996. **81**(1): p. 156-159.

36. Morris, R., K. Nigon, and E. Reed, *Evidence that the severity of depletion of inorganic phosphate determines the severity of the disturbance of adenine nucleotide metabolism in the liver and renal cortex of the fructose-loaded rat.* The journal of clinical investigation, 1978. **61**(1): p. 209-220.

37. Gravelyn, T., et al., *Hypophosphatemia-associated respiratory muscle weakness in a general inpatient population.* The American journal of medicine, 1988. **84**(5): p. 870-876.

38. Bregendahl, K., et al., *Fractional Protein Synthesis Rates Are Similar When Measured by Intraperitoneal or Intravenous Flooding Doses of L-[ring-2H5]Phenylalanine in Combination with a Rapid Regimen of Sampling in Piglets.* The Journal of Nutrition, 2008. **138**: p. 1976– 1981.

39. Bregendahl, K., et al., *Fractional Protein Synthesis Rates Measured by an Intraperitoneal Injection of a Flooding Dose of L-[ring-2H5]Phenylalanine in Pigs.* Journal of Nutrition, 2004. **134**: p. 2722-2728.

40. D'Alessandro, C., et al., *"Dietaly": practical issues for the nutritional management of CKD patients in Italy.* BMC Nephorlogy, 2016. **17**(1).

41. Buraczewska, L., et al., *Measurement of albumin synthesis in the pig by using L-[ring-2H5] Phenylalanine as a stable isotope tracer.* FAL, 2005. **55**: p. 245-253.

42. Sève, B., et al., *Protein synthesis and retention in some tissues of the young pig as influenced by dietary protein intake after early-weaning. Possible connection to the energy metabolism.* Reproduction Nutrition Développement, 1986. **26**(3): p. 849-861.

43. Nuttall, F. and M. Gannon, *Metabolic Response to Egg White and Cottage Cheese Protein in Normal Subjects* Metabolism: clinical and experimental, 1992. **39**(7): p. 749-755.

44. Hettleman, B., et al., *Defective adenosine triphosphate synthesis. An explanation for skeletal muscle dysfunction in phosphate-deficient mice.* Journal of Clinical Investigation, 1983. **72**(2): p. 582-589.