A Nutritional Evaluation and Optimisation of Infant Foods using Microencapsulation

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DECLARATION

"I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than that of Doctor of Philosophy being studied at the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others."

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Date: **16/01/2016**

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ABSTRACT

A Nutritional Evaluation of Infant Foods and Optimisation using Microencapsulation

Over recent decades the modern lifestyle dynamic has lead to an increased parental reliance on commercially marketed complementary infant foods in the UK, which has been highlighted by the Diet and Nutriton Survey of Infants and Young Children. The current nutritional labelling formats for ready-to-eat complementary infant foods are a duplicate of the legislative requirements for manufacturing of ready meals, intended for the general population, the implication of this is that a number of important nutrients maybe limiting or excessive, which will affect their nutritional quality and suitability as an infant food. Furthermore nutritional databases, such as McCance and Widdowson provide limited data on the composition of these types of food products. The European Food Safety Authority has highlighted that nutrient intake data after six months of life is currently inadequate as well as insufficient and urgently needs to be addressed. Therefore the nutritional content of these food products needs to be assessed to ascertain whether or not infants are meeting dietary requirements when consuming such products.

The aims of this study were to evaluate the nutritional suitability of infant food products currently available on the UK market, according to the most up to date recommendations and recent relevant legislation and to explore the microencapsulation of docasahexaenoic acid (DHA) to optimise the nutrient content of infant food products.

New protocols were developed for the quantitative analysis of certain key nutrients including High Pressure Liquid Chromatography (HPLC) – Charged Aerosol Detection for essential fatty acids, HPLC and UV spectrophotometry for fat soluble vitamins A and E, competitive enzyme immunoassay (Vitakit D™) for vitamin D and Inductively Coupled Plasma Optical Emission Spectroscopy for essential elements (Ca, Cu, Fe, K, Mg, Na, P and Zn) in commercial infant foods in the UK. The estimated daily intakes of these products were compared against current dietary recommendations for infants. In addition the Ca:P ratio was also determined in a range of commercial infant foods and compared with recommendations in relation to bone health. Furthermore, the effects of commonly practiced re-heating treatments used by parents were examined to establish whether different preparation methods affected the fatty acid content of manufactured infant formula milks. Finally, through the nutritional evaluation of these infant food products, the infant's diet was found to be low in

DHA, which provided opportunities for scope and product optimisation to improve the nutritive value of infant food products. Therefore microencapsulation of DHA was explored as a potential way to improve the nutritional quality of infant food products.

The nutritional evaluation of the essential fatty acid content of a 6-9 month old infant's diet highlighted that pre-formed long chain polyunsaturated fatty acids (LCPUFA) DHA and arachidonic acid (AA) intakes (at 23.3 mg/day and 36.7 mg/day, respectively) were below recommendations set by the US, at 103.3 mg/day and 147.5 mg/day, respectively. This provides scope for product optimization to improve the nutritive value of commercial infant food products. With respect to the precursor essential fatty acids, the dietary intake of the *n*-6 fatty acid linoleic acid (LA) was found to be above recommendations at 3147.9 mg/day, whereas the *n*-3 fatty acid α-linolenic acid (ALA) was found to be below recommendations at 296.4 mg/day, which increases the LA:ALA ratio of the diet; this may have implications for allergy. As the fortified infant formula was identified as the major dietary contributor and due to the fact that unsaturated fatty acids are prone to oxidation, the impact of re-heating treatments used by parents on the fatty acid content of formula milk was investigated and a degree of statistically significant changes were observed. In relation to the transparency of the nutritional information declared on the labels by the manufacturers, infant formula milks were all within the limits of EU regulations although there was a degree of significant variation between the quantitative values analyzed in this study and the declared values on the labels.

With regards to the vitamin A and E analysis, normal phase HPLC was employed for the simultaneous quantification of retinyl acetate, retinyl palmitate, α-tocopherol and γ-tocopherol; reverse phase HPLC was used for the quantification of β-carotene and UV spectrophotometry for the quantification of carotenoids from selected meat and vegetable 'ready-to-feed' commercial infant meals. Based on the results of the study, the estimated total daily intake for a 6-9 month old infant of vitamin A (retinol equivalents, RE) and vitamin E (α-tocopherol equivalents, ATE) from both infant food and formula milk were 1.74 mg RE/day and 10.4 mg ATE/day, respectively. These intakes exceed the recommendations set by the Department of Health (1991). The main dietary contributor was highlighted as being the fortified infant formula which highlights the importance of nutrient dense foods in situations where infant formula is reduced or compromised.

The study into the essential elemental content of dairy based commercial infant food products found that the Ca:P ratio of a 7-12 month old infant's diet was 1.49:1, which was within the recommended range of 1-2:1. However, the level of intake for each of the elements analyzed, with the exception of sodium, were found to be above the Recommended Nutrient Intake (RNI) set by the Department of Health (1991), which warrants further investigation in relation to both micronutrient interactions, and in situations where the intake of fortified infant formula milk is compromised. In addition, as this study was the first to include consumption of infant snack products, the level of total calorie intake was also assessed, which indicated that energy intakes exceed recommendations set by the Scientific Advisory Committee of Nutrition (2011) by 42%, which may have implications for obesity. This highlights that parents need to select appropriate snack products. In relation to bone health, vitamin D was also quantified in a range of commercial infant meals. The total dietary intake of vitamin D3 was determined to be 9.61 μg/day, which is 137% higher than the RNI set by the Department of Health (1991) for 7-12 month old infants. However 120% is contributed from fortified infant formula, which may raise a cause for concern over deficiency issues, in situations where infant formula is reduced or compromised or the infant is breastfed. Furthermore the National Diet and Nutriton Survey have shown evidence for an increased risk of vitamin D deficiency in all age and sex groups in the UK.

Consequently, following the nutritional evaluation of commercial infant food products, an infant's diet is not meeting recommendations for the pre-formed long chain polyunsaturated fatty acids, DHA and AA. DHA may be of more significance due to endogenous production. Therefore, two approaches have been explored for the encapsulation of DHA in the pH dependent polymer hydroxypropyl methylcellulose acetate succinate (HPMCAS). In the first approach direct spray drying was implemented for the microencapsulation of DHA/HPMCAS organic solutions, while in the second approach solid lipid nano-emulsions of DHA, produced by high pressure homogenization, were subsequently spray dried in HPMCAS aqueous solutions. The direct spray drying approach resulted in significantly higher quantities of DHA being encapsulated, at 2.09 g/100 g compared to 0.60 g/100 in the spray dried solid lipid nano-emulsions. DHA stability was increased by the direct spray drying approach and the release of DHA was analysed by a dissolution methodology. The encapsulated powders produced by the desired method offer a source of DHA that has the potential to be incorporated into infant foods to increase their dietary DHA consumption.

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ABBREVIATIONS

Abbreviation	Description
AA	Arachidonic Acid
AI	Actual Intake
ALA	α-Linolenic acid
ANOVA	Analysis of Variance
ATE	α-Tocopherol Equivalents
ATR	Attenuated Total Reflectance
BCMO	β-Carotene Monooxygenase
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
Ca:P	Calcium-to-Phosphorus Ratio
CAD	Charged Aerosol Detector
COMA	Committee of Medical Aspects of Food and Nutrition Policy
COX	Cyclooxygenase
CRBP	Cellular Retinol Binding Protein
D2	Ergocalciferol
D3	Cholecalciferol
DGLA	Dihomo γ-Linolenic Acid
DHA	Docosahexaenoic Acid
DNSIYC	Diet and Nutrition Survey of Infants and Young Children
DOH	Department of Health
DRI	Dietary Reference Intake
DSC	Differential Scanning Calorimetry
EAR	Estimated Average Requirement
EEI	Estimated Energy Intake
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
ELSD	Evaporative Light Scattering Detection
EPA	Eicosapentaenoic Acid
ESPGHAN	European Society for Paediatric Gastroenterology Hepatology and Nutrition
FADS	Fatty Acid Desaturase

FAO	Food and Agriculture Organisation
FGF	Fibroblast Growth Factor
FTIR	Fourier Transform Infrared
GLA	γ-Linolenic Acid
HPMCAS	Hydroxy Propyl Methyl Cellulose Acetate Succinate
ICPOES	Inductively Coupled Plasma Optical Emission Spectroscopy
IDA	Iron Deficiency Anaemia
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IOM	Institute of Medicine
LA	Linoleic Acid
LCPUFA	Long Chain Polyunsaturated Fatty Acids
LOD	Limit of Detection
LOQ	Limit of Quantification
LOX	Lipoxygenase
LRNI	Lower Reference Nutrient Intake
LT	Leukotrienes
MTBE	Methyl Tert-Butyl Ether
NDNS	National Diet and Nutrition Survey
NFκB	Nuclear Factor Kappa B
NHANES	National Health and Nutrition Examination Survey
NIH	National Institute of Health
NP-HPLC	Normal Phase High Performance Liquid Chromatography
NPT	Sodium/phosphate co-transporter
PE	Petroleum Ether
PG	Prostaglandins
PPAR	Peroxisome Proliferator Activated Receptor
PSD	Particle Size Distribution
PTH	Parathyroid Hormone
PUFA	Polyunsaturated Fatty Acids
RAE	Retinol Activity Equivalents
RAR	Retinoic Acid Receptor

RBP	Retinol Binding Protein
RDA	Recommended Daily Allowance
RE	Retinol Equivalents
RNI	Recommended Nutrient Intake
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RSD	Relative Standard Deviation
RTF	Ready-to-Feed
RXR	Retinoid X Receptor
S/N	Signal to Noise
SD	Standard Deviation
SEM	Standard Error of Mean
SEM	Scanning Electron Microscopy
SLN	Solid Lipid Nanoparticles
T_h	T Helper
T reg	Regulatory T cell
TGA	Thermal Gravimetric Analysis
TGF	Transforming Growth Factor
THF	Tetrahydrofuran
TNF	Tumour Necrosis Factor
α-TTP	α-Tocopherol Transport Protein
TX	Thromboxane
UL	Tolerable Upper Intake Level
VDR	Vitamin D Receptor
VEP	Visual Evoked Potential
WHO	World Health Organisation
XRDP	X Ray Powder Diffraction

Chapter 1

Overview of the thesis

Pregnancy, lactation and infancy are specific times in life when essential nutrients are indispensable and where their requirements are different to any other life stages to support rapid growth and development (Bryhn, 2006). With foetal life and childhood being periods of rapid growth and development, an imbalanced nutrient supply at this age may alter body structure and function in a way that increases the risk of chronic disease (Scientific Advisory Committee on Nutrition, 2011). During the first 4-5 months of life the weight of an infant doubles, this results in a relatively high requirement of energy and nutrients/kg of body weight; which are proportionally higher than at any other time during life (Scientific Committee on Food, 2003). Growth and development is absolutely dependent on an adequate supply of energy and nutrients which are sufficient to match the variable needs of the infant as growth progresses, with any limitation in nutrient supply likely to constrain the pace and pattern of development (Scientific Advisory Committee on Nutrition, 2011).

Based on extensive scientific evidence, the World Health Organisation (World Health Organisation, 2004) recommends exclusive breastfeeding for the first 6 months of life (Food Standards Agency, 2008). At around 6 months, infants require more iron, energy and other nutrients which breast milk can no longer supply independently. The Department of Health therefore recommends that solid foods or complementary foods are introduced at 6 months of age; along with the continuation of breastfeeding or the use of an appropriate breast milk substitute (Caroli, et al., 2012). Complementary or weaning foods are semi-solid or solid foods used to transition an infant from the sole source of breast milk or infant formula to an adult diet (Bond, et al., 2005). Weaning is the gradual introduction of beverages and foods other than breast milk or infant formula, which should result in the consumption of foods that are nutritionally balanced and supply the essential micro and macro nutrients required for the optimal growth and development of an infant (Schwartz, et al., 2011).

The Diet and Nutrition Survey of Infants and Young Children has identified that 58% of children who are fed foods other than milk have eaten a commercial baby or toddler meal (Lennox et al., 2013), indicating that commercial ready-made baby meals are important contributors to the infant's nutrient supply. The use of ready-made baby foods is most common between 5-10 months (McAndrew, et al., 2012). An increasing number of mothers

feed their infants with industrially processed infant foods and in recent years the baby food market and the range of products has grown significantly. The first year of an infant's life is a sensitive period for the development of the nervous, reproductive, digestive, respiratory and immune systems. Therefore the nutritional quality of commercial infant foods urgently needs to be assessed in order to evaluate whether they are meeting the infants nutritional requirements at a critical life stage (Pandelova, et al, 2012).

In addition, there is currently no clear and complete analytical nutritional data available for ready to feed complementary infant foods in the UK. The nutritional database used in the UK, McCance and Widdowson, contains limited data on the composition of complementary infant foods, in addition the analytical techniques and nutrient data contained may now be outdated. It is essential that food composition tables are regularly updated with the current foods available on the market, especially with the regular introduction of new foods to the market (Food Standards Agency, 2002).

Establishing the current intake of nutrients in infancy is a priority step to issuing dietary guidelines (Nissensohn, et al., 2011). The European Food Safety Authority has highlighted that nutrient intake data after 6 months of age is insufficient in most European countries (EFSA, 2009). Observational studies have raised a cause for concern over later health consequences bought about by deficient or excessive nutrient intakes during infancy (Caroli, et al., 2012). A major health challenge in developed countries such as Europe and the United States is the rising prevalence of diet related diseases including obesity, type 2 diabetes, cardiovascular disease and cancer, which emphasises the need to assess the effects of specific nutrients in early life and their contribution to chronic diseases in later life (Friedl, et al., 2014; Schwartz, et al., 2011).

1.1 Research Questions

The issues that this study will address are:

1. What is the content of essential fatty acids, vitamins A, E and D and essential elements (with special attention to the calcium to phosphorus ratio (Ca:P) in relation to bone health) in selected commercial infant foods currently available in the UK?
2. Are the nutrient compositions of commercial infant foods assessed in line with the current nutritional guidelines set for infants?

3. What is the effect of common heat treatment practices used by parents for the preparation of infant formula milk on the essential fatty acid content of formula milk products?
4. Can microencapsulation of fish oil offer protection against degradation of essential fatty acids and offer a source of DHA that can be used to fortify infant food products?

1.2 Aims and Objectives

This study aims to:

1. Evaluate the nutritional suitability of infant food products currently available on the UK market, according to the most up to date recommendations and recent relevant legislation, with careful consideration of contemporary aspects of infant nutrition, such as nutritional quality, allergens and essential fatty acids.
2. Explore the microencapsulation of essential fatty acids to optimise the nutrient content of 'ready-to-feed' infant food products.

The objectives of the studies reported in this thesis are as follows:

1. To quantitatively establish the concentration of essential fatty acids (Chapter 3), vitamins A, E (Chapter 4) and D and essential elements (Chapter 5) in commercially prepared infant foods in the UK and compare estimated daily intakes of these products against current dietary recommendations for infants.
2. Examine the effects of commonly practiced re-heating treatments used by parents on essential fatty acids in manufactured infant formula milks (Chapter 3).
3. Establish the Ca:P ratio in a range of commercially prepared infant foods currently sold in the UK and compare to recommendations in relation to bone health (Chapter 5).
4. Explore the microencapsulation of essential fatty acids using two approaches: direct spray drying and solid lipid nano-emulsions produced by high pressure homogenization, subsequently spray dried, followed by characterisation using moisture content, water activity, encapsulation efficiency, particle size distribution, scanning electron microscopy, X-ray powder diffraction, Fourier-transform infrared, differential scanning calorimetry, thermogravimetric analysis, dissolution and stability testing (Chapter 6).

In summary this project will develop new protocols for the quantitative analysis of certain key nutrients including High Pressure Liquid Chromatography (HPLC) – Charged Aerosol detection for essential fatty acids, HPLC and UV spectrophotometry for vitamins A and E, competitive enzyme immunoassay (Vitakit D™) for vitamin D and Inductively Coupled Plasma Optical Emission Spectroscopy for essential elements (Ca, Cu, Fe, K, Mg, Na, P and Zn) in commercial infant foods in the UK. The results from the quantitative analysis will be used to evaluate the total daily intake of nutrients based upon the consumption of commercial 'ready to feed' infant foods and infant formula. An evaluation of the nutritional quality of these products in relation to nutritional requirements for infants will then be undertaken. In addition the Ca:P ratio was also determined in a range of commercial infant foods and compared with recommendations in relation to bone health. Furthermore, the effect of home processing methods used by parents for the preparation of infant formulas will also be evaluated to determine any nutrient losses due to certain thermal processes, focusing in particular on the essential fatty acid content. Finally microencapsulation of essential fatty acids will be explored to improve the nutritional quality of infant food products.

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Chapter 2

Introduction

2.1 Infant Feeding

Early life nutrition can influence long term health and play a role in the development of chronic disease in later life. The European Food Safety Authority (EFSA) has highlighted that nutrient intake data after 6 months of age is currently insufficient and inadequate in European countries (EFSA, 2009). Observational studies have identified a cause for concern over later health consequences resulting from deficient or excessive nutrient intake during infancy (Caroli, et al., 2012). A major health challenge in developed countries, such as Europe and the United States is the rising prevalence of diet related diseases including obesity, type 2 diabetes, cardiovascular disease and cancer, which emphasises the need to assess the effects of specific nutrients in early life and their contribution to chronic diseases in later life (Friedl, et al., 2014; Schwartz, et al., 2011).

Foetal life and childhood are periods of rapid growth and development and an imbalanced nutrient supply at this age may alter body structure and function in a way that increases the risk of chronic disease (Scientific Advisory Committee on Nutrition, 2011). The weight of an infant doubles in the first 4-5 months, which results in a relatively high requirement of energy and nutrients/kg of body weight; which are proportionally higher than at any other time during life (Scientific Committee on Food, 2003). Experimental studies in animals have identified critical periods in early development when an alteration in the nutrient supply may alter structure and function irreversibly, which is known as nutritional programming. Early nutrient supply also influences both the composition and distribution of tissues deposited during growth and the achievement of optimal functional and metabolic capacity of the whole body at a cellular level. Growth and development is absolutely dependent on an adequate supply of energy and nutrients which are sufficient to match the variable needs of an infant as growth progresses, with any limitation in nutrient supply likely to constrain the pace and pattern of development (Scientific Advisory Committee on Nutrition, 2011). The metabolic requirements of an infant are counterintuitive with their limited capacity to compensate for unbalanced nutrient supplies due to infant's having small body stores of nutrients and immature homeostatic mechanisms (Scientific Committee on Food, 2003). Furthermore, infants may not be able to synthesise enough of certain nutrients due to insufficient amount of

certain substrates related to the limited capacity of certain metabolic synthesis pathways; therefore, they are required from the diet (Thompkinson & Kharb, 2007).

After birth infants receive either breast milk or infant formula meaning that their nutritional intake is solely dependent on the nutritional composition of these milks which should provide the infant with sufficient macro and micro nutrients (Schwartz, et al., 2011). Breastfeeding has been associated with numerous health benefits for both the infant and mother; for the infant a reduction in lower respiratory and gastrointestinal infections and otitis media occurrence, cognitive and behavioural advantages and obesity reduction; and for the mother lower incidence of certain cancers have been observed (McAndrew, et al., 2012). Breast milk is a biological fluid and the best form of nutrition for infants, as it provides all the nutrients in a form that is easily absorbed that an infant requires for healthy growth and development during the first 6 months of life. Breast milk contains proteins such as lactoferrin, which binds to iron to prevent bacteria utilising it, immunoglobulins and macrophages to protect the infant against infection, fatty acids, growth factors, anti-viral factors, anti-bacterial substances and living white blood cells (Crawley & Westland, 2014). Based on extensive scientific evidence the World Health Organisation (WHO) recommends exclusive breastfeeding for the first 6 months of life, with infant formula being the only alternative to breast milk suitable for infants during the first year of life (Food Standards Agency, 2008). Around 6 months infants require more iron, energy and other nutrients which breast milk can no longer supply independently. Therefore the Department of Health (DOH) recommends that solid foods or complementary foods are introduced at 6 months of age; along with the continuation of breastfeeding or the use of an appropriate breast milk substitute. The introduction of solid foods before 6 months when the neuromuscular co-ordination and gut and kidney maturation are insufficient is thought to increase the risk of infection and allergy development (Caroli, et al., 2012).

Numerous risk factors have also been associated with early introduction of complementary feeding (<6 months), which include mothers that smoke, mothers who have not breastfed, infants who have been introduced to formula milk early in life, rapid weight gain in the first 6 weeks, low socioeconomic status and parents perception that the infant is hungry. Although recommendations focus on the age of introduction to solid foods; information regarding the quality and amount of solid foods is limited (Caroli, et al., 2012).

During the first year of life many physiological changes occur that allow the infant to consume foods of varying composition and texture. As the infant's mouth, tongue and digestive tract mature the infant shifts from being able to suckle, swallow and take in liquid foods to being able to chew and consume a variety of different solid complementary foods (US Department of Agriculture, 2009). Complementary or weaning foods are semi-solid or solid foods used to transition an infant from the sole source of breast milk or infant formula to an adult diet (Bond, et al., 2005). Weaning is the gradual introduction of beverages and foods other than breast milk or infant formula, which should result in the consumption of foods that are nutritionally balanced and supply the essential micro and macro nutrients required for optimal growth and development of an infant. Different countries have different weaning practices, for instance many European authorities have agreed that complementary feeding between 4 – 6 months is safe (Schwartz, et al., 2011). Furthermore complementary foods in the UK are labelled from 4 months, which produces confusion for parents (The Association of UK Dietetics, 2013). Food preferences in early life have been tracked into adulthood, which can impact on the development of eating habits for both long and short term (Schwartz, et al., 2011).

Infant formula aims to provide formulations which have the compositional and biochemical characteristics similar to breast milk; the data in **Table 2.1** shows the composition of breast milk, cow's milk and ready to feed first infant milks available in the UK and the data in **Table 2.2** compares the fat content of breast milk and cow's milk (Thompkinson & Kharb, 2007). Major differences remain between breast milk and infant formula due to the fact that the exact chemical properties of breast milk are still unknown and therefore cannot be reproduced; breast milk contains over 300 components whereas infant formula only contains about 75. In addition breast milk composition changes over time and in response to the mothers feeding habits, therefore the mother's diet is also important to consider. Furthermore breast milk contains the mother's antibodies and other defensive factors which cannot be added to manufacture infant formula. Issues also arise due to the manufacturing of infant formula; such as storage, delivery and quality control problems. Infant formula composition must comply with the infant formula and follow on formula regulations 2007 (Department of Health, 2013). As some vitamins and minerals are more easily absorbed from breast milk (such as calcium and zinc) they will or can be added in higher concentrations to infant formula to compensate for this. Also certain vitamins are known to deteriorate during storage, so may also be added in higher concentrations. The Royal College of Nursing recommends

infants consume 600 mL of milk between 7-9 months and 500 mL between 10-12 months (Crawley & Westland, 2014). From the 2010 Infant Feeding Survey in the UK 19% of mothers did not initiate breastfeeding at birth, 31% introduced infant formula on the first day, at 1 week 52% had given their infant formula and 73% by 6 weeks, by 4-6 months 60% were entirely on infant formula and by 9 months 95% has received infant formula, this indicates that infant formulas are widely used by mothers in the UK and infant formula content should also be considered when assessing infant's diets (McAndrew, et al., 2012).

Maclean, et al. (2010) conducted a survey on nutrient levels in infant milks. The formulas met the minimum requirements, however levels in some milks exceeded the guidance on upper levels; these included vitamin A, K, B6, B12, C, thiamine, riboflavin, niacin, folic acid, iron, copper, manganese, potassium and iodine. The data also showed considerable variability between products which shows the difficulties in manufacturing a product which has to contain minimum amounts of certain nutrients from manufacturing to the end of the products shelf life. The complex nature of the degradation of nutrients in relation to storage and interactions between components makes it difficult to know the exact amount of nutrients which will be available upon consumption (Crawley & Westland, 2014).

Karlberg (1987) modelled the pattern of human growth from conception to completion as a set of 3 overlapping curves, known as the infancy-childhood-puberty model of human growth. During infancy relative growth is more rapid than at any other time and any restriction of nutrient supply will have the greatest effect at these early stages. The brain grows at peak velocity around birth and completes most of its growth by 2 years of age. Tissues and organs appear to undergo critical or sensitive periods of development where insults or stimuli at a certain time can lead to lifelong changes in organ structure or function. Animal studies have also shown that different organs have different critical periods of development (Scientific Advisory Committee on Nutrition, 2011). During infancy the diet is not only required for maintenance but to cover energy and substrate requirements for the synthesis and deposition of newly formed tissues. In addition large metabolic requirements contrast with the infant's limited capacity to compensate for an unbalanced nutrient supply due to small body stores and immature homeostatic mechanisms (Scientific Committee on Food, 2003).

Table 2.1 Breast milk, cow's milk and nutritional composition of leading UK brands of first infant milks.

Per 100 mL	Hipp Organic First Milk	SMA First Infant Milk	Aptamil First Milk	C & G First Infant Milk	Breast Milk	Cow's milk
Energy kJ	280	280	275	275	285[1]	285[1]
kcal	67	67	66	66	68[1]	68[1]
Fat, g	3.5	3.6	3.4	3.4	3.9[1]	3.8[1]
Of which saturates, g	1.1	1.6	1.4	1.5	2[1]	2.3[1]
Unsaturates, g	ND	2	ND	1.9	1.9[1]	1.4[1]
Of which LCP, g	0.019	ND	0.024	0.015	ND	ND
ALA, mg	90	42	ND	ND	32[1]	55[1]
LA, mg	640	520	ND	ND	285[1]	52[1]
AA, mg	ND	12.3	11	6.5	ND	ND
DHA, mg	ND	7.1	10	6.4	ND	ND
Carbohydrate, g	7.3	7.3	7.4	7.3	7.2[1]	4.7[1]
Of which sugars, g	7.2	7.3	7.3	7.2	ND	ND
Of which lactose, g	ND	ND	7.1	7	7.0[2]	4.8[2]
Fibre, g	0.4	ND	0.6	0.6	ND	ND
Protein, g	1.4	1.3	1.3	1.3	1.0[1]	3.3[1]
Whey, g	0.8	ND	0.8	0.8	0.72[2]	0.64[2]
Casein, g	0.6	ND	0.5	0.5	0.31[2]	2.60[2]
Salt, g	ND	0.04	0.04	ND	ND	ND
Vitamin A, µg	70	66	55	55 (RE)	67[1]	30[1]
Vitamin D, µg	1.1	1.2	1.2	1.2	0.055[1]	0.06[1]
Vitamin E, mg	0.9	0.74	1.1	1.1 (ATE)	0.66[2]	0.10[2]
Vitamin K, µg	6.0	6.7	4.5	4.5	2.1[1]	4.9[1]
Vitamin C, mg	10	9	8.3	8.3	4[1]	1.7[1]
Thiamin (B1), mg	0.1	0.1	0.05	0.05	0.02[1]	0.03[1]
Riboflavin (B2), mg	0.14	0.11	0.10	0.10	0.035[1]	0.2[1]
Niacin (B3), mg	0.6	0.5	0.43	0.43	0.15[1]	0.08[1]
Pantothenic Acid, mg	0.75	0.35	0.33	0.33	0.18[1]	0.35[1]
Vitamin B6, mg	0.07	0.06	0.04	0.04	0.009[1]	0.047[1]
Folic Acid, µg	15	11	12	12	8.5[1]	5.0[1]
Vitamin B12, µg	0.15	0.18	0.18	0.18	0.1[1]	0.4[1]

Per 100 mL	Hipp Organic First Milk	SMA First Infant Milk	Aptamil First Milk	C & G First Infant Milk	Breast Milk	Cow's milk
Biotin, µg	2.5	2	1.5	1.5	0.4[1]	3.5[1]
Sodium, mg	30	16	17	18	18[1]	48[1]
Potassium, mg	62	65	66	68	52.5[1]	157[1]
Chloride, mg	44	43	41	41	42[1]	102[1]
Calcium, mg	52	42	49	50	28[1]	120[1]
Phosphorus, mg	31	24	27	28	14[1]	92[1]
Magnesium, mg	5	4.5	5	5	3.5[1]	12[1]
Iron, mg	0.5	0.64	0.5	0.55	0.03[1]	0.046[1]
Zinc, mg	0.7	0.6	0.5	0.5	0.12[1]	0.35[1]
Copper, mg	0.04	0.03	0.04	0.04	0.025[1]	0.01[1]
Manganese, µg	10	10	6	7.5	ND	ND
Fluoride, mg	0.01	<0.005	<0.003	<0.003	ND	ND
Selenium, µg	1.4	1.4	1.2	1.5	ND	ND
Iodine, µg	13	10	13	12	7[2]	ND
L-carnitine, mg	ND	1	1.1	0.9	ND	ND
Choline, mg	10	10	11.1	10	9.0[2]	13.0[2]
Inositol, mg	5	4.5	3.5	3.3	39[2]	13[2]
Taurine, mg	ND	4.7	5.9	5.3	ND	ND
Nucleotides, mg	ND	2.6	3.2	3.2	ND	ND
IgA, mg	0	0	0	0	148[1]	ND
IgM, mg	0	0	0	0	1[1]	ND
IgG, mg	0	0	0	0	1[1]	ND
Lactoferrin, mg	0	0	0	0	150[2]	Trace[2]
Alpha lactalbumin, g	0	0	0	0	0.15[2]	0.12[2]
Beta lactoglobulin, g	0	0	0	0	ND	0.3[2]
Lysozyme, g	0	0	0	0	0.05[2]	Trace[2]
Serum albumin, g	0	0	0	0	0.05[2]	0.03[2]

[1]. International Union of Food Science and Technology, 2000-2010, [2]. Thompkinson & Kharb, 2007, AA - arachidonic acid, ALA - α-linolenic acid, ATE - α-tocopherol equivalents, , DHA - docosahexaenoic acid, , LA - linoleic acid, LCP - long chain polyunsaturated fatty acids, ND – no data, RE - retinol equivalents

Table 2.2 Fat content of human milk and cow's milk (Journal of Tropical Paediatrics, 2014).

Per 100 mL of milk	Human Milk	Cow's milk
Fat, g	4.2	3.9
Cholesterol, mg	16	14
Total saturated fatty acids, mg	2001	2330
Butyric 4:0, mg	0	118
Caporic 6:0, mg	0	74
Caprylic 8:0, mg	Trace	44
Capric 10:0, mg	54	103
Lauric 12:0, mg	213	129
Myristic 14:0, mg	290	413
Palmitic 16:0, mg	1051	959
Stearic 18:0, mg	393	413
Total monounsaturated fatty acids, mg	1612	1244
Myristoleic 14:1, mg	Trace	52
Palmitoleic 16:1, mg	160	100
Oleic 18:1, mg	1408	1026
Total polyunsaturated fatty acids, mg	317	107
Linoleic acid 18:2, mg	285	52
α-Linolenic acid 18:3, mg	32	55

The National Diet and Nutrition Survey (NDNS) evaluates the diet, nutrient intake and nutrient status of the population over 18 months in the UK (Public Health England, 2014). However there is a lack of national data on dietary intake and nutritional status of infants younger than 18 months from this survey (Scientific Advisory Committee on Nutrition, 2011). The Infant Feeding Survey has been carried out every 5 years since 1975 to assess the incidence, prevalence and duration of breast feeding and other feeding practices used by mothers in the UK on infants aged between 4-10 weeks up to 10 months (McAndrew, et al., 2012). Establishing current intake of nutrients in infancy is a priority step to issuing dietary guidelines. There is however debate over which method is best to collect data related to nutrient intake in the paediatric population; food frequency questionnaires and weighted food records are commonly used (Nissensohn, et al., 2011). Over the last 50 years breast feeding rates in the UK have been amongst the lowest in the world. The results from the latest Infant Feeding Survey 2010 indicates that mothers in the UK are breastfeeding their babies for longer; with ⅓ of mothers breastfeeding at 6 months in 2010 compared to only ¼ in 2005. However only 1/100 breastfed exclusively for 6 months, which is the current recommendation set by the DOH. Initial breast feeding has also increased; 62% in 1990, 76% in 2005 and 81% 2010. A higher incidence of breastfeeding was found to be associated with older mothers (>30 years), ethnic minority groups, higher education (left >18 years of age),

managerial or professional occupation and living in the least deprived areas (McAndrew, et al., 2012).

In addition the Infant Feeding Survey showed that 51% of infants were introduced to solid foods by 4 months in 2005; however, in 2010 this reduced to 30% which indicated that more mothers were following recommendations. However by 5 months 75% of mothers had introduced solid foods and by 6 months 94% had been introduced; which indicates that only 6% are following the DOH recommendation to introduce solids at 6 months. The diet and nutrition survey of infants and young children (DNSIYC) 2011 has identified that 58% of children who are fed foods other than milk have eaten a commercial baby or toddler meal (Lennox et al., 2013), indicating that ready-made baby meals are important contributors to an an infant's nutrient supply. The use of ready-made baby foods is most common between 5-10 months. Most frequent foods that are first introduced are fruit and cereals. In England baby rice is the commonest first food (74%) (McAndrew, et al., 2012). However local food traditions will also influence solid food consumption (Caroli, et al., 2012).

After 6 months vitamin drops are recommended for breastfeeding infants or infants receiving less than 500 mL of infant formula per day containing vitamin A, C and D (Food Standards Agency, 2008). Data from the infant feeding survey indicates that a small percentage (<15%) are actually receiving these supplements which may suggest that many infants may be receiving insufficient intakes of these particular vitamins (McAndrew, et al., 2012).

