EVALUATION OF STORAGE CONDITIONS ON THE AMINO ACIDS, FATTY ACIDS, VITAMINS AND ELEMENTAL PROFILES OF AQUAFEED

THESIS SUBMITTED
FOR PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD
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DOCTOR OF PHILOSOPHY
IN
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Submitted by:

PARUL PURI
(Ph.D. Roll No. 2K19/PHDBT/07)

Under the Supervision of

Prof. Jai Gopal Sharma
Supervisor
Department of Biotechnology
Delhi Technological University
Delhi -110042

Prof. Ram Singh
Co-Supervisor
Department of Applied Chemistry
Delhi Technological University
Delhi -110042

To the
DEPARTMENT OF BIOTECHNOLOGY
(Formerly Delhi College of Engineering)

DELHI TECHNOLOGICAL UNIVERSITY
SHAHBAD DAULATPUR, MAIN BAWANA ROAD, DELHI-110042, INDIA

SEPTEMBER, 2023

Dedicated to

'The Almighty'

&

My Family

Who made it all possible

DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi College of Engineering)

Shahbad Daulatpur, Bawana Road, Delhi-110042, India

CERTIFICATE

This is to certify that the Ph.D. thesis entitled "**Evaluation of Storage Conditions on the Amino Acids, Fatty Acids, Vitamins and Elemental Profiles of Aquafeed"** submitted to Delhi Technological University, Delhi-110042, in the fulfilment of the requirement for the award of **Doctor of Philosophy** has been carried out by the candidate, **Ms. Parul Puri** (Reg. No. 2K19/PHD/BT/07) under the supervision of Prof. Jai Gopal Sharma, Department of Biotechnology, Delhi Technological University, Delhi, India and co-supervision of Prof. Ram Singh, Department of Applied Chemistry, Delhi Technological University, Delhi, India. It is further certified that the work embodied in this thesis is neither partially nor fully submitted to any other university or institution for the award of any degree or diploma.

Prof. Jai Gopal Sharma
Supervisor
Department of Biotechnology
Delhi Technological University
Delhi -110042

Prof. Ram Singh
Co-Supervisor
Department of Applied Chemistry
Delhi Technological University
Delhi -110042

Prof. Pravir Kumar
Head of Department
Department of Biotechnology
Delhi Technological University
Delhi -110042

DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi College of Engineering)

Department of Biotechnology

Shahbad Daulatpur, Bawana Road, Delhi-110042, India

DECLARATION

This is to declare that the research work embodied in this thesis entitled **"Evaluation of storage conditions on the amino acids, fatty acids, vitamins and elemental profiles of aquafeed"** submitted to the Department of Biotechnology, Delhi Technological University, Delhi, India is an original work and carried out by me for the degree of Doctor of Philosophy under the supervision of Prof. Jai Gopal Sharma, Department of Biotechnology, Delhi Technological University, Delhi, India and co-supervision of Prof. Ram Singh, Department of Applied Chemistry, Delhi Technological University, Delhi, India. This thesis is a contribution to my original research work. The extent of information derived from the existing literature has been indicated in the body of the thesis at appropriate places giving the source of information. Every effort has been made to make sure that the scientific contributions of others are appropriately cited. To the best of my knowledge, this research work has not been submitted in part or full for award of any degree or diploma in Delhi Technological University or in any other University/Institution.

Date:

Place:

Parul Puri

Research Scholar

Reg. No. 2K19/PHD/BT/07

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ABSTRACT

Aquafeed performance improvement provides an opportunity to enhance the sustainability of aquafarming practices, sourcing the expanse of this 'fast-growing food sector' catering to global nutrition requirements. Aquaculture productions are largely dependent on availability of quality aquafeeds that govern fish nutrition. World over, nearly half of the aquaculture production is feed-based. With growing food fish demand, feed-based aquaculture will dictate future aquaculture growth and sustainability. Moreover, sustainable aquaculture growth will be dependent on finding alternative feed ingredients as suitable replacements to compensate fishmeal, fish oil scarcity and impelled increments in feed costs. Judicious storage of feed can suitably meet nutritional demands of fed-aquaculture. Largely, the nutrient profile of aquafeed determines fish welfare and consequent consumer health. Additionally, proper storage of feed have important role in the economic, health and welfare aspect of aquaculture production. Appropriate storage and timely utilization of feed can prevent induction of food linked hazards in the food chain. Storage conditions, especially duration and temperature are important factors affecting biochemical profile viz; fatty acid, protein, amino acid and vitamin composition as well as microbiological quality of feeds. Effects of duration and temperature variables on stored feed rations requires timely assessments as a measure for assurance of feed quality.

Present work is designed to evaluate effect of different storage conditions on feed nutrient quality. The study aims at assessing incurred losses in nutritional quality of compounded feeds stored over long-term duration under variable condition of storage temperatures. This work also aims at determining the effects of feed composition and feed processing technique on the nutritional quality of feeds.The study is based on evaluation of impact of storage variables, temperature and duration, on the quality parameters of

formulated aquafeeds. Greater duckweed, *Spirodela polyrhiza* was used as alternative feed ingredient for partial substitution of fishmeal in extruded diet (diet1) and non-substituted pelleted diet (diet 2) is taken as control. Feeds were stored at four different temperature conditions comprising low temperatures (LT1= -20^{o} C, LT2= 4^{o} C); ambient (AT = 17^{o} C - 31.5^{o} C for diet1; 15.8 ^{o} C -31 ^{o} C for diet 2) and high temperature (HT= 45^{o} C), for six months. Bimonthly assessment of nutritional profile for biochemical composition; ash, moisture, crude lipid, crude protein, carbohydrates as nitrogen free extract (NFE), is performed along with changes in storage profile of essential, non-essential, free amino acids; saturated, unsaturated, free fatty acids; fat and water-soluble vitamins; micro and macro-elements, gross energy (GE), assessing aflatoxin incidences, if any; at variable temperature conditions during storage. From the results it is noteworthy that storage temperature and duration have highly significant effects ($P< 0.05$) on changes in crude protein, crude lipid, moisture, NFE, and GE content of feeds. Incurred losses in vitamins A, E, K, B2, B12 and C are noteworthy across storage duration 60-day onwards, at all temperatures, for both diets. Elemental interaction and moisture notedly impact element profile changes. Aflatoxin incidences unreported in the assessments denote effects of dietary combats, good storage, and packaging conditions. Significant losses, from initial to six months are noteworthy for total saturated, monounsaturated and polyunsaturated fatty acids (n-3PUFA, n-6PUFA) for both diets at all storage temperatures. There is an overall decrease in total essential and total non-essential amino acids along storage duration. Overall best restorative conditions for most nutrients is determined as, within two months of LT1 storage. The study helps understand timely utilization of stored feeds for maximizing their nutritional benefit to the fish. Through this work, assessed percentage loss of each nutrient in compounded diets may help specify need to develop feed formulae accounting incurred loss of nutrients during the storage period. Substantially, these findings may provide helpful information for fish farmers in managing feed storage of formulated feeds with an aim to

prolong their shelf-life; safeguarding significant amount of the total production costs of fed-aquaculture.

ABBREVIATIONS

ALA	Alpha linoleic acid
AOAC	Association of Official Analytical Chemists
ARA	Arachidonic acid
ANOVA	Analysis of Variance
CL	Crude Lipid
CP	Crude Protein
DHA	Docosahexaenoic acid
DP	Dietary protein
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAME	Fatty acid methyl esters
FCR	Feed conversion ratio
FFA	Free fatty acid
FM	Fish Meal
FO	Fish Oil
FTIR	Fourier transform infrared
GC	Gas chromatography
GE	Gross energy
ICP-MS	Inductively coupled plasma-mass-spectrometry
MANOVA	Multivariate Analysis of Variance
MUFA	Monounsaturated fatty acid/s
N %	Nitrogen percentage
PUFA	Polyunsaturated fatty acid/s
SD	Standard Deviation
SE	Standard Error
SFA	Saturated fatty acid/s
SGR	Specific growth rate
TGA	Triglyceride
UPLC	Ultra-performance liquid chromatography

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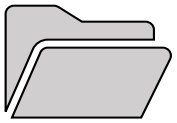


Chapter 1

INTRODUCTION

Global dietary need of quality protein and essential nutrients for human consumption are met from fish diet. Fish is a valued source of quality protein with relatively high amount of most essential amino acids (EAAs); omega-n3 essential fatty acids eicosapentaenoic, docosahexaenoic (EPA and DHA); micro, macro minerals; and vitamins; thus, play quintessential role in human diet as well as global nutrition supply. Digestibility and bioavailability of proteins, minerals and vitamins obtained from fish foods is higher compared to any plant-based food product. Amino acid composition, polyunsaturated acid (PUFA) profile, presence of bioactive peptides with protein digestibility greater than 90%, provides added health benefits of fish to human diet. Fish is growingly becoming global staple; in fulfilling food requirement of world population rural as well as urban.

Aquaculture has significantly contributed to global food fish demand with 52% share of total fish produce. Aquaculture and fisheries production reached recording high at 179 million tonnes (MT) in 2018 with an estimate to reach 186 MT in 2030 (FAO, 2022; World Bank, 2013). Compared to capture fisheries, aquaculture has contributed more fish for human consumption. Among aquaculture harvest, finfish dominated world fish production in 2018, amounting to 54.3 million tonnes (MT); largely 47 MT contributed from inland while the remaining 7.3 MT from marine sources (Puri et al., 2022; FAO, 2020). Finfish production in Asia amounts to >80% of the global total cultured fish yield (FAO, 2018). Aquaculture productions are largely dependent on availability of quality aquafeeds that govern fish nutrition. World over, nearly half (50%) of the aquaculture production is feed-based. With growing food fish demand, feed-

based aquaculture will dictate future aquaculture growth and sustainability. Aquaculture feeding practices followed in South Asia are of intensive (industrially produced pelleted feed), semi-intensive (mix of industrial and farm-made feed) and extensive / traditional type (on-farm-made feed as mixture of locally available ingredients). India is one of the largest and vastly upsurging compound feed markets in the world. In India, farm-made feeds account 70% of the total aquaculture feeds used (Giri, 2017) grossly representing over 97 percent of carp feeds used by farmers in India (Tacon et al., 2011).

Nutrition requirements in aquaculture can be accomplished through quality feeds as compounded diets contributing to growth and health of aquaculture species (Puri et al., 2022). Quality feed is significant for scaling up fisheries output by fulfilling the balanced nutritional requirements of fish. Formulation of aquafeeds for aquaculture development world over, is of great importance to fulfil dietary requirements of proteins, essential fatty acids, minerals, amino acids and vitamins in human diet; that itself is dependent largely on availability of quality feed ingredients (Hua et al., 2019). Although fishmeal and fish oil (FMFO) have remained gold standards for aquafeed formulations, sustainable aquaculture growth will be dependent on finding alternative feed ingredients as suitable replacements to compensate FMFO impelled increments in feed costs. Increments in feed performance along with the use of non-conventional feed sources can enhance environmental, economic (cost), and societal footprint (nutritional fulfilment, increased fish produce), adding sustainability to aquaculture practices, accredited to be "fastest-growing food-producing sector" (FAO, 2018).

Duckweeds, freshwater macrophytes belonging to family- Lemnaceae; due to their high nutritional value are sought as promising alternative source for protein (Chakrabarti et al., 2018). Nutritional content of S*pirodela* and Lemnaceae in general is relatable to that of animal feed (Pípalová, 2003). Additionally, essential amino acid compositions of duckweed species are comparably higher to most cereal grains and FAO reference intake values for humans (Xu et al., 2021). Inclusion of duckweed in fish diet can significantly improve growth parameters as well as nutrimental quality of fish (Shrivastav et al., 2022). Prospected for their valuable resource potential, duckweeds can contribute to sustainable aquaculture productions, with minimized environmental impact; assuring global food security.

Feed composition, quality of raw materials used, moisture content, processing technique, storage practices involved, evidently impact the overall feed quality. Nutrient composition of aquafeeds influences utilization of feed by fish and consequently fish growth. There is a larger scope towards increase in fish production as well as their nutrient value for human consumption by judicious use of fishmeal and fish oil regraded as gold standards in aquaculture and fisheries practices, as well improving feed formulations and management of processed feed during storage, handlings; in farming environment. While employing choice of ingredients for aquafeeds; quality of feed and feedstuffs, storage, as well as storage handlings are essential considerations (Giri, 2017). Feed storage is imperative to overcome scarcities of feed supply, produce; maintaining continuous resource of ration to meet timely demands of aquaculture. Sustainability in aquaculture thus, can be achieved by using equitably sustainable food sources in feed compositions, as well as provisioning

quality nutrition from stored rations, required over time. Storage help administer timely feed requirements in aquaculture; nonetheless, improper storage or storage handlings incur feeds susceptible to accelerated deterioration (Kop et al., 2019).

Changes in the standard feed characteristics is usually indicative of problems regarding quality. Largely, problems associated with resulting low-quality fish feeds are improper uptake, stunted growth, increased feed conversion rate (FCR) and decreased fish survival. Proper storage of feed is thus imperative for safeguard of feed quality and ensuring overall fish health and consequent consumer benefit. Moreover, the nutritional quality of feed ingredients and fish feeds are significantly influenced by storage conditions such as temperature, humidity, moisture content of feeds, storage duration, light and post-handling procedures (Aanyu and Ondhoro, 2017). Produced feeds are stored under variable storage conditions during distribution and farming with less contemplation on the impact of alterations on nutrient value (Solomon et al., 2016a). Effects of duration and temperature variables on stored feed rations requires timely assessments as a measure for assurance of feed quality. Storage conditions, largely temperature and humidity are critical influences affecting biochemical profile viz; fatty acid, protein, amino acid and vitamin composition as well as microbiological quality of feeds throughout storage duration (Riaz et al., 2009; Hossen et al., 2011; Zmysłowska and Lewandowska, 2000). Environmental temperatures greater than 27°C, humidity high above 62%, and feed moisture exceeding 14%, promotes aflatoxins production in feeds (Dirican, 2015). Feed stored at ambient temperature greater than three months is predisposed to the cessation of vitamins and oils causing

lipidic rancidity following peroxidation (Solomon et al., 2016a). Compounded diets are highly susceptible to storage effects due to variable temperature and humidity (Peitsch, 2020). High temperatures denature nutrients and cause loss of nutrients (Aanyu and Ondhoro, 2017). Feed ingredients composed of highly polyunsaturated fatty acids, including FM, FO are predisposed to oxidative effects (Ahmed et al., 2016). High fat content feedstuffs are equivocally predisposed to storage effects (Chow, 1980). Feed storage at elevated temperature can increase rancidity leading to off-flavors and malodors resulting from lipid oxidation, with consequent loss in feed quality. Feed ingredients comprising long chain polyunsaturated fatty acids are susceptible to oxidations (Chow,1980). At temperatures > 30^{o}C fats are inherently unstable yielding ketonic acids upon hydrolysis, further undergoing auto-oxidation to hydroperoxide with generation of hazardous free radical polymerization products (Solomon et al., 2016a). Oxidative loss of lipids and proteins are most outstanding deteriorative changes in quality during processing and storage of aquaculture feeds. Autooxidation (= *in situ* oxidation) of lipids, proteins in feed can decrease their digestibility and biological availability (Geng et al., 2023) as well deplete abundance of natural antioxidants in feed ingredients such as vitamins (Kołakowska and Bartosz, 2014). Largely, diminution of feed vitamins is accentuated due to high temperature, humidity, extreme pH, light exposure, presence of elements and lipidic free radicals. Loss of vitamins owing to increased temperature and oxidation at time of processing and storage of feed is well noteworthy (Kavitha et al., 2004). Unfavorable environmental conditions render fish feeds to microbial attacks causing feed decomposition with disease incidence in fed fish (FAO, 2001). Unsuitable storage temperatures and

humidity may support pathogenic growth and survival in the feed, or even favour production of harmful fungal toxins such as aflatoxins, patulins and trichotecens, ochratoxin A (OTA), with potential teratogenic, carcinogenic, hepatotoxic, mutagenic and immuno-suppressive effects (Solomon et al., 2016a; Pietsch et al., 2020; Zmysłowska and Lewandowska, 2000). Aflatoxins (AFs) are the utmost hazardous natural contaminants in compounded feeds. Of 18 different known aflatoxins; AFB1, B2, G1 and G2 are of notable importance with AFB1 being prevalently toxic (Dirican, 2015). Concomitantly, contaminated feeds can potentially transmit carry over hazards of mycotoxins to tissues (ovary, muscle, serum, hepatopancreas) of farmed fish; incidentally posing health risk to the consumers (Wonzy et al., 2013; Han et al., 2010; Yang et al., 2020).

RESEARCH GAP

There is insufficient information on effects of storage periods on biochemical composition of animal feeds. There is an information gap on the effect of storage duration on the nutritional composition of food-stuffs significantly non-conventional foodstuffs some of these materials undergo processing exposing them to high temperatures and humidity that affect shelf-life. Appropriate shelf-life for such ingredients under available environmental conditions and good storage practices is not much documented (Aanyu and Ondhoro, 2017). There is paucity of data on study of vitamin, amino acid, element profiles of fish feeds stored under impacts of variable temperature conditions over longer durations in diverse farming environments. Literature regarding such works are few specifically for changes in vitamins, both fat- and water-soluble. Available data

on the effect of aflatoxins in aquaculture are very limited. It is to be ascertained whether exposures to unfavourable storage conditions can possibly augment mycotoxin incidence in fish feeds (Dirican, 2015). In terms of Indian scenario, Bureau of Indian Standard (BIS) specifications for fish feed standards is limited and there is requirement for set guidelines on acceptable levels of quality parameters of aquafeeds (Ebeneezar et al., 2018).

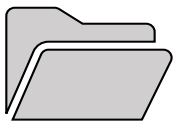
Evidently, the nutrient profile of aquafeed determines fish welfare and consequent consumer health; the study is outlined to evaluate impact of storage variables on feed nutrient quality. Present work is based on evaluation of impact of variables, temperature and storage duration on quality parameters of formulated aquafeeds using non-conventional feed source to compensate fishmeal requirement and fishmeal-based control diet; stored for six months under low temperatures (LT1= -20^{o} C, LT2 = 4^{o} C); ambient (AT = 17^{o} C - 31.5^{o} C for diet1; 15.8 ^{o} C -31 ^{o} C for diet 2) and high temperature (HT= 45^{o} C) conditions. The study aims at assessing incurred losses in nutritional quality of stored feeds over long-term storage duration under variable condition of storage temperatures. This work also aims at determining the effects of feed composition and feed processing technique on the nutritional quality of feeds.

Following objectives are undertaken in the study,

Objective 1: Formulation of aquafeeds (extruded, pelleted diet) using various locally available ingredients; packaging and storage of feeds.

Objective 2: Analysis of biochemical composition (moisture, protein, lipids, ash, amino acids, fatty acids, vitamins); element profile and calorific value of formulated feeds.

Objective 3: Study of impact of storage conditions (viz., temperature, duration) on the biochemical composition, element profile and calorific value of feeds.



Chapter 2

REVIEW OF LITERATURE

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2.1 Nutrient and Energy Requirements of fish

Feed is a source of nutrients and energy, fundamental for growth, reproduction, and fish health (NRC, 1993). Feed is the foremost important input for fish welfare and health in sustainable intensive aquaculture productions. Nutrient quality and safety are two most important attributes of healthy diet (HLPE, 2017). Quality feed is important for scaling up fisheries outturn by fulfilling the balanced nutritional requirements of fish (Puri et al., 2022). Sustainable, quality feeds can confer health benefits to aquaculture and consequently to humans (consumers). Aquafeed performance improvement provides an opportunity to enhance the sustainability of aquafarming practices, sourcing the expanse of this fast-growing food sector catering to global nutrition requirements (Ghamkhar and Hicks, 2021). In terms of quantity as well as quality, dietary requirements of fish vary as per life stage of the species, feeding habits and environmental fluctuations of temperature, salinity and natural food availability in the culture environment (Giri, 2017). Formulating balanced, least expense feed based on the nutrition and health needs of fish is thus an essential prerequisite (Ahmed and Ahmad, 2020). For cost-effective, economical production, diets must be formulated in agreement with the elementary nutritional needs of the specific species, containing accurate apportionment of protein, lipid, carbohydrate as well as energy gains.

2.1.1 Protein and amino acid requirement

Protein stands as the most valuable and expensive constituent in fish feed compositions, in terms of levels required for incorporation and quality it offers, directing the feed cost (Fatma and Ahmed, 2020). Fish require protein for

growth accrual and survival. Proteins comprise 50% of dietary constituents in fish feeds (Benitez,1989). Dietary needs of protein for fish is nearly 2 to 4-fold higher than other vertebrates (Wilson, 2003). Carnivorous species have greater protein requirements (40-50% crude protein) than omnivore and herbivore (25-35%) fishes (Jauralde et al., 2021; NRC,1993; Gatlin, 2010). According to Tacon and Cowey (1985); Wilson (1989) for maximal growth dietary crude protein requirement of cultured fish varies between 300-550 g per kg of diet. In a meta-analysis approach Teles et al. (2020), enlist that fishes require 624g protein to achieve 1kg weight gain nearing protein retention efficiency (PER) to 32 percent. Dietary protein necessities are relatable to trophic level, salinity, rearing temperature, stock size, frequency of feeding, non-protein source of dietary energy, diet quality of protein (Fatma and Ahmed, 2020; Teles et al., 2020; Shimeno et al.,1980). Proteins comprise mix of amino acids as building blocks. Fish requirement for protein is in terms of utilization of amino acids specifically those it cannot synthesize. These amino acids are nutritionally essential (EAA) and offered exogenously from diet source. Amino acid profile (AA profile) thus is a marked indicator of nutritional quality of dietary proteins (Benitez,1989). According to Nunes et al. (2014), nutritional and economic gains from a protein are dynamics of digestibility and AA composition. Moreover, Peres and Oliva-Teles (2008), suggest AA profile of whole body of fish to be largely interrelated to their EAA needs. Besides, in an “ideal protein”, ratio between EAA to non-essential AA (NEAA) remains constant; despite variations in each AA requirement across life stages of fish (Bicudo and Cyrino, 2014; Ogino, 1980). NEAA in fish nutrition includes alanine, proline, asparagine, cystine, aspartate, glycine, serine, glutamine, tyrosine and glutamate (Li et al.,

2011; Gatlin, 2010). NEAAs such as cystine (cys) can be synthesized from methionine (met); tyrosine (tyr) synthesis occurs from phenylalanine (phe). Thus, appropriate EAAs can supplement NEAA requirement (Wilson, 2003; Nunes et al., 2014). High amounts of NEAA present in animal origin proteins can reduce energy cost and EAA requirements for their *de novo* synthesis in animals, causing improved feed efficiencies (Li and Wu 2018, 2020). Dietary protein requirement has twin purpose; to provide both EAA (that fish cannot synthesize) and NEAA (as pool of NH_2 nitrogen to synthesize AAs). Since NEAA biosynthesis is energy driven, dietary proteins that suffice the requirements of fish for both EAA and NEAA will contribute to most efficient fish growth (NRC,1993; Li and Wu, 2020).Together EAA and NEAA comprise proteinogenic amino acids (PAAs).

Adequate met and lys levels can augment uptake and utilization of other EAAs since they can lower oxidative rate of other AAs (Kerr and Easter, 1995). Met is involved in glutathione biosynthesis, together they function as antioxidant system (Wang et al., 2023). High quality fishmeal (FM) has balanced amount of all EAAs predominantly lysine (Miles and Chapman, 2006); n3 omega polyunsaturated FAs chiefly, DHA as well as EPA; essential minerals and vitamins; with 85% of aquaculture species relying on fish meal (FM), from feed (Jeyasanta and Patterson, 2020). Fishmeal replacement owing to its scarcity and incremental feed cost (Naylor et al., 2009) has shifted focus to marine, plant-based sources of equivalent protein provisions. Although, many plant-based feedstuffs and harshly processed ingredients of animal origin (used in preparation of artificial diets for fish) are deficient in met and lys enlisted to be initial-limiting AA (Gatlin et al., 2007; NRC, 2011). Non-proteinogenic amino

acids (NPAA) (listed in table 4.2b, 4.3b) are not directly involved in protein synthesis and are themselves result of indirect conversions (CHEBI; Waarde, 1988). NPAAs contribute to the biosynthesis of PAAs, enable ammonia detoxification, and supply auxiliary form of transportable nitrogen. Additionally, NPAAs such as taurine, creatine in animal-source feedstuffs are significantly involved in antioxidant mechanisms and energy metabolism in tissues predominantly brain, skeletal muscle, heart, and gonads (Wu, 2013). Amino acids thus have multifarious role in fish functions involving protein synthesis, growth, metabolic processes, synthesis of neurotransmitters (val, leu, ile, phe, tyr, tryp, glu-NH_2); ammonia detoxification (orn, Glu-NH_2, arg), lipid oxidation (arg), inhibition of protein degradation (val, leu, ile); immunostimulation, antioxidant (met, cys) and osmolytic properties (sarcosine, sar; taurine, tau) (Andersen et al., 2016; Ahmed and Khan, 2006; Waarde, 1988).

Table2.1 Indispensable amino acid, EAA requirements of fishes (as g/100g diet = % of diet,dry weight basis).

Amino Acids	*L. rohita*	*C. catla*	*C. carpio*	*I. punctatus*	*S. salar*	*A. japonica*	*O. niloticus*	*O. mykiss*	*O. tshawytscha*
Arginine	2.30	1.9	1.6	1.0	2.0	1.7	1.18	2.0	2.4
Histidine	0.90	1.0	0.8	0.4	0.7	0.8	0.48	0.7	0.7
Isoleucine	1.20	0.9	0.9	0.6	0.8	1.5	0.87	0.8	0.9
Lysine	1.50	2.5	2.2	1.2	1.8	2.0	1.43	1.8	2.0
Leucine	2.27	1.5	1.3	0.8	1.4	2.0	0.95	1.4	1.6
Methionine	1.42	1.4	1.2	0.6	1.0	1.2	0.75	1.0	1.6
Phenylalanine	1.48	1.5	2.5	1.2	1.2	2.2	1.05	1.2	2.1
Threonine	1.71	2.0	1.5	0.5	0.8	1.5	1.05	0.8	0.9
Tryptophan	0.45	0.4	0.3	0.12	0.2	0.4	0.28	0.2	0.2
Valine	1.50	1.4	1.4	0.71	1.3	1.5	0.78	1.3	1.3

Table 2.1 lists indispensable EAA requirements of juvenile stages of *Labeo rohita*, *Ictalurus punctatus, Catla catla*, *C. carpio*, *Oncorhynchus niloticus,*

Anguilla japonica, O. tshawytscha, Salmo salar and *O. mykiss* (FAO, 2013; Kaushik,1995 ; NRC 1993).

2.1.2 Lipid and fatty acid requirement

Lipids have significant role in fulfilling essential fatty acid (EFA) and energy requirements for fish physiological functions. They are essential constituents of cell membranes, mediators in reproduction, stress response, dictating immune state, survival and overall health of fish (Arts and Kohler, 2009). Fatty acids (FAs) are organic acids with a carbon chain containing terminal carboxyl group. According to the presence, inclusion of total double bonds, FAs are classified as saturated, monounsaturated (SFAs, MUFAs) and polyunsaturated (PUFAs) (Chen and Liu, 2020); depending on chain length of C1-C6 carbons as short chain (SC), C7-12 medium chain (MC), more than C14 as long or highly chained (LC or HC) fatty acids (Schönfeld and Wojtczak, 2016; NRC,1989).

Dietary lipids are primarily incorporated in feed formulations in order to augment sparing effect on dietary protein (Hasan, 2001; Watanabe,1982). Among PUFA types, n3,n6,n9 FAs are found in all animals, including fish; with regulated ability to synthesize *de novo* only n9 PUFAs. Both n3 and n6 PUFA are essential (EFAs) and need to be obtained from diet sources to fish. EFA needs of fish vary across species. Marine fish require n3LC-PUFA (EPA, DHA); rainbow trout n-3 fatty acids such as ALA 18:3 n3 and n3HC-PUFA; carp nutrition needs are for both n3, n6 FAs and tilapia requires n6 LA (18:2 n6) (Takeuchi et al., 1991; Hasan, 2001; Opstvedt, 1985). Plant-derived oils used in aquafeeds (such as sunflower, linseed, soybean) are rich sources of n6-series of SC-PUFA, and MC-PUFAs (LA C18:2, n6). n3 LC-PUFAs (= heavy chain PUFA, HC-PUFA) of larger interest DHA 22:6 and EPA 20:5 are opulently found in marine origin

oils (such as FOs, algal oils) and animal fats (Gonzalez-Silvera, 2016). n3PUFA, EPA and DHA are important for growth, cardiovascular health, anti-inflammatory response, neural and brain development. PUFAs have biological role in plasma membranes composition and fluidity, gene transcription regulation, cell signal modulation (Czumaj and Sledzinski, 2020; Arts and Kohler, 2009). Table 2.2 lists EFA deficiency symptoms reported in fishes. All fishes require EFA at 1-2% of diet, as per dry weight (Hasan, 2001). Recommended intake of EPA and DHA for humans lies between 200-500 mg/day (Strobel, 2012) with need of dietary n3:n6 ratio above or at least equal to 1:1 (Simopoulos,1991). Dietary percentage of lipids required by fish is dependent on lipid type as well as digestible energy to protein ratio, DE:P in diet (NRC,1993).

Table 2.2 EFA deficiency symptoms reported in fishes

Fish	Deficiency symptom
C. carpio	High mortality, fatty liver
Ctenopharyngodon idella	Lordosis, decreased growth and feed efficiency, shock syndrome
O. mykiss	shock syndrome, liver degeneration, high mortality
O. niloticus	Swollen pale liver
Lates calcarifer	Effects growth, feed efficiency, fin reddening
Scophthalmus maximus	High mortality, decreased growth, gill epithelium degeneration

Source: Tacon (1992); Takeuchi et al. (1991),(1990).

2.1.3 Vitamins

Vitamins are vital organic substances required in trace quantities for important physiological functions by fish. As per their miscibility in aqueous environment,

vitamins are classified as water and lipid-miscible forms. Water-soluble vitamins comprise vitamin B-group including in them B1, 2, 3, 5, 6, 7, 9,12 (namely thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folate, cobalamin) and ascorbate (vit C). Due to their hydrophilic property these vitamins are miscible in aqueous solutions. They function mainly as coenzymes contributing to metabolic processes (B1, B2); in red blood cell formation, fatty acid- and amino-acid metabolism (B6, B12), amino acid interconversions (B6); bone formation, collagen synthesis and immunoregulatory functions (vitamin C) (NRC, 2011). Vitamin C is the most labile water-soluble vitamin, known for its antioxidant properties. Dietary inclusions of vit. C is found to improve growth, hematological parameters, carcass nutrients and feed conversion ratio in *Labeo rohita* (Afrin and Rahman, 2019). Fish like humans lack capability of vit. C biosynthesis and can fulfill it through dietary supplementation. In fishes, dietary deficiency of vit. C can impair collagen synthesis leading to scurvy, lordosis, scoliosis, impaired wound healing, lethargy and anemia symptoms (Sandnes and Utne,1982; Tucker and Halver,1986). Vit C is a co-substrate for enzymes involved in the biosynthesis carnitine, and neurotransmission. Nasar et al. (2021), recommend 67.17 mg/kg of ascorbate in grass carp feed for optimum growth parameters of juveniles. Fat-miscible vitamins A, D (calciferols), E (tocopherol), K are bound to lipophilic fraction in feeds and are transported, as well absorbed, in similar fashion as fats (NRC,1989). These vitamins have antioxidant properties (vit. A, E), hematological (vit. K) and immunostimulatory functions (A,D) hence contribute to fish and consumer health. Vitamin A has essential function in vision, embryonic development, growth, reproduction, and differentiation (Hernandez and Hardy, 2020). Vitamin E can prevent fragility of

erythrocytes and due to its anti-oxidant mechanism prevent free radical oxidation of dietary (PUFA) and membrane lipids (Sau et al., 2004). Vit E requisite for rohu fry is determined as 131.91 $mgkg^{-1}$ dry weight of feed (Sau et al., 2004), and for Atlantic salmon at diet conc. 120 $mgkg^{-1}$ (Hamre and Lie, 1995).

2.1.4 Elemental Requirement

Minerals are inorganic elements obtained from diet and aquatic surroundings by fish. In addition to dietary elements, fish absorbs minerals from surrounding waters (Watanabe et al.,1997). Therefore, dietary requirement for particular element to fish will largely be affected by its available concentrations from aquatic source to fish. Elements are of types macro-elements, those required in large amounts accounting sodium, calcium, chlorine, potassium, magnesium and phosphorus. Micro-elements are minerals with requirements in trace amounts to fish, less than 100mg/kg dry diet. Elements have role in regulating acid base; balance, formation of skeleton, bone density, colloidal regulation (osmotic balance, viscosity) and to aid hormones and enzymes in biofunctions (Chanda et al., 2015; NRC,1993). P insufficiency signs comprise reduced feed efficiency, and bone mineralization and retarded growth. Vit D plays vital role in metabolism of Ca. P and Ca are required by the fish for bone formation (Braga et al., 2016). Both Ca and P have antagonistic interaction, affecting their bioavailability (Chavez-Sanchez et al., 2000). Magnesium is essential for cartilage and bone formation. K is essential for synthesis of protein and glycogen, along with Na it regulates acid base balance, osmotic pressure inside cells (NRC, 1993; FAO, 2017; Webster and Lim, 2002).

Iron, cobalt, chromium, zinc, copper, iodine, manganese and selenium are of great importance in small amounts to fish. Trace element requirements of fish for Fe ranges 30-170 mg/kg dry diet, selenium 0.15-0.5 mg/kg, Zn 15-40 mg/kg, Mn 2 to 20, Cu accounts between 1to 5 mg per kg and cobalt 0.05 to1 mg per kg dry feed (Watanabe et al., 1997). Copper takes part in activity of enzyme cytochrome oxidase; while Mn is essential for development of brain, metabolism of dietary lipid, carbohydrate for fish. Zinc is a cofactor for various enzymes and assists wound healing (FAO, 2017). Fe is involved in redox reaction and respiratory electron transfer processes. It has catalytic role in free radical sequestration, and in presence of unsaturated FAs can cause lipid oxidations, generating lipid radicals and hydroperoxides. Thus, under aerobic conditions, Fe interaction can deteriorate feed lipid, causing rancidity. Vitamin C supplementation at 143 and 573 mg/kg diet to catfish (*Ictalurus punctatus*) diet, is found to ameliorate iron-induced toxic effects; improving hemoglobin content and hematocrit values (Yadav et al., 2020).

Selenium exists as seleno-proteins with their role in maintenance of redox homeostasis and in glutathione peroxidase enzyme system (Lall and Kaushik, 2021). Interaction of selenium with Vit E has role in antioxidant mechanisms pertaining to hydroperoxide detoxification (Kołakowska and Bartosz, 2014; Hilton,1989).

2.1.5 Energy Requirement

Energy itself is a non-nutrient and is obtained from metabolization of dietary nutrient sources of fats, proteins and carbohydrates (NRC,1993). Gross energy (GE) value of feed, is the heat generated on complete ignition of feed

compounds. GE is a measure of absolute energy offered from organic dietary constituents to the organism. It is measured by burning compounded feed under controlled oxygen pressure in a thick metal walled container known as 'bomb' through which the technique finds name as bomb calorimetry (NRC, 2011). Determination of GE is the beginning point of evaluation of other energy estimates from feed, metabolizable (ME) and digestible energy, DE (Weiss and Tebbe, 2018). Total intake energy (IE), of fish from diet is relatable to GE content of feeds. In a compounded diet, GE is a contribution of its constituents each with their mean energy contribution; protein (23.6 kilojoules per g diet), lipids and carbohydrates (39.5,17.2 kilojoules per g) (NRC, 2011). Thus, it is important to evaluate energy estimates of diet available for utilization by fish. Formulation of feed based on knowledge of energy utilizations of fish at various stages of life history would be cost effective approach preventing over addition of nutrients (NRC,1993). GE of feed ingredients is fundamental to precise feed formulation for fish performance and sustainable environment effects (Sayed et al., 2018). Retention of energy by fish for growth and other physiological functions (ME) is partly dependable on available energy from feed (GE), along with type, life history stage and culture conditions of fish. Feed formulations ideal for one species may not be optimal for other species and is also a function of culture conditions for fish development (Sales, 2009). Optimization of digestible protein (DP) and DE content (DE = GE of feed subtracting GE lost to feces) (Zuidhof, 2019), of fish diet is the foremost consideration in feed formulation (White, 2013; Happel, 2020). For cultured fish species, these ratios range between 81 -117 mg DP per kcal of DE (NRC, 2011). DE requirements for fry to fingerling stage of fishes such as carp, rohu, mrigal, silver and grass

carp scopes between 3500 to 4500 Kcal/kg of diet at level of 37%- 47% protein, 30% carbohydrate, and 7% lipid in diet (FAO, 2017; Giri, 2017). Alongside, biochemical-proximate considerations; energy digestibility values are important in choice of dietary ingredients; for least cost formulation; feed quality assessment for ingredients, feed processing and storage conditions.

2.2 Formulation of feeds

2.2.1 Formulated feeds and fishmeal replacement

Conventional feed ingredients in feed formulation including wheat, corn, oilseeds; agricultural by products such as rice bran, soybean and groundnut oil-cake, are competitively priced commodities lurching to fulfil agricultural food demands alongside livestock feed developments from parallel land use (Pahlow et al., 2015). Fishmeal is a sought for animal protein used in aquaculture feed formulation. Aquaculture dependence on FM and FO accounts 63%, 81% of their respective global supplies specifically concerning valued farmed carnivorous varieties such as salmon (HLPE, 2014; WRI, 2013). Demanding pursuit for alternative sources of protein and oil, wholly or partly substituting FM and FO in aquafeeds (Silva et al., 2010) is in accordance with the FAO recommendations on lessening their consumption from extract fishing, and to meet sustainable UN goals for fisheries and aquaculture. In this direction much focus has shifted in finding and use of non-conventional alternative feed source towards aquaculture growth and developments (Giri, 2017; HLPE, 2017). Terrestrial ingredients such as plant proteins, plant-derived oils, rendered animal by-products (insect meal and poultry product meal) for aquaculture can pave way for sustainable productions, minimizing economic and environmental

footprint (Pleic et al., 2022; Naylor et al., 2009). Potential of aquatic origin macrophytes such as duckweeds have been explored as alternative feed ingredient. Duckweed belong to family Lemnaceae. Nutritional content of *Spirodela* and Lemnaceae in general shows rich EAA and fatty acid profiles of n3 and n6 (Chakrabarti, 2018) and is relatable to that of animal feed (Pípalová, 2003).Partial inclusion of duckweed in fish diet can significantly improve growth parameters as well as nutrimental quality of fish (Shrivastav et al., 2022). Marine algae is a source of omega-3 fatty acids comparable to FM, FO (Camacho-Rodríguez, 2018). Microalgae and algae oil have benefits of high growth rate alongside high antioxidation potential, probiotic, prebiotic effects in restoring healthy gut microbiota for fish (Nagappan et al., 2021). Prebiotic effects of orally administered of insoluble microalgal polysaccharide-enriched extracts (MAe), and whole *Phaeodactylum tricornutum* cells to Senegalese sole (*Solea senegalensis*) are studied (Puri et al., 2022; Carballo et al., 2019). MAe effected transient systemic anti-inflammatory response in sole. Whole microalgae *P. tricornutum* delayed activation of IL-1β, largely influencing intestinal microbial diversity of fish. In aquaculture, particulate β-glucans (prebiotics) are known for their enhancement effect on both non-specific and specific immune parameters (Vetvicka et al., 2013; Carballo et al., 2019).

2.2.2 Feed processing: Extruded and Non-extruded pelleted feeds

Extrusion is a sought after technique for aquafeed processing with merits of feed nutritional quality improvement. Extruded diets provide enhanced feed digestibility owing to diminution of anti-nutritional factors (tannins, phytates, phenols) and pathogenic interferences from diet (Sorensen, 2009). Hot-extrusion is performed at temperatures above 100°C forming floating diets for

surface feeders such as, *O. niloticus* ; cold extrusion identifies at ambient temperatures producing sinking pellets for bottom dwelling fish including *Clarius gariepinus* (Choton et al. 2020). Were et al. (2021), demonstrated benefits of hot extrusion processing of feeds in reducing microbial interferences in insect and larval meal replacement of FM diet for Nile tilapia and African catfish.

Extrusion cooking affects nutritional quality of diet at various degrees. In its beneficial aspect, in addition to destruction of antinutrient factors, extrusion diets contain gelatinized starch, improved soluble dietary fiber; increasing its digestibility to fish and feed physical properties in water (Welker et al., 2018; Glencross et al., 2011). On the contrary, depending on extrusion conditions heat susceptible vitamins can be lost, with protein-sugar reactions at high extrusion temperatures deteriorating feed nutrient profiles (Singh et al., 2007). During extrusion process feed is mixed, sheared and heated under high temperature, high pressure with extrudate leaving the die of proper dimension. Extruder parameters temperature, shear, screw type, pressure, size of die; and diet composition have great influence of physical and nutritional quality of extruded feed (Sorensen et al., 2005). Riaz et al. (2009) evaluated vitamin stability due to extrusion, and pelleting procedures at three months storage, reporting extrusion loss of thiamine (88%) compared to pelleting loss (60-96%). Even coated forms of ascorbate are largely lost at high rates to extrusion. Most susceptible vitamins to extrusion were thiamine (due to high shear rates of extrusion), vit A, C and folate. Yang et al. (2020b), evaluated impact of extrusion temperatures and encapsulation procedure on vitamin stability to conclude high stability of micro-encapsulated vitamin compared to un-encapsulated forms in feed. Storage loss of vit A is reported due to oxidative influences (Harper,1988).

Since many parameters need to be optimized for extrusion processing of feeds, the technique require precise controlled operating conditions to restore maximal nutrient benefits from feeds. Pelleting aggregates ingredients into large homogenized particles under effect of heat, pressure and moisture (Lovell,1980). Pelleted diets in comparison to extruded diets are dense with low floatability. Pelleted diets are less costly than extruded feeds thus provides cost effective feeds. Pelletization process require steam water addition to diet mixture and have high moisture content that must be reduced for their use as fish diets. Extruded feed are more resilient to dissociation in water and produces low density floatable diets (Khater et al., 2014), that allow aqua culturist to account amount of feed consumption by fish.

2.3 Storage loss of quality

Proper storage of feed is imperative for safeguard of feed quality, ensuring overall fish health and consumer beneficence. Storage loss of lipids, proteins and moisture are most evident biochemical changes in feed. While changes in ash content is also indicative of feed nutritional quality changes. Total ash percentage provides extent of mineral content of the feed (DuPonte, 2009), high ash content is indicative of adulteration or presence of impurities. Larger problems associated with resulting low-quality fish feeds are poor uptake, stunted growth, increased FCR and decreased fish survival.

Aflatoxin are reported to be most toxic natural contaminants in compounded diets. Toxins can pose risk of bioaccumulation in farmed species and thereby to human health and safety. Several works have revealed retention of AFB1 residues in tissues of aquatic organisms, indicating likely consumer health risks

(Han et al., 2010; Santacroce et al., 2011). Aflatoxins can cause disease incidence in fish by imposing nutritional deficit upon destroying essential nutrients in the diet, that include fat and water soluble antioxidants, vitamin A, C and thiamine. Aflatoxins depress the immune system of fish making it susceptible to pathogenic incidences. This decreases efficiency of aquaculture production due to stunted fish growth, reduced produce weight, increase of feed input and therapeutic costs, enhancing overall cost of fish production in aquaculture farming (Dirican, 2015).

2.3.1 Oxidative loss of proteins and lipids

Feed storage at elevated temperatures can increase oxidative and hydrolytic rancidity leading to off-flavors and malodors resulting from lipid oxidation, with consequent loss in feed quality. At temperatures >30^{o}C fats are inherently unstable yielding hydroperoxides that further undergo auto-oxidation generating free radical products. Oils with peroxide conc. <10 mg per kg diet are fresh, while between 20-40 mg per kg are rancid (Solomon et al., 2016a). Oxidative protein decrease parallels that of lipidic loss, on oxidation. This is due to free radical release of reactive oxygen entities (ROEs) to which both lipids and proteins are similarly prone to. These losses are more pronounced at high temperature storage compared to ambient and low temperatures (Hossen et al., 2011). Autooxidation of lipids, proteins in feed can decrease their digestibility and biological availability (Geng et al., 2023) as well deplete abundance of vitamins as natural antioxidants in feed ingredients (Kołakowska and Bartosz, 2014). Largely, diminution of feed vitamins is accentuated due to

high temperature, humidity, extreme pH, light exposure, presence of elements and lipidic free radicals.

2.3.2 Storage effects on vitamins

Vitamin depletion in feeds is accentuated by high temperature, humidity, photosensitization, high pH, presence of elements such as iron, copper and lipid oxidation polymerization reaction. Vitamin loss owing to high temperature and oxidation during processing and storage of feed is thoroughly examined in work by Kavitha et al., (2004). Among the vit, C and E (α-tocopherol) are readily oxidized by nascent oxygen generation. B2 is highly sensitive to photosensitization. Photosensitization (visible or UV light exposure) of riboflavin, under oxygenated conditions can form reactive singlet oxygen further capable of oxidative degradation of dietary proteins, lipids and other vitamins A,C,D and E (Choe, 2005).

Increasing temperatures relate to decreases in vit B2 retentions (Athar et al., 2006), with extrusion losses on storage up to 50%. Kubitza and Cyrino (1998) described higher B2 loss owing to heat, compared with thiamine. Extrusion losses are dependent on increasing shear rates with increase in screw speed, moisture (Yang et al., 2020b). Giannakourou and Taoukis (2021); Soliman et al (1987); reported that extrusion and pelleting feed processing procedures through moisture and heat addition, can result in decreased vit. C levels in formulated feeds causing rapid losses at extreme conditions. Vit C in aquaculture feeds is susceptible to processing, storage losses (Tucker and Halver,1986). Additionally, oxidative, non-oxidative and leaching losses of vit. C in fish feeds are reported. Use of high levels of PUFA fats in the feed

necessitate an increased level of vit C supplementation (Hung and Slinger, 1980); to utilize reducing potential of vit. C for preventing oxidation of fats. Vit E along with vit A, C have strong antioxidant properties owing to their free radical sequestration potential. Thus, are prone alike to oxidative effects during storage.

2.3.3 Storage and Elemental changes

Availability of minerals from diet is a function of their chemical state of occurrence, variability in feed ingredient and feed composition at large (Watanabe et al., 1997; Chanda et al., 2015). Moreover, it depends on dietary particle dimension and interactions from other diet constituents. Elemental availability is increased or decreased depending on cooperative or antagonistic interactions from diet. These include interactions of type, element to element (some as Ca-P, Zn-Cu, Mg-Ca, Na-K), vitamin-element (vitamin E with Se, vit C-Fe, vit D-Ca), protein-element (selenium-cystine), lipid-element interaction (Fe-PUFA) (Watanabe et al., 1997; Lall and Kaushik, 2021; Tacon and De Silva, 1983; Hilton, 1989). Anti-nutrients such as tannins, polyphenols can also change element availabilities out of antagonistic interactions in diet. Phytate chelates Ca and other microminerals including Zn, Cu, Fe reducing their biological availabilities to fish (Samtiya et al., 2020).

2.4 Storage strategies and management practices

Feed composition, nature of raw materials used, processing technique, storage and management practices evidently impact the overall feed quality. While feed composition as well as processing technique involved is the prerogative of

manufacturer; storage and management involves the responsibility of farmers and proper feed storage conditions (Cruz, 1994). Produced feeds are stored under different storage conditions by distributors as well as farmers with less consideration to their impact on the nutrient profiles of the feeds (Solomon et al., 2016a). Aquaculture feeds are prone to accelerated deterioration if not stored and managed properly.

2.4.1 Biochemical changes and assessments

Biochemical analyses are considered to be important indicators of feed quality. Biochemical assessments of lipids, proteins, FA, AA and elements are auxiliary measures to precisely assess overall wellbeing and underlying health condition of fish. Storage conditions, especially storage temperatures, duration and humidity are important factors affecting biochemical profile viz; fatty acid, protein, amino acid, vitamin and elemental composition, as well as microbiological quality of feeds. Important biochemical measurements include proximate composition for estimation of feed moisture, crude lipid (CL) and protein (CP), ash, carbohydrates and dry matter (DM), amino acid (AA), fatty acid (FA), vitamin and element profiles (Riaz et al., 2009; Hossen et al., 2011). Various works evaluating effects of storage conditions such as temperature, storage duration and humidity variables on biochemical parameter changes have been reported. Table 2.3 lists storage studies based on evaluation of nutrition profile and quality of fish feeds and feedstuffs. Hossen et al. (2011), compared effects of low and ambient temperature storages on proximate composition and physical characteristics of feeds. Deterioration of nutrient quality of feed is reportedly high at higher temperature, compared to cold

condition storages, suggesting better restoration of feed nutrients at low temperatures. Aanyu and Ondhoro (2017), investigated farm made feed as well as feed ingredients stored under airtight and open-air conditions over four months for proximate composition crude ash, CP, CL, DM and moisture. Notable trend in reduction of CP, CL, fibre, and DM of the feedstuffs was observed; while for feeds, decrease in crude protein, lipid was non-concomitant to increased ash and fibre levels from second to fourth month of storage. The study emphasises on the need to utilise farm-made feeds within less than two months post their storage. Nutrient profile of commercial feeds stored under open-air and airtight conditions were evaluated for six months by Solomon et al. (2016b). Magnitude of oxidative changes in feeds peroxide value and free FA levels, increased in all diet groups over the time of storage. Compared to airtight conditions feed stored under open-air was more prone to physical deterioration indicated by unacceptable changes in texture, odour, colour, insect as well as mold growth within third month of storage. Variability in feed response to storage parameters was seen among different commercial feeds, indicating role of packaging and feedstuff composition on individual feed behaviours during storage. Solomon et al. (2016b) investigated effect of feed storage on growth performance of African catfish, *C. gariepinus.* It was observed that sealed condition storage of feeds supported better fish growth as compared to open air storage for most feeds. While in case of locally produced fish feed severe mortality of fed fish was observed irrespective of feed storage conditions indicating poor feed acceptability of ingredients used for feed manufacture. Variable storage temperatures (-1.1^{o} to -15^{o}, 10^{o}, 20.8^{o}C) were explored to study (Khan and Seyhan, 2021) their impact on growth accrual and

feed utilization by trout (*O. mykiss*). Although, no significant change in rate of specific growth and FCR was reported for trout.