Pregnancy, lactation and infancy are specific times in life when essential nutrients are indispensable and where most requirements are different to any other life stages to support the rapid growth and development (Bryhn, 2006). Most infant nutrient intake recommendations are based on the nutrient composition of breast milk of healthy well-nourished mothers (Hoffman, et al., 2009). This may be inadequate due to differing bioavailability's of nutrients between those present in breast milk and infant formula, as well as breast milk composition varying across the course of lactation, the composition varying between each individual and the variability in the volume of milk (Powers, 1997). Establishing precise daily requirements of nutrients is difficult as there is considerable individual variation. Adult recommendations are based on observational intakes in healthy populations, nutrient balance studies and laboratory blood and tissue status associated with a particular level of intake, activity of an enzyme or functional marker of adequacy (Shergill-Bonner, 2013). There is a need to develop biological, physiological and clinical markers of a

defined specific function. This requires a better understanding of the absorption and metabolism of nutrients specific to the infant population (Powers, 1997). Furthermore many European countries have established their own dietary recommendations, which on the macronutrient level are comparable, however, with regards to the micronutrient level large differences exist and alignment is necessary. Most of the current guidelines on complementary feeding are not evidence based; further research is required to clarify the effects of specific foods or nutrients on growth, development and metabolic status (Nissensohn, et al., 2011).

The total amount of nutrients in a food does not reflect the amount that is available for the body to utilise (Watzke, 1998). Bioavailability is the proportion of a nutrient that can be utilised for normal bodily functions; both physiological and dietary factors influence nutrient bioavailability (Food Standards Agency, 2002). Bioavailability can be divided into 3 phases; phase 1 availability for absorption, phase 2 and 3 retention in the body and utilization in the body dependent on homeostatic control and physiological needs of the individual. Dietary factors include the physical form of the nutrient within the food structure, ease with which the nutrient can be released from the food structure, chemical form of the nutrient, solubility in the lumen and the presence of enhancers and inhibitors of absorption, presence of competitive inhibitors for transport binding proteins or absorption sites and competitive interactions between nutrients. Physiological factors include gastric acidity, luminal redox state, intestinal secretions, the individual's nutrient status, anabolic demands, endocrine effects, genetics, gut micro flora and disease state. Nutritional bioavailability cannot be evaluated in isolation, but must take into account the overall composition of the diet (Watzke, 1998).

Over the past 10,000 years major changes have occurred in our diet, however, our genes have not changed. Nutrition is classed as an environmental factor. The interaction of genetics and environment, nature and nurture is the foundation for health and disease. For instance genes affect nutrient absorption, metabolism and excretion; and also nutrients can affect the expression of genes (Simopoulos, 2002). The data in **Fig. 2.1** illustrates some of the changes that have occurred in our diets.

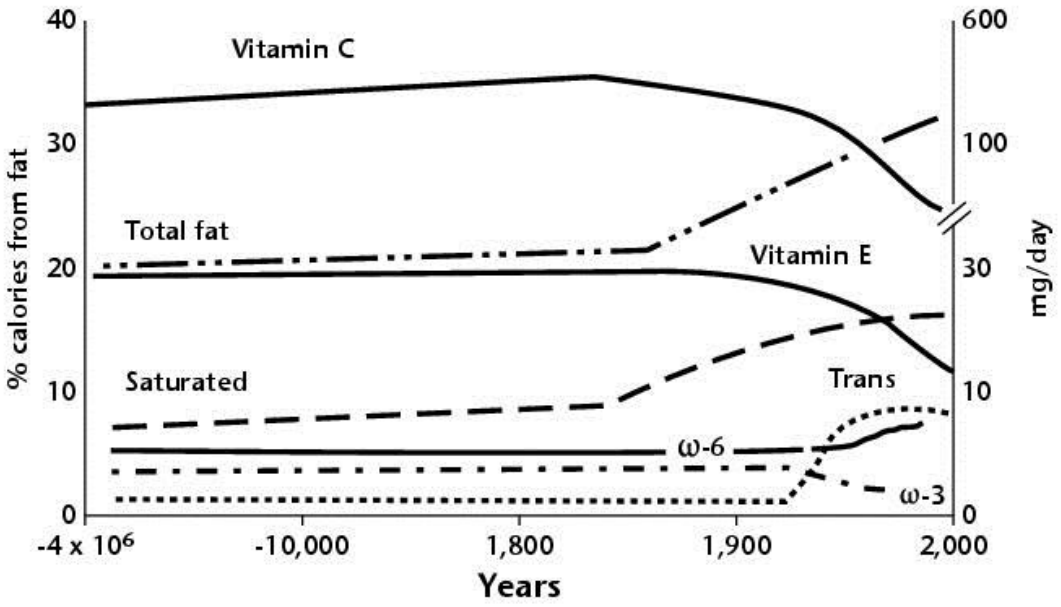

Figure 2.1 Hypothetical scheme of fat, fatty acid (ω6, ω3, *trans* and total) (as percentage of calories from fat), vitamins E and C (mg/d) dietary intake changes (Simopoulos, 2002).

The composition of commercial infant foods can be very different to the diet of the general population and therefore information is required to establish the levels of nutrients present in these food groups. Furthermore the range of commercial infant foods is constantly growing and changing (Committee on Toxicity, 2003). An increasing number of mothers feed their infants with industrially processed infant foods and in recent years the baby food market and the range of products has grown significantly. The first year of an infant's life is a sensitive period for the development of the nervous, reproductive, digestive, respiratory and immune systems. Therefore the composition of nutrient intake of an infant is critical (Pandelova, et al., 2012).

There is currently no clear and complete analytical nutritional data available for 'ready-to-feed' complementary infant foods in the UK. The nutritional database in the UK, McCance and Widdowson contains limited data on the composition of complementary infant foods, in addition the analytical techniques and nutrient data contained may now be outdated. It is essential that food composition tables are regularly updated with the current foods available on the market, especially with the regular introduction of new foods to the market (Food Standards Agency, 2002).

There are many sources of information for mothers regarding infant feeding; such as baby food company websites, blogs, e-forums and books written by mothers; these sources of information often provide recommendations and recipes which are often nutritionally inadequate for the infant's needs (Caroli, et al., 2012).

In summary with numerous diseases being diet related and evidence to associate early infant feeding with long term health outcomes the nutrient intake of an infant's diet is of paramount significance. In addition the lack of data concerning infant's nutrient intake means that it urgently needs to be evaluated. Furthermore as commercial weaning foods are increasingly being incorporated into the infant's diet and nutritional databases lack nutrient content for these particular products, their nutritional adequacy for the rapidly developing infant needs to be assessed.

2.2 Essential Fatty Acids

Over the past two decades there has been a drastic change in the fatty acid composition of the diets in industrialised countries with increased intakes in the *n*-6 fatty acid linoleic acid (LA); this is mainly due to the replacement of saturated fats (from butter and lard) with plant based polyunsaturated fatty acids (PUFA) (from vegetable oils) in an attempt to lower plasma cholesterol (Gibson, et al., 2011). However, at the time the implications this would have on *n*-3 fatty acid metabolism were not considered (Innis, 2008). These changes in diet have been paralleled to the increased rates of numerous diseases that involve inflammatory responses such as cardiovascular disease, obesity, inflammatory diseases and certain psychiatric disorders (Wall, et al., 2010).

Dietary fat is the predominant source of fuel energy for infants, supplying 40-60% of the energy for the first 6 months of life which is gradually reduced to 30-35% by 3 years (Uauy & Dangour, 2009). Both breast milk and the majority of infant formulas provide around 50% of energy as fat. Dietary lipids provide essential fatty acids that are necessary for the absorption of fat soluble vitamins and cholesterol and are also carriers of flavours and contribute to the satiety value (Thompkinson & Kharb, 2007). Traditionally the main focus of research has been upon the total amount of fat in the diet; however it has now been recognised that the quality of dietary lipids in early life can impact on infant growth and development and contribute to long term health consequences (Uauy & Dangour, 2009). Hansen, et al. (1963) firmly established the essentiality of LA in normal infant nutrition, with essential fatty acid deficiency causing dryness and thickening of the skin and growth

faltering. The roles of other essential fatty acids are still being established however their presence in breast milk indicates essential roles for infant development (Uauy & Dangour, 2006).

PUFA are important structural components of all cell membranes and organelles, they can influence membrane fluidity and the behaviour of membrane bound enzymes and receptors. They are also important for a wide variety of other functions including blood pressure, blood clotting, development and function of brain and nervous systems and regulating inflammatory responses (Wall, et al., 2010). Long chain polyunsaturated fatty acids (LCPUFA) are precursors for eicosanoid and docosanoid production for the formation of prostaglandins (PG), prostacyclins, thromboxanes (TX), leukotrienes (LT), resolvins and neuropectins (Uauy & Dangour, 2009). The types of fat in the diet will influence the body composition and ultimately its function and health (Morse, 2012).

2.2.1 Metabolism of Essential Fatty Acids

PUFA contain at least 2 double bonds, they are classified as *n*-3 or *n*-6 depending on the location of the last double bond relative to the terminal end of the molecule (Wall, et al., 2010). Humans must obtain the essential fatty acids α-linolenic acid (ALA) 18:3 (*n*-3) and LA 18:2 (*n*-6) from dietary sources as they lack Δ-15 and Δ-12 desaturase enzymes to produce them. ALA is then metabolised by Δ-6 desaturation, elongation and Δ-5 desaturation to form eicosapentaenoic acid (EPA) 20:5 (*n*-3) in the endoplasmic reticulum; LA is also metabolised by the same enzymes to form arachidonic acid (AA) 20:4 (*n*-6) (Innis, 2008); **Fig. 2.2** illustrates essential fatty acid metabolism. The synthesis of docosahexaenoic acid (DHA) 22:6 (*n*-3) requires a second Δ-6 desaturase step and β oxidation in the peroxisomes. The Δ-5 and Δ-6 desaturases and elongases exhibit higher affinity to metabolize the *n*-3 fatty acids (Wall, et al., 2010). Fatty acid desaturase (FADS1 and FADS2) enzymes are responsible for the expression of the Δ-5 and Δ-6 desaturase enzymes and polymorphisms of these enzymes may alter the conversion capability of an individual (Janssen & Kiliaan, 2014). The majority of PUFA conversion takes place in the liver (Guesnet & Alessandri, 2011). In addition micronutrients such as iron, zinc, vitamin B6 and vitamin E are required for the conversion (Shek, et al., 2012).

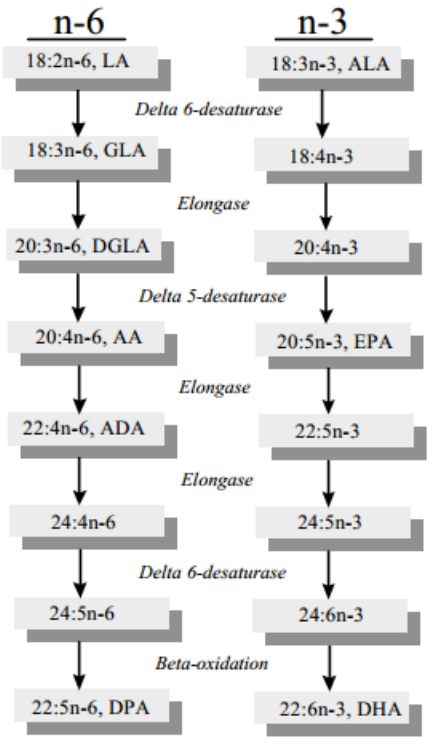

Figure 2.2 Synthesis pathways of *n*-6 and *n*-3 long chain polyunsaturated fatty acids (Minich, 1999). AA – arachidonic acid, ADA – adrenic acid, ALA – α-linolenic acid, DGLA – dihomo-gamma-linolenic acid, DHA – docosahexaenoic acid, DPA – docosapentaenoic acid, EPA – eicosapentaenoic acid, GLA – γ-linolenic acid, LA – linoleic acid.

Endogenous synthesis of LCPUFA from essential fatty acid precursors is inefficient and can vary widely among individuals, approaching zero for certain LCPUFA in some infants. Stable isotope tracer studies have indicated 0.2-8% for EPA and <0.05 – 4% for DHA from ALA; <0.1% for AA from LA (Heaton, et al., 2013). This suggests that LCPUFA may need to be supplied in their pre-formed source in the infant's diet as conversion from their fatty acid precursors is potentially insufficient. There are various areas of evidence that support inefficiency of conversion in infants. For instance, β oxidation of ALA is used for energy rather than conversion to DHA (Innis, 2008) and ALA has a higher affinity for the Δ-6 desaturase enzyme compared to tetracosapentaenoic acid during the conversion to DHA (Gibson, et al., 2011). High levels of LA in the diet generally result in low *n*-3 LCPUFA status, due to simple competitive inhibition. The synthesis of AA is not subject to the same regulatory steps as the synthesis of DHA which requires a second Δ-6 desaturase step and β-oxidation, meaning that synthesis of DHA will be more affected by the inefficient conversion pathway (Makrides, et al., 2000). The fatty acid desaturation pathways are

saturated when the concentration of LA exceeds 3% of total energy; and infant formulas will typically provide more than 3% LA (Innis, 2008). Synthesis of LCPUFA may also be influenced by genetic polymorphisms; therefore a direct dietary supply may be required, which has already been shown to be successful in infants (Agostoni, et al., 2009); for example numerous studies have indicated that DHA status in the blood is responsive to the content of DHA within the diet regardless of the precursor ALA content of the diet (Hoffman, et al., 2009). Conversion depends on the intake of other fatty acids along with the LA:ALA ratio of the diet (Heaton, et al., 2013).

ALA is found mainly in plant oils, including canola, soybean and from walnuts, green leafy vegetables and flaxseed (Heaton, et al., 2013). Whereas LA is widely distributed in the Western diet and found in plant oils such as sunflower, safflower and corn oils along with cereals, animal fat and wholegrain bread, **Table 2.3** shows food sources of essential fatty acids (Wall, et al., 2010). The low dietary intakes of *n*-3 LCPUFA, EPA and DHA, in Western nations are a concern due to their limited availability in the diet. In particular there are few plant sources of DHA and EPA, which may in particular affect vegetarian populations. The synthesis of DHA occurs in phytoplankton and animals but not in plants, therefore DHA is absent in foods of plant origin (Innis, 2008). Infants mainly obtain them from oily fish, fish oil supplements, breast milk and fortified infant formulas (Heaton, et al., 2013). Essential fatty acids are present in all natural lipid structures but are relatively more common in the storage lipids of plants and of marine animals rather than land animals (Department of Health, 1991). The fatty acid content of meat is dependent on the breed, feeding regime, age of slaughter and weight of the animal (Nudda, et al., 2011). Animal feed grains are often high in LA which produces animals high in LA available for meat consumption (Simopoulos, 2002). From an analysis of Italian baby foods, DHA and AA were not detected in beef, veal, chicken or turkey products (Nudda, et al., 2011). Therefore assessment of commercial 'ready-to-feed' infant foods in the UK is required.

Most if not all fish contain at least trace amounts of methyl mercury, which is a neurotoxin that can harm the nervous system; a developing infant's nervous system can be particularly sensitive to it. Substantial evidence over the last decade has shown that fish consumption during pregnancy can benefit the development of the nervous system; whether this is due to the *n*-3 content or other nutrients remains unclear (Food and Drug Administration, 2014). Fish and eggs are natural sources of DHA however due to allergy issues tend to be introduced later into the infant's diet (Agostoni, et al., 2009). Furthermore many women in Western

countries are failing to meet DHA recommendations during pregnancy and lactation (Heaton, et al., 2013).

Table 2.3 Common dietary sources of *n*-3 and *n*-6 essential fatty acids (Heaton, et al., 2013).

Essential fatty acid	Dietary Source
α-linolenic acid	Vegetable oils (linseed, canola, flaxseed, soybean and walnut), fish (herring, salmon and tuna), dark green leafy vegetables
Linoleic acid	Vegetable oils (corn, sunflower, safflower), pork, walnut, peanut, wheat, fish (herring, salmon, tuna)
Docosahexaenoic acid and eicosapentaenoic acid	Oily fish (herring, salmon, trout, tuna), fish oil supplements
Arachidonic acid	Beef, pork, poultry, eggs, whole grain wheat

In the gastrointestinal tract dietary lipids are hydrolysed by lipases to form less water insoluble constituents, these with bile salts form mixed micelles which are absorbed into enterocytes of the small intestine mucosa. Fatty acids are re-acylated into triacylglycerides and assembled into chylomicrons to be transported into the lymph; **Fig. 2.3** illustrates the absorption of fatty acids (Thompkinson & Kharb, 2007).

Lipids in the form of triglycerides are stored in adipose tissue as an energy reserve and contain the highest concentration of lipids in the body. Within adipose tissues LA is the most abundant PUFA, with ALA being the most abundant *n*-3 PUFA, whereas there are only minute amounts of LCPUFA which suggests a limited storage capacity and the need for a continuous dietary supply (Arterburn, et al., 2006a). The brain has the 2nd highest concentration of lipids but in the form of phospholipids of cell membranes (Innis, et al., 2001). EPA is not stored in significant quantities in the brain and therefore is implicated in cardiovascular and immunological health (Ryan, et al., 2010). Lipids such as phospholipids and cholesterol are integral structural components of cell membranes with important physiological functions such as fluidity, permeability, activity of membrane bound enzymes and receptors; and they also can play a direct role in gene regulation (Scientific Committee on Food, 2003).

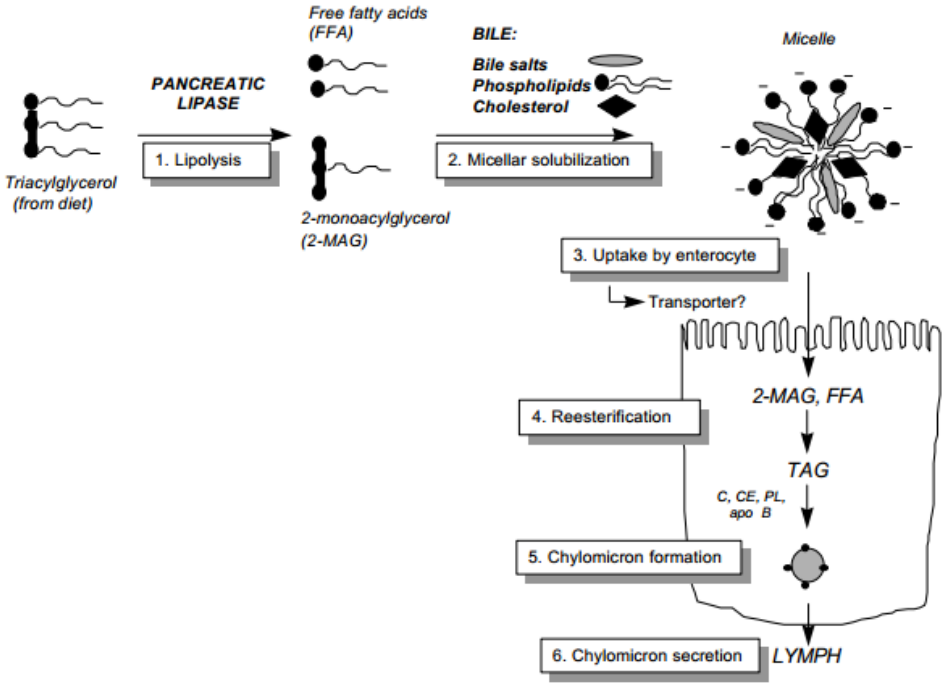

Figure 2.3 Representation of the major steps involved in lipid absorption (Minich, 1999).

2.2.2 Essential fatty acids in breast milk and infant formula

Fats are better absorbed from breast milk rather than infant formula; this may be due to the presence of palmitic acid in the β position in the triglyceride configuration in comparison to the terminal or free fatty acid form found in infant formula. Breast milk PUFA levels vary widely depending on the type of fat consumed by the mother (Thompkinson & Kharb, 2007). Breast milk DHA concentrations have been found to vary considerably between 0.06% and 1.4% of total fatty acids; **Fig. 2.4** shows the variation in DHA in breast milk worldwide. However AA concentrations in breast milk have been found to be less varied, between 0.24% and 1% of total fatty acids possibly due to the regulatory steps involved in their conversion. Mean worldwide averages of DHA and AA found in breast milk are 0.32% and 0.47% of total fatty acids respectively (Hoffman, et al., 2009). Fatty acid effects depend on the length of the carbon chain, degree of unsaturation, *cis* or *trans* configuration, position of double bonds and to a certain extent the position in the triglyceride molecule (Thompkinson & Kharb, 2007).

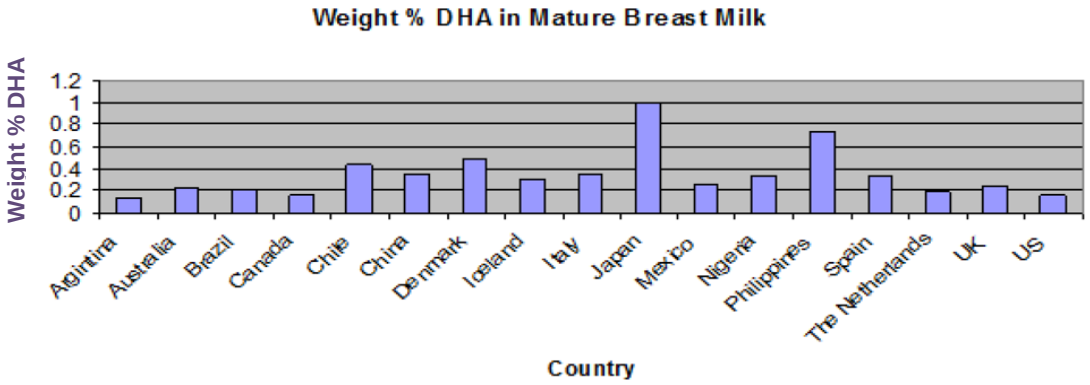

Figure 2.4 Variation in breast milk DHA concentrations worldwide (Morse, 2012).

The fat source in infant formulas is mainly vegetable oils but can also be fish and fungal sources. EU regulations state a minimum of LA (0.3 g/100 kcal) and ALA (0.05 g/100 kcal) to be added to infant formulas as laid down by the Directive 2006/141/EC (Crawley & Westland, 2014). Most LCPUFA in breast milk are supplied as triglycerides (98-99%), with the remainder as phospholipids (Agostoni, et al., 2005).

2.2.3 LA:ALA ratio

Several sources of information suggest that humans evolved on a diet of LA:ALA of around 1, Western diets of today are around 15-16.7:1 (Simopoulos, 2002). The LA:ALA ratio is important as it determines the rate at which LCPUFA are synthesised. It has been shown that by increasing the concentration of ALA in infant formulas the DHA status of the infant is improved, however not to the levels observed in breastfed infants, thought to be due to the direct supply of DHA available in breast milk (Gibson & Makrides, 2000).

The LA:ALA ratio is recommended to be between 5-15:1 (Thompkinson & Kharb, 2007). However these recommendations have been made in the absence of functional or clinical data. Furthermore the ability of infants to convert essential fatty acids (LA and ALA) into LCPUFA has been subject to considerable debate. Early work conducted on infant liver samples measuring Δ-6 desaturase activity indicated that infants may have negligible ability to synthesise LCPUFA. However others have showed that ALA and LA can be converted to their LCPUFA (Makrides, et al., 2000). The majority of data suggests that infants have the endogenous capacity to synthesis LCPUFA but that this capacity is low in comparison to the total need of the rapidly growing and developing infant (Guesnet & Alessandri, 2011). Makrides, et al. (2000) looked at feeding infants with formulas containing different LA:ALA

ratios (10:1 and 5:1) along with breastfed infants to assess infant growth, fatty acid status and visual evoked potential (VEP) acuity. Infants fed the 5:1 formula had higher DHA in plasma and erythrocyte phospholipids, but this was still observed to be less than breastfed infants. There was no difference seen with respect to infant growth or VEP acuity at 16 and 34 weeks. Studies have shown that the LA:ALA ratio needs to be below 6:1 to improve DHA status in formula fed infants, studies varying the LA:ALA in infant formula are shown in **Table 2.4**. Furthermore the feeding of infants after 6 months with adequate ALA but with no DHA does not sustain the same cerebral accretion of DHA as breastfed infants (Guesnet & Alessandri, 2011). This suggests that the LA:ALA ratio does have an impact on LCPUFA status in infancy however to reach the same status as breastfed infants a direct dietary supply is required. In addition even though DHA status was increased this did not have any functional effects with regards to growth or vision. Increased DHA status needs to be associated with a functional outcome to have relevance in infant growth and development. Adequate growth is an important indicator of health in infants, however meta-analysis have shown that LCPUFA have no effect on weight, length or head circumference (Makrides, et al., 2005). The effect of LCPUFA, especially DHA and AA, in relation to benefits on infant's vision and neurodevelopment have given mixed results and will be discussed in the following chapters.

2.2.4 Essential fatty acids: vision and neurodevelopment

The concentration of DHA has been found to be higher in the plasma, erythrocyte membranes and brains (from autopsies of infants) of breastfed infants or infants fed with DHA supplemented infant formula compared to those fed infant formula only containing the precursors (LA and ALA) (Hoffman, et al., 2009; Thompkinson & Kharb, 2007). This suggests that infants require DHA to be provided in its pre-formed source. In breastfed infants a higher mental ability, differences in visual function and neurodevelopment have been observed in comparison to un-supplemented formula fed infants, this is thought to be due to the differences in DHA content (Agostoni, et al., 2009). The question over whether a dietary supply of DHA and/or AA is advantageous for visual or cognitive development have been debated for numerous years (Hoffman, et al., 2009).

Table 2.4 Studies assessing the effect of the LA:ALA in infant formulas with effects on blood composition and visual function in term infants.

Reference	Study Group	Intervention	Assessment	Outcomes
Clark, et al. (1992)	38 term infants	IF 10W supplementation: LA:ALA 19:1 (14% LA, 0.7% ALA) LA:ALA 4:1 (13% LA, 3.3% ALA LA:ALA 3:1 (3.5% LA, 1.1% ALA) BF	Blood composition at 10W.	Decreasing LA:ALA ratio increased *n*-3 C20 and C22 FA in erythrocytes, did not reach BF infant levels (DHA and AA).
Jensen, et al. (1997)	Unknown	Birth – 4M LA:ALA: 44:1, 18.2:1, 9.7:1, 4.8:1	Fatty acid pattern of phospholipids at 21, 60 and 120D. Anthropometric data same as above and 240D. Transient visual evoked responses at 120 and 240D.	4.8:1 had higher plasma phospholipids DHA and lower AA at 21, 60 and 120 days. Weight in 4.8:1 was less than the 44:1. Neither visual evoked response latency nor amplitude differed in any groups.
Makrides, et al. (2000)	176 term infants	Birth – 34W IF LA:ALA 10:1 or 5:1 or BF	FA status, growth assessed at 6, 16 and 34W. Visual evoked potential assessed at 16 and 34W.	5:1 had higher plasma and erythrocyte phospholipids DHA concentrations, still less than BF. Visual evoked potential similar in formula groups and BF. Infant growth did not differ between groups.

AA - arachidonic acid, , ALA - α-linolenic acid, BF – breastfed, DHA - docosahexaenoic acid, IF - infant formula, LA - linoleic acid, , D – days, M – months, W – weeks.

In animals neurophysiological alterations have been observed in *n*-3 deficient animals (Guesnet & Alessandri, 2011). DHA deficiency in animal models is detrimental to the developing brain leading to deficits in neurogenesis, neurotransmitter metabolism, altered learning and visual function (Innis, 2008). Young monkeys fed a deficient *n*-3 PUFA diet have poor visual acuity. Evidence from animal studies suggests that DHA in particular is important for visual and neurodevelopment, however if or to what extent this translates into infants is unknown (Ruxton, et al., 2004).

At birth the infant's brain is fully developed; however, with only 25% of its definitive volume. After birth the brain expands by an increase in glial cells, outgrowth of axons and dendrites and myelination of nerve fibres. The lipid bilayer of neuronal tissues consists of phospholipids with DHA, AA and EPA as the main components (Janssen & Kiliaan, 2014). In addition infants are born with a poorly developed visual system but during the first year of life vision rapidly develops (Uauy & Dangour, 2009).

DHA is the predominant fatty acid in the phospholipid of the photoreceptor cells of the retina (Scientific Committee on Food, 2003) and animal studies show that DHA is essential for the normal development of vision (Hoffman, et al., 2009). DHA is a structural component of the retinal photoreceptor membrane, where it makes up over 50% of the phospholipid content of the retinal membrane bilayer. The DHA concentration affects the enzyme activity of membranes of retinal photoreceptors and therefore their function. DHA is also required for photoreceptor differentiation and the activity of rhodopsin, which is the essential pigment needed for photo transduction in retinal photoreceptors. In animal studies rats fed *n*-3 deprived diets have reduced DHA levels and electroretinogram amplitudes (Qawasmi, et al., 2013).

For pre-term infants DHA has been related to visual function and AA to growth probably due to the insufficient supply of lipids during the final trimester; therefore they are added to pre-term infant formulas; however in term infants the evidence is not as clear which may be due to larger endogenous pools of LCPUFA and slower rates of accretion (Decsi, et al., 1995).

AA and DHA are found in high concentrations in neural tissue and the retina of the eye, which is another factor that has directed the interest of LCPUFA in visual and neurodevelopment, **Fig. 2.5** illustrates the distribution of essential fatty acids in the body. Due to their presence in breast milk, EU regulations permit the addition of DHA and AA to infant formula but inclusion is not mandatory, **Table 2.5** illustrates expert positions on the

DHA and AA concentrations to be added to infant formulas. Since around 2000 LCPUFA have been added to infant formulas at concentrations generally found in breast milk (Crawley & Westland, 2014). There is a dose response relationship between the level of DHA and AA supplemented to infants with the level of DHA and AA present in erythrocyte membrane phospholipids (Qawasmi, et al., 2012). **Fig. 2.6** illustrates the effect of DHA supplementation in mothers and concentrations of LCPUFA in breast milk. Trials examining the potential beneficial effects of DHA and or AA have had mixed results on visual function and neurodevelopment, results from these studies are shown in **Table 2.6**.

Table 2.5 Expert positions for DHA and AA levels in term infant formulas (Hoffman, et al., 2009).

Organisation	**DHA (% fatty acids)**	**AA (% fatty acids)**
British Nutrition foundation	~0.4	~0.4
FAO/WHO expert panel	~0.35	~0.7 (total *n*-6 LCPUFA)
Expert panel convened by ISSFAL	~0.35	~0.5
Child health foundation, Germany	≥0.2	≥0.35
ADA and Dieticians of Canada	≥0.2	≥0.2
World Association of Perinatal Med./Early Nutrition Academy/ Child Health Foundation	0.2-0.5	≥0.2

AA – arachidonic acid, ADA – American dietetic association, DHA – docosahexaenoic acid, FAO – food and agriculture organisation, , ISSFAL – international society for the study of fatty acids and lipids, LCPUFA – long chain polyunsaturated fatty acid, WHO – world health organisation

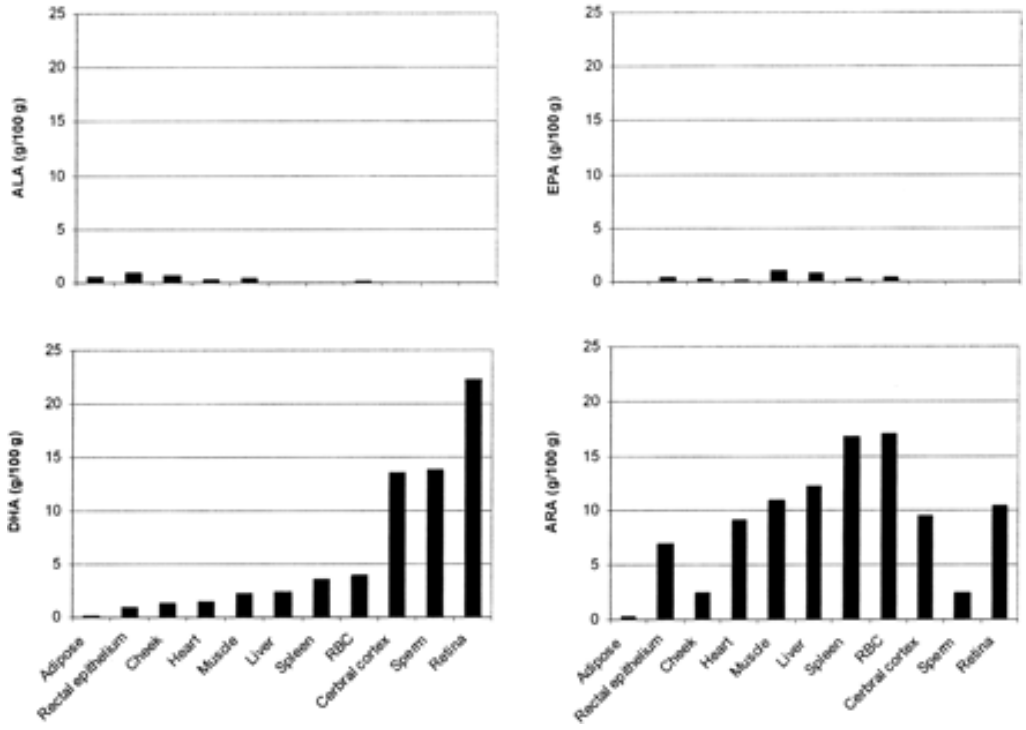

Figure 2.5 Distribution of α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA) in the body (Arterburn, et al., 2006).

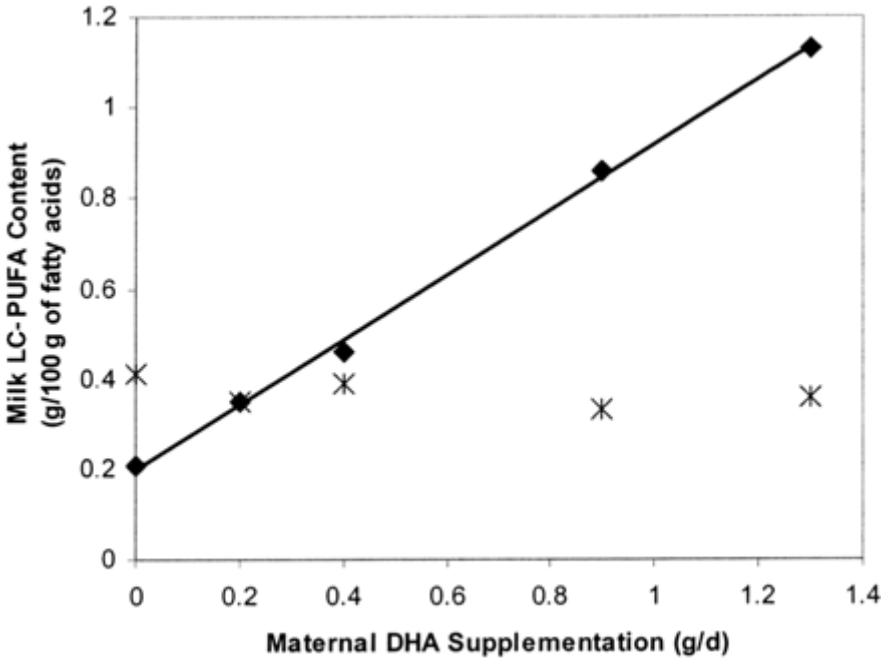

Figure 2.6 Maternal DHA supplementation and effects on breast milk DHA (■) and AA concentrations (X) (Arterburn, et al., 2006).