Improper storage temperatures and humidity may support pathogenic growth and survival in the feed, or even favour production of harmful microbial toxins with potential teratogenic, carcinogenic, hepatotoxic, mutagenic and immuno-suppressive effects (Pietsch et al., 2020; Zmysłowska and Lewandowska 2000). Microbiological evaluation of aquafeeds provides qualitative and quantitative assessment of the microbial load present in feeds. Microbiological quality of fish feeds have also been evaluated in many works. Zmysłowska and Lewandowska (2000) emphasised on requirement of microbiological analysis in accordance to standards for classifying fish feeds suitable for use. Ebeneezar et al. (2018) analysed, microbial parameters including total plate and fungal counts of commercial aquafeeds. The results indicated contamination of feeds with micro-organisms, demanding compliance of manufactured feeds with the quality regulations set in accordance to Bureau of Indian Standards.

Pietsch et al. (2020), reported that feeds stored under unsuitable conditions (suboptimal temperature and humidity) for shorter durations are equally prone to deterioration in quality. This indicates impact of humidity and temperature conditions even over short duration storage, on feed characteristics. Fourteen different mycotoxins with high prevalence of deoxynivalenol DON (92.9%), aflatoxins (64.3%) were reported from local feeds and feed ingredients in comparison to imported commercial feeds (Marijani *et al*., 2017). The study highlighted that simultaneous occurrence of mycotoxins in aquafeeds and feed material implicates synergistic and augmented hazard upon fish physiology and consequently, incidental consumer health. Among recognized toxigenic fungi

most genera belong to *Aspergillus*, *Fusarium*, *Penicillium, Cladosporium* and *Alternaria spp*., with ability to produce potential mycotoxins such as aflatoxins, zearalenone (ZEN), T-2 toxin, DON, ochratoxin A, fumonisins and patulin; based on fungal prevalence (Greco et al., 2015; Pietsch et al., 2020). Of 18 different known aflatoxins; AFB1, B2, G1and G2 are of notable importance with AFB1 being prevalently toxic (Dirican, 2015).

Table 2.3 Storage studies based on evaluation of nutrition profile and quality of fish feeds and feedstuffs.

Feed type /feed ingredients analyzed	Storage material; duration of storage/ sampling Interval	Place of study/ feed, ingredients source	Nutritional Parameter Analyzed	References
wheat pollard, sunflower seed cake, cotton seed cake , maize bran, blood meal	polythene sacks; four-months (June-October)/ two months	Uganda	**Biochemical** *Proximate*: MC, DM, CA, CL, CP, CF	Aanyu et al., 2017
Local Feed ingredient: fish meal	One year/ every three months (January, June and December)	South Khartoum, Sudan	**Biochemical** *Proximate*: MC, DM, CP, Fat, CA	
Commercial Feed	poly-propylene and polythene bag; two months (July-August)/ 15 days	Mymensingh, Bangladesh	**Biochemical** *Proximate*: MC, DM,CP, CL, CA,CF, TN, NP **Physical**: color, odor, texture, infestation, fines	Hossen et al., 2011
Commercial Feed	air tight /7 days	Ilorin, Nigeria	**Biochemical** *Proximate*: MC, CA, CL, CP, NFE *Anti-nutritional factors*: Tannins, oxalate, phytate	et al., 2014
Commercial Feed	Open air and air tight conditions; six months (November-April) /monthly	Makurdi, Nigeria	**Biochemical** *Proximate*: MC, CA, CL, CP, NFE, CF *Oxidation status*: POV, FFA *Macroelements*: P, Ca, Mg, Fe **Physical** *Organoleptic*: color,	Solomon et al., 2016b

			smell, caking, infestation	
Commercial Feed	Bags wide open, seal open, sealed; six months/ post six-month storage	Makurdi, Nigeria	**Biochemical** *Proximate:* MC, CA, CL, CP, NFE, CF *Oxidation status*: POV, FFA **Microbiological**: Mold count, mold type	Solomon et al., 2016a
Commercial Feed	Sacks; 45 days/15 days	Turkey	**Biochemical** *Proximate*: CP,CL,CA,CF *Macroelements*: P, Ca, Na *Oxidation status:* POV, p-anisidine, totox value	Yildirim et al., 2020
Feed ingredient: fish meal	six months/ monthly	India	**Biochemical** *Proximate*: MC,CP, , AIA,NPN, calorie content; AA, FA, cholesterol, vitamin content, salt content, protein solubility, *elements*: P, Mg, Ca, Na, K, S, Cl, Cu, Mn, Se, I, Co,F, Cr, Hg, Cd, Fe, Pb, As, Zn,Ni *Biological amines*: TVB-N,TMA-N, Histamine *Oxidation status*: POV, FFA,TBA **Microbiological:** TPC,TFC	Jeyasanta and Patterson, 2020
Commercial Feeds	Not Mentioned	Kochi, India	**Microbiological:** TPC, *Escherichia coli* count, coliforms count, Enterobacteriaceae count, yeast count, mold count	Ebeneezar et al., 2018
Commercial Feeds	food-grade oxygen barrier polyethylene , aluminum bags, airtight bottles; one week feed storage/35 days feeding trial for growth, feed performance of juvenile *O. mykiss*	Turkey/ feed from *Skretting Aquaculture, Norway*	**Biochemical** *Proximate*: CP,CF,CL,CA *Macro, micro elements*: Ca, P, Na, Fe, Cu, Zn **Fish growth parameters**: FCR,SGR,TGC	Khan and Seyhan, 2021

Formulated feeds (lab-made)	Not Mentioned	Greece/ fish oil (*Austral Group, Lima, Peru*), sardine oil (*Panama)*	**Biochemical** FA *Oxidation status*: TBA, TBARs, volatile compounds	Grigorakis et al., 2010
Feed ingredients: meal of Anchovy, Peruvian fish and poultry, oil of Salmon by-products, Black Sea fish and anchovy, soybean meal, Salmon fish oil, aquaculture by-products and sprat oil	oil 60 days storage, other ingredients 30 days	Turkey	*status*: POV	K et al., 2019
Feed ingredients: Fish oil	150 days of storage/intervals 1,15, 30,60,120, 150 days	Turkey	*Oxidation status*: EV,IV, PV,SV, TBA,AV,USM	Bo et al., 2006
Commercial Feeds, feed ingredient: fish meal EQ treated	original packages, disposable open to air aluminium foil pans; 90 days /one month	Canada/ herring fish meal (*Connors Bros Ltd, Blacks Harbour*), EQ-treated fish meal, two fish feeds (*EWOS Ltd, Surrey)* commercial salmon feeds (*Moore-Clark, St Andrews; Corey Feed Mill Ltd, Fredericton*)	**Biochemical** *Oxidation status*: EQ,DM,QI	He and an, 2000
Commercial feed supplemented with probiotics	25 days/5days	Nigeria	**Microbiological:** Probiotic survival *L. brevis1, L. plantarum, P. pentosaceus2*	Am et al., 2016
Formulated feed	72 days/8 days	Poland	**Microbiological:** bacteria, fungi	Zmysłowska and Lewandowska, 2000
Formulated feed	days	Poland	**Microbiological:** bacteria	Zmy owska, 1999
Commercial feeds, feed ingredients: Indian fishmeal, Danish fish meal, wheat flour	Polyethylene bags; six months /two months for all parameters; three month interval for FA	Cochin, India	**Biochemical** MC,CP,CA,CL, FA,AA *Biolo mine*: Histamine	Kavitha et al., 2004

Abbreviations AA: Amino acid composition; **AIA:** acid insoluble ash; **AV:** acid value; **DM:** dihydroethoxytrimethylquinoline; **EQ:** ethoxyquin; **FA:** fatty acids; IV: Iodine value; MC: Moisture content; NFE: nitrogen free extract; NP: Nitrogen in protein; **NPN:** Non-

protein nitrogen;

BA: thiobarbituric acid; **TBARs:** TBA reactive substances; **TFC:** total fungal-count; **TGC:** thermal-unit growth coefficient; **TMA:** trimethylamine; **totox:** total oxidation; **TN:** Total nitrogen; **TVB-N:** Total volatile bases nitrogen; **USM:** unsaponifiable matter.

2.4.2 Mitigation to storage changes

2.4.2.1 Natural Antioxidants

Activation of oxidants,

rancidity leading to malodors and off flavors, deteriorating nutritive value of fats during storage processing. Synthetically derived antioxidants such as butyl-hydroxytoluene, and ethoxyquin have been used; although, works demonstrating their suppressive health effects have been reported (Camacho-Rodríguez, 2018; Lundebye et al., 2010). This has shifted focus towards use of natural antioxidant source in diets including microalgae, duckweeds and bioactive extracts of *Aspergillus.*

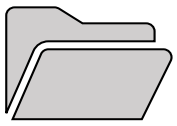
Fermented bioactive enzymes (FB, extracted from *A. ibericus*) supplemented fish feeds illustrated lowering of lipid oxidation rates up to 19.5 percent at low temperature 4^{o}, and ambient storages (Filipe et al., 2023). Mohamed et al. (2017), tested effectiveness of clove oil as a strategy to combat growth of aflatoxigenic strain. Dose-dependent increase, in formation of fungal inhibition zone, indicated promising fungal control of toxicogenic strains using clove oil. Yeast cell wall extract was used as a mycotoxin mitigation strategy (Yang et al., 2020a), decreasing AFB1 residues in aflatoxin contaminated diet with restoration of immune response; counteracting liver damage, intestinal microbiota disruption in turbot .

2.4.2.2 Functional storage-feed augmentation and mycotoxin mitigation

Bioaugmentation of feed with natural therapeutics such as probiotic, prebiotics and their mixture in synbiotics have founded numerous prospects for aquaculture improvements. Functional feeds based on probiotics and prebiotics, as a combination in synbiotics, aim at establishing the modalities of microbial dynamics to maximize host fitness. In an aquaculture scenario, probiotic feed supplementation is considered to confer immunoprophylactic control to disease incidence and improving gut-microbial ecology contributing to the overall health status of fish (Puri et al., 2022, 2023). Synbiotic addition effectively enhanced anti-oxidative enzymes SOD, catalase (CAT), and glutathione peroxidase (GPX) fed *O. niloticus*; during a 60-day trial (Dawood et al., 2020). These enzymes are compromised on increase in oxidative cellular stress, as in peroxidation and free radical attacks of lipids and proteins during feed storage (Kołakowska Bartosz, 2014). Synbiotic or probiotic treatment was counterproductive on oxidative enzyme malonaldehyde, suggesting an improved antioxidant response as well as minimized cell damage in treated fish when compared to control fed populations. Early development of prolonged beneficial anti-oxidative effects of synbiotic galacto-oligosaccharide (GOS) and *Bacillus subtilis* was evident in *L. rohita* (Devi et al., 2019; Puri et al., 2022).

Probiotics have role in improving zootechnical parameters of FCR, SGR and fish growth (Nathanailides et al., 2021; Puri et al., 2022). Probiotics additionally due to their antibacterial and antifungal activity, NH_3 detoxification potential, maintenance of redox status, metal chelating effects improve feed nutritional parameters for improved fish physiology. Production of fishmeal based on

probiotics and prebiotics is an essential development by Hendalia et al. (2019). FM produced based on pro-, pre-biotics inhibited microbial growth in storage feeds, with improved protein and energy structure of feeds. Probiotics are upcoming mycotoxin mitigation strategy in stored feeds with better stability and viability at low temperatures (Ragoubi et al., 2021). Storage at 4◦C maintained probiotic viability and feed quality even towards long-duration storage (Chomova et al., 2023). Probiotic suspension of *Lactiplantibacillus plantarum* strain provided more fibre, Ca, fat, and AAs (val, lys, isole, leu, arg and phe) to stored probiotic-coated fish diet (Chomova et al., 2023).



Chapter 3

MATERIALS AND METHODS

3.1 Feed ingredients and feed composition

The study was carried over a period of 180-days for each feed formulation. Locally available feed ingredients were used for feed preparation as listed in Table 3.1. Fishmeal was prepared from dry fish *Harpadon nehereus*, Bombay duck obtained from Ghazipur, New Delhi, India (Figure 3.1). Wheat flour (Aashirvaad Atta, Kolkata, India), corn flour (Brown and Polson, Hindustan Unilever limited Andheri, Maharashtra, India) and sunflower oil (Sundrop superlite advance, AgroTechFoods ltd. Secundrabad, India) were purchased from local market in Delhi, India. Greater duckweed, *Spirodela polyrhiza* was cultured at Department outdoor facility in cemented tanks with organic manure (Figure 3.2), comprising equally mixed mustard oil cake, poultry droppings and cattle manure @ 1.052kg/m^3 (Chakrabarti, 2018; Sharma et al., 2019).

Figure 3.1: Fishmeal production from dry fish *Harpadon nehereus*, Bombay duck.
Source: Author (2023)

(a) **(b)**

Figure3.2: *Spirodela polyrhiza* (a)at outdoor culture tank facility,(b)fronds enlarged.
Source: Author (2023)

3.2 Feed formulation and preparation

Extrusion based compounded diet was formulated using Interactive Fish Feed Designer (IFFD) software version 2 (Chakraborty et al., 2020); comprising fishmeal as major protein source with substitution of *S. polyrhiza* and wheat flour as minor protein sources, plant-oil sunflower oil was substituted for fish oil. All dry ingredients were well mixed and oil was added just before sieving of mixed feed through fine mesh and then subjected to extrusion pelleting with addition of Milli-Q ultrapure water (Synergy ultrapure, Millipore, Germany). Two-kilogram sinking pellet extruded feed was prepared based on twin-screw extrusion technique (BTPL lab model twin-screw food extruder, Kolkata, India) using 2.5 mm die as per set conditions with extruder rpm 214, cutter rpm 545, feeder rpm 10, extruder torque 13.9, heater1 (65°C) and 2 (70°C), temperature of final mass 101°C. Extruded feed was dried in feed drier (Hicon, India) for 4h at 40°C, then kept at ambient conditions for 1h and packaged for storage conditions. Non-extruded pelleted diet was formulated based on IFFD software

version 2, using pelletizer machine (Kent, India) with 2 mm die. All ingredients were added as per feed composition (table 1), and dry mixed. After sieving, mixture was transferred to pelletizer and hot water (60^{o}C) was added. Dough formed through automatic kneading process was pelleted. Diet pellets were collected into feed trays and dried, packaged for storage; as per conditions similar to extruded diet. Prepared diets in figure 3.C and 3.D.

Table 3.1: Composition of formulated feeds

Ingredients (g/kg)	Diets	
	Aquafeed 1 (Extruded diet)	Aquafeed 2 (Non-extruded Pelleted diet)
Fish meal	432	528.3
Wheat flour	276.45	471.69
Spirodela polyrhiza	276.45	-
Corn flour	-	75
Sunflower oil (mL)	10	10
Vitamin-mineral premix*	4	4

* Multivitamin Supradyn tablet (mg/kg in diet)

3.3 Feed storage and storage duration

Three replicates (n=3) per temperature condition were kept for storage. Sealable were used for low temperature and ambient storage; while, screw- bottles (Borosil, 500 mL) were used to store experimental feed at high temperature for six months, during October 2021 to April 2022 and October 2022 to March 2023. Daily temperature record was maintained towards ambient storage during storage months using thermo-

hygrometer (ThermoPro TP53, USA) shown in Figure 3.3. Temperature records are given in table 3.2. Replicates (n = 3; for four temperature conditions under study) were drawn bimonthly at 0, 60th, 120th, 180th day during each assessment sampling.

Figure 3.3: Thermo-hygrometer, ThermoPro TP53 for ambient temperature recording.

Source: Author (2023)

Table 3.2: Temperature record for ambient storage conditions for formulated aquafeeds

Storage Period (2021-2022 & 2022-2023)	Aquafeed 1 (Extruded diet)		Aquafeed 2 (Non-extruded Pelleted diet)	
	Maximum (° C)	Minimum (° C)	Maximum (° C)	Minimum (° C)
October		12	31	25.9
November	2	12	27	21.6
December	25	18.5	22	17.2
January	20	17	19.6	15.8
February	23.5	18	24.1	18.4
March	30.8	22.5	27.1	23.5
April	31.5	27.5	-	-

Source: Author (2023)

3.4 Methodology

3.4.1 Preparation of Feed Sample

All samples were ground as per AOAC method 950.02 to fine particles then sieved (1mm) to obtain fine, powdered feed which was mixed thoroughly for biochemical assessments and analysis of fatty acids, amino acids, vitamin and elemental profile (AOAC,1990).

3.4.2 Proximate Analysis

Estimation of proximate parameters moisture, ash, crude lipid and protein, carbohydrates as nitrogen free extract (NFE), for all sample replicates was performed and mean was calculated for the interpretation of results.

3.4.2A. Moisture

Moisture in feed was determined as percentage, by standard method (AOAC, 2000). Petridish and lid were oven dried 3h. Their weights were recorded after cooling in desiccator. 3g of feed sample was weighed. Sample was uniformly spread and sample dish is dried for 3h at 105°C in the oven. After Dried dish with partly covered lid was kept in desiccator. Thereafter, dried sample in dish was reweighed.

Moisture percent was determined as per given formula;

$$\text{Moisture (\%)} = \frac{(W_w - W_d) \times 100}{W_w}$$

W_w= sample

W_d = dry wt. of sample

3.4.2B. Ash

Standard AOAC method for ash determination was followed. Dry crucibles were weighed. 2g sample in crucible was oven dried at 100°C for 24h. After bringing

to room temperature, crucibles with sample were transferred to muffle furnace. Temperature of furnace was raised to 550±5°C for 8h, until white ash appeared. After cooling, crucibles were reweighed for determining ash content (figure 3.4) on wet wt. basis, using formula;

$$\% \text{ Ash (wet wt. basis)} = \frac{(\text{Crucible} - \text{Crucible}}{\text{crucible with sample weight} - \text{crucible weight}} \times 100$$

(a) **(b)**

Figure 3.4 a): Crucible with sample pre-ashing , b) crucible with ash

Source: Author (2023)

3.4.2C. Crude Protein

Crude protein (C.P.) was determined according to AOAC 2001.11 for C.P. in animal feed, official block digestion method (Thiex and Manson, 2002). 0.25 g test portion was added with 3.5 g K_2SO_4 and 0.5g Cu catalyst mixture and 12 mL conc. H_2SO_4 (98%) in 250 mL Kjeldahl tube, temperature of block digestor (Kelplus KES 06L, Pelican equipments, Chennai, Tamil Nadu India) was increased stepwise for 30 min to reach 420°C and maintained for 2h until complete digestion of samples to green clear solution. Samples after removal from block digestor were rested to cool at ambient temperature and diluted with

10 mL Milli Q ultrapure water. Automated distillation (Kelplus-Classic DX VA, Pelican equipments, India) was performed using 40% NaOH and 2.5% H_3BO_3 solution containing methyl red and green indicator. NH_3 liberated in the receiving solution was titrated with standardized acid 0.1N HCl to end point in an autotitration unit (Metrohm Titrando, Herisau, Switzerland). Percent Kjeldahl Nitrogen (N%) and C.P. were calculated by preinstalled software program TIAMO version 2.2-81(Metrohm AG, Switzerland), figure 3.5.

$$N\ (\%) = \frac{14 \times \text{HCl Normality} \times \text{value of titrant} \times 100}{\text{Sample wt.} \times 1000}$$

% C.P. was calculated by multiplying 'N' with 6.25 (factor for converting N to protein).

Figure 3.5: Micro Kjeldahl Method Kelplus, Pelican equipments

Source: Author (2023)

3.4.2D. Crude Lipid

Estimation of crude lipid (C.L.) is in accordance to gravimetric analysis (Folch et al., 1957). Weigh 500mg test sample in centrifuge tube and add chloroform/ :1 (v/v) ratio. Vortex for 1min, then centrifuge at 2057g, for 15 min. Collect the supernatant in fresh tube. Re-extract thrice as

above and pool the supernatant. Filter the pooled extract through Whatman filter paper 1. Wash with 2 mL Milli-Q water and centrifuge (min at 25° C). Aspirate to discard the upper aqueous phase and collect the lower organic phase in a pre-weighed empty petridish. After complete drying of organic phase (6 h) reweigh the petridish to determine the final weight of dried extract as crude lipid, figure 3.6.

Wt. of crude lipid = Final wt. of petridish with lipid - Initial wt. of empty petridish

$$\% \text{ C.L.} = \frac{\text{Wt. of C.L. X 100}}{\text{Wt. of sample}}$$

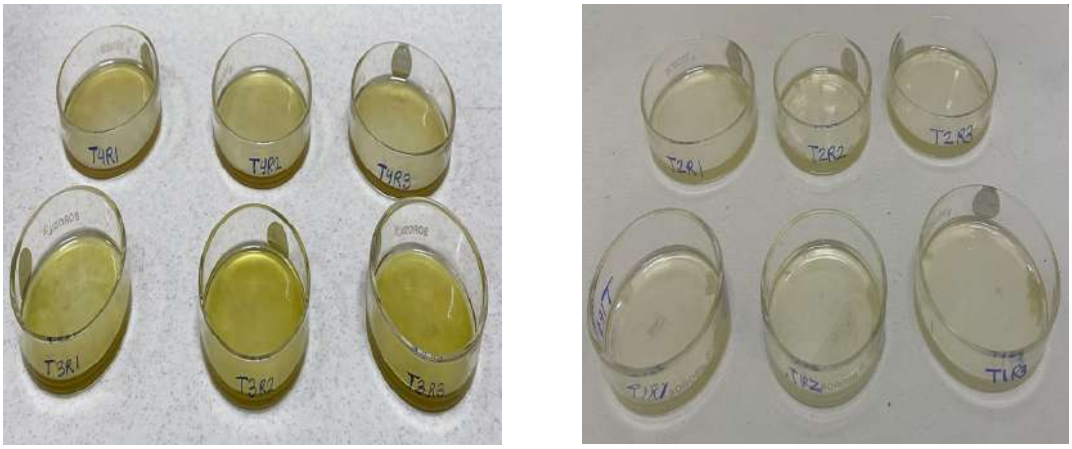


Figure 3.6: Gravimetric determination of Crude lipid a) extruded diet; b) Non-extruded diet

Source: Author (2023)

3.4.2E. Carbohydrates (Nitrogen free extract = NFE)

Carbohydrates as NFE was evaluated 'by difference' as percent present after all other components are determined (Noh et al., 2020), according to formula;

% Carbohydrates = 100 – (% Moisture + % . + % Ash)

3.4.3 Gross Energy/ Calorific value of stored feed

Gross energy (GE) value of feed, the heat generated on complete oxidation of feed compounds was determined in an automated O_2 bomb calorimeter (Parr 6400, , US) based on substitution procedure

(Figure 3.7). Oxygen (at purity 99.5%) and nitrogen (oil and water free) pressure are set at psig; respectively. Calorimeter is used in operating mode, heater and pump are set on. temperature is allowed to reach 30°C± 0.5 °C followed by pretest run for preconditioning. Thereafter, test process is carried for sample estimation. Capsule holder is removed from combustion vessel and heating wire above it is dried. Sample is pre-weighed to 0.0001g in stainless-steel capsule (diameter 1", height 7/16") and placed in capsule holder. Cotton ignition thread (length 4") is looped over heating wire to double-over; twisted to lay in sample cup. Vessel head containing sample capsule is transferred to combustion vessel and rotated leftwards to 20° for closure. Test process is run. From display, GE value is recorded as cal/g (=kcal/kg). GE of replicates is averaged and reported with value of standard deviation

Figure 3.7: Gross energy determination using Bomb calorimeter Parr6400

Source: Author (2023)

3.4.4 Amino Acid Analysis

Amino acid (AA) profiles of sample replicates of storage diets, 1(extruded), 2 (non-extruded); were estimated using automated AA analyzer (,

Japan) shown in Figure 3.8. Fresh glutamine, tryptophan standard solutions were added (Sigma-Aldrich, US) prior to analysis of standard mixture. Samples for all amino acids (except tryp, cys, met), were acid hydrolyzed

HCl (Fountoulakis and Lahm, 1998 ; Dai et al., 2014). Based on performic cysteine is estimated as cysteic acid; as methionine sulfone (Macdonald et al.,1985). For analysis of tryptophan, samples are hydrolyzed with 10 ml 4M methanesulfonic acid and 0.2% tryptamine [2-(3-indolyl) ethylamine] (Simpson et al., 1976). Digested samples are dried under N_2 pressure (PCi Analytic pvt. ltd., Mumbai, India) at 60°C to evaporate HCl. Sample is reconstituted in HCl adjusting protein concentration at 0.5 mg/ml. After filtration with micro syringe filter Whatman PES Filter), sample is placed in autosampler (Hitachi L- 8900 AAA auto sampler rack) for analysis.

Figure 3.8: Automatic Amino Acid Analyzer (Hitachi L-8900)

Source: Author (2023)

Analysis is based on separation of amino acids in a cation-exchange resin column with dimensions 4.6 mm x 60 mm (id, length) with size of particle 3 mm temperature 135°C, column temperature between 30° - 70°C, ninhydrin flow rate 0.35 ml min^{-1}. All amino acids with α-amino group form Ruhemann's purple

on ninhydrin reaction and are determined at a wavelength of 570 nm. Detection of imino acids, proline and hydroxyproline is due to yellow colour ninhydrin product at absorbance wavelength 440 nm. Chromatogram peak areas are used for quantification of amino acid concentrations in comparison to that of standard concentrations.

3.4.5 Vitamin Analysis

For analysis of vitamin profile changes, sample replicates n=3 was considered for both diets. Preparation and analysis of lipid- miscible vitamins (A,D,E,K) is in accordance to

(2014), with slight modifications was considered.

3.4.5A. Chemicals and Reagents

Vitamin standards, retinyl acetate (A) (No.46958, Sigma-Aldrich Chemicals private limited, India), B1 as thiamine hydrochloride, B2 riboflavin, B6 as pyridoxine hydrochloride, cyanocobalamin form of B12 and C as L-Ascorbate (No.47858, 47861, 47862,47863, 47869 respectively), cholecalciferol (D3), ergocalciferol (D2), K1 (No. 95271 Bio-xtra sum of isomers, HPLC grade) at high purity >98% were purchased from Sigma-Aldrich, USA. E-acetate (DL-α-tocopherol acetate) was obtained from Himedia laboratories private limited, India at purity >97%.Taka-diastase, source *Aspergillus oryzae* (No. 86247) was obtained from Sigma-Aldrich (USA). n-butanol, chloroform (SRL, Sisco Research Laboratories, India), acetone, sodium acetate (extrapure AR SRL, Sisco Research Laboratories, India), sulphuric acid (Merck, India), methanol,

acetonitrile (HPLC grade, Merck, India), metaphosphoric acid (No.239275, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were also procured. Standard solutions were prepared using Milli-Q water (Synergy ultrapure, Millipore, Germany) at pH7.

3.4.5B Water-soluble vitamins sample preparation

3.4.5B1. Vitamins- B1, B2, B6, B12

To 2g finely powdered feed sample, 25 mL of 0.1N sulphuric acid solution was added in 50 mL conical flask, and incubated at 121°C up to min in heated oven. Contents were brought at room temperature than adjusted to pH 4.5 (Orion Versatar, Thermoscientific, USA ; Figure 3.9) using 2.5M sodium acetate. -diastase enzyme powder was added to cooled contents. After gentle vortex for 1min preparation was stored overnight in oven maintained at 35°C. Digested mix was filtered using Whatman filter paper No.4. Extract was diluted with 50 mL Milli-Q ultrapure water (Synergy ultrapure, Millipore, Germany) and re-filtered through 0.45μm syringe filter (Merck Millipore ltd., India) into HPLC vial.

Figure 3.9: pH setting for Vitamin B samples (pH meter Orion Versatar, Thermoscientific)

Source: Author (2023)

3.4.5.B2. Vitamin C

10 g powdered feed samples were blended and homogenized with 25 mL extracting solution of 0.3M meta acid and 1.4M C COOH. After centrifuging for rpm mixture was filtered out using Whatman filter 4. Extraction was performed for triplicates.

3.4.5C. Fat soluble vitamins A/D/E/K sample preparation

0.5 g grounded sample was taken in 50mL falcon tube. 8mL chloroform : acetone mixture (70: 30 ratio) was added. Nitrogen gas was flushed through the sample tube and tube was tightly capped. Contents were vortexed for 1 min and falcon was left to stand (for 5 min); then vortexed again (for 30s). Mixture was subjected to centrifugation (, 25^{o}C) for 5 min. After centrifugation, 1mL supernatant is transferred into nitrogen evaporator tube. Extract is completely dried in nitrogen evaporator and dissolved in 1 mL n-butanol. Finally, reconstituted extract is filtered using 0.4μm syringe filter, into HPLC vial.

3.4.5D. Determination and quantification of vitamins

Stock solutions of vitamin standards 1.0mg/mL were prepared. Standard calibration was performed to obtain linearity at concentrations 0.25 mg per mL, 0.5 and 1 mg/mL. Chromatographic analysis of vitamins was conducted by ultra-high pressure liquid chromatography (UHPLC Thermo Fisher, model Dionex Ultimate 3000), Figure 3.10; with stationary phase C18 column (3μm,150x4.6mm). 20μL of extract was introduced for HPLC determination. Simultaneous separation for B1, B2, B6, B12 was performed at a solvent flow of 0.5 mL/min set at wavelength of 270 nm and MeOH H_3PO_4 (33:67

ratio, pH 3.54) as mobile phase. For estimation of vitamin C, flow rate is maintained at 1 mL/min, wavelength of 254 nm considering acetonitrile (ACN, 100%) as mobile phase solvent.

Fat-soluble vitamins (A,D,E,K) were determined at flow speed of 1.8 mL/min, UV absorbance 290 nm, MeOH as mobile phase.

Figure 3.10: UHPLC analysis of Vitamins using Dionex Ultimate3000,Thermofischer

Source: Author (2023)

3.4.6 Fatty acid profile

Stored diets were analyzed in replicates for their fatty acid (FA) composition. Briefly, total lipid extraction was performed according to Folch et al. (1957). Methyl esters of fatty acid (FAME) were prepared by acid catalytic transesterification of total lipids (1mg) in 1% (v/v) methanolic sulphuric acid solution, 1mL toluene was added to dissolve neutral lipids and reaction mixture was heated overnight in stoppered tube at 50°C for 16h (Christie, 2003; Schlechtriem et al., 2008). After addition of 2 mL 2% potassium bicarbonate,

methyl esters were extracted in 5mL iso-hexane / diethylether (1:1 ratio) containing 0.01% butylated hydroxytoluene.

Crude FAME was dissoluted in 100μl isohexane and after thin layer chromatographic separation (Tocher and Harvie,1988), examined for FA profile by gas chromatography using flame-ionization detector (GC-FID, Clarus 580, Perkin Elmer, US), shown in Figure 3.11. ZB-wax GC column (Phenomenex, Hyderabad, India) 60 m x 0.32 mm, thickness of film 0.25µm, was used for determination in on-column injector mode using nitrogen as carrier gas at flow speed 2mL/min. Injector temperature was kept at 260°C; oven ramping initial setting conditions were set to °C @ 2°C/min, 2min hold then raising @ 1°C per min to 220°C, holding time 15 min. Heptadecanoic acid C17:0 was taken as reference standard. Fatty concentration obtained (TotalChrom Workstation Version 6.3, PerkinElmer pre-installed software, USA), was expressed in mg/100g.

Figure 3.11: Fatty acid analysis using GC-FID, Clarus 580, PerkinElmer

Source: Author (2023)

3.4.7 Elemental Profile Analysis

Mineral profile changes for all samples in triplicates were performed by inductively coupled plasma mass-spectrometric analysis (ICP-MS, Agilent 7900 USA) at central facility, Indian Inst. of Technology, Delhi (CRF, IIT Delhi). Briefly, 300 mg ground samples were digested at 200^{o}C (with 8 mL conc. HNO_3 for 30 min) in microwave system (AntonPaar, MultiwavePRO). After make-up of volume (to 40 mL using MQ water), samples were passed through 0.2μm membrane and subjected to ICP-MS analysis at following conditions of nebulizer and auxillary gas flow at 1 L/min; plasma flow at 15 L/min; He flow at 0.2 mL/min in reaction cell; reflected and forward power 45W,1500W; vacuum analyser at 6 x10^{-5}. Linearity of standard solutions of elements was established at 10μg, 20μg, 50μg,100μg, 200 μg per L.

3.4.8 Aflatoxin analysis

Aflatoxin analysis of stored feed was performed for all replicates to estimate levels of aflatoxins B1,B2,G1and G2, if present. Briefly, 25g homogenate was blended with 100 mL, 80% methyl alcohol and 5g sodium chloride at increased speed for 2 to 3min. Extract was filtered using Whatman filter paper no. 42.To 2mL filtrate, 10 mL phosphate buffer saline (PBS) was added and clean-up was performed through SPE immunoaffinity column (Alfarhone wide, R-Bioform P116/100). Column was washed with 20 mL PBS solution (at a rate 1drop/sec); dried, and rewashed with 1.5mL MeOH followed by 1.5mL milli-Q water (Indian Standards IS-13427, Annexure G). Final extract was vortexed in glass tube, filtered with 0.45μm syringe filter and collected in injection vial. Liquid chromatographic (LC) analysis of samples was performed using Agilent 1260 Infinity, FLD (Model No-G1321B, 1260FLD) detector system on Inertsil ODS-3V column (5μm, length 150mm, i.d. 4.6mm). Aflatoxin standards for B1 (n'TOX, France 99.9% purity), B2, G1 and G2 (Fermentek Ltd., Israel) at purity 100, 99.0, 99.03% respectively, are diluted in 50% MeOH. Linearity is obtained at 0.5, 1, 2, 5 ppb standard concentrations with acceptance criterion 0.99.

3.4.9 Fourier Transform Infrared Spectrophotometric Analysis (FTIR)

FTIR analysis was performed using Shimadzu, Model IRAffinity-1S Kyoto, Japan (in figure 3.12), for analysis of changes in IR spectra for lipids, proteins, carbohydrates and moisture under storage conditions. FTIR is equipped with beam splitter comprising germanium coated potassium bromide (Ge-KBr) used as standard with wavenumber range 7,800 - 350 cm^{-1} , a temperature-controlled detector, DLATGS and interferometer at 30° incidence with moisture drying system. Test sample is located in sample compartment and set for assessment

at wavenumber range 4000-400 cm^{-1}. Intensity data, transmittance (%T) of sample triplicates were averaged to analyse spectral plots drawn using XY scatter plot Microsoft® Excel version16.66.1(22101101).

Figure 3.12: FTIR analysis Shimadzu, Model IRAffinity-1S

3.5 Statistical Analysis

Data is estimated as mean ± std. deviation (SD) for three replicates.Two-way analysis of variance (two-way ANOVA) with interaction effects of temperature, storage duration on proximate parameters, vitamins, elements, amino acid and gross energy content of formulated aquafeed is performed using IBM SPSS statistics (SPSS, version 25). Statistical difference in values is reported by honestly significant difference (HSD). Post-hoc analysis based on Tukey's, at level of significance for $P < 0.05$ is considered. Univariate regression analysis for effects of moisture on gross energy value of stored feed is performed using Microsoft® Excel version16.66.1(22101101). Multivariate test (MANOVA) is used to assess storage effects of temperature and duration on changes in fatty acid profile of formulated aquafeed using SPSS analysis (SPSS, version 25).

Post-hoc analysis for multivariate comparison of fatty acid profile is based on Tukey's HSD at $P < 0.05$.

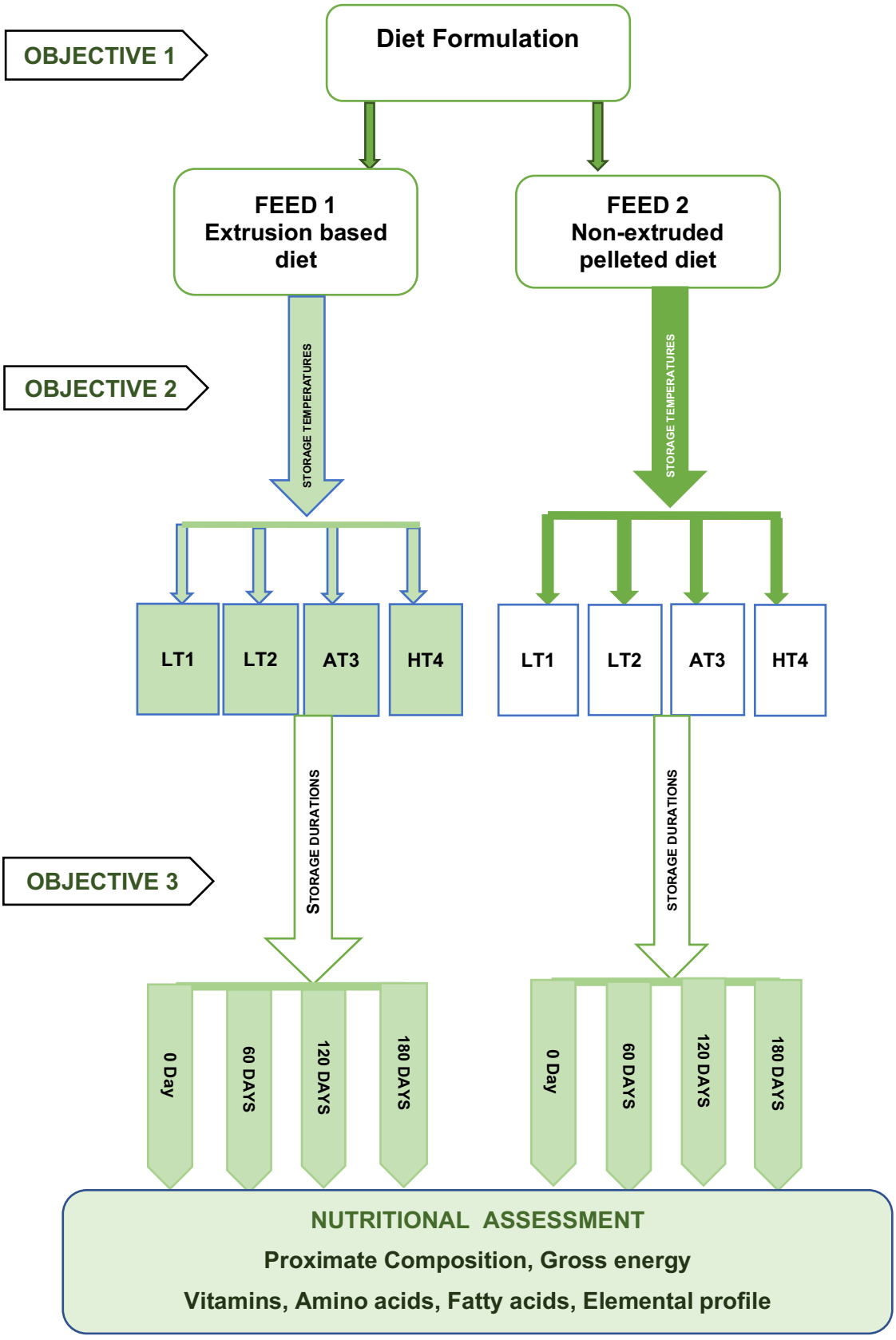
3.A. OUTLINE OF METHODOLOGY

- **Diet Formulations**
  - **Extrusion based fishmeal-substituted diet = Feed formulation 1**
  - **Non-Extruded control diet = Feed formulation 2**

- **Storage Temperatures**
  - **Low temperatures** **LT1** = -20^{o}C
    LT2 = 4^{o}C
  - **Ambient** **AT3**
  - **High temperature** **HT4** = 45^{o}C

- **Storage Durations**
  - **Initial** = 0 day
  - **Two-months**= 60-days
  - **Four months**= 120-days
  - **Six-months**= 180-days

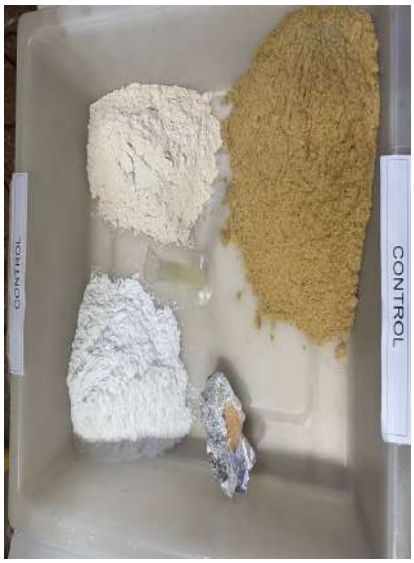
3.B. FLOWCHART: METHODOLOGY WORKFLOW

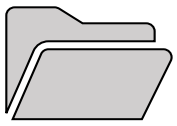


Source: Author (2023)

3.C. AQUAFEED 1 - EXTRUDED DIET

3.D. AQUAFEED 2: NON-EXTRUDED PELLETED DIET





Chapter 4

RESULTS

4.1 Proximate composition storage changes

Formulation of feeds are on dry matter basis, but weighing out or mixing of ingredients is on an as-fed basis (DuPonte, 2009). Since feed formulation is conducted at 'as-fed basis's and dry matter conversions are performed, in practice; it is essential to evaluate impact of both aspects for understanding apparent (as-fed basis) as well as actual changes (dry matter basis) during storage of feeds (Lee et al., 2016; Parish, 2007).

4.1.1 Moisture

Largely, there is a decrease in moisture content from initial to end of storage duration with greatest moisture losses at HT4 storage (9.15±0.30 for diet 1, and 9.66±0.31 for diet 2) table 4.1a),e). There are noteworthy effects of both storage duration and temperature on moisture of feeds. Significant difference in moisture content of feeds was found between all temperatures except for that between AT and HT for feed 1. Duration effects on feed 1 moisture are significant among initial, four- (120 days) and six-month (180 days) storages. For feed 2, significant difference in moisture ($P<0.05$) exist between HT and other temperatures (LT, AT); similar trend is noticeable for duration effect on feed 2 moisture content.

4.1.2 Ash

As-fed basis

Diet 1 and diet 2: There is increase in ash content at all storage temperatures at end of 180-day storage for both extruded (diet 1) and non-extruded pelleted

diet (diet 2). Difference in ash value are significant (P<0.05) between low temperatures and AT, low temperatures and HT for diet 1; while no significant difference in ash percentage is noteworthy between low temperatures LT1 and LT2, or between AT and HT conditions.

For diet 2, significant difference in ash percent is obtained between HT and other temperatures (LT, AT), but significantly there is no difference between LT1, LT2 and AT storage. Duration dependent changes in ash value are significant between initial and 180-day storage at all temperature conditions for both diets.

Dry matter basis

Diet 1: For diet 1, ash content decreased over storage duration at all temperature conditions with gross decrease at end of 180-day storage for diet 1.There is a significant (P<0.05) difference in ash amount between 0 and 60-day, between 60 and 120, 120 and 180 day. Major effects of duration and interaction are significant at P< 0.05,while effects of temperature conditions on ash value are non-significant.

Diet 2: Increase in ash value is significant (P<0.05) between 0 and 60, 0 and 120, 0 and 180 days. No significance (P>0.05) in ash content exist between 60 and 120 days; or between 120 and 180 days. Moreover, between-subject effects of temperature and duration hold significance (P<0.05); whereas interaction effects are insignificant for changes in ash content. As compared to low temperature LT1, LT2; significantly high ash content is found at HT4. While no significant difference exist between AT3 and HT4 ash values. Evidently, among all storage conditions highest ash value is determined at HT4 conditions.

4.1.3 Crude lipid

As-fed basis

Diet 1 and diet 2: Crude lipid; as-fed basis, for both diets show constant decrease along storage duration (0<60<120<180 day) at all temperatures, except at 60th day AT and HT conditions for diet 1. Compared to LT2, AT and HT, significantly higher C.L. contents (P<0.05) are obtained at LT1 throughout storage. It is noteworthy, that greatest losses in C.L. are incurred at 180th day storage of feeds, up to 26.75% at LT1; 31.92% (LT2); 39.12 % (AT); 42.62% at HT for feed 1, and 13.18%, 14%, 21.75% and 27.68% (at LT1, LT2, AT, HT respectively) for feed 2, tables 4.1 e)-f). Significant difference due to duration effects is obtained between initial day and end of storage period. Although, interaction effects for temperature x duration are insignificant (for changes in storage content of lipids.

Dry matter basis

Diet 1: Incurred losses in lipid content follow duration-wise decrease as observed during as-fed basis. Compared to LT2, AT3 and HT4, at all durations highest C.L. contents are obtained at LT1, table 4.1c). Percentage of loss at end of 180-day duration at LT1=28.03%, LT2=35.03%, AT3= 42.04%, HT4 = 45.8%. Major effects of temperature and duration on lipid content have high significance (P = 0.000), while interaction effects are not significant (P>0.05), table 4.1d).

Diet 2: Marked difference in lipid content between 0 and 180 day is noteworthy at all storage temperatures. Between-subject-effects of temperature and duration on diet 1 lipids are significant at P = 0.005 and 0.000 respectively. Relatable difference due to interaction effects temperature x duration, on C.L.

changes in diet 2, is not noteworthy. Temperature effects are significant between low temperatures (LT1, LT2) and HT4 table 4.1g),h). Highest C.L. content is found at initial storage (6.86 ± 0.55%) while subsequent decrease at all durations is noteworthy with greatest losses at 180-days. At end of storage duration percentage loss are determined as LT1=13.12%, LT2= 14.29%, AT3=21.57%, HT4 = 29.15%, highest C.L. loss at HT4.

4.1.4Crude protein

As-fed basis

Diet 1 , 2: For diet 1, there is an overall decrease in crude protein content with increasing duration, at all storage temperatures. Incurred losses in C.P content is significantly high for HT compared to LT and AT storages. Duration dependent changes in C.P. values are significant ($P<0.05$) between 0,120 and 180 days. While there is no significant difference for C.P. content between 0-day and 60- day as well as between 60- and 120-day storage. Notedly, interaction effects are insignificant ($P>0.05$) for C.P. during storage. Significant difference in protein content is noteworthy between initial compared to two-, four-, sixth month storage for diet 2.

Dry matter basis

Diet 1: Decrease in protein content is noteworthy at all temperatures with increment in storage time. Along storage durations, C.P. losses are in accordance to increase in temperature for storage in order LT1 (7.02% loss) < LT2 (7.77%) < AT3 (10%) <HT4 (11.66%) with greatest decreases at HT4. Significant decrease in protein percent ($P<0.05$), due to temperature and duration effects exist while interaction effects on protein value are insignificant.

Diet 2: Significant decrease in protein values due to duration effects exist between 0 and 60 days, between 0 and 120 days, 0 and180 days at all temperature storages.

4.1.5 Carbohydrates as Nitrogen free extract (NFE)

As-fed basis

Diet 1: Significant difference due to temperature effects on carbohydrates is found between LT1 and AT3, LT1 and HT4 storage; while there is no significant difference between LT1 and LT2, or among LT2, AT3 and HT4. Time-dependent storage changes in percentage carbohydrates are significant between all durations except between 60 and 120 days. Major effects of temperature, duration and interaction are significant ($P<0.05$).

Diet 2: Significant difference due to temperature effects on carbohydrates is found between LT1 and AT, LT1 and HT storage,LT2 and HT. There being no significant difference between LT1 and LT2, LT2 and AT3 on NFE values. Marked difference between 0 and 60-day, 0 and 120-day , 0 and 180-day NFE content exist. Difference among 60,120 and 180-day carbohydrate content is non-significant. Two-way ANOVA reveals significant ($P<0.05$) difference for between subject effects of temperature (T), duration (D) and interaction (I) on NFE.

Dry matter basis

Diet 1: Significant difference due to temperature effects on carbohydrates is found between low temperature (LT1, LT2) and HT storage; while there is no significant difference between LT1 and LT2, LT2 and AT. Time-dependent storage changes in percentage carbohydrates are significant between all

durations except between 60 and 120 days. Duration and temperature effects are significant whereas, interaction impact on NFE is insignificant ($P>0.05$).

Diet 2: NFE on DM basis, in diet 2 shows marked difference between 0 and 60,60 and 180,120 and 180 days. No significant difference exist between 60- and 120- days values. For temperature effects difference is noteworthy between LT1 and AT, LT1 and HT, LT2 and HT. But not between LT1and LT2, LT2 and AT. As for diet 1; between subject effects are significant ($P<0.05$) towards temperature and duration and not for storage x duration interaction.

Table 4.1a): Effect of temperature and storage duration on proximate parameters of formulated aquafeed 1 (as-fed basis).

Temperature	Duration (day/s)	Crude (%)	Crude Protein (%)	Moisture (%)	Carbohydrates (%)	Crude Ash (%)	Gross Energy (kcal/kg)
LT1	0	5.42± 0.44^{aA}	39.09± 0.26^{abA}	13.66± 0.49^{aA}	30.99± 0.66^{bC}	10.83± 0.10^{bBC}	3830.13±13.92^{bD}
	60						3846.99±20.74^{bC}
	120	4.38± 0.21^{aB}	37.84± 0.03^{abB}	13.22± 0.06^{aB}	33.82± 0.28^{bB}	10.73± 0.06^{bB}	3855.46±5.33^{bB}
	180	3.97± 0.30^{aC}	36.98± 0.16^{abC}	12.16± 0.24^{aC}	36.01± 0.73^{bA}	10.89±0.08^{bA}	3888.19±13.20^{bA}
LT2	0	5.42± 0.44^{bA}	39.09± 0.26^{aA}	13.66± 0.49^{bA}	30.99± 0.66^{abC}	10.83± 0.10^{bBC}	3830.13±13.92^{aD}
	60	3.88± 0.09^{bBC}	39.12± 0.32^{aAB}	12.98± 0.33^{bB}	33.42± 0.26^{abB}	10.60± 0.13^{bC}	3883.67±26.81^{aC}
	120	3.84± 0.18^{bB}	38.40± 0.22^{aB}	12.16± 0.12^{bB}	34.78± 0.15^{abB}	10.82± 0.12^{bB}	3894.40±21.70^{aB}
	180	3.69± 0.25^{bC}	37.76± 0.34^{aC}	9.58± 0.16^{bC}	37.87± 0.57^{abA}	11.10± 0.06^{bA}	3974.62±12.04^{aA}
AT3	0	5.42± 0.44^{bA}	39.09± 0.26^{aA}	13.66± 0.49^{cA}	30.99± 0.66^{aC}	10.83± 0.10^{aBC}	3830.13±13.92^{aD}
	60	3.59± 0.38^{bBC}	39.04± 0.39^{aAB}	11.63± 0.17^{cB}	34.91± 0.90^{aB}	10.83± 0.09^{aC}	3890.95±26.03^{aC}
	120						
	180	3.30± 0.16^{bC}	36.98± 0.19^{aC}	9.35± 0.33^{cC}	39.22.± 0.28^{aA}	11.15± 0.03^{aA}	3976.02±15.05^{aA}
HT4	0	5.42± 0.44^{bA}		13.66± 0.49^{cA}	30.99± 0.66^{aC}	10.83± 0.10^{aBC}	3830.13±13.92^{aD}
	60	3.49± 0.15^{bBC}	37.68± 1.34^{bAB}	11.64± 0.41^{cB}	35.92± 1.66^{aB}	10.72± 0.16^{aC}	3839.73±15.37^{aC}
	120	3.55± 0.06^{bB}	37.15± 1.83^{bB}	11.15± 0.41^{cB}	35.70± 0.73^{aB}	11.07± 0.12^{aB}	
	180	3.11± 0.32^{bC}	36.34± 1.10^{bC}	9.15± 0.30^{cC}	40.16± 2.72^{aA}	11.23± 0.05^{aA}	3981.74±9.88^{aA}

Values represented as Mean ± SD(n=3) LT1 = -20^{o}C; LT2 = 4^{o}C; AT3 = 17^{o}C - 31.5^{o}C; HT4 = 45^{o}C. Statistical differences (Tukey's, P< 0.05) are given by changed letters: changed small letters 'a - c' in similar column denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar column denote difference is significant (P< 0.05) among feed storage durations.