Table 2.6 Studies assessing the effect of DHA +/- AA supplementation on visual and neurodevelopment in term infants during infancy.

Reference	Study Group	Intervention	Assessment	Outcomes
Agostoni, et al. (1995)	90 term infants (29 supplemented; 31 un-supplemented; 30 exclusively BF)	Supplemented formula contained 0.3% FA DHA + 0.44% FA AA 3D – 4M	Brunet-Lezine psychomotor development test at 4M.	BF and supplemented infants performed significantly higher in Brunet-Lezine test.
Makrides, et al. (1995)	79 term infants (13 supplemented, 18 un-supplemented IF, 23 fully BF and 24 partially BF)	Supplemented IF- blend of fish oil (providing EPA and DHA) and evening primrose oil (providing GLA) until 7.5M	VEP acuity at 16 and 30 weeks BSID.	VEP acuities better in supplemented and BF infants at 16 and 30 weeks. No differences in MDI/PDI.
Carlson, et al. (1996)	58 term infants (19 supplemented ; 20 un-supplemented; 19 BF)	IF supplemented with egg yolk lecithin (DHA 0.1%; AA 0.43%) for 12M	2, 4, 6, 9, 12 M: Grating visual acuity (Teller acuity card), blood FA composition.	At 2M BF and supplemented group had higher grating acuity, no difference at 4, 6, 9 and 12M.
Agostoni, et al. (1997)	81 toddlers (26 supplemented; 30 un-supplemented; 25 exclusively BF)	Supplemented formula contained 0.3% FA DHA + 0.44% FA AA 3D – 4M	Brunet-Lezine psychomotor development test at 24M.	No differences found.
Auestad, et al. (1997)	197 term infants (134 FF; 63 BF).	DHA formula: 0.23 wt % from fish oil. DHA + AA formula: 0.12% DHA and 0.43% AA from egg yolk phospholipids from 1W - 12M	Visual acuity: acuity card procedure and VEP at 2, 4, 6, 9 and 12M.	No difference in visual function.
Gibson, et al. (1997)	52 term infants exclusively BF for 4M	Maternal supplementation with 0, 0.2, 0.4, 0.9 or 1.3 g DHA from algal oil for 4M.	VEP acuity (12 and 16W).	No difference in VEP by diet or DHA status at 12 and 16W.
Birch, et al. (1998)	108 term infants (79 exclusively FF from birth, 29 exclusively BF)	IF: DHA (0.35% FA) or DHA (0.36%) + AA (0.72%) from birth - 4M	6, 17, 26, 52W: sweep VEP acuity, forced choice preferential looking acuity.	DHA/DHA+AA better sweep VEP acuity at 6, 17 and 52 weeks. No difference in forced choice preferential looking acuity.
Jørgensen, et al. (1998)	54 term infants (37 FF and 17 BF)	2W – 4M: Formula 1: 0.3 wt % DHA + 0.5 wt % GLA; formula 2: DHA 0.3 wt %; formula 3: un-supplemented	VEP at 4M.	No difference in visual acuity.

Reference	Study Group	Intervention	Assessment	Outcomes
Scott, et al. (1998)	134 infants	DHA formula: 0.23 wt % from fish oil. DHA + AA formula: 0.12% DHA and 0.43% AA from egg phospholipids from 1W - 12M	Mental and motor scales of the BSID at 12M. Vocabulary and gesture communication scores from the Macarthur Communicative Development Inventories at 14M.	No differences in BSID scores. DHA group had lower scores on 2 of the MacArthur scales (vocabulary comprehension and vocabulary production scales).
Willats, et al. (1998)	44 term infants (21 supplemented; 23 un-supplemented)	Supplemented formula: DHA 0.15-0.2% FA + AA 0.3-0.4% FA from birth - 4M	At 10M means end problem solving test – the intentional execution of a sequence of steps to achieve a goal.	Increase in 3 step problem solving at 10M by supplemented group. No difference in 2 step means end problem solving at 9M.
Lucas, et al. (1999)	447 term infants (155 supplemented; 154 un-supplemented; 138 BF)	Supplemented formula: 0.3% AA and 0.32% DHA from 1st week to 6M	BSID MDI and PDI at 18M Knobloch, Passamanick, Sherrards test at 9M.	No difference in cognitive and motor development.
Birch, et al. (2000)	56 term infants	IF DHA (0.35% FA) or DHA (0.36%) + AA (0.72%) from 5D - 4M.	BSID-II at 18 M.	DHA+AA increase of 7 points on the MDI. DHA/ DHA+AA cognitive and motor MDI subscales age advantage. PDI and BRS showed no difference.
Hoffman, et al. (2000)	116 term infants (87 FF, 29 exclusively BF)	Control formula, DHA supplemented (0.35% FA), DHA + AA (0.36%; 0.72%) from birth – 17W	Electroretinographic responses, VEP at birth, 7, 17 and 52W.	Supplemented formula group had more mature electroretinographic responses at 6W than controls. VEP better in BF and DHA supplemented at 17 and 52W.
Makrides, et al. (2000)	114 term infants (23 DHA; 24 DHA+AA; 21 un-supplemented; 46 BF)	From 1st week of life. DHA: 0.35% from fish oil or DHA + AA: 0.34% both from egg phospholipid	VEP acuity at 16 and 34W. BSID MDI and PDI at 1 and 2Y.	No difference in VEP in formula groups at 34W, better VEP at 4M in supplemented group. BF had better VEP at 34W. No difference in MDI and PDI in formula groups at 1 or 2Y. BF had higher MDI at 2Y.
Auestad, et al. (2001)	404 term infants (77 control, 162 supplemented, 165 BF)	From 1W to 1Y. Egg: 0.14% DHA + 0.45% AA; fish/fungal: 0.13% DHA + 0.46% AA	Visual acuity, information processing, general development, language and temperament at 14M.	No differences at 14M. No difference on Fagan test of intelligence at 6/9M, MDI/PDI at 6/12M, MacArthur CDI at 9/14M No difference in teller acuity card at 2,4,6 and 12M.

Reference	Study Group	Intervention	Assessment	Outcomes
Birch, et al. (2002)	65 term infants FF (32 supplemented, 33 un-supplemented)	Supplemented IF: 0.36% FA DHA + 0.72% AA from 6W to 12M	VEP and stereo acuity at 6, 17, 26 and 52W.	Poorer visual acuity at 17, 26 and 52W and poorer stereo acuity at 17W in un-supplemented.
Auestad, et al. (2003)	276 term infants (65 DHA; 66 DHA + AA; 65 un-supplemented; 80 BF)	From 1W to 1Y. DHA formula: 0.23 wt % from fish oil. DHA + AA formula: 0.12% DHA and 0.43% AA from egg yolk phospholipid	At 39M Stanford Binet IQ, Peabody Picture vocabulary test, mean length of utterance, Beery visual motor index and acuity card procedure.	No difference in IQ, receptive and expressive language, visual motor function and visual acuity.
Bouwstra, et al. (2003)	397 term infant (119 supplemented; 131 un-supplemented; 147 BF)	Supplementation for 2M. Supplemented: 0.18% ALA, 0.03% DGLA, 0.39% AA, 0.06% EPA, 0.23% DHA	General movements at 3M.	Infants in control group had more mildly abnormal general movements.
Helland, et al. (2003)	341 women, 84 children	Mothers to take 10 mL of cod liver oil (1183 mg DHA, 803 mg EPA) or corn oil from 18W of pregnancy until 3M after birth.	Cognitive function tests at 6 and 9M. K-ABC at 4Y.	Children from the cod liver oil group scored higher on the Mental Processing Composite (K-ABC) at 4Y.
Hoffman, et al. (2003)	61 term infants	Infants BF 4-6M and then assigned to supplemented IF (DHA 0.36% + AA 0.72%) or un-supplemented IF until 12M.	VEP acuity and stereo acuity.	VEP acuity was more mature in supplemented group at 1Y. No differences in stereo acuity.
Ben, et al. (2004)	271 term infants (69 supplemented; 124 supplemented + BF; 52 un-supplemented; 26 BF)	From birth – 6M Supplemented: LA, ALA, AA, DHA; 435, 62, 69, 69 mg/dm^3. Unsupplemented: LA, ALA; 440, 44 mg/dm^3	BSID.	No difference in MDI/PDI at 3/6M.
Hoffman, et al. (2004)	51 infants (25 supplemented, 26 un-supplemented)	At 6M infants were assigned 1 jar of baby food containing egg yolk enriched with DHA (116 mg DHA/100g) or control for 1Y	VEP acuity.	Supplemented group were 1.5 lines on the eye chart better than controls at 12M.
Laurite, et al. (2004)	144 mothers (44 olive oil, 53 fish oil and 47 high fish intake mothers)	Low fish intake mothers to receive fish oil (1.3 g/day *n*-3 LCPUFA) or olive oil for 4M	VEP at 2 and 4M.	No difference in visual acuity between the groups.
Ünay, et al. (2004)	80 infants	Birth – 16W: supplemented with 0.5% DHA.	Brainstem auditory evoked potentials at 1 and 16W.	More rapid maturation of auditory brainstem at 16W in supplemented.

Reference	Study Group	Intervention	Assessment	Outcomes
Birch, et al. (2005)	103 term infants	At 5D infants received supplemented (DHA 0.36% + AA 0.72%) or un-supplemented for 1Y.	VEP acuity and stereo acuity.	VEP was better at 6, 17, 26 and 52W in the supplemented group. Stereo acuity was better at 17W but not 39 or 52W.
Bouwstra, et al. (2005)	474 term infants (146 supplemented; 169 un-supplemented; 159 BF)	0.3% FA DHA + 0.45% AA in supplemented from birth – 2M	Follow up at 18M: Hempel assessment. BSID MDI and PDI.	No differences.
Lauritzen, et al. (2005)	122 mothers (low fish intake) randomised plus 53 mothers with high fish intake.	Mothers received fish oil (4.5 g/day) or olive oil (control) from 2W – 4M.	Problem solving ability at 9M. Language at 1 and 2Y.	No difference in problem solving. Passive vocabulary lower in fish oil group at 1Y, no difference at 2Y.
Jensen, et al. (2005)	227 mothers (230 infants)	BF mothers received either high DHA algal oil (200 mg DHA/day) or vegetable oil (no DHA) from birth - 4M	Visual function (teller acuity cards and VEP) at 4 and 8M. BSID at 12 and 30M.	No difference in visual function at 4 or 8M. No difference in visual acuity and neurodevelopmental indexes at 12M. PDI was higher in the supplemented group at 30M.
Birch, et al. (2007)	52 infants available for follow up at 4 Y + 32 BF infants.	Infant formula DHA (0.36% FA) or DHA (0.36%) + AA (0.72%).	At 4Y visual acuity and Wechsler Preschool and Primary Scale of Intelligence.	At 4Y supplemented and BF groups had better visual acuity and verbal IQ than the control. DHA and BF did not differ significantly on verbal IQ scores. Control had poorer IQ scores than BF.
Singhal, et al. (2007)	262 infants (184 FF, 78 BF)	From 1W - 6M. Supplemented IF: 0.32% DHA + 0.3% AA.	At 4-6Y stereo acuity (random dot E test) and visual acuity (Sonksen-Silver system).	Stereo acuity did not differ between formula groups. None of the groups differed in Sonksen Silver visual acuity.
Helland, et al. (2008)	Unknown	Mothers to take 10 mL of cod liver oil (1183 mg DHA, 803 mg EPA) or corn oil from 18W of pregnancy until 3M after birth.	K-ABC at 7Y.	No difference in K-ABC.
Agostoni, et al. (2009)	1160 term infants (580 supplemented; 580 control)	For 1Y 20 mg liquid DHA once a day.	4 gross motor milestones.	Time to achievement of sitting without support was shorter in supplemented group (1 week). No difference in other milestones.
Drover, et al. (2009)	229 infants	Infants fed DHA (0.36% FA) + AA (0.72% FA) formula or control 1-5D -12M; 6W -12 M.	2 step problem solving task.	In 12M and 6W supplemented children had more intentional solutions (successful tasks) and higher attention scores than controls.

Reference	Study Group	Intervention	Assessment	Outcomes
Pivik, et al. (2009)	Unknown	Birth – 6M. 0.15% DHA + 0.4% AA; 0.32% DHA + 0.64% AA.	BSID.	No difference in BSID at 3 or 6M.
De Jong, et al. (2010)	475 term infants (169 control, 146 supplemented, 160 BF)	Birth – 2M Supplemented formula: 0.45% AA and 0.3% DHA	9Y Neurological optimality score, severity and type of minor neurological dysfunction.	No differences between 2 formula groups.
Birch, et al. (2010a)	343 term infants	0% DHA (control), 0.32% DHA, 0.64% DHA, 0.96% DHA (DHA sup formulas also contain 0.64% AA) from birth to 1Y.	VEP acuity at 12M.	Control infants had poorer visual acuity than supplemented infants. No difference between different concentrations of DHA.
Jensen, et al. (2010)	Unknown	BF mothers received either high DHA algal oil (200 mg DHA/day) or vegetable oil (no DHA) for 4M.	At 5Y assessment of gross and fine motor function, perceptual/visual motor function, attention, executive function, verbal skills, visual function.	No difference in visual function (Bailey Lovie acuity chart, transient/sweep VEP). DHA group performed better on the sustained attention subscale of the Leiter International performance scale.
Van Goor, et al. (2010)	119 mothers (42 DHA; 41 DHA + AA; control)	From 17W gestation until 12W postnatal. DHA (220 mg/day); DHA + AA (220 mg/day)	General movements assessed at 2 and 12W.	General movement quality of infants in the DHA group was lower than the other groups, especially at 12W. Infants in the DHA group had more mildly abnormal general movements compared to other 2 groups.
Cheatham, et al. (2011)	122 mothers + 53 high fish intake group	Mothers randomised to fish oil (1.5g/d *n*-3 LCPUFA) or olive oil during first 4M of lactation.	Processing speed, age appropriate Stroop task, strength and difficulties questionnaire at 7Y.	Boys whose mothers consumed fish oil had lower prosocial scores and inhibitory control/working memory scores.
Colombo, et al. (2011)	122 term infants	Birth – 12M 0% DHA (control), 0.32% DHA, 0.64% DHA, 0.95% DHA (DHA formulas also contained 0.64% AA).	At 4, 6 and 9M assessed on visual habituation protocol.	The 2 lowest DHA groups spent proportionately more time engaged in active stimulus processing than un-supplemented, highest dose were intermediate.
Drover, et al. (2011)	181 infants	12M supplementation: 0% DHA (control), 0.32% DHA, 0.64% DHA, 0.95% DHA (DHA formulas also contained 0.64% AA).	BSID II at 18M.	No diet group differences in MDI, PDI, BRS. MDI scores of DHA supplemented infants were higher than control.
Van Goor, et al. (2011)	114 infants (41 DHA; 39 DHA + AA; 34 placebo)	From 17W gestation until 12W postnatal. DHA (220 mg/day); DHA + AA (220 mg/day).	18M minor neurological dysfunction and BSID,	No effect on any outcomes.

Reference	Study Group	Intervention	Assessment	Outcomes
Meldrum, et al. (2012)	420 term infants	From birth – 6M DHA enriched fish oil supplement (250 mg DHA/day, 60 mg EPA/day) or placebo (olive oil).	At 18M BSID-III and child behaviour checklist. Language assessment (Macarthur-Bates communicative developmental inventory) at 12 and 18M.	No difference in BSID-III. Children in fish oil group had higher percentile ranks of later developing gestures at 12 and 18M and total number of gestures.
Colombo, et al. (2013)	81 children (62 supplemented ; 19 control)	0% DHA (control), 0.32% DHA, 0.64% DHA, 0.95% DHA (DHA formulas also contain 0.64% AA).	Tested from 18M every 6M until 6Y with age appropriate standardized and specific cognitive tests.	No difference at 18M on tests of language and performance. Positive effects observed 3-5Y on rule learning and inhibition tasks, the Peabody picture vocabulary test at 5Y and the Weschler Primary Preschool Scales of intelligence at 6Y. No effect on special memory, simple inhibition or advanced problem solving.
Willatts, et al. (2013)	235 children (71 supplemented; 76 control; 88 BF)	1W - 4M. Supplemented: AA 0.35 g/100 g fat; DHA 0.21 g/100 g fat. Unsupplemented: AA <0.1 g/100 g fat	At 6 Y IQ, attention control (day-night test), speed of processing on the matching familiar figures test.	No difference in IQ or attention control. Matching familiar figures test error scores were the same but LCPUFA group was faster at making correct responses.

AA – arachidonic acid, ALA- α-linolenic acid, BF – breastfed, , BRS – behaviour rating scale, BSID – bayley scale of infant development, CDI – communicative development inventories, D – days, DGLA – dihomo-γ-linolenic acid, DHA – docosahexaenoic acid, EPA – eicosapentaenoic acid, FA – fatty acid, FF – formula fed, GLA – γ-linolenic acid, IF – infant formula, IQ – intelligence quotient, K-ABC – Kaufman assessment battery for children, LA – linoleic acid, M – months, MDI – mental development index, PDI – psychomotor development index, VEP – visual evoked potential W – weeks, Y – years

DHA and AA accumulate in the brains grey matter for at least the first 2 years of life. During the final trimester and early post-natal life the brain has a large increase in growth and cellular proliferation (Hoffman, et al., 2009), known as the brain growth spurt, where there is a 30 – 40 fold increase in DHA and AA in the infant forebrain (Qawasmi, et al., 2012); during this time the brain is highly sensitive to extreme variations in DHA supply (Heaton, et al., 2013). **Fig. 2.7** shows DHA accumulation in the infant brain during the first year of life. In humans the foetal and infant brain DHA content appears to be more effected by diet than the AA content, this is potentially due to the endogenous conversion process where DHA requires additional regulatory steps for its synthesis (Agostoni, et al., 2005). The accretion of DHA in the membranes of the central nervous system is required for the optimal development of the retina and brain functions (Guesnet & Alessandri, 2011).

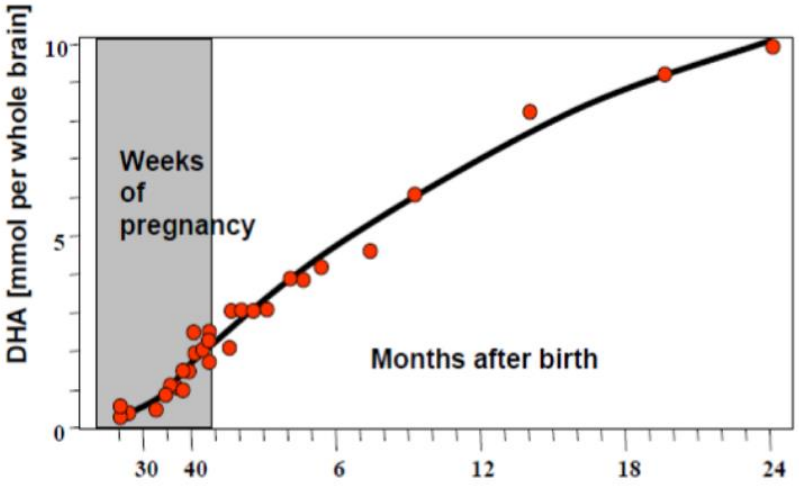

Figure 2.7 DHA accumulation in the infant brain during the first year of life (Morse, 2012).

Both *n*-3 and *n*-6 fatty acids have roles in neuronal growth and affect the development of synaptic processes for neural cell interaction. AA is present in growth cones and synaptosomes. It is released from membrane phospholipids by phospholipase A2, to influence signal transduction events which regulate cone growth and activity which ultimately leads to the conversion of growth cones into mature synaptic endings (Uauy & Dangour, 2006).

The concentration of LCPUFA can alter neuronal membrane fluidity and physical structures of neurons. Both *n*-3 and *n*-6 fatty acids are implicated in the production and activity of several neurotransmitters, such as dopamine and serotonin; effect synaptic transmission and substrate binding to membrane receptors. Neurite outgrowth in hippocampal neurons is enhanced by DHA; this may promote learning due to the hippocampus being a critical region for memory formation. DHA has many roles in the structure of the brain and its function, with the potential to effect neurodevelopment and performance (Heaton, et al., 2013).

Variations in study design may have caused the conflicting results from LCPUFA supplementation studies, shown in **Table 2.6**. Factors that may influence the variation in results include the duration of supplementation, sources of LCPUFA, concentration of LCPUFA supplementation, age that intervention was commenced, age of infant at time of assessment, population characteristics, genetic differences in fatty acid metabolism, other intakes of fatty acids in the maternal and infant diet, infants DHA status at birth and the test method used to assess visual or neurodevelopment as they may not be sensitive enough to evaluate infant development (Guesnet & Alessandri, 2011). Confounding factors may also influence results, for example social economic status has been positively associated with maternal and infant IQ and decision to breast feed (Heaton, et al., 2013). Furthermore factors influencing brain development are complex and multifactorial with numerous potential confounders; such as birth weight, parental education, socio economic status, smoking, DHA levels at birth and PUFA ratios (Agostoni, et al., 2005). It has been suggested that trials using formulas close to the worldwide average are more likely to yield positive results. Also there is some clinical evidence to suggest that an AA:DHA ratio greater than 1:1 is associated with improved cognitive outcomes. Lastly trials involving PUFA may require sufficient amounts of vitamin E, as unsaturation is highly susceptible to peroxidation (Ruxton, et al., 2004).

There are other key micronutrients that have been implicated in brain development in early life such as folate, retinol, iodine, iron, zinc and copper which may also be important to assess when evaluating brain development (Uauy & Dangour, 2006).

ADHD, schizophrenia, autism, depression, dyslexia and anxiety have also been associated with *n*-3 PUFA deficiency; thought to be due to a reduction in neurotransmission processes, for instance involving dopamine and serotonin (Janssen & Kiliaan, 2014; Riediger, et al., 2009).

2.2.5 Essential fatty acids: allergy

Foetal and neonatal periods are key times for immunological adaptation. Components of both the innate and acquired immune systems are present at birth, but in reduced amounts and at reduced activities. Therefore the infant is regarded as immunocompetent but immunologically naïve. The infant must down regulate the predominant T helper (T_h) 2 state which is present at birth, which is believed to be a hall mark for allergy development. The regulation of tolerance and active immune responses are critical to health and failure to regulate these responses can lead to recurrent infections, inflammatory diseases and allergic reactions (Gottrand, 2008).

The idea that *n*-3 LCPUFA are protective against allergic diseases initially arose from epidemiological evidence that Greenland Eskimos have a low prevalence of asthma; which is thought to be a consequence of their diets containing large amounts of oily fish and seafood which are rich in *n*-3 LCPUFA. *n*-3 PUFA have generally been regarded as having anti-inflammatory roles whilst *n*-6 PUFA are regarded as having pro-inflammatory roles (Calder & Miles, 2000). It has therefore been suggested that there is a link between the *n*-3 and *n*-6 PUFA in allergic disease. PUFA act via several mechanisms to modulate immune function. *n*-3 PUFA may alter the T_h cell balance by inhibiting cytokine production, immunoglobulin (Ig) E synthesis and T_h 2 differentiation. In addition they can alter membrane composition, eicosanoid metabolism and gene expression (Shek, et al., 2012). Furthermore observational studies have linked low levels of LCPUFA in breast milk to increased risk of infant atopy (Gottrand, 2008).

The belief that *n*-6 LCPUFA are pro-inflammatory stems from the classic role of membrane phospholipid liberated AA being a precursor for cyclooxygenase (COX) and lipoxygenase (LOX) enzymes (Harbige, 2003). COX and LOX are present in epithelial and inflammatory cells which give rise to different mediators depending on the substrates present, which in turn will regulate the severity and length of the inflammatory response (Shek, et al., 2012). AA can act as a substrate for COX for the formation of 2 series PG and TX, LOX for the formation of 4 series LT and cytochrome P450 monooxygenases to form 19 and 20-hydroxyeicosatetraenoic acid, shown in **Fig. 2.8**. PGE2 has been discussed extensively for its roles in inflammation. EPA and DHA can replace AA in cell membranes, which reduces the conversion of AA derived eicosanoids. EPA is a substrate for COX and 5-LOX; producing 3 series PG and 5 series LT; which are less potent than AA derived eicosanoids (Calder &

Miles, 2000). In addition E series and D series resolvins from EPA and DHA have anti-inflammatory and inflammation resolving actions, **Fig. 2.8**. Resolvins are endogenous, local acting mediators with potent anti-inflammatory and immunomodulatory functions; at a cellular level these include reducing neutrophil infiltration, regulation of the cytokine chemokine axis and reactive oxygen species; to reduce the extent of the inflammatory response. *n*-3 LCPUFA can induce direct alteration of gene expression via modification of transcription factors as they are ligands for nuclear receptors such as peroxisome proliferator activated receptors (PPARα and PPARγ). Nuclear Factor (NF) κB also plays a major role in many inflammatory signalling pathways, controlling numerous cytokines (interleukin (IL)-1, IL-2, IL-6, IL-12, tumour necrosis factor (TNF)-α), chemokine IL-8, monocyte chemo attractant protein 1, adhesion molecules and inducible effector enzymes (nitric oxide synthase and COX2). EPA has the ability to block the actions of NFκB by decreasing the degradation of the inhibitory subunit IκB (Wall, et al., 2010).

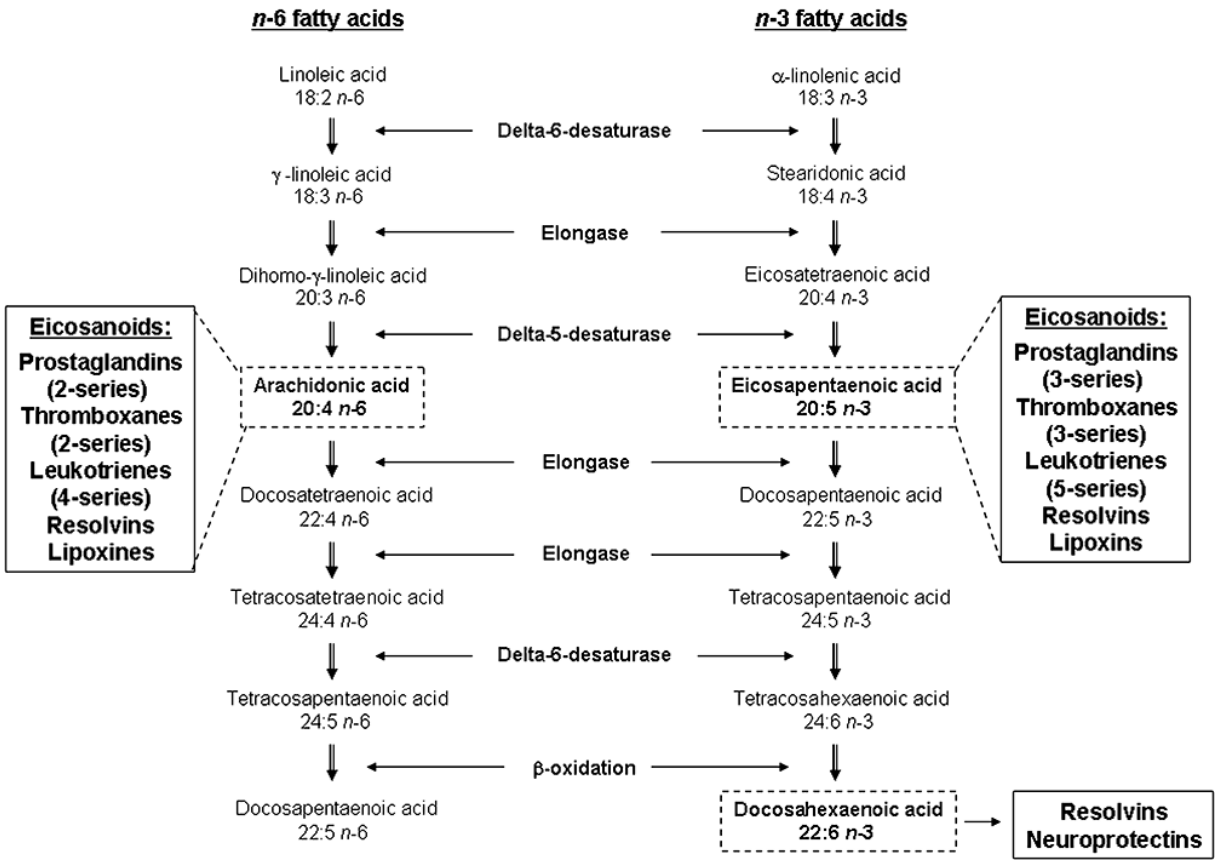

Figure 2.8 Inflammatory mediators derived from AA, EPA and DHA (Wall, et al., 2010).

Mechanisms by which *n*-3 and *n*-6 fatty acids may potentially affect the development of allergy are extensive. *n*-3 fatty acids, depending on the level of intake, reduce the pro-inflammatory cytokines TNF-α, IL-1α, IL-1β, IL-2, IL-6 and IL-8 (Harbige, 2003; Calder, et al., 2006). By altering the membrane phospholipid AA/EPA ratio in favour of EPA the production of TXB2, PGE2 and LTB4 are decreased and the production of LTC5, LTB5 and TXB3 are increased (Calder, et al., 2006). *n*-3 LCPUFA can also affect the expression of cell surface molecules, such as CD25 (IL-2 receptor) and adhesion molecules such as intracellular adhesion molecule 1, vascular cell adhesion molecule 1 and lymphocyte function associated antigen on lymphocytes and monocytes (Harbige, 2003). EPA and DHA can inhibit the inflammatory cascade of Toll-like receptors and TNF-α signalling through the G-protein coupled receptor (GPR120) activation thus elevating intracellular calcium and activation of extracellular signal-regulated kinase cascade (Oh, et al., 2010). Platelet activating factor can also be reduced by fish oil feeding and neutrophil chemotaxis can be inhibited by chemotaxin-stimulated inositol triphosphate (Sperling, 1998) and DHA gives rise to the protectins DP1 (Calder, 2012). *n*-6 fatty acids can alter the DGLA/AA ratio giving rise to an increase in anti-inflammatory PGE1 and decrease LTB4 and in some cases decrease PGE2. Increased PGJ2 by γ-linolenic acid (GLA) 18:3 (*n*-6) feeding has anti-inflammatory effects and with the lipoxins, such as LTA4 have resolving-like effects. GLA feeding has also been shown to increase anti-inflammatory transforming growth factor (TGF) β production and reduce IL-1β and monocyte chemotactic protein 1 (Harbige, 2003; Xiang, et al., 2010). Furthermore reduced adhesion molecule expression, such as CD62+, CD36+ and CD54+ on T cells and monocytes has been observed with GLA feeding (Xiang, et al., 2010). There is also evidence that *n*-3 and *n*-6 fatty acids can affect tolerance induction and via their membrane phospholipid incorporation and metabolites may also affect gut permeability and closure (Harbige & Fisher, 2001).

PGE2 acts on T lymphocytes to reduce the formation of T_h1 cytokines (interferon (IFN) γ and IL-2) with no effect on IL-4 and IL-5 production. IL-4 can promote the synthesis of IgE which may lead to allergic sensitization (Black & Sharpe, 1997). PGE2 has been found to be immunosuppressive at high concentrations whereas at low concentrations it is required for normal T cell function (Gottrand, 2008). It has also been suggested to be protective against airway inflammation and re-exposure to allergens. It inhibits 5-LOX which down regulates LT production and induces 15-LOX which produces lipoxin A4 which has anti-inflammatory roles (Kremmyda, et al., 2011). Therefore it seems that PG such as PGE2 may have dual roles

in allergy. Furthermore the AA metabolite PGJ2 has potent anti-inflammatory effects on inflammatory cytokines (Harbige, 2003). Dihomo-γ-linolenic acid (DGLA) 20:3 (*n*-6) is also a substrate for COX producing PGE1 and 15-LOX which produces 15-hydroxyDGLA. 15-HydroxyDGLA inhibits 5 and 12-LOX producing an anti-inflammatory response. DGLA can also decrease lymphocyte responsiveness and suppress the production of IL-2, IL-1β and TNF-α (Calder & Miles, 2000).

Wright & Bolton, (1989) observed higher proportions of LA and lower amounts of DGLA in the breast milk lipids of mothers of children with atopic eczema. In agreement with Ioppi, et al. (1994), Thijs, et al. (2000) found lower levels of AA in the breast milk of atopic mothers compared with non-atopic mothers. Thijs, et al. (2000) also found that supplementation of atopic mothers with GLA, which bypasses the rate limiting Δ6 desaturase step, increased DGLA but not AA in breast milk. Low *n*-6 breast milk fatty acids in atopic mothers were also reported by Duchen, et al. (2000) and low AA levels were found in a study by Johansson, et al. (2011). In the Netherlands an intervention study in infants with a maternal history of atopy were supplemented with *n*-6 rich borage oil (100 mg GLA/day) or sunflower oil as placebo, for the first 6 months of life. Although the use of sunflower oil is questionable because it contains the precursor *n*-6 PUFA LA, the results did not show any difference in total IgE and positive serum IgE food allergen tests. There was also no association between the presence of atopic dermatitis and supplementation at 1 year. GLA, DGLA and AA were higher in the plasma phospholipids of the intervention group. This study was conducted due to the finding that low concentrations of *n*-6 LCPUFA in umbilical cord blood may precede the development of atopic dermatitis, potentially due to reduced conversion of LA to GLA by Δ6 desaturase (Van Gool, et al., 2003). It has also been observed that mothers of atopic infants have lower concentrations of *n*-6 LCPUFA in their breast milk. It is important to consider that if levels of *n*-6 LCPUFA are lower in certain allergic individuals then reducing the *n*-6 content in the diet may cause detrimental effects.