Table 4.1b): Two-Way ANOVA with between subject effects of temperature, storage duration on proximate parameters and gross energy content of formulated aquafeed 1 (as-fed basis).

Parameter	Source	SS	df	MS	F	P-value
Moisture (%)	**T**	18.72	3	6.240	53.78	**0.000**
	D	79.53	3	26.509	228.46	**0.000**
	I	11.66	9	1.296	11.17	**0.000**
Crude Ash (%)	**T**	0.26	3	0.09	7.23	**<0.001**
	D	0.80	3	0.27	22.45	**<0.001**
	I	0.40	9	0.05	3.77	**0.003**
Crude Lipid (%)	**T**	2.99	3	1.00	11.53	**0.000**
	D	26.39	3	8.80	101.83	**0.000**
	I	1.09	9	0.12	1.40	0.231
Crude Protein (%)	**T**	7.56	3	2.52	5.29	**0.004**
	D	29.20	3	9.73	20.43	**0.000**
	I	3.97	9	0.44	0.93	0.517
Carbohydrates (%)	**T**	37.31	3	12.44	23.30	**0.000**
	D	322.34	3	107.45	201.27	**0.000**
	I	20.84	9	2.27	4.24	**0.001**
Gross Energy (kcal/kg)	**T**	14819.52	3	4939.84	18.77	**0.000**
	D	100199.06	3	33399.69	126.91	**0.000**
	I	14957.21	9	1661.91	6.32	**0.000**

Post-hoc analysis based on Tukey's HSD. Temperature (T), Duration (D), Interaction of Temperature*Duration (I).

P-value for between subject effects ; Significant **P-values, P< 0.05 in highlight.**

Table 4.1c): Effect of temperature and storage duration on proximate parameters of formulated aquafeed 1 (dry matter basis).

Temperature	Duration (day/s)	Moisture (%)	Dry Matter (%)	Crude Lipid (%)	Crude Protein (%)	Carbohydrates (%)	Crude Ash (%)	Gross Energy (kcal/kg)
LT1	0	13.66± 0.49^{aA}	86.34±0.49^{cC}	6.28±0.51^{aA}	45.28±0.54^{aA}	35.89±0.59^{cC}	12.55±0.05^{aA}	4436.19±9.67^{aA}
	60	13.03± 0.33^{aB}	86.97±0.33^{cB}	5.06±0.22^{aB}	44.57±0.89^{aB}	37.89±1.20^{cB}	12.36±0.12^{aB}	4423.51±8.63^{aB}
	120	13.22± 0.06^{aB}	86.78±0.06^{cB}	5.05±0.25^{aB}	43.60±0.06^{aB}	38.98±0.30^{cB}	12.37±0.06^{aA}	4442.97±6.52^{aA}
	180	12.16± 0.24^{aC}	87.84±0.24^{cA}	4.52±0.35^{aC}	42.10±0.29^{aC}	41.03±0.78^{cA}	12.40±0.12^{aB}	4426.44±7.14^{aB}
LT2	0	13.66± 0.49^{bA}				35.89±0.59^{bcC}	12.55±0.05^{aA}	4436.19±9.67^{aA}
	60	12.98± 0.33^{bB}	87.02±0.33^{bB}	4.46±0.09^{bB}	44.95±0.27^{aB}	38.40±0.38^{bcB}	12.18±0.11^{aB}	
	120	12.16± 0.12^{bB}	87.84±0.12^{bB}	4.37±0.21^{bB}	43.71±0.20^{aB}	39.59±0.16^{bcB}	12.32±0.15^{aA}	4433.34±19.92^{aA}
	180	9.58± 0.16^{bC}	90.42±0.16^{bA}	4.08±0.28^{bC}	41.76±0.37^{aC}	41.89±0.62^{bcA}	12.28±0.05^{aB}	4395.73±9.53^{aB}
AT3	0	13.66± 0.49^{cA}	86.34±0.49^{aC}	6.28±0.51^{bcA}	45.28±0.54^{aA}	35.89±0.59^{bC}	12.55±0.05^{aA}	4436.19±9.67^{bA}
	60	11.63± 0.17^{cB}	88.37±0.17^{aB}				12.26±0.12^{aB}	4402.85±25.24^{bB}
	120	11.79± 0.19^{cB}	88.21±0.19^{aB}	4.13±0.20^{bcB}	43.97±0.19^{aB}	39.26±0.22^{bB}	12.64±0.21^{aA}	4418.36±4.50^{bA}
	180	9.35± 0.33^{cC}	90.65±0.33^{aA}	3.64±0.19^{bcC}	40.75±0.03^{aC}	43.27±0.22^{bA}	12.30±0.08^{aB}	4386.13±11.32^{bB}
HT4	0	13.66± 0.49^{cA}	86.34±0.49^{aC}	6.28±0.51^{cA}	45.28±0.54^{bA}	35.89±0.59^{aC}	12.55±0.05^{aA}	4436.19±9.67^{bA}
	60	11.64± 0.41^{cB}	88.36±0.41^{aB}	3.95±0.15^{cB}	42.65±1.67^{bB}	41.27±1.74^{aB}		
	120	11.15± 0.41^{cB}	88.85±0.41^{aB}	3.99±0.08^{cB}	43.31±0.57^{bB}	40.25±0.75^{aB}	12.46±0.17^{aA}	4407.22±12.13^{bA}
	180	9.15± 0.30^{cC}	90.85±0.30^{aA}	3.43±0.36^{cC}	40.0±0.63^{bC}	44.21±0.77^{aA}	12.37±0.01^{aB}	4382.78±12.82^{bB}

Values represented as Mean ± SD(n=3) LT1 = -20^{o}C; LT2 = 4^{o}C; AT3 = 17^{o}C - 31.5^{o}C; HT4 = 45^{o}C. Statistical differences (Tukey's, P< 0.05) are given by changed letters: changed small letters 'a - c' in similar column denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar column denote difference is significant (P< 0.05) among feed storage durations.

Table 4.1d): **Two-Way ANOVA with between subject effects of temperature, storage duration on proximate parameters and gross energy content of formulated aquafeed 1 (dry matter basis).**

Parameter	Source	SS	df	MS	F	P-value
Moisture (%)	**T**	18.72	3	6.24	53.78	**0.000**
	D	79.53	3	26.51	228.46	**0.000**
	I	11.66	9	1.30	11.17	**0.000**
Dry Matter (%)	**T**	18.72	3	6.24	53.78	**0.000**
	D	79.53	3	26.51	228.46	**0.000**
	I	11.66	9	1.30	11.17	**0.000**
Crude Ash (%)	**T**	0.06	3	0.02	1.68	0.191
	D	0.64	3	0.21	17.59	**0.000**
	I	0.29	9	0.03	2.65	**0.020**
Crude Lipid (%)	**T**	4.69	3	1.56	13.70	**0.000**
	D	39.39	3	13.13	114.99	**0.000**
	I	1.64	9	0.18	1.60	0.159
Crude Protein (%)	**T**	9.65	3	3.22	7.47	**0.001**
	D	107.96	3	35.99	83.53	**0.000**
	I	8.33	9	0.93	2.15	0.054
Carbohydrates (%)	**T**	25.22	3	8.41	14.42	**0.000**
	D	270.32	3	90.11	154.60	**0.000**
	I	15.59	9	1.73	2.97	0.011
Gross Energy (kcal/kg)	**T**	12921.96	3	4307.32	27.54	**0.000**
	D	10561.39	3	3520.46	22.51	**0.000**
	I	14440.72	9	1604.52	10.26	**0.000**

Post-hoc analysis based on Tukey's HSD. Temperature (T), Duration (D), Interaction of Temperature*Duration (I)**.**
P-value for between subject effects ; Significant **P-values, P< 0.05 in highlight.**

Table 4.1e): Effect of temperature and storage duration on proximate parameters of formulated aquafeed 2 (as-fed basis).

Temperature	Duration (day/s)	Crude Lipid (%)	Crude Protein (%)	Moisture (%)	Carbohydrates (%)	Crude Ash (%)	Gross Energy (kcal/kg)
LT1	0	6.07±0.47^{aA}	35.6±0.09^{aA}	11.59±0.33^{aA}	36.16±0.63^{cB}	10.58±0.05^{bB}	3931.39± 6.11^{aB}
	60	5.91±0.15^{aAB}	34.96±0.45^{aB}	11.78±0.28^{aA}	36.70±0.70^{cA}	10.65±0.13^{bAB}	3900.80±12.33^{aB}
	120	5.66±0.63^{aBC}	35.02±0.45^{aB}	12.24±0.36^{aA}	36.49±0.70^{cA}	10.59±0.17^{bA}	3902.53±23.30^{aB}
	180	5.27±0.22^{aC}	34.7± 0.71^{aB}	11.52±0.19^{aB}	37.85±0.49^{cA}	10.65±0.06^{bA}	3933.24±17.40^{aA}
LT2	0	6.07±0.47^{aA}	35.6±0.09^{abA}	11.59±0.33^{aA}	36.16±0.63^{bcB}	10.58±0.05^{bB}	3931.39± 6.11^{aB}
	60	5.7±0.31^{aAB}	35.27±0.16^{abB}	11.60±0.26^{aA}	36.70±0.19^{bcA}	10.72±0.02^{bAB}	3932.85±18.29^{aB}
	120	5.43±0.71^{aBC}	35.01±0.27^{abB}	11.79±0.22^{aA}	37.14±0.69^{bcA}	10.63±0.04^{bA}	3926.04±20.45^{aB}
	180	5.22±0.11^{aC}	33.77±1.64^{abB}	11.27±0.16^{aB}	39.12±1.86^{bcA}	10.62±0.10^{bA}	3938.84±14.71^{aA}
AT3	0	6.07±0.47^{abA}	35.6±0.09^{bA}	11.59±0.33^{aA}	36.16±0.63^{bB}	10.58±0.05^{bB}	3931.39± 6.11^{aB}
	60	5.51±0.35^{abAB}	34.39±0.30^{bB}	11.76±0.04^{aA}	37.66±0.69^{bA}	10.69±0.03^{bAB}	3925.72±7.63^{aB}
	120	5.13±0.18^{abBC}	33.95± 0.38^{bB}	12.08±0.18^{aA}	38.13±0.41^{bA}	10.72±0.08^{bA}	3907.51±31.10^{aB}
	180	4.75±0.04^{abC}	32.79±0.74^{bB}	11.77±0.02^{aB}	39.99±0.75^{bA}	10.71±0.04^{bA}	3941.92±8.54^{aA}
HT4	0	6.07±0.47^{bA}	35.6±0.09^{abA}	11.59±0.33^{bA}	36.16±0.63^{aB}	10.58±0.05^{aB}	3931.39± 6.11^{bB}
	60	5.46±0.23^{bAB}	33.72 ±0.68^{abB}	11.60±0.21^{bA}	38.45±0.67^{aA}	10.77±0.02^{aAB}	3924.29±4.67^{bB}
	120	4.56±0.46^{bBC}	34.21±1.80^{abB}	10.64±0.13^{bA}	39.64±1.40^{aA}	10.95±0.22^{aA}	3992.72±38.6^{bB}
	180	4.39±0.79^{bC}	34.52±0.96^{abB}	9.66±0.31^{bB}	40.29±0.31^{aA}	11.14±0.28^{aA}	4067.91±51.16^{bA}

Values represented as Mean ± SD (n=3).LT1 = -20°C; LT2 = 4°C; AT3 = 15.8°C - 31°C; HT4 = 45°C. Statistical differences (Tukey's, P< 0.05) are given by changed letters: changed small letters 'a - c' in similar column denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar column denote difference is significant (P< 0.05) among feed storage durations.

Table 4.1f): Two-Way ANOVA with between subject effects of temperature, storage duration on proximate parameters and gross energy content of formulated aquafeed 2 (as-fed basis).

Parameter	Source	SS	df	MS	F	P-value
Moisture (%)	**T**	6.81	3	2.27	35.96	**0.000**
	D	3.30	3	1.10	17.42	**0.000**
	I	6.16	9	0.68	10.85	**0.000**
Crude Ash (%)	**T**	0.44	3	0.15	11.59	**0.000**
	D	0.25	3	0.08	6.43	**0.002**
	I	0.35	9	0.04	3.04	**0.010**
Crude Lipid (%)	**T**	2.63	3	0.88	4.63	**0.008**
	D	9.35	3	3.12	16.50	**0.000**
	I	1.38	9	0.15	0.81	0.610
Crude Protein (%)	**T**	5.81	3	1.94	3.39	**0.030**
	D	16.94	3	5.65	9.88	**0.000**
	I	7.94	9	0.88	1.54	0.175
Carbohydrates (%)	**T**	28.71	3	9.57	16.61	**0.000**
	D	22.90	3	7.63	13.25	**0.000**
	I	11.55	9	1.28	2.30	**0.046**
Gross Energy (kcal/kg)	**T**	27459.72	3	9153.24	19.94	**0.000**
	D	17063.09	3	5687.70	12.39	**0.000**
	I	27943.19	9	3104.80	6.76	**0.000**

Post-hoc analysis based on Tukey's HSD. Temperature (T), Duration (D), Interaction of Temperature*Duration (I).

P-value for between subject effects ; Significant **P-values P< 0.05 in highlight.**

Table 4.1g): Effect of temperature and storage duration on proximate parameters of formulated aquafeed 2 (dry matter basis).

Temperature	Duration (day/s)	Crude Lipid (%)	Crude Protein (%)	Moisture (%)	Dry Matter (%)	Carbohydrates (%)	Crude Ash (%)	Gross Energy (kcal/kg)
LT1	0	6.86± 0.55^{aA}	40.27±0.15^{aA}	11.59±0.33^{aA}	88.41±0.33^{bB}	40.90±0.58^{cC}	11.97±0.01^{bB}	4446.8±13.38^{bAB}
	60	6.70±0.16^{aAB}	39.63±0.59^{aB}	11.78±0.28^{aA}	88.22±0.28^{bB}	41.60±0.73^{cB}	12.07±0.15^{bA}	4421.55±24.13^{bB}
	120	6.45±0.69^{aBC}	39.91±0.41^{aB}	12.24±0.36^{aA}				4446.64±14.43^{bAB}
	180	5.96±0.25^{aC}	39.22±0.82^{aC}	11.52±0.19^{aA}	88.48±0.19^{bA}	42.78±0.53^{cA}	12.04±0.08^{bA}	4445.51±18.37^{bA}
LT2	0	6.86± 0.55^{aA}	40.27±0.15^{abA}	11.59±0.33^{aA}	88.41±0.33^{bB}	40.90±0.58^{bcC}	11.97±0.01^{bB}	4446.8±13.38^{abAB}
	60	6.44±0.34^{aAB}					12.13±0.04^{bA}	4449.08±12.34^{abB}
	120	6.15±1.16^{aBC}					12.05±0.07^{bA}	4450.95±20.54^{abAB}
	180	5.88±0.13^{aC}	38.06±1.92^{abC}	11.27±0.16^{aA}	88.73±0.16^{bA}	44.09±2.01^{bcA}	11.97±0.08^{bA}	4439.12±8.99^{abA}
AT3	0	6.86±0.55^{abA}	40.27±0.15^{bA}	11.59±0.33^{aA}	88.41±0.33^{bB}	40.90±0.58^{abC}	11.97±0.01^{abB}	4446.8±13.38^{abAB}
	60	6.24±0.40^{abAB}	38.97±0.36^{bB}	11.76±0.04^{aA}	88.24±0.04^{bB}	42.67±0.77^{abB}	12.11±0.03^{abA}	4448.91±9.75^{abB}
	120	5.83±0.22^{abBC}	38.62±0.40^{bB}	12.08±0.18^{aA}	87.92±0.18^{bB}	43.36±0.41^{abB}	12.19±0.12^{abA}	4444.35±26.57^{abAB}
	180	5.38±0.05^{abC}	37.16±0.85^{bC}	11.77±0.02^{aA}	88.23±0.02^{bA}	45.32±0.84^{abA}	12.14±0.04^{abA}	4467.78±9.25^{abA}
HT4	0	6.86± 0.55^{bA}	40.27±0.15^{bA}	11.59±0.33^{bB}	88.41±0.33^{aB}	40.90±0.58^{aC}	11.97±0.01^{aB}	4446.8±13.38^{aAB}
	60	6.17±0.24^{bAB}						
	120	5.10±0.53^{bBC}	38.28 ±1.96^{bB}	10.64±0.13^{bB}	89.36±0.13^{aB}	44.36±1.63^{aB}	12.25±0.25^{aA}	4467.93±38.50^{aAB}
	180	4.86±0.88^{bC}	38.21± 0.99^{bC}	9.66±0.31^{bB}	90.34±0.31^{aA}	44.60±0.39^{aA}	12.33±0.28^{aA}	4502.81±43.30^{aA}

Values represented as Mean ± SD (n=3). LT1 = -20°C; LT2 = 4°C; AT3 = 15.8°C - 31°C; HT4 = 45°C. Statistical differences (Tukey's, P< 0.05) are given by changed letters: changed small letters 'a - c' in similar column denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar column denote difference is significant (P< 0.05) among feed storage durations.

Table 4.1h): Two-Way ANOVA with between subject effects of temperature, storage duration on proximate parameters and gross energy content of formulated aquafeed 2 (dry matter basis).

Parameter	Source	SS	df	MS	F	P-value
Moisture (%)	**T**	6.81	3	2.27	35.96	**0.000**
	D	3.30	3	1.10	17.42	**0.000**
	I	6.16	9	0.68	10.85	**0.000**
Dry Matter (%)	**T**	6.81	3	2.27	35.96	**0.000**
	D	3.30	3	1.10	17.42	**0.000**
	I	6.16	9	0.68	10.85	**0.000**
Crude Ash (%)	**T**	0.19	3	0.06	4.47	**0.010**
	D	0.22	3	0.07	5.27	**0.005**
	I	0.15	9	0.02	1.14	0.366
Crude Lipid (%)	**T**	3.81	3	1.27	5.20	**0.005**
	D	12.41	3	4.14	16.94	**0.000**
	I	2.07	9	0.23	0.94	0.505
Crude Protein (%)	**T**	9.72	3	3.24	4.45	**0.010**
	D	26.53	3	8.84	12.16	**0.000**
	I	7.91	9	0.88	1.21	0.324
Carbohydrates (%)	**T**	20.90	3	6.97	9.02	**0.000**
	D	66.95	3	22.32	28.90	**0.000**
	I	11.60	9	1.29	1.67	0.138
Gross Energy (kcal/kg)	**T**	3773.11	3	1257.70	2.97	**0.047**
	D	3722.76	3	1240.92	2.93	**0.049**
	I	6200.44	9	688.94	1.63	0.150

Post-hoc analysis based on Tukey's HSD. Temperature (T), Duration (D), Interaction of Temperature*Duration (I).

P-value for between subject effects ; Significant **P-values P< 0.05 in highlight.**

4.2 Gross Energy/ Calorific value storage changes

As-fed basis

Diet 1 and diet 2: Storage temperature, duration, and their interaction (temperature x duration) have significant effect on moisture, ash and GE content of feed. For diet1, highest GE value (3981.74 kcal/kg) is found at lowest moisture content (9.15%), at end of storage at HT4 condition; compared to those at 180th day AT3 (9.35% moisture and 3976.02 kcal/kg GE)< LT2 (9.58% and 3974.62 kcal/kg)< LT1 (12.16% and 3888.19kcal/kg).

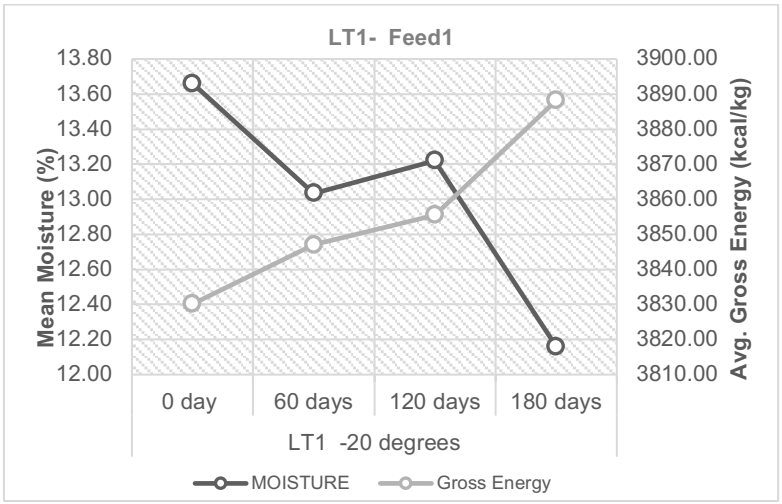
Reciprocal effects of moisture are notable on gross energy values (as-fed basis) with decrease in GE, on moisture increase. Univariate regression of moisture effects on GE values, of diet 1 depict coefficient of determination R^2 =0.8927 (at LT1), 0.9140 (at LT2), 0.954 (for AT3), 0.8255 (HT4). Diet 2; R^2 = 0.3958 (at LT1), 0.4087 (LT2), 0.4151 (AT3), 0.8887 (HT4), refer figure 4.1(a)-(d). In diet 1, moisture impact on GE are more pronounced at LT1,LT2, AT; compared to that for diet 2. While at HT conditions, for both diets; moisture has greater impact on GE as depicted by high R^2 values.

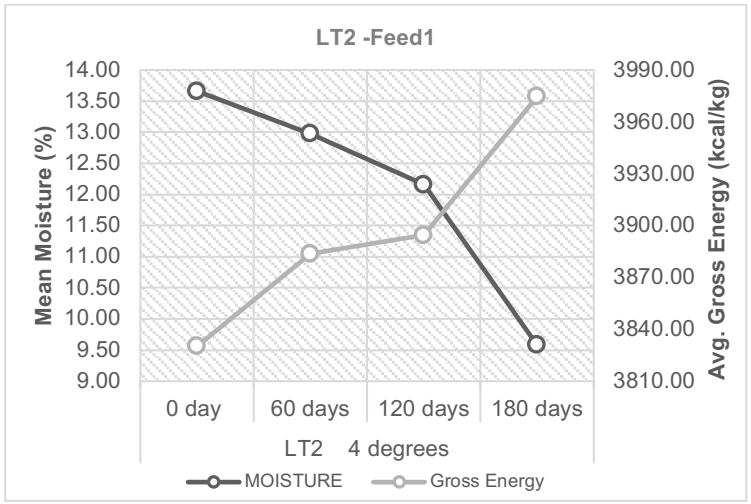
Dry matter basis

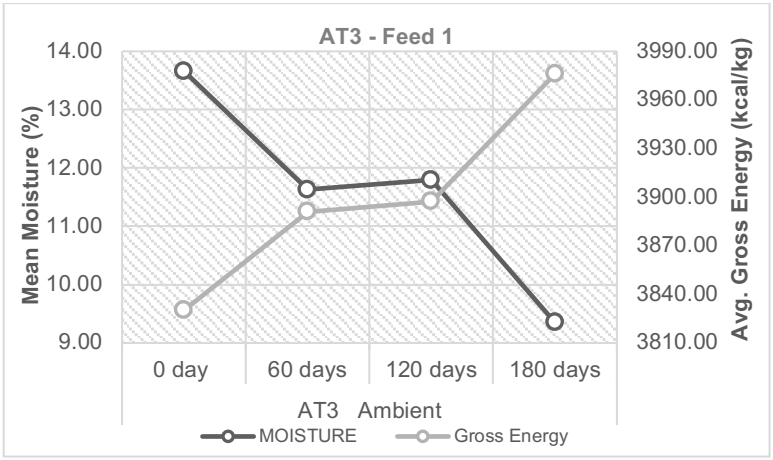
Diet 1: As for diet1, higher GE values are found at low temperatures compared to AT3 and HT4. Between-subject-effects of temperature, duration, and both are highly significant (P= 0.000).

Diet 2 : No significant difference between low temperatures and AT3, between LT2 and HT4 GE values exist. Difference between LT1 and HT4 values for GE is noteworthy, P<0.05. Interaction effects are non-significant; rather, temperature and duration impacts GE significantly.

Figure 4.1a): Impact of mean moisture on average gross energy value (as-fed basis) of formulated feed 1 stored at variable storage temperatures and durations.







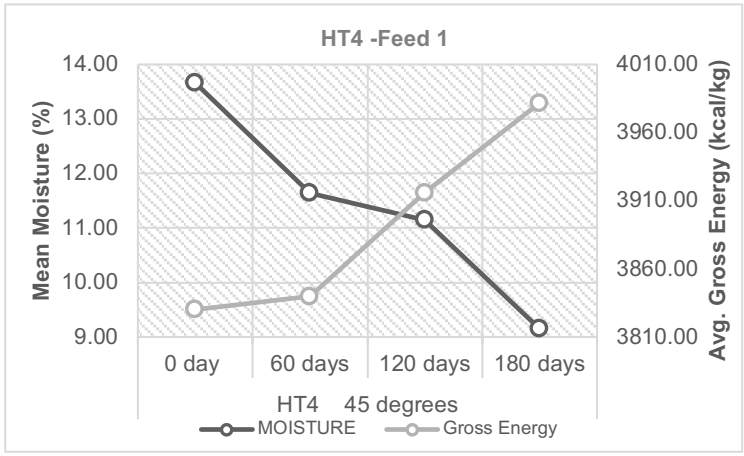
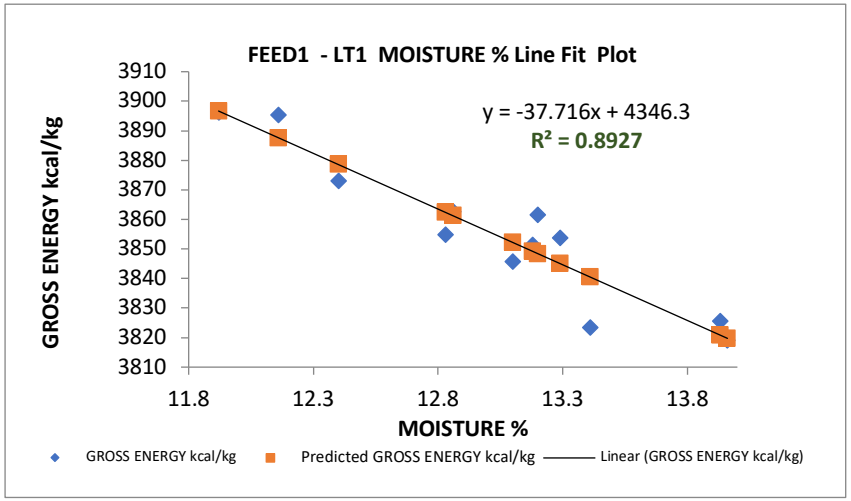
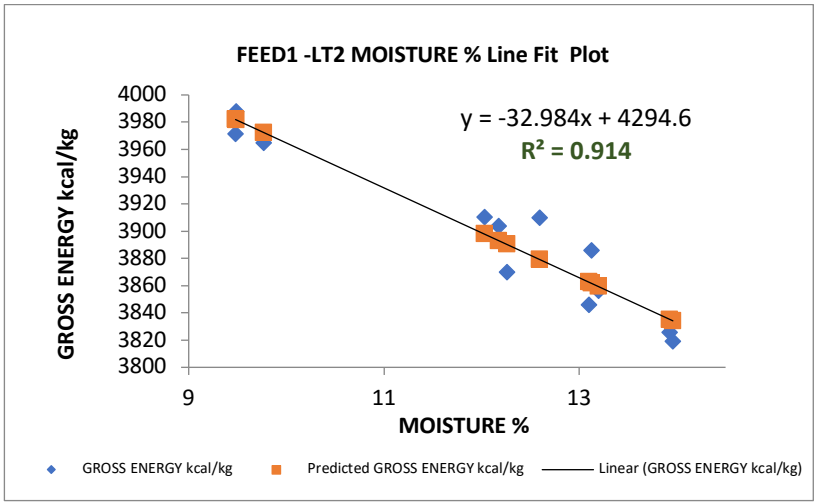
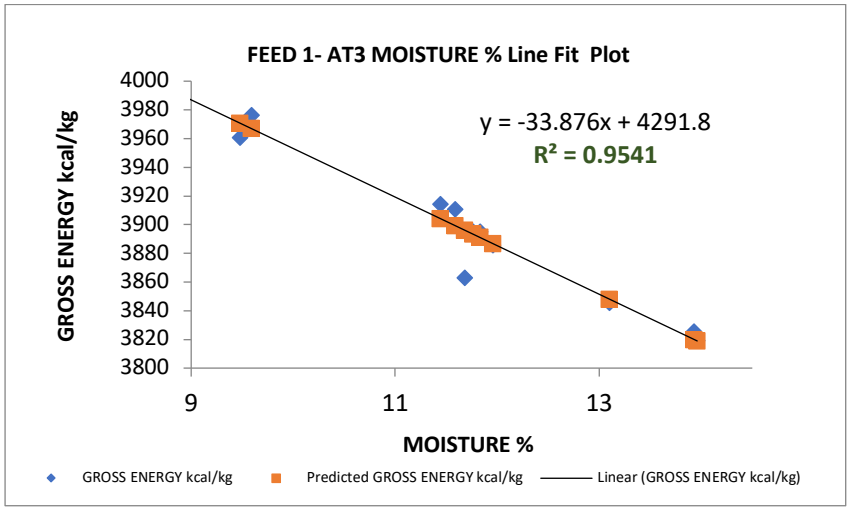


Figure 4.1b): Univariate Regression Analysis depicting reciprocal impact of moisture on gross energy value (as-fed basis) of storage feed 1.







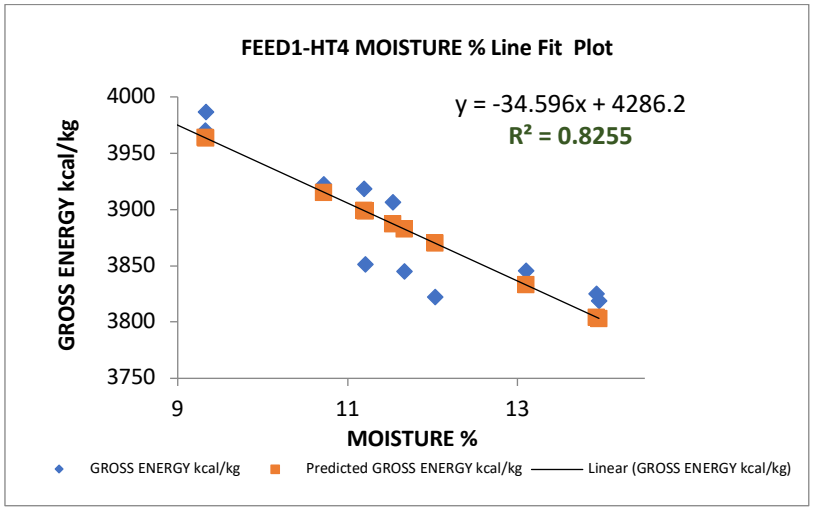
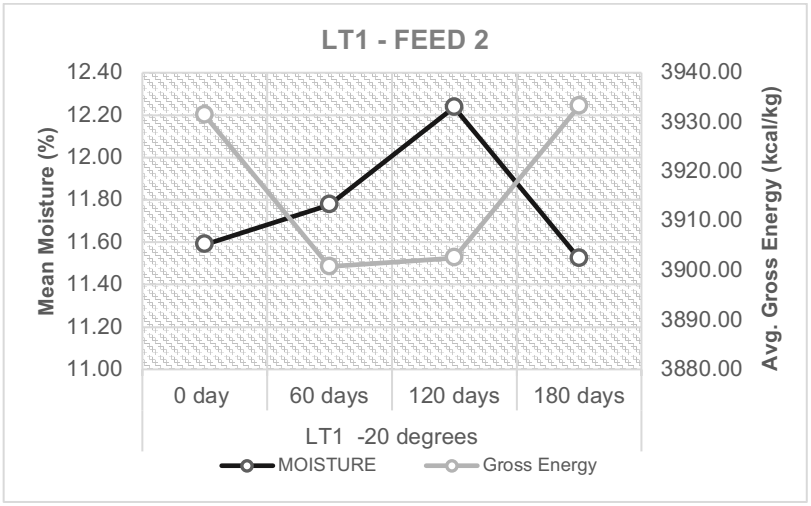
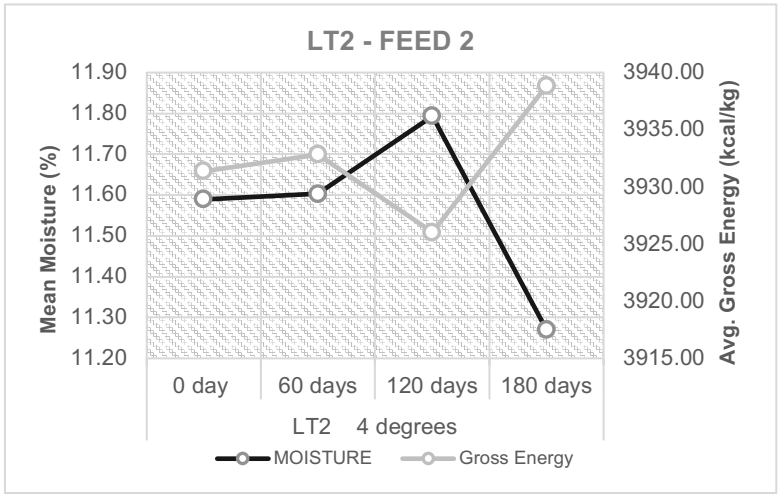
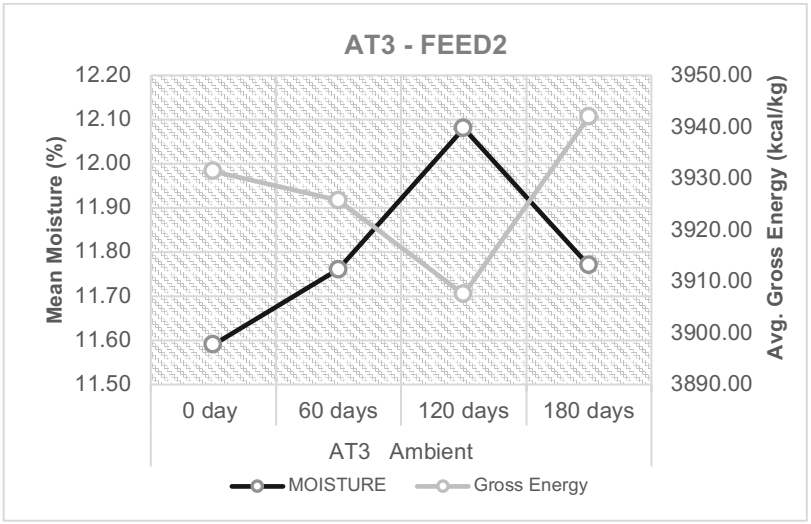


Figure 4.1c): Impact of mean moisture on average gross energy value (as-fed basis) of formulated feed 2 stored at variable storage temperatures and durations.







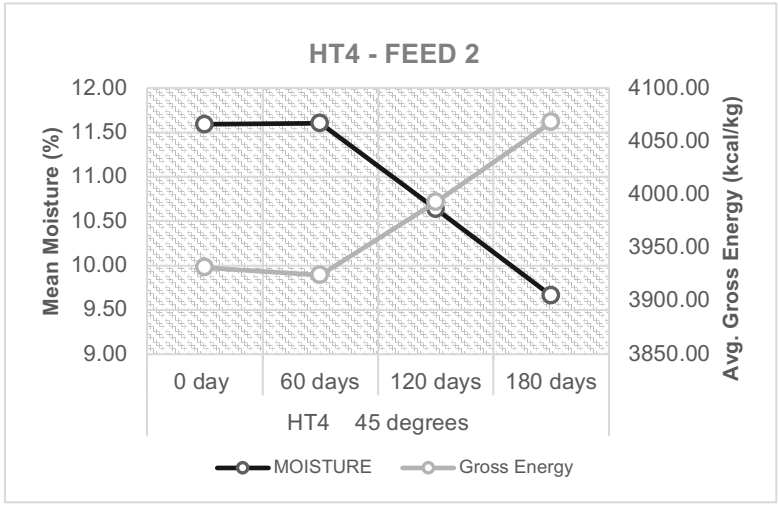
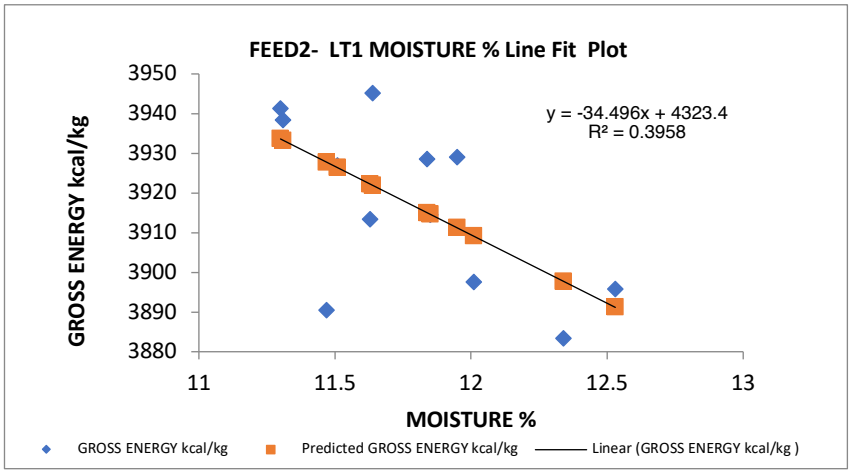
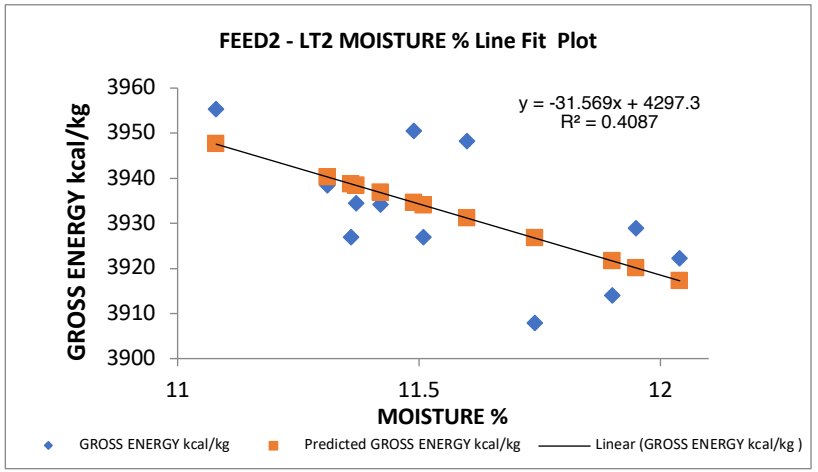
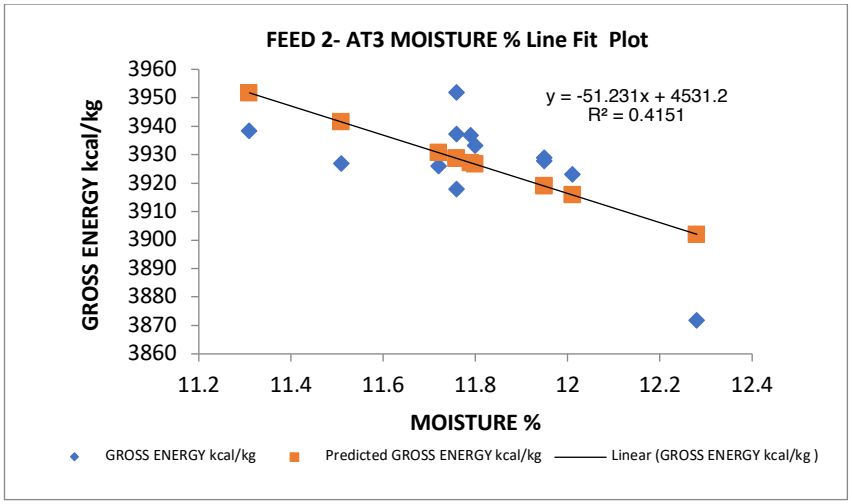
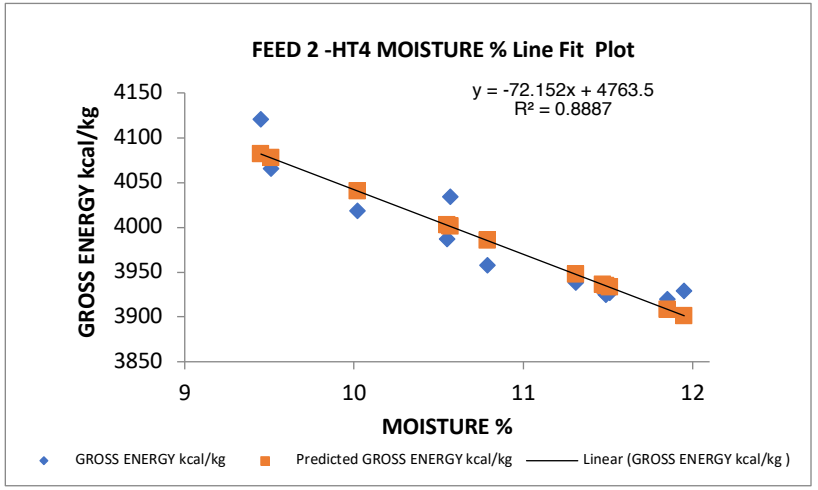


Figure 4.1d):Univariate Regression Analysis depicting reciprocal impact of moisture on gross energy value (as-fed basis) of storage feed 2.









4.3 Amino acid Profile storage changes

Amino acids are released as products of feed protein digestion in animals. Amino acid profile is a marked indicator of nutritional quality of dietary proteins (Benitez,1989). AAs were resolved with post-column derivatization for chromatographic separation. Figure 4.2 graphically elucidates chromatogram of mixture of 38 AA standards. 37 AAs were characterized and separated in diet 1, and 28 AAs in diet 2; comprising 10 EAAs, 8 NEAAs (in both diets) while 19,10 NPAAs in diet 1 and 2, respectively.

4.3.1 Essential Amino acids

According to their abundance at initial day of storage leucine is most abundant EAA in both diets at 2.73±0.09 g/100g of diet 1, 2.49±0.08 g/100g of diet 2; followed by lys >val >thr >Ile >phe >met >arg> his> trp in diet 1, and arg> val> lys> ile> thr> phe> met> his> trp in diet 2 (table 4.2a).

All EAAs show significant ($P<0.05$) storage loss due to temperature effects for both diets with highest decrease at end of storage duration at 180 day. Additionally, total EAAs show gradual bimonthly decrease at all temperatures from initial to final storage for both diets. AA losses for diet 1 at 180th day account 28.97%, 25.35%, 28.08%, at , AT, HT conditions. Incurred loss of EAAs being highest at high temperature storage at sixth month. Estimated AA losses are 13.96% (LT1), 26.88% (LT2), 17.23% (AT3), 25% (HT4) at end of storage in diet 2.

For diet 1, between subject effects of duration have high significance ($P=0.000$) including significant effects ($P<0.05$) of interaction for all EAAs (table 4.2a). Major effects of temperature are significant ($P<0.05$) for arginine, isoleucine,

lysine, phenylalanine, tryptophan but non-significant (P>0.05) for histidine, leucine, methionine, threonine and valine. In case of diet 2, highly significant (P=0.000) between subject effects of duration are notable with significant effects of interaction for all EAAs except, arginine and histidine. While with temperature, between subject impacts are noteworthy (P<0.05) for arg, his, lys, met, thr and trp only.

4.3.2 Non-Essential Amino acids

Glutamic acid is the most prevalent NEAA at concentration 5.76 ± 0.27 g/100g diet1, 6.12 ± 0.18g/100g diet 2. Initial contents are higher for aspartate followed by proline, alanine, glycine, serine, tyrosine, and cysteine in diet 1. In diet 2, initial concentrations following glutamic acid are in order of decrease as asp>gly>pro>ala>ser>tyr>cys (table 4.2a,4.3a).

There is an overall loss in total NEAAs at end of storage compared to initial concentration in diets. Similar to total EAAs, storage losses of total NEAAs are highest at HT condition at 180th day for diet 2, but at LT2 followed by HT for diet 1. Individual NEAAs for both diets, depict gross reduction at 180th day storage at all temperature conditions. Likewise, for all in both diets; duration dependent between subject effects are relevant (P<0.05). Significant effects of interaction are present for NEAAs excluding ser in diet 1 and except ala, gly, pro, ser, tyr for diet 2. Major effects of temperature are non-significant (P>0.05). for asp, glu, ser, tyr towards diet 1 and ala, gly, ser, tyr in diet 2.

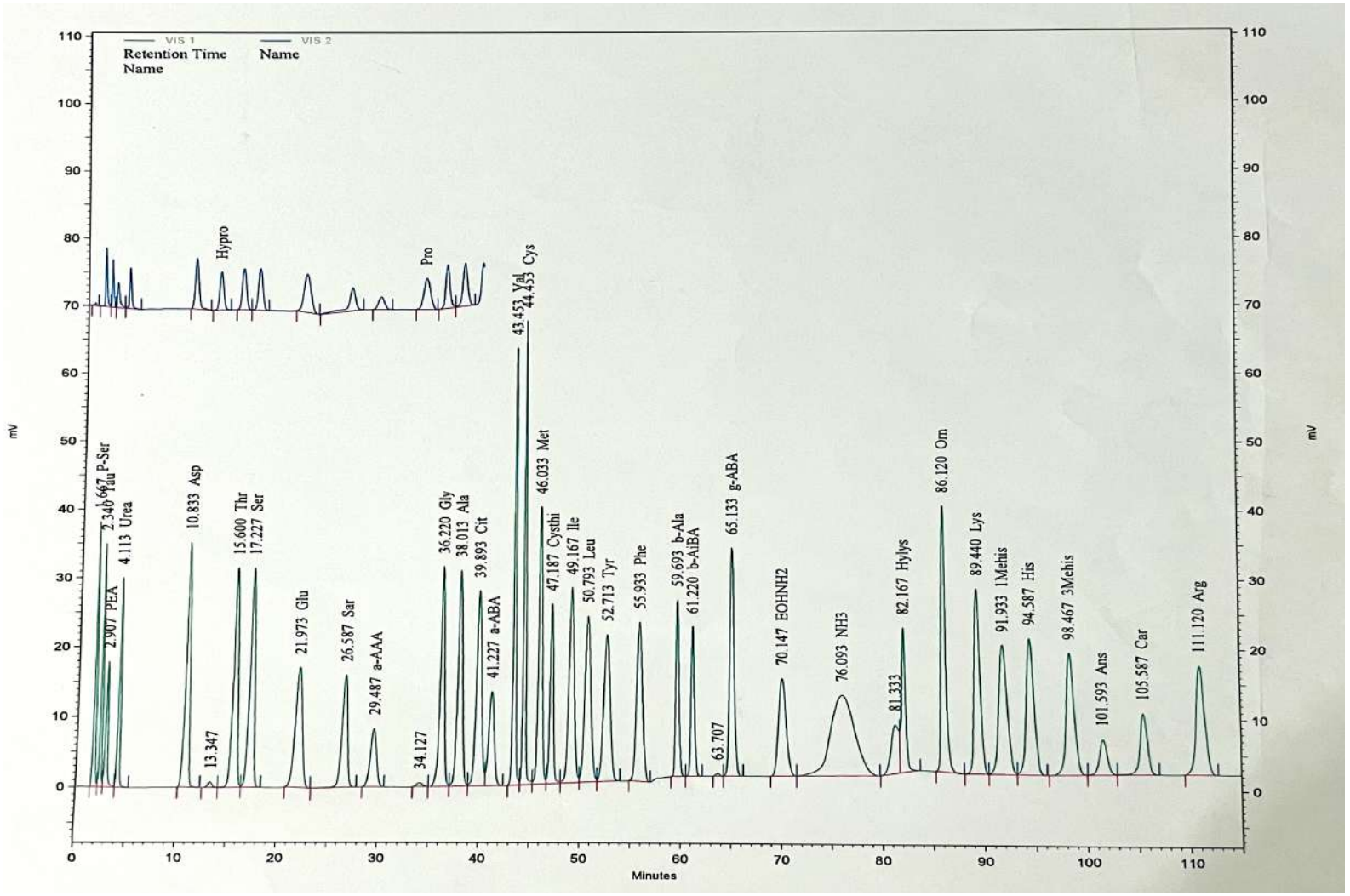
Together with comprise proteinogenic AAs (PAAs). PAAs depict incurred decrements at end of storage at all temperature conditions, a trend similar to EAA .

Non-proteinogenic AAs (NPAAs)

Diet 1: There is an overall decrease in total NPAA at the end of storage time. α-AAA is totally absent 120-day onwards at LT1, and 60-day further at LT2, AT3, HT4 storages. 3-methylhistidine depicts absence at 180-day determination at LT1, with complete onwards at all other temperatures. Hydroxylysine and anserine amounts are lost to null, second month onwards at all storage temperatures. Duration based between subject effects are highly significant; except for hylys and ans.

Diet 2: There is significant decrease in amounts of p-ser, tau, ϒ-ABA, ß-ala, orn, cit, 1-mehis, ethanolamine, hypro, ß-AiBA, at all temperatures at the end of storage regime (table 4.3b). Bimonthly (at 0, 60,120,180 day) for all, total NPAAs is notable (except, for AT3 60 day compared to initial 0 day). Major effects of duration (D), temperature (T) and interaction (I) are noteworthy ($P<0.05$) except for between subject effects of 'T' for tau ($P= 0.588$), orn ($P= 0.259$) and that of 'I' for ß-ala and ornithine.

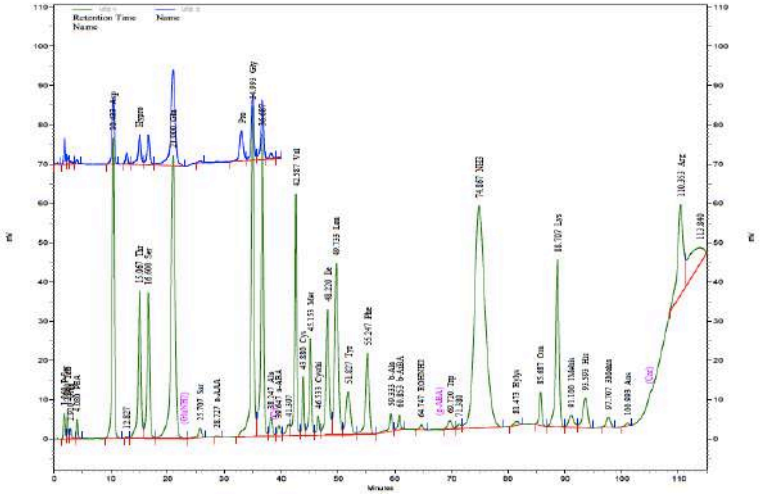
Figure 4.2: Chromatogram of Amino acid standards.