There have also been published reports of lower *n*-3 LCPUFA in the breast milk of atopic mothers (Duchen, et al., 2000; Hoppu, et al., 2005; Johansson, et al., 2011) whilst some studies have found no differences between breast milk *n*-6 and *n*-3 fatty acids from atopic and non-atopic mothers (Lauritzen, et al., 2006; Wijga, et al., 2002). Thus most, but not all studies, have found changes in the *n*-6 and *n*-3 LCPUFA in the breast milk of atopic mothers. Cord blood *n*-3 and *n*-6 LCPUFA have also been shown to be lower in several studies (Beck, et al., 2000). However total *n*-3 LCPUFA and total *n*-6 LCPUFA from cord serum

phospholipids were found to be higher in subjects who later went on to develop respiratory allergy or atopic eczema compared to controls that did not develop allergy (Barman, et al., 2013). Furthermore no association could be confirmed between the FADS1 and FASD2 gene polymorphism and eczema (Rzehak, et al., 2010). It is however possible that the abnormalities in breast milk fatty acids reported in some studies may be related to other factors. For example zinc is known to be an important factor in the desaturation of the precursor fatty acid LA to AA and the conversion of LA to AA is normally correlated in breast milk (Xiang, et al., 2007). To our knowledge there have been no published studies of breast milk zinc and fatty acids levels in atopic mothers.

Maternal fish intake during pregnancy has consistently demonstrated a protective effect on atopic disease and allergy in infants and children (Best, et., 2015). However when fish intake has been studied during infancy the effects have been inconsistent, nevertheless many studies have demonstrated protective effects. Fish oil supplementation studies in infants have caused a higher *n*-3 LCPUFA status; however, currently it is unclear what clinical significance this has. Fish studies have provided more promising outcomes, possibly due to other nutrients being present in comparison to fish oil intervention studies (Shek, et al., 2012). **Table 2.7** summarises the fatty acid intervention studies that have been conducted in term infants with effects on allergy.

There have been numerous epidemiological based studies looking at fish intake during infancy and its effects on allergic outcomes. If the hypothesis that a lower consumption of *n*-3 LCPUFA plays a role in the rise in allergy then higher intakes from foods containing *n*-3 LCPUFA such as fish, seafood or fish oil supplementation should clinically alleviate allergic outcomes. There is some evidence to support this hypothesis however other studies have given contradictory outcomes.

There are numerous limitations to the studies conducted so far. In many of the studies there is a distinct lack of information on the type of fish consumed and the actual consumption, thus the dietary intake of various *n*-3 LCPUFA is likely to vary considerably between these studies. However Jong, et al (2012) concluded that the amount of fish consumed had no effect on the study outcome. Whereas contradictory conclusions on the type of fish have been noted; Alm, et al. (2009) found no effect with the type of fish consumed, whereas Storro, et al. (2010) found that oily fish showed a stronger association. Furthermore, if the effects from studies are due to the *n*-3 LCPUFA content of the fish then the type of fish would be expected

to make a difference as oily fish contain much higher levels of EPA and DHA. However fish contain numerous other nutrients which may also be associated with differential effects, such as vitamins, minerals and proteins. In addition, cod liver oil has a high content of *n*-3 LCPUFA therefore if the beneficial effects from fish were due to *n*-3 LCPUFA then cod liver oil should produce beneficial effects which have not been the case (Nja, et al., 2005; Storro, et al., 2010). However, these studies do indicate that the timing of introduction of fish maybe important within the first year of life, this strengthens the idea of a critical window of opportunity in early life. Most of the results are from parental questionnaires and parental diagnosis rather than clinical diagnosis which could be inaccurate. There is also the issue of reverse causation; fish introduced later in infants who are prone to allergy. The study group itself will also be important due to the risk of allergy. Further studies are needed to look at whether the timing of introduction or the amount or type of fish, has any effect and whether the total dietary intake of *n*-3 fatty acids and *n*-6 fatty acids have an impact on allergic disease and to establish whether fish oil supplementation has any long term protective effects. Furthermore problems with some studies also include changes made in diet after diagnosis. Therefore although LCPUFA are known to be immunomodulatory their role in allergy based on the studies at present are controversial.

2.2.6 Essential fatty acids: bone health

LCPUFA have also been implicated in bone metabolism and bone health. *n*-3 fatty acids have been shown to enhance the activity of osteoblasts (cells that synthesise bone) and inhibit the activity of osteoclasts (cells that resorb bone tissue). Bone formation and resorption are locally regulated by eicosanoids, cytokines and growth factors and systemically through hormones. LT are stimulators of bone resorption and inhibit osteoblast proliferation. PGE2 is a potent stimulator of bone resorption (Committee on Toxicity, 2004).

Table 2.7 Fatty acid studies in term infant's effects on allergy and infection.

Nutrient/ Food	Study Design	Study Group	Intervention	Diagnosis	Outcomes	Reference
CLO	Cross sectional study	502 infants	No intervention	Clinical examination, SPT and parental questionnaire. CLO supplementation 0-11 months	Daily intake of CLO supplements in 1st Y of life did not decrease the risk of asthma. Increased prevalence of allergic sensitization with early CLO supplements.	Nja, et al. (2005) Norway
CLO and fish	Prospective birth cohort study	3086 infants	No intervention	Information on exposure to fish during pregnancy and 1st Y assessed at 1Y. Parental questionnaire at 2Y. Semi quantitative FFQ.	Negative association between infant consumption of fish at 1Y and parental reported eczema at 2Y, strongest association with oily fish; no association with asthma. No significance with CLO consumption during 1st Y and eczema and asthma at 2Y.	Storro, et al. (2010) Norway
DHA/AA	Observation study	1342 infants	DHA (17 mg/100kcal) + AA (34 mg/100kcal); DHA (8 mg/100kcal) + AA (13 mg/100 kcal); DHA (16 mg/100 kcal) + AA (6 mg/100kcal) or unsupplemented formulas.	Anthropometric assessments, medical history, illnesses during the 1st Y of life	Higher incidence of bronchiolitis/ bronchitis in lower DHA/AA and unsupplemented formula groups at 5, 7 and 9M. Higher incidence of AR at 1M and upper airway infection at 1 and 12M in unsupplemented group.	Pastor, et al. (2006) Spain
DHA/AA	Randomised double blind study	89 infants	Infants received either DHA/AA supplemented formula (DHA 17 mg/100kcal, AA 34 mg/100kcal) or placebo from ≤5D – 1Y.	Nurses reviewed children's medical charts.	DHA/AA group had delayed onset and reduced occurrence of upper respiratory infections and common allergic disorders by 3Y.	Birch, et al. (2010b) US
DHA/AA	Observational study	325 infants	Infants received formula containing DHA (17 mg/100kcal) + AA (34 mg/100kcal) or unsupplemented.	Respiratory illnesses, otitis media, eczema and diarrhoea during 1st Y.	Infants fed DHA/AA had lower incidence and lower odds ratio of having at least 1 episode bronchiolitis/ bronchitis, croup, nasal congestion, cough and diarrhoea requiring medical attention.	Lapillonne, et al. (2014)

Nutrient/ Food	Study Design	Study Group	Intervention	Diagnosis	Outcomes	Reference
Fish	Prospective birth cohort study	2531 Infants	No intervention	Parental questionnaires including food exposure during 1st Y of life. AR, AD and asthma at 4Y diagnosed by physician.	At 4Y AR was lower in children who had fish during the 1st Y of life compared to those who had it later; similar trend for asthma but not statistically significant. All children introduced to fish in the 1st 6M of life did not develop asthma or AR. AE at 4 years lower among children with early fish introduction.	Nafstad, et al. (2003) Norway
Fish	Prospective birth cohort	3670 infants	No intervention	Parental questionnaires including fish introduction and consumption in 1st Y of life. At 4Y clinical examination and blood sampling.	Regular fish (≥ twice/month) consumption during the 1st Y of life associated with reduced risk for allergic disease (asthma, eczema, AR) and sensitization at 4Y. Inverse association between age of fish introduction; children who received fish between 3-8 months had reduced allergic disease outcomes compared to children who were introduced from 9 months onwards. IgE sensitization to fish was rare at 4Y (18/2614).	Kull, et al. (2006a) Sweden
Fish	Prospective longitudinal cohort	4921 infants	No intervention	Parental questionnaire at 6 and 12M.	Beneficial effects of introducing fish before 9M on eczema. No influence on the type of fish (lean/white or fat/oily).	Alm, et al. (2009) Sweden
Fish	Birth cohort	184 infants with parental history of allergic disease	No intervention	Diaries documented feeding practices. Parental interviews at 6 and 12M. Clinical examination and sensitization tests at 18M.	Earlier fish introduced lower frequency of eczema. Asthma showed similar pattern, not statistically significant. Sensitization not affected.	Hesselmar, et al. (2010) Sweden
Fish	Birth cohort	994 infants (susceptible to type I diabetes)	None	Questionnaire and blood samples at 5Y. Dietary questionnaires up to 5Y.	Late introduction of fish (>8.2M) associated with sensitization to food allergens and inhalant allergens.	Nwaru, et al. (2010) Finland

Nutrient/ Food	Study Design	Study Group	Intervention	Diagnosis	Outcomes	Reference
Fish	Birth cohort	1293 infants (susceptible to type 1 diabetes)	None	Structured dietary questionnaires up to 2Y. Persistent asthma and AR assessed at 5Y by questionnaire.	Early introduction of fish showed a dose dependent association with decreased risk of AR.	Virtanen, et al. (2010) Finland
Fish	Prospective longitudinal cohort study	4496 infants	No intervention	Parental postal questionnaires at 6, 12M and 4.5Y.	Risk of AR at 4.5Y reduced by fish introduction before 9M.	Alm, et al. (2011) Sweden
Fish	Prospective longitudinal cohort study	4496 infants	No intervention	Parental postal questionnaires at 6, 12M and 4.5Y.	Introduction of fish before 9M reduced the risk of recurrent wheeze at 4.5Y.	Goksor, et al. (2011) Sweden
Fish	Population based birth cohort	7210 infants	No intervention	12 and 14M timing of introduction of fish assessed. Semi quantitative FFQ at 14M to look at fish consumption. Physician interview for asthma like symptoms assessed at 36 and 48M.	Fish introduction at 6-12M associated with lower risk of wheeze at 48M compared to later introduction. Introduction of fish before 6M associated with increased risk of wheeze at 48M. Amount of fish not associated with asthma like symptoms at 36/48M.	Jong, et al. (2012) Netherlands
Fish	Birth cohort	3781 infants (susceptible to Type 1 diabetes)	None	Dietary questionnaires up to 2Y. At 5Y questionnaire on allergy history and blood sample for IgE analysis.	Introduction to fish <9M inversely associated with asthma, especially atopic asthma, AR and atopic sensitization.	Nwaru, et al. (2013) Finland
Fish	Prospective birth cohort	3285 infants	No intervention	Parental questionnaires, clinical examination and blood sampling.	Children who consumed fish regularly (≥2-3/M) in the 1st Y had reduced risk of asthma, rhinitis and eczema compared to those who had irregular intakes (≤1/M).	Magnusson, et al. (2013) Sweden
Fish	Prospective study	256 infants with family history of allergy (parent or sibling)	3 intervention groups: dietary counselling + probiotic (LB rhamnosus), dietary intervention + placebo (microcrystalline cellulose and dextrose anhydrate), control + placebo.	Semi structured interviews up to 4Y. Food diary about introduction of complementary foods. SPT. Physician examination for eczema.	Introduction of fish > 7M, compared to before increased atopic eczema. After controlling for parental perceptions the results were no longer significant.	Niinivirta, et al. (2014) Finland

Nutrient/ Food	Study Design	Study Group	Intervention	Diagnosis	Outcomes	Reference
Fish oil	Randomised trial	64 healthy infants	Infants received CM/IF alone or with FO (571 mg EPA, 381 mg DHA/day) (3.4 ± 1.1 mL/day) from 9-12M	FA composition of erythrocyte membranes, plasma IgE, CRP and soluble IL-2 receptor concentrations measured. Parental interviews.	FO supplementation increased erythrocyte *n*-3 concentrations and decreased *n*-6. No effect on plasma CRP, IgE, soluble IL-2 receptor concentrations at 12M or fecal IgA at 10M. Higher IFN-γ and lower IL-10 production in whole blood after FO supplementation.	Damsgaard, et al. (2007) Denmark
Fish oil	RCT	145 infants with family history of allergy	Mothers received either *n*-3 group (1.6g EPA and 1.1g DHA/day) or placebo group (soybean oil capsules) from 25 weeks gestation until 3.5 months of BF	Clinical examinations, SPT, analysis of plasma phospholipid fatty acids, IgE asc eczema and food allergy.	At 1Y decreased risk of positive SPT (15 v 32%), allergic sensitization to egg (12 v 29%), food allergy (2 v 15%) and IgE asc eczema (8 v 24 %) in *n*-3 group.	Furujelm, et al. (2009) Sweden
Fish oil	RCT	145 infants with family history of allergy	Mothers received either *n*-3 group (1.6 g EPA and 1.1 g DHA) or placebo group (soybean oil) from 25W gestation until 3.5M of BF	Clinical examinations, SPT, analysis of plasma phospholipid fatty acids.	At 2Y no difference in prevalence of allergic symptoms. Cumulative incidence of IgE associated disease lower in *n*-3 group (13% v 19%). Higher DHA and EPA associated with lower prevalence of IgE associated disease in dose dependent manner.	Furuhjelm, et al. (2011) Sweden
Fish oil	Double blind RCT	150 infants with allergic mothers	Infants received 650 mg FO (280 mg DHA, 110 mg EPA) or placebo (650 mg olive oil) from birth – 6M	Blood samples at 6M. FA levels, induced cytokine responses, T cell subsets and monocyte HLA-DR expression assessed at 6M. Allergy assessed by questionnaires and clinical examination at 6 and 12M. SPT at 12M.	FO group DHA/EPA higher, AA lower in erythrocytes. Plasma DHA/EPA levels higher in the FO group. Eczema at 6/12M did not differ between groups, lower plasma DHA levels predictive of eczema at 12M, erythrocyte EPA predictive of eczema at 12M. FO reduced allergen specific HDM IL-13 responses, increased mitogen (PHA) induced IFN-γ and TNF.	D'Vaz, et al. (2012a) Australia

Nutrient/ Food	Study Design	Study Group	Intervention	Diagnosis	Outcomes	Reference
Fish oil	Double blind RCT	420 (218 FO, 202 placebo) infants with allergic mothers.	Infants received FO supplement (280 mg DHA and 110 mg EPA) or placebo (olive oil) for 6M	Blood samples taken at 6M. Clinical examination at 6 and 12M. SPT at 12M.	Plasma/ erythrocyte DHA/EPA higher in FO group, erythrocyte AA lower. No difference in allergic outcomes between 2 groups at 12M. Infants with higher erythrocyte EPA and higher EPA:AA, higher DHA at 6M less likely to develop eczema at 12M. Higher plasma DHA and total *n*-3 PUFA at 6M associated with reduced risk of recurrent wheeze at 12M.	D'Vaz, et al. (2012b) Australia
Full cream/ milk products/ butter/ Fish	Prospective birth cohort study	3291 infants	No intervention	FFQ data at 2Y and asthma reported symptoms at 3Y.	Daily consumption of full cream milk, milk products and butter at 2Y significantly associated with low rates of asthma and wheeze at 3Y. Eating fish at least once a week at 2Y not associated with asthma or wheeze at 3Y.	Wijga, et al. (2003) Netherlands
GLA	Double blind randomized placebo controlled trial	118 infants with maternal history of atopic disease	Infants received borage oil supplement (100 mg GLA) or placebo (sunflower oil) daily for the first 6M of life (FF from 2W)	Blood collected at 1W, 3, 6 and 12M. Test for total IgE and allergen tests at 1Y. Clinical diagnosis of AD at 1Y by dermatologist.	At 3 and 6 M GLA, DGLA and AA were higher in the borage oil group. IgE concentrations and positive allergen tests did not differ. No association with presence of AD.	Van Gool, et al. (2002) Netherlands
n-3 supplements and oils	RCT	554 infants at high risk of asthma	Dietary intervention group: *n*-3 rich tuna fish oil supplement (500 mg; 184 mg *n*-3; 30 mg EPA, 128mg DHA) to give to child from 6M to 5Y (if bottle fed < 6M added to formula). Canola oils and spreads for food preparation. Control: Sunola oil supplements and poly unsaturated oils and spreads for food preparation	At 18M clinical assessment by a nurse, interview administered questionnaire to parents, 3D weighed food record. SPT, blood collection for total serum IgE, PBMC and plasma FA.	*n*-3 FA proportion higher in plasma of the intervention group, *n*-6 FA proportion higher in control group. *n*-3:*n*-6; intervention 1:5, control 1:7.14. The intervention group showed 9.8% reduction in prevalence of any wheeze, 7.8% reduction in prevalence of wheeze > 1/W. No effect on serum IgE concentration, atopy or doctor diagnosed asthma.	Mihrshahi, et al. (2003) Australia

Nutrient/ Food	Study Design	Study Group	Intervention	Diagnosis	Outcomes	Reference
n-3 supplements and oils	RCT	526 infants at high risk of asthma	Same as above	At 3Y clinical assessment and interview administered questionnaire to parents, semi quantitative FFQ. SPT, blood samples for total IgE.	*n*-3 in plasma higher in the intervention group. *n*-3:*n*-6; intervention 1:5.6, control 1:7.7. 10% reduction in the prevalence of cough in atopic children in intervention group; no effect in non-atopic children. No significant difference on wheeze. No difference in mean total IgE.	Peat, et al. (2004) Australia
n-3 supplements and oils	RCT	516 infants at high risk of asthma	Same as above	At 5Y children examined for eczema, spirometric lung function and respiratory system resistance measured. SPT. Parental interview.	*n*-3:*n*-6; intervention 1:5.8, control 1:7.4. No difference on any of the clinical lung function or allergic outcomes at 5Y. No reduction in asthma, wheeze, cough, rhinitis, eczema or atopic sensitization at 5Y.	Marks, et al. (2006) Australia
n-3 supplements and oils	RCT	450 infants at high risk of asthma	Same as above	At 8Y parental interview questionnaire, spirometry lung function test, SPT. NO levels (airway inflammation). Blood samples for total and specific IgE and plasma FA levels.	*n*-3:*n*-6; intervention 1:6.9, control 1:7.3. Prevalence of all clinical outcomes at 8Y did not differ between groups.	Toelle, et al. (2010) Australia
Organic Foods	KOALA birth cohort study	2764 infants	No intervention	Parental questionnaires at 3, 7, 12 and 24M. Venous blood sampling at 2Y for total and specific IgE.	No association between organic meat, vegetables or egg consumption in the first 2Y of life and eczema, wheeze or atopic sensitization. Organic dairy consumption associated with reduced eczema risk.	Kummeling, et al. (2008) Netherlands

AA – arachidonic acid, AD – atopic dermatitis, AE – allergic eczema, AR – allergic rhinitis, CFU – colony forming unit, CLO – cod liver oil, CM – cow's milk, CRP – C reactive protein, D – days, DGLA – dihomo-γ-linolenic acid, DHA – docosahexaenoic acid, EPA – eicosapentaenoic acid, FA – fatty acid, FF – formula fed, FFQ – food frequency questionnaire, FO – fish oil, GLA – γ-linolenic acid, HDM – house dust mite, HLA-DR – human leukocyte antigen D related, IF – infant formula, IFN-γ – interferon γ, M – months, NO – nitric oxide, PBMC – peripheral blood mononuclear cell, PHA – phytohemaglutinin, RCT – randomised control trial, SPT – skin prick test, TNF – tumour necrosis factor, W – weeks, Y – year,

2.2.7 Essential fatty acids: cardiovascular and obesity

In early studies, Greenland Eskimos were found to have lower cardiovascular disease risk, thought to be due to their high fish intake, which is rich in *n*-3 LCPUFA. Furthermore Japanese populations consume more fish than North America and they have lower rates of myocardial infarction and atherosclerosis (Wall, et al., 2010). Individual's cardiovascular disease risk profile in later life is influenced by dietary habits in childhood (Uauy & Dangour, 2009). *n*-6 fatty acids also have the ability to promote the differentiation of pre-adipocytes and exposure to high intakes during the period of fat cell formation either in utero or infancy has the potential to cause excess fat deposition which could lead to obesity (Gibson, et al., 2011). Forsyth, et al. (2003) conducted a study evaluating DHA and AA supplemented infant formula and found lower blood pressure at 6 years of age.

Research has shown that either by increasing EPA though increasing the dietary ratio of *n*-3:*n*-6 or by directly increasing dietary intake with EPA or DHA reduces the incidence of numerous chronic diseases which involve inflammatory responses, such as cardiovascular disease (Wall, et al., 2010).

2.2.8 Essential fatty acid recommendations

The human need for dietary PUFA is unquestioned but accurate definition of requirements is needed. Most expert nutrition committees have based fatty acid recommendations on the composition present in breast milk, the Institute of Medicine and the Food and Agriculture Organisation recommendations are shown in **Table 2.8** and suggested Actual Intakes (AI) for infant formula and diet illustrated in **Table 2.9**. Recommendations for LCPUFA are difficult to determine as they can be synthesized from precursors. Furthermore there are no plasma concentrations or clinical tests recognised for LCPUFA deficiency or insufficiency. There is a need to associate a functional outcome to a LCPUFA status for recommendations to be made. An absence of LA in the diet results in growth retardation and dermatological abnormalities and its essentiality has been recognised for many years (Gibson & Makrides, 2000). DHA may be more important for direct dietary supply due to its reduced endogenous conversion from its precursor compared to other LCPUFA.

Table 2.8 Institute of Medicine and Food and Agriculture Organisation recommended dietary intakes of total fat and fatty acids during infancy (Huffman, et al., 2011).

Age	Total Fat	ALA	LA	LA:ALA	DHA
IOM (2005) 0-6M	-	500 mg/day (AI)	4.4 g/day (AI)	5-15:1	
7-12M	-	500 mg/day (AI)	4.6 g/day (AI)		
1-3Y	30-40%	0.6-1.2% E (AI) 700 mg/day (AI)	5-10% energy (AI) 7 g/day (AI)		
FAO (2010) 0-6M	40-60% E	0.2-0.3% E (AI)			0.1-0.18% E (AI)
6-24M	35% E	0.4-0.6% E (AI) 0.3-0.6 g/day	3-4.5% E (AI) 2.1-4.2 g/day		10-12 mg/kg (AI)
2-4Y	25-35% E	≥0.5% E (AI)	2-3% E (AI)		EPA + DHA 100-150 mg/day (AI)

AI – actual intake, ALA – α-linolenic acid, DHA – docosahexaenoic acid, E – energy, FAO – food and agriculture organisation, IOM – institute of medicine, LA – linoleic acid, M – month, Y – year

Table 2.9 Actual intakes recommended for infant formula/diet (Simopoulos, 2002).

Fatty Acid	Percentage of fatty acids
LA	10
ALA	1.5
AA	0.5
DHA	0.35
EPA	<0.1 (upper limit)

AA – arachidonic acid, ALA – α-linolenic acid, DHA – docosahexaenoic acid, EPA – eicosapentaenoic acid, LA – linoleic acid

In 2003 the Scientific Committee on Food stated ‘whilst DHA may have potentially beneficial effects on visual acuity, no consensus could be reached that DHA or AA or both are indispensable nutrients for term infants nor that a direct dietary supply is beneficial.’ However in 2010, the EFSA made the following statement ‘DHA has a structural and functional role in the retina and DHA intake contributes to visual development of infants up to 12 months.’

2.3 Fat Soluble Vitamins

Vitamins are indispensable micronutrients which have numerous important roles and regulate many biochemical functions within the body. There are 13 vitamins in total; vitamin A, D, E and K are lipid soluble vitamins and can be stored in the body; whilst the others are water soluble vitamins and cannot be stored in the body and therefore require a daily supply, the functions and recommended intakes are shown in **Table 2.10**. Lipid soluble vitamins are stored in fat deposits in the body, such as adipose tissue. High intakes over time should be treated with caution as they may lead to tissue accumulation (Thompkinson & Kharb, 2007). Vitamins are either not synthesised by the body or synthesized in inadequate amounts so are therefore required from the diet (Fellows, 2009)

Vitamin A has functions in cell and tissue differentiation, vision and immunity. Vitamin E primarily protects cell membranes from lipid peroxidation and has antioxidant roles. Vitamin D's main role has traditionally been related to bone health; however other potential functions are now emerging. Vitamin K has blood clotting functions, (Fellows, 2009).

In the 1991/1992 NDNS half of toddlers (1.5-3 years) were receiving below Recommended Nutrient Intake (RNI) for vitamin A, with 8% below the Lower Reference Nutrient Intake (LRNI). However from the most recent NDNS there has been a drastic change, with excessive feeding. At least 75% of boys over 7 months and 78% of girls are above the 50^{th} percentile for weight compared against the UK WHO growth standards (Lennox et al., 2013). Consequently diet alone now provides close to or above the RNI for all vitamins, with the exception of vitamin D (Public Health England, 2014). For the purpose of this thesis vitamin A, D and E will be discussed in the following sections.

Table 2.10 Vitamin functions, sources and recommendations (Fellows, 2009).

			DOH			IOM (DRI)	
Vitamin	Function	Sources	4-6M RNI	7-9M RNI	10-12M RNI	0-6M AI	7-12M AI
Water Soluble Vitamins							
Vitamin C (ascorbic acid)	Synthesis of hydroxyproline, a component of collagen and connective tissues. Essential for growth of cartilage, bones, teeth and wound healing. Antioxidant. Helps absorb iron and maintain healthy tissues.	Fresh fruit and vegetables	25 mg/day	25 mg/day	25 mg/day	40 mg/day	50 mg/day
Thiamine (vitamin B1)	Thiamine pyrophosphate is a coenzyme for several enzymes involved in decarboxylation reactions for the metabolism of carbohydrates to energy. Essential for correct heart function and healthy nerve cells.	Meats, dairy, leafy green vegetables, grains and legumes	0.2 mg/day	0.2 mg/day	0.3 mg/day	0.2 mg/day	0.3 mg/day
Riboflavin (vitamin B2)	Precursor for the coenzymes FAD and FMN – hydrogen carriers for numerous oxidation-reduction reactions within mitochondria. Important for growth and production of red blood cells.	Milk, meats and grains	0.4 mg/day	0.4 mg/day	0.4 mg/day	0.3 mg/day	0.4 mg/day
Niacin (nicotinamide)	Precursor to coenzymes NAD and NADPH - hydrogen carriers in glycolysis, Krebs cycle and oxidative phosphorylation.	Meats, fish, dairy, green leafy vegetables, potatoes, nuts	3 mg/day (NE)	4 mg/day (NE)	5 mg/day (NE)	2 mg/day	4 mg/day
Pyridoxine (vitamin B6)	Precursor to pyridoxal phosphate, a coenzyme for reactions involving protein metabolism, transamination processes for the synthesis of amino acids. Helps form red blood cells and maintain brain function.	Pyridoxal and pyridoxamine – milk, meat, fish, poultry Pyridoxine and pyridoxal – vegetables	0.2 mg/day	0.3 mg/day	0.4 mg/day	0.1 mg/day	0.3 mg/day
Pantothenic acid	Precursor to coenzyme A, enzyme required for the oxidation and or synthesis of carbohydrates and fatty acids. Plays a role in the production of hormones and cholesterol.	grains, brassicas, legumes, egg yolk, fish, yeast, meat	-	-	-	1.7 mg/day	1.8 mg/day

			DOH			IOM (DRI)	
Vitamin	Function	Sources	4-6M RNI	7-9M RNI	10-12M RNI	0-6M AI	7-12M AI
Biotin	Coenzyme for numerous enzymes that catalyse carboxylation, decarboxylation and deamination processes, metabolism of proteins and carbohydrates and production of hormones and cholesterol.	Egg yolk, legumes, nuts, potatoes, liver	-	-	-	5 mg/day	6 mg/day
Cyanocobalamin (vitamin B12)	Cobalt containing coenzyme involved in numerous metabolic processes. Helps form red blood cells and maintain the central nervous system.	Animal products	0.3 µg/day	0.4 µg/day	0.4 µg/day	0.4 µg/day	0.5 µg/day
Folic acid	Coenzyme in the synthesis of several amino acids, DNA purines and thymine. Required for energy production, protein metabolism, formation of red blood cells and normal growth and development.	Beans, pulses, meat, organ meat, bran, cheese, chicken, dates, dark green leafy vegetables, milk, oranges, root vegetables, fish, whole grain, yeast, eggs	50 µg/day	50 µg/day	50 µg/day	65 µg/day	80 µg/day
Fat Soluble Vitamins							
Vitamin A (retinol)	Required for the production of vision pigments, resistance to infectious agents, and maintenance of epithelial cells in soft tissues, mucous membranes and skin. Maintenance of healthy teeth and bones.	Animal tissues especially fish, liver and dairy products	350 µg/day (RE)	350 µg/day (RE)	350 µg/day (RE)	400 µg/day (UL 600 µg/day) RAE	500 µg/day (UL 600 µg/day) RAE
Vitamin D (cholecalciferol)	Steroid hormone that facilitates the absorption of calcium in the intestine, maintain blood calcium homeostasis for healthy teeth and bones.	Fish, eggs, dairy, oatmeal, sweet potatoes	8.5 µg/day	7 µg/day	7 µg/day	10 µg/day (UL 25 µg/day)	10 µg/day (UL 38 µg/day)
Vitamin E (tocopherol)	Antioxidants particularly to prevent the oxidation of unsaturated fatty acids. Maintain the integrity of cell membranes. Role in red blood cell formation.	vegetable oils, herring, whole grains, unrefined cereals, wheat germ, oatmeal, dark leafy green vegetables, nuts, eggs, milk, organ meats, sweet potatoes and soybeans	-	-	-	4 mg/day	5 mg/day
Vitamin K	Blood clotting and bone formation.	Dark green leafy vegetables, soybeans, egg yolk, liver, oatmeal, rye, wheat	-	-	-	2 µg/day	5 µg/day

AI – actual intake, DNA – deoxyribonucleic acid, DOH – Department of Health, DRI – dietary reference intakes, FAD – Flavin adenine dinucleotide, FMN – Flavin mononucleotide, IOM – institute of medicine, M – month, NAD – nicotinamide adenine dinucleotide, NADPH – nicotinamide adenine dinucleotide phosphate, NE – niacin equivalent, RAE – retinol activity equivalent, RE – retinol equivalent, RNI – recommended nutrient intake, UL – upper limit

2.3.1 Vitamin A

Vitamin A is a group of lipid soluble compounds metabolically related to *all-trans*-retinol (European Food Safety Authority, 2006). Vitamin A modulates the growth and differentiation of epithelial and bone cells, vision and is required for reproduction, testosterone synthesis and the integrity of numerous immune functions (Thompkinson & Kharb, 2007).

There are 2 sources of vitamin A. Pre-formed vitamin A which includes retinol, retinal, retinyl esters and retinoic acid and are collectively known as retinoids. Pre-formed vitamin A is only present in animal products, mainly as retinyl palmitate (European Food Safety Authority, 2006). Whereas provitamin A carotenoids are precursors of vitamin A, which are converted to retinol within the body. Carotenoids are mainly present in fruit and vegetables. In the Western diet, it is estimated that 70% of vitamin A is from pre-formed sources and 30% from provitamin A sources (Committee on Toxicity, 2014). **Fig. 2.9** illustrates the different forms of vitamin A and their conversion pathways.

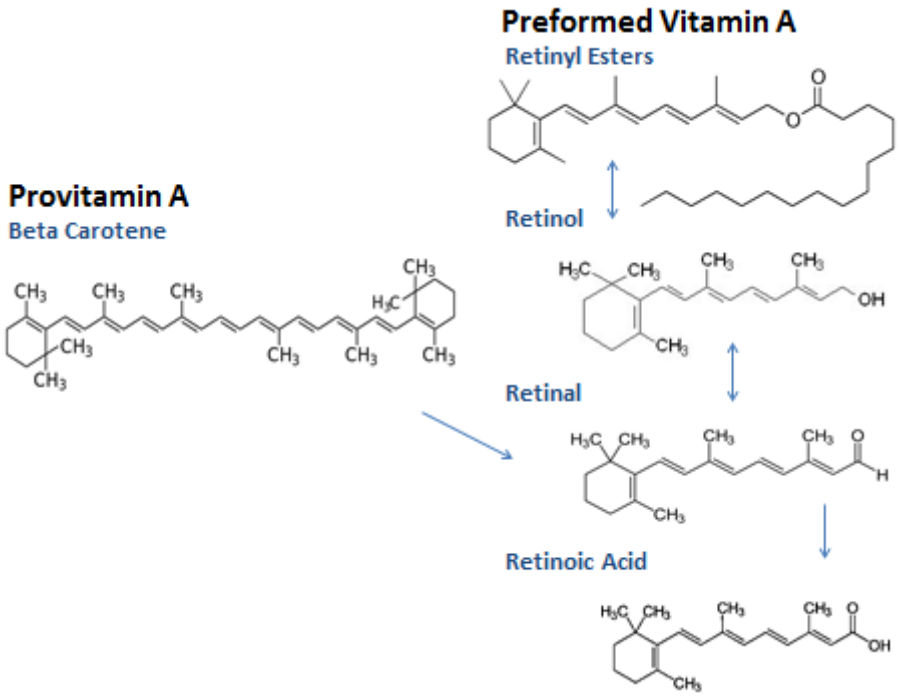

Figure 2.9 Forms of vitamin A and their conversion pathways.