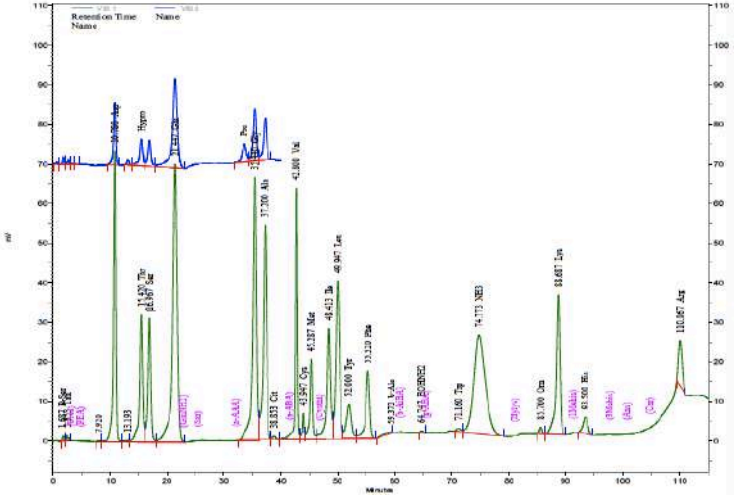
Source: Author (2023)

Figure 4.3a): Representative amino acid profiles of aquafeed 1 at LT1 storage durations.

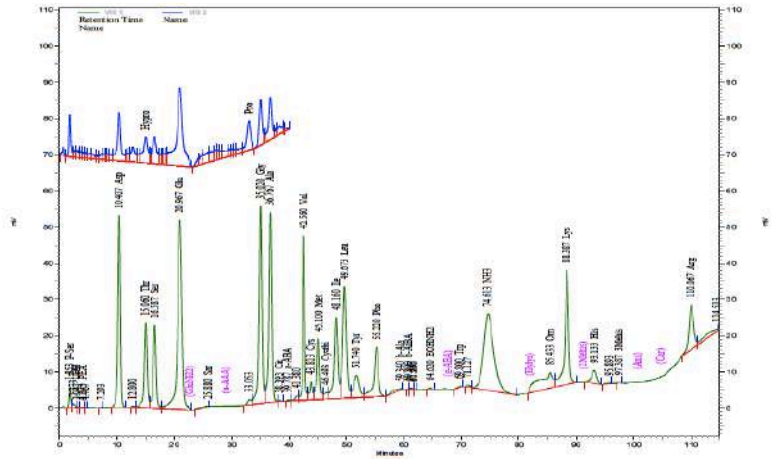
FEED 1 - ZERO DAY



FEED 1- 60 DAY T1



FEED 1 - 120 DAY T1



FEED 1 - 180 DAY T1

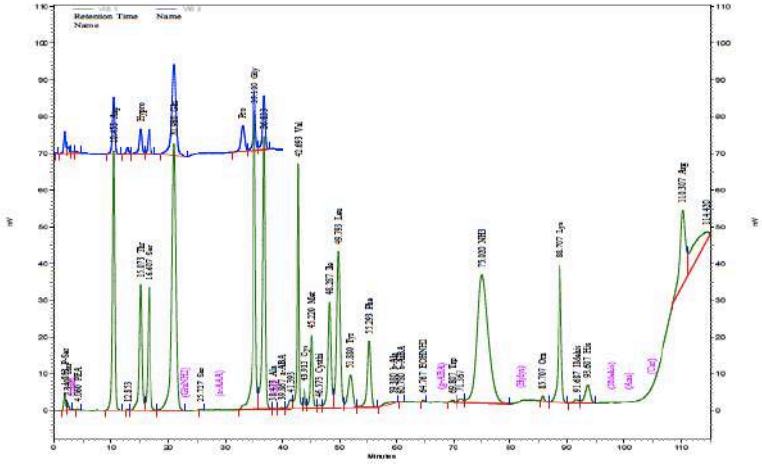
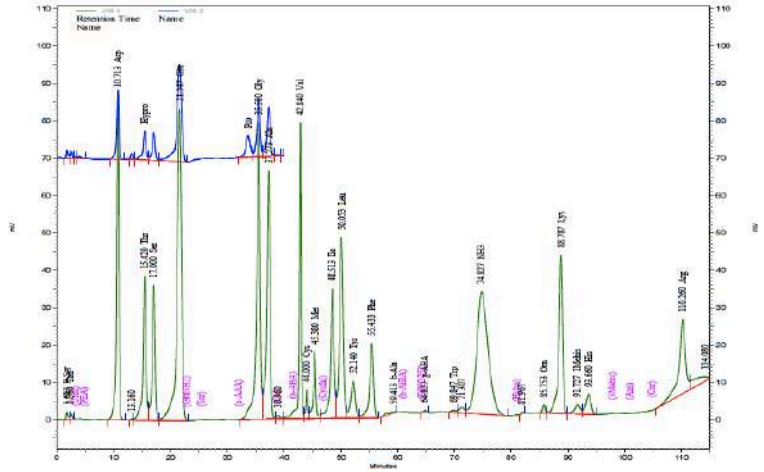
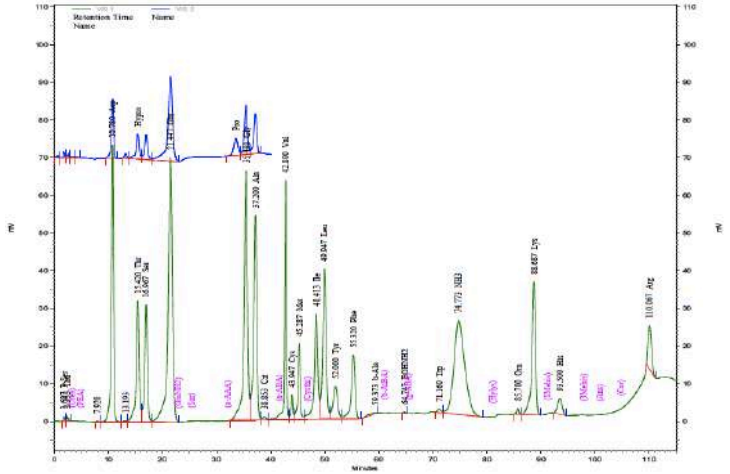


Figure 4.3b): Representative amino acid profiles of aquafeed 2 at LT1 storage durations.

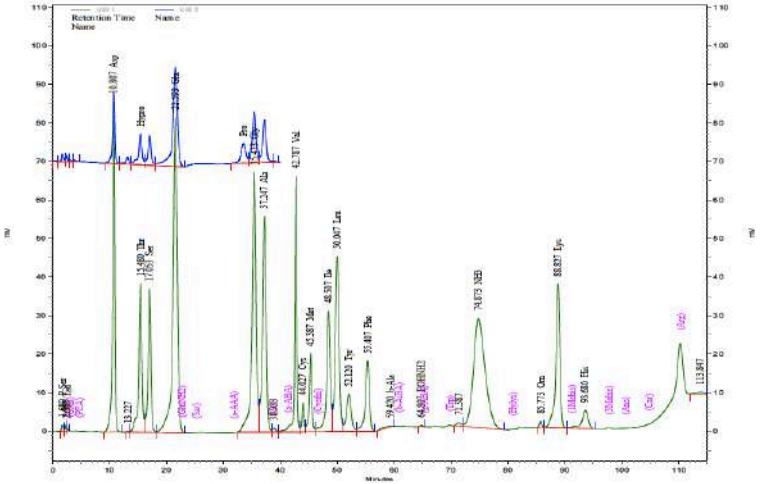
FEED 2 - ZERO DAY



FEED 2 - 60 DAY T1



FEED 2 - 120 DAY T1



FEED 2 - 180 DAY T1

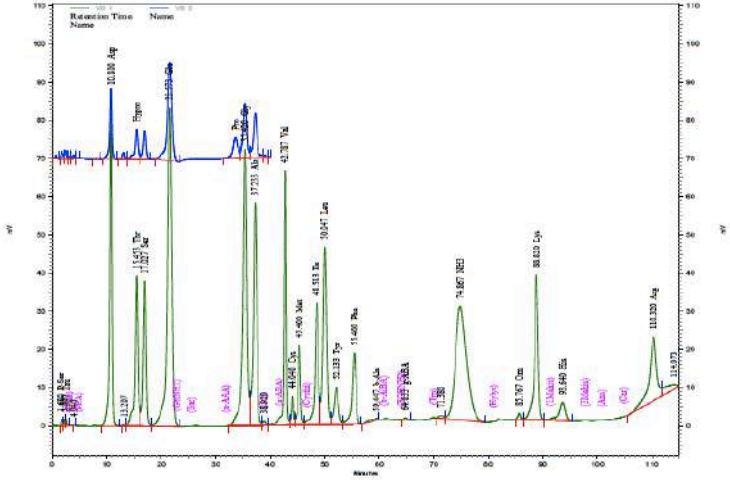


Table 4.2a): Amino acid profile changes during storage for aquafeed 1 (extruded diet).

Amino Acids (AA) g/100g	Storage Conditions																		
Proteinogenic AA	**LT1**				**LT2**				**AT3**				**HT4**				**P-value**		
Essential AA	**0day**	**60day**	**120day**	**180day**	**0day**	**60day**	**120day**	**180day**	**0day**	**60day**	**120day**	**180day**	**0day**	**60day**	**120day**	**180day**	**T**	**D**	**I**
Arginine (Arg)	0.93±0.11^{bB}	0.97±0.01^{bA}	0.75±0.05^{bC}	0.40±0.05^{bD}	0.93±0.11^{bB}	1.26±0.14^{bA}	0.44±0.03^{bC}	0.63±0.01^{bD}	0.93±0.11^{aB}	1.47±0.01^{aA}	0.81±0.02^{aC}	0.59±0.01^{aD}	0.93±0.11^{bB}	1.33±0.21^{bA}	0.70±0.01^{bC}	0.36±0.03^{bD}	**.000**	**.000**	**.000**
Histidine (His)	0.78±0.09^{aA}	0.46±0.03^{aB}	0.50±0.03^{aB}	0.50±0.08^{aB}	0.78±0.09^{aA}	0.45±0.00^{aB}	0.43±0.02^{aB}	0.49±0.03^{aB}	0.78±0.09^{aA}	0.45±0.02^{aB}	0.46±0.01^{aB}	0.44±0.01^{aB}	0.78±0.09^{aA}	0.39±0.01^{aB}	0.58±0.01^{aB}	0.37±0.02^{aB}	.521	**.000**	**.014**
Isoleucine (Ile)	1.54±0.02^{abA}	1.52±0.04^{abA}	1.28±0.01^{abB}	1.20±0.03^{abC}	1.54±0.02^{abA}	1.52±0.02^{abA}	1.17±0.02^{abB}	1.25±0.07^{abC}	1.54±0.02^{bA}	1.49±0.11^{bA}	1.23±0.04^{bB}	1.12±0.07^{bC}	1.54±0.02^{aA}	1.55±0.13^{aA}	1.41±0.02^{aB}	1.20±0.07^{aC}	**.016**	**.000**	**.013**
Leucine (Leu)	2.73±0.09^{aA}	2.68±0.08^{aB}	2.16±0.05^{aC}	1.96±0.03^{aD}	2.73±0.09^{aA}	2.69±0.00^{aB}	1.92±0.00^{aC}	2.17±0.13^{aD}	2.73±0.09^{aA}	2.62±0.18^{aB}	2.18±0.00^{aC}	2.07±0.01^{aD}	2.73±0.09^{aA}	2.38±0.05^{aB}	2.59±0.03^{aC}	1.99±0.11^{aD}	.519		
Lysine (Lys)	2.48±0.05^{aA}	2.27±0.19^{aB}	2.01±0.06^{aC}						2.48±0.05^{bcA}	2.04±0.01^{bcB}	1.93±0.01^{bcC}	1.81±0.12^{bcD}	2.48±0.05^{cA}	2.16±0.03^{cB}	1.80±0.04^{cC}	1.62±0.05^{cD}	**.000**	**.000**	**.022**
Methionine (Met)	0.95±0.08^{aA}	0.82±0.02^{aB}	0.77±0.04^{aB}	0.78±0.09^{aC}	0.95±0.08^{aA}	0.85±0.05^{aA}	0.80±0.04^{aB}	0.55±0.07^{aC}	0.95±0.08^{aA}	0.82±0.07^{aA}	0.70±0.09^{aB}	0.65±0.04^{aC}	0.95±0.08^{aA}	0.66±0.08^{aA}	0.86±0.02^{aB}	0.81±0.08^{aC}	.193	**.000**	**.000**
Phenylalanine (Phe)	1.52±0.03^{aA}	1.39±0.01^{aB}	1.26±0.08^{aC}	1.21±0.07^{aD}	1.52±0.03^{abA}	1.41±0.01^{abB}	1.26±0.00^{abC}	1.16±0.06^{abD}	1.52±0.03^{bA}	1.38±0.10^{bB}	1.19±0.03^{bC}	1.08±0.00^{bD}	1.52±0.03^{aA}	1.32±0.05^{aB}	1.36±0.01^{aC}	1.28±0.04^{aD}	**.003**	**.000**	**.002**
Threonine (Thr)	1.60±0.02^{aA}	1.52±0.02^{aA}	1.20±0.05^{aB}	1.08±0.05^{aB}	1.60±0.02^{aA}	1.63±0.02^{aA}	1.03±0.03^{ab}	1.20±0.08^{aB}	1.60±0.02^{aA}	1.55±0.14^{aA}	1.10±0.11^{aB}	1.35±0.19^{aB}	1.60±0.02^{aA}	1.43±0.09^{aA}	1.44±0.00^{aB}	1.02±0.03^{aB}	.436	**.000**	**.000**
Tryptophan (Trp)	0.34±0.00^{aA}	0.10±0.03^{aB}	0.21±0.01^{aB}	0.08±0.00^{aB}	0.34±0.00^{aA}	0.11±0.01^{aB}							0.34±0.00^{bA}	0.10±0.03^{bB}	0.08±0.00^{bB}	0.06±0.01^{bB}	**.000**	**.000**	**.000**
Valine (Val)	1.77±0.05^{aA}	1.78±0.05^{aA}	1.52±0.01^{aB}	1.31±0.05^{aC}	1.77±0.05^{aA}	1.81±0.01^{aA}	1.33±0.02^{aB}	1.53±0.05^{aC}	1.77±0.05^{aA}	1.79±0.13^{aA}	1.39±0.08^{aB}	1.33±0.07^{aC}	1.77±0.05^{aA}	1.64±0.04^{aA}	1.68±0.02^{aB}	1.28±0.07^{aC}	.377	**.000**	**.000**
Total EAA	**14.64**	**13.52**	**11.67**	**10.40**	**14.64**	**13.91**	**10.63**	**10.93**	**14.64**	**13.68**	**11.08**	**10.53**	**14.64**	**12.96**	**12.50**	**10.00**	**P-value**		
Non-Essential AA																	**T**	**D**	**I**
Alanine (Ala)	2.41±0.11^{aA}	2.45±0.14^{aA}	1.97±0.07^{aB}	1.70±0.06^{aB}	2.41±0.11^{aA}	2.49±0.10^{aA}	2.01±0.12^{aB}	1.59±0.01^{aB}	2.41±0.11^{aA}	2.40±0.16^{aA}	1.92±0.03^{aB}	1.99±0.00^{aB}	2.41±0.11^{bA}	2.06±0.02^{bA}	1.65±0.09^{bB}	1.00±0.47^{bB}	**.002**	**.000**	**.036**
Aspartate (Asp)	3.36±0.02^{aA}	3.41±0.10^{aA}	2.78±0.08^{aB}	2.44±0.06^{aB}	3.36±0.02^{aA}	3.26±0.06^{aA}	2.80±0.18^{aB}	2.24±0.03^{aB}	3.36±0.02^{aA}	3.19±0.19^{aA}	3.10±0.44^{aB}	2.59±0.23^{aB}	3.36±0.02^{aA}	2.95±0.14^{aA}	2.35±0.15^{aB}	3.20±0.02^{aB}	.199		**.000**
Cysteine (Cys)	0.55±0.12^{abA}	0.21±0.00^{abC}					0.22±0.02^{aC}	0.34±0.04^{aB}	0.55±0.12^{bA}	0.23±0.02^{bC}	0.18±0.01^{bC}	0.26±0.07^{bB}	0.55±0.12^{aA}	0.23±0.00^{aC}	0.35±0.01^{aC}	0.27±0.01^{aB}	**.026**		**.001**
Glutamic Acid (Glu)	5.76±0.27^{aB}	6.85±0.80^{aA}	4.75±0.14^{aC}	4.34±0.07^{aD}	5.76±0.27^{aB}	6.10±0.07^{aA}	4.78±0.28^{aC}	3.97±0.01^{aD}	5.76±0.27^{aB}	5.98±0.38^{aA}	4.53±0.01^{aC}	4.45±0.41^{aD}	5.76±0.27^{aB}	5.58±0.25^{aA}	5.59±0.06^{aC}	4.08±0.24^{aD}	.138		**.000**
Glycine (Gly)	2.37±0.13^{aA}	2.34±0.05^{aA}	1.87±0.06^{aB}	1.54±0.03^{aB}	2.37±0.13^{aA}	2.29±0.01^{aA}	1.87±0.11^{aB}	1.52±0.10^{aB}	2.37±0.13^{aA}	2.27±0.10^{aA}	1.82±0.06^{aB}	1.95±0.08^{aB}	2.37±0.13^{aA}	2.25±0.02^{aA}	1.50±0.07^{aB}	2.26±0.02^{aB}	**.034**	**.000**	**.000**
Proline (Pro)	2.63±0.03^{aA}	2.09±0.03^{aB}	2.71±0.01^{aA}	3.21±0.39^{aA}	2.63±0.03^{bA}	1.86±0.15^{bB}	2.70±0.12^{bA}	2.39±0.09^{bA}	2.63±0.03^{aA}	1.94±0.19^{aB}	2.64±0.05^{aA}	3.07±0.20^{aA}	2.63±0.03^{bA}	1.88±0.05^{bB}	2.50±0.16^{bA}	2.40±0.07^{bA}	**.000**	**.000**	**.000**
Serine (Ser)	1.55±0.00^{aA}	1.35±0.02^{aAB}	1.43±0.22^{aB}	1.05±0.03^{aC}	1.55±0.00^{aA}	1.70±0.33^{aAB}	1.17±0.08^{aB}	0.97±0.00^{aC}	1.55±0.00^{aA}	1.65±0.25^{aAB}	1.46±0.33^{aB}	1.07±0.12^{aC}	1.55±0.00^{aA}	1.37±0.19^{aAB}	1.41±0.00^{aB}	0.97±0.06^{aC}	.352	**.000**	.095
Tyrosine (Tyr)	1.21±0.07^{aA}	1.06±0.03^{aB}	0.85±0.02^{aC}	0.72±0.14^{aC}	1.21±0.07^{aA}	1.10±0.00^{aB}	0.84±0.01^{aC}	0.80±0.04^{aC}	1.21±0.07^{aA}	1.05±0.00^{aB}	0.85±0.10^{aC}	0.90±0.10^{aC}	1.21±0.07^{aA}	0.99±0.11^{aB}	1.04±0.01^{aC}	0.90±0.00^{aC}	.099	**.000**	**.009**
Total NEAA	**19.86**	**19.75**	**16.66**	**15.32**	**19.86**	**19.05**	**16.39**	**13.83**	**19.86**	**18.72**	**16.49**	**16.28**	**19.86**	**17.30**	**16.38**	**15.08**			
Total EAA + NEAA	**34.50**	**32.88**	**28.59**	**25.85**	**34.50**	**32.62**	**27.18**	**24.92**	**34.50**	**32.20**	**27.68**	**26.93**	**34.50**	**29.72**	**29.25**	**25.08**			

*Data represented as Mean± SD (n=3). Statistical differences (Tukey's, P< 0.05) in means are given by changed letters: changed small letters 'a - c' in similar row denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar row denote difference is significant (P< 0.05) among feed storage durations.

Table 4.2b): Amino acid profile changes during storage for aquafeed 1 (extruded diet).

Amino Acids g/100g	Storage Conditions																P-value		
	LT1				LT2				AT3				HT4						
Non-Proteinogenic AA	**0day**	**60day**	**120day**	**180day**	**0day**	**60day**	**120day**	**180day**	**0day**	**60day**	**120day**	**180day**	**0day**	**60day**	**120day**	**180day**	**T**	**D**	**I**
Cystathionine (Cysthi)	0.14± 0.02^{abB}	0.18± 0.06^{abB}	0.29± 0.03^{abA}	0.14± 0.00^{abB}	0.14± 0.02^{aB}	0.13± 0.01^{aB}	0.38± 0.00^{aA}	0.14± 0.00^{aB}	0.14± 0.02^{bB}	0.14± 0.01^{bB}	0.12± 0.01^{bA}	0.25± 0.11^{bB}	0.14± 0.02^{aB}	0.20± 0.06^{aB}	0.35± 0.02^{aA}	0.15± 0.00^{aB}	**.016**	**.000**	**.000**
Phosphoserine (p-Ser)	0.12± 0.02^{aB}	0.21± 0.04^{aA}	0.17± 0.07^{aAB}	0.23± 0.01^{aA}	0.12± 0.02^{aB}	0.16± 0.02^{aA}	0.19± 0.04^{aAB}	0.22± 0.02^{aA}	0.12± 0.02^{aB}	0.18± 0.05^{aA}	0.14± 0.07^{aAB}	0.14± 0.05^{aA}	0.12± 0.02^{aB}	0.12± 0.04^{aA}	0.14± 0.06^{aAB}	0.19± 0.03^{aA}	**.080**	**.003**	.271
Phosphoethanolamine (PEA)	0.01± 0.00^{aC}	0.00^{a}	0.03± 0.00^{aA}	0.01± 0.00^{aB}	0.01± 0.00^{aC}	0.00^{a}	0.02± 0.00^{aA}	0.01± 0.00^{aB}	0.01± 0.00^{aC}		0.02± 0.01^{aA}	0.01± 0.00^{aB}	0.01± 0.00^{aC}	0.00^{a}	0.03± 0.00^{aA}	0.02± 0.00^{aB}	.168	**.000**	.341
Taurine (Tau)	0.15± 0.02^{abA}	0.17± 0.00^{abA}	0.06± 0.00^{abB}			0.16± 0.02^{aA}	0.06± 0.00^{aB}	0.04± 0.00^{aB}	0.15± 0.02^{bcA}	0.15± 0.06^{bcA}	0.04± 0.00^{bcB}	0.05± 0.01^{bcB}	0.15± 0.02^{cA}	0.07± 0.01^{cA}	0.03± 0.01^{cB}	0.06± 0.00^{cB}	**.005**	**.000**	**.002**
ϒ-Amino-n-butyric acid (ϒ-ABA)	0.01± 0.00^{aC}	0.00^{aB}	0.07± 0.01^{aA}	0.03± 0.00^{aB}	0.01± 0.00^{aC}	0.00^{aB}	0.07± 0.01^{aA}	0.03± 0.00^{aB}	0.01± 0.00^{bC}	0.00^{bB}	0.03± 0.00^{bA}	0.03± 0.00^{bB}	0.01± 0.00^{bC}	0.02± 0.01^{bB}	0.03± 0.00^{bA}	0.01± 0.00^{bB}	**.000**	**.000**	**.000**
β -Alanine (β -Ala)	0.20± 0.05^{bA}	0.14± 0.03^{bB}	0.10± 0.00^{bC}	0.11± 0.00^{bC}	0.20± 0.05^{aA}	0.22± 0.02^{aB}	0.14± 0.00^{aC}	0.10± 0.01^{aC}	0.20± 0.05^{bA}	0.17± 0.09^{bB}	0.01± 0.00^{bC}	0.10± 0.02^{bC}	0.20± 0.05^{bA}	0.10± 0.00^{bB}	0.12± 0.00^{bC}	0.10± 0.00^{bC}	**.010**	**.000**	**.006**
β Amino isobutyric acid (β-AiBA)	0.04± 0.02^{abB}	0.09± 0.04^{abA}	0.00^{abB}	0.03± 0.00^{abB}	0.04± 0.02^{abB}	0.09± 0.01^{abA}	0.00^{abB}	0.00^{abB}	0.04± 0.02^{aB}	0.12± 0.01^{aA}	0.04± 0.00^{aB}	0.03± 0.00^{aB}	0.04± 0.02^{bB}	0.00^{bA}	0.00^{bB}	0.02± 0.01^{bB}	.089	**.000**	.398
Ornithine (Orn)	0.08± 0.02^{bA}	0.09± 0.03^{bA}	1.00± 0.09^{bB}	0.09± 0.00^{bA}				0.09± 0.00^{aA}	0.08± 0.02^{cA}	0.09± 0.02^{cA}	0.08± 0.00^{cB}	0.08± 0.00^{cA}	0.08± 0.02^{bA}	0.04± 0.00^{bA}	0.94± 0.05^{bB}	0.05± 0.00^{bA}	**.000**	**.000**	**.000**
1Methyl histidine (1 Mehis)	0.14± 0.00^{aB}	0.00^{a}	0.34± 0.00^{aA}	0.17± 0.10^{aB}	0.14± 0.00^{aB}	0.00^{a}	0.33± 0.04^{aA}	0.32± 0.03^{aB}	0.14± 0.00^{bB}	0.00^{b}					0.00^{bA}	0.09± 0.01^{bB}	.340	**.000**	.323
3Methyl histidine (3 Mehis)	0.27± 0.00^{bA}	0.01± 0.00^{bB}	0.01± 0.00^{bB}	0.00^{b}	0.27± 0.00^{aA}	0.00^{aB}	0.00^{aB}	0.00^{a}	0.27± 0.00^{aA}	0.00^{aB}	0.00^{aB}	0.00^{a}	0.27± 0.00^{aA}	0.00^{aB}	0.00^{aB}	0.00^{a}	1.000	**.000**	ND
Sarcosine (Sar)	0.01± 0.00^{aB}	0.15± 0.02^{aA}	0.04± 0.00^{aB}	0.04± 0.01^{aB}	0.01± 0.00^{bB}	0.00^{bA}	0.00^{bB}	0.02± 0.01^{bB}	0.01± 0.00^{aB}	0.25± 0.09^{aA}	0.01± 0.00^{aB}	0.04± 0.00^{aB}	0.01± 0.00^{bB}	0.08± 0.05^{bA}	0.06± 0.00^{bB}	0.01± 0.00^{bB}	**.025**	**.000**	**.000**
Hydroxyproline (Hypro)	2.11± 0.15^{bA}	2.02± 0.07^{bA}	2.65± 0.42^{bA}	1.45± 0.04^{bB}	2.11± 0.15^{aA}	2.25± 0.47^{aA}	2.90± 0.49^{aA}	1.43± 0.12^{aB}	2.11± 0.15^{cA}	1.97± 0.39^{cA}	1.19± 0.21^{cA}	1.32± 0.13^{cB}	2.11± 0.15^{bcA}	1.74± 0.06^{bcA}	1.71± 0.45^{bcA}	1.70± 0.00^{bcB}	**.000**	**.001**	**.000**
α Amino adipic acid (α-AAA)	0.03± 0.00^{a}	0.19± 0.03^{a}	0.00^{a}	0.00^{a}	0.03± 0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.03± 0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.03± 0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	1.000	**.000**	ND
a-ABA	0.04± 0.01^{bB}	0.23± 0.06^{bA}	0.02± 0.01^{bB}	0.03± 0.00^{bB}	0.04± 0.01^{aB}			0.04± 0.00^{aB}	0.04± 0.01^{bB}	0.23± 0.02^{bA}	0.03± 0.00^{bB}	0.03± 0.01^{bB}	0.04± 0.01^{aB}	0.36± 0.01^{aA}	0.01± 0.00^{aB}	0.04± 0.00^{aB}			
Ethanolamine(EOHNH2)	0.01± 0.00^{abB}	0.02± 0.01^{abA}	0.01± 0.00^{abB}	0.01± 0.00^{abB}	0.01± 0.00^{aB}	0.02± 0.01^{aA}	0.01± 0.00^{aB}	0.01± 0.00^{aB}	0.01± 0.00^{abB}	0.02± 0.01^{abA}	0.01± 0.00^{abB}	0.01± 0.00^{abB}	0.01± 0.00^{abB}	0.01± 0.00^{abA}	0.01± 0.00^{abB}	0.01± 0.00^{abB}			
Hydroxylysine (Hylys)	0.14± 0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.14± 0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.14± 0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.14± 0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.000	ND	ND
Ans	0.31± 0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.31± 0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.31± 0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.31± 0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.000	ND	ND
Glutamine (Glu-NH2)	0.01± 0.00^{aC}	1.65± 0.31^{aB}	0.00^{aA}	0.00^{a}	0.01± 0.00^{bC}	0.00^{bB}	0.00^{bA}	0.00^{b}	0.01± 0.00^{bC}	0.00^{bB}	0.00^{bA}	0.00^{b}	0.01± 0.00^{aC}	0.00^{aB}	2.28± 0.62^{aA}	0.00^{a}	1.000	**.000**	ND
Cit	0.02± 0.00^{bB}	0.01± 0.00^{bB}	0.03± 0.00^{bA}	0.02± 0.00^{bB}	0.02± 0.00^{aB}	0.03± 0.02^{aB}	0.07± 0.03^{aA}	0.02± 0.00^{aB}	0.02± 0.00^{bB}	0.03± 0.00^{bB}	0.04± 0.01^{bA}	0.02± 0.00^{bB}	0.02± 0.00^{bB}	0.01± 0.00^{bB}	0.02± 0.00^{bA}	0.00^{bB}	**.000**	**.000**	**.001**
Total NPAA	**3.83**	**5.16**	**4.80**	**2.42**	**3.83**	**3.42**	**5.27**	**2.47**	**3.83**	**3.36**	**1.75**	**2.27**	**3.83**	**2.76**	**5.74**	**2.44**			

*Data represented as Mean± SD (n=3). Statistical differences (Tukey's, P< 0.05) in means are given by changed letters: changed small letters 'a - c' in similar row denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar row denote difference is significant (P< 0.05) among feed storage durations.

Table 4.3a): Amino acid profile changes during storage for aquafeed 2 (Non-extruded diet).

Amino Acids g/100g	Storage Conditions																		
Proteinogenic AA	**LT1**				**LT2**				**AT3**				**HT4**				**P-value**		
Essential AA	**0day**	**60day**	**120day**	**180day**	**0day**	**60day**	**120day**	**180day**	**0day**	**60day**	**120day**	**180day**	**0day**	**60day**	**120day**	**180day**	**T**	**D**	**I**
Arginine (Arg)	2.18± 0.13^{aA}	1.99± 0.31^{aB}	2.00± 0.11^{aB}	0.98± 0.03^{aC}	2.18± 0.13^{bA}	1.52± 0.49^{bB}	1.36± 0.07^{bB}	0.81± 0.11^{bC}	2.18± 0.13^{abA}	1.77± 0.49^{abB}	1.74± 0.12^{abB}	1.08± 0.04^{abC}	2.18± 0.13^{bA}	1.37± 0.24^{bB}	1.20± 0.04^{bB}	1.17± 0.52^{bC}	**.007**	**.000**	.061
Histidine (His)	0.38± 0.01^{aA}	0.38± 0.07^{aA}	0.39± 0.00^{aA}	0.37± 0.08^{aB}	0.38± 0.01^{abA}	0.39± 0.02^{abA}	0.35± 0.00^{abA}	0.31± 0.01^{abB}	0.38± 0.01^{aA}	0.38± 0.05^{aA}	0.37± 0.02^{aA}	0.34± 0.00^{aB}	0.38± 0.01^{bA}			0.28± 0.01^{bB}	**.003**	**.000**	.361
Isoleucine (Ile)	1.60± 0.03^{aA}	1.46± 0.01^{aA}	1.42± 0.13^{aB}	1.35± 0.01^{ac}	1.60± 0.03^{aA}	1.61± 0.01^{aA}	1.45± 0.00^{aB}	1.30± 0.05^{aC}	1.60± 0.03^{aA}	1.53± 0.08^{aA}	1.47± 0.16^{aB}	1.37± 0.03^{aC}	1.60± 0.03^{aA}			1.20± 0.02^{aC}	.343	**.000**	**.032**
Leucine (Leu)	2.49± 0.08^{aA}	2.32± 0.35^{aA}	2.30± 0.03^{aB}	2.23± 0.15^{aB}	2.49± 0.08^{aA}	2.53± 0.02^{aA}	2.31± 0.01^{aB}	1.94± 0.25^{aB}	2.49± 0.08^{aA}	2.56± 0.04^{aA}	2.48± 0.11^{aB}	2.27± 0.03^{aB}	2.49± 0.08^{aA}			2.11± 0.15^{aB}	.087		**.002**
Lysine (Lys)	1.72± 0.05^{aA}	1.60± 0.26^{aA}	1.56± 0.02^{aB}	1.46± 0.04^{aB}	1.72± 0.05^{aA}	1.76± 0.02^{aA}	1.56± 0.00^{aB}	1.25± 0.21^{aB}	1.72± 0.05^{aA}	1.75± 0.07^{aA}	1.63± 0.06^{aB}	1.53± 0.04^{aB}	1.72± 0.05^{bA}			1.37± 0.03^{bB}	**.000**		**.002**
Methionine (Met)	0.70± 0.02^{aA}	0.74± 0.01^{aA}	0.78± 0.02^{aAB}	0.69± 0.04^{aB}	0.70± 0.02^{bA}	0.79± 0.21^{bA}		0.52± 0.00^{bB}	0.70± 0.02^{aA}	0.76± 0.08^{aA}	0.67± 0.20^{aAB}	0.58± 0.12^{aB}	0.70± 0.02^{abA}			0.55± 0.09^{abB}	**.001**		**.009**
Phenylalanine (Phe)	1.39± 0.04^{aA}	1.32± 0.17^{aA}	1.28± 0.02^{aB}	1.25± 0.07^{aB}	1.39± 0.04^{aA}	1.39± 0.01^{aA}	1.28± 0.00^{aB}	1.16± 0.08^{aB}	1.39± 0.04^{aA}			1.21± 0.00^{aB}	1.39± 0.04^{aA}			1.34± 0.02^{aB}	.225		**.000**
Threonine (Thr)	1.44± 0.01^{bcB}	1.43± 0.02^{bcA}	1.16± 0.03^{bcBC}	1.35± 0.10^{bcC}	1.44± 0.01^{bB}	1.55± 0.03^{bA}	1.42± 0.01^{bBC}	1.33± 0.03^{bC}	1.44± 0.01^{aB}	1.87± 0.25^{aA}	1.63± 0.00^{aBC}	1.38± 0.02^{aC}	1.44± 0.01^{cB}			1.19± 0.27^{cC}	**.000**		**.001**
Tryptophan (Trp)	0.10± 0.01^{aBC}	0.09± 0.01^{aB}	0.06± 0.00^{aC}	0.58± 0.02^{aA}	0.10± 0.01^{cBC}	0.11± 0.03^{cB}	0.09± 0.04^{cC}	0.06± 0.00^{cA}	0.10± 0.01^{cBC}	0.07± 0.01^{cB}	0.10± 0.00^{cC}	0.10± 0.01^{cA}	0.10± 0.01^{bBC}			0.08± 0.00^{bA}	**.000**		**.000**
Valine (Val)	1.76± 0.02^{aA}	1.62± 0.02^{aAB}	1.57± 0.18^{aB}	1.59± 0.15^{aC}	1.76± 0.02^{aA}	1.72± 0.05^{aAB}	1.62± 0.01^{aB}	1.41± 0.10^{aC}	1.76± 0.02^{aA}	1.68± 0.10^{aAB}	1.66± 0.16^{aB}	1.54± 0.02^{aC}	1.76± 0.02^{aA}			1.36± 0.00^{aC}	.635		.057
Total EAA	**13.76**	**12.95**	**12.52**	**11.84**	**13.76**	**13.09**	**11.99**				**13.16**	**11.39**	**13.76**			**10.32**			
																	T		**I**
Alanine (Ala)	2.00± 0.04^{aA}	1.89± 0.31^{aA}	1.84± 0.05^{aA}	1.82± 0.08^{aB}	2.00± 0.04^{aA}	2.05± 0.08^{aA}	1.87± 0.00^{aA}	1.56± 0.19^{aB}	2.00± 0.04^{aA}	2.06± 0.05^{aA}	2.01± 0.05^{aA}	1.83± 0.06^{aB}	2.00± 0.04^{aA}			1.72± 0.16^{aB}	.115		.144
Aspartate (Asp)	2.96± 0.05^{abA}	2.92± 0.10^{abA}	2.88± 0.04^{abA}	2.85± 0.28^{abB}	2.96± 0.05^{bA}	3.12± 0.05^{bA}	2.89± 0.01^{bA}	2.36± 0.34^{bB}	2.96± 0.05^{aA}	3.01± 0.20^{aA}	3.23± 0.04^{aA}	2.75± 0.04^{aB}	2.96± 0.05^{abA}	3.13± 0.13^{abA}	3.00± 0.04^{abA}	2.34± 0.13^{abB}	**.038**		**.001**
Cysteine (Cys)	0.33± 0.00^{aA}	0.29± 0.04^{aB}	0.30± 0.00^{aB}	0.28± 0.00^{aC}	0.33± 0.00^{bA}	0.27± 0.04^{bB}	0.27± 0.03^{bB}	0.18± 0.03^{bC}	0.33± 0.00^{aA}	0.32± 0.0^{aB}	0.27± 0.04^{aB}	0.27± 0.01^{aC}	0.33± 0.00^{aA}	0.33± 0.01^{aB}	0.32± 0.01^{aB}	0.26± 0.13^{aC}	**.000**		**.000**
Glutamic Acid (Glu)	6.12± 0.18^{bA}	5.77± 0.05^{bA}	5.61± 0.07^{bB}	5.40± 0.52^{bC}	6.12± 0.18^{abA}	6.32± 0.12^{abA}	5.65± 0.04^{abB}	5.32± 0.06^{abC}	6.12± 0.18^{aA}	6.44± 0.16^{aA}	6.11± 0.24^{aB}	5.29± 0.14^{aC}	6.12± 0.18^{bA}	6.22± 0.22^{bA}	5.88± 0.07^{bB}	4.56± 0.24^{bC}	**.004**		**.000**
Glycine (Gly)	2.17± 0.06^{aA}	2.22± 0.09^{aA}	2.09± 0.38^{aA}	2.03± 0.08^{aB}	2.17± 0.06^{aA}	2.28± 0.08^{aA}	2.00± 0.01^{aA}	1.70± 0.20^{aB}	2.17± 0.06^{aA}	2.33± 0.11^{aA}	2.21± 0.02^{aA}	2.00± 0.09^{aB}	2.17± 0.06^{aA}	2.18± 0.02^{aA}	2.13± 0.06^{aA}	1.84± 0.20^{aB}	.087	**.000**	.344
Proline (Pro)	2.01± 0.16^{bAB}	1.91± 0.41^{bA}	1.94± 0.04^{bAB}	1.92± 0.01^{bB}	2.01± 0.16^{abAB}	2.05± 0.10^{abA}	2.01± 0.02^{abAB}	1.10± 0.08^{abB}	2.01± 0.16^{aAB}	2.29± 0.24^{aA}	2.12± 0.04^{aAB}	2.01± 0.07^{aB}	2.01± 0.16^{abAB}	2.15± 0.05^{abA}	1.86± 0.01^{abAB}	1.81± 0.35^{abB}	**.022**	**.049**	.128
Serine (Ser)	1.54± 0.30^{aA}	1.73± 0.48^{aA}	1.64± 0.11^{aA}	1.02± 0.02^{aB}	1.54± 0.30^{aA}	1.79± 0.45^{aA}	1.64± 0.39^{aA}	0.77± 0.03^{aB}	1.54± 0.30^{aA}	1.39± 0.03^{aA}	1.39± 0.02^{aA}	1.15± 0.03^{aB}	1.54± 0.30^{aA}	2.09± 0.80^{aA}	1.31± 0.05^{aA}	1.01± 0.06^{aB}	.671	**.000**	.483
Tyrosine (Tyr)	1.05± 0.17^{aA}	1.04± 0.23^{aA}	0.94± 0.05^{aAB}	0.94± 0.18^{aB}	1.05± 0.17^{aA}	1.00± 0.18^{aA}	0.91± 0.03^{aAB}	1.80± 0.02^{aB}	1.05± 0.17^{aA}	1.06± 0.12^{aA}	0.95± 0.06^{aAB}	0.92± 0.10^{aB}	1.05± 0.17	1.04± 0.10	0.86± 0.16	0.89± 0.04	.645	**.008**	.985
Total NEAA	**18.18**	**17.77**	**17.23**	**16.25**	**18.18**	**18.88**	**17.23**	**14.78**	**18.18**	**18.91**	**18.28**	**16.22**	**18.18**	**19.09**	**17.31**	**14.44**			
Total EAA + NEAA	**31.94**	**30.71**	**29.75**	**28.09**	**31.94**	**31.97**	**29.22**	**24.87**	**31.94**	**32.66**	**31.44**	**27.61**	**31.94**	**31.78**	**28.17**	**24.76**			

*Data represented as Mean± SD (n=3). Statistical differences (Tukey's, P< 0.05) in means are given by changed letters: changed small letters 'a - c' in similar row denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar row denote difference is significant (P< 0.05) among feed storage durations.

Table 4.3b) Amino acid profile changes during storage for aquafeed 2 (Non-extruded diet).

Amino Acids (conc. g/100g)	Storage Conditions																P-value		
	LT1				LT2				AT3				HT4						
Non-Proteinogenic AA	**0day**	**60day**	**120day**	**180day**	**0day**	**60day**	**120day**	**180day**	**0day**	**60day**	**120day**	**180day**	**0day**	**60day**	**120day**	**180day**	**T**	**D**	**I**
Phosphoserine (p-Ser)	0.14± 0.06^{bA}	0.06± 0.01^{bAB}	0.07± 0.00^{bBC}	0.07± 0.01^{bC}	0.14± 0.06^{aA}		0.14± 0.06^{aBC}	0.07± 0.00^{aC}	0.14± 0.06^{bA}	0.09± 0.00^{bAB}	0.10± 0.01^{bBC}	0.05± 0.01^{bC}	0.14± 0.06^{bA}	0.09± 0.01^{bAB}	0.07± 0.00^{bBC}	0.05± 0.01^{bC}	**.000**	**.000**	**.000**
Taurine (Tau)	0.19± 0.00^{aA}	0.05± 0.00^{aB}	0.03± 0.00^{aB}	0.03± 0.01^{aC}	0.19± 0.00^{aA}		0.05± 0.01^{aB}	0.02± 0.00^{aC}	0.19± 0.00^{aA}	0.04± 0.01^{aB}	0.04± 0.00^{aB}	0.03± 0.01^{aC}	0.19± 0.00^{aA}	0.03± 0.01^{aB}	0.04± 0.01^{aB}	0.03± 0.00^{aC}	.588	**.000**	**.004**
ϒ-Amino-n-butyric acid (ϒ-ABA)	0.04± 0.00^{aA}	0.03± 0.01^{aB}	0.03± 0.01^{aAB}	0.03± 0.00^{aA}	0.04± 0.00^{aA}		0.00^{aAB}	0.00^{aB}	0.04± 0.00^{aA}	0.01± 0.01 ^{aB}	0.00^{aAB}	0.00^{aA}	0.04± 0.00^{aA}	0.01± 0.00^{aB}	0.00^{aAB}	0.00^{aA}	**.027**	**.000**	**.027**
β -Alanine (β -Ala)	0.12± 0.02^{bA}	0.09± 0.02^{bAB}	0.07± 0.01^{bBC}	0.07± 0.01^{bC}	0.12± 0.02^{abA}		0.09± 0.00^{abBC}	0.05± 0.01^{abC}	0.12± 0.02^{bA}	0.08± 0.01^{bAB}	0.06± 0.02^{bBC}		0.12± 0.02^{aA}	0.15± 0.08^{aAB}	0.14± 0.05^{aBC}	0.07± 0.00^{aC}	**.010**	**.000**	.185
β Amino isobutyric acid (β-AiBA)	0.06± 0.02^{bB}	0.06± 0.00^{bA}	0.05± 0.00^{bB}	0.05± 0.01^{bB}	0.06± 0.02^{bB}		0.05± 0.02^{bB}	0.03± 0.00^{bB}	0.06± 0.02^{bB}	0.07± 0.00^{bA}	0.08± 0.00^{bB}		0.06± 0.02^{aB}	0.22± 0.01^{aA}	0.08± 0.01^{aB}	0.07± 0.01^{aB}	**.000**	**.000**	**.000**
Ornithine (Orn)	0.05± 0.00^{aA}	0.05± 0.02^{aB}	0.03± 0.00^{aB}	0.03± 0.01^{aB}	0.05± 0.00^{aA}		0.04± 0.00^{aB}	0.03± 0.00^{aB}	0.05± 0.00^{aA}	0.04± 0.00^{aB}	0.04± 0.00^{aB}		0.05± 0.00^{aA}	0.03± 0.00^{aB}	0.03± 0.00^{aB}	0.03± 0.00^{aB}	.259	**.000**	.723
1Methyl histidine (1 Mehis)	0.18± 0.01^{bA}	0.01± 0.01^{bB}	0.00^{bB}	0.00^{bC}	0.18± 0.01^{aA}	0.22± 0.05^{aB}	0.15± 0.01^{aB}	0.08± 0.01^{aC}	0.18± 0.01^{abA}	0.15± 0.07^{abB}	0.10± 0.02^{abB}	0.10± 0.03^{abC}	0.18± 0.01^{abA}	0.13± 0.07^{abB}	0.13± 0.01^{abB}	0.05± 0.00^{abC}	**.000**	**.000**	**.001**
Hydroxyproline (Hypro)	1.92± 0.27^{abA}	1.88± 0.01^{abAB}	1.75± 0.12^{abB}	1.72± 0.05^{abC}	1.92± 0.27^{bA}	1.74± 0.03^{bAB}	1.76± 0.10^{bB}	1.50± 0.02^{bC}	1.92± 0.27^{aA}	2.33± 0.27^{aAB}	2.01± 0.08^{aB}	1.62± 0.13^{aC}	1.92± 0.27^{bA}	1.91± 0.15^{bAB}	1.57± 0.30^{bB}	1.29± 0.06^{bC}	**.006**	**.000**	**.034**
Ethanolamine(EOHNH2)	0.01± 0.00^{bA}	0.01± 0.00^{bA}	0.00^{bB}	0.00^{bB}	0.01± 0.00^{aA}	0.01± 0.00^{aA}	0.01± 0.00^{aB}	0.00^{aB}	0.01± 0.00^{aA}	0.01± 0.00^{aA}	0.01± 0.00^{aB}	0.00^{aB}	0.01± 0.00^{aA}	0.01± 0.00^{aA}	0.01± 0.00^{aB}	0.00^{aB}	**.001**	**.000**	**.002**
Cit	0.10± 0.01^{aA}	0.06± 0.01^{aB}	0.05± 0.00^{aC}	0.05± 0.01^{aC}	0.10± 0.01^{aA}	0.07± 0.01^{aB}	0.06± 0.00^{aC}	0.05± 0.01^{aC}	0.10± 0.01^{aA}	0.08± 0.00^{aB}	0.06± 0.00^{aC}	0.04± 0.00^{aC}	0.10± 0.01^{aA}	0.06± 0.00^{aB}	0.04± 0.01^{aC}	0.03± 0.00^{aC}	.075	**.000**	**.326**
Total NPAA	**2.79**	**2.30**	**2.10**	**2.07**	**2.79**	**2.55**	**2.34**	**1.84**	**2.79**	**2.89**	**2.49**	**1.99**	**2.79**	**2.63**	**2.08**	**1.61**			

*Data represented as Mean± SD (n=3). Statistical differences (Tukey's, P< 0.05) in means are given by changed letters: changed small letters 'a - c' in similar row denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar row denote difference is significant (P< 0.05) among feed storage durations.

4.4 Vitamin Profile storage changes

4.4.1 Water-soluble vitamins

Vitamin B1 (thiamin)

Gradual decrease in vitamin B1 is evident bi-monthly at all storage temperatures for diet 1, table 4.4a). Incurred losses being higher at high temperatures HT4 (55.81% vit. B1 present) >AT3 (61.88% present) compared to retention at low temperature LT1 (66.06%), LT2 (66.17%) at the end of storage duration. Although, non-extruded diet 2, show initial increase in thiamin concentration up to 120 days, with decreases, at later duration between fourth to sixth month across all storage temperatures. Duration effects on storage thiamin, are significant for both diets; while temperature and interaction significantly impact ($P<0.05$) thiamin concentration only in diet 2.

Vitamin B2 (Riboflavin)

Vitamin B2 in diet 1 show significant ($P<0.05$) duration effects with decrease from initial to two-month storage at all temperatures. Effects of temperature and interaction on B2 changes are insignificant for diet1; whereas temperature, duration but not interaction impacts vit. B2 concentrations in diet 2, significantly (at $P<0.05$).

In diet1 at 60^{th} day, LT1 vit. B2 value decreased from initial concentration of 0.352 mcg/g to 0.003 mcg/g (with loss of 99.15%, retention 0.85%) and remained undetected beyond two months. At LT2, 60-day conc. depicts 0.054 mcg/g (15.34% retention), AT3 value 0.023 mcg/g (6.57% retained), HT4 7.95% retention at 0.028 mcg/g. During fourth month (at all temperatures) and during sixth month at LT1, HT4, vit. B2 could not be determined in extruded diet. For non-extruded diet, B2 show significant loss at each storage interval compared

to initial concentration. Retention values of B2 at second, fourth and sixth months were determined respectively at LT1 as 96.49%, 84.21%, 57.89%; LT2 as 96.49%, 78.95%, 71.93%; AT3 as 66.67%, 38.60%, 42.11%; and, HT as 29.82%, 0%, N.D (not defined at end of storage).

Vitamin B6 (pyridoxine)

In diet 1, vit B6 retention at end of 60,120,180 day followed 69.48 %, 61.49%, 56.76% respectively at LT1; 88.63%, 72.52%, 83.44% at LT2; 98.53%, 75.90%, 74.54% at AT3; 90.20%, 73.19%, 46.51% at high temperature HT4 conditions. Diet 1, results show significant ($P<0.05$) effects of temperature, duration and interaction. Diet 2 depicts significant decrease in retention of B6 at end of duration at all temperatures LT1 (80.37%), LT2 (80.74%), AT (71.48%), except with slight chromatographic overestimations at HT.