Dietary vitamin A is absorbed in the upper part of the small intestine similar to that of lipid absorption. Protein, protein calorie malnutrition, zinc deficiency and certain food constituents such as nitrites have been associated with vitamin A malabsorption (Food Standards Agency, 2002). Retinyl esters undergo hydrolysis by pancreatic lipase and an enzyme in the intestinal brush border to form unesterified retinol. Released retinol is incorporated into mixed micelles and absorbed into enterocytes where it is bound to an intra-cellular protein, cellular retinol binding protein (CRBP) II. Intracellular retinol is re-esterified by lecithin:retinol acyltransferase and packaged into chylomicrons and released into the general circulation via the lymphatic system. The liver is the main storage site for vitamin A in stellate cells. Vitamin A absorbed in excess of immediate needs is stored in the liver in its esterified form (Department of Health, 1991). Retinyl esters can be metabolised by retinly ester hydrolase to retinol which binds to CRBP-1 for storage. Alternatively retinol can bind to retinol binding protein (RBP) for release into the plasma to be distributed to peripheral tissues as retinol RBP transthyretin complex. Once retinol reaches its target cells retinol can be converted to retinoic acid. The absorption and metabolism of vitamin A are illustrated in **Fig. 2.10**. Pre-formed vitamin A has absorption rates between 70 – 90%, whereas provitamin A are absorbed less efficiently at rates ranging between 10 - 90% (European Food Safety Authority, 2006). Serum retinol concentrations are tightly homeostatically controlled despite variations in the dietary supply of retinol (Committee on Toxicity, 2014).

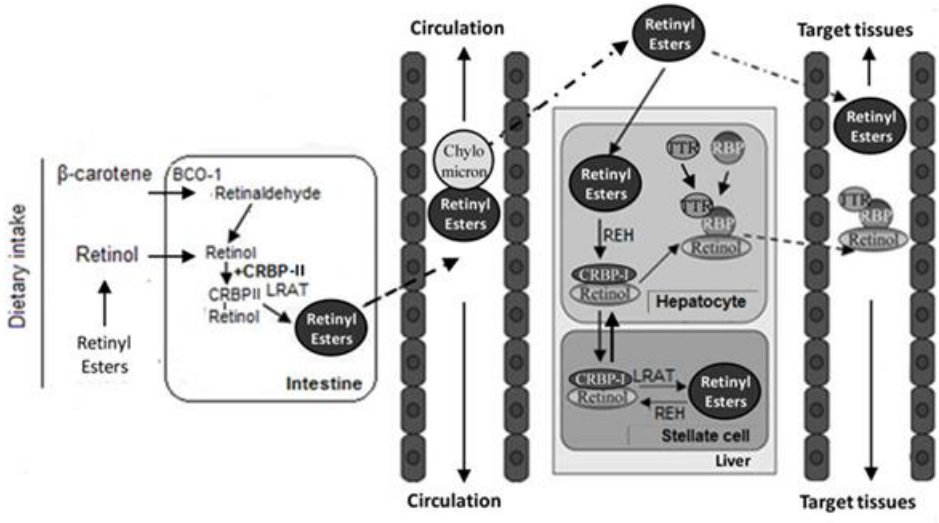

Figure 2.10 Schematic diagram of vitamin A absorption, transport, and metabolism (Ruiz, et al., 2012). BCO-1 – β carotene oxygenase 1, CRBP – cellular retinol binding protein, LRAT – lecithin:retinol acyltransferase, RBP – retinol binding protein, REH – retinyl ester hydrolase, TTR – transthyretin.

Retinol can be oxidised to retinal by the actions of cytosolic and microsomal retinol dehydrogenases (alcohol dehydrogenase); and retinal can be converted back to retinol (retinal reductase). Retinal can be irreversibly oxidised to retinoic acid by retinalaldehyde dehydrogenase and cytochrome P450 enzymes (European Food Safety Authority, 2006). Retinoic acid is transported to cells via cellular retinoic acid binding protein to interact with nuclear receptors (Committee on Toxicity, 2014). Retinoic acid regulates gene expression by binding to specific nuclear receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR); upon ligand binding nuclear receptors bind to specific response elements on DNA. Activated receptors bind with themselves, with each other and other receptors of the same superfamily (vitamin D receptor (VDR), thyroid hormone receptor, PPAR) (European Food Safety Authority, 2006).

After cellular uptake and binding to specific cytosolic proteins retinol acts as a ligand and activator of several nuclear receptors; modulating gene expression and regulating the synthesis of proteins and enzymes (Scientific Committee on Food, 2003).

Retinal is the chromophore of rhodopsin, which is the visual pigment of cone cells of the pigmented epithelium of the retina. The photo induced isomerization of *11-cis* retinal into *all-trans* retinal is the initial event in the photo transduction cascade, which results in the production of a signal to the ocular nerves (European Food Safety Authority, 2006). Retinoic acid maintains normal differentiation of the cornea in the eye, therefore is also important for vision (Committee on Toxicity, 2012).

Rodent studies have linked vitamin A nutrition and altered retinoid signalling with cognitive behaviour due to retinoid receptors being located within the hippocampus of the brain, which is an area that is important for memory. Retinoids control the differentiation of neurons and a role in memory and sleep has been suggested (Benton, 2008). Bone health has also been linked to vitamin A, as both vitamin A receptors and the VDR will compete for dimerization with RXR, affecting the influence of vitamin D on bone mineralization and calcium uptake (European Food Safety Authority, 2006).

Vitamin A has been reported to have an important role in inducing the expression of the gut homing chemokine CCR9 and α4β7 integrin during T cell activation; impaired gut immune responses in early infancy may contribute to the development of atopic sensitization. In vitro retinoic acid can inhibit IL-4 mediated proliferation and IgE production by B cells. Retinoic acid is also required by dendritic cells for the induction of forkhead box P3 regulatory T cells

(T reg) from $CD4^+$ T cells and is a regulator of the induction of IgA secretion (Cunningham-Rundles, et al., 2009). Pesonen, et al. (2007) looked at plasma retinol concentrations of Finnish infants and their subsequent development of allergic symptoms in childhood and adulthood; they found that lower retinol concentrations at 2 months were inversely correlated with positive skin prick tests at 5 and 20 years and allergic symptoms at 20 years. Infants in the study received 1000 IU/day of vitamin A which is the national recommended dose in Finland for infants, therefore dietary deficiency of vitamin A was unlikely. The infants in the study were all on a similar diet (exclusive breastfeeding) at the time that retinol differences were measured. It is possible, therefore, that a new-born infant with low concentrations of retinol could cause $CD4^+$ T cell homing to the gut to be inhibited, which could cause T_h1 type responses to become weak and produce an environment favourable towards the development of allergic sensitization.

Vitamin A deficiency is common in young children in developing countries resulting in blindness and infant mortality (Department of Health, 1991). However in developed countries such as the UK deficiency does not appear to be an issue according to the NDNS, where excess intakes may pose more of a threat to health. Mean intakes of vitamin A from food sources were close to or above the RNI for all age groups, the major contributor being milk and milk products (Public Health England, 2014). Also in the US 35% of toddlers exceed the Tolerable Upper Intake Level (UL) (600 μg retinol activity equivalent/day), which was additionally found to be consistent with results from the National Health and Nutrition Examination Survey III survey (Butte, et al., 2010). Classical symptoms of vitamin A deficiency include night blindness, xerophthalmia, growth retardation, skin disorders and impaired immune function. Hypervitaminosis can occur when the capacity of the stellate cells is saturated or when the amount of vitamin A in plasma exceeds the capacity of the RBP. High intakes of pre-formed vitamin A can be toxic, however high intakes from provitamin A such as β-carotene have not been found to cause toxicity but can lead to a yellow appearance. Clinical features of toxicity include anorexia, bulging fontanelles which are caused by increased cerebrospinal fluid volume, drowsiness, lethargy, irritability, vomiting, raised intracranial pressure, alopecia, bone and joint pain, hepatotoxicity, anaemia, photophobia, conjunctivitis and skin desquamation. It can also decrease vitamin C storage, antagonise the action of vitamin K's blood clotting functions and have anti-thyroid effects (Committee on Toxicity, 2014). Toxicity of vitamin A usually arises from chronic ingestion over a period of time, which will build up stocks that exceed the livers ability to destroy or store them. A

relationship has been found between high intakes of vitamin A during pregnancy and birth defects in infants (Department of Health, 1991).

In the UK vitamin A requirements are expressed as retinol equivalents (RE), which takes into account the activity of different forms of vitamin A in comparison to retinol activity, **Table 2.11** shows the conversion factors of the different forms of vitamin A (Food Standards Agency, 2002). Vitamin A recommendations for infants are based on concentrations from breast milk as breastfed infants do not show signs of vitamin A deficiency. The breast milk of well-nourished women in Europe ranges between 40-70 μg RE/100 mL. Infant and follow on formula regulations allow 60-180 μg RE/100 mL to be added (Committee on Toxicity, 2014). Retinol, retinyl acetate and retinyl palmitate are used in infant formula (Thompkinson & Kharb, 2007).

Table 2.11 Conversion factors for the different forms of vitamin A into retinol equivalents (Food Standards Agency, 2002).

Vitamin A	Retinol Equivalents
1 μg Retinyl palmitate	0.55 μg RE
1 μg Retinyl acetate	0.87 μg RE
1 μg β carotene	0.17 μg RE
1 μg other carotenoids	0.083 μg RE

RE – retinol equivalents

Retinol is teratogenic (Department of Health, 1991). In the UK Pregnant women are advised not to consume liver or liver products as they contain large amounts of retinol which can be harmful to the baby, furthermore infants over 6 months are advised not to have more than 1 portion of liver per week (Committee on Toxicity, 2014).

During food processing retinoids and carotenoids can undergo isomerization due to exposure with acids, heat and light. Thermal treatment can either cause isomerization from *trans* to *cis* isomers or oxidation. However heating can also promote carotenoid absorption, possibly due to the release of carotenoids from its matrix. Homogenization of foods can also improve the availability of carotenoids. Processing methods such as dehydration, blanching and canning can cause either oxidation or isomerization of carotenoids (Klein & Kurilich, 2000). However retinol and carotenoids have been found to be relatively stable throughout most cooking procedures with industrial food processes such as pasteurization and sterilization generally only resulting in small losses (Committee on Toxicity, 2012).

2.3.2 Carotenoids

β-Carotene and carotenoids are isoprenoid compounds which are not synthesised in animals, but biosynthesised in plants and microorganisms. Around 700 naturally occurring carotenoids have been identified, with around 10% being present in the human diet and around 50 having provitamin A activity. The predominant carotenoids present in human plasma are β-carotene, α-carotene, lycopene, lutein and β-cryptoxanthin (European Food Safety Authority, 2006). The carotenoid β-carotene has the most vitamin A activity as it can be converted into 2 retinal molecules and it is also widely available in the diet (Committee on Toxicity, 2014).

β-Carotene is a hydrocarbon ($C_{40}H_{56}$). Carotenoids which contain at least one unsubstituted β-ionone ring and a polyene chain are potential precursors of vitamin A. Carotenoid functions include radical quenching, antioxidant and anti-carcinogenic activities and regulators of cell function. Carotenoids can act as free radical and reactive species scavengers. They have been reported to modulate cytochrome P450 metabolism, inhibit AA metabolism and inhibit chromosome instability (European Food Safety Authority, 2006). Epidemiological studies have associated high carotenoid intake with lower cancer and cardiovascular disease incidence (Scientific Committee on Food, 2006).

Initially β-carotene is dissolved in lipid droplets in the stomach and passes into the duodenum where it is taken up into micelles (Committee on Toxicity, 2014). β-Carotene is absorbed in the intestine via passive diffusion of mixed micelles, which have been formed during fat digestion in the presence of bile acids, **Fig. 2.10** (European Food Safety Authority, 2006). Like retinol provitamin A are taken up by enterocytes (Committee on Toxicity, 2014). In general the types and amounts in the plasma reflect those in the diet. In the literature the absorption of β-carotene varies widely between 10-90%, with the most critical step being the release from the food matrix into the lipid phase and solubilization within mixed micelles. They are transported in association with lipoproteins with similar distribution to cholesterol. The adipose tissue and liver are the main storage sites for carotenoids (European Food Safety Authority, 2006).

In mammalian tissues β-carotene can be cleaved mainly at the central double bond (C15,15') to yield 2 retinal molecules; which can either be reduced to retinol or further oxidised to retinoic acid. Alternatively a non-central cleavage at eccentric double bonds to form retinoids or apo-B-carotenoids can occur (European Food Safety Authority, 2006). Carotenoids are converted to retinal by β-carotene monooxygenase (BCMO) 1; they can also be converted to apocarotenal by BCMO2, which is subsequently converted to apro carotenoic acid and finally retinoic acid (Committee on Toxicity, 2014).

The bioavailability of carotenoids is dependent on the food structure, species of carotenoid, linkages to alkyl groups, amount of carotenoid present in the food, matrix properties of the plant the carotenoid is contained in, nutritional status of the individual, genetics and food preparation method (Committee on Toxicity, 2014). For example cooking and chopping of foods containing carotenoids may enhance their absorption due to increasing the ease that carotenoids are extracted from the food matrix (Food Standards Agency, 2002).

One study among smokers and asbestos workers associated an increase in lung cancer with high dose of β-carotene supplements. However the toxicity of carotenoids differs to that of retinoids and they are not considered to be toxic due to reduction in their conversion when vitamin A levels are high (European Food Safety Authority, 2006).

The rate of absorption and bioequivalence to retinol of β-carotene and other carotenoids is unknown in infants (Scientific Committee on Food, 2003), partly due to varied release of carotenoids from different food matrixes and different processing effects, along with the biological effects of absorption and conversion. Therefore there is insufficient evidence to set recommendations for carotenoids and conversion factors should be used with caution (Department of Health, 1991).

2.3.3 Vitamin E

There are 8 naturally occurring fat soluble compounds, known as tocochromanols, with vitamin E activity which exhibit antioxidant activity and are nutritionally essential; **Fig. 2.11** illustrates the 8 different forms of vitamin E. There are 2 types of tocochromonals: tocopherols (α, β, γ and δ) and tocotrienols (α, β, γ and δ), which are biologically less potent. The basic structural unit is composed of a chroman ring system (2-methyl-6-hydroxychroman) with an isoprenoid side chain of 16 carbon atoms. α, β, γ and δ differ in number and position of the methyl substituents on the chroman ring. Tocopherols differ to tocotrienols by having a saturated side chain. The presence of the phenolic hydroxyl group is important for vitamin E's antioxidant activity. α-tocopherol is the most active isomer and accounts for the majority of vitamin E present in human tissues, followed by β, γ and then δ. Animal models and epidemiological studies have suggested that vitamin E may protect against cancer and prevention of coronary heart disease (European Food Safety Authority, 2006).

Vitamin E is synthesised by plants, one of the richest sources being plant oils. The most biologically active form of vitamin E is α-tocopherol, which is also the most abundant form in the European diet due to the use of olive and sunflower oil. Whereas in the US diet γ-tocopherol is the most abundant form of vitamin E due to the use of soybean and corn oil. The least abundant forms of vitamin E in foods are the tocotrienols (Stone, et al., 2003).

The biological activity of vitamin E is mainly related to its antioxidant properties and acts as a major lipid soluble antioxidant in membranes. The first vitamin E deficiency state was clearly demonstrated in premature infants with clinical states displaying haemolytic anaemia, thrombocytosis and oedema (Department of Health, 1991).

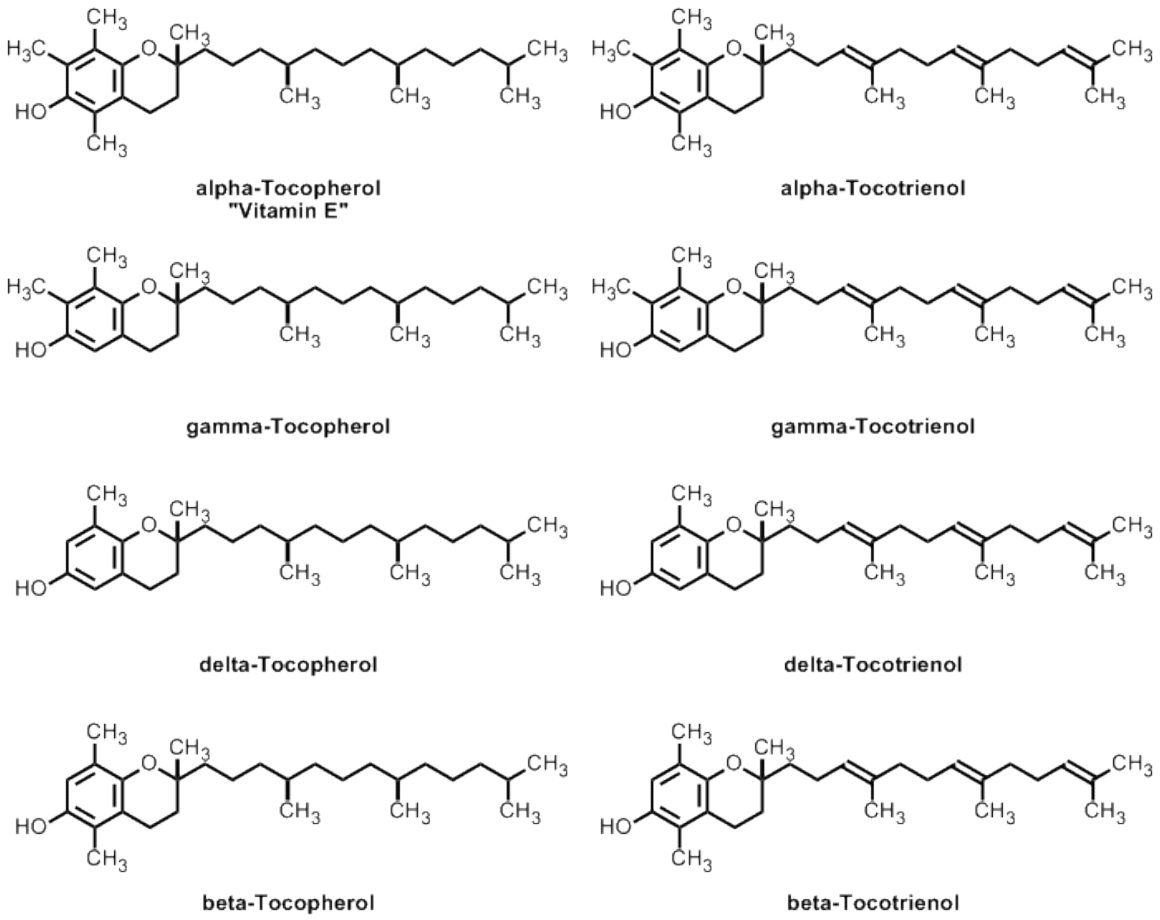

Figure 2.11 Different forms of vitamin E (Vita-dose.com, ND).

Vitamin E primarily functions as a chain breaking antioxidant. It protects membrane phospholipids from peroxidation and prevents free radical generation in cell membranes; **Fig. 2.12** shows vitamin E in cell membranes (Greene, et al., 1988). During lipid peroxidation a vitamin E radical is formed, which can be regenerated by vitamin C (Diplock, et al., 1998). Oxidative stress and inflammation play a central role in cardiovascular disease and cancer, however, current knowledge does not provide clear conclusions for the role of vitamin E in these disease areas (Brigelius-Flohe & Traber, 1999). Immune system cells contain the highest amount of vitamin E due to their exposure to high levels of oxidative stress (Suskind & Tontisirin, 2001). Therefore vitamin E may play a role in the prevention or protection against asthma and allergic disease inflammation, which are currently increasing in the UK. A maternal feeding study observed an association between increased intakes of vitamin E during pregnancy and decreased childhood wheeze and asthma in the infants (Devereux, et al., 2006).

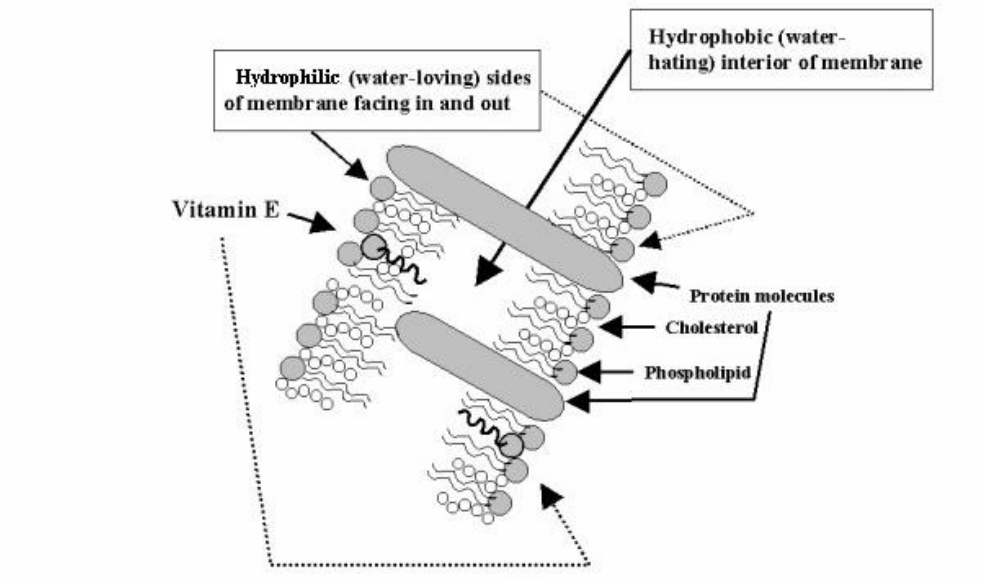

Figure 2.12 Vitamin E in cell membranes (Papas, 1999).

Absorption of vitamin E in the lumen part of the small intestine into enterocytes is via passive diffusion which finally enters the circulation via the lymphatic system (European Food Safety Authority, 2008). Vitamin E is absorbed with lipids, which are subsequently packed into chylomicrons and transported to the liver (Brigelius-Flohe & Traber, 1999). After passage through the liver α-tocopherol transport protein (α-TTP) selects α-tocopherol (Stone, et al., 2003). Intestinal absorption of fat soluble vitamins depends on pancreatic function, biliary secretions to form micelles and transfer across the intestinal membrane. Dietary vitamin E is absorbed in the small intestine following its uptake into mixed micelles, which requires the presence of bile acids and pancreatic enzymes to form monoglycerides that are incorporated into micelles. Prior to absorption vitamin E esters need to be hydrolysed by the action of bile salt stimulated lipase or pancreatic lipolytic activity. The absorption of vitamin E appears to be passive with no specific carrier; **Fig. 2.13** illustrates the absorption and excretion of vitamin E (Thompkinson & Kharb, 2007). After absorption tocopherols are incorporated into triglyceride rich proteins, such as lipoproteins, chylomicrons and very low density lipoproteins. After triglyceride lipolysis most circulating vitamin E is found in cholesterol rich lipoproteins, such as low density lipoproteins and high density lipoproteins allowing uptake into tissues (Scientific Committee on Food, 2003). Absorption of vitamin E varies widely in the literature between 20-80%, which is highly dependent on the dose and lipid content of the diet. High pectin, wheat bran and PUFA intakes reduce vitamin E absorption (Food Standards Agency, 2002). Nearly all of the vitamin E absorbed across the intestinal mucosa is free tocopherol which can be transported via the lymphatic system into the blood

stream where it is distributed into lipoproteins on passage into the liver (European Food Safety Authority, 2006).

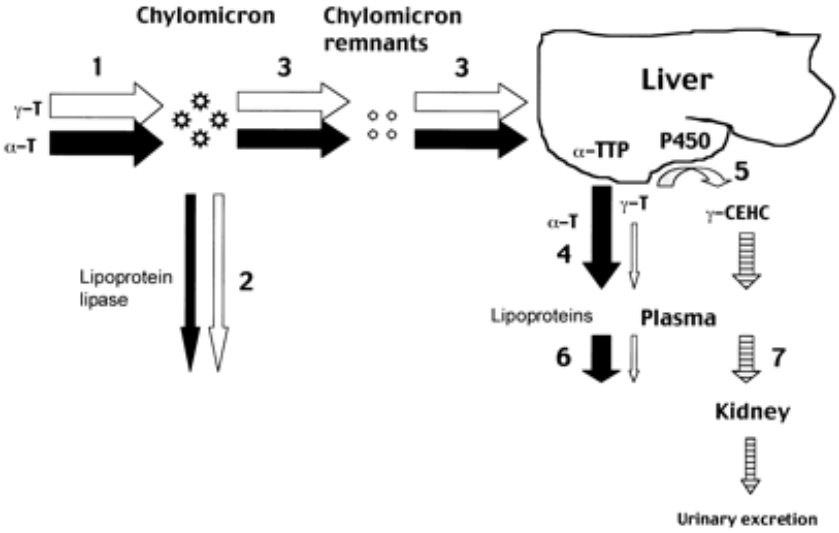

Figure 2.13 Absorption and excretion of α-tocopherol and γ-tocopherol (Jiang, et al., 2001). 1. α-Tocopherol (α-T) and γ-Tocopherol (γ-T) are absorbed by the intestine along with dietary fat and are secreted into chylomicron particles. 2) Some of the chylomicron-bound vitamin E is transported to peripheral tissues with the aid of lipoprotein lipase. 3) The resulting chylomicron remnants are subsequently taken up by the liver. 4) In the liver, most of the remaining α-T but only a small fraction of γ-T is reincorporated into nascent very low density lipoproteins (VLDL) by α-Tocopherol transfer protein (α-TTP) 5) Substantial amounts of γ-T are probably degraded by a cytochrome P450 3A–mediated reaction to 2,7,8-trimethyl-2-(ß-carboxyethyl)-6-hydroxychroman (γ-CEHC). 6) Plasma vitamin E is further delivered to tissues by low density lipoproteins (LDL) and high density lipoproteins (HDL). 7) γ-CEHC is excreted into urine.

Tocopherols are transported in the body by non-specific associations with lipoproteins and tissue concentrations are directly related to fatty acid content. Dietary PUFA intake influences tissue fatty acid content and the dose of vitamin E required to prevent lipid peroxidation is directly related to PUFA intake (Greene, et al., 1988). Fats are an essential part of cell membranes, which are highly susceptible to oxidation by free radicals. α-Tocopherol can intercept free radicals and prevent the oxidation of lipids (Brigelius-Flohe & Traber, 1999). Vitamin E's lipid solubility allows its broad diffusion into numerous cells and tissues (Baydas, et al., 2002).

Less attention has been focused on γ-tocopherol; however it may also be important to human health and possesses unique features that differ from the roles of α-tocopherol. It is mainly metabolised to γ-CEHC which has been shown to have natriuretic activity in the kidney. Blood levels of γ-tocopherol in general are ten times lower than α-tocopherol levels due to the selectivity of α-TTP for α-tocopherol and it also possesses lower antioxidant activity due to its structure. However γ-tocopherol appears to be better at trapping electrophiles, such as reactive nitrogen species (Jiang, et al., 2001).

Processing of foods, such as drying in the presence of air and sunlight, addition of organic acids, milling and refining, irradiation and canning have been shown to reduce the vitamin E content; along with high temperatures, prolonged storage and high moisture content. Seasonal changes in vitamin E levels have also been shown in food products (Eitenmiller & Lee, 2004). Therefore the actual content of vitamin E in commercial infant foods may vary due to processing and seasonality of products used for production.

Vitamin E deficiency is a major cause for concern in preterm or low birth weight infants, where excessive production of free radicals can cause broncho-pulmonary dysplasia, intraventricular haemorrhage and retinopathy due to the low antioxidant status. It has also been well documented that placental transfer of vitamin E is limited, with cord blood levels of infants being lower than maternal blood (Baydas, et al., 2002). In addition intestinal absorption of vitamin E is impaired in premature infants (Davis, 1972). Furthermore the developing nervous system appears to be sensitive to vitamin E deficiency (Brigelius-Flohe & Traber, 1999). Although there is evidence that excess vitamin E is harmful all such data originates from supplementation studies in preterm infants (Powers, 1997).

The bioavailability and metabolism of vitamin E is largely unclear; however factors have been identified that will affect vitamin E within the body; such as the food matrix and the amount and type of fat (Borel, et al., 2013).

The NDNS from 2008/2009 – 2010/2011 indicated that mean intakes of vitamins (including vitamin E) either met or exceeded the recommended nutrient intake (Public Health England, 2014). In the US vitamin E intakes exceed the recommended adequate intakes for vitamin E in infants below 12 months, however after 12 months they appear to be below the recommended adequate intakes (Butte, et al., 2010). This may be due to the reduction in milk intake and increase intake of complementary foods.

The main function of vitamin E in human tissues is to protect PUFA from oxidation by trapping free radicals and donating hydrogen. It protects lipids and phospholipids in membranes. Vitamin E is also known to interact with other nutrients involved in the oxidation process. Vitamin C, selenium and zinc interact synergistically with vitamin E. Iron overload has been associated with lowering serum levels of vitamin E. High intakes of vitamin E may also have effects on other fat soluble vitamins such as vitamin K with potential effects on blood clotting (European Food Safety Authority, 2006).

Vitamin E content can be expressed as α-tocopherol equivalents (ATE), which takes into account the varying activity of the different vitamin E forms, **Table 2.12** shows the conversion factors for the different forms of vitamin E (Food Standards Agency, 2002). There is no RNI or LRNI set for vitamin E. Adults are recommended to consume > 3 - 4 mg/day (Public Health England, 2014). Vitamin E requirements are largely dependent on the PUFA intake which varies widely and poses a problem for setting dietary reference values (Powers, 1997). Furthermore the relationship between PUFA intake and vitamin E requirements is not linear therefore difficult to estimate. In America, 0.4 mg ATE/g of dietary PUFA appears to be satisfactory and is used in the UK for the ratio used in infant formulas (Department of Health, 1991). Vitamin E in infant formula is in the form of tocopheryl acetate and labelled in terms of ATE (Powers, 1997). Foods rich in PUFA also tend to be high in vitamin E content. Vitamin E content in breast milk varies during early lactation but then remains constant (Department of Health, 1991).

Table 2.12 Conversion factors for vitamin E activity in α-tocopherol equivalents (Food Standards Agency, 2002).

Form of vitamin E	Conversion factor
α-tocopherol	1
β-tocopherol	0.4
γ-tocopherol	0.1
δ-tocopherol	0.01
α-tocotrienol	0.30
β-tocotrienol	0.05
γ-tocotrienol	0.01

2.4 Bone Health

Micronutrients are essential dietary components; 30 are essential and cannot be synthesised by the body on a daily basis therefore they are required from dietary sources; **Table 2.13** shows examples of micronutrients, their functions and dietary requirements. Micronutrients have a wide range of biochemical functions which are fundamental in the homeostatic regulation of bodily function, physiological functioning, metabolic pathways and normal growth and development (Shergill-Bonner, 2013). They are grouped into major minerals (>100 mg/day required from the diet) and trace elements (<100 mg/day required from the diet) (Fellows, 2009). Both low intakes and reduced bioavailability can lead to deficiencies of micronutrients (Melø, et al., 2008).

During the first year of life an infant requires adequate intake of both calcium and phosphorus for the rapid growth and development of teeth and bones. Calcium and phosphorus are the main components of hydroxyapatite which is the major constituent of teeth and bones. In addition the dietary intake of vitamin D and other micronutrients, such as magnesium, are involved in bone health and their intakes at this crucial stage of life will be of great importance.

Table 2.13 Mineral function, sources and recommendations (Fellows, 2009).