Vitamin B12 (cobalamin)

B12 changes in both diets estimate duration based significant effects ($P<0.05$) between initial and final storage at each temperature conditions. For diet 2, interaction effects are also significant for changes in B12 value, but no noteworthy effect of temperature lies for both diets during storage. LT1 retention for diet 1= 45.27%, diet 2 =74.07%; LT2=32.92%, 55.56%; AT= 28.81%, 49.38%; HT= 65.84%, 77.78% clearly with higher retentions in diet 2 over storage.

Vitamin C (Ascorbic acid)

Percentage of vit. C retention at end of 180 days is highest at LT1 conditions following LT1 (89% diet1, diet1,81.02% diet 2) >AT (51.38% diet1,79.56% diet 2) >HT (39.45% diet1, 51.82% diet 2). Significant

difference (P<0.05) due to temperature, duration and interaction effects was found for vit C values in diet 1, table 4.4a).

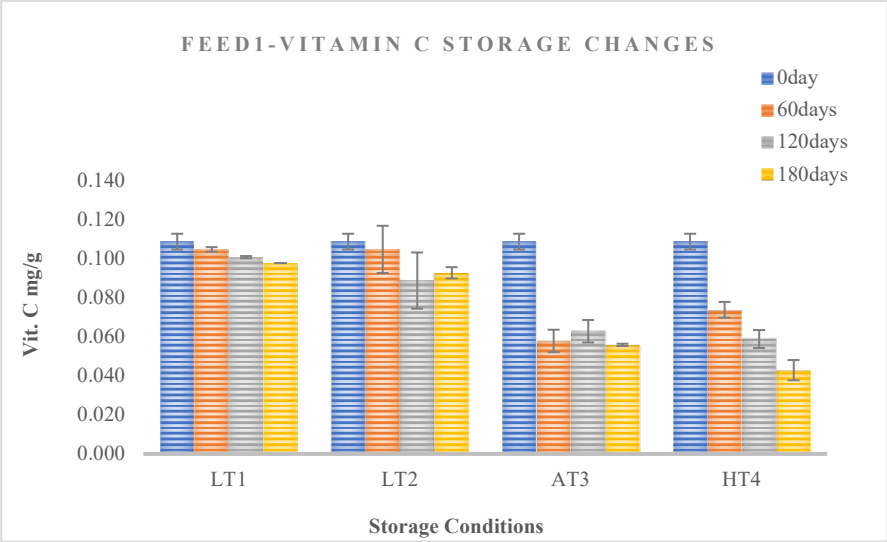


Figure 4.4 (a)

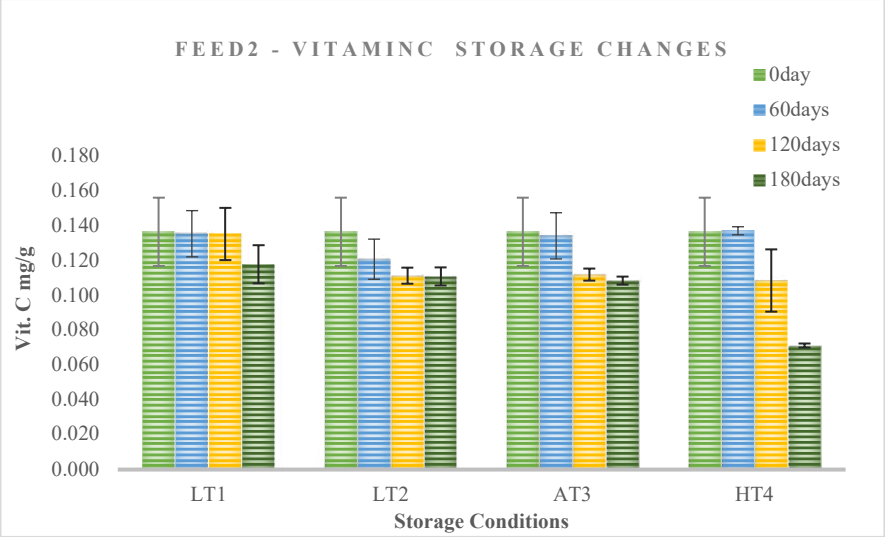


Figure 4.4 (b)

For diet 2 these effects were significant for duration only. Although diet 1 and 2 depict gradual loss of vitamin C over low temperature; losses are evidently higher at HT4 due to intense temperatures, figures 4.4(a),(b) .

4.4.2 Fat-soluble vitamins

4.4.2.1 Vitamin A (retinyl acetate)

At each bimonthly assessment, vitamin A decreased at all storage temperatures, in both diets, figure 4.4 (c),(d). In diet 1, LT1 retentions are 72.52% > LT2 (38.65%), >AT 3.26%) at the end of storage duration.

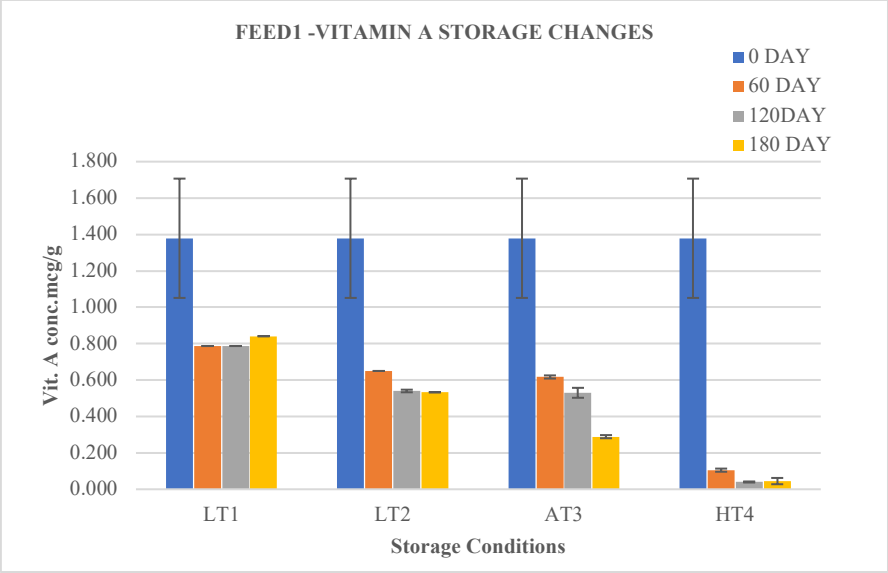


Figure 4.4 (c)

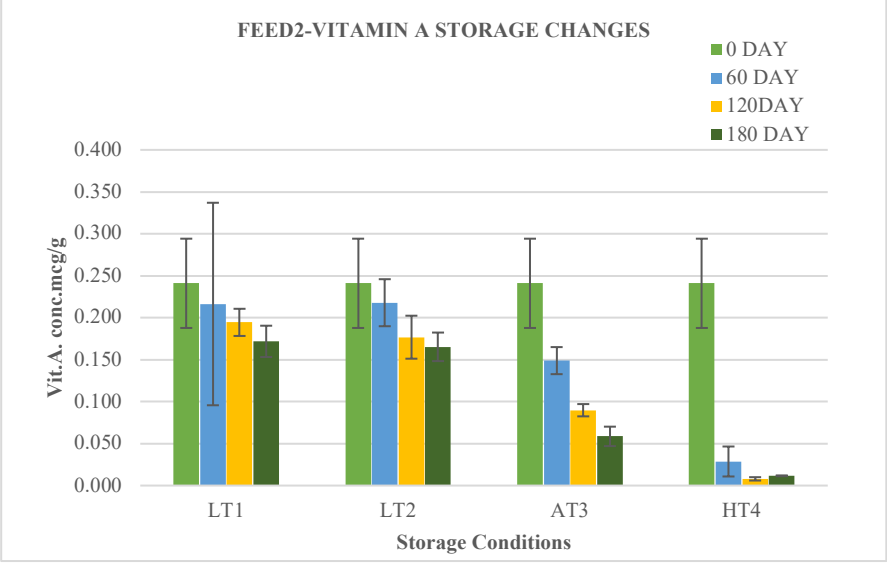


Figure 4.4 (d)

In diet 2, retention rates at storage completion being 71.37%, 68.46%, 24.48%, 4.98% at LT1, LT2, AT, HT respectively. There is no significant difference between vit. A content at any temperature condition for diet 1. Temperature affects A- values significantly (P<0.05) between low ,LT2) and other temperature conditions (ambient and high), between AT and HT for diet 2. Between subject effects of temperature and duration on vit A storage depletion are highly significant (P=0.000) for both diets, with significant interaction effects only towards diet 2; table 4.4b).

4.4.2.2 Vitamin D (D2 Ergocalciferol, D3 cholecalciferol)

Detectable vit. D in diet 1 was plant-based form ergocalciferol (vit D2), figure 4.4(e). For diet 2 animal-based form cholecalciferol (D3) was quantifiable. Estimated D2 retention (diet 1) at end of storage accounted 99.40% (LT1), 50.57% (LT2), 30.21% (AT3), 39.54% (HT4).

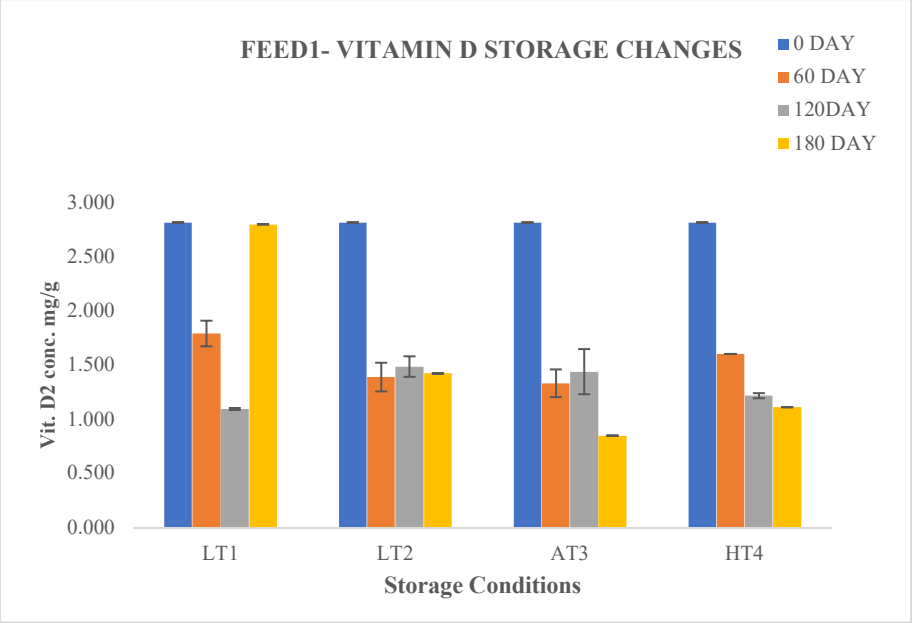


Figure 4.4 (e)

Severe losses of D3 were encountered in pelleted stored diet 2 with incurred losses accounting 100%, fourth storage month onwards at high temperature

(HT4) storage figure 4.4(f). Even at LT1, LT2 and AT3 accountable D3 retentions were low as 38.46%, 26.92%, 7.69% respectively.

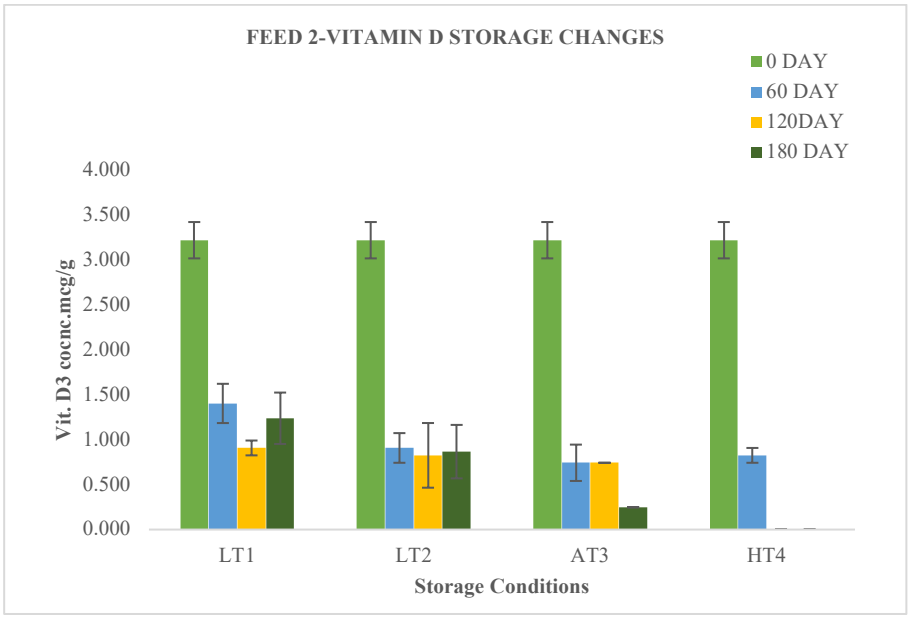


Figure 4.4 (f)

4.4.2.3 Vitamin E (α-tocopherol)

Losses ranging from 39.41% to 51.36% are estimated for diet1 at sixth month, figure 4.4(g). For pelleted month, incurred loss accounted between 59.78% - 80.43%, figure 4.4(h).

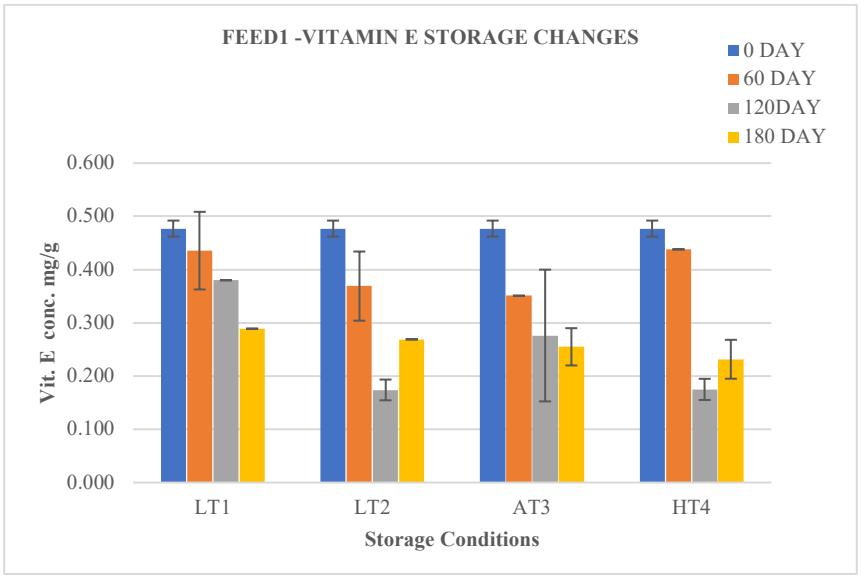


Figure 4.4 (g)

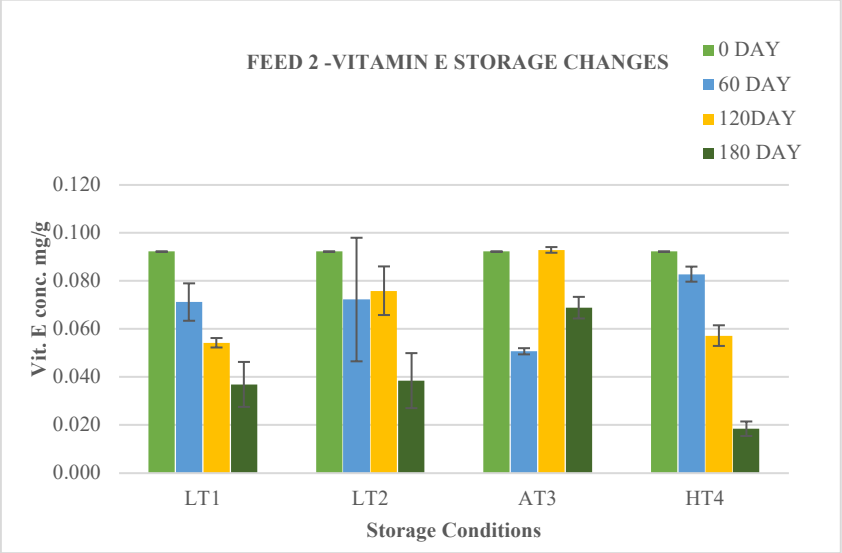


Figure 4.4 (h)

4.4.2.4 Vitamin K1 (Phylloquinone)

Vit K1 in feeds was identified as cis- and trans- isomers at chromatographic retention times (RTs) 1.09 - 1.15 for cis; 1.77-1.807 for trans-isomer. Of these, only trans- form of vitamin K1 is biologically relevant (Berger et al., 2013).

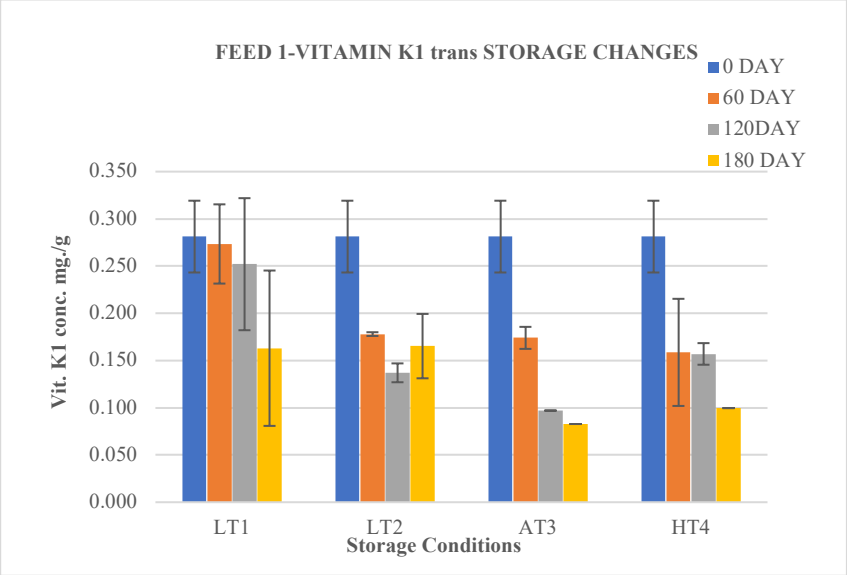


Figure 4.4 (i)

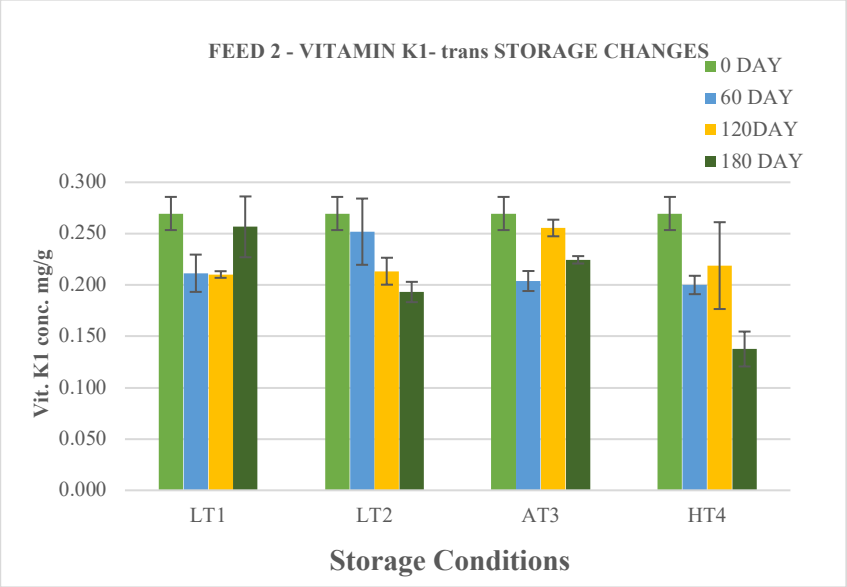


Figure 4.4 (j)

K1-trans, during storage, in both diets show significant between-subject-effect of duration; but not for storage temperature or interaction. Incurred losses are higher towards extruded diet, .41% than pelleted diet 2 (4.81%-49.89%), figures 4.4(i)-(j).

Table 4.4a): Storage profile changes of water-soluble and fat-soluble vitamins of Aquafeed 1(extruded diet).

WATER-SOLUBLE VITAMINS (conc.)	Storage Temperatures	Storage Durations (days)				P-Value		
		0	60	120	180	T	D	T * D
C (mg/g)	LT1	0.109±0.004^{aA}	0.105±0.001^{aB}	0.101±0.001^{aBC}	0.098±0.000^{aC}	**0.000**	**0.000**	**0.000**
	LT2	0.109±0.004^{aA}	0.105±0.012^{aB}	0.089±0.014^{aBC}	0.093±0.003^{aC}			
	AT3	0.109±0.004^{bA}	0.058±0.006^{bB}	0.063±0.006^{bBC}	0.056±0.001^{bC}			
	HT4	0.109±0.004^{bA}	0.074±0.004^{bB}	0.059±0.005^{bBC}	0.043±0.005^{bC}			
B1 (mcg/g)	LT1	4.664±0.632^{aA}	4.547±2.020^{aAB}	3.817±0.162^{aAB}	3.081±0.293^{aB}	0.439	**0.011**	0.970
	LT2	4.664±0.632^{aA}	4.545±0.826^{aAB}	3.485±0.083^{aAB}	3.086±0.092^{aB}			
	AT3			3.276±0.160^{aAB}	2.886±0.647^{aB}			
	HT4			4.065±0.143^{aAB}	2.603±1.176^{aB}			
B2 (mcg/g)	LT1	0.352±0.163^{aA}	0.003±0.001^{aB}	ND	ND	0.999	**0.015**	1.000
	LT2	0.352±0.163^{aA}	0.054±0.000^{aB}	ND	0.008±0.004^{aB}			
	AT3	0.352±0.163^{aA}	0.023±0.001^{aB}	ND	0.009±0.005^{aB}			
	HT4	0.352±0.163^{aA}	0.028±0.000^{aB}	ND	ND			
B6 (mg/g)	LT1	0.888±0.010^{bA}	0.617±0.127^{bB}	0.546±0.014^{bC}	0.504±0.027^{bC}			**0.047**
	LT2	0.888±0.010^{aA}	0.787±0.016^{aB}	0.644±0.019^{aC}	0.741±0.021^{aC}			
	AT3	0.888±0.010^{aA}	0.875±0.106^{aB}	0.674±0.038^{aC}	0.662±0.001^{aC}			
	HT4	0.888±0.010^{abA}	0.801±0.019^{abB}	0.650±0.018^{abC}	0.413±0.129^{abC}			
B12 (mg/g)	LT1	0.243±0.088^{aA}	0.100±0.011^{aB}	0.034±0.011^{aB}	0.011±0.004^{aB}	0.829	**0.000**	0.959
	LT2	0.243±0.088^{aA}	0.087±0.015^{aB}	0.030±0.000aB	0.008±0.001^{aB}			
	AT3	0.243±0.088^{aA}	0.026±0.005^{aB}	0.025±0.009^{aB}	0.007±0.000^{aB}			
	HT4	0.243±0.088^{aA}	0.026±0.001^{aB}	0.040±0.001^{aB}	0.016±0.000^{aB}			
FAT SOLUBLE VITAMINS (conc.)	**Storage Temperatures**	**0**	**60**	**120**	**180**	**T**	**D**	**T * D**
A (mcg/g)	LT1	1.379±0.328^{aA}	0.787±0.000^{aB}				**0.000**	0.404
	LT2	1.379±0.328^{aA}	0.650±0.000^{aB}					
	AT3	1.379±0.328^{abA}	0.617±0.009^{abB}					
	HT4	1.379±0.328^{bA}	0.105±0.009^{bB}	0.040±0.003^{bB}	0.045±0.017^{bB}			
D (mg/g)	LT1	2.820±0.000^{aA}	1.795±0.118^{aB}	1.098±0.009^{aC}	2.803±0.000^{aB}	**0.000**		**0.000**
	LT2	2.820±0.000^{bA}	1.393±0.133^{bB}	1.490±0.095^{bC}	1.426±0.000^{bB}			
	AT3	2.820±0.000^{cA}	1.336±0.128^{cB}	1.443±0.208^{cC}	0.852±0.000^{cB}			
	HT4	2.820±0.000^{bcA}	1.607±0.000^{bcB}	1.221±0.024^{bcC}	1.115±0.000^{bcB}			
E (mg/g)	LT1	0.477±0.015^{aA}	0.436±0.073^{aB}	0.380±0.000^{aC}	0.289±0.000^{aC}	**0.025**		0.057
	LT2	0.477±0.015^{bA}	0.369±0.065^{bB}	0.174±0.019^{bC}	0.269±0.000^{bC}			
	AT3	0.477±0.015^{abA}			0.255±0.035^{abC}			
	HT4	0.477±0.015^{abA}	0.438±0.000^{abB}	0.175±0.020^{abC}	0.232±0.037^{abC}			
K1 trans (mg/g)	LT1	0.281±0.038^{aA}	0.273±0.042^{aB}	0.252±0.070^{aB}	0.163±0.082^{aB}	0.263		0.718
	LT2	0.281±0.038^{aA}	0.178±0.002^{aB}	0.137±0.010^{aB}	0.165±0.034^{aB}			
	AT3	0.281±0.038^{aA}	0.174±0.012^{aB}	0.097±0.000^{aB}	0.083±0.000^{aB}			
	HT4	0.281±0.038^{aA}	0.159±0.057^{aB}	0.157±0.011^{aB}	0.100±0.000^{aB}			

Values are Mean ± SE (n=3)**.** Statistical differences (Tukey's, P< 0.05) in means are given by changed letters: changed small letters 'a - c' in similar column denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar row denote difference is significant (P< 0.05) among feed storage durations.

Temperature (T), Duration (D), Interaction of Temperature*Duration (I). # P-value for between subject effects ; **Significant P-values P< 0.05 in highlight.**

Table4.4b): Storage profile changes of water-soluble and fat-soluble vitamins of Aquafeed 2 (Non-extruded diet).

WATER SOLUBLE VITAMINS (conc.)	Storage Temperatures	Storage Durations (days)				P-Value		
		0	60	120	180	T	D	T * D
C (mg/g)	LT1	0.137±0.020^{aA}	0.136±0.013^{aA}	0.135±0.016^{aAB}	0.118±0.011^{aB}	0.281	**0.009**	0.386
	LT2	0.137±0.020^{aA}	0.121±0.012^{aA}	0.111±0.004^{aAB}	0.111±0.005^{aB}			
	AT3	0.137±0.020^{aA}	0.134±0.013^{aA}	0.112±0.004^{aAB}	0.109±0.003^{aB}			
	HT4	0.137±0.020^{aA}	0.137±0.002^{aA}	0.109±0.018^{aAB}	0.071±0.001^{aB}			
B1 (mcg/g)	LT1	0.395±0.074^{abB}	0.516±0.026^{abA}	1.342±0.049^{abA}	0.443±0.003^{abAB}	**0.007**	**0.006**	**0.000**
	LT2	0.395±0.074^{bB}	0.517±0.082^{bA}	0.468±0.009^{bA}	0.441±0.086^{bAB}			
	AT3	0.395±0.074^{abB}	1.321±0.023^{abA}	0.489±0.017^{abA}	0.330±0.140^{abAB}			
	HT4	0.395±0.074^{aB}	0.747±0.192^{aA}	1.154±0.408^{aA}	1.443±0.511^{aAB}			
B2 (mcg/g)	LT1	0.057±0.008^{abA}	0.055±0.014^{abB}	0.048±0.000^{abB}	0.033±0.006^{abB}	**0.000**		0.053
	LT2	0.057±0.008^{aA}						
	AT3	0.057±0.008^{bcA}						
	HT4	0.057±0.008^{cA}	0.017±0.001^{cB}	0.000^{cB}	ND			
B6 (mg/g)	LT1	0.270±0.016^{aAB}	0.270±0.057^{aA}	0.279±0.011^{aAB}	0.217±0.057^{aB}	0.461		0.114
	LT2	0.270±0.016^{aAB}	0.354±0.005^{aA}	0.220±0.058^{aAB}	0.218±0.074^{aB}			
	AT3	0.270±0.016^{aAB}	0.382±0.005^{aA}	0.313±0.007^{aAB}	0.193±0.053^{aB}			
	HT4	0.270±0.016^{aAB}	0.288±0.004^{aA}	0.278±0.059^{aAB}	0.311±0.073^{aB}			
B12 (mg/g)	LT1	0.027±0.001^{aA}	0.025±0.003^{aA}	0.024±0.002^{aA}	0.020±0.008^{aB}	0.588		**0.000**
	LT2	0.027±0.001^{aA}	0.038±0.001^{aA}	0.018±0.008^{aA}	0.015±0.005^{aB}			
	AT3	0.027±0.001^{aA}	0.058±0.002^{aA}	0.015±0.002^{aA}	0.012±0.005^{aB}			
	HT4	0.027±0.001^{aA}	0.013±0.001^{aA}	0.021±0.000^{aA}	0.021±0.000^{aB}			
FAT SOLUBLE VITAMINS (conc.)	**Storage Temperatures**	**0**	**60**	**120**	**180**	**T**		**T * D**
A (mcg/g)	LT1	0.241±0.053^{aA}	0.216±0.121^{aB}	0.195±0.016^{aBC}	0.172±0.019^{aC}	**0.000**	**0.000**	**0.000**
	LT2	0.241±0.053^{aA}	0.218±0.028^{aB}	0.177±0.026^{aBC}	0.165±0.017^{aC}			
	AT3	0.241±0.053^{bA}	0.149±0.016^{bB}	0.090±0.007^{bBC}	0.059±0.012^{bC}			
	HT4	0.241±0.053^{cA}	0.029±0.018^{cB}	0.008±0.002^{cBC}	0.012±0.000^{cC}			
D (mcg/g)	LT1	3.224±0.203^{aA}	1.405±0.219^{aB}	0.909±0.083^{aB}	1.240±0.286^{aB}	0.084	**0.000**	0.442
	LT2	3.224±0.203^{aA}	0.909±0.165^{aB}	0.827±0.360^{aB}	0.868±0.298^{aB}			
	AT3	3.224±0.203^{aA}	0.744±0.203^{aB}	0.744±0.000^{aB}	0.248±0.000^{aB}			
	HT4	3.224±0.203^{aA}	0.827±0.083^{aB}	0.000^{aB}	0.000^{aB}			
E (mg/g)	LT1	0.092±0.000^{abA}	0.071±0.008^{abB}	0.054±0.002^{abB}	0.037±0.009^{abC}	**0.008**	**0.000**	**0.000**
	LT2	0.092±0.000^{abA}		0.076±0.010^{abB}	0.039±0.011^{abC}			
	AT3	0.092±0.000^{aA}	0.051±0.001^{aB}	0.093±0.001^{aB}	0.069±0.004^{aC}			
	HT4	0.092±0.000^{bA}	0.083±0.003^{bB}	0.057±0.004^{bB}	0.018±0.003^{bC}			
K1 cis (mg/g)	LT1	0.159±0.036^{aA}	0.123±0.008^{aA}	0.159±0.050^{aA}	0.128±0.001^{aA}	0.613	0.240	0.839
	LT2	0.159±0.036^{aA}	0.139±0.007^{aA}	0.145±0.060^{aA}	0.127±0.001^{aA}			
	AT3	0.159±0.036^{aA}	0.138±0.044^{aA}					
	HT4	0.159±0.036^{aA}	0.133±0.016^{aA}	0.075±0.000^{aA}	ND			
K1 trans (mg/g)	LT1	0.270±0.016^{aA}	0.211±0.018^{aB}	0.210±0.003^{aB}	0.257±0.030^{aB}	0.246	**0.000**	0.188
	LT2	0.270±0.016^{aA}		0.213±0.013^{aB}	0.193±0.010^{aB}			
	AT3	0.270±0.016^{aA}		0.255±0.008^{aB}	0.224±0.004^{aB}			
	HT4	0.270±0.016^{aA}	0.200±0.009^{aB}	0.219±0.042^{aB}	0.138±0.017^{aB}			

Values are Mean ± SE. Statistical differences (Tukey's, P< 0.05) in means are given by changed letters: changed small letters 'a - c' in similar column denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar row denote difference is significant (P< 0.05) among feed storage durations. # Temperature (T), Duration (D), Interaction of Temperature*Duration (I). # P-value for between subject effects ; Significant **P-values P< 0.05 in highlight.**

4.5 Fatty acids profile storage changes

Fatty acids were quantitatively determined as methyl esters derived from lipids by gas chromatographic analysis at concentration in milligrams per 100g of diet. Accordingly, 17 fatty acids are identifiable in extruded diet 1 and 12 fatty acids in pelleted non-extruded control diet 2.

In diet 1, initial contents of linoleic acid (C18:2n6) predominates at 864.23±7.90 mg/100g, followed by palmitic acid (C16:0) 826.23±10.07 mg/100g, and oleic acid (C18:0) 450.19±24.67 mg/100g. Amount of palmitic acid being highest (1694.4±2.81) followed by linoleic (922.50±12.06) and oleic (604.95±2.91), among polyunsaturated, saturated, monounsaturated fatty acids respectively, at initial duration for diet 2. Figures 4.5a), b) shows fatty acid chromatographic profiles of initial day extruded and non-extruded diets.

4.5.1 Saturated Fatty acids (SFAs)

Diet 1 : Among SFAs, C16:0 (palmitate), C18:0 (stearate), C14:0 (myristic), C6:0 (caproate), C8:0 (octanoic), C4:0 (butyric), C24:0 (lignoceric) and C12:0 (laurate) are quantified as listed in decreasing order of their presence at initial storage of diet 1, table 4.5a). Significant increase ($P< 0.05$) of butyrate, caproate, octanoic, laurate at end of storage duration is determined at all temperatures LT1 (except 4:0); LT2; AT3 (except 8:0) and HT4.Temperature effects are also significant for butyric, caproic, caprylic acid changes among all storage temperatures. C14:0,C16:0 decreased from initial to final storage at all temperature conditions. C18:0 increased at 180th day at LT1, LT2 but decreased at storage culmination at AT3 and HT4 conditions compared to initial concentrations. Lignoceric acid decreased significantly ($P<0.05$) between initial

to 60-day, at all storage temperatures. Between subject effects of temperature, duration, temperature x duration are significant (P<0.05) for all SFAs except for C12:0 showing insignificant duration (P=0.98) and interaction effects (P=0.126). Overall, there is a significant decrease in total SFA at end of duration compared to initial, 60- and 120-day contents. SFA retentions at 180th day accounts 100% at LT1 > 95.51% (LT2) > 89.29% (HT4) > 83.58% (AT3).

Diet 2: SFAs reported in order of their initial concentration in pelleted diet are 16:0 >14:0 >18:0 >12:0 >24:0.Comparatively lower SFA values are reported at AT3, HT4 and LT2 conditions, table 4.5d). Between subject effects on SFA are significant (P<0.05) due to temperature, duration and interaction. Retentions of all individual and total SFA being highest during LT1 initial storage (-20^{O}C, 0-day values).Total SFA at the end of storage at LT1= 95.32% decreasing to 64.51% (AT3), 60.51% (LT2), 57.47% (HT4).

4.5.2 Monounsaturated Fatty acids (MUFAs)

Diet 1: Oleic (C18:1n9), hexadecenoic (C16:1n9) and nervonic (C24:1) acids are present at initial values of 450.19± 24.67,122.31±2.37,10.12±1.22 mg/100g diet. C16:1n9 conc. decreased significantly between all temperatures; among all bimonthly storages, being lowest determined at 180-day HT4. Losses are significant among all durations at all temperatures for oleic acid. While temperature effects are significant for C18:1n9 losses, higher at LT2 than LT1; AT3 than LT1; HT4 than LT1; HT4 than AT3. Signifying highest contents of C18:1n9 at LT1. Between subject effects of C24:1 are insignificant towards temperature or interaction. Significant major effects of temperature, duration and interaction exist for oleic and hexadecenoic acid. Total MUFA depicts

decrease in retention LT1 onwards (97.25%) > LT2 (84.53%) > AT3 (62.91%) > HT4 (49.51%). Best values being restored at end of storage at LT1, table 4.5b).

Diet 2: C18:1n9 and C16:1n9 MUFA represented in diet 2 depict highest individual concentrations at initial storage of LT1, table 4.5d). Sum of MUFA represents similar trend, with best values at zero-day LT1 conditions. MUFA estimates at 180-day LT1 = 97.16%, LT2 = 69.20%, AT = 38.53%, HT4=15.95%.

4.5.3 Polyunsaturated Fatty acids (PUFAs)

Diet 1: EPA (20:5n3), DPA (22:5n3) and DHA (22:6n3) are among overall n-3 PUFA present. EPA contents increased significantly at LT2, AT3 followed by HT4 conditions between initial and 180-day with least changes at LT1.There is significant increase in DPA at end of duration compared to initial, at all temperatures; highest values being at AT3 storage. Significantly, higher content of DHA are obtained at all bimonthly durations when compared to zero-day values. Sum of n-3 PUFA 60-and 120-day storage. Among n-6; ALA (C18:2n6)) are maximally retained at LT1 storage zero-day duration. Total n-6 PUFA depicts best fatty acid restorations at initial day LT1 as compared to LT2, AT, HT4. Significant, between subject effects exist for all parameters for n3, n6 contents as well for their ratios (n6:n3, n3:n6) in diet1; table4.5c).

Diet 2: EPA, DHA, ALA values are highest during LT1 storage, table 4.5e). Their content being greatest among durations, at zero-day compared to 60, 120-,180-day LT1 values. Respective retentions of total n-3, n-6 PUFA

estimates graded decrease from LT1(n-3 =87.69%; n-6= 93.04%) >LT2 (57.78%;74.64%) >AT3 (41.95%; 50.31%) > HT4 (20.30%; 37.81%) to the end of storage. Significant between subject effects of temperature, duration, interaction exist for MUFA, n-3-PUFA, n6-PUFA and their ratios (n3:n6, n6:n3) for diet 2, table 4.5f).

Table 4.5a): Fatty acid profile changes of SFA under temperature conditions and duration effects of stored aquafeed 1 (extruded diet).

Fatty Acid conc. (mg/ 100g)	Storage temperatures and durations															
	LT1				LT2				AT3				HT4			
	0day	60day	120day	180day	0day	60day	120day	180day	0day	60day	120day	180day	0day	60day	120day	180day
Butyric **4:0**	20.20± 0.05^{dC}	20.20± 0.05^{dB}	20.20± 0.05^{dA}	20.20±0. 05^{dA}	20.20± 0.05^{bC}	43.26± 0.14^{bB}	43.26±0 .14^{bA}	43.26± 0.14^{bA}	20.20± 0.05^{cC}	33.14± 1.11^{cB}	33.14± 1.11^{cA}	33.14± 1.11 ^{cA}	20.20± 0.05^{aC}	20.20± 0.05^{aB}	58.94± 0.58^{aA}	58.94± 0.58^{aA}
Caproic **6:0**	76.72± 1.89^{dC}	77.98± 0.99 ^{dB}	77.98± 0.99 ^{dA}	77.98±0. 99 ^{dA}	76.72± 1.89 ^{bC}	100.78±0. 05^{bB}	100.78± 0.05 ^{bA}	100.78±0. 05 ^{bA}	76.72± 1.89^{cC}	91.49± 2.66^{cB}	91.49± 2.66^{cA}	91.49± 2.66^{cA}	76.72± 1.89^{aC}	77.98± 0.99^{aB}	151.23±1. 78^{aA}	151.23± 1.78^{aA}
Caprylic **8:0**	25.78± 0.57 ^{cC}	26.16± 0.31^{cB}	26.16± 0.31 ^{cA}	26.16± 0.31 ^{cA}	25.78± 0.57 ^{bC}	31.82± 0.04 ^{bB}	31.82± 0.04^{bA}	31.82± 0.04 ^{bA}	25.78± 0.57 ^{dC}	23.60± 0.02 ^{dB}	23.60± 0.02 ^{dA}	23.60± 0.02^{dA}	25.78± 0.57 ^{aC}	26.16± 0.31 ^{aB}	64.19± 0.38 ^{aA}	64.19± 0.38^{aA}
Lauric **12:0**	9.61± 1.72^{bA}	10.73± 0.60^{bA}	10.73± 0.60^{bA}	10.73±0. 60 ^{bA}	9.61± 1.72^{bA}	10.97± 0.49 ^{bA}	10.97± 0.49 ^{bA}	10.97± 0.49 ^{bA}	9.61± 1.72^{bA}	11.84± 0.94^{bA}	11.84± 0.94^{bA}	11.84± 0.94 ^{bA}	9.61± 1.72^{aA}	10.73± 0.60^{aA}	33.27± 23.95^{aA}	33.27± 23.95^{aA}
Myristic **14:0**	92.59± 1.52 ^{aA}	92.45± 1.39 ^{aB}	92.45± 1.39 ^{aC}	92.45± 1.39 ^{aC}	92.59± 1.52 ^{dA}	80.70± 0.20 ^{dB}	80.70± 0.20 ^{dC}	80.70± 0.20 ^{dC}	92.59± 1.52 ^{bA}	88.06± 2.96 ^{bB}	88.06± 2.96 ^{bC}	88.06± 2.96 ^{bC}	92.59± 1.52 ^{cA}	92.45± 1.39 ^{cB}	80.26± 1.11 ^{cC}	80.26± 1.11 ^{cC}
Palmitic **16:0**	826.23± 10.07 ^{aA}	823.46± 6.77 ^{aB}	823.46±6. 77 ^{aC}	823.46± 6.77 ^{aD}	826.23± 10.07 ^{cA}	734.94± 1.62 ^{cB}	734.94± 1.62 ^{cC}	734.94± 1.62 ^{cD}	826.23± 10.07 ^{bA}	791.42± 15.76 ^{bB}	803.24± 20.71 ^{bC}	657.35± 0.08 ^{bD}	826.23± 10.07 ^{dA}	824.98± 7.49^{dB}	701.55± 5.77^{dC}	581.48± 1.92^{dD}
Stearic **18:0**	95.73± 5.43 ^{aA}	96.73± 4.62 ^{aA}	99.05± 5.05 ^{aB}	99.05± 5.05 ^{aC}	95.73± 5.43 ^{aA}	96.07± 2.15 ^{aA}	96.07± 2.15 ^{aB}	96.07± 2.15 ^{aC}	95.73± 5.43 ^{bA}	102.39± 1.61 ^{bA}	81.75± 2.46 ^{bB}	50.62± 0.16 ^{bC}	95.73± 5.43 ^{bA}	91.42± 2.79 ^{bA}	76.64± 0.13 ^{bB}	51.77± 2.27 ^{bC}
Lignoceric **24:0**	15.93± 1.18 ^{aA}	15.32± 0.22 ^{aB}	15.32± 0.22 ^{aAB}	15.32± 0.22 ^{aAB}	15.93± 1.18 ^{bA}	12.05± 1.38 ^{bB}	12.05± 1.38 ^{bAB}	12.05± 1.38 ^{bAB}	15.93± 1.18 ^{aA}	15.77± 0.65 ^{aB}	15.77± 0.65 ^{aAB}	15.77± 0.65 ^{aAB}	15.93± 1.18 ^{aA}	15.32± 0.22 ^{aB}	17.16± 1.25 ^{aAB}	17.16± 1.25 ^{aAB}
∑SFA	**1162.78± 13.70^{aA}**	**1163.03± 13.80^{aA}**	**1165.34± 15.36^{aA}**	**1165.34 ±15.36 ^{aB}**	**1162.78±1 3.70^{bcA}**	**1110.60± 3.31^{bcA}**	**1110.60 ±3.31 ^{bcA}**	**1110.60± 3.31^{bcB}**	**1162.78± 13.70^{cA}**	**1157.70± 22.48^{cA}**	**1148.88± 26.57^{cA}**	**971.86± 8.40^{cB}**	**1162.78 ±13.70 ^{bA}**	**1159.24 ±13.83 ^{bA}**	**1183.24± 13.21^{bA}**	**1038.30 ±18.49 ^{bB}**

*Data represented as Mean± SD (n=3). Statistical differences (Tukey's, P< 0.05) in means are given by changed letters: changed small letters 'a - c' in similar row denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar row denote difference is significant (P< 0.05) among feed storage durations.

Table 4.5b): Fatty-acid profile changes of MUFA and PUFA under temperature conditions and duration effects of stored aquafeed 1(extruded diet).

Fatty Acid conc. (mg/100g)	Storage temperatures and durations															
	LT1				LT2				AT3				HT4			
	0day	60day	120day	180day	0day	60day	120day	180day	0day	60day	120day	180day	0day	60day	120day	180day
Hexadecenoic **16:1n9**	122.31± 2.37 aA	120.85± 0.34 aB	120.85 ±0.34 aC	120.85 ±0.34 aD	122.31± 2.37 bA	110.79± 1.03 bB	110.79 ±1.03 bC	110.79± 1.03 bD	122.31± 2.37 cA	120.28± 2.52 cB	121.37± 1.96 cC	49.14± 5.49 cD	122.31± 2.37 dA	120.54± 1.25 dB	105.57± 1.36 dC	45.33± 1.08 dD
Oleic **18:1n9**	450.19± 24.67 aA	451.17± 26.35 aB	435.19 ±2.86 aC	435.19 ±2.86 aD	450.19± 24.67 bA	370.84± 2.11 bB	370.84 ±2.11 bC	370.84± 2.11 bD	450.19± 24.67 bA	404.96± 12.44 bB		306.58 ±0.58 bD	450.19± 24.67 cA	466.97± 5.33 cB	311.07± 4.27 cC	231.14± 0.50 cD
Nervonic **24:1**	10.12± 1.22 aB	10.53± 0.69 aAB	10.53± 0.69 aA	10.53± 0.69 aA	10.12± 1.22 aB	10.86± 0.26 aAB	10.86± 0.26 aA	10.86± 0.26 aA	10.12± 1.22aB	10.80± 0.49 aAB		10.80± 0.49 aA	10.12± 1.22 aB	10.53± 0.69 aAB	12.00± 0.79 aA	12.00± 0.79 aA
∑MUFA	**582.62± 26.06 aA**	**582.55± 25.94 aB**							**582.62± 26.06 bA**	**536.05± 14.47 bB**		**366.52 ±5.40 bD**	**582.62± 26.06 cA**	**598.04± 5.89 cB**	**428.63± 2.13cC**	**288.47± 2.37cD**
α-linolenic **18:3n3**	32.02± 0.48 aA	32.60± 0.73 aA	32.33± 0.38 aB	32.33± 0.38 aC	32.02± 0.48 aA	33.14± 1.30 aA	33.14± 1.30 aB	33.14± 1.30 aC	32.02± 0.48 bA	33.43± 0.86 bA		11.16± 1.13 bC	32.02± 0.48 cA	30.26± 0.52 cA	12.62± 0.57 cB	9.20± 0.55 cC
Eicosapentaenoic **20:5n3**	71.16± 1.67 cB	70.2± 0.00 cA		70.2±0.00 cA	71.16±1.67 aB	86.96± 1.40 aA	86.96± 1.40 aA	86.96±1.40 aA	71.16±1.67 aB	88.52±3.73 aA		82.78± 1.63 aA	71.16±1.67 bB	68.32±0.88 bA		
Docosapentaenoic **22:5n3**	15.21± 2.15 cB	15.92± 1.25 cA		15.92± 1.25 cA	15.21± 2.15 bB	18.61± 0.26 bA	18.61± 0.26 bA	18.61± 0.26 bA	15.21± 2.15 aB	21.67± 1.25 aA		21.67± 1.25 aA	15.21± 2.15bB	15.92± 1.25bA	19.29± 0.74 bA	19.29± 0.74 bA
Docosahexaenoic **22:6n3**	136.50± 1.60 cB	136.49± 1.59 cA		136.49 ±1.59 cA	136.50± 1.60aB	169.00± 0.56aA	169.00 ±0.56 aA	169.00± 0.56aA	136.50± 1.60 bB	164.59± 3.66 bA		136.87 ±3.50 bA	136.50± 1.60 dB	112.95± 1.65 dA	134.88± 4.61 dA	134.88± 4.61dA
∑n-3PUFA	**254.89± 0.67 aC**	**255.20± 1.02 aA**		**254.94 ±0.71 aB**	**254.89± 0.67 cC**	**307.71± 0.20cA**	**307.71 ±0.20 cA**	**307.71± 0.20 cB**	**254.89± 0.67 bC**	**308.22± 7.01bA**		**252.48 ±2.75 bB**	**254.89± 0.67dC**	**227.44± 1.80dA**	**248.39± 8.35dA**	**244.97± 8.32dB**
Linoleic **18:2n6**	864.23± 7.90 aA	861.51± 4.37 aB	861.47 ±4.33 aC	861.47 ±4.33 aD	864.23± 7.90 bA	718.09± 2.20 bB	718.09 ±2.20 bC	718.09± 2.20 bD	864.23± 7.90 bA	780.79± 20.52 bB	723.21± 13.48 bC	618.32 ±4.99 bD	864.23± 7.90 cA	826.07± 5.08 cB	507.48± 7.64 cC	433.87± 4.97 cD
Arachidonic **20:4n6**	53.88± 0.55 aA	54.07± 0.31 aB	54.07± 0.31 aB	54.07± 0.31 aB	53.88± 0.55 bA	62.69± 0.89 bB	62.69± 0.89 bB	62.69± 0.89 bB	53.88± 0.55 cA	62.76± 1.86 cB	54.99± 0.91 cB	54.99± 0.91 cB	53.88± 0.55 dA	47.53± 0.34 dB	54.18± 1.09 dB	54.18± 1.09 dB
∑n-6PUFA	**918.11± 7.35 aA**	**915.58± 4.07 aB**	**915.53 ±4.03 aC**	**915.53 ±4.03 aD**	**918.11± 7.35 bA**	**780.79± 3.10 bB**	**780.79 ±3.10 bC**	**780.79± 3.10 bD**	**918.11± 7.35 cA**	**843.55± 22.37 cB**	**778.20± 12.57 cC**	**673.32 ±4.08 cD**	**918.11± 7.35 dA**	**873.60± 5.42 dB**	**561.66± 8.73 dC**	**488.06± 6.05 dD**

*Data represented as Mean± SD (n=3). Statistical differences (Tukey's, P< 0.05) in means are given by changed letters: changed small letters 'a - c' in similar row denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar row denote difference is significant (P< 0.05) among feed storage durations.

Table 4.5c): Multivariate analysis of variance (MANOVA), between subject effects on fatty acid profile of formulated aquafeed 1 (extruded diet).