Mineral	Properties and functions	Sources	DOH			IOM (DRI)	
			4-6M RNI	7-9M RNI	10-12M RNI	0-6M AI	7-12M AI
Calcium	Maintains blood electrolyte balance, structure and rigidity of bones and teeth. Involved in blood clotting, transmission of impulses from nerves to muscles, regulation of PTH and heartbeat, functioning of muscles, skin, soft tissues and circulatory, digestive, enzymatic and immune systems.	Dairy, molasses, nuts, cereals, fruits, tofu, seafood's, green leafy vegetables, seaweeds	13.1 mmol/d 525 mg/day	13.1 mmol/day 525 mg/day	13.1 mmol/day 525 mg/day	200 mg/day (UL 1000 mg/day)	260 mg/day (UL 1500 mg/day)
Copper	Numerous protein and enzymes contain copper, some essential for the utilisation of iron. Involved in protein metabolism, respiration, healing and formation of myelin sheaths that protect nerve fibres. Blood antioxidant. Combines with zinc and vitamin C to form elastin and formation of bones and red blood cells.	Avocado, barley, cauliflower, nuts, lamb, oranges, organ meats, raisins, salmon, legumes, green leafy vegetables, soybeans	5 umol/day 0.3 mg/day	5 umol/day 0.3 mg/day	5 umol/day 0.3 mg/day	200 ug/day	220 ug/day
Iodine	Functioning of the immune system, brain and thyroid gland. Involved in the conversion of carotene to vitamin A, protein and cholesterol synthesis.	Iodised salts, seafood's, asparagus, fish, garlic, beans, mushrooms, sesame seeds, soybeans, spinach	0.5 umol/day 60 ug/day	0.5 umol/day 60 ug/day	0.5 umol/day 60 ug/day	110 ug/day	130 ug/day
Iron	Required for the production of haemoglobin, myoglobin, certain enzymes, oxygenation of red blood cells, healthy growth and resistance to disease, healthy immune system and energy.	Liver, red meat, dark green leafy vegetables, eggs, seafood, fish, dates, legumes, peaches, pears, pumpkins, raisins, rice, wheat bran, sesame seeds and soybeans	80 umol/day 4.3 mg/day	140 umol/day 7.8 mg/day	140 umol/day 7.8 mg/day	0.27 mg/day (UL 40 mg/day)	11 mg/day (RDA) (UL 40 mg/day)
Magnesium	Essential for enzyme activity and protein synthesis. Assists in calcium and potassium uptake to maintain blood electrolyte balance. Important role in bone formation, carbohydrate and mineral metabolism, functioning of arteries, bones, cells, digestive, immune and reproductive systems heart, nerves and teeth. Extracellular magnesium role in maintenance of electrical potentials of nerve and muscle membranes and transmission of nerve impulses to muscles.	Dairy, fruits, fish, meat, seafood, garlic, lima beans, sesame seeds, tofu, green leafy vegetables, whole grains	2.5 mmol/day 60 mg/day	3.2 mmol/day 75 mg/day	3.3 mmol/day 80 mg/day	30 mg/day	75 mg/day

Mineral	Properties and functions	Sources	DOH			IOM (DRI)	
			4-6M RNI	7-9M RNI	10-12M RNI	0-6M AI	7-12M AI
Phosphorus	Essential component of bone and to utilise vitamins to metabolise food and maintain electrolyte balance, correct functioning of brain cells, circulatory and digestive systems, eyes, liver, muscles, nerves, teeth and bones.	Most foods especially asparagus, maize, dairy, eggs, fish, fruits, garlic, sunflower seeds, meats, wheat bran and whole grains	13.1 mmol/day 400 mg/day	13.1 mmol/day 400 mg/day	13.1 mmol/day 400 mg/day	100 mg/day	275 mg/day
Potassium	Important in a balance with sodium for cellular metabolism and regulating transfer of nutrients to cells, maintaining blood pressure and electrolyte balance, transmitting electrochemical impulses. Correct functioning of blood, endocrine, digestive and nervous systems, heart, kidneys, muscle and skin.	Dairy, fish, fruit, meat, poultry, vegetables, whole grains, beans, nuts, potatoes, wheat bran, yams	22 mmol/day 850 mg/day	18 mmol/day 700 mg/day	18 mmol/day 700 mg/day	0.4 g/day	0.7 g/day
Selenium	Antioxidant that protects all cell membranes enhances immune system. Required with vitamin E for production of antibodies, binding of toxic metals, amino acid metabolism and promotion of growth and fertility. Production of prostaglandins. Enzyme co factor for glutathione peroxidase.	Seafood's, organ meats, cereals	0.2 umol/day 13 ug/day	0.1 umol/day 10 ug/day	0.1 umol/day 10 ug/day	15 ug/day (UL 45 ug/day)	20 ug/day (UL 60 ug/day)
Sodium	With potassium for maintaining cellular water balance and blood electrolyte balance/pH. Required for stomach, lymphatic system, nerve and muscle function.	Most foods	12 mmol/day 280 mg/day	14 mmol/day 320 mg/day	15 mmol/day 350 mg/day	0.12 g/day	0.37 g/day
Zinc	Needed to maintain required functioning of immune system, thymus and spleen, maintaining concentrations of vitamin E in the blood, correct functioning of blood, bones, eyes, heart, joints, liver and prostate gland. Component of insulin and many enzymes involved in metabolism of phosphorus and proteins.	Fish, meats, seafood, whole grains, liver, egg yolks, beans, mushrooms, nuts, pumpkin seeds, sardines, soybeans, wheat germ	60 umol/day 4 mg/day	75 umol/day 5 mg/day	75 umol/day 5 mg/day	2 mg/day (UL 4 mg/day)	3 mg/day (RDA) (UL 5 mg/day)

AI – actual intake, DOH – Department of Health, DRI – dietary reference intake, PTH – parathyroid hormone, RDA – recommended daily allowance, RNI – recommended nutrient allowance, UL – tolerable upper intake level

2.4.1 Calcium

Calcium is the 5th most abundant element in the human body. Over 99% of calcium in the body is located in the bones and teeth, mainly as hydroxyapatite $Ca_5(PO_4)_3(OH)$, with less than 1% present in soft tissues and bodily fluids (European Food Safety Authority, 2006).

Calcium is a vital mineral for the developing infant and is the most abundant mineral in the human body. Calcium is transported through the placenta to the foetus during pregnancy, where the largest amount of foetal accumulation is during the 3rd trimester. Calcium is important for intracellular metabolism, bone growth, blood clotting, nerve conduction, muscle contraction and cardiac functions. Bone calcium accretion and accumulation is positive during the first 18 years of life, being highest in the first year. During the first 6 months of life bone mass accretion is directly related to mineral intake. Calcium accretion and bone mineralization are greatest during the first year of life and many nutritionists' believe that early bone mass accumulation is important for preventing poor childhood growth and later adult osteoporosis (Bass & Chan, 2006).

The bone mineral provides structure and strength to the body and a reservoir of calcium to maintain the constant concentration within the blood. Cell surface calcium sensing receptors maintain a constant calcium concentration in the extracellular fluid and blood. Calcium is present in the blood in 3 different forms; free Ca^{2+} ions, bound to protein or complexed to citrate, phosphate, sulphate or carbonate. Ionised calcium is kept within narrow limits via the action of parathyroid hormone (PTH), calcitriol ($1,25(OH)_2D$) and calcitonin. Intracellular calcium varies between tissues and is predominantly bound to intracellular membrane structures or contained in special storage vesicles. Intracellular calcium rises due to stimuli interacting with cell surface receptors, which activates different responses including hormone or neurotransmitter release and muscle contraction (European Food Safety Authority, 2006). Within cells calcium acts as a second messenger to modulate the transmission of hormonal signals and regulating enzyme function (Thompkinson & Kharb, 2007).

Reduced serum calcium levels are sensed by the parathyroid gland and result in the secretion of PTH. PTH actions on the bone cause resorption of calcium and phosphorus by stimulating osteoblasts (bone synthesising cells) to release factors to increase osteoclasts (cells that induce resorption of bone) number and activity. In the kidney PTH increases calcium resorption and also up regulates 1-α-hydroxylase which results in an increase in $1,25(OH)_2D$. $1,25(OH)_2D$ increases the intestinal absorption of calcium by stimulating the biosynthesis of

specific intestinal calcium binding proteins (Department of Health, 1991). In states of increased serum calcium levels the actions of PTH are reversed to reduce calcium levels. In addition the thyroid gland stimulates the secretion of calcitonin which increases the deposition of calcium in the bones and reduces uptake in the kidneys. Magnesium also stimulates the secretion of calcitonin (Bass & Chan, 2006).

The absorption of calcium mainly occurs in the jejunum, but also in the ileum and colon, by active transport (vitamin D dependent) and paracellular passive diffusion, active transport being more important under suboptimal dietary intakes (Greer & Krebs, 2006). In infants calcium absorption is highly variable ranging between 10 - 80% (Bass & Chan, 2006). Urinary calcium excretion is high when protein and sodium intakes are high (Department of Health, 1991).

2.4.2 Phosphorus

Phosphorus in the form of phosphate is involved in numerous physiological processes; cells energy cycle, regulation of the body's acid-base balance, component of phospholipids, nucleotides, nucleic acids, DNA and RNA, cell regulation, signalling by phosphorylation of catalytic proteins, second messenger cAMP, and the mineralization of teeth and bones as hydroxyapatite (European Food Safety Authority, 2006). Around 80% of phosphorus in the body is present in the bones (Department of Health, 1991). In the blood 70% is present as organic phosphates in phospholipids and the other 30% present as inorganic phosphate (European Food Safety Authority, 2006).

Inorganic phosphate in the extracellular fluid is under tight control. When phosphate levels are increased the bone secretes fibroblast growth factor-23 (FGF-23) which decreases the expression of NPT (sodium/phosphorus co-transporter) 2 in the proximal tubule to decrease the reabsorption of phosphorus and decrease 1-α hydroxylase, therefore reducing the formation of $1,25(OH)_2D$ (Liu & Quarles, 2007). In addition high phosphate levels also increase the secretion of PTH, which reduces the concentration of serum phosphate by increasing the urinary excretion of itself. Phosphate intestinal absorption is increased by $1,25(OH)_2D$ (European Food Safety Authority, 2006). **Fig. 2.14** illustrates the regulation of phosphorus.

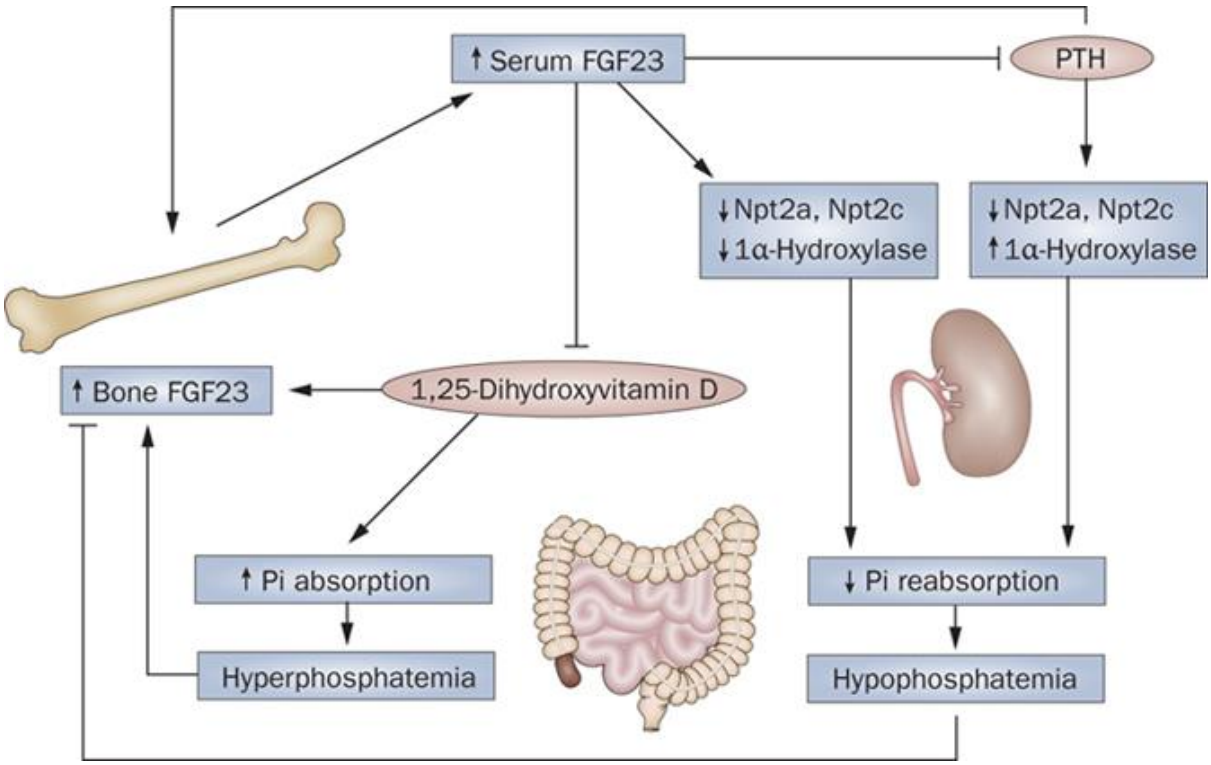

Figure 2.14 Regulation of phosphorus (Farrow & White, 2010). FGF23 - fibroblast growth factor, Npt2a/c (sodium/phosphorus co-transporter) Pi- phosphate, PTH – parathyroid hormone.

In infants 65-90% of phosphorus is absorbed. Absorption is mainly in the jejunum by a saturable, active transport mechanism facilitated by 1,25$(OH)_2$D and also by passive diffusion. Excretion of phosphorus is mainly via the kidney. The majority of phosphorus reabsorption occurs in the proximal tubule in the kidney via the type 2 NPT2 co transporter; high phosphorus intake can result in the down regulation of the transporter so that more phosphorus is excreted, vice versa (European Food Safety Authority, 2006).

Phosphorus requirements are conventionally set as equal to calcium in terms of mass. Therefore recommendations are made so that phosphorus is equal to calcium in mmol (Department of Health, 1991).

2.4.3 Ca:P

It has been suggested that the ratio of Ca:P is important for proper bone growth and development in infancy (Sax, 2001). Inadequate intake of calcium and phosphorus in infancy has been associated with the pathogenesis of bone disease in infants. Infants have a lower renal excretion capacity and therefore have higher serum organic phosphate values, which favours skeletal mineralization; however high levels adversely affect bone accretion. During infancy at a time of rapid growth the Ca:P may be important to enable optimal growth (European Food Safety Authority, 2006). In breast milk the Ca:P is normally around 2:1 (Thompkinson & Kharb, 2007).

Excessive phosphorus intakes (1-1.5 g/d) with low Ca:P molar ratio (1:3) alter calcium metabolism causing hypocalcaemia (low serum calcium) and secondary hyperparathyroidism (high PTH) (Department of Health, 1991). The Ca:P ratio is important for infants and should be between 1-2:1 (weight/weight) (Koletzko, et al., 2005)

2.4.4 Micronutrient Interactions

Micronutrient intakes cannot be assessed independently due to numerous interactions that occur between them, for instance the formation of insoluble complexes and competition for absorption sites. The physico-chemical form of metal ions also has an important influence on the absorption within the body. Foods contain a large number of ligands for metal ions, including proteins, amino acids, carbohydrates, lipids, peptides and organic ions some of which can form insoluble complexes, whereas others can make the mineral more absorbable (Watzke, 1998). **Table 2.14** shows examples of micronutrient interactions.

It is evident that calcium, phosphorus and magnesium are essential for bone health. However it is also important to take into consideration other dietary factors which may affect the utilisation of micronutrients and therefore their intakes are also required to be assessed.

Table 2.14 Examples of micronutrient interactions (Booth & Aukett, 1997; Crawley & Westland, 2014; European Food Safety Authority, 2006; Food Standards Agency, 2002).

Micronutrient	Beneficial	Detrimental
Calcium	Lactose, vitamin D, inulin, fructo-oligosaccharides, medium chain triacylglycerols	Phytate, oxalate, protein, iron, zinc
Phosphorus		Phytic acid, calcium
Copper	Histidine	Sucrose, fructose, ferrous iron, zinc, vitamin C, calcium, phosphorus, cadmium, molybdenum, histidine
Magnesium		Phytate, dietary fibre, calcium
Iron	Vitamin C, citrate, fumurate, cysteine, oligopeptides	Polyphenols, phytate, bovine casein, calcium, tannin, phosphorus, copper, zinc, oxalates
Zinc	Histidine, methione, cysteine, vitamin A	Iron, calcium, phytate, copper, cadmium

2.4.5 Food labelling of micronutrients

In the UK micronutrient labelling is non-mandatory; it is only required to be declared when 15% of the reference values are supplied per 100 g/ 100 mL of the product according to the Commission Directive 2006/125/EC. Therefore there is a need to assess the nutritional composition of commercial 'ready-to-feed' infant foods especially with regards to micronutrient content in relation to recommendations.

2.4.6 Vitamin D

Vitamin D refers to a group of lipid soluble vitamins with anti-rachitic (prevention of rickets) activity, which generally refers to the prohormones ergocalciferol (D2) and cholecalciferol (D3). Vitamin D3 is of animal origin which can be supplied by dietary intake or synthesized endogenously in the skin. Vitamin D2 is of plant origin and is formed by UVB radiation from its precursor ergosterol, which is found in plants such as yeast and fungi (European Food Safety Authority, 2006). 10 – 20 % of vitamin D is required from the diet, whereas 80- 90% from exposure to sunlight (Bozzetto, et al., 2012).

Skin exposure to UVB energies, ranging between 290-315 nm, catalyses the photolytic conversion of 7-dehydrocholesterol to previtamin D3, which occurs in the plasma membrane of human skin keratinocytes (European Food Safety Authority, 2006). After which, spontaneous isomerization due to the temperature of the skin creates cholcalciferol (Eyles, et al., 2013). Initially vitamin D is hydroxylated in the liver to 25-hydroxy-vitamin-D3 (25(OH)D), which is catalysed by cytochrome P450 enzymes, this is then released into the circulation bound to α-2 globulin. In the kidney 25(OH)D is further hydroxylated by the

mitochondrial enzyme 1-α hydroxylase (CYP27B1) into calcitriol ($1,25(OH)_2D$), which is the biologically active form of vitamin D (Thompkinson & Kharb, 2007). Magnesium is also required as a cofactor for vitamin D metabolising enzymes. $1,25(OH)_2D$ is transported in the body bound to vitamin D binding protein (European Food Safety Authority, 2006). The concentration of $1,25(OH)_2D$ is subject to various regulatory mechanisms; synthesis and concentration are increased in the plasma due to poor availability of calcium and phosphorus and high PTH concentrations; on the other hand it is reduced under conditions of sufficient calcium and phosphorus and by its own concentration (Scientific Committee on Food, 2003). 24-hydroxylase (CYP24A1) is the major catabolic mitochondrial enzyme for both 25(OH)D and $1,25(OH)_2D$ (European Food Safety Authority, 2006). **Fig. 2.15** illustrates the metabolism of vitamin D and its regulatory mechanisms.

In the mid 1600's numerous children living in crowded and polluted industrialised cities in northern Europe developed a bone deforming disease now known as rickets which had classical characteristics of deformed legs, growth retardation, bending of the spine and weak and toneless muscles. In 1822 Sniadecki recognised the importance of sun exposure for the prevention and cure of rickets. Followed by Palm in the 1980's recommending sun baths to prevent rickets and in 1919 Huldschinski found that exposing children to UV radiation was effective at treating rickets (Wacker & Holick, 2013). During the past decade there has been a resurgence of vitamin D deficiency worldwide (Perez-Rossello, et al., 2012).

The primary physiological role of vitamin D has been to maintain extracellular calcium ion levels, playing a crucial role in the development and maintenance of a healthy skeleton throughout life (Wacker & Holick, 2013).

Vitamin D is absorbed from dietary sources in the duodenum and jejunum. Fat soluble vitamins such as vitamin D are emulsified by bile for absorption; therefore adequate bile secretions are required for vitamin D absorption (Bass & Chan, 2006). Only a few food sources contain vitamin D3 naturally in quantities to make an impact on intake, these include fish liver, fish liver oils, fatty fish and egg yolks; this is one of the reasons why countries fortify (European Food Safety Authority, 2006).

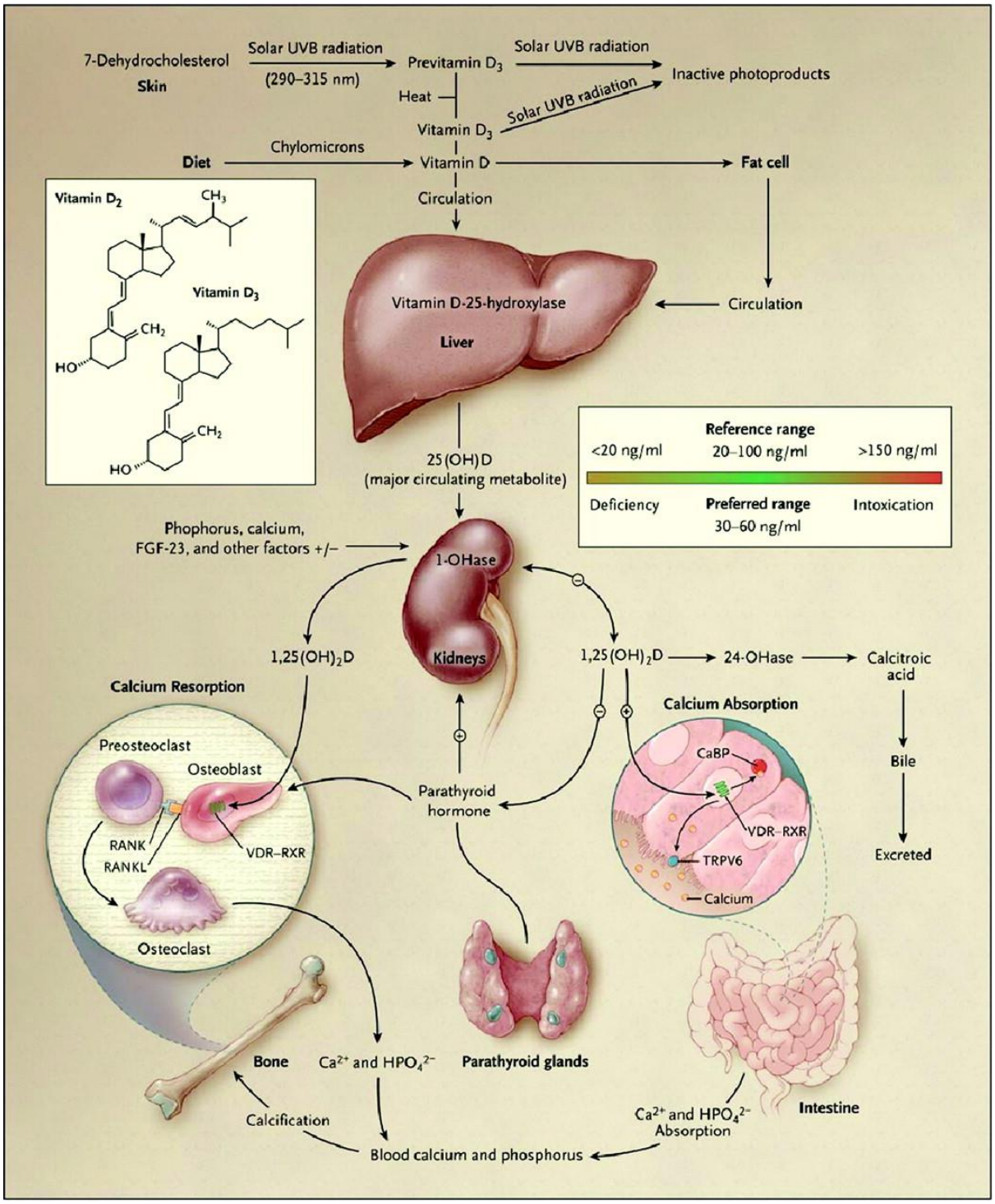

Figure 2.15 Metabolism and regulation of vitamin D (Wallis, et al., 2008). CaBP – calcium binding protein, FGF23 – fibroblast growth factor, RANK – receptor activator of NFκB, RANKL – receptor activator of NFκB ligand, RXR – retinoid X receptor, TRPV6 – transient receptor potential cation channel, VDR – vitamin D receptor.

In the small intestines 1, 25$(OH)_2$D enhances the absorption of calcium and phosphorus. It also decreases the secretion of PTH and can enhance the mobilisation of calcium and phosphorus from the bone at times of calcium deprivation (European Food Safety Authority, 2006), by binding to the VDR in osteoblasts biochemical signals are released which lead to the formation of mature osteoclasts. Osteoclasts release collagenases and hydrogen chloride to dissolve the matrix and mineral to release calcium into the blood. When vitamin D levels are low calcium absorption is insufficient to satisfy the requirements of bone health and for most metabolic functions and neuromuscular activity (Holick, 2006). 1,25$(OH)_2$D also results in the secretion of FGF-23 from the bone which reduces the reabsorption of phosphorus in the kidneys and also decreases the concentration of 1-α hydroxylase and thus its own production (Liu & Quarles, 2007).

1,25$(OH)_2$D is a steroid and binds to genomic receptors in the intestinal epithelial cells and increases the synthesis of the calcium binding protein, calbindin. Increased levels of calbindin will bind a larger proportion of intracellular calcium, which will decrease the intracellular ionized calcium levels, causing a gradient change between the intestinal lumen and intestinal epithelial cell; therefore calcium will diffuse intracellularly and cause an increase in calcium absorption. In the bone, 1,25$(OH)_2$D increases the differentiation of precursor cells to osteoclasts to mobilize calcium and phosphorus from the bone (Bass & Chan, 2006). 1,25$(OH)_2$D induces bone formation by regulation of matrix proteins, including osteocalcin, osteopontin, alkaline phosphatase, matrix gla proteins and collagen; and mineral apposition. 1,25$(OH)_2$D can act as a ligand and bind to the steroid nuclear receptor; VDR. Following ligand binding the VDR heterodimerises with RXR (antagonistic effects with vitamin A); this then binds to vitamin D response elements in vitamin D regulated genes. Numerous genes can be both up and down regulated (European Food Safety Authority, 2006).

Vitamin D deficiency impairs the absorption of calcium and phosphorus; resulting in poor mineralization of the skeleton (Scientific Advisory Committee on Nutrition, 2011), which can cause growth disturbances, bone abnormalities and muscle weakness (Powers, 1997). Vitamin D deficiency is clinically associated with lower serum ionized calcium levels and increased PTH levels (Wacker & Holick, 2013). This alters the Ca:P product in the bone and decreases mineralization of collagen with the development of osteomalacia or rickets in the immature skeleton. Decreased bone mineralization leads to thinning of the cortical bone, coarse trabeculation and diffuse radiolucency (Perez-Rossello, et al., 2012). Vitamin D deficiency during skeletal maturation disrupts chondrocyte maturation and inhibits the normal

mineralization of the growth plates, which causes widening of the epiphyseal plates at the end of long bones and bulging of costochondral junctions. The inadequate Ca:P product results in poor mineralization making the skeleton less rigid (Holick, 2006). Insufficient vitamin D during infancy can result in reduced bone mineralization, slower growth, bone deformities and increased risk of fractures (Grant & Holick, 2005).

There are numerous factors that contribute to the vitamin D status of an individual. The season, time spent outdoors, dress (coverage by clothing), skin pigment (melanin content), geographical altitude (>37° North and <37° south) and use of sunscreen will all effect the amount of vitamin D that can be produced through the skin. Dietary intake of vitamin D will be affected by the consumption of foods containing vitamin D, fortified foods, supplements and the concentrations in breast milk and infant formula. Lastly body mass index and the amount of body fat has also been inversely associated with serum 25(OH)D levels, likely to be due to the sequestration of body fat compartments (Holick, 2006).

2.4.6.1 Vitamin D: Recommendations

Vitamin D recommendations vary widely between countries (Thompkinson & Kharb, 2007). This in part is due to the differences in UVB exposure between countries (European Food Safety Authority, 2006). Current vitamin D requirements are based upon calcium absorption and bone health. In breast milk vitamin D is present as D2, D3 and 25(OH)D and unlike the majority of other recommendations for infants the concentrations present in breast milk are not classed as the gold standard and mothers are advised to take supplements (Powers, 1997). In breast milk the concentration of vitamin D varies widely between 0.1-1.2 ug/L in the literature which can also vary considerably due to season (European Food Safety Authority, 2006).

In the 1930's milk, soda and bread were fortified with vitamin D; however after several cases of presumed vitamin D intoxication in infants during the 1950's, the UK implemented strict regulations limiting the vitamin D fortification to margarine (Wacker & Holick, 2013).

Infant formula is fortified with vitamin D mainly in the form of vitamin D3 in the UK (Crawley & Westland, 2014). 25(OH)D plasma concentration is the conventional method used to assess vitamin D status (Powers, 1997). Serum 25(OH)D concentrations < 30 nmol/L (12 ng/mL) are considered to be vitamin D deficient and associated with the development of rickets; however much debate over the concentration to determine deficiency has been

debated in the literature (European Food Safety Authority, 2006). Insufficiency has been associated with serum concentrations of 25(OH)D between 30-50 nmol/L (12-20 ng/ml) and adequacy associated with 50 nmol/L (20 ng/ml). Vitamin D insufficiency was termed in the 1980's as subjects with sub optimal vitamin D status in the absence of rachitic bone disease. Some studies have also described a U shaped association between 25(OH)D levels and poor health outcomes (Jones, et al., 2012).

According to the most recent NDNS there is evidence of an increased risk of vitamin D deficiency in all age and sex groups. Twenty seven percent of preschool childrens (aged between 1.5-3 years) mean intakes of vitamin D were found to be below the RNI. Furthermore 7.5% of pre-school children were found to be below the threshold for vitamin D adequacy, measured by 25(OH)D. The major dietary contributor of vitamin D, being from milk and milk products, most of which are typically fortified (Public Health England, 2014).

Vitamin D3 is the main form used to fortify foods worldwide (European Food Safety Authority, 2006). Until recently vitamin D2 and D3 were believed to be equipotent based on anti-rachitic findings, however there is now evidence to suggest that the two forms may vary in their bioefficacy. For instance vitamin D3 has been found to be more effective at raising the 25(OH)D levels, however other studies have shown conflicting results (O'Mahony, et al., 2011).

Infants and toddlers are a population that are highly susceptible to vitamin D deficiency for numerous reasons. Breast feeding without supplementation, toddlers drinking juice rather than milk, more time spent indoors and infants often in blankets (reduced cutaneous vitamin D synthesis) are all contributing factors (Perez-Rossello, et al., 2012). Furthermore there is no UV radiation of the appropriate wavelength in Britain between October and March. Vitamin D intakes usually decline during weaning as most weaning foods are low in vitamin D; except those that are fortified. Between 6 months to 3 years infants and toddlers are particularly vulnerable to vitamin D depletion due to the high rate of calcium deposition in the bone (Department of Health, 1991).

2.4.6.2 Vitamin D: other roles

The primary and well established role of vitamin D is to maintain intra and extra cellular calcium concentrations and its essentiality for the development and maintenance of a mineralized skeleton. In the last couple of decades numerous functions other than those of mineral metabolism have been identified. Vitamin D affects cell differentiation and proliferation, plays a role in the process of insulin secretion and $1,25(OH)_2D$ regulates the transcription of numerous genes (European Food Safety Authority, 2006). Vitamin D deficiency has been associated with psoriasis, multiple sclerosis, inflammatory bowel disease, diabetes, hypertension, cardiovascular disease, metabolic syndrome and various cancers (O'Mahony, et al., 2011).

There is a historical link between vitamin D and innate immune function from the use of cod liver oil for tuberculosis. Individuals with lower 25(OH)D may be less able to support monocyte induction of antibacterial activity and therefore put the individual at risk of infection. Vitamin D can influence the innate response to pathogens via effects on antigen presentation by macrophages and dendritic cells. Treatment with $1,25(OH)_2D$ inhibits dendritic cell maturation, suppresses antigen presentation and promotes tolerogenic T cell responses. T and B cells express the VDR. $1,25(OH)_2D$ has anti-proliferative effects. In vitro, $1,25(OH)_2D$ has been shown to inhibit T_h1 cytokine production and induce T_h2 cytokine production. Vitamin D has been shown to inhibit IL-17 from T_h17 secreting cells. $1,25(OH)_2D$ also induces T reg which suppress the immune responses of other T cell functions (Jones, et al., 2012).

In 1969 the nuclear VDR was discovered which motivated a vast amount of research. Furthermore the VDR was found to be in at least 38 human tissues and organs and controls over 900 genes. The VDR is a phosphoprotein which enables a variety of biological functions upon binding by $1,25(OH)_2D$ and subsequent regulation of gene expression (O'Mahony, et al., 2011). The presence of VDR in other tissues suggests that vitamin D may also be important for other non-skeletal processes (Holick, 2006).

2.4.6.3 Vitamin D: Asthma

Epidemiological evidence suggests that low serum 25(OH)D is associated with a higher risk of upper and lower respiratory infections in children. Vitamin D has potent immunomodulatory properties. It exerts its action on cells of the innate immune system to inhibit pro-inflammatory cytokine production and induce antimicrobial peptide synthesis. Vitamin D also modulates the adaptive immune system by direct effects on T cell activation and on the phenotype and function of antigen presenting cells (Bozzetto, et al., 2012).

Many studies report a protective effect of vitamin D against asthma however, some suggest that vitamin D is a risk factor for asthma and atopy (Bozzetto, et al., 2012). Birth cohort studies in Boston, Scotland, Japan and Finland have shown that maternal dietary intake of vitamin D during pregnancy is inversely related to wheeze, recurrent wheeze and asthma in early childhood (Paul, et al., 2012).

2.4.6.4 Vitamin D: Allergy

The vitamin D receptor and vitamin D metabolizing enzymes have been identified in immune cells. Vitamin D can induce T reg production (Litonjua, 2009). Vitamin D3 inhibits T cell proliferation, in particular T_h1 cells, macrophages and T_h17 cells; and can amplify $CD4^+CD25^+$ T reg cells to increase production of IL-10 (Peroni, et al., 2011). Vitamin D induces a shift towards the allergic T_h2 response; reducing secretion of IL-2 and IFN-γ and increasing secretion of IL-4 (Hypönnen, et al., 2004). In addition animal models and studies in human foetal tissue show that vitamin D plays a role in the growth and maturation of the foetal lung (Litonjua, 2009).

A prospective Swedish birth cohort study looked at the relationship between low (0.6-13 μg/day) and high (13.2-25.1 μg/day) vitamin D3 intake in infancy and atopic disease at 6 years. Infants in the study were recommended to take 400 IU/day of vitamin D3 from 6 weeks to 2 years. The study found that in the group with a higher intake of vitamin D3, atopic manifestations were more prevalent (Bäck, et al., 2009). A Finnish study conducted by Hypönnen, et al. (2004) assessed vitamin D supplementation in infancy and found that the prevalence of allergic conditions at 31 years was higher in those who received vitamin D supplementation regularly compared to those who received it irregularly or not at all during the 1st year of life. In this particular study the vitamin D content was considerably higher than the RNI, at 2000 IU/day. The actual intakes as well as the recommended intakes vary

between countries and can make it difficult to compare studies. However both studies published in relation to high vitamin D intake in infancy suggest a detrimental effect on allergy development.

Kull, et al. (2006b) conducted a study in Sweden looking at early life supplementation of vitamin A and D in different forms; infants supplemented with vitamin A and D in the water soluble form in the 1st year of life had an almost 2 fold increased risk for asthma, food hypersensitivity and IgE sensitization to common allergens at 4 years compared to supplements in peanut oil. This suggests that the forms in which vitamins are administered is important, possibly due to differences in delivery and absorption.

In New Zealand Camargo, et al. (2010) found an inverse relationship between cord blood levels of 25(OH)D at birth and risk of wheezing by 5 years, however there was no effect on incidence of asthma. Allen, et al. (2013) found that 1 year old Australian born infants with vitamin D insufficiency were 3 times more likely to have egg allergy, 11 times more likely to have peanut allergy and overall more likely to have multiple food allergies. It has been suggested that vitamin D insufficiency in a critical developmental period increases the susceptibility to colonization with abnormal intestinal microbial pathogens which contribute to abnormal intestinal barrier permeability and excess exposure to dietary antigens.

Breast milk has lower concentrations of vitamin D than formula; therefore it is important for investigators to assess how the infant is being fed during any study. The above findings suggest a double edged sword phenomenon of vitamin D; where potentially both excess and deficient levels causing adverse immunological effects. A study by Rothers, et al. (2011) assessed cord blood levels of 25(OH)D in Tuscon infants and found that both low (<50 nmol/L) and high (≥100 nmol/L) concentrations were associated with higher IgE and increased aeroallergen sensitization at 5 years. In addition high 25(OH)D was associated with increased skin prick tests at 5 years but there was no association linked to allergic rhinitis or asthma (Rothers, et al., 2011). Furthermore it is difficult to compare vitamin D studies in different countries due to different levels of sunlight and dietary exposure, which is therefore important to consider when studying the effects of vitamin D. From an immunological prospective increasing vitamin D should decrease T_h1 responses and increase T_h2 responses, theoretically likely therefore to increase atopic disease. It has been observed that countries further from the equator and therefore exposure to lower UV radiation have higher paediatric admissions to hospital for food allergy related events (Allen, et al., 2013). **Table 2.15**

summarises the findings from vitamin D studies in term infants and the effect on atopic disease.

2.4.6.5 Vitamin D: Brain Development

Vitamin D has also been implicated in brain development and mental health (Morse, 2012). Vitamin D deficiency in early life has effects on neuronal differentiation, axonal connectivity, dopamine ontogeny and brain structure and function. Schizophrenia is considered a neurodevelopmental disorder and those born in winter and spring and at higher altitudes have increased risk of developing schizophrenia (Eyles, et al., 2013). A study in England has also linked birth seasonality to bipolar disorder (Grant & Holick, 2005). Therefore vitamin D may also play a role in mental development and disorders.

Table 2.15 Studies assessing the effects of vitamin D during infancy on atopic diseases.

Study Type	Study Group	Intervention	Assessment	Outcomes	Reference
Birth cohort	7648 infants	None – majority of infants received 2000 IU/day of vitamin D	Data on vitamin D supplementation obtained during 1st year of life. At 31 years current asthma and AR reported and SPT.	Prevalence of atopy, AR and asthma at 31 years higher in subjects who received vitamin D supplementation regularly during 1st year of life.	Hyponnen, et. (2004) Finland
Prospective birth cohort study	123 infants	None – infants received 1000 IU/day RP and 400 IU/day vitamin D3 from 6 weeks – 2 years	At 6 years postal questionnaire on AD, AR and asthma. Questionnaire on diet and supplements. Vitamin D3 intake assessed at 5, 7 and 10 months.	Atopic manifestations, especially AD more prevalent in group taking higher (13.2-25.1 μg/day) vitamin D3 compared to lower (0.5-13 μg/day).	Back, et al. (2009) Sweden
Population based birth cohort	823 infants	None	Cord blood taken at birth. Questionnaires up to 5 years.	Cord blood 25(OH)D levels inverse association with risk of respiratory infection and wheeze up to 5 years. No association with asthma by 5 years.	Camargo, et al. (2010) New Zealand
Prospective birth cohort	3670 infants	None – vitamins based in peanut oil/ water soluble form/ combination or no vitamins. Recommended 1000 IU/day Vitamin A and 400 IU/day vitamin D	Parental questionnaires at 4 years.	Infants supplemented with vitamins in water soluble form in the 1st year had almost a 2 fold increase risk for asthma, food hypersensitivity, multiple allergic disease and sensitization to common allergens at 4 years compared to vitamins in peanut oil.	Kull, et al. (2006b) Sweden

AD – atopic dermatitis, AR – allergic rhinitis, RP – retinyl palmitate, SPT – skin prick tests

2.4.6.6 Vitamin D: Diabetes

Vitamin D may play a role in glucose metabolism and the development of both type 1 and type 2 diabetes mellitus. Epidemiological evidence suggests a link between exposure to vitamin D in early life and development of type 1 diabetes mellitus. Animal studies have shown that $1,25(OH)_2D$ plays an essential role in the normal secretion of insulin. Vitamin D deficiency has been associated with poor B cell function. In a type 2 diabetes mellitus model low vitamin D levels have been observed. In humans glucose intolerance and hyperinsulinaemic responses in oral glucose tolerance tests have been associated with low vitamin D concentrations; in addition a positive correlation between circulating 25(OH)D and insulin sensitivity has been observed (Flores, 2005). Sufficient levels of 25(OH)D in infants appear to greatly reduce the risk of type 1 diabetes mellitus and VDR alleles have been associated with the risk of type 1 diabetes mellitus (Grant & Holick, 2005).

There is some evidence to suggest that increasing vitamin D intake in infancy may reduce the development of type 1 diabetes mellitus. Vitamin D has also been associated with several contributing factors associated with the development of type 2 diabetes mellitus, such as pancreatic B cell function, insulin sensitivity and systemic inflammation (Kulie, et al., 2009). B islet cells express the VDR and respond to $1,25(OH)_2D$ by increasing insulin production (Holick, 2006).

The VDR is expressed in pancreatic B cells and $1,25(OH)_2D$ stimulates the secretion of insulin. Vitamin D status also improves insulin sensitivity by up regulating insulin receptors. Ecological studies have also associated the prevalence of auto immune disease with latitude. There is an inverse association between maternal 25(OH)D and type 1 diabetes mellitus risk. Furthermore supplementation with vitamin D during the first year of life has been associated with a reduction in type 1 diabetes mellitus in later life; however other studies have shown no effect (Wacker & Holick, 2013).

Therefore as national feeding surveys have indicated that there is evidence for an increased risk of vitamin D deficiency in all age and sex groups in the UK, which will partly be due to limited foods being rich in vitamin D. In particular, for infants, mother's breast milk is an insufficient source of vitamin D. It is vital to assess the infant's vitamin D dietary intake due to its primary role in the growth and development of bones and with mounting evidence to suggest roles in numerous other major disease areas.

2.5 Processing and Storage

The food industry has mainly been concerned with food hygiene in relation to food manufacture. Where traditionally heat treatments, such as pasteurization and sterilization, are used to inactivate harmful micro-organisms to make a food product safe to eat for consumers. However food processing treatments can lead to a loss of nutrients and deterioration of organoleptic properties (Chemat, et al., 2011). The main functions of food preservation are to extend the shelf life of food products, remove and inactivate toxins or microbes in foods with maintaining the original sensory and nutritional properties as high as possible. Therefore there needs to be a balance between food safety and nutritional quality (Henry & Heppell, 2002).

Food spoilage can be defined in a number of ways; the food is no longer acceptable due to changes in sensory properties, the number or activity of pathogenic micro-organisms make them unsafe to eat or when deterioration of one or more nutrients no longer has its declared nutritional value. In most countries there is a legal requirement to identify the best before, sell-by or use by date on food products. In Europe the Food Labelling Directive (2000/13/EEC) requires most pre-packed foods to carry a minimum durability on the packaging. The best before date indicates when a food will retain its optimum condition, whereas use by labelling is employed for foods that are microbiologically highly perishable and are likely to cause a danger to health (Fellows, 2009).

Numerous studies have reported variable and in some cases substantial losses of nutrients in foods due to physical removal, chemical reactions during postharvest, industrial processes, storage and domestic preparation (International Union of Food Science and Technology, 2000-2010).

The nutritional content in plant sources at harvest can vary due to the variety of cultivar, maturity stage, geographical or climate effects, agricultural practices and soil composition. Furthermore the nutritional content in animal sources can also vary depending on breed and age of animal, composition of the feed and breeding practices (International Union of Food Science and Technology, 2000-2010). Therefore the nutritional content of foods can vary from the beginning of production depending on numerous factors relating to the ingredients used before any processing has even occurred.

The heating of foods can destroy microbes and inactivate enzymes and toxins. Microorganisms and enzymes are primarily responsible for food deterioration. Conventional thermal processes kill vegetative microorganisms and some spores and inactivate enzymes (Chemat, et al., 2011). Thermal processing has long been known to cause significant losses of vitamins. Higher temperatures and longer exposure times decrease the retention of nutrients (International Union of Food Science and Technology, 2000-2010). Nutrients can be sensitive to pH, oxygen, moisture, light and heat (Henry, et al., 2009). Thermal processing can also cause isomerization of certain nutrients, for example *trans* to *cis* β-carotene, which can affect the function of nutrients within the body (Rickman, et al., 2007).

Pasteurization kills most microorganisms, whereas sterilization is a more severe process and destroys all microorganisms (Food a Fact of Life, 2015). Pasteurization and sterilization are the most common techniques used to inactivate microorganisms; however treatment temperature and time tend to be proportional to nutrients lost (Chemat, et al., 2011).

The physical removal of certain nutrients such as vitamins, carotenoids and flavonoids can occur if the peel is discarded during preparation, due to nutrients being highly concentrated in certain regions. Grinding and fractionation in the milling of cereal grains causes major losses of vitamins and minerals due to the removal of the embryo and bran in the process, where they are concentrated. In plant tissues vitamins are protected, actions such as cutting, chopping and shredding can destroy this protection and increase exposure to oxidation (International Union of Food Science and Technology, 2000-2010).

Transport and storage can also lead to losses in nutrients due to exposure to temperature, and light. Furthermore losses also tend to decrease over then length of storage time. Processing results in the most nutrient losses, whereas storage in comparison, has fewer losses, potentially due to modified atmosphere packaging (exclusion of oxygen), storage at low temperatures (slower reaction rates) and protection from light (International Union of Food Science and Technology, 2000-2010).

Minerals are not destroyed by exposure to heat, light, oxidising agents or extreme pH, and losses due to processing, storage and cooking are minimal. They are however removed by leeching and physical removal (International Union of Food Science and Technology, 2000-2010). Water soluble vitamins can also be leeched into water during processing (Kalt, 2005). On the other hand lipid soluble vitamins are not significantly lost to leeching, but are sensitive to oxidation (Department of Health, 2011).

Home preparation of foods with heat treatments such as microwaving, boiling, steaming, freezing and deep frying can also affect nutrient content (International Union of Food Science and Technology, 2000-2010).

Non-thermal processing technologies such as high pressure processing and high intensity pulsed electric field processing have been reported to inactivate microorganisms without the adverse thermal effects (International Union of Food Science and Technology, 2000-2010). However new processing techniques such as food irradiation, high pressure processing, ohmic heating and pulse electromagnetic techniques have not been used extensively due to high costs, lack of regulatory approval and consumer acceptance (Chemat, et al., 2011).

Studies that have looked at the effects of certain processing methods on nutrients have often given variable results (Rickman, et al., 2007). In addition the wide variation of processing methods used on foods and their parameters, such as temperature and time of exposure has made it difficult to draw clear conclusions. Therefore it is essential to assess the effects that processing and storage have on the nutrient content of foods, as if they are having a detrimental effect and resulting in substantial nutrient losses this will affect the actual intake which could lead to nutritional deficiencies. If this is the case then methods may need to be employed to protect certain nutrients against losses. For infants this will be particularly important as the limited volume of food that they can consume needs to satisfy adequate nutritional requirements for optimal growth and development. Both infant foods and infant formula need to be assessed.

2.6 Fortification

There is now great interest to improve health and wellbeing in the form of dietary means by consumers, public health organisations and the medical community mainly due to increased rates of adverse, diet related health conditions in Western societies which cause huge social and economic costs (Kalt, 2005). Food fortification has been used by the food industry for over 50 years. Fortified or enriched foods are foods that have one or more nutrients added to them to maintain, improve or enhance the quality of a diet consumed by a specific population (Henry & Heppell, 2002). Fortification is an effective, efficient and sustainable alternative to control and reduce the health consequences of micronutrient deficiencies (Shamah & Villalpando, 2006). Furthermore it can also be used to restore nutrients lost during processing (Department of Health, 2011).

In developing countries micronutrient deficiencies are a public health problem especially for infants and children during the first 2 years of life. Iron deficiency has resulted in anaemia and vitamin A deficiency has been associated with higher rates of infection and mortality (Eichler, et al., 2012).

There has been numerous fortification programmes implemented worldwide. For example vitamin D in milk for the prevention of rickets, thiamine and niacin in wheat and cereals for beriberi and pellagra, iodization of salt for mental retardation, iron for anaemia, vitamin A for vision and infections and zinc for growth and infections (Shamah & Villalpando, 2006).

The food used for fortification must be consumed by the population in adequate quantities and on a regular basis to reach required intakes. There must also be a low risk of excessive consumption. The nutrient added must be well absorbed and utilised (bioavailability) by the body, with a balance between enhancers and inhibitors of absorption and cause minimal effects on other nutrients within the diet with no changes to organoleptic properties. Furthermore the cost of a fortified product should be low so that it is available to a wide population. Other nutritional substances can also be added to foods that are considered to be beneficial for health, such as probiotics, omega 3's and taurine (Shamah & Villalpando, 2006).

2.7 Microencapsulation

Microencapsulation generally refers to a process where a core material (active agent) is entrapped within a shell or coating (wall material) or embedded in a homogenous or heterogeneous matrix (Gharsallaoui, et al., 2007). It has also been defined as a technology of packaging, solids, liquids or gases in small capsules that release their contents at a controlled rate over prolonged periods and under specific conditions. The particles produced are typically a few nm – mm in diameter. In the food industry functional compounds are added to food products, these compounds are typically highly susceptible to environmental, processing or gastrointestinal conditions, therefore encapsulation aims to effectively protect them (Nedovic, et al., 2011).

There are numerous reasons and benefits to encapsulating food ingredients, such as (Nedovic, et al., 2011):

- To provide protection against degradation of the core material i.e. from oxygen and water.
- To provide a physical barrier between sensitive bioactive core materials and the environment.
- Prevent undesirable interactions with the food matrix.
- Prevent the loss of flavour.
- Controlled release of the core material.
- Mask undesirable tastes and aromas.
- Increase bioavailability.
- Stabilize food ingredients.
- Improve stability of the final food product during storage and processing.
- Modification of the original material to improve physical characteristics, i.e. easier handling.

The wall material used for the shell or coating must be food grade, biodegradable, must not react with the active agent and have the ability to form a barrier between the internal phase and surroundings. Polysaccharides, lipids and proteins can be used as wall materials (Nedovic, et al., 2011).

The encapsulation technique most commonly used in the food industry is spray drying as it is flexible, continuous and economical. Other techniques used include spray chilling, spray cooling, coacervation, fluid-bed coating, freeze-drying, melt extrusion, melt injection and extrusion methods (Gharsallaoui, et al., 2007). There are also more expensive techniques such as molecular inclusion in cyclodextrins and liposomal vesicles which are less commonly used (Nedovic, et al., 2011). The physico chemical properties of the core, the composition of the wall materials and microencapsulation technique used will determine the type of particles produced, **Fig. 2.16** (Gharsallaoui, et al., 2007).

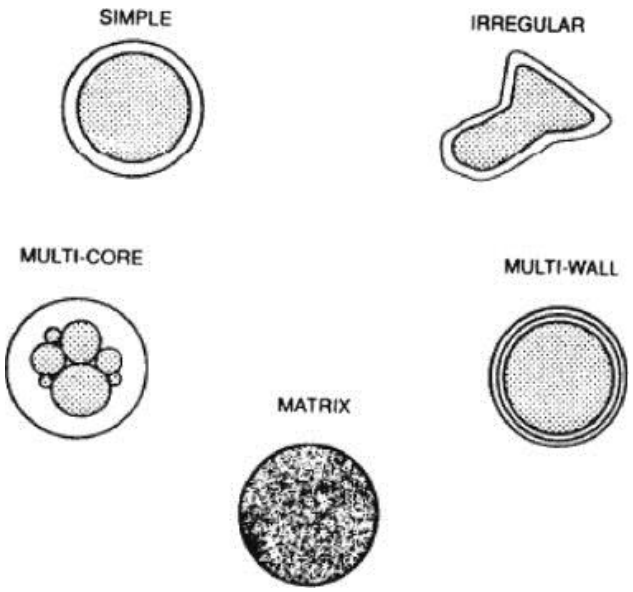

Figure 2.16 Morphology of different types of microparticles (Gharsallaoui, et al., 2007).

2.8 Assessing Dietary Intake

Zand, et al., (2012) have adopted a combination of approaches to estimate the total daily intake of an infant based on the consumption of commercial 'ready-to-feed' infant foods and manufactured infant formulas.

Estimation of the gastric capacity of an infant (30g / kg body weight / day) is based upon the WHO expert Consultation on Complementary feeding data. The average infant weight for different age ranges is taken from the report by the Committee of Medical Aspects of Food and Nutrition Policy (COMA): Weaning and the Weaning Diet. The gastric capacity of an 8 month old infant, with an average weight of 8.3 kg is estimated to be 249 g per day, which would normally be divided by 3 to take into account breakfast, lunch and dinner. However in this particular system the daily intake is based upon intake from infant formula and commercial 'ready-to-feed' infant foods, omitting cereal based breakfast porridges. Therefore the gastric capacity is divided by 2 for lunch and dinner, resulting in 124.5 g portion sizes for lunch and dinner. The estimated milk consumption was set at 600 mL as recommended by COMA for infants up to 12 months.

Consequently the total daily intake will be calculated by adding the contribution of a particular nutrient from 600 mL of infant formula and 124.5 g of the nutrient for lunch and dinner from the experimental analysis of commercial 'ready-to-feed' infant foods. This value can then be compared to recommendations to assess whether the infant is deficient, in excess or meeting requirements.

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Chapter 3

An investigation into the fatty acid content of selected fish based commercial infant foods in the UK and the impact of commonly practiced re-heating treatments used by parents for the preparation of infant formula milks

3.1 Introduction

Traditionally lipids have been considered as a major contributor to dietary energy, however recently the importance of the nature of dietary lipids ingested during infancy has been highlighted (Innis, 2007). Long chain polyunsaturated fatty acids (LCPUFA) are thought to be a specific dietary requirement during infancy due to a rapid rate of physiological and immunological development (Department of Health, 1991). Currently research has focused upon the LCPUFA, arachidonic acid (AA) and docosahexaenoic acid (DHA) for their essentiality and roles in the visual and brain development of infants (Simmer, et al., 2011). In addition, with the rise in the prevalence of allergies during recent years, a particular interest has also been focused on *n*-3 LCPUFA, such as eicosapentaenoic acid (EPA) and DHA for their possible protective roles in restricting the development of allergic disease (Calder & Miles, 2000).

Humans must obtain the essential fatty acids α-linolenic acid (ALA) 18:3 (*n*-3) and linoleic acid (LA) 18:2 (*n*-6) from dietary sources as they lack Δ-15 and Δ-12 desaturase enzymes. ALA is then metabolised by Δ-6 desaturation, elongation and Δ-5 desaturation to form EPA 20:5 (*n*-3); LA is metabolised by the same enzymes to form AA 20:4 (*n*-6) (Innis, 2008). The synthesis of DHA 22:6 (*n*-3) however requires a second Δ-6 desaturase step and β-oxidation. There is, however, much evidence to suggest that during infancy conversion of the essential fatty acid precursors (ALA and LA) into LCPUFA is insufficient due to the immaturity of the desaturase and elongase enzymes and therefore meeting the essential daily requirements through pre-formed dietary sources for infants is paramount (Makrides, et al., 2000).

Several randomized controlled infant formula milk supplementation trials have demonstrated that infant feeding with DHA levels similar to the worldwide average (0.32% of fatty acids) and AA levels higher than DHA provide visual and cognitive benefits to infants (Birch et al., 2010). Expert bodies now recommend that infants who do not receive breast milk should receive infant formula milk supplemented with DHA and AA (Hoffman, et al., 2009).

In the UK exclusive breastfeeding is recommended for the first 6 months of an infant's life, with the only suitable substitute being infant formula milk. From the diet and nutrition survey of infants and young children (DNSIYC) conducted in 2011, 87-89% of infants aged 4-11 months are being given a type of infant formula milk. After 6 months complementary infant foods are recommended to be introduced. The DNSIYC has also identified that 58% of children who are fed foods other than milk have eaten a commercial baby or toddler meal (Lennox, et al., 2013). The results from the DNSIYC indicate that infant formula milk and commercial baby and toddler meals are major dietary contributors to an infant's nutritional intake; therefore these products need to be assessed for their nutritional adequacy. Furthermore there is currently no clear and complete analytical nutritional data available for commercial 'ready-to-feed' complementary infant foods in the UK. The nutritional database used in the UK, McCance and Widdowson contains limited data on the composition of complementary infant foods, in addition the analytical techniques and nutrient data contained may now be outdated. It is essential that food composition tables are regularly updated with the current foods available on the market, especially with the regular introduction of new infant food products to the market (Food Standards Agency, 2015).

The oxidation of lipids is induced by oxygen in the presence of initiators including heat, free radicals, light, photosensitizing pigments and metal ions through sequential free radical chain reaction mechanisms. Numerous compounds can be formed during these reactions such as hydroperoxides and conjugated dienes (Martínez-Yusta, et al., 2014). Alterations in food products due to lipid oxidation can deteriorate the quality of the product both in terms of sensory and nutritional characteristics and can generate undesirable compounds which can have effects on human health. In particular unsaturated fatty acids are especially susceptible to oxidation (Santos-Fandila, et al., 2014). Infant formula milks are recommended to be served warm to the infant; meaning they will typically be subjected to a heat treatment prior to being given to the infant. Therefore it is important to assess any effect that a particular heat treatment has on the fatty acid content of the infant formula milk.

The study herein, aims to investigate the following.

a) Investigate the polyunsaturated fatty acid content of commercial 'ready-to-feed' complementary infant foods available on the UK market in order to evaluate whether they are nutritionally adequate in relation to recommendations for a 6-9 month old infant, also taking into consideration the consumption of infant formula milk.

b) Assess whether commonly practiced re-heating treatments used for the preparation of infant formula milks by parents affect the polyunsaturated fatty acid content of infant formula milk.

c) Examine the transparency of nutritional labels, by comparing the analytically quantified concentrations of polyunsaturated fatty acids in infant formula milk to those provided on the packaging.

d) Evaluate the content of polyunsaturated fatty acids in four infant formula milk brands available on the UK market based on their nutritional label.

3.2 Materials and methods

3.2.1 Chemicals

Linolenic acid, linoleic acid, arachidonic acid, docosahexaenoic acid and eicosapentaenoic acid, butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) were obtained from Sigma Aldrich (Bellefonte, PA). HPLC-grade acetone, acetonitrile, tetrahydrofuran, formic acid, absolute ethanol, isopropanol, methanol and laboratory reagent grade chloroform and potassium hydroxide (KOH) were purchased from Fisher (UK).

3.2.2 Sample collection and analysis of complementary infant foods

Four fish-based commercial 'ready-to-feed' complementary infant food samples representative of the four leading brands in the UK (Statista, 2008a) were selected from leading supermarkets (Statista, 2008b) between June and July 2014. The main ingredients of the baby food samples and their characteristics are presented in **Table 3.1**. Three independent replicates were analyzed before the use by date of the product and within one month of purchase. The samples were stored unopened at room temperature, similar to their distribution and market environment.

Each of the food samples were mixed and homogenised using a domestic blender (Multi-quick, Braun 300) and three independent replicates of 1 g (wet weight) were weighed prior to the addition of 1.2 mL of chloroform/methanol (3:1, v/v) with 1 mg/mL BHT. The mixture was vortexed for 15 minutes followed by centrifugation at 35, 500 RCF for 5 minutes to remove solids. 1 mL of the liquid phase extract was dissolved in 4 mL isopropanol/water (3:2, v/v) with 10 mg/L BHA and 1 mL 5M KOH, which were then heated in an 80°C water bath for 1 hour with occasional stirring. After cooling at room temperature a 500 µL aliquot was removed and 25 µL of formic acid was added to neutralise the sample. 50 µL of sample was then added to 50 µL of a standard solution (containing 0.5 mg/mL EPA, ALA, DHA and AA and 2.5 mg/mL LA) to prepare the spiked sample. Finally 15 µL of the spiked sample was injected into the high performance liquid chromatography-charged aerosol detector (HPLC-CAD).

Table 3.1 Infant complementary food sample characteristics.

	Product name (*n*=4)	Ingredients	Nutritional information (per 100g)
A	Creamy vegetables and cod	Baby-grade vegetables (40%) [carrot (20%), pumpkin (8%), onion, peas, celeriac, spinach], cooking water, rice, cod (8%), potato (7%), cream, corn starch, vegetable oils (rapeseed, sunflower), thyme, garlic.	**Energy** 266kJ/63kcal, **Protein** 2.3g, **Carbohydrate** 8.5g, of which sugars 1.4g, **Fat** 1.9g, of which saturates 0.8g, omega 3 (ALA) 59mg, **Fibre** 1.4g, **Sodium** 0.03g, iron 0.24g
B	Fish pie with mash	Organic whole milk 28%, organic peas 14%, organic potatoes 14%, organic broccoli 14%, organic onions 14%, organic salmon 11%, organic unsalted butter 4%, organic parsley <1%.	**Energy** 518kJ/124kcal, **Protein** 6g, **Carbohydrate** 8.6g, of which sugars 1.5g, F**at** 6.9g, of which saturates 2.9g, **Fibre** 1.9g, **Sodium** trace
C	Mediterranean paella with salmon	Organic vegetables 36% [onion 8%, tomato 6%, sweet potato 6%, peas 5%, red pepper 5%, tomato paste 5%], water, organic cooked rice 18%, organic salmon 12%, organic quinoa flakes 3%, organic parsley 1%, organic turmeric, organic garlic, organic thyme.	**Energy** 306kJ/73kcal, **Protein** 3.2g, **Carbohydrate** 9.6g, of which sugars 2.4g, **Fat** 2.1g, saturated fat 0.5g, **Fibre** 1.3g, **Sodium** trace
D	Salmon with root vegetables	Organic vegetables (61%) [carrot, potato, butternut squash, leek], water, organic salmon (10%), organic apple (10%), organic cheese (from cow's milk), organic tarragon.	**Energy** 249kJ/59kcal, **Protein** 402g, **Carbohydrate** 6.2g, of which sugars 3.5g, **Fat** 1.6g, of which saturates 0.8g, **Fibre** 1.5g, **Sodium** 0.04g

3.2.3 Sample collection and analysis of infant formula milk

A single brand of infant follow-on-formula milk was selected for the quantitative analysis in both the ready-to-feed (RTF) and the powdered form, in order to determine the impact of preparation methods on fatty acid content of formula milks. The selected infant formula milk was suitable for an infant aged 6 months + and available in the supermarkets in the UK between June and July 2014. The selection of this particular brand was based on the inclusion of ALA, LA, DHA and AA content being present on the nutritional label of the infant formula milk, where other brands on the UK market did not provide such information. Three independent replicates were analyzed before the use by date of the product within one month of purchase. The selected infant formula milk was stored, unopened, at room temperature, similar to their distribution and market environment.

Four different commonly practiced re-heating treatment methods of the selected infant formula milk were assessed, which are commonly practiced by parents and in accordance with the manufacturer's instructions including:

a) Control RTF infant formula milk had no heat treatment applied prior to analysis.

b) Heated RTF infant formula milk was prepared by placing the bottle of RTF infant formula milk in previously boiled water (15 minutes after boiling) as advised by the manufacturer. The infant formula milk reached a maximum temperature of 50 ± 0.5°C, which was left to cool down to 37°C prior to analysis.

c) Microwaved RTF infant formula milk was prepared by microwaving 120 mL of RTF infant formula milk for 30 seconds, which reached a maximum temperature of 50 ± 0.5°C, which was left to cool down to 37°C prior to analysis. It is important to note that this type of preparation method is not advised by manufacturers; however it is commonly used by parents for the preparation of infant formula milk and therefore its inclusion is warranted.

d) Powdered infant formula milk was prepared by reconstituting 6 scoops of powdered milk to 180 mL of previously boiled water at the temperature of 88 ± 0.5°C as advised by the manufacturer. The reconstituted infant formula milk reached a maximum temperature of 81 ± 0.5°C and was cooled down to 37°C after being subjected to running cold water.

It is important to mention that a control could not be used for the powdered form of the brand of infant formula milk selected as it would not dissolve in cold water.

Samples from each of the different preparation methods were left to cool to 37°C prior to analysis, the recommended temperature for the consumption of milk by infants. 1 mL of each sample was dissolved in 5 mL isopropanol/water (3:2, v:v) with 10 mg/L BHA and 1 mL 5 M KOH, which were then heated in an 80°C water bath for 1 hour with occasional stirring. After cooling at room temperature a 500 μL aliquot was removed and 25 μL of formic acid was added to neutralize the sample prior to 15 μL being injected into the HPLC-CAD.

3.2.4 Sample collection for nutritional labelling evaluation of infant formula milks

Four of the leading brands of follow-on infant formula milk available at leading supermarkets in the UK were also selected for nutritional evaluation based on the information provided on the nutritional labels with respect to fatty acid content.

3.2.5 HPLC–CAD analysis

A reverse phase (RP) HPLC-CAD methodology was employed for the analysis of fatty acids in this study (Acworth, et al., 2011). CAD has successfully been used for the detection of various different lipids (Lisa, et al., 2007; Moreau, 2006; 2009; Plante, et al., 2009). CAD offers low detection limits, a dynamic range over four orders of magnitude, good reproducibility, gradient compatibility, and similar analyte responses for non-volatile species independent of chemical structure. Lipids typically lack chromophores and therefore do not exhibit any significant response to UV absorption. CAD and evaporative light scattering detection (ELSD) involve similar nebulisation stages with different methods of detection; however CAD avoids the complex mixture of light scattering mechanisms which creates a limited dynamic range and non-uniformity of responses using ELSD detection. Furthermore CAD is typically ten times more sensitive compared to ELSD. With respect to gas chromatography analysis, fatty acids require derivatisation and auto-oxidation of fatty acids may be an issue, in addition to thermal degradation of certain lipids (Plante et al., 2009).

An Agilent 1200 series RP-HPLC system was used for the analysis, equipped with a quaternary pump, thermostated autosampler set to 15°C, and a Thermo Scientific™ Dionex™ Corona™ ultra RS™ Charged Aerosol Detector. An Acclaim™ C30 column (250 x 3 mm), with a 3 μm particle size from Thermo Scientific (UK) was used for analysis.

Separation was performed at 30°C using a gradient system consisting of Mobile phase A [water, formic acid, mobile phase B (900:3.6:100)], Mobile phase B [acetone, acetonitrile, tetrahydrofuran, formic acid (675:225:100:4)] at a flow rate of 0.5 mL/min. The gradient system used had a A:B starting composition of 29:71 (v/v), respectively, which was gradually increased over 20 minutes to reach 73% B. At 20 minutes the composition was changed to 95% B and held at this composition for 10 minutes. Finally the composition of B was decreased back to its original composition of 71% for 10 minutes with a stop time at 40 minutes. Injection volume of 15 µL, nebulizer temperature of 15°C and the corona filter set to high was used for analysis. The retention time for the fatty acids were 9.8, 10.2, 11.8, 12.7 and13.6 min for EPA, ALA, DHA, AA and LA, respectively **(**shown in **Fig. 3.1)**.

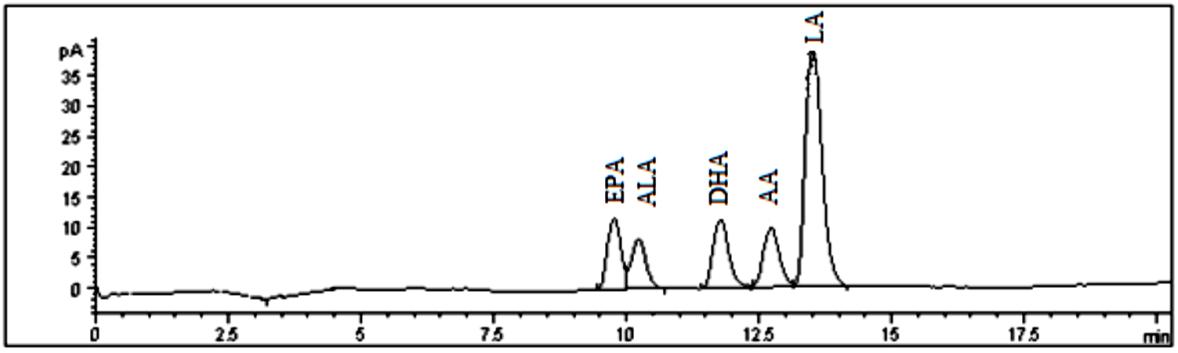

Figure 3.1 HPLC-CAD chromatogram of fatty acids: EPA (9.772 min), ALA (10.238 min), DHA (11.778 min), AA (12.735 min) and LA (13.547 min).

3.2.6 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Six different standard solutions of EPA, ALA, DHA, AA (0.02 – 2 mg/mL) and LA (0.1 – 10 mg/mL) were prepared following dilution of stock solution (10 mg/mL) using absolute ethanol with 1 mg/mL BHT. Calibration curves were fitted using the power function. The limit of detection (LOD) and limit of quantification (LOQ) were determined using the signal-to-noise ratio (S/N) of the lowest three concentrations (**Table 3.2).**

Table 3.2 Range, Limit of Detection (LOD) and Limit of Quantification (LOQ) for EPA, ALA, DHA, AA and LA.

Compound	Range (mg/mL)	LOD (mg/mL)	LOQ (mg/mL)
EPA	0.02 – 2.00	0.004	0.011
ALA	0.02 – 2.00	0.005	0.014
DHA	0.02 – 2.00	0.003	0.009
AA	0.02 – 2.00	0.003	0.010
LA	0.1 – 10.00	0.009	0.027

AA – arachidonic acid, ALA – α-linolenic acid, DHA –docosahexaenoic acid, EPA – eicosapentaenoic acid, LA – linoleic acid, LOD – limit of detection, LOQ – limit of quantification

3.2.7 Quality Assurance

The accuracy of the method was verified by analysing a Wild Alaskan full spectrum omega soft gel, a fish oil rich in EPA (202 mg/capsule) and DHA (180 mg/capsule). The % recovery for EPA was 99.85% and DHA 100.12%, which demonstrates the validity of the method.

Spiking of samples was also used to verify the recovery of the fatty acids tested. 111%, 105%, 105%, 108% and 119% recoveries were obtained for EPA, ALA, DHA, AA and LA respectively (Trisconi, et al., 2012).