Fatty Acid	Source	df	F	P-value
C4:0	T	3	3344.42	**<0.001**
	D	3	3500.10	**<0.001**
	I	9	1277.05	**<0.001**
C6:0	T	3	1003.95	**<0.001**
	D	3	846.66	**<0.001**
	I	9	424.56	**<0.001**
C8:0	T	3	8753.19	**<0.001**
	D	3	3324.76	**<0.001**
	I	9	2930.14	**<0.001**
C12:0	T	3	4.95	**0.006**
	D	3	2.29	0.098
	I	9	1.71	0.126
C14:0	T	3	59.78	**<0.001**
	D	3	48.92	**<0.001**
	I	9	15.29	**<0.001**
C16:0	T	3	213.30	**<0.001**
	D	3	422.98	**<0.001**
	I	9	120.45	**<0.001**
C16:1 n-9	T	3	284.33	**<0.001**
	D	3	943.87	**<0.001**
	I	9	282.06	**<0.001**
C18:0	T	3	75.41	**<0.001**
	D	3	90.07	**<0.001**
	I	9	35.55	**<0.001**
C18:1 n-9	T	3	57.33	**<0.001**
	D	3	133.66	**<0.001**
	I	9	38.27	**<0.001**
C18:2 n-6	T	3	1269.83	**<0.001**
	D	3	1563.52	**<0.001**
	I	9	445.67	**<0.001**
C18:3n-3	T	3	571.45	**<0.001**
	D	3	486.77	**<0.001**
	I	9	235.76	**<0.001**
C20:4 n-6	T	3	207.67	**<0.001**
	D	3	30.88	**<0.001**
	I	9	55.48	**<0.001**
C20:5 n-3	T	3	128.57	**<0.001**
	D	3	75.72	**<0.001**
	I	9	28.51	**<0.001**
C24:0	T	3	26.62	**<0.001**
	D	3	3.64	**0.023**
	I	9	3.53	**0.004**
C24:1	T	3	1.86	0.156
	D	3	3.74	**0.021**
	I	9	0.78	0.633
C22:5 n-3	T	3	19.65	**<0.001**
	D	3	18.84	**<0.001**
	I	9	3.17	**0.008**
C22:6 n-3	T	3	336.73	**<0.001**
	D	3	33.25	**<0.001**
	I	9	80.73	**<0.001**
∑SFA	T	3	29.40	**<0.001**
	D	3	97.63	**<0.001**
	I	9	30.69	**<0.001**
∑MUFA	T	3	83.79	**<0.001**
	D	3	217.12	**<0.001**
	I	9	60.30	**<0.001**
∑n-6 PUFA	T	3	1224.98	**<0.001**
	D	3	1456.74	**<0.001**
	I	9	415.78	**<0.001**
∑n-3 PUFA	T	3	417.44	**<0.001**
	D	3	64.27	**<0.001**
	I	9	85.66	**<0.001**
n6/n3	T	3	3732.36	**<0.001**
	D	3	4960.33	**<0.001**
	I	9	1658.52	**<0.001**
n3/n6	T	3	1620.08	**<0.001**
	D	3	2136.00	**<0.001**
	I	9	778.65	**<0.001**

Table 4.5d):Fatty-acid profile changes of SFA and MUFA under temperature conditions and duration effects of stored aquafeed 2 (Non-extruded diet).

Fatty Acids (conc. mg/100g)	Storage temperatures and durations															
	LT1				LT2				AT3				HT4			
	0day	60day	120day	180day	0day	60day	120day	180day	0day	60day	120day	180day	0day	60day	120day	180day
Lauric **12:0**	67.79± 0.02^{aA}	64.74± 0.99^{aB}	64.66± 1.65^{aC}	53.31± 5.00^{aD}	67.79± 0.02^{aA}	61.51± 1.34^{aB}	62.71± 0.21^{aC}	45.71± 2.68^{aD}	67.79± 0.02^{bA}	54.49± 0.95^{bB}	40.31± 1.27^{bC}	38.03±4 .20^{bD}	67.79± 0.02^{cA}	44.83± 1.57^{cB}	34.274± 1.45^{cC}	18.46± 1.70^{cD}
Myristic **14:0**	210.35± 0.27^{aA}	210.0± 0.13^{aB}	211.15± 0.87^{aC}	188.39± 2.45^{aD}	210.35± 0.27^{bA}	142.00± 0.40^{bB}	142.46± 0.88^{bC}	133.08± 1.31^{bD}	210.35± 0.27^{cA}	128.74± 0.59^{cB}	118.68± 1.20^{cC}	91.33± 6.70^{cD}	210.35± 0.27^{dA}	162.23± 5.37^{dB}	84.03± 2.41^{dC}	60.04± 2.61^{dD}
Palmitic **16:0**	1694.47± 2.81^{aA}	1688.2 2±8.03 ^{aB}	1680.20 ±2.28a^{B} C	1680.29 ±2.87^{aC}	1694.47 ±2.81^{cA}		1072.26± 0.59^{cBC}	1045.91± 27.20^{cC}	1694.47± 2.81^{bcA}	1243.4± 152.04^{bc} B	1224.8± 102.43^{bc} BC	1153.1± 107.50^{bc} C	1694.47± 2.81^{bA}	1602.7± 13.00^{bB}	1164.6± 96.04^{bBC}	1107.11± 92.73^{bC}
Stearic **18:0**	193.92±1 .36^{aA}	196.1± 1.93^{aB}	212.93± 9.91^{aB}	154.11± 2.82^{aC}	193.92± 1.36^{bA}		108.17± 0.68^{bB}	93.05± 2.66^{bC}	193.92± 1.36^{bA}	137.98± 28.57^{bB}	124.32± 19.67^{bB}	125.20± 15.14^{bC}	193.92± 1.36^{bA}	217.25± 40.87^{bB}	121.57± 18.64^{bB}	70.05± 10.32^{bC}
Lignoceri c **24:0**	31.14± 0.31^{aA}	30.96± 1.14^{aA}	26.87± 1.30^{aB}	18.78± 0.13^{aC}	31.14± 0.31^{bA}		19.18± 2.27^{bB}	12.11± 0.57^{bC}	31.14± 0.31^{abA}	19.35± 4.06^{abA}	35.29± 7.75^{abB}		31.14± 0.31^{bA}	38.67± 4.09^{bA}		7.29± 1.86^{bC}
∑SFA	**2197.67± 4.11^{aA}**	**2190.0 5±6.39 ^{aB}**	**2195.8± 7.14^{aC}**	**2094.89 ±2.47^{aC}**	**2197.67 ±4.11^{cA}**		**1404.78± 2.72^{cC}**	**1329.85± 24.23^{cC}**	**2197.67± 4.11^{bcA}**	**1584.04 ±183.87^{b} cB**	**1543.46 ±130.18^{b} cC**		**2197.67± 4.11^{bA}**	**2065.7± 54.09^{bB}**		**1262.95± 105.92^{bC}**
Hexadec enoic **16:1n9**	231.72±0 .20^{aA}	231.0± 0.61^{aB}	231.46± 0.48^{aC}	219.90± 0.11^{aD}	231.72± 0.20^{bA}	141.47± 17.94^{bB}	160.12±0 .69^{bC}	137.37± 14.05^{bD}	231.72±0 .20^{cA}	129.36± 17.97^{cB}	111.31± 2.42^{cC}		231.72±0. 20^{cA}	140.80± 10.47^{cB}	77.06± 5.13^{cC}	49.79± 4.88^{cD}
Oleic **18:1n9**	604.95±2 .91^{aA}	606.87 ±2.57^{a} B	599.03± 11.57^{aB}	592.99± 2.14^{aC}	604.95± 2.91^{bA}	444.77± 68.36^{bB}	504.62±1 3.36^{bB}	441.63± 56.90^{bC}	604.95±2 .91^{cA}	341.75± 17.76^{cB}	319.11± 27.18^{cB}		604.95±2. 91^{cA}	362.66± 52.53^{cB}	264.82± 26.40^{cB}	83.63± 13.61^{cC}
∑MUFA	**836.67± 2.71^{aA}**	**837.9± 2.35^{aB}**	**830.49± 11.10^{aB}**	**812.89± 2.24^{aC}**	**836.67± 2.71^{bA}**	**586.25± 86.26^{bB}**	**664.74±1 2.67^{bB}**	**579.00± 70.95^{bC}**	**836.67± 2.71^{cA}**	**471.11± 37.72^{cB}**	**430.42± 27.72^{cB}**		**836.67± 2.71^{cA}**	**503.47± 62.99^{cB}**	**341.89± 28.71^{cB}**	**133.42± 16.59^{cC}**

*Data represented as Mean± SD (n=3). Statistical differences (Tukey's, P< 0.05) in means are given by changed letters: changed small letters 'a - c' in similar row denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar row denote difference is significant (P< 0.05) among feed storage durations.

Table 4.5e): Fatty-acid profile changes of PUFA under temperature conditions and duration effects of stored aquafeed 2 (Non-extruded diet).

Fatty Acids (conc. mg/100g)	Storage temperatures and durations															
	LT1				LT2				AT3				HT4			
	0day	60day	120day	180day	0day	60day	120day	180day	0day	60day	120day	180day	0day	60day	120day	180day
Alpha-linolenic **18:3n3**	30.24± 0.10^{aA}	34.79± 0.73^{aB}	33.47± 0.37^{aC}	31.79± 0.13^{aD}	30.24± 0.10^{bA}	23.48± 2.43^{bB}	24.75± 0.36^{bC}	23.26± 1.37^{bD}	30.24± 0.10^{cA}	21.54± 0.73^{cB}	24.3± 0.50^{cC}	6.84± 2.24^{cD}	30.24± 0.10^{dA}	26.52± 3.31^{dB}	4.50± 0.50^{dC}	4.84± 0.67^{dD}
Eicosapentaenoic **20:5n3**	88.81± 0.30^{aA}	90.07± 1.43^{aB}	82.52± 3.77^{aC}	81.26± 0.25^{aD}	88.81± 0.30^{bA}	60.70± 1.23^{bA}	56.89±2. 50^{bB}	55.12± 0.45^{bC}	88.81± 0.30^{bA}	56.80± 2.98^{bD}		43.7± 3.69^{bB}	88.81± 0.30^{cA}	74.08± 9.41^{bC}	39.15± 2.31^{cD}	9.88± 1.04^{cA}
Docosahexaenoic **22:6n3**	189.7± 0.31^{aA}	198.76± 1.14^{aB}	190.45± 6.58^{aC}	157.66± 3.02^{aD}	189.7± 0.31^{bA}	130.84± 1.38^{bB}		99.98± 2.70^{bD}	189.7± 0.31^{cA}	111.67± 8.62^{cB}		78.9± 5.35^{cD}	189.7± 0.31^{dA}		72.29± 1.86^{dC}	47.95± 2.28^{dD}
∑n-3 PUFA	**308.7± 0.09^{aA}**	**323.61± 1.63^{aB}**	**306.44± 10.50^{aC}**	**270.70± 2.76^{aD}**	**308.74±0 .09^{bA}**	**215.01± 5.68^{bB}**		**178.36± 4.09^{bD}**	**308.74± 0.09^{bA}**	**190.01±1 2.12^{bB}**		**129.53± 11.15^{bD}**	**308.74± 0.09^{cA}**		**115.9± 4.58^{cC}**	**62.67± 3.62^{cD}**
Linoleic **18:2n6**	922.5± 12.06^{aA}	913.92± 2.30^{aB}	913.43± 5.33^{aC}	856.39± 16.87^{aD}	922.5±12 .06^{bA}	710.60± 22.75^{bB}		689.43± 23.06^{bD}	922.5±1 2.06^{cA}	588.39± 14.54^{cB}		474.2± 38.67^{cD}	922.5±12 .06^{cA}		459.9± 24.78^{cC}	363.1±20 .52^{cD}
Arachidonic **20:4n6**	76.58± 0.14^{aA}	77.54± 1.08^{aB}	77.42± 0.37^{aC}	73.19± 0.13^{aD}	76.58± 0.14^{bA}	50.27± 2.71^{bB}		50.35± 1.67^{bD}	76.58± 0.14^{cA}	48.41± 3.72^{cB}	54.05± 1.89^{cC}	28.41± 3.50^{cD}	76.58± 0.14^{dA}		24.61± 1.12^{dC}	14.58± 1.12^{dD}
∑n-6 PUFA	**999.12 ±12.16 ^{aA}**	**991.46± 2.08^{aB}**	**990.85± 5.70^{aC}**	**929.58± 16.95^{aD}**	**999.12± 12.16^{bA}**	**760.87± 25.46^{bB}**	**778.51±5 .47^{bC}**	**739.79± 24.72^{bD}**	**999.12± 12.16^{cA}**	**636.80± 11.06^{cB}**	**525.95±2 2.00^{cC}**	**502.70± 42.15^{cD}**	**999.12± 12.16^{cA}**	**801.3± 42.90^{cB}**	**484.6± 24.6^{7cC}**	**377.71± 21.11^{cD}**

*Data represented as Mean± SD (n=3). Statistical differences (Tukey's, P< 0.05) in means are given by changed letters: changed small letters 'a - c' in similar row denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar row denote difference is significant (P< 0.05) among feed storage durations.

Table 4.5f): Multivariate analysis of variance (MANOVA),between subject effects on fatty-acid profile of formulated aquafeed 2 (Non-extruded diet).

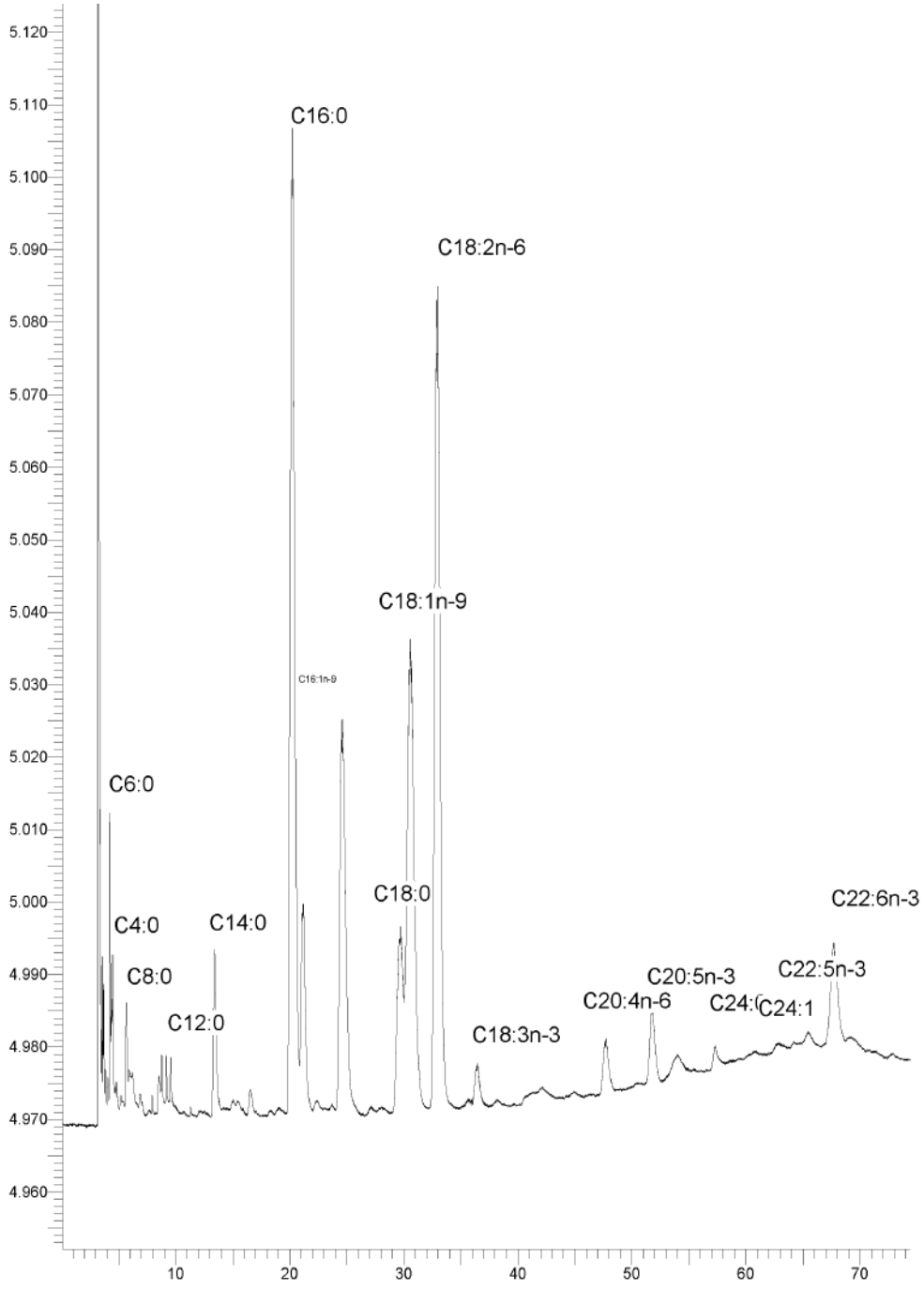
Fatty Acid	Source	df	F	P-value
C12:0	T	3	90.68	**0.000**
	D	3	142.23	**0.000**
	I	9	13.96	**0.000**
C14:0	T	3	755.82	**0.000**
	D	3	1022.11	**0.000**
	I	9	142.64	**0.000**
C16:0	T	3	39.57	**0.000**
	D	3	40.38	**0.000**
	I	9	7.24	**0.000**
C16:1 n-9	T	3	128.15	**0.000**
	D	3	135.26	**0.000**
	I	9	19.128	**0.000**
C18:0	T	3	13.038	**0.000**
	D	3	20.24	**0.000**
	I	9	5.21	**0.000**
C18:1 n-9	T	3	73.77	**0.000**
	D	3	60.47	**0.000**
	I	9	11.71	**0.000**
C18:2 n-6	T	3	164.53	**0.000**
	D	3	183.75	**0.000**
	I	9	28.84	**0.000**
C18:3n-3	T	3	116.15	**0.000**
	D	3	85.182	**0.000**
	I	9	33.67	**0.000**
C20:4 n-6	T	3	207.30	**0.000**
	D	3	278.89	**0.000**
	I	9	73.40	**0.000**
C20:5 n-3	T	3	78.43	**0.000**
	D	3	126.74	**0.000**
	I	9	24.80	**0.000**
C24:0	T	3	4.12	**0.014**
	D	3	39.48	**0.000**
	I	9	9.40	**0.000**
C22:6 n-3	T	3	173.16	**0.000**
	D	3	301.32	**0.000**
	I	9	39.38	**0.000**
∑SFA	T	3	46.11	**0.000**
	D	3	58.30	**0.000**
	I	9	9.55	**0.000**
∑MUFA	T	3	89.35	**0.000**
	D	3	79.33	**0.000**
	I	9	13.81	**0.000**
∑n-6 PUFA	T	3	190.11	**0.000**
	D	3	216.26	**0.000**
	I	9	34.892	**0.000**
∑n-3 PUFA	T	3	146.91	**0.000**
	D	3	229.67	**0.000**
	I	9	36.22	**0.000**
n6/n3	T	3	17.11	**0.000**
	D	3	25.69	**0.000**
	I	9	8.72	**0.000**
n3/n6	T	3	11.44	**0.000**
	D	3	17.08	**0.000**
	I	9	7.90	**0.000**

Temperature (T), Duration (D), Interaction of Temperature*Duration (I). # P-value for between subject effects ; **Significant P-values P< 0.05 in highlight.**

Table 4.5g): Multivariate test showing effect of temperature and storage duration on change in fatty-acid profile of formulated aquafeeds 1 and 2.

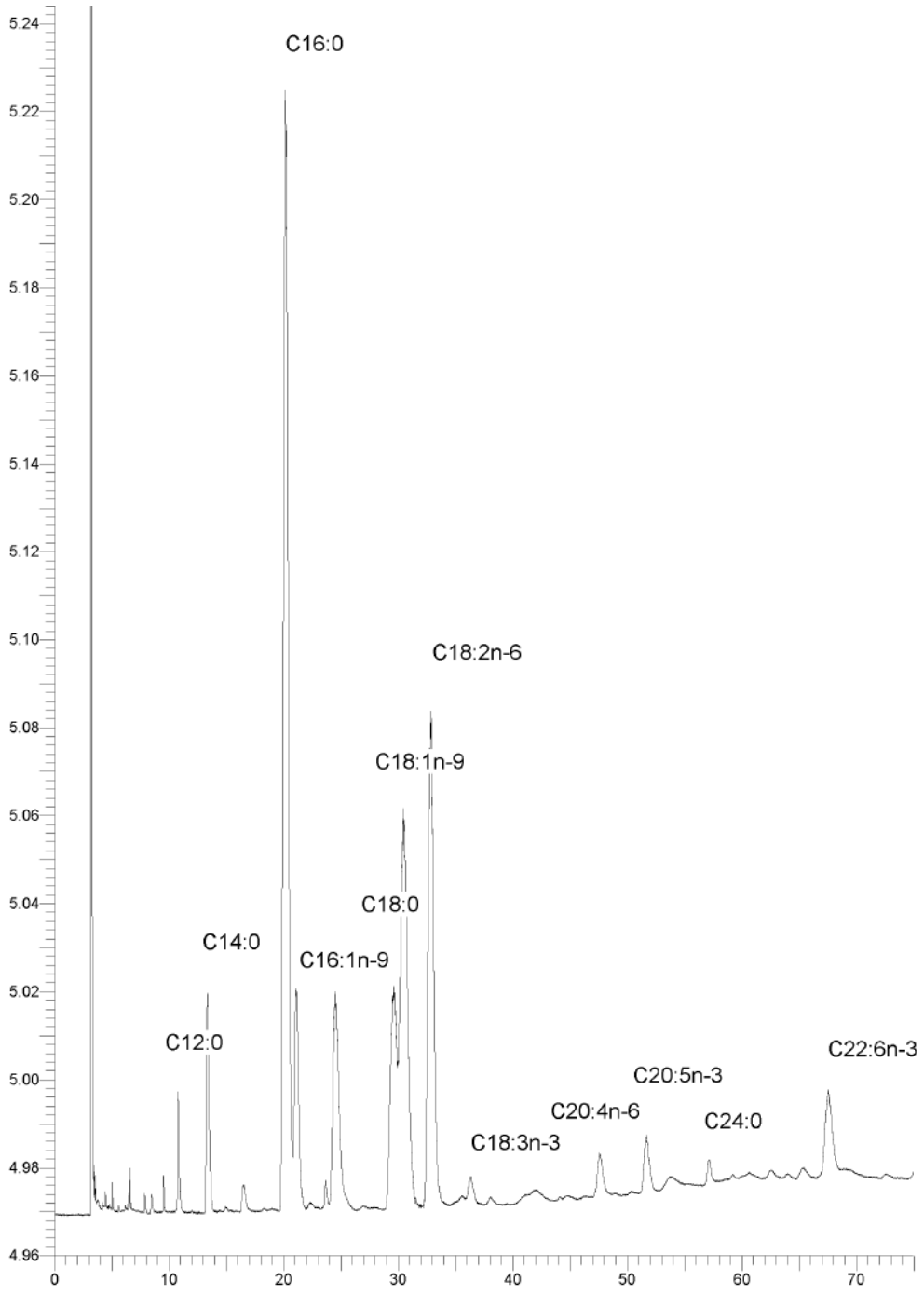
Effect		Value	F (Aquafeed1)	F (Aquafeed2)	P-value (Aquafeed1)	P-value (Aquafeed2)
T	Wilks' Lambda	0.000	26976.97	62.687	**< 0.001**	**0.000**
D		0.000	6391.83	79.709	**< 0.001**	**0.000**
I		0.000	209.19	14.750	**< 0.001**	**0.000**

Figure 4.5a): Fatty acid chromatographic profile of initial day extruded diet 1.



Source: Author (2023)

Figure 4.5b):Fatty acid chromatographic profile of initial day non-extruded diet 2.



Source: Author (2023)

4.6 Elemental Profile Analysis

Five macro-elements are determined in both diet 1 and diet 2. While 43 minor element profiles are analyzed in aquafeed 1, out of which 13 micro-elements are of direct importance to fish physiology. In aquafeed 2, 38 minor elements are assessed with reportedly 12 micro-elements of relevance to fish diet. Figures 4.6 a)-f) show standard calibration graphs obtained to determine linearity at 0.999.

4.6.1 Macro-elements

Among macro-elements, potassium is present at highest amount accounting 0-day initial value of 12.04±0.50 mg/g, followed by phosphorous (6.84± 0.35 mg/g), sodium (6.38± 0.32 mg/g), magnesium (2.98± 0.14 mg/g) and

is potassium at 6.67± 0.41 mg/g, other elements being magnesium 4.47± 0.19 mg/g, sodium 3.33± 1.11 mg/g, calcium 1.98± 0.10 mg/g, and phosphorous 1.49± 0.82 mg/g. All macro-elements in diet 1 and 2 show significant increase beyond initial storage duration except for potassium (in diet 1) where no significant ($P>0.05$) difference exist between initial and final values at all storage temperatures, table 4.6a). In both diets, between subject effects of duration are significant ($P<0.05$) for all major elements.

Temperature effects are not noteworthy ($P>0.05$) for Mg, P and K in extruded diet 1 and those for P, K, Ca of pelleted diet 2. Significant effects ($P<0.05$) of interaction on diet 1 elements exist towards sodium, magnesium, potassium and calcium. In diet 2, interaction is significant ($P<0.05$) for Na values.

4.6.2 Microelements

Copper and subsequently iron, are the two abundant microelements at initial concentrations in both diets. Concentrations of micro-elements Cu, Fe, Al, Zn, Sr, Mn (diet1), B (in diet1), have been reported in mg/g in both diets, tables 4.6 a)-a.1). Chromium and Al could not be detected at initial duration in diet 1 with subsequent values reported in microgram and mg/g respectively. All other minor elements are determined as mcg/g of diets (tables 4.6a.1,2 and 4.6b.1,2). Micro-elements of relevance to fish diet are listed in table 4.6a) for diet 1, and table 4.6 b) for diet 2 these namely being zinc, selenium, nickel, aluminum, copper, As, Sn (only in diet 1), molybdenum, vanadium, Mn, chromium, cobalt and iron.

Significant ($P<0.05$) decrease in iron and manganese is noteworthy at end of storage duration in diet 1. Cr and Zn decreased ($P<0.05$) over storge duration in diet 2, while Fe decreased significantly ($P<0.05$) over storage at all temperatures in diet 1. No significant ($P>0.05$) overall effects of storage is found for diet 2 Mo, Cu, As values and that of duration on Zn of diet 1. This holds true for other microelements consisting Fe, Se, V, Al in diet 2.

Table 4.6a): Elemental profile 'macro- and micro-element' changes during storage aquafeed1.

Temperature	Storage days	Macro-elements (conc.)						Micro-elements (conc.)											
		Atomic Mass/Symbol																	
		23 Na mg/g	24 Mg mg/g	31 P mg/g	39 K mg/g	43 Ca mg/g	52 Cr mcg/g	55 Mn mg/g	56 Fe mg/g	63 Cu mg/g	66 Zn mg/g	78 Se mcg/g	27 Al mg/g	51 V mcg/g	59 Co mcg/g	60 Ni mcg/g	75 As mcg/g	95 Mo mcg/g	118 Sn mcg/g
LT1	0	6.38± 0.32^{aC}	2.98± 0.14^{aC}	6.84± 0.35^{aC}	12.04± 0.50^{aA}	1.10± 0.04^{aC}	0.00^{aC}	0.22± 0.01^{aA}	1.37± 0.01^{aA}	9.64± 1.01^{aB}	0.05± 0.01^{aA}	0.69± 0.07^{aB}	0.00^{aB}	1.72± 0.07^{aA}	0.2± 0.03^{aB}	0.00^{aB}	0.6± 0.07^{cBC}	1.3± 0.12^{bA}	0.8± 0.89^{aA}
	60	10.93± 0.75^{aB}	5.19± 0.52^{aB}	12.77± 1.22^{aB}	11.38± 1.01^{aB}	3.06± 0.25^{aB}	9.72± 0.95^{aB}	0.19± 0.02^{aC}	1.08± 0.09^{aB}	11.03± 1.65^{aB}	0.06± 0.00^{aA}	5.49± 1.46^{aAB}	0.3± 0.05^{aA}	1.72± 0.18^{aB}	0.4± 0.10^{aA}	1.1± 0.41^{aA}	0.2± 0.00^{cAB}	0.8± 0.21^{bB}	ND
	120	11.72± 0.35^{aA}	5.82± 0.23^{aA}	14.88± 0.35^{aA}	12.22± 0.41^{aA}	3.45± 0.07^{aA}	13.23± 0.05^{aA}	0.20± 0.01^{aB}	1.19± 0.08^{aB}	11.00± 0.85^{aA}	0.07± 0.01^{aA}	2.14± 0.61^{aAB}	0.3± 0.03^{aA}	1.83± 0.15^{aA}	0.6± 0.16^{aA}	1.2± 0.25^{aA}	ND	0.6± 0.12^{bB}	ND
	180	11.19± 0.67^{aA}	5.57± 0.38^{aA}	12.17± 0.70^{aAB}	11.52± 0.72^{aA}	3.09± 0.17^{aA}	11.38± 1.46^{aAB}	0.19± 0.01^{aB}	1.15± 0.12^{aB}	10.77± 1.13^{aAB}	0.06± 0.01^{aA}	4.61± 1.60^{aA}	0.3± 0.05^{aA}	1.52± 0.02^{aAB}	0.50± 0.07^{aA}	2.8± 1.13^{aA}	ND	0.5± 0.11^{bC}	ND
LT2	0	6.38± 0.32^{abC}	2.98± 0.14^{aC}	6.84± 0.35^{aC}	12.04± 0.50^{aA}	1.10± 0.04^{aC}	0.00^{abC}	0.22± 0.01^{abA}	1.37± 0.01^{aA}	9.64± 1.01^{aB}	0.05± 0.01^{abA}	0.69± 0.07^{aB}	0.00^{aB}	1.72± 0.07^{aA}	0.21± 0.03^{aB}	0.00^{aB}	0.6± 0.07^{bcBC}	1.3± 0.12^{abA}	0.8± 0.89^{aA}
	60	10.14± 0.19^{abB}	4.83± 0.15^{aB}	11.79± 0.33^{aB}	10.44± 0.25^{aB}	2.98± 0.07^{aB}	10.18± 0.41^{abB}	0.17± 0.01^{abC}	0.96± 0.03^{aB}	9.88± 0.87^{aB}	0.06± 0.01^{abA}	2.76± 1.59^{aAB}	0.3± 0.07^{aA}	1.38± 0.12^{aB}	0.49± 0.01^{aA}	1.3± 0.08^{aA}	0.4± 0.13^{bcAB}	0.8± 0.07^{abB}	ND
	120	11.28± 0.27^{abA}	5.59± 0.17^{aA}	14.10± 0.76^{aA}	12.45± 0.25^{aA}	3.65± 0.05^{aA}		0.21± 0.01^{abB}	1.22± 0.04^{aB}	14.00± 1.55^{aA}	0.07± 0.00^{abA}	5.07± 2.35^{aAB}	0.3± 0.02^{aA}	1.98± 0.15^{aA}	0.59± 0.10^{aA}	1.6± 0.31^{aA}	1.1± 0.32^{bcA}	0.8± 0.18^{abB}	ND
	180	11.46± 0.33^{abA}	5.79± 0.18^{aA}	13.07± 0.32^{aAB}	11.82± 0.44^{aA}	3.35± 0.07^{aA}		0.20± 0.01^{abB}	1.23± 0.07^{aB}	11.80± 0.87^{aAB}	0.05± 0.00^{abA}	2.15± 0.62^{aA}	0.3± 0.02^{aA}	1.70± 0.17^{aAB}	0.50± 0.04^{aA}	1.2± 0.16^{aA}	0.00^{bcC}	0.6± 0.16^{abC}	ND
AT3	0	6.38± 0.32^{abC}	2.98± 0.14^{aC}	6.84± 0.35^{aC}	12.04± 0.50^{aA}	1.10± 0.04^{abC}		0.22± 0.01^{abA}	1.37± 0.01^{abA}	9.64± 1.01^{aB}	0.05±0. 01^{abA}	0.69± 0.07^{aB}	0.00^{aB}	1.72± 0.07^{abA}	0.21± 0.03^{aB}	0.00^{aB}	0.6± 0.07^{abBC}	1.3± 0.12^{abA}	0.8± 0.89^{aA}
	60	9.11± 0.28^{abB}	4.46± 0.11^{aB}	11.72± 0.39^{aB}	9.29± 0.29^{aB}	2.46± 0.08^{abB}		0.16± 0.00^{abC}	0.92± 0.03^{abB}	8.67± 0.25^{aB}		0.65± 0.13^{aAB}	0.3± 0.01^{aA}	1.27± 0.03^{abB}	0.41± 0.01^{aA}	1.8± 0.32^{aA}	1.1± 0.06^{abAB}	1.2± 0.09^{abB}	0.0± 0.01^{aA}
	120	11.18± 0.18^{abA}	5.53± 0.09^{aA}	13.41± 0.47^{aA}	12.10± 0.18^{aA}	3.42± 0.16^{abA}		0.20± 0.00^{abB}	1.03± 0.05^{abB}	13.23± 0.71^{aA}	0.05± 0.00^{abA}	4.59± 0.51^{aAB}	0.3± 0.01^{aA}	1.62± 0.02^{abA}	0.48± 0.03^{aA}	2.1± 0.54^{aA}	0.4± 0.00^{abA}	0.9± 0.24^{abB}	ND
	180	11.11± 0.53^{abA}	5.59± 0.31^{aA}	13.10± 0.55^{aAB}	11.52± 0.66^{aA}	3.31± 0.09^{abA}		0.19± 0.01^{abB}	1.01± 0.03^{abB}	10.52± 1.54^{aAB}	0.04± 0.00^{abA}	3.70± 1.43^{aA}	0.3± 0.01^{aA}	1.63± 0.23^{abAB}	0.49± 0.08^{aA}	1.2± 0.28^{aA}	0.00^{abC}	0.5± 0.06^{abC}	ND
HT4	0	6.38± 0.32^{bC}	2.98± 0.14^{aC}	6.84± 0.35^{aC}	12.04± 0.50^{aA}	1.10± 0.04^{bC}	0.00^{bC}	0.22± 0.01^{cA}	1.37± 0.01^{bA}	9.64± 1.01^{aB}	0.05± 0.01^{bA}	0.69± 0.07^{aB}	0.00^{aB}	1.72± 0.07^{bA}	0.21± 0.03^{aB}	0.00^{aB}	0.6± 0.07^{aBC}	1.3± 0.12^{aA}	0.8± 0.89^{aA}
	60	9.27± 0.18^{bB}	4.55± 0.09^{aB}	11.70± 0.10^{aB}	9.45± 0.18^{aB}	2.48± 0.11^{bB}	7.03± 0.24^{bB}	0.15± 0.00^{cC}	0.85± 0.01^{bB}	8.15± 0.27^{aB}	0.04± 0.00^{bA}	0.39±0. 16^{aAB}			0.39± 0.01^{aA}	2.0± 0.45^{aA}	1.1± 0.06^{aAB}	1.1± 0.06^{aB}	0.0± 0.01^{aA}
	120	9.50± 0.09^{bA}	4.68± 0.01^{aA}	12.22± 0.01^{aA}	9.58± 0.06^{aA}	2.42± 0.05^{bA}	8.22± 0.20^{bA}	0.16± 0.00^{cB}	0.89± 0.02^{bB}	10.11± 1.07^{aA}	0.04± 0.00^{bA}	0.45± 0.07^{aAB}	0.3± 0.01^{aA}	1.29± 0.05^{bA}	0.40± 0.01^{aA}	1.8± 0.06^{aA}	1.0± 0.02^{aA}	1.2± 0.06^{aB}	0.0± 0.02^{aA}
	180	12.08± 0.08^{bA}	6.10± 0.06^{aA}	13.49± 0.04^{aAB}	12.58± 0.18^{aA}	3.66± 0.15^{bA}	9.90± 0.27^{bAB}	0.20± 0.00^{cB}	0.94± 0.02^{bB}	10.14± 0.25^{aAB}	0.05± 0.00^{bA}	2.78± 1.86^{aA}	0.3± 0.01^{aA}	1.57± 0.10^{bAB}	0.59± 0.07^{aA}	1.3± 0.10^{aA}	0.00^{aC}	0.6± 0.10^{aC}	ND
	T	**.029**	.126	.385	.051	**.000**	**.003**	**.027**	**.002**	.109	**.008**	.064	.150	**.013**	.413	.761	**.003**	**.037**	1.000
P- value	**D**	**.000**	**.000**	**.000**	**.000**	**.000**	**.000**	**.000**	**.000**	**.007**	.168	**.011**	**.000**	**.002**	**.000**	**.000**	**.034**	**.000**	.425
	I	**.005**	**.024**	.062	**.005**	**.000**	**.037**	**.017**	.422	.465	.390	.106	.729	**.044**	.482	.056	**.000**	.330	.973

Statistical differences (Tukey's, P< 0.05) in means are given by changed letters: changed small letters 'a - c' in similar column denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar column denote difference is significant (P< 0.05) among feed storage durations.

Temperature (T), Duration (D), Interaction of Temperature*Duration (I). # P-value for between subject effects; **Significant P-values P< 0.05 in highlight.**

Table 4.6a.1): Elemental profile 'micro-element' changes during storage aquafeed 1.

Temperature	Storage days	Micro-elements (conc.) Atomic Mass / Symbol																	
		7 Li mcg/g	9 Be mcg/g	11 B [mg/g]	45 Sc mcg/g	47 Ti mcg/g	71 Ga mcg/g	72 Ge mcg/g	88 Sr mg/g	90 Zr mcg/g	93 Nb mcg/g	101Ru mcg/g	103 Rh mcg/g	105 Pd mcg/g	107 Ag mcg/g	111 Cd mcg/g	115 In mcg/g	118 Sn mcg/g	121 Sb mcg/g
LT1	0	0.87± 0.22	0.03±0. 00	0.31±0. 01	3.85±0. 13	24.61± 1.96	0.04±0. 00	0.38±0. 07	0.14±0. 01	0.20±0. 14	0	0	0.004± 0.001	0.26±0. 04	0.01±0. 00	0.07±0. 03	0	0.89±0. 89	0.05±0. 03
	60	0.71±0. 20	0.06±0 .03	0.28±0 .01	8.92±0 .99	30.13± 3.40	0.15±0 .00	0.41±0 .11	0.24±0 .03	0.45±0 .11	0.81±0 .32	0.05±0 .02	0.05±0 .01	2.59±0 .76	0.28±0 .12	0.17±0 .14	0.04±0 .00	ND	ND
	120	1.31±0 .32	0.53±0 .45	0.31±0 .00	7.52±1 .00	19.52± 2.46	0.51±0 .14	0.74±0 .19	0.26±0 .01	0.14±0 .04	12.82± 2.30	0.02±0 .01	0.04±0 .01	1.69±0 .25	0.23±0 .03	0.23±0 .03	0.03±0 .01	ND	0.06±0 .00
	180	0.96±0 .24	0.07±0 .01	0.28±0 .01	6.23±0 .26	23.10± 4.57	0.29±0 .14	0.33±0 .11	0.23±0 .02	0.19±0 .11	12.89± 2.16	0.02±0 .00	0.02±0 .00	0.91±0 .22	0.13±0 .11	0.23±0 .06	0.03±0 .01	ND	ND
LT2	0	0.87±0 .22	0.03±0 .00	0.31±0 .01	3.85±0 .13	24.61± 1.96	0.04±0 .00	0.38±0 .07	0.14±0 .01	0.20±0 .14	0	0	0.004± 0.001	0.26±0 .04	0.01±0 .00	0.07±0 .03	0	0.89±0 .89	0.05±0 .03
	60	1.44±0 .36	0.05±0 .01	0.27±0 .00	8.91±0 .81	18.61± 3.41	0.24±0 .00	0.50±0 .13	0.21±0 .01	0.20±0 .07	0.38±0 .06	0.04±0 .04	0.04±0 .01	2.03±0 .03	0.06±0 .00	0.14±0 .03	0.04±0 .01	ND	ND
	120	0.69±0 .03	0.06±0 .03	0.30±0 .00	6.59±0 .41	22.28± 2.11	0.60±0 .52	0.83±0 .23	0.26±0 .01	0.21±0 .00	18.01± 6.75	0.02±0 .01	0.03±0 .01	1.39±0 .22	0.47±0 .16	0.26±0 .10	0.01±0 .01	ND	ND
	180	0.61±0 .03	0.03±0 .02	0.29±0 .01	5.57±0 .97	29.26± 3.79	0.16±0 .00	0.33±0 .20	0.24±0 .01	0.16±0 .08	7.53±0 .37	0.03±0 .02	0.02±0 .01	0.94±0 .06	0.03±0 .03	0.26±0 .09	0.01±0 .01	ND	0.03±0 .00
AT3	0	0.87±0 .22	0.03±0 .00	0.31±0 .01	3.85±0 .13	24.61± 1.96	0.04±0 .00	0.38±0 .07	0.14±0 .01	0.20±0 .14	0	0	0.004± 0.001	0.26±0 .04	0.01±0 .00	0.07±0 .03	0	0.89±0 .89	0.05±0 .03
	60	1.61±0 .19	0.07±0 .05	0.25±0 .01	N.D.	19.52± 0.83	0.12±0 .01	0.16±0 .02	0.21±0 .00	0.24±0 .03	0.37±0 .29	0	0.01±0 .00	0.76±0 .08	0.03±0 .01	0.14±0 .01	0	0.03±0 .01	0.11±0 .02
	120	0.65±0 .03	0.11±0 .02	0.30±0 .00	7.38±1 .0	28.52± 0.70	0.24±0 .00	0.19±0 .09		0.20±0 .07	5.04±1 .14	0.02±0 .02	0.02±0 .00	1.36±0 .12	0.06±0 .00	0.20±0 .08	0.02±0 .01	ND	0.03±0 .00
	180	0.48±0 .32	0.06±0 .01	0.28±0 .02	4.75±0 .26	29.26± 3.39	0.12±0 .03	0.41±0 .11		0.22±0 .07	5.83±0 .23	0.01±0 .01	0.01±0 .00	1.10±0 .14	0	0.12±0 .03	0.03±0 .01	ND	0.09±0 .00
HT4	0	0.87±0 .22	0.03±0 .00	0.31±0 .01	3.85±0 .13	24.61± 1.96	0.04±0 .00	0.38±0 .07		0.20±0 .14	0	0	0.004± 0.001	0.26±0 .04	0.01±0 .00	0.07±0 .03	0	0.89±0 .89	0.05±0 .03
	60	0.67±0 .01	0.02±0 .02	0.25±0 .00	N.D.	19.50± 0.55	0.11±0 .01	0.16±0 .03		0.21±0 .03	0.04±0 .00	0	0.01±0 .00	0.63±0 .04	0.05±0 .03	0.15±0 .03	0	0.08±0 .01	0.11±0 .02
	120	0.69±0 .01	0.01±0 .01	0.25±0 .00	N.D.	20.20± 1.73	0.14±0 .01	0.12±0 .00		0.15±0 .03	0.03±0 .01	0	0.01±0 .00	0.60±0 .00	0.03±0 .01	0.13±0 .02	0	0.05±0 .02	0.09±0 .02
	180	0.48±0 .32	0.03±0 .01	0.31±0 .00	3.55±0 .28	26.09± 2.70	0.24±0 .09	0.38±0 .19		0.18±0 .05	0.86±0 .36	0.02±0 .02	0.02±0 .01	1.01±0 .06	0.21±0 .17	0.12±0 .08	0.02±0 .01	ND	ND
P-value	T	.252	.276	**.028**	.177	.405	.676	**.009**		.704	**.004**	.466	**.002**	**.000**	**.022**	.182	**.003**	1.000	.673
	D	**.048**	.236	**.000**	**.000**	**.035**	.135	.412		.373	**.000**	.156	**.000**	**.000**	**.001**	**.002**	**.000**	.425	.128
	I	.057	.433	**.000**	.397	**.027**	.929	**.027**	**.003**	.733	**.011**	.964	**.003**	**.000**	**.003**	.754	**.000**	.973	.626

P-value for between subject effects; **Significant P-values P< 0.05 in highlight.** # Temperature (T), Duration (D), Interaction of Temperature*Duration (I).

Table 4.6a.2): Elemental profile 'micro-element' changes during storage aquafeed 1.

Temperature	Storage days	Micro-elements (conc.)											
		Atomic Mass / Symbol											
		133 Cs mcg/g	137 Ba mcg/g	178 Hf mcg/g	181 Ta mcg/g	182 W mcg/g	185 Re mcg/g	193 Ir mcg/g	195 Pt mcg/g		205 Tl mcg/g	208 Pb mcg/g	209 Bi mcg/g
LT1	0	0.07±0.01	0.01± 0.00	0.01±0.00	0.004±0.001	0	0.01±0.00	0	0		0.002±0.001	0.27±0.04	0.004±0.002
	60	0.16±0.00	0.02±0.00	0.03±0.01	0.94±0.21	0.49±0.18	0.03±0.01	0.18±0.04	0.04±0.00		0.41±0.08	0.32±0.00	0
	120	0.03±0.01	0.02±0.00	0.02±0.01	0.42±0.04	0.29±0.12	0.02±0.00	0.06±0.02	0.02±0.00		0.18±0.01	0.27±0.21	0
	180	0.15± 0.12	0.02±0.00	0	0.18±0.01	0.09±0.00	0.02±0.01	0.03±0.01	ND		0.12±0.02	0.01±0.00	0
LT2	0	0.07±0.01	0.01±0.00	0.01±0.00	0.004±0.001	0	0.01±0.00	0	0		0.002±0.001	0.27±0.04	0.004±0.002
	60	0.06±0.01	0.02±0.00	0.02±0.01	0.64±0.03	0.43±0.05	0.02±0.01	0.12±0.03	0		0.23±0.05	0.12±0.00	0
	120	0.08±0.04	0.02±0.00	0.02±0.01	0.38±0.02	0.24±0.01	0.01±0.00	0.07±0.02	0.03±0.00	25.15±2.04	0.15±0.01	1.23±0.15	0
	180	0.02±0.00	0.02±0.00	0.01±0.01	0.19±0.03	0.05±0.03	0.02±0.00	0.06±0.03	ND	12.34±1.46	0.11±0.01	ND	0
AT3	0	0.07±0.01	0.01±0.00	0.01±0.00	0.004±0.001	0	0.01±0.00	0	0	0.34±0.06	0.002±0.001	0.27±0.04	0.004±0.002
	60	0.06±0.01	0.02±0.00	0.02±0.00	0.02±0.01	0.25±0.09	0	0	0.05±0.01	0.17±0.03	0.01±0.00	0.59±0.05	0
	120	0.10±0.01	0.03±0.00	0.01±0.01	0.31±0.02	0.26±0.01	0.01±0.00	0.04±0.01	ND	14.52±0.98	0.15±0.03	1.30±0.00	0
	180	0.06±0.01	0.02±0.00	0	0.13±0.03	0.01±0.00	0.02±0.01	0.16±0.12	ND	8.02±0.22	0.06±0.01	ND	0
HT4	0	0.07±0.01	0.01±0.00	0.01±0.00	0.004±0.001	0	0.01±0.00	0	0	0.34±0.06	0.002±0.001	0.27±0.04	0.004±0.002
	60	0.06±0.01	0.02±0.00	0.01±0.00	0.01±0.00	0.13±0.01	0	0	0.06±0.01	0.14±0.00	0.01±0.00	0.56±0.02	0.01±0.00
	120	0.06±0.00	0.02±0.00	0.01±0.00	0.01±0.00	0.10±0.01	0	0	0.04±0.02	0	0.01±0.00	0.56±0.03	0.01±0.00
	180	0.06±0.00	0.02±0.00	0.03±0.02	0.14±0.03	0.06±0.03	0.00±0.00	0.07±0.03	ND	9.21±0.19	0.06±0.01	ND	0
P-value	T	.135	.209	.649	**.000**	.051	**.007**	**.035**	.339	**.000**	**.000**	**.000**	**.000**
	D	.600	**.000**	**.008**	**.000**	**.000**	.766	**.000**	**.000**	**.000**	**.000**	**.000**	**.000**
	I	.071	.161	.117	**.000**	.219	.238	**.001**	.740	**.000**	**.000**	**.000**	**.000**

P-value for between subject effects; **Significant P-values P< 0.05 in highlight.** # Temperature (T), Duration (D), Interaction of Temperature*Duration (I).

Table 4.6b): Elemental profile 'macro- and micro-element' changes during storage aquafeed 2.

Temperature	Storage days	Macro-elements (conc.)							Micro-elements (conc.)									
		Atomic Mass/Symbol																
		23 Na mg/g	24 Mg mg/g	31 P mg/g	39 K mg/g	43 Ca mg/g	52 Cr mcg/g	55 Mn mcg/g	56 Fe mg/g	63 Cu mg/g	66 Zn mg/g	78 Se mcg/g	27 Al mg/g	51 V mcg/g	59 Co mcg/g	60 Ni mcg/g	75 As mcg/g	95 Mo mcg/g
LT1	0	3.33± 1.11^{cC}	4.47± 0.19^{bB}	1.49± 0.82^{aB}		1.98± 0.10^{aB}	4.69± 1.07^{aA}	50.16± 2.66^{aB}	0.26± 0.01^{aA}	9.51± 0.40^{aA}	0.11± 0.01^{aAB}	3.02± 0.73^{aA}	0.22± 0.03^{aA}	0.60± 0.14^{aA}	0.27± 0.02^{aB}	1.09± 0.16^{aAB}	1.03± 0.37^{aA}	0.32± 0.05^{aA}
	60	3.59± 0.52 ^{cB}	4.71± 0.07^{bA}	1.61± 0.22^{aA}		2.11± 0.06^{aA}	4.42± 0.53^{aA}	51.92± 1.07^{aA}	0.28± 0.05^{aA}	9.46± 0.17^{aA}	0.11± 0.04^{aA}	2.45± 0.61^{aA}	0.22± 0.03^{aA}	0.60± 0.04^{aA}	0.28± 0.04^{aA}	2.16± 0.89^{aA}	1.07± 0.22^{aA}	0.31± 0.03^{aA}
	120	3.73± 0.55 ^{cA}	4.77± 0.11^{bA}	1.61± 0.33^{aA}		2.07± 0.09^{aA}	3.89± 0.28^{aAB}	51.60± 2.18^{aAB}	0.28± 0.03^{aA}	9.78± 0.91^{aA}	0.11± 0.05^{aB}	2.64± 0.53^{aA}	0.21± 0.04^{aA}	0.73± 0.21^{aA}	0.29± 0.03^{aAB}	0.89± 0.07^{aB}	1.18± 0.16^{aA}	0.31± 0.05^{aA}
	180	3.85± 0.68 ^{cA}	4.70± 0.13^{bA}	1.57± 0.65^{aA}		1.97± 0.02^{aAB}	3.26± 0.21^{aB}	48.61± 0.82^{aAB}	0.28± 0.02^{aA}	8.57± 0.80^{aA}	0.09± 0.03^{aC}	2.42± 1.03^{aA}	0.22± 0.05^{aA}	0.59± 0.13^{aA}	0.28± 0.04^{aAB}	0.66± 0.17^{aB}	1.16± 0.12^{aA}	0.26± 0.03^{aA}
LT2	0	3.33± 1.11^{abC}	4.47± 0.19^{abB}	1.49± 0.82^{aB}		1.98± 0.10^{aB}	4.69± 1.07^{aA}	50.16± 2.66	0.26± 0.01^{aA}	9.51± 0.40^{aA}	0.11± 0.01^{aAB}	3.02± 0.73^{aA}	0.22± 0.03^{aA}	0.60± 0.14^{aA}	0.27± 0.02^{aB}	1.09± 0.16^{aAB}	1.03± 0.37^{aA}	0.32± 0.05^{aA}
	60	3.75± 0.29^{abB}	4.97± 0.04^{abA}	1.69± 0.15^{aA}	7.26± 0.07^{aA}	2.18± 0.07^{aA}	5.19± 0.32^{aA}	52.81± 0.46^{aB}	0.27± 0.01^{aA}	9.36± 0.37^{aA}	0.13± 0.05^{aA}	3.87± 0.52^{aA}	0.23± 0.04^{aA}	0.61± 0.11^{aA}	0.31± 0.02^{aA}	1.15± 0.43^{aA}	1.13± 0.22^{aA}	0.34± 0.01^{aA}
	120	3.86± 0.85^{abA}	4.91± 0.10^{abA}	1.66± 0.56^{aA}	7.15± 0.18^{aA}	2.11± 0.07^{aA}			0.28± 0.03^{aA}	9.17± 0.27^{aA}	0.12± 0.04 ^{aB}	2.75± 0.60^{aA}	0.23± 0.04^{aA}	0.65± 0.06^{aA}	0.29± 0.01^{aAB}	1.01± 0.36^{aB}	1.17± 0.11^{aA}	0.31± 0.04^{aA}
	180	4.02± 0.69^{abA}	4.95± 0.10^{abA}	1.66± 0.44^{aA}	7.24± 0.16^{aA}	2.13± 0.08^{aAB}	3.80± 0.21^{aB}	52.32± 1.58^{aAB}	0.25± 0.03^{aA}	8.56± 0.38^{aA}	0.10± 0.02^{aC}	2.26± 0.23^{aA}	0.22± 0.04^{aA}	0.50± 0.04^{aA}	0.30± 0.04^{aAB}	0.79± 0.21^{aB}	0.99± 0.20^{aA}	0.26± 0.04^{aA}
AT3	0	3.33±1. 11^{bcC}	4.47± 0.19^{abB}	1.49± 0.82^{aB}	6.67± 0.41^{aB}	1.98± 0.10^{aB}	4.69± 1.07^{aA}	50.16± 2.66^{aB}	0.26± 0.01^{aA}	9.51± 0.40^{aA}	0.11± 0.01^{aAB}	3.02± 0.73^{aA}	0.22± 0.03^{aA}	0.60± 0.14^{aA}	0.27± 0.02^{aB}	1.09± 0.16^{aAB}	1.03± 0.37^{aA}	0.32± 0.05^{aA}
	60	3.76± 1.19^{bcB}	4.84± 0.17^{abA}	1.63± 0.45^{aA}	7.11± 0.25^{aA}	2.16± 0.15^{aA}	4.12± 0.42^{aA}	52.70± 0.43^{aA}	0.26± 0.01^{aA}	9.03± 0.11^{aA}	0.12± 0.01^{aA}	2.52± 0.54^{aA}	0.23± 0.01^{aA}	0.61± 0.09^{aA}	0.32± 0.05^{aA}	0.84± 0.02^{aA}	1.12± 0.20^{aA}	0.31± 0.02^{aA}
	120	3.84± 0.92^{bcA}	4.79± 0.18^{abA}	1.61± 1.04^{aA}	6.90± 0.30^{aB}	2.08± 0.22^{aA}	4.60± 0. 2^{aAB}	49.67± 1.72^{aAB}	0.27± 0.01^{aA}	8.62± 0.67^{aA}	0.10± 0.01^{aB}	3.06± 0.70^{aA}	0.27± 0.07^{aA}	0.53± 0.08^{aA}	0.30± 0.01^{aAB}	0.92± 0.17^{aB}	0.95± 0.17^{aA}	0.31± 0.02^{aA}
	180	3.8± 0.17^{bcA}	4.69± 0.14^{abA}	1.58± 0.62^{aA}	6.97± 0.20^{aA}	2.08± 0.20^{aAB}	3.10± 0.44^{aB}	49.72± 1.30^{aAB}	0.26± 0.01^{aA}	8.61± 0.50^{aA}	0.10± 0.01^{aC}	2.24± 1.17^{aA}		0.54± 0.06^{aA}	0.28± 0.01^{aAB}	1.03± 0.13^{aB}	0.74± 0.18^{aA}	0.30± 0.05^{aA}
HT4	0	3.33± 1.11^{aC}	4.47± 0.19^{aB}	1.49± 0.82^{aB}	6.67± 0.41^{aB}	1.98± 0.10^{aB}	4.69± 1.07^{aA}	50.16± 2.66^{aB}	0.26± 0.01^{aA}	9.51± 0.40^{aA}	0.11± 0.01^{aAB}	3.02± 0.73^{aA}	0.22± 0.03^{aA}	0.60± 0.14^{aA}	0.27± 0.02^{aB}		1.03± 0.37^{aA}	0.32± 0.05^{aA}
	60	3.7± 1.30^{aB}	4.82± 0.18^{aA}	1.65± 0.63^{aA}	6.95± 0.23^{aA}	2.21± 0.07^{aA}	4.29± 0.71^{aA}	52.22± 1.79^{aA}	0.29± 0.02^{aA}	10.05± 2.12^{aA}	0.12± 0.01^{aA}	2.35± 0.78^{aA}	0.24± 0.04^{aA}	0.53± 0.06^{aA}	0.31± 0.05^{aA}		1.15± 0.10^{aA}	0.33± 0.04^{aA}
	120	4.02± 1.14^{aA}	5.01± 0.13^{aA}	1.69± 0.37^{aA}	7.24± 0.17^{aA}	2.18± 0.10^{aA}	3.73± 0.24^{aAB}	52.56± 2.08^{aAB}	0.27± 0.01^{aA}	8.77± 0.23^{aA}	0.10± 0.00^{a}	2.84± 0.34^{aA}	0.22± 0.02^{aA}	0.62± 0.13^{aA}	0.32± 0.02^{aAB}		1.06± 0.25^{aA}	0.33± 0.03^{aA}
	180	4.1± 1.29^{aA}	5.02± 0.16^{aA}	1.66± 0.52^{aA}	7.37± 0.24^{aA}	2.06± 0.06^{aAB}	3.47± 0.36^{aB}	51.69± 2.58^{aAB}	0.27± 0.01^{aA}	9.01± 0.40^{aA}	0.10± 0.00^{aC}	2.77± 0.84^{aA}	0.24± 0.03^{aA}	0.51± 0.05^{aA}	0.31± 0.01^{aAB}		1.11± 0.22^{aA}	0.33± 0.06^{aA}
P- value	T	**.001**	**.014**	.070	.178	.360	.494	.305	.716	.382	.267	.677	.476	.490	.309		.443	.430
	D	**.000**	**.000**	**.000**	**.001**	**.002**	**.000**	**.037**	.361	.356	**.000**	.237	.796	.236	**.019**		.636	.179
	I	**.037**	.427	.920	.651	.961	.668	.582	.908	.421	.315	.432	.949	.837	.838		.859	.774

Statistical differences (Tukey's, P< 0.05) in means are given by changed letters: changed small letters 'a - c' in similar column denote difference is significant (P < 0.05) among temperature conditions; changed Capital letters 'A-D' in similar column denote difference is significant (P< 0.05) among feed storage durations.

Temperature (T), Duration (D), Interaction of Temperature*Duration (I). # P-value for between subject effects; **Significant P-values P< 0.05 in highlight.**

Table 4.6b.1): Elemental profile 'micro-element' changes during storage aquafeed 2.

Temperature	Storage days	Micro-elements (conc.) Atomic Mass / Symbol																
		7 Li mcg/g	9 Be mcg/g	11 B mcg/g	45 Sc mcg/g	47 Ti mcg/g	71 Ga mcg/g	72 Ge mcg/g	85 Rb mcg/g	88 Sr mg/g	90 Zr mcg/g	93 Nb mcg/g	101Ru mcg/g	103 Rh mcg/g	105 Pd mcg/g	107 Ag mcg/g	111 Cd mcg/g	115 In mcg/g	133 Cs mcg/g
LT1	0	0.49± 0.01	0.024± 0.007	10.26± 0.86	0.71± 0.18	24.13± 5.82	0.07± 0.02	0.07± 0.02	5.73± 0.49	96.41± 6.32	0.19± 0.04	0.06± 0.02	0.01± 0.00	0.004± 0.001	0.21± 0.05	1.88± 1.79	0.07± 0.05	0.004± 0.004	0.09± 0.01
	60	0.50± 0.02	0.016± 0.016	13.13± 1.73	1.17± 0.25	23.16± 9.41	0.09± 0.02	0.06±0. 00	5.87± 0.36	99.64± 3.84	0.13± 0.02	0.09± 0.06	0.01± 0.00	0.002± 0.002	0.20± 0.03	0.54± 0.00	0.07± 0.01	0.007± 0.002	0.05± 0.01
	120	0.37± 0.02	0.010± 0.007	10.65± 0.23	1.72± 0.07	13.52± 2.15	0.05± 0.03	N.D.	5.56± 0.15	93.69± 2.22	0.07± 0.01	0.05± 0.03	0.01± 0.00	0.003± 0.00	0.17± 0.04	N.D.	0.05± 0.01	0.005± 0.000	0.04± 0.01
	180	0.38± 0.04	0.022± 0.006	9.71± 0.36	1.42± 0.14	13.04± 1.88	0.04± 0.02	0.05±0. 03	5.52± 0.22		0.19± 0.25	0.07± 0.05	0.01± 0.00	0.002± 0.00	0.15± 0.02	N.D.	0.03± 0.04	0.001± 0.002	0.04± 0.02
LT2	0	0.49± 0.01	0.024± 0.007	10.26± 0.86	0.71± 0.18	24.13± 5.82	0.07± 0.02	0.07± 0.01	5.73± 0.49		0.19± 0.04	0.06± 0.02	0.01± 0.00	0.004± 0.001	0.21± 0.05	1.88± 1.79	0.07± 0.05	0.004± 0.004	0.09± 0.01
	60	0.47± 0.01	0.019± 0.007	11.28± 0.32	1.03± 0.21	18.05± 1.74	0.09± 0.01	0.02±0. 00	5.94± 0.40		0.14± 0.03	0.05± 0.01	0.01± 0.00	0.003± 0.003	0.19± 0.03	0.23± 0.00	0.06± 0.08	0.003± 0.000	0.05± 0.01
	120	0.38± 0.03				16.72± 2.57	0.06± 0.00	N.D.	5.55± 0.24		0.18± 0.07	0.06± 0.02	0.01± 0.00					0.003± 0.003	0.07± 0.03
	180	0.39± 0.02	0.013± 0.010	9.37± 0.44		16.22± 2.12	0.07± 0.01	0.04±0. 00	5.64± 0.10		0.07± 0.02	0.01± 0.01	0.01± 0.00	0.003± 0.003	0.14± 0.02	N.D.	0.02± 0.01	0.003± 0.003	0.05± 0.00
AT3	0	0.49± 0.01	0.024± 0.007	10.26± 0.86		24.13± 5.82	0.07± 0.02	0.07± 0.01	5.73± 0.49		0.19± 0.04	0.06± 0.02	0.01± 0.00	0.004± 0.001	0.21± 0.05	1.88± 1.79	0.07± 0.05	0.004± 0.004	0.09± 0.01
	60	0.38± 0.07	0.018± 0.005	12.80± 1.61		16.28± 2.90	0.12± 0.06	0.06± 0.00	5.73± 0.02	98.36± 3.90	0.12± 0.03	0.04± 0.02	0.01± 0.00	0.003± 0.00	0.18± 0.05	N.D.	0.07± 0.03	0.002± 0.002	0.05± 0.01
	120	0.38± 0.01	0.015± 0.002	10.06± 0.87		14.13± 1.44	0.05± 0.02	0.58± 0.00	5.26± 0.25	93.68± 6.44	0.08± 0.03	0.02± 0.01	0.02± 0.00	0.004± 0.001	0.16± 0.03	N.D.	0.03± 0.02	0.001± 0.002	0.03± 0.01
	180	0.32± 0.02	0.016± 0.009	8.59± 0.19		13.55± 2.15	0.06± 0.02	0.03± 0.01	5.75± 0.36	93.57± 6.88	0.05± 0.02	0.01± 0.01	0.01± 0.00	0.004± 0.004	0.15± 0.02	N.D.	N.D.	0.002± 0.002	0.03± 0.01
HT4	0	0.49± 0.01	0.024± 0.007	10.26± 0.86		24.13± 5.82	0.07± 0.02	0.07± 0.00	5.73± 0.49	96.41± 6.32	0.19± 0.04	0.06± 0.02	0.01± 0.00	0.004± 0.001	0.21± 0.05	1.88± 1.79	0.07± 0.05	0.004± 0.004	0.09± 0.01
	60	0.40± 0.02	0.018± 0.009	11.69± 0.42	1.33± 0.09	14.32± 1.02	0.06± 0.02	0.06± 0.04	5.55± 0.18	97.78± 0.95	0.10± 0.04	0.02± 0.00	0.01± 0.00	0.002± 0.002	0.18± 0.02	0.70± 0.78	0.05± 0.03	0.009± 0.010	0.05± 0.01
	120	0.40± 0.01	0.008± 0.005	9.93± 0.26	1.55± 0.07	14.63± 0.86	0.08± 0.03	0.02± 0.00	5.71± 0.34	95.93± 1.23	0.13± 0.03	0.06± 0.01	0.01± 0.00	0.003± 0.003	0.18± 0.01	N.D.	0.02± 0.02	0.002± 0.002	0.04± 0.02
	180	0.35± 0.02	0.015± 0.006	8.95± 0.59	1.47± 0.35	15.80± 2.88	0.09± 0.04	0.03± 0.01	5.71± 0.31	96.21± 3.00	0.09± 0.02	0.01± 0.01	0.00	0.002± 0.002	0.18± 0.04	N.D.	0.07± 0.03	0.001± 0.002	0.04± 0.01
P-value	T	**.000**	.817	.148	.810	.654	.582	**.000**	.910	.406	.418	.937	.553	.601	.716	.998	.754	.535	.986
	D	**.000**	**.002**	**.000**	**.000**	.000	**.044**	**.000**	.302	**.016**	.494	**.017**	.312	.168	**.004**	.439	.115	.136	**.013**
	I	**.000**	.945	.401	.341	.515	.126	**.000**	.779	.873	.345	1.000	.516	.994	.904	.979	.892	.534	1.000

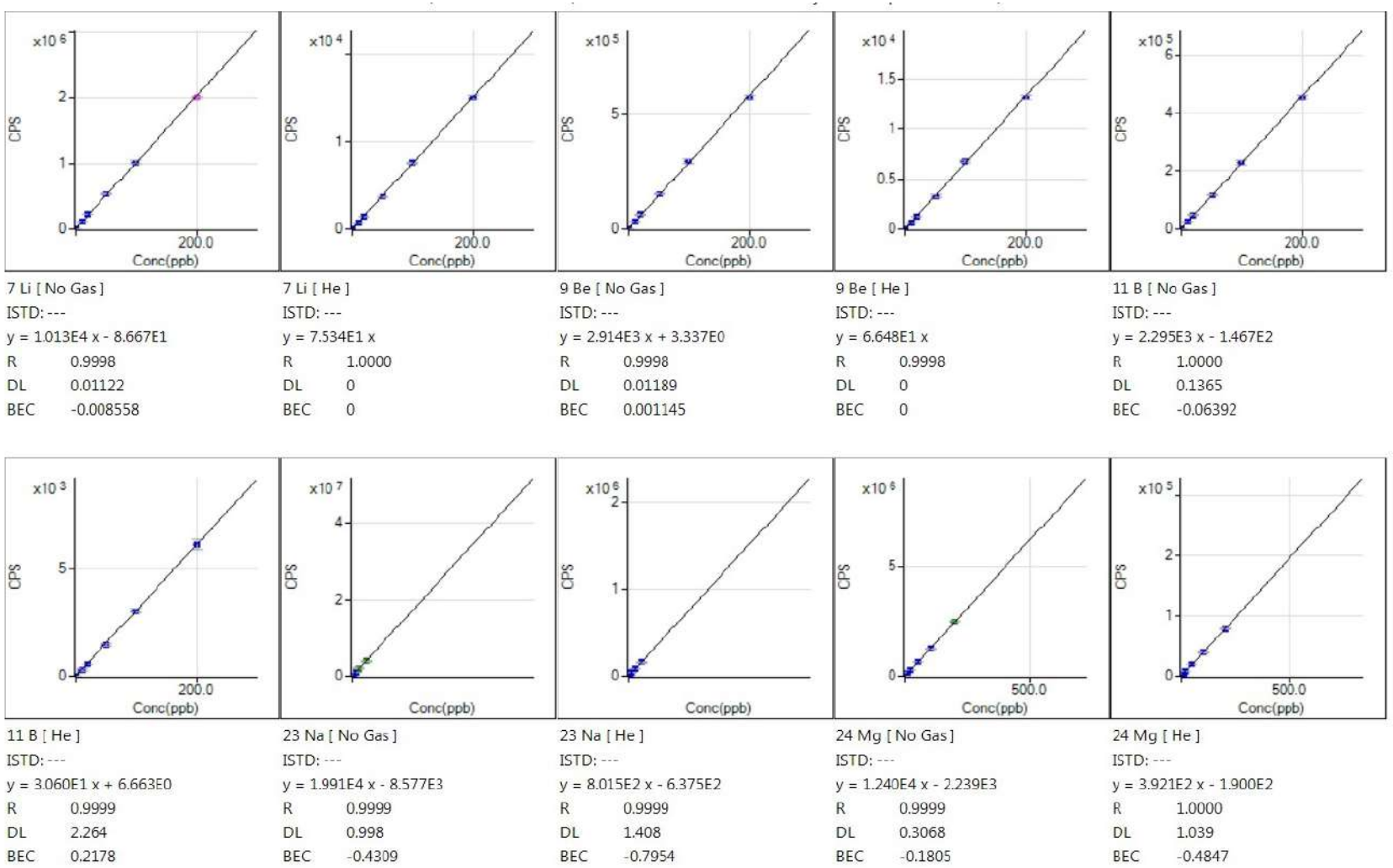
P-value for between subject effects; **Significant P-values P< 0.05 in highlight** # Temperature (T), Duration (D), Interaction of Temperature*Duration (I).

Table 4.6b.2): Elemental profile 'micro-element' changes during storage aquafeed 2.

Temperature	Storage duration (days)	Micro-elements (conc.)							
		Atomic Mass / Symbol							
		137 Ba mcg/g	178 Hf mcg/g	181 Ta mcg/g	182 W mcg/g	185 Re mcg/g	195 Pt mcg/g	197 Au mcg/g	209 Bi mcg/g
LT1	0	3.22± 0.04	0.005±0.003	0.017±0.005	0.08±0.03	0.019±0.000	0.028±0.002	0.76±0.32	0.07±0.02
	60	3.31±0.34	0.004±0.001	0.005±0.003	0.03±0.01	0.018±0.006	0.026±0.013	0.51±0.10	0.01±0.01
	120	3.43±0.23	0.000	0.003±0.000	0.01±0.00	0.018±0.013	0.013±0.007	0.21±0.03	N.D.
	180	3.42±0.41	0.009±0.008	0.004±0.001	N.D.	0.022±0.004	0.009±0.001	0.10±0.02	N.D.
LT2	0	3.22±0.04	0.005±0.003	0.017±0.005	0.08±0.03	0.019±0.000	0.028±0.002	0.76±0.32	0.07±0.02
	60	3.55±0.63	0.005±0.003	0.005±0.004	0.04±0.01	0.008±0.005	0.026±0.011	0.54±0.32	N.D.
	120	3.15±0.20	0.018±0.02	0.005±0.000	0.02±0.01	0.026±0.009	0.010±0.002	0.19±0.05	0.01±0.00
	180	3.45±0.22	0.003±0.000	0.002±0.002	N.D.	0.025±0.008	0.004±0.001	0.09±0.03	0.00
AT3	0	3.22±0.04	0.005±0.0003	0.017±0.005	0.08±0.03	0.019±0.000	0.028±0.002	0.76±0.32	0.07±0.02
	60	3.18±0.27	0.025±0.023	0.002±0.002	0.03±0.02	0.025±0.005	0.013±0.005	0.30±0.11	0.00
	120	2.89±0.09	0.001±0.00	0.009±0.006	0.07±0.01	0.022±0.008	0.014±0.005	0.24±0.14	0.09±0.00
	180	2.92±0.14	0.002±0.002	0.002±0.002	0.02±0.00	0.028±0.006	0.008±0.005	0.09±0.06	N.D.
HT4	0	3.22±0.04	0.005±0.003	0.017±0.005	0.08±0.03	0.019±0.000	0.028±0.002	0.76±0.32	0.07±0.02
	60	3.54±0.17	0.004±0.001	0.002±0.002	0.01±0.01	0.017±0.004	0.008±0.003	0.17±0.02	0.04±0.00
	120	2.82±0.28	0.002±0.002	0.005±0.000	0.01±0.00	0.023±0.007	0.009±0.001	0.13±0.01	N.D.
	180	3.14±0.58	0.004±0.004	0.004± 0.001	0.01±0.01	0.031±0.003	0.004±0.001	0.05±0.02	N.D.
P-value	T	.055	.624	.370	.432	.508	**.044**	.404	.199
	D	.082	.577	.708	**.000**	**.001**	**.000**	**.000**	**.009**
	I	.398	.103	.829	.617	.482	.051	.793	.121

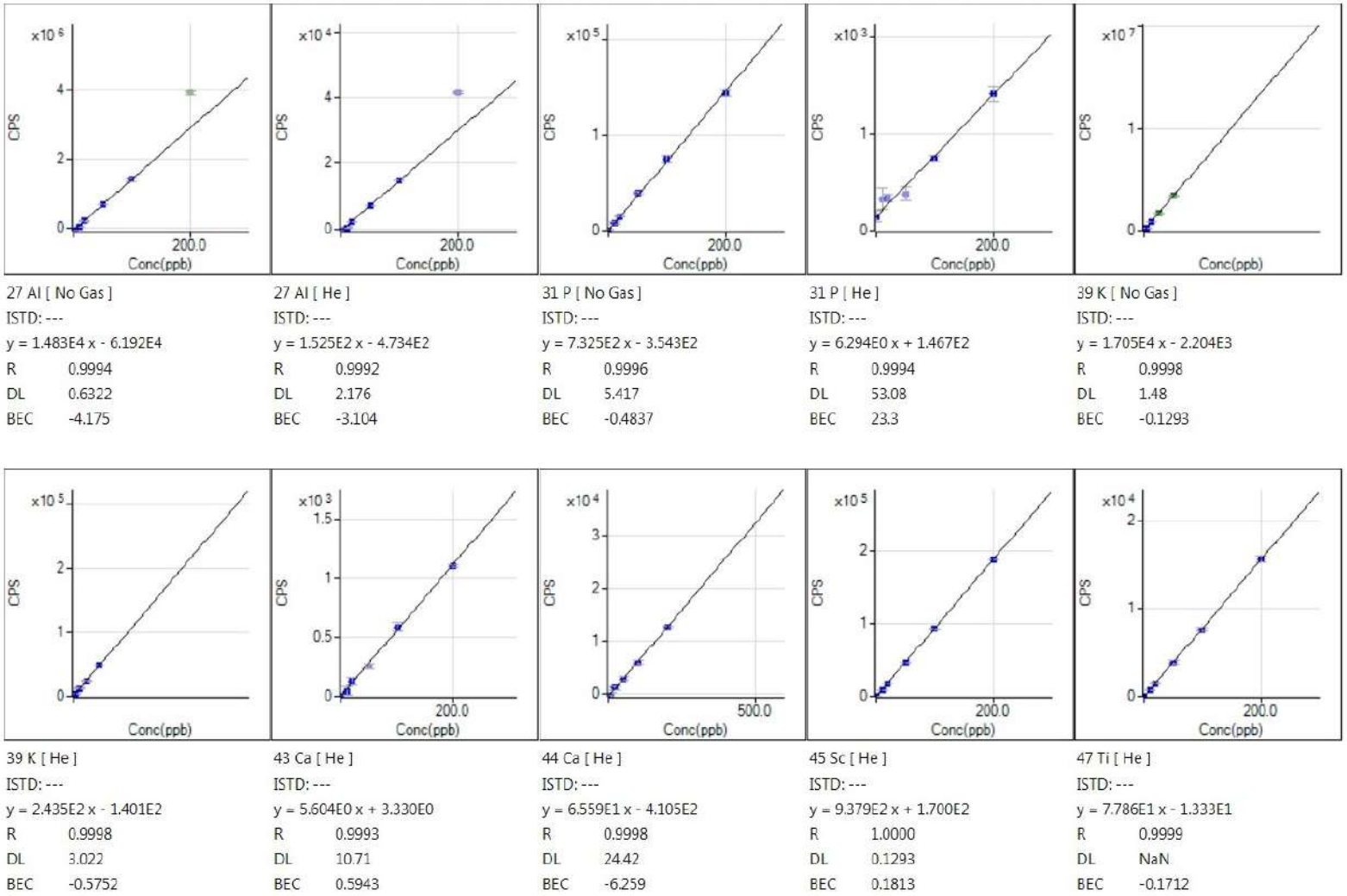
P-value for between subject effects; **Significant P-values P< 0.05 in highlight.** # Temperature (T), Duration (D), Interaction of Temperature*Duration (I).

Figure 4.6 a): ICP-MS calibration graphs of elements



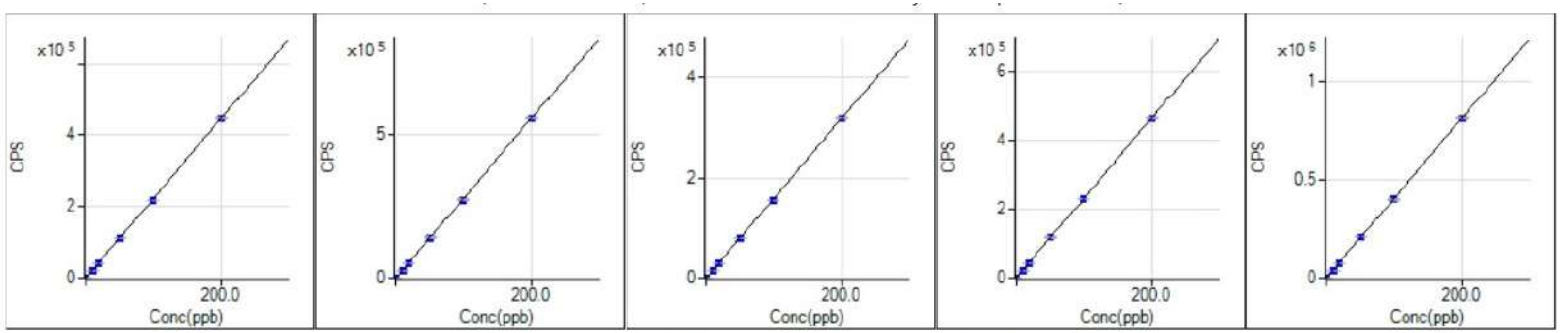
Source: Author (2023)

Figure 4.6 b) ICP-MS calibration graphs of elements



Source: Author (2023)

Figure 4.6 c): ICP-MS calibration graphs of elements



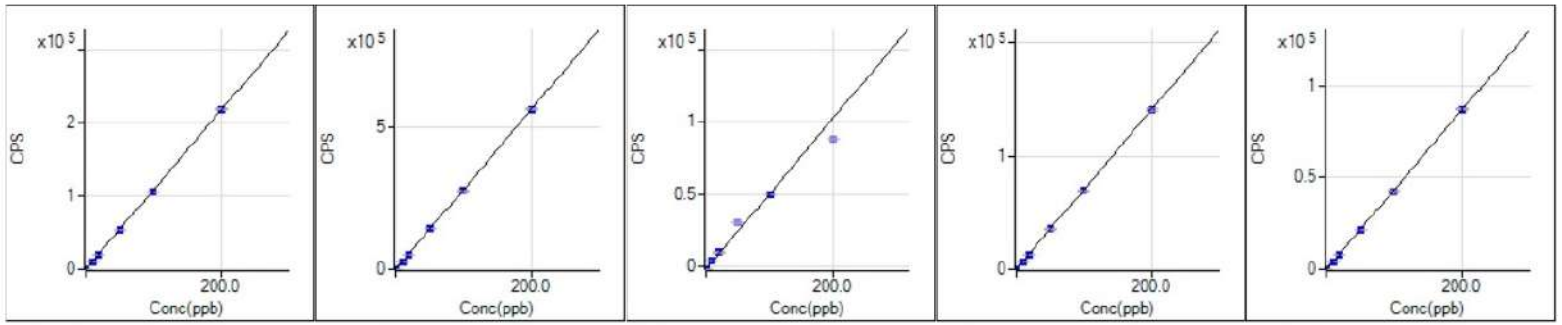
51 V [He]
ISTD: ---
y = 2.228E3 x + 1.667E1
R 0.9999
DL 0.02057
BEC 0.007482

52 Cr [He]
ISTD: ---
y = 2.769E3 x - 7.000E1
R 0.9999
DL 0.1067
BEC -0.02528

55 Mn [He]
ISTD: ---
y = 1.591E3 x - 5.667E1
R 0.9999
DL 0.08639
BEC -0.03561

56 Fe [He]
ISTD: ---
y = 2.327E3 x - 8.271E2
R 0.9999
DL 0.0846
BEC -0.3554

59 Co [He]
ISTD: ---
y = 4.059E3 x - 6.663E0
R 0.9999
DL 0.004267
BEC -0.001642



60 Ni [He]
ISTD: ---
y = 1.088E3 x - 5.334E1
R 0.9999
DL 0.1361
BEC -0.04904

63 Cu [He]
ISTD: ---
y = 2.797E3 x - 5.001E1
R 0.9999
DL 0.1218
BEC -0.01788

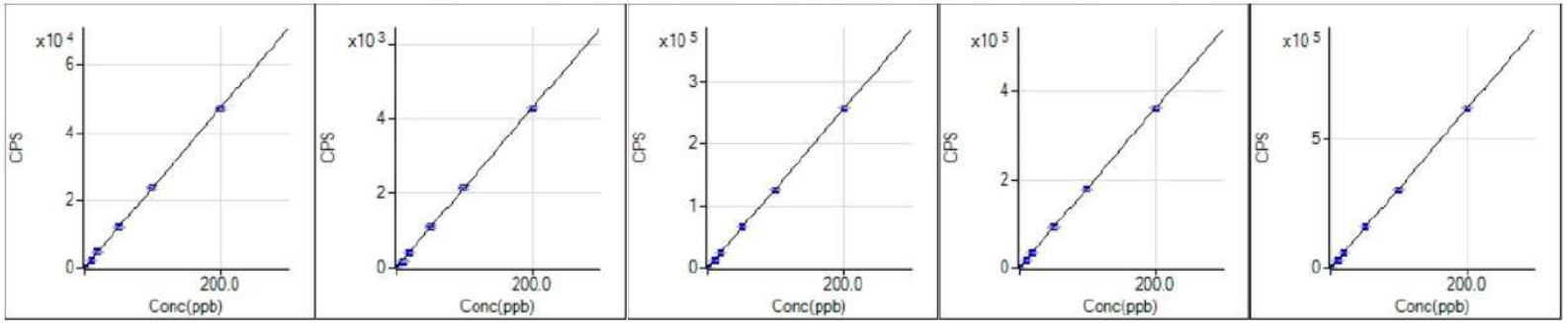
66 Zn [He]
ISTD: ---
y = 5.209E2 x - 1.627E3
R 0.9997
DL 4.068
BEC -3.123

71 Ga [He]
ISTD: ---
y = 7.033E2 x
R 0.9999
DL 0
BEC 0

72 Ge [He]
ISTD: ---
y = 4.343E2 x + 6.667E0
R 0.9999
DL 0.03988
BEC 0.01535

Source: Author (2023)

Figure 4.6 d): ICP-MS calibration graphs of elements



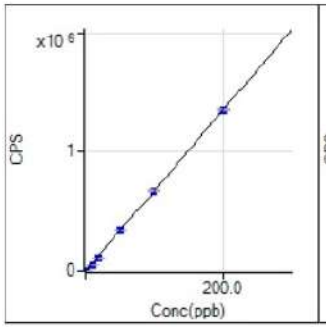
75 As [He]
ISTD: ---
y = 2.366E2 x + 6.670E0
R 1.0000
DL 1.512E-09
BEC 0.02819

78 Se [He]
ISTD: ---
y = 2.138E1 x - 3.333E-3
R 0.9999
DL 0.4679
BEC -0.0001559

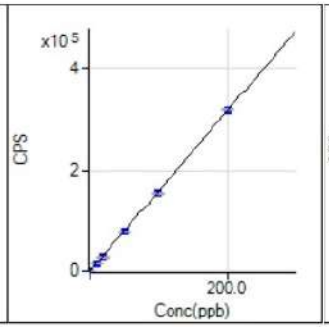
85 Rb [He]
ISTD: ---
y = 1.287E3 x + 1.000E1
R 0.9998
DL 0.04661
BEC 0.007769

88 Sr [He]
ISTD: ---
y = 1.806E3 x + 1.000E-2
R 0.9999
DL 0.07853
BEC 5.538E-06

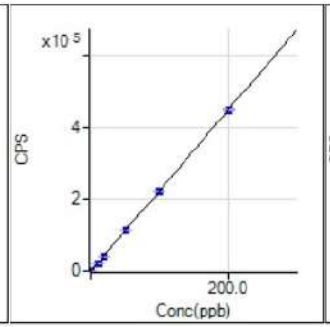
90 Zr [He]
ISTD: ---
y = 3.105E3 x + 3.333E0
R 0.9999
DL 0.01476
BEC 0.001073



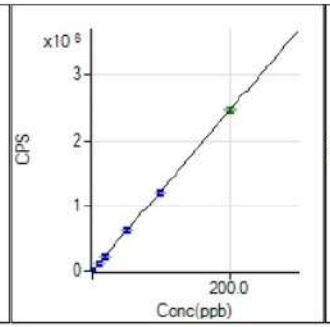
93 Nb [He]
ISTD: ---
y = 6.775E3 x + 2.000E1
R 0.9998
DL 0.006764
BEC 0.002952



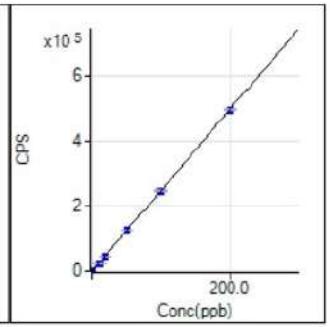
95 Mo [He]
ISTD: ---
y = 1.587E3 x + 1.667E1
R 0.9999
DL 0.01891
BEC 0.01051



101 Ru [He]
ISTD: ---
y = 2.245E3 x - 6.663E0
R 0.9999
DL 0.007713
BEC -0.002967



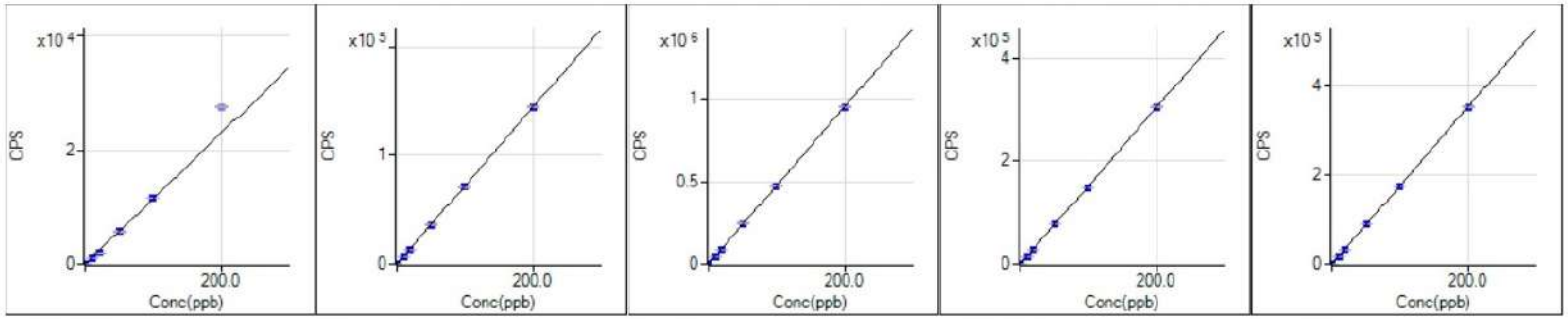
103 Rh [He]
ISTD: ---
y = 1.230E4 x
R 0.9998
DL 0
BEC 0



105 Pd [He]
ISTD: ---
y = 2.482E3 x - 1.000E1
R 0.9999
DL 0
BEC -0.004029

Source: Author (2023)

Figure 4.6 e): ICP-MS calibration graphs of elements



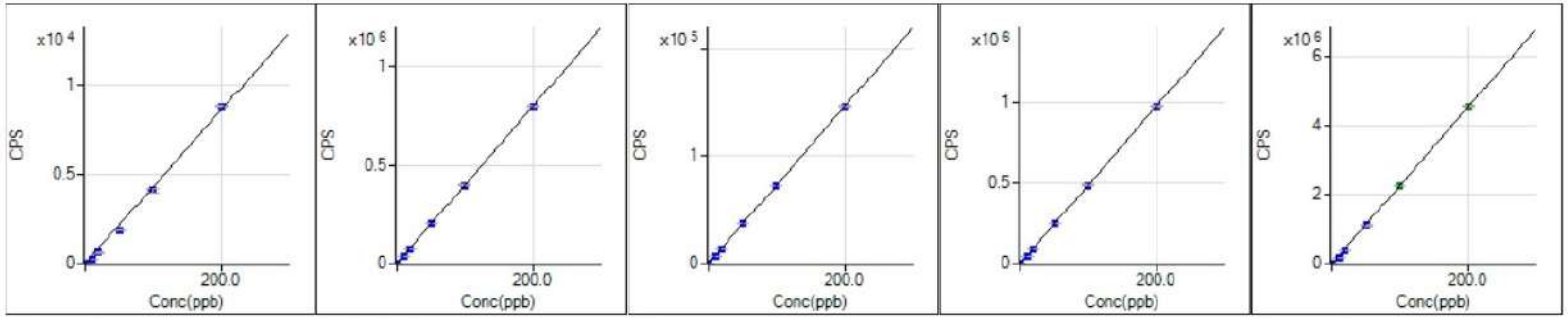
107 Ag [He]
ISTD: ---
y = 1.147E2 x + 3.333E-3
R 0.9994
DL 0.3997
BEC 2.907E-05

111 Cd [He]
ISTD: ---
y = 7.200E2 x + 1.666E1
R 0.9999
DL 0.1049
BEC 0.02314

115 In [He]
ISTD: ---
y = 4.762E3 x
R 0.9999
DL 0
BEC 0

118 Sn [He]
ISTD: ---
y = 1.521E3 x + 6.660E0
R 0.9999
DL 0.05695
BEC 0.004379

121 Sb [He]
ISTD: ---
y = 1.756E3 x - 1.067E2
R 0.9999
DL 0.07111
BEC -0.06073



125 Te [He]
ISTD: ---
y = 4.321E1 x - 6.670E0
R 0.9990
DL 8.276E-09
BEC -0.1543

133 Cs [He]
ISTD: ---
y = 3.993E3 x + 6.663E0
R 0.9999
DL 0.008676
BEC 0.001669

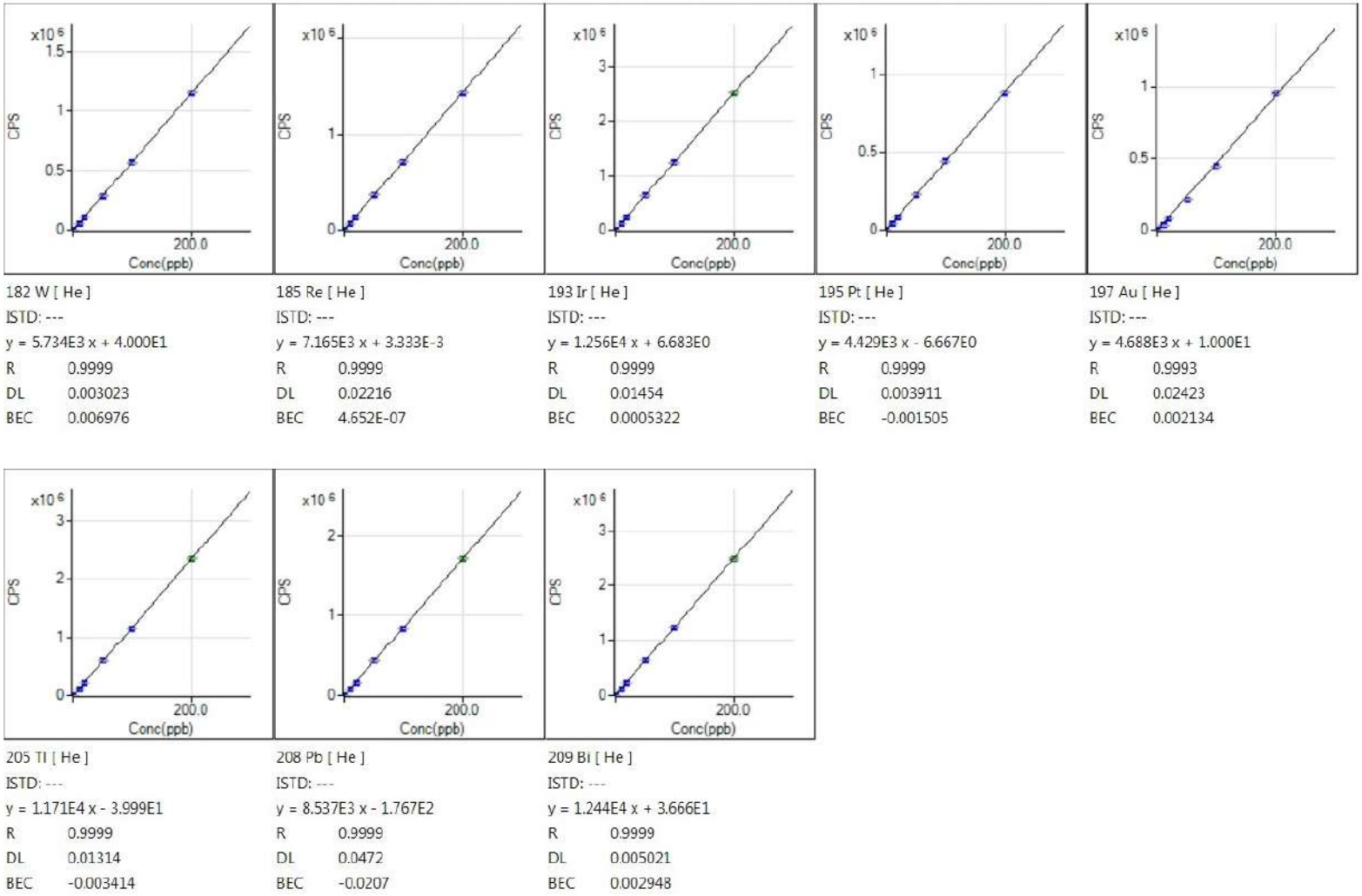
137 Ba [He]
ISTD: ---
y = 7.316E2 x + 2.333E1
R 0.9999
DL 0.1253
BEC 0.03188

178 Hf [He]
ISTD: ---
y = 4.867E3 x - 3.333E-3
R 0.9999
DL 0.007118
BEC -6.849E-07

181 Ta [He]
ISTD: ---
y = 2.267E4 x - 3.333E0
R 0.9999
DL 0.006619
BEC -0.0001471

Source: Author (2023)

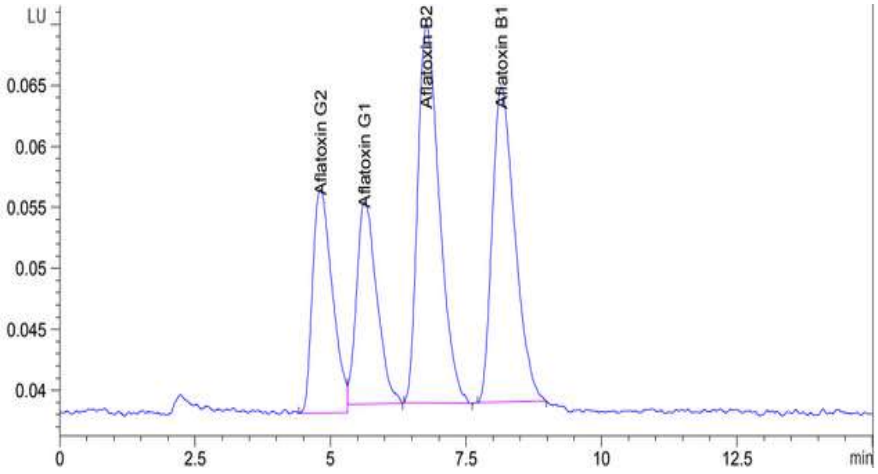
Figure 4.6 f): ICP-MS calibration graphs of elements



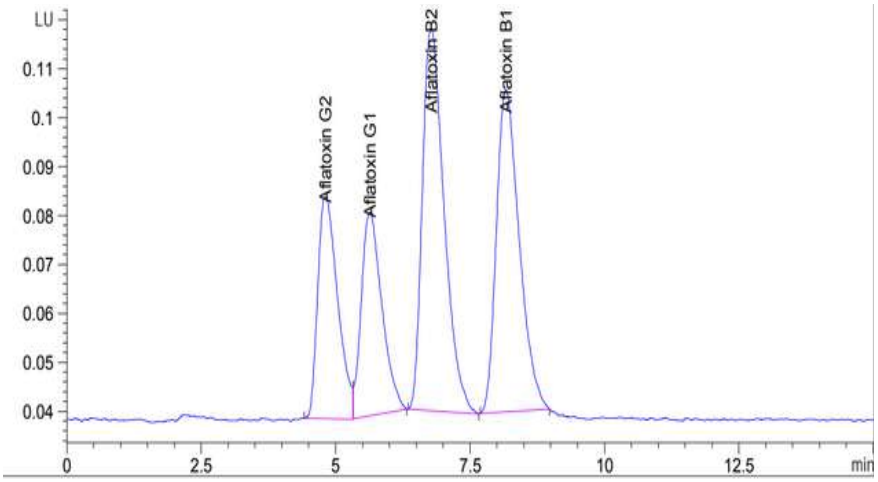
Source: Author (2023)

4.7 Aflatoxin analysis

All samples were found below limit of quantification (BLOQ < 0.5) for aflatoxins B1, B2, G1 and G2 and below quantitation limits BLOQ < 2.0, for total aflatoxins (summation of B1, B2, G1, and G2). Hence no incidences of aflatoxins were reported in feeds over storage temperature and duration regime. Figure 4.7(a), (b) depicts aflatoxin standard at concentrations 2ppb, 5ppb.



(a)



(b)

Figure 4.7: Aflatoxin standard concentrations (a) 2ppb, (b) 5ppb

Source: Author, (2023)

4.8 FTIR Spectra analysis

FTIR analysis was performed in transmission mode in the spectral range from 4000 to 400 cm^{-1} at spectrum resolution of 1cm^{-1}.The following results represent and elaborate the potential of spectroscopic analysis in formulated compounded diet. The results are compared with biochemical results for storage changes of lipids, proteins, carbohydrates and moisture in both feeds. Three characteristic spectral regions 1(

moisture between 3700 - et al., 2019; Ratti et al., 2023; Jáuregui-López et al., 2020).

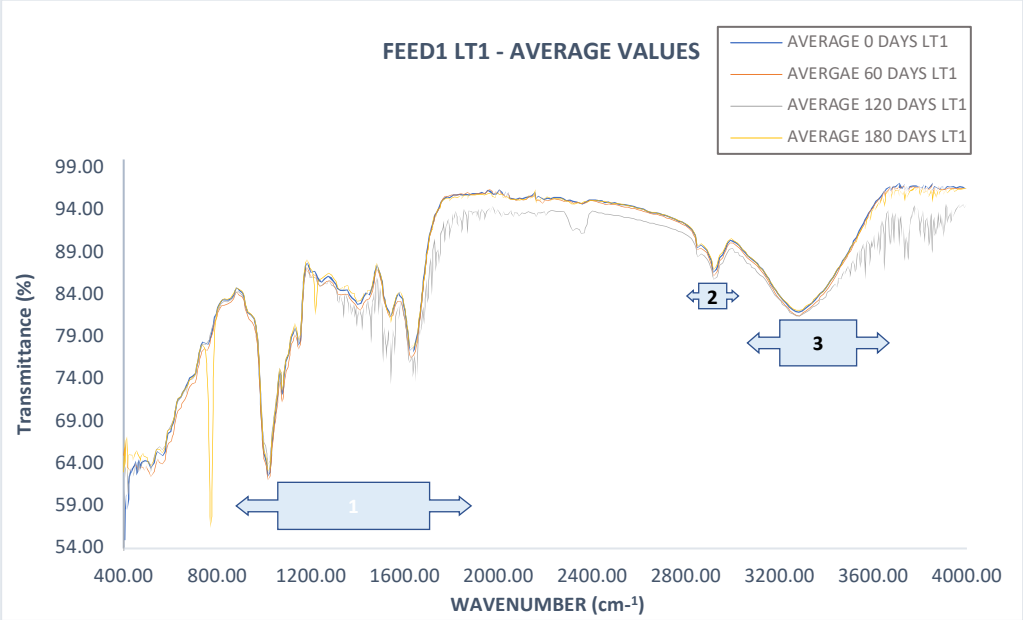
Vibrational spectrum is complex with overlapping vibrational bands for different groups. For example, 1790 - 900 cm^{-1} (spectral region of overlap for spectra arising from carbohydrates, protein and lipids. Spectral overlaps for lipids (comprising 3000 - -1700 cm^{-1}) and water (3700 - cm^{-1}) are at intensities covering at all three regions considered.

Characteristic IR peaks obtained for proteins are and at 1541 cm^{-1} as amide II; related bonds (CO-NH) associated with primary structure information of proteins. ~1457 cm^{-1} arising from protein side chains (Zarantoniello et al. 2020; Hernández-Martínez et al., 2013). Peaks at ~1080 cm^{-1} due to OH- stretching of carbohydrates (corresponding to 1078.21 cm^{-1} obtained in feed1), 3300 to 2500 cm^{-1} O-H stretching of carboxylate. Regions for fats and oils 3000 of carbonyls of triglycerides (TGAs) (Andrade et al., 2019; Upadhyay et al., 2018).

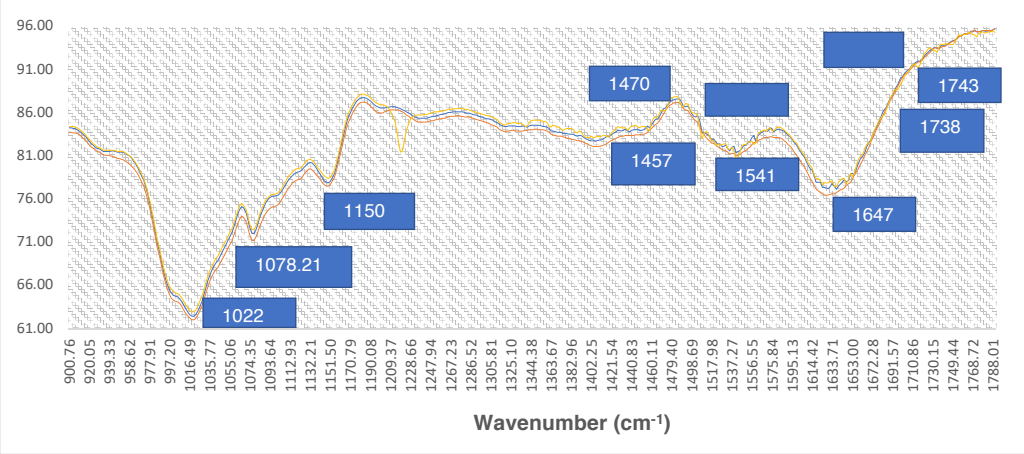
Strong bands for lipids at 2925 and 2853 cm^{-1} (C-H stretching of CH_2 groups). Oxidation status of lipids due to free fatty acids FFAs (1710 cm^{-1}) and triglycerides (1743 cm^{-1}, carbonyl stretching of ester, aldehyde, ketone) (Sherazi et al., 2007).

O-H stretch peaking at 3360 cm^{-1} and 1640 cm^{-1} relate to in diets (Nesakumar et al., 2018). Moisture and related starch retrogradation during storage (specifically in diet 2), figures 4.9(A)-(D) is associated to FTIR characteristic bands arising at 928, 9

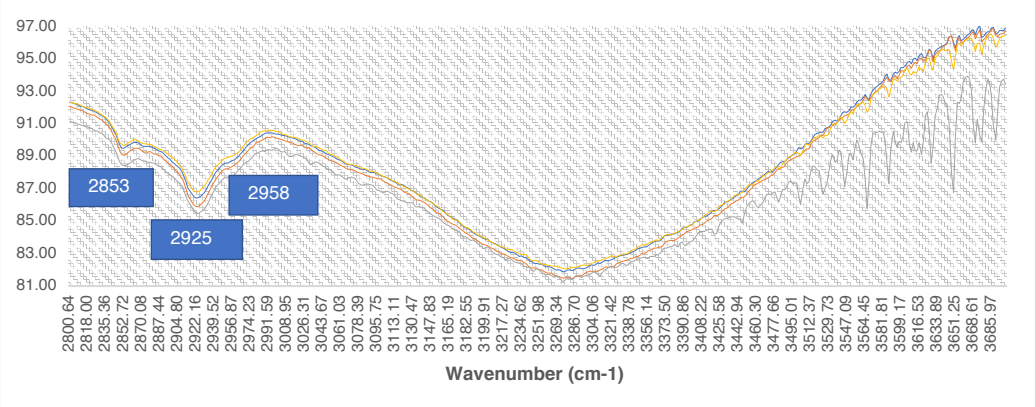
ds mainly due to the vibrational modes of molecule in retrogradation of starch. Peaks around 1600 cm^{-1} are attributed to amorphous starch; while that at 1022 cm^{-1} is delicate to changes in crystalline structure of starch.1506 cm^{-1} band is because of skeletal mode vibrational spectrum of 1,4 linkage (C–O–C) in amylose and amylopectin of starch. Skeletal mode vibrational spectra are known to arise from strongly linked stretching/ bending movements of atoms in straight, branched chains.



(A)



(A.1)



(A.2)

Figure 4.8(A):

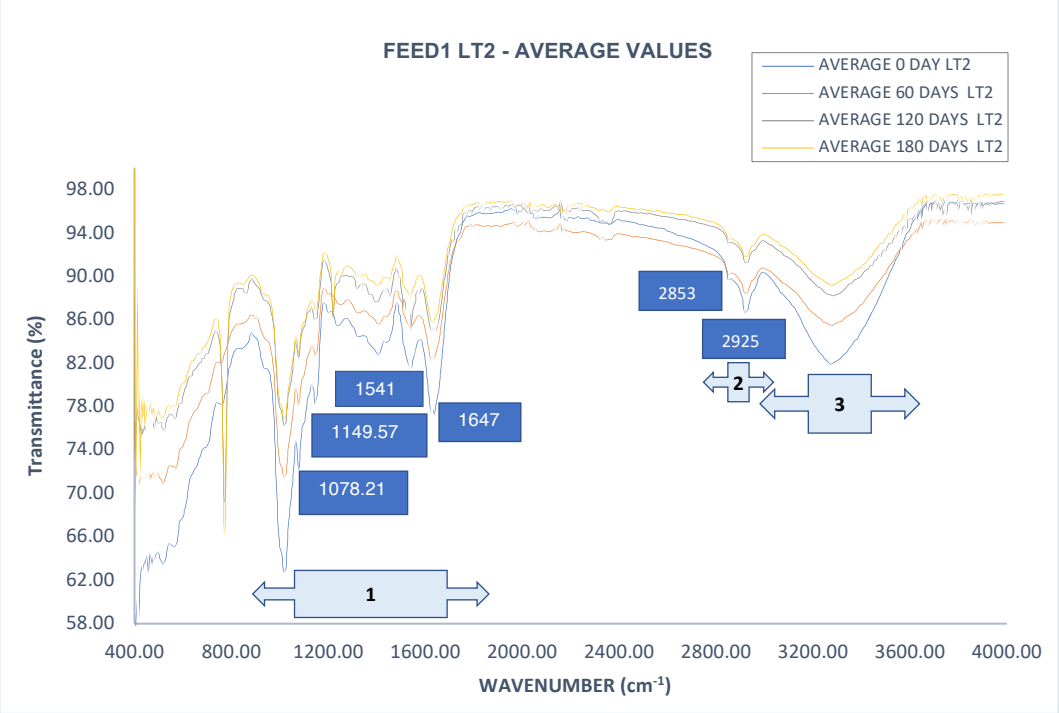


Figure 4.8(B): **. Source:** Author (2023)

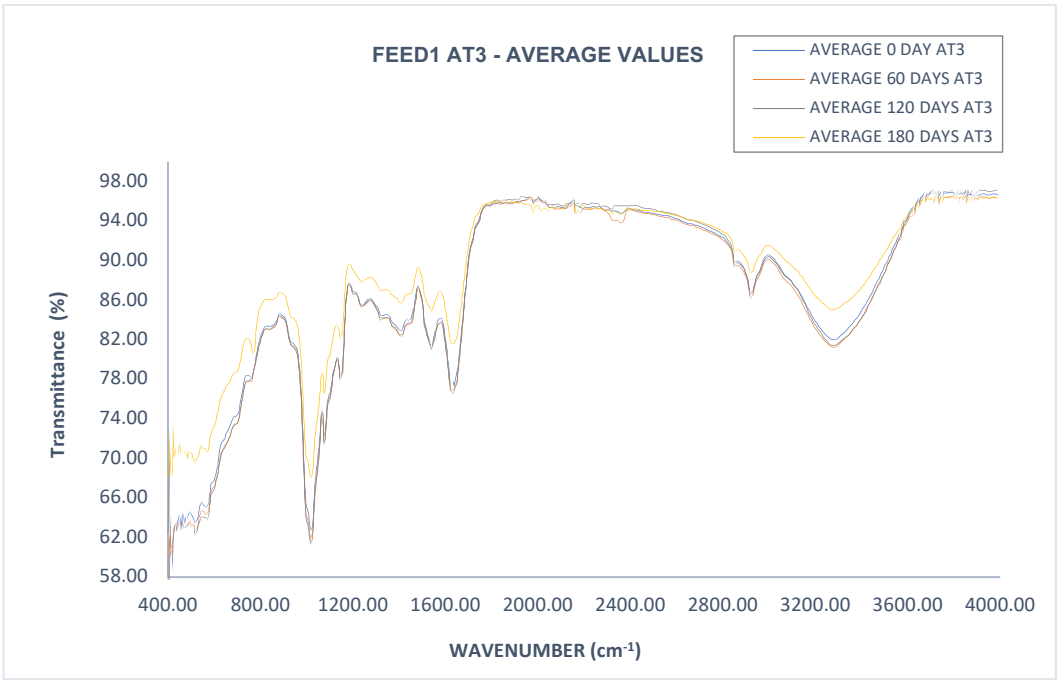


Figure 4.8(C): FTIR spectra feed 1 **storage. Source:** Author (2023)

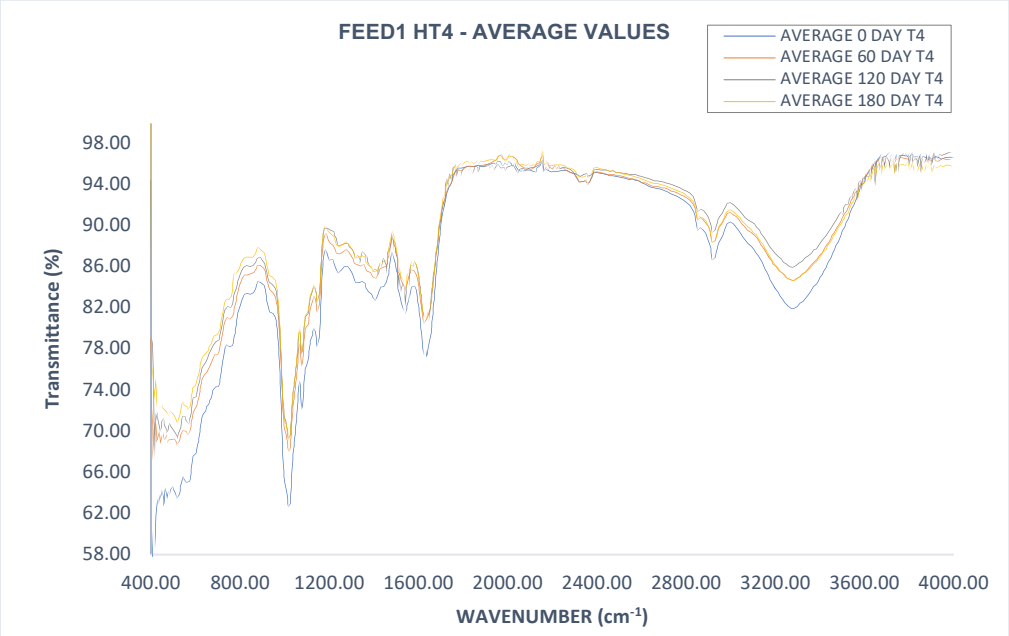


Figure 4.8(D): FTIR spectra feed 1 at high temperature storage. Source: (Author, 2023)

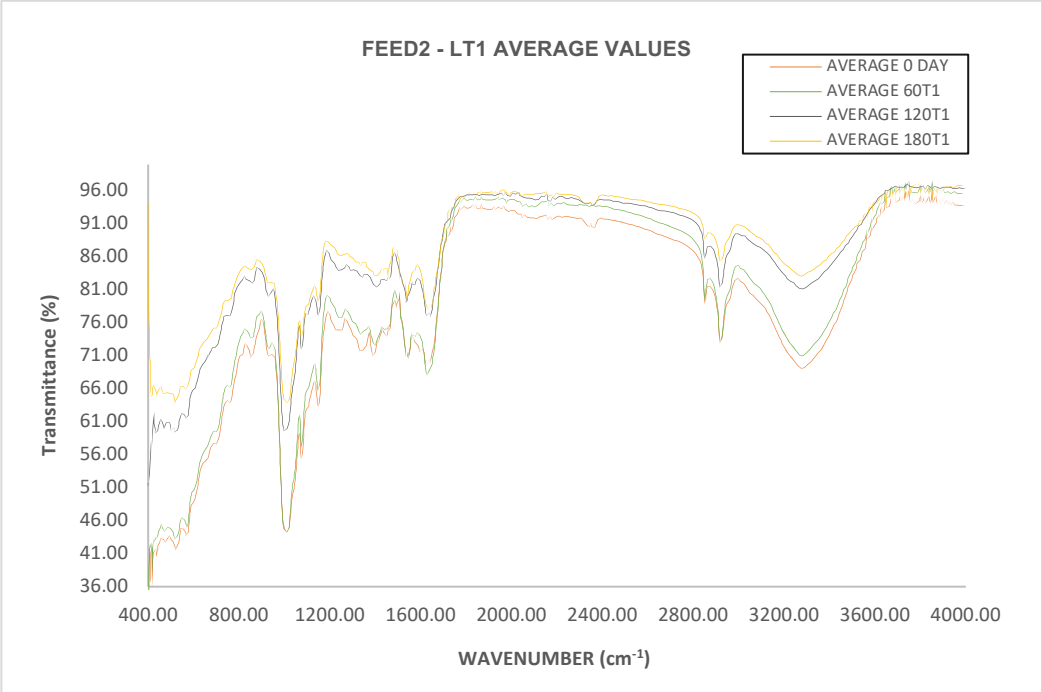


Figure 4.9(A): FTIR spectra feed 2 at - . Source: (Author, 2023)

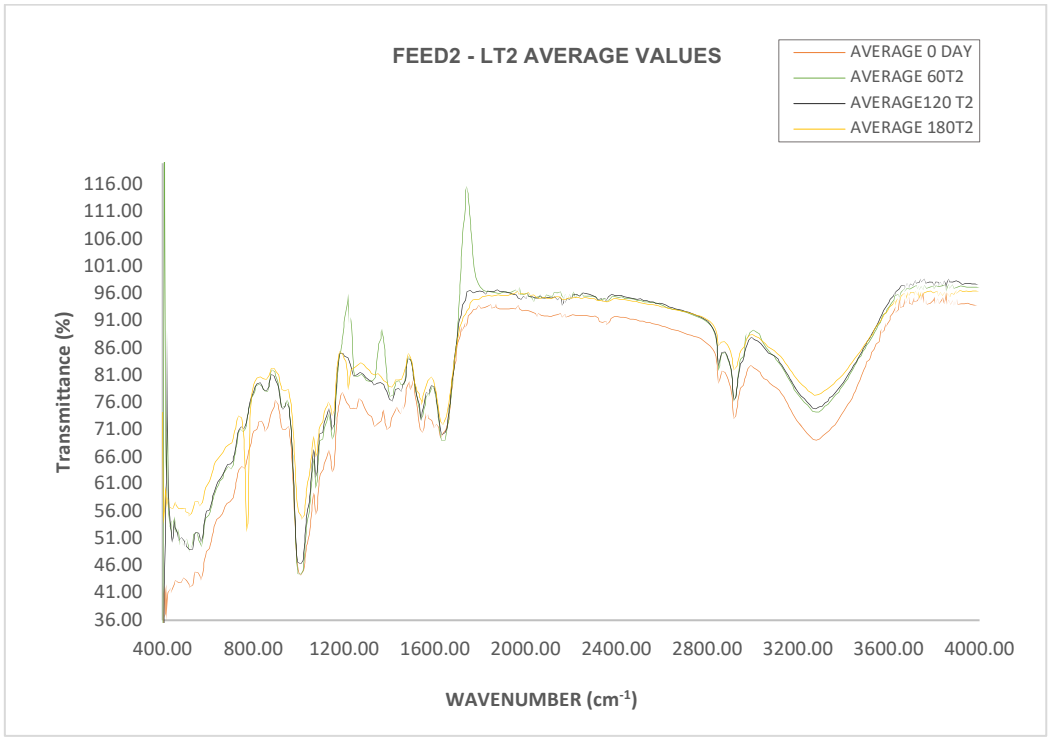


Figure 4.9(B): FTIR spectra feed 2 at low storage. Source: (Author, 2023)

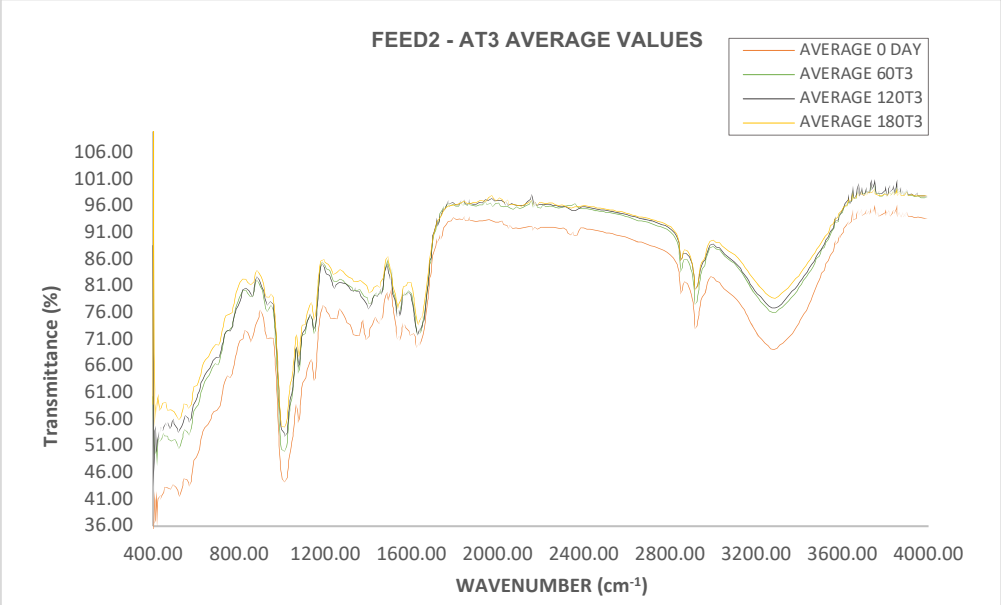


Figure 4.9(C): FTIR spectra feed 2 **temperature storage. Source:** (Author, 2023)

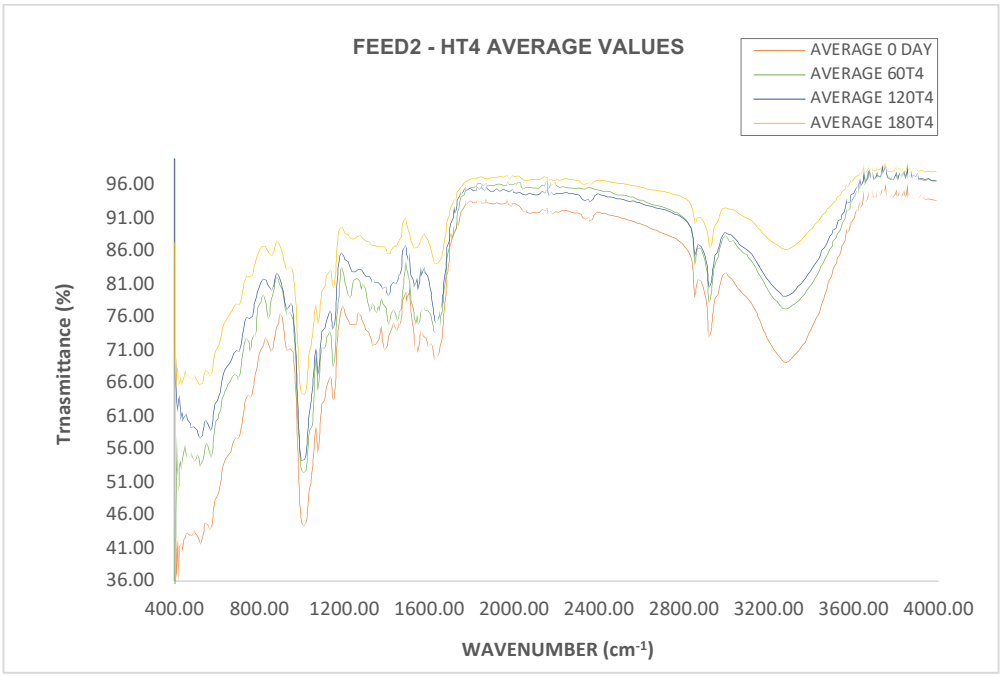
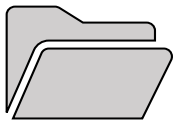


Figure 4.9(D): FTIR spectra feed 2 at **storage. Source:** (Author, 2023)



Chapter 5

DISCUSSION

5.1 Storage quality changes in biochemical profile

5.1.1 Moisture changes

There are significant effects ($P<0.05$) of both storage duration and temperature on moisture of feeds. Significant difference in moisture content of feeds was found between all temperatures except for that between AT and HT for feed 1. Duration effects on feed 1 moisture are significant among initial, four-months and six-month storages. For feed 2, significant difference in moisture ($P<0.05$) exist between HT and other temperatures (LT1, LT2, AT3); similar trend is noticeable for duration effect on feed 2 moisture content. Increase in moisture content at 60^{th} and 120^{th} day (compared to initial) at low (LT1, LT2) and ambient temperatures can be because

al., 2019).

Retrogradation is a process of rearrangement of gelatinized starch components mainly amylose, and in smaller proportion amylopectin, on lowering of temperature and consequent storage (Kibar et al., 2011). Heating or extrusion cooking of starch in the presence of water forms starch gel. During gelatinization starch absorbs water from the medium and swells up. Retrograded starch tends to release water on recrystallisation i.e., syneresis; due to lowering of water holding potential. It is found that water holding capacity of flours with high starch content is more (

of maize bran mixed

wheat flours at increasing maize bran substitution at 5,10,15%. Water holding capacity of mixed flours increased in order of inclusion, higher compared to that of pure wheat flour. This is the trend similar to that in our diet 2 which consists

of corn starch and wheat flour mixture. Water release by syneresis is proportional to degree of starch retrogradation. High amylose containing starch (example, maize starch) retrograde more than low amylose starch (Miller and Whistler, 2009). Since retrogradation occurs at low cooling and ambient conditions during prolonged storage of starch, moisture increase due to syneretic water loss was evident only at LT1, LT2 and AT3 conditions but not at high temperature HT4 for diet 2.

). According to Scott and Awika (2023), certain extrusion parameters (time of extrusion, temperature) restrict retrogradation that would have prevented intermittent water (moisture) increase in extruded diet 1.

5.1.2 Ash changes

Ash is the inorganic residue

Ash content is a valuable parameter for the nutritional assessment of feeds (Chow,1980). Overall decrease in ash values on dry matter basis compared to initial (12.55±0.05) is found through LT1, LT2, AT3 and HT4 (12.40±0.12, 12.28±0.05, 12.30±0.08, 12.37±0.01 respectively) at the end of 180-day storage for diet 1. Ash value decreased significantly ($P<0.05$) between 0 to 60-day, increasing between 60 to 120-day and finally decreased significantly between initial to final, as well between 120 -and 180-day. These results are in line with Hossen et al., 2011 where similar changes in ash over storage at low and ambient conditions were reported. Percentage of ash in fish feeds ranges between 7%-12% (Chapman and Miles, 2009). Ash increased noteworthily ($P<0.05$) from initial (11.97±0.01) to end of storage time, at LT1 (12.04±0.08),

LT2 (11.97±0.08), AT3 (12.14±0.04) and HT4 (12.33±0.28) for diet 2. Content of ash differs largely within compounded diets; specifically, animal by-product-based feeds, due to graded differences in types of fish meal (FM) or other animal-based ingredients such as insect meal (IM), blood meal (BM), poultry product (PP). IM, FM and PP-based substitution of plant diets exhibited increments in ash content of animal-product-based feed substitution (Pleic et al., 2022) . Ash contents of these diets increased at 7.7% (IM substitution); 9.5% (IM+PPB); 12.1% (FM+IM); 11.9% (FM substitution) compared to plant based diet alone with lower ash values at 7.1% .

5.1.3 Crude Lipid changes

Marked difference in lipid content between 0 and 180 day is noteworthy at all storage temperatures for both diets. Arising losses in lipid content follow duration-wise decrease (0<60<120<180 day) at all temperatures. According to Solomon et al. (2016b); lipid oxidation occurs at varying degrees depending on the susceptibility of lipidic composition in the feed ingre of lipids in feed or feed ingredients can cause deterioration of nutritional and biochemical quality. Lipids in foods, during processing and on storage; mainly unsaturated fatty acids such deterioration due to intrinsic (fatty acid, diet composition) and external factors such as light, temperature, oxygen (ard et al., 2015). Highest C.L. loss are at high temperatures HT4, seconded by ambient AT3 losses. While relative f lipids at low temperatures are less severe. These results are in strong accordance to reported work by Hossen et al., 2011; determining gradual storage decline in feed lipids (at low 5^{o} to 8^{o}C; and ambient temperatures 25^{o}-30^{o}C) assessed

over 15 day intervals up to two-month evaluations. Additionally, these outcomes are confirmed by work of Nepote et al. (2006) describing higher oxidative loss of lipids at 40°C in peanuts stored at variable low freezing (-15°C), ambient (23°C) and high (40°C) temperatures. Degree of lipid oxidation increase with increase in storage temperature and duration; additionally, high temperature storage compared to low and moderate temperature storages heighten lipid losses due to thermal oxidation at decreased oxygen pressure (Liu et al., 2019; Márquez-Ruiz, 2014). At high temperatures, oxidation of lipids is accelerated due to fastidious free radical polymerization. Lipidic loss into free fatty acid with hydroperoxide formation leads to off-flavors, malodors in stored feed causing rancidity with deterioration of nutritional quality. High temperature storage instigate oxidative rancidity, free fatty acid release through hydrolytic rancidity and enzymatic rancidity are outcomes of deterioration of unsaturated fatty acids in processed feeds. Lipid oxidation amounts largely to loss of essential fatty acids (Pazos and Medina, 2014).

5.1.4 Crude Protein changes

Decrease in protein content DM basis is noteworthy at all temperatures with increment in storage time. Along storage durations, C.P. losses are in accordance to increase in temperature for storage in order LT1<LT2<AT3<HT4, with greatest decreases at HT4. According to Solomon et al. (2016b), these decreases can be attributed to protein aging along duration of storage. There is no noteworthy difference in protein value between LT1 and LT2, between AT3 and HT4, as well as among LT2, AT3 and HT4 in diet 2. Temperature effects are significant for protein changes between LT1 and AT3, between LT1 and

HT4. For protein content best values for both diets are restored at LT1 condition along storage duration and least values at HT4. Protein decrease is higher at high temperature storage compared to that at low temperatures (Hossen et al., 2011). It is also noteworthy that storage changes of protein and lipids follow similar trends in decrease over durations at all temperatures. Direct oxidation of proteins occur, alike lipid oxidation mechanisms based on free radical generation from reactive oxygen entity. Lipid autooxidation can consequently accelerate oxidation of proteins from release (Geng et al., 2023) a form of indirect oxidation of proteins. Lipidic free radical release mechanisms also trigger aggregation of proteins through crosslinking; distorting protein structure, function, depleting their biological availability and nutritive value in foods.

5.1.5 Carbohydrates (Nitrogen free extract = NFE) changes

Storage duration and temperature conditions have significant effects on dietary carbohydrates whereas, interaction impact of both variables taken together is insignificant on NFE of both experimental diets. Since estimation of NFE is based on subtraction, differences in content is under influence of changes in C.P., C.L., moisture and ash (Solomon et al., 2016).

5.1.6 Gross Energy changes

Gross energy is the calorific value of feed and its compounding ingredients. Since gross energy estimates are relatable to total intake energy from diet, it provide a criteria to calculate actual available energy (as metabolizable energy) for fish functions (NRC, 2011). Gross energy changes on storage, depict impact

of moisture content on notable differences in obtained GE values. Calorific value (Szymajda and Łaska, 2019), the energy currency of biomass is affected by moisture correlations. From results of GE obtained 'as-fed' basis in both diets strong correlation of decreasing moisture in diets on storage is obtained towards significant ($P<0.05$) increments in GE values. This is furthered from reported works of Szymajda and Łaska, 2019; Burubai and Okpala, 2017 depicting increase in calorific value of biomass with moisture decrease.

5.1.7 Aflatoxin Analysis

Presence of aflatoxins was not reported in any sample of stored feed indicating effects of good packaging and storage conditions. All samples were found below limit of quantification (< 0.5ppm). Additionally for diet 1 known antifungal, antimicrobial properties of duckweed *S. polyrhiza* (Nova et al., 2019) may have prevented mycotoxin growth under storage regime.

5.2 Assessment of Protein and lipid oxidation

Oxidative loss of lipids and proteins during processing and storage are the most prominent diminutive changes in nutrition quality of aquaculture feeds. Auto-oxidation of lipids, proteins in feed can decrease their digestibility and biological availability as well deplete abundance of natural antioxidants in feed ingredients such as vitamins (Geng et al., 2023; Kołakowska and Bartosz, 2014).

5.2.1 Assessment of Lipid oxidation

Oxidative loss of lipids is evident from crude lipid changes discussed in prior section. Fatty acid profile changes provide strong support to reduction of lipids over storage (Ahmad et al., 2021). Storage lipids undergo lipolysis producing

free fatty acids, lipo-oxidation (due to peroxide formation) producing hydroperoxides and associated oxidation products; reducing lipid contents (Liang et al., 2020). Significant loss in most SFAs, MUFAs, PUFAs (n-3, n6, n3/n6 diet 2, n6/n3 diet1) with decrease in their total contents in diets depict oxidative loss of fatty acids (constituting dietary lipids), during storage regime (figure 5.1a-b). Unsaturated FAs (as found in fish meal and oils) are largely susceptible to peroxidation (Jeyasanta and Patterson, 2020; Awada et al., 2012). FTIR analysis provisions to these understanding, with prominent spectra of oxidative strains 1710 cm^{-1} , 1743 cm^{-1} due to free fatty acids and ester, aldehyde, ketone stretching in TGAs (Andrade et al.,2019; Upadhyay et al., 2018). Absorbance decrease being higher at high temperature storage due to prominent thermal oxidation at elevated temperatures.

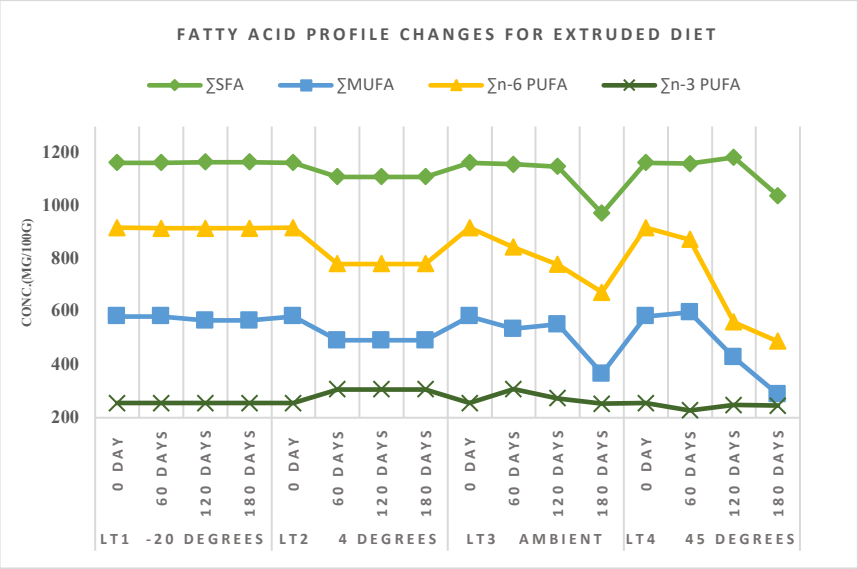


Figure 5.1(a)

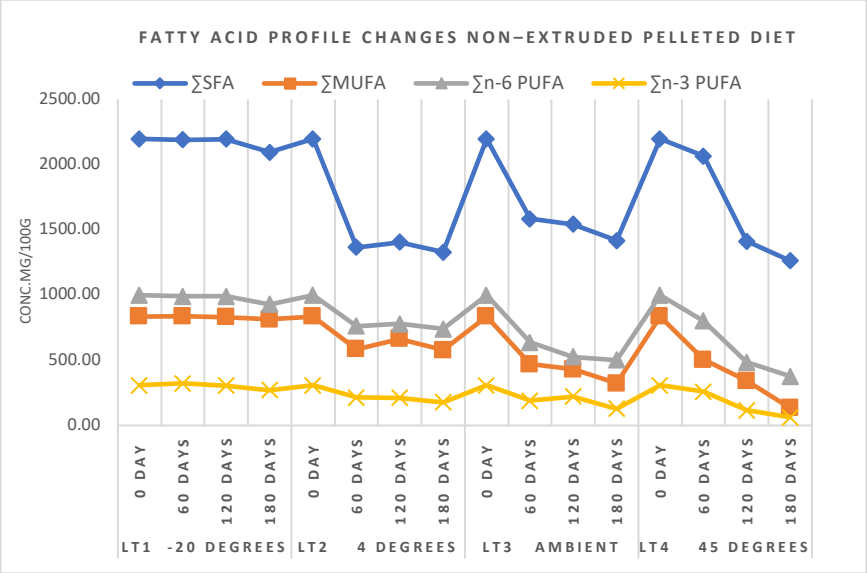


Figure 5.1(b)

Figure 5.1a-b) Changes in total SFA, MUFA, n-3 & n-6 PUFA content of diet1 and 2 during storage conditions. Source: (Author, 2023)

Significant Wilk's Lambda P-values (table 4.5g) explains fatty acid interaction effects owing to nature and type of fatty acid in dietary composition; on prospective changes during storage. As according to Solomon et al. (2016b) lipid oxidation occurs at varying degrees depending on the susceptibility of lipids in the feed ingredients.

5.2.2 Assessment of Protein oxidation

Oxidative protein decrease parallels that of lipidic loss, on oxidation. This is due to free radical release of reactive oxygen entities (ROEs) to which both lipids and proteins are similarly prone to. Crude protein decline as discussed earlier, depicts higher incurred bimonthly loss in proteins. Moreover, these losses are more pronounced at high temperature storage compared to > ambient > and low temperature values (Hossen et al., 2011). Amino acid profile changes, assess underlying decline of total proteins. Evident decrease of total EAAs and NEAAs explains protein degradation. Among EAAs lys, met tryp, his, phe and

cys , tyr among NEAAs show significant depletion at end of storage in both diets compared to initial concentrations. It is known that side chain modification of amino acid with most susceptible AAs to oxidation being lysine (epsilon group under free radical attack); histidine (amine group); cysteine, methionine (sulfur group), tryptophan and tyrosine (, 2014; Xiong and Guo, 2021). Additionally, FTIR spectra 1457 cm^{-1} supports side chain stress (Zarantoniello et al. 2020; Hernández-Martínez et al., 2013) with absorbance changes observed at various storage conditions.

5.3 Storage changes in Vitamins

5.3.1 Water-soluble vitamin changes

Water-soluble vitamins comprise vitamin B-group and C. These vitamins are prone to storage effects showing losses due thermal, processing, storage and feed matrix properties. This is evident from the storage profile changes of these vitamins at various temperature conditions over time.

Vitamin B1 decrease is evident bi-monthly at all storage temperatures for extruded diet 1 with greater loss in order HT>AT>LT2>LT1 at end of six month storage. Thiamine is thermolabile and susceptible to losses at high temperature processing (extrusion) as well as low moisture conditions. Extrusion loss of thiamine up to 50% is reported by Riaz et al. (2009). As per NRC (2011), thiamine retentions ranged between 60-80% in extruded feeds stored for 3 months at ambient temperatures. Non-extruded diet 2, shows initial increase in thiamin concentration up to 120 days; with decreases, at later duration between fourth to sixth month across all storage temperatures. These findings are in good agreement to study based on frozen foods (DiSabatino et al., 2021),

reporting initial increase in thiamine content during storage, processing; followed by storage decrease of thiamine, yet no proposed mechanism could be explained. We believe that this effect may be due to interaction effects of thiamine with dietary antinutrients mainly phytochemicals such as tannins, which would have overestimated B1 peaks in chromatographic run. Additional work by

of adducts (weak bonds) between thiamine and oxidized tannin to be co-eluted at same retention values during thin layer chromatographic analysis. Compared to extruded diets, non-extruded diets are prone to interaction influence . Extrusion processing can reduce tannins and their interference on bioavailability of dietary nutrients (proteins, lipids, vitamins and minerals). Duration effects on storage thiamine are significant for both diets while temperature and interaction impact thiamin concentration only in pelleted non-extruded diet.

Cyrino (1998) described higher heat loss of B2, riboflavin compared to thiamine. Increasing temperatures relate to decreases in riboflavin retention (Bjorck and Asp, 1983; Athar et al., 2006). It is noteworthy that vit B2 decline intensely over storage durations in extruded diet 1 compared to non-extruded diet 2. Extrusion f riboflavin is large up to 50%. These losses due to extrusion are dependent on increasing shear rates due to increase in screw speed, moisture (Yang et al., 2020). Extrusion does not have direct thermal effect on B2 recoveries, rather it increases B2 loss by reducing residence time achieved by increasing screw speed and decrease (Riaz et al,2009; Morin et al.,2021). Being thermostable, B2 is less prone to temperature effects compared to thiamin; unless affected by processing parameters, composition

of food matrix, oxidation, pH, moisture and presence of oxygen. Riboflavin is a light sensitive vitamin. Its stability is affected by oxygen, moisture and water activity (Choe, 2005). Presence of oxygen during storage can severely accelerate B2 deterioration rate (Dennison et al., 1977). Packaging material plays an important role in the photostability. Intense losses of B2 can occur due to improper exposure to light from package material even when other conditions of storage (pH, temperature, moisture) are optimum (Sheraz et al.,2014). Photosensitization (visible or UV light exposure) of riboflavin, under oxygenated conditions can form reactive singlet oxygen further capable of oxidative degradation of dietary proteins, and other vitamins A,C,D and E (Choe, 2005).

Vitamin B6 exists as six distinct forms of which three vitamers pyridoxal, pyridoxine and pyridoxamine (PL, PN, PM) are relevant in chromatographic determinations. Variable forms and properties of B6 vitamers complicate analysis of pyridoxine (Kall, 2003). Thus, applications reporting food estimations determine B6 concentration as sum of vitamers PL+PM+PN (Yaman, 2019) PL and PM being interconvertible forms (Snell,1945). In plant-based foods PN dominates; while PM, PL are found in animal origin foods. In compounded diets both forms exist. Presence of several bioactive forms of the vitamins, along with their physicochemical heterogeneity make simultaneous analysis difficult task. According to Ndaw et al. (2000), enzyme protocol in B6 estimation increased their content due to the release of bound forms from food matrix; this release being under effect of enzyme concentration, purity and differential ability of enzyme combinations used to release specific forms of B6 vitamers.

Although for B12, low temperature retentions are more compared to ambient, in both diets at the end of storage; HT4 values at 180th day (in both diets) are highest this increase at high temperatures is unexplainable. As per Morin et al. (2021) few studies are available on cobalamin, and possibly fewer about its storage behavior in fish feeds; limiting our knowledge on the possible effects of variables on cobalamin changes. Present work may provide some insights regarding this gap in literature.

Gradual loss of vitamin C over low temperature is depicted in both diets; losses are evidently higher at HT4 due to intense temperatures. This is in agreement with works of Giannakourou and Taoukis (2021); Soliman et al (1987); reporting that extrusion and pelleting feed processing procedures through moisture and heat addition, can result in decreased vit. C levels in formulated feeds causing rapid losses at extreme conditions. Vit C in aquaculture feeds is susceptible to processing, storage losses (Tucker and Halver,1986). Additionally,

. Fish like humans lack capability of vit. C biosynthesis and can fulfill it through dietary supplementation. In fishes, dietary deficiency of vit. C can impair collagen synthesis leading to scurvy, lordosis, scoliosis, impaired wound healing, lethargy and anemia symptoms (Sandnes and Utne,1982; Tucker and Halver,1986). Use of high levels of polyunsaturated in the feed necessitate an increased level of supplementation (Hung and Slinger, 1980) to utilize reducing potential of vit. C for preventing oxidation of fats. Dietary inclusions of vit. C is found to improve growth, hematological parameters, carcass nutrients and feed conversion ratio in *Labeo rohita* (, 2019).

5.3.2 Fat-soluble vitamin changes

Fat-soluble vitamins are also prone to effects of storage and feed processing variables. Hydroperoxides produced on lipid oxidation in stored feeds reduce the availability of fat-soluble vitamins (Kop et al., 2019). High temperature above 100^{o}C, moisture, extrusion conditions (such as screw speed) have extreme impact on vit A depletion (Camire,2001; Harper, 1988; Riaz et al.,2009). At each bimonthly assessment vitamin, A decreased at all temperature storages in both diets; largely, since vit A is extremely labile to oxidative loss arising from lipid, protein oxidations following free radical attacks.Only duration effects (P=0.000) are noteworthy for D3 changes over storage, with no significant effect due to temperature (P=0.084) or interaction (P=0.442). Alterations in D2 are significantly (P<0.05) under impact of temperature, duration and their interactions. Vitamin D assessments are in alignment to Kubitza and Cyrino (1998) depicting high pelleting losses of D3 nearing 80-90%, with stable extrusion changes.

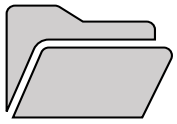
Biologically relevant trans- form of vitamin K1 (Berger et al., 2013), show higher incurred losses towards extruded diet compared to pelleted diet. Variabilities in dietary loss of tocopherols may be with effect to thermal degree of lipid rancidity, presence of light, or metal chelators typically zinc, iron, and copper (Charlton and Ewing, 2007) in diets. This is explainable from higher obtainable concentrations of these micro-elements in elemental profiles at later storage durations. Vit E along with vit have strong antioxidant properties owing to their free radical sequestration potential. Thus, are prone alike to oxidative effects during storage. Obtained results are in strong agreement to these changes.

5.4 Storage changes in elemental profile

Total ash percentage provides extent of mineral contents of the feed, and not the amount of each mineral contribution (DuPonte, 2009). For analysis of each mineral content in diets elemental profile (=mineral) analysis based on ICPMS is performed. ICPMS is standard multi-element determination method by reference laboratory of European Union (EN 17053:2018). It is known for ease of sample preparation, precision, low detection limit, resolution and ability to analyze several elements in simultaneous run (Wilschefski and Baxter, 2019). Macro elements sodium, Mg, Ca and P in both diets show significant increase ($P<0.05$) between initial and final storage except for potassium in diet 1.This increase in available macro elements over storage could be because of moisture differences over storage regime. Loss of moisture in feeds increase dry matter content and consequently available mineral content. These increase are supported by results of Irungu et al. (2018) considering increase in calcium with loss in feed moisture of insect based extrusion diets from 30% to 20%, similar increase in Mg, Na and K were reported owing to moisture changes in diet. Increase in micro-minerals such as , Mn followed moisture dependent effects. Our results align to these findings. Data obtained for macro elements in the feeds appear to be mg/g amounts but there is no suggestion of their bio-availability to fish or their existing physiochemical form. Availability of minerals from diet is a function of their chemical state of occurrence, variability in feed ingredient and feed composition at large. Moreover, it depends on dietary particle dimension and interactions from other diet constituents (Watanabe et al.,1997; Chanda et al., 2015). Elemental availability is increased

or decreased depending on cooperative or antagonistic interactions from diet. These include element to element interactions (some as Ca-P, Zn-Cu, Mg-Ca, Na-K), , lipid-element interaction

Anti-nutrients such as tannin, polyphenols can also change element availabilities out of such interactions in diet. It is therefore noteworthy, that estimated amounts of minerals during storage are outcomes of all above mentioned impact on elemental profiles of feed stored over variable durations and temperatures.



Chapter 6

CONCLUSIONS & FUTURE OUTLOOK

CONCLUSIONS

Aquafeed performance improvement provides an opportunity to enhance the sustainability of aquafarming practices, sourcing the expanse of this fast-growing food sector catering to global nutrition requirements. Nutrient profile of aquafeed determines fish welfare and consequent consumer health. While employing choice of ingredients for aquafeeds; storage, as well as storage handlings are essential considerations. Feed storage is imperative to overcome scarcities of feed supply, produce; maintaining continuous resource of ration to meet timely demands of aquaculture. Present work is designed to evaluate effect of different storage conditions on feed nutrient quality. The study aims at assessing incurred losses in nutritional quality of compounded feeds stored over long-term duration under variable condition of storage temperatures. This work also aims at determining the effects of feed composition and feed processing technique on the nutritional quality of feeds.

The study is based on evaluation of impact of variables, temperature and storage duration, on quality parameters of formulated aquafeeds. Greater duckweed, *Spirodela polyrhiza* was used as alternative feed ingredient for partial substitution of fishmeal in extruded diet (feed /diet1) and non-substituted pelleted diet (feed/diet 2) is taken as control. Feeds were stored at four different temperature conditions comprising low temperatures (LT1= -20^{o} C, LT2= 4^{o} C); ambient (AT) and high temperature (HT= 45^{o} C) conditions for six months. Bimonthly assessment of nutritional profile for biochemical composition

proximate parameters; ash, moisture, crude lipid, crude protein, carbohydrates as nitrogen free extract (NFE), is performed along with changes in storage profile of essential, non-essential, free amino acids; saturated, unsaturated, free fatty acids; fat and water-soluble vitamins; micro and macro-elements, gross energy (GE), assessing aflatoxin incidences, if any; at variable storage temperature conditions. It is determined that overall, storage temperature and duration have highly significant effect ($P< 0.05$) on CP, CL, moisture, NFE, and GE content of feeds. Interaction effects are significant ($P< 0.05$) for moisture in both feeds while interaction for ash and GE significantly impacts feed 1. Largely, there is decrease in crude lipid, crude protein and moisture content from LT to HT condition (LT1< LT2< AT3< HT4), across assessment duration (0<60<120<180 days), at all temperatures for diet 1 (extruded diet). For crude protein, crude lipid (diet 1 and 2); and NFE (diet1), temperature and duration effects are noteworthy ($P< 0.05$) with no significant effect due to interaction ($P>0.05$).

There is significant difference in GE value between low temperature storages and other temperatures (ambient and 45^{o}C) with higher GE at low temperature storage, for feed 1. For diet 2, differences in GE are significant between LT1 and HT4 storages. Incurred losses in vitamins A, E, K, B2, B12 and C are noteworthy across storage duration 60-day onwards, at all temperatures, for both diets. While, vitamin D losses are pronounced in pelleted diet (diet 2). These losses are most noteworthy during fourth month; at HT storage, followed by that at AT.

Elemental interaction and moisture noteworthily impact element profile changes. Significant losses, from initial to six months was notable for most, total

saturated, monounsaturated and polyunsaturated fatty acids (n-3PUFA, n-6 PUFA) for both diets at all storage temperatures.

There is an overall decrease in total essential and total non-essential amino acids along storage duration for extruded and pelleted diet. Oxidative loss of protein and lipids is assessed through respective amino acid and fatty acid profiles. Supporting confirmations from FTIR spectra indicate at storage losses with absorbance changes and peculiar spectra for side chain amino acid modifications, amide bond spectra, indicating protein deterioration. TGA and FFA spectra confirm lipid declines with supportive confirmation from crude lipid dry matter values. Aflatoxin incidences have not been reported in the assessments indicating effects of good storage and packaging conditions.

The results determine percentage loss of each nutrient in compounded diets; thus, specifying need to develop feed formulae accounting their incurred losses during the storage period.

From outcomes of this study, it is evident that for best possible nutritional gains appropriate storage temperature and timely utilization of feed are necessary considerations. Interplay of temperature and storage duration can foster several deteriorative changes in dietary nutrients; mainly feed lipids, proteins and vitamins; significantly depleting shelf-life of stored feeds. In this aspect, best nutritional outcomes from feed can be obtained from storage at low freezing temperature at -20^{o}C with utilization of feed earlier up to two months of storage. This study additionally, helps understand impact of temperature and duration variables on feed quality at farm conditions. Comparison of formulated feeds to ascertain their response to storage variables. Secondary effect of these variables on level of feed deterioration to help ascertain strategies required for

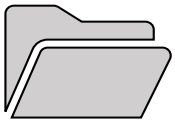
feed utilization when storage is prolonged. Maintain the nutritional quality of feed ingredients during storage period. Need to develop feed formulae taking into consideration the percentage loss of each nutrient from the foodstuffs during the storage period. Understanding feed utilization for maximizing it's nutritional benefit to the fish. Additionally, these findings may provide helpful information for fish farmers in managing feed storage of formulated feeds with an aim to prolong their shelf life; safeguarding significant amount of the total production costs of fed aquaculture.

Future Outlook

Storage improvement provides an opportunity to enhance aquaculture sustainable productions contributing to matchable fish demands. Storage inflicted changes in inventories and storage farms must be thoroughly monitored and assessed to design hazard prevention strategies as well as nutrition restoration of feeds.

In this aspect biochemical assessments alongside, microbial, physical assessment paves way for maximizing feed benefits even from stored rations.

Moreover, storage hazard mitigation through bio-actives and probiotic feed supplementation of healthy microbiota can improve feed quality as well nutritional, health benefits to the fish restoring overall global consumer gains from the fish and fisheries production.



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LIST OF PUBLICATIONS

RESEARCH PUBLICATIONS

(A) Articles

Puri P, Singh R, Sharma JG* (2023). Micro-/bio-/nano-, syn-encapsulations and co-treatments of bioactive microbial feed supplementation in augmenting finfish health and aquaculture nutrition: a review. ***Beneficial Microbes*** **(Wageningen Academic Publishers)** 1-22. **Impact Factor = 5.4** (Online published, In press) https://doi.org/10.3920/BM2022.0087

Puri P, Sharma JG*, Singh R (2022). Biotherapeutic microbial supplementation for ameliorating fish health: developing trends in probiotics, prebiotics, and synbiotics use in finfish aquaculture. ***Animal Health Research Reviews*** **(Cambridge University Press)** 23(2): 113-135. **Impact Factor = 2.5** https://doi.org/10.1017/S1466252321000165

(B) Conference Paper Presentations

Parul Puri, Ram Singh*, JaiGopal Sharma* (2022). Evaluation of Nutritional profile and Storage Temperature influence on Moisture content of aquafeed prepared from non-conventional food source: Quality assessments for Sustainable Aquaculture Productions. Oral paper presentation at CSIR & ICSSR sponsored *International online*

Conference on Science and Society organized by Miranda House University of Delhi.

Parul Puri, Ram Singh*, Jaigopal Sharma* (2022). Improving Nutritional Profile of Stored Aquaculture feeds for promoting human health: Biochemical Assessments and biotherapeutic augmentation strategies poster presentation at *International Symposium Chemical Wisdom by Her* organized by Department of Chemistry, Deshbandhu College, University of Delhi.