3.2.8 Statistical methods

The experimental results were subject to statistical analysis using Excel 2010 and SPSS package v.17.0. A 2-sided unpaired *t*-test at $p = 0.05$ level of significance (with 95% confidence interval) was used to examine mean differences between heat treatments and the nutritional label of the infant formula milk.

3.3 Results and Discussion

3.3.1 Evaluation of infant's dietary daily intake of fatty acids in relation to recommendations

The concentration of fatty acids determined from the commercial 'ready to feed' complementary infant food samples analyzed is summarized in **Table 3.3**. All the samples were deliberately selected from a range of fish based complementary foods with an average of 10.25% fish content, as essential fatty acids are more commonly found in the storage lipids of marine animals rather than land animals. It is however important to note the inclusion of vegetable oils (blend of rapeseed and sunflower oil) in Brand A, as well as whole milk and cheese in Brand B as contributing factors.

Table 3.3 Concentration of fatty acids in four brands of commercial 'ready to feed' complementary infant foods in the UK (mean (*n*=3) ± SD).

Fatty acid	Brand A	Brand B	Brand C	Brand D	Mean
EPA (mg/g)	<LOD*	0.014 ± 0.009	ND	0.033 ± 0.013	0.024
ALA (mg/g)	0.012 ± 0.002	0.036 ± 0.007	ND	0.054 ± 0.021	0.034
DHA (mg/g)	ND	ND	ND	0.014	-
AA (mg/g)	ND	<LOD**	ND	0.020 ±0.011	0.020
LA (mg/g)	ND	<LOD***	ND	0.112 ± 0.065	0.112

LOD – limit of detection, ND = not detected, * < LOD (0.004 mg/mL), ** < LOD (0.003 mg/mL), *** < LOD (0.009 mg/mL)

The nutritional information and ingredient declaration on the labelling of the selected food samples does not include specific fatty acid content apart from Brand A where the concentration on ALA was stated to be 0.59 mg/g, as opposed to the analytically determined 0.012 mg/g. The variation between the label and the analytically quantified values in studies as such usually highlights the adverse impact of food processing which is not usually considered when labelling information is calculated using a nutritional data base such as Diet Plan 6; in addition to differing soils, climates, agricultural practices or the different varieties of ingredients used which has also been highlighted by Fernandez, et al. (2002).

Although the data is insightful, it is important to examine the entire daily nutrient intake when studying the nutrient quality of complementary foods. In **Table 3.4** an example of a standard menu suggested by Zand, et al. (2012), which includes recommended daily intake of infant formula milk and commercial 'ready to feed' complementary infant foods tested in this study, is provided to calculate the total daily intake of fatty acids for an infant aged 6-9 months.

The limitation with the standardised menu approach is that it does not take into consideration wastage of foods and milk, and contributions made from the consumption of breast milk, home-made foods, breakfast and snacks. The exclusion of breakfast and snack products are purely due to the fact that these products are not typically fortified with polyunsaturated fatty acids and it is unlikely they would make a significant contribution. As for the total dietary intake also, there is currently no recommended guidelines for the daily intake of fatty acids in the UK in order to evaluate the significance of the findings.

However the US National Institute of Health provides recommendations for the composition of infant formula/diet based upon studies that demonstrate support for both the growth and neural development of infants in a manner particular to that of the breastfed infant (Simopoulos, 2000). In **Table 3.4**, the calculation of fat intake is based on 40% (EFSA NDA Panel, 2013) of the Estimated Average Requirements (EAR) for energy of an infant breast milk substitute-fed, aged between 6-9 months at 664.23 kcal/day (Scientific Advisory Committee on Nutrition, 2011), which equates to 29.5 g of fat per day (1g fat = 9 kcal). Based on this calculation % of fatty acids were calculated based upon the US National Institute of Health recommendations.

Based on the results of the current study, the total daily intake of pre-formed sources of AA (0.037 g/day) and DHA (0.023 g/day) were found to provide only 24.89% and 22.58% respectively of the recommended intakes (0.148 g/day for AA, 0.103 g/day for DHA). These findings are despite the fact that infant formula milks are currently being fortified and since during infancy the conversion of the LCPUFA is suggested to be insufficient, the latter poses a significant question in relation to the adequacy of the intake of LCPUFA and warrants further investigation. With respect to the recommended relative proportions of AA to DHA, AA was found to be equal to or higher than DHA as recommended.

Furthermore the total daily intake of LA (3.148 g/day) was found to provide 106.71% of the recommended intake and ALA (0.296 g/day) to only provide 67.0% of the recommended intake (LA 2.950 g/day; ALA 0.443 g/day); which increases the LA:ALA ratio from the recommended 6.7:1 up to 10.6:1. However it is important to mention that the 10.6:1 ratio is within EU regulations of 5-15:1 (Koletzko et al., 2005). It is important to note that the conversion to LCPUFA from these essential fatty acids is thought to be insufficient in infants; therefore it may not significantly affect the concentrations of LCPUFA. It may, however, still be important especially with respect to the development of allergic disease which are currently on the increase in the UK and therefore warrants further attention.

The total daily intake of EPA (0.005 g/day) provided 19.91% of the recommended intake, which is below the recommended upper limit of 0.1% of fatty acids. EPA is not added to infant formula milks in the UK therefore all pre-formed sources must come from the foods consumed by an infant.

Table 3.4 Evaluation of total daily dietary intake of fatty acids by an infant aged 6-9 months old[a], based on a standard feeding regime composed of commercial 'ready-to-feed' infant foods and formula milk in relation to recommended total daily intakes.

Fatty Acid	Infant Formula		Lunch and Dinner		Total Daily Intake[d (b+c)]	Recommended fatty acid %[e]	Recommended Total Daily Intake (AI)
(mg)	100 mL	600 mL [b]	100 g	249 g [c]	mg/day	%	mg/day
EPA	-	-	2.36	5.872	5.872	<0.1 (UL)	<29.5
ALA	48	288	3.38	8.423	296.423	1.5	442.5
DHA	3.3	19.8	1.41	3.518	23.318	0.35	103.25
AA	5.3	31.8	1.98	4.917	36.717	0.5	147.5
LA	520	3120	11.19	27.854	3147.854	10	2950

[a] Average weight about 8.3 kg.
[b] Daily recommended volume of milk intake for a 6-9 month old infant, as recommended on the packaging of infant formula in the UK.
[c] The portion size is based on the gastric capacity of an infant aged 6-9 months old (30 g/kg of body weight) to make up for lunch and dinner (30 x 8.3 = 249 g)
[d] Daily intake is simply calculated by the sum of milk (b) and non-milk intake (c)
[e] The calculation of fat intake is based on 40% of the Estimated Average Requirements (EAR) for energy of an infant aged between 6-9 month at 664.23 kcal/day, which equates to 29.5 g of fat per day (1g = 9 kcal). The % of fatty acids is according to Simopoulos (2000) for adequate intakes (AI) of infant formula/diet.

Formula milk alone provides 0% of EPA, 65.09% of ALA, 19.18% of DHA, 21.56% of AA and 105.76% of LA in the infant's daily diet. The contribution from commercial 'ready to feed' complementary infant foods (249 g per day) in this study were found to be low with only 19.91% contribution from EPA, 1.90% ALA, 3.41% DHA, 3.33% AA and 0.94% LA. To meet the recommended requirements the intake from sources other than infant formula milk need to provide 100% of EPA, 34.92% of ALA, 80.82% of DHA, 78.44% of AA, with no contribution of LA. This is clearly not being met from the commercial 'ready to feed' complementary infant foods tested in this study. This may become an issue if milk intake is compromised or when infants consume less infant formula milk which is the case after the first year of life. Therefore parents may need to choose fortified food products to meet requirements. This also provides opportunities and scope for product optimization to improve the nutritive value of commercial infant food products.

The LA intakes found to be above recommendations may in part be due to the use of cooking oils containing high levels of the *n*-6 fatty acid LA as well as grain based animal feeds rich in LA. In addition low intakes of oily fish rich in long chain *n*-3 fatty acids in the infant's diet could offer some explanation for the lower intakes of *n*-3 LCPUFA (Simopoulos, 2000).

3.3.2 Impact of commonly practiced re-heating treatments for the preparation of infant formula milk and the transparency of the nutritional labels

Infant formula milk has been identified as the main source of essential fatty acids in an infant's diet, it is therefore important to consider the detrimental impact which processing or domestic preparation of the infant formula milk could impose both on the quantity and also the quality of the daily intake, for instance oxidation and isomerization of the long chains of unsaturated fatty acids. The second part of this study was to investigate the commonly practiced re-heating treatments used by parents for the preparation of infant formula milk and its effects on fatty acid content.

The concentration of fatty acids for each re-heating treatment is shown in **Table 3.5**. The data shows a significant 12.62% decrease in the concentration of LA in the control RTF formula milk to the heated RTF formula milk (p = 0.04). There was also a significant decrease of 22.22% in ALA and 15.24% decrease in LA from the microwaved RTF treatment to the heated RTF treatment (p = 0.002; p = 0.01 respectively). Overal microwaving appears to be least influential on the retention of fatty acids, which may be due to the shortened length of time that the formula milk is subjected to elevated temperatures. These findings indicate that

the mode of heating can have a significant impact on the concentration of certain fatty acids in infant formula milk, where differences could possibly be due to the adverse effects of temperature. However there does not seem to be a statistically significant effect on the concentration of LCPUFA in any of the heat treatments analyzed. A study of formation of oxidation products such as hydroperoxides could prove useful in determining the adverse impact of heat treatments (Michalski, et al., 2008). In addition further investigation into the structural changes and formation of isomers as a result of heat treatment by using mass spectrometry is also recommended (Herzallah, et al., 2005).

Table 3.5 Concentration of fatty acids in infant formula milk subjected to different re-heating treatments (mean (n=3) ± SD) and concentrations stated on the manufacturer's label.

	Experimental				Label
Fatty acid (mg/mL)	Control RTF*	Microwave RTF	Heated RTF	Powder	
EPA	ND	ND	ND	ND	-
ALA	0.82 ± 0.16^{a}	0.81 ± 0.04^{b}	0.63 ± 0.03^{c}	0.99 ± 0.15^{d}	0.48^{e}
DHA	0.05 ± 0.03	0.04 ± 0.02	0.02 ± 0.01	0.02 ± 0.01^{d}	0.03^{e}
AA	0.11 ± 0.05	0.08 ± 0.02	0.05 ± 0.01	0.07 ± 0.01	0.05
LA	8.40 ± 0.55^{a}	8.66 ± 0.47^{b}	7.34 ± 0.29^{c}	9.05 ± 0.30^{d}	5.20^{e}

RTF – ready to feed, * No heat treatment, ND = not detected
[a, b, c, d,] – Statistically significant difference between heat treatments at the 95% confidence limit; ALA (a&e, p=0.021; b&c, p=0.002; d&e, p=0.004), DHA (d&e, p=0.039), LA (a&c, p=0.043 a&e, p=2.456E-05; b&c, p=0.014; d&e, p=0.001).

Furthermore a comparison between the analytically determined fatty acid concentrations and those provided by the manufacturer on the nutritional label were also evaluated. The result of the comparison between the control RTF formula milk concentrations from this study and the manufacturer's label is illustrated in **Table 3.5**. The concentrations of ALA and LA that were analytically quantified were found to be significantly higher, 70.83% and 61.54% increase respectively, in comparison to what is stated on the label provided by the manufacturer (p = 0.02, p = 2.456E-05, respectively).

In addition the powdered formula milk concentrations quantified in this study and those provided on the manufacturer's label were also compared, illustrated in **Table 3.5**. There was a significant difference between the concentration detected in ALA (p = 0.004), DHA (p = 0.04) and LA (p = 0.001) compared to what was stated on the label at 95% CF. There is a 106.25% increase in ALA and 74.4% increase in LA detected in the analytically quantified concentrations of the powdered formula milk analyzed in this study compared to the label, whereas there is a 33.33% decrease in the detected concentration of DHA in this study compared to the label. It is important to mention that heated water was used to reconstitute

the powdered infant formula milk. An un-heated control using cold water could not be used as the powder would not fully dissolve. Therefore effects could be due to the heat treatment used to prepare the infant formula milk.

The majority of fatty acids precursors (ALA and LA) are obtained from the infant formula milk in an infant's diet, 65.09% and 105.76%, respectively (**Table 3.4)**; therefore, any detrimental effects arising from preparation using elevated temperatures could have an impact on the nutritional adequacy of the infant's diet and subsequent development. It is important to be aware that infant formula milk provides 5.76% over the recommended LA intake and it is necessary to be aware that the Western diet is known to already be higher in LA and lower in *n*-3 polyunsaturated fatty acids. This could have an impact on allergy development. High proportions of AA and DHA are required to be supplied by the diet (78.44% and 80.82% respectively), which based on this study is not being met by the contribution from commercial 'ready to feed' complementary infant foods. This could have detrimental effects on the infant's cognitive and visual development. If recommendations are to be met complementary infant foods may need to be fortified with LCPUFA.

Fatty acids can have major differences in both physical and biological characteristics depending on their isomers being *cis* or *trans*. The *cis* formation is mainly used for metabolic and structural purposes. It has been observed that the *trans* formation is less absorbed in comparison to its *cis* counterpart. Unsaturated fatty acids from vegetable oil and most animal sources adopt the *cis* formation. The synthesis of LCPUFA may be impaired by *trans* fatty acids, this is important to consider for infants growth and development. Recently there has been increased interest in *trans* fatty acids as they have been linked to cardiovascular disease (Uauy & Castillo, 2003). High temperatures make fats susceptible to direct isomerization (Department of Health, 1991). One of the limitations with this study was the fact that the mass based detection of compounds in this study (CAD) was not useful in determining the types of isomers. However a study using gas chromatography mass spectrometry by Precht, et al. (1999) looked at the isomerization of milk fats with heating; strongest isomerization effects were found after heating at 300°C, however at 200°C only slight changes were observed. Temperatures did not exceed 90°C in the heat treatments used to prepare infant formula milks in this study; therefore *cis* and *trans* isomerization should be low, if any in the treatments used to make up infant formula milks by parents.

There have also been some studies conducted looking at the cooking effects on fish in relation to fatty acids. Gladyshev, et al. (2006; 2007) examined the effect of boiling and heating in common fish species to look at the effect on DHA and EPA, as they are susceptible to oxidation during heating and other cooking preparations. The fish were boiled to 85-90°C for 10-15 minutes; in comparison the infant formula milks in this study were initially subjected to similar temperatures but then left to cool, so any effects seen could be due to the time the fats were subjected to high temperatures. However, boiling was found to not significantly decrease the DHA or EPA content of the fish analyzed. Therefore, indicating that the heat treatments used to make up infant formula milks do not oxidize EPA or DHA. Gall, et al. (1983) also looked at different cooking methods on fish fillets; both boiling (177°C) and microwaving (15 second intervals) had no effect on the fatty acid concentration.

3.3.3 Labelling of infant formula milks in the UK

The labeling of four leading brands of follow-on infant formula milk on the UK market was examined for their fatty acid content (**Table 3.6**). There appears to be a variation between the popular brands on the UK market according to the nutritional labelling information, which has also been highlighted by Zunin, et al. (2015) from infant formula milks in Italy. The source of fats also differs between vegetable oils and fish oils between brands. In some cases, however, it is not clear whether the source of LA and ALA present in the product is from the addition of vegetable oils. Only two formula milk brands were found to be fortified with LCPUFA according to their labels (Brand A and C). From the labelling information the LA:ALA ratio of infant formula milks is within the EU regulations of between 5-15:1, in addition where DHA and AA information is available the fatty acids are within recommended ratios.

Table 3.6 Fatty acid characteristics of 6+ months follow on RTF infant formula available in the UK market.

Fatty acid (mg/100ml)	Brand A	Brand B	Brand C	Brand D
EPA	-	-	-	-
ALA	48	-	-	80
DHA	3.3	-	8.7	-
AA	5.3	-	8.7	-
LA	520	-	-	500
Source of fat	Vegetable oil (fungal and algal)	Vegetable oils	Vegetable oils and fish oil	Organic vegetable oils (algal)

In relation to the EPA content, none of the formula milk brands on the UK market declare that they contain EPA; therefore any EPA will need to be converted endogenously from its precursor ALA or ingested directly from complementary foods. It is however important to note that EPA in amounts > 0.1% may antagonize AA and interfere with infant growth (Simopoulos, 2000).

3.4 Conclusions

This study concludes that the major dietary contributor of LCPUFA in an infant's diet based on the standard menu composed of daily consumption of commercial 'ready-to-feed' infant foods and formula milk products is infant formula milk and the current range of infant food product lacks LCPUFA. The latter highlights an important issue in relation to situations where consumption of formula milk is compromised. This provides opportunities and scope for product optimization to improve the nutritive value of commercial infant food products. In addition, the study highlights that the impact of commonly practiced re-heating treatments used by parents for the preparation of infant formula milks introduces significant changes in certain fatty acids, and therefore future assessment of the oxidation products following these methods of preparation is suggested. Furthermore, in relation to the transparency of the nutritional information declared on the labels by the manufacturers, infant formula milks were all within the limits of EU regulations, although there was a degree of significant variation between the quantitative values analyzed in this study and the declared values on the labels.

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Chapter 4

Vitamin A and E content of commercial infant foods in the UK: a cause for concern?

4.1 Introduction

Vitamin A consists of a group of lipid soluble vitamins that have an important role in growth and development, vision and immune function. Dietary vitamin A is obtained from two sources: pre-formed vitamin A and provitamin A. Pre-formed vitamin A includes retinol, retinal, retinoic acid and retinyl esters, which are available from animal sources mainly in the form of retinyl esters. Conversely provitamin A's are precursors of vitamin A in the form of carotenoids, which are available from plant derived sources (Department of Health, 1991). Plants synthesise hundreds of carotenoids, however only some act as vitamin A precursors. β-Carotene is the most important carotenoid due to its increased vitamin A activity compared to other carotenoids and its widespread availability in the diet. The increased vitamin A activity of β-carotene is due to its conversion into two retinal molecules. Absorption rates of pre-formed vitamin A range between 70 – 90%, whereas provitamin A are absorbed less efficiently at rates ranging between 20 – 50% (Preedy, 2012).

Vitamin E is another group of lipid soluble vitamins consisting of eight isomers: four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ). The most biologically active form of vitamin E is α-tocopherol, which is also the most abundant form present in the European diet due to the use of olive and sunflower oil (Wagner, et al., 2004).

Vitamin E primarily functions as a chain breaking antioxidant. It protects membrane phospholipids from peroxidation and prevents free radical generation in cell membranes (Greene, et al., 1988). α-Tocopherol can intercept free radicals and prevent the oxidation of the lipids in cell membrane (Baydas, et al., 2002). Immune system cells contain the most vitamin E due to their exposure to high levels of oxidative stress (Suskina & Tontisirin, 2001). Therefore, vitamin E may play a role in the prevention or protection against asthma and allergic disease inflammation, which currently are increasing in the UK. A maternal feeding study observed an association between increased intakes of vitamin E during pregnancy and decreased childhood wheeze and asthma (Devereux, et al., 2006). Oxidative stress and inflammation may have a role in cardiovascular disease and cancer however the

current knowledge on the role of vitamin E in in these diseases is not conclusive (Lee et al., 2005).

Dietary polyunsaturated fatty acid (PUFA) intake influences tissue fatty acid content and the dose of vitamin E required to prevent lipid peroxidation is directly related to PUFA intake (Greene, et al., 1988). The bioavailability and metabolism of vitamin E is largely unclear and ranges between 10 - 79%; nevertheless factors have been identified that will affect vitamin E uptake and utilisation within the body such as the food matrix and the amount and type of fat (Borel, et al., 2013).

During food processing, retinoids and carotenoids can undergo isomerization due to exposure with acids, heat and light. Thermal treatments can either cause isomerization, from *trans* to *cis,* or oxidation. However, heating can also promote carotenoid absorption, possibly due to the release of carotenoids from its matrix. Homogenization of foods can also improve the availability of carotenoids. Processing methods such as dehydration, blanching and canning can cause either oxidation or isomerization of carotenoids (Klein & Kurilich, 2000). However retinol and carotenoids have been found to be relatively stable throughout most cooking procedures with industrial food processes such as pasteurization and sterilization generally only resulting in small losses (Committee on Toxicity, 2012). Processing of foods, such as drying in the presence of air and sunlight, addition of organic acids, milling and refining, irradiation and canning have been shown to reduce vitamin E content of foods, along with high temperatures, prolonged storage and high moisture contents. Seasonal changes in vitamin E levels have also been shown in food products (Eitenmiller & Lee, 2004). Therefore the actual content of vitamin E in commercial infant foods may vary due to processing and seasonality of products used for production.

The diet and nutrition survey of infants and young children (DNSIYC) has identified that 58% of children who are fed foods other than milk have eaten a commercial baby or toddler meal (Lennox, et al., 2013). These results indicate that commercial baby and toddler meals are major dietary contributors to an infant's nutritional intake, therefore these products need to be assessed for their nutritional adequacy. Furthermore there is currently no clear and complete analytical nutritional data available for commercial 'ready-to-feed' complementary infant foods in the UK. The nutritional database in the UK, McCance and Widdowson, contains limited data on the composition of commercial infant foods, in addition the analytical techniques and nutrient data contained may now be outdated. It is essential that

food composition tables are regularly updated with the current foods available on the market (Food Standards Agency, 2015).

The DNSIYC has shown that mean intakes of vitamin A and E are close to or in excess of the Recommended Nutrient Intake (RNI) (Lennox, et al., 2013). Furthermore in the US 35% of toddlers exceed the Tolerable Upper Intake Level (UL) of 600 μg/day for vitamin A and vitamin E intakes were found to exceed the recommended adequate intakes of infants below 12 months (Butte, et al., 2010).

Vitamin A deficiency is common in developing countries with common symptoms to include visual impairments (Department of Health, 1991). However, in developed countries such as the UK, excess vitamin A intakes may be more of an issue. Excess retinol can cause liver and bone damage, hair loss, double vision, vomiting and headaches. It can also decrease vitamin C storage, antagonise the action of vitamin K's blood clotting function and have anti-thyroid effects (Committee on Toxicity, 2014). Toxicity usually arises from chronic ingestion, which exceeds the ability of the liver to store of metabolise excess vitamin A.

High vitamin E intakes could potentially have antagonistic effects on the other fat soluble vitamins such as vitamin A, D and K in relation to absorption. It has been observed that infants have low vitamin K levels and studies have indicated that individuals with low vitamin K levels should not be consuming excessive vitamin E due to blood clotting effects (Diplock, et al., 1998). High intakes of vitamin E could cause pro-oxidant effects due to the production of α-tocopherol radicals, however if antioxidant systems are balanced for example by the sparing effect of vitamin C detrimental effects will not be exhibited (Rietjens, et al., 2002). However vitamin E is one of the least toxic vitamins and no evidence of negative side effects of vitamin E that occurs naturally in foods has been observed (Eitenmiller & Lee, 2004).

This study assessed the intake of vitamin A (pre-formed vitamin A, retinyl palmitate and retinyl acetate and provitamin A, β-carotene and total carotenoid content as retinol equivalents, RE) and vitamin E (α-tocopherol and γ-tocopherol as α-tocopherol equivalents, α-TE) based on a diet consisting of commercial 'ready-to-feed' infant foods and infant formula in the form of a standardized menu as suggested by Zand, et al., (2012), and the results were compared with current recommendations to assess the adequacy of intake.

4.2 Materials and methods

4.2.1 Chemicals

Retinyl acetate, retinyl palmitate, β-carotene, methyl tert-butyl ether (MTBE), α-tocopherol and γ-tocopherol were obtained from Sigma-Aldrich (Poole, UK). The reference material (ERM – BD600) was purchased from the Institute for Reference Materials and Measurements for Certified Reference materials (Geel, Belgium). High performance liquid chromatography (HPLC)-grade iso-hexane, ethyl acetate, absolute ethanol, methanol, tetrahydrofuran (THF), and laboratory reagent grade sodium chloride (NaCl), petroleum ether (PE) (bp 40-60°C) and anhydrous sodium sulphate were obtained from Fisher Scientific (Loughborough, UK).

4.2.2 Sample collection

Eight different baby food samples were obtained from leading supermarkets in the UK between June and July 2014. The samples represented four popular brands available on the market (Statista, 2008). All brands were represented by two different product categories: (i) meat based and (ii) vegetable based. The main ingredients of the baby food samples and their characteristics are presented in **Table 4.1.** Three independent replicates of all samples were analyzed. The samples were stored unopened at room temperature, similar to their distribution and market environment.

4.2.3 Sample preparation for retinyl acetate, retinyl palmitate, and α-tocopherol and γ-tocopherol analysis

Each of the food samples were mixed and homogenized using a domestic blender (Multi-quick, Braun 3000) and three independent replicates of 1 g (wet weight) were transferred into a centrifuge tube. The samples were then treated according to the methods described by Chávez-Servín, et al. (2006). Three millilitres of absolute ethanol were added and the mixture shaken mechanically for 3 minutes prior to adding 1 mL of hexane and the mixture was shaken for a further minute. The samples were then left to stand for 5 minutes, after which 3 mL of saturated NaCl was added to aid solvent separation. Samples were inverted manually before being centrifuged for 5 minutes at 3000 rpm at room temperature. The hexane phase was recovered and directly filtered through a 0.22 μm nylon filter and collected in a 1 mL amber glass vial. 0.4 mL of sample was spiked with 0.4 mL of a standard solution prior to 20 μL being injected into the HPLC system.

Table 4.1 Infant complementary food sample characteristics.

Brand Code	Product name (*n*=8)	Ingredients	Nutritional Information (per 100g)
A (meat)	Succulent pork casserole	Baby grade vegetables (50%), carrot (30%), tomato, butternut squash, onion, garlic, cooking water, potato (12%), pork (9%), tapioca starch, rapeseed oil, parsley, black pepper.	**Energy** 259kJ/62kcal, **Protein** 2.6g, **Carbohydrate** 7.9g, of which sugars 1.9g, **Fat** 1.8g, of which saturates 0.3g, **Fibre** 1.8g, **Sodium** 0.02g.
B (meat)	Zingy lamb and cous cous	Organic vegetable stock 35% (water and organic vegetables: carrots, parsnips, Swedes, onions leeks), organic carrots 14%, organic lamb 10%, organic tomatoes 9%, organic mangoes 8%, organic butternut squash 8%, organic green beans 6%.	**Energy** 196kJ/48kcal, **Protein** 2.8g, **Carbohydrate** 6.7g, of which sugars 4.3g, **Fat** 1g, of which saturates 0.4g, **Fibre** 1.6g, **Sodium** 0.02g
C (meat)	Fruity chicken casserole	Apple juice from concentrate (23%), carrot, potato, water, chicken (8%), onion, corn flour, peach (3%), unsalted butter, natural flavouring, herb, iron sulphate.	**Energy** 280kJ/66kcal, **Protein** 2.5g, **Carbohydrate** 9.9g, of which sugars 3.5g, **Fat** 1.6g, of which saturates 0.8g, Fi**bre** 1.2g, **Sodium** 0.09g, **Iron** 1.2mg
D (meat)	Parsnip, potato and turkey casserole	Organic vegetables (52%), [potatoes (25%), parsnips (19%), carrots], water, organic turkey (8%), organic cooked rice, organic rapeseed oil (1.5%), organic vegetable stock [salt, organic ground rice, organic vegetables (carrots, onions, celery), organic yeast extract, organic vegetable oil, organic spices], organic herbs and spices (rosemary and pepper).	**Energy** 284kJ/68kcal, **Protein** 2.7g, **Carbohydrate** 8.1g, of which sugars 1.3g, **Fat** 2.4g, of which saturates 0.4g, of which linolenic acid (*n*-3) 0.11g, **Fibre** 1.5g, **Sodium** 0.05g
A (veg)	Creamy cauliflower cheese	Baby-grade cauliflower (36%), cooking water, skimmed milk, cheddar cheese (8%), rice, corn starch, parsley.	**Energy** 263kJ/63kcal, **Protein** 3.3g, **Carbohydrate** 5.8g, of which sugars 1.5g, **Fat** 2.7g, of which saturates 1.5g, **Fibre** 1.0g, **Sodium** 0.07g
B (veg)	Vegetable bake with lentils	Organic tomatoes 47%, organic vegetable stock 14%, (water and organic vegetables: carrots, parsnips, Swedes, onions, leeks, peppercorns, marjoram, parsley and thyme), organic sweet potatoes 9%, organic onions 9%, organic red lentils 9%, organic carrots 5%, organic red peppers 5%, organic olive oil 1%, organic cumin <1%, organic oregano <1%.	**Energy** 278kJ/65kcal, **Protein** 3.8g, **Carbohydrate** 10.4g, of which sugars 3.8g, **Fat** 0.5g, of which saturates 0.3g, **Fibre** 2.3g, **Sodium** 0.02g
C (veg)	Cheesy tomato pasta stars	Water, tomato (20%), pasta (18%) (water, durum wheat semolina), vegetarian cheddar cheese (8%), corn flour, natural flavouring (contains celery, celeriac), iron sulphate.	**Energy** 285kJ/68kcal, **Protein** 2.9g, **Carbohydrate** 8.5g, of which sugars 0.7g, **Fat** 2.4g, of which saturates 1.8g, **Fibre** 0.3g, **Sodium** 0.1g, **Iron** 0.9mg
D (veg)	Tomato, courgette and potato bake	Organic vegetables (79%) [potatoes (31%), carrots, tomatoes (19%), courgettes (8%), onions], skimmed milk, mozzarella (from cow's milk), cooked rice, tomato puree, rapeseed oil (1%), vegetable stock [salt, rice flour, vegetables (carrots, onion, celeriac), yeast extract, vegetable oil, spices], herbs (oregano, basil).	**Energy** 285kJ/68kcal, **Protein** 2.6g, **Carbohydrate** 9.3g, of which sugars 2.7g, **Fat** 1.9g, of which saturates 0.6g, of which linolenic acid (n-3) 0.08g, **Fibre** 1.5g, **Sodium** 0.06g

4.2.4 Sample preparation for β-carotene and total carotenoid analysis

The samples were treated according to the methods described by HarvestPlus (Rodriguez-Amaya & Kimura, 2004). Each of the food samples was mixed and homogenized using a domestic blender (Multi-quick, Braun 3000) and 3 independent replicates between 0.5-2.5 g (wet weight) were weighed. Fifty millilitres of THF:methanol (1:1) was added to the sample for 5 minutes, which was then homogenized using a polytron for 1 minute and subsequently filtered through a porosity 2-sintered glass funnel under vacuum and rinsed with THF:methanol until there was no colour left in the filtrate. Twenty millilitres of PE was added to a 500 mL separating funnel, the extracts were then poured into the separating funnel, followed by 50 mL of 10% NaCl to aid separation. After washing thrice with 200 mL of deionised water the upper PE phase which contained the extract was collected in a 100 mL flask. The PE phase was then dewatered by the addition of anhydrous sodium sulphate, then filtered into a 50 mL volumetric flask through glass wool and made up with PE. At this stage spectrophotometer readings were analyzed for total carotenoid content. Twenty millilitres of extract were collected in a glass vial and brought to dryness using nitrogen. Prior to injection into the HPLC 0.5 mL of THF:methanol (1:1) was added to reconstitute the dried sample, finally 5 µL of reconstituted sample was injected into the HPLC system.

4.2.5 HPLC analysis

An Agilent 1200 series normal phase high performance liquid chromatography (NP-HPLC) system was used, equipped with an isocratic pump, thermostated austosampler set to 6°C and multiple wavelength detector. A Pinnacle II silica short narrow-bore column (50 mm x 2.1 mm I.D.), with a 3 µm particle size from Restek (Bellefonte, USA) was used for the analysis. Separation was performed at 30°C using an isocratic mixture of 0.5% ethyl acetate in hexane, delivered at a flow rate of 0.4 mL/min. Detection for α and γ-tocopherol was at 296 nm whereas detection of retinyl acetate and retinyl palmitate was at 326 nm. The injection volume was 20 µL and total run time for each routine injection was 30 minutes. Retention times for retinyl palmitate, retinyl acetate, α-tocopherol and γ-tocopherol were 1.1 minutes, 2.2 minutes, 3.5 minutes and 8.3 minutes respectively (**Fig. 4.1**).

For the analysis of β-carotene an Agilent 1200 series reverse phase high performance liquid chromatography (RP-HPLC) system was used equipped with a quaternary pump, thermostated autosampler and diode array detector. A YMC C30 column (250 mm x 4.6 mm) with a 5µm particle size from YMC (Europe GmbH) was used for the analysis. Separation

was performed at 25°C using an isocratic mixture of 80:20 methanol:MTBE delivered at a flow rate of 1 mL/min. Detection of β-carotene was at 450 nm. The injection volume was 5 μL and total run time for each routine injection was 40 minutes. Retention time for β-carotene was 30 minutes (**Fig. 4.2**).

4.2.6 Spectrophotometer analysis

A UV visible Cecil's Super Aquarius CE9200 9000 series spectrophotometer was used to measure absorbance at 450 nm in glass cuvettes for total carotenoid analysis.

4.2.7 Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Six standard solutions of retinyl acetate, retinyl palmitate (0.2 – 5 μg/mL), α-tocopherol (5 – 75 μg/mL) and γ-tocopherol (1 – 50 μg/mL) were prepared at different concentrations by diluting the stock solutions using hexane. Linear calibration curves, at six different concentrations were defined by the best-fit line equations (**Table 4.2)**. The limit of detection (LOD) and limit of quantification (LOQ) were determined by using the signal-to-noise ratio (S/N) as demonstrated in **Table 4.2**.

In the case of β-carotene four standard solutions of β-carotene (0.001 – 0.1 mg/mL) were prepared at different concentrations following the dilution of stock solutions using THF:methanol. A linear concentration curve was defined by the line of best fit (**Table 4.2)**. LOD and LOQ were calculated using LINEST function in Excel and shown in **Table 4.2**.

Table 4.2 Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ) of vitamin A and E.

Compound	r2	% Recovery*	U (mg/g)	LOD (μg/mL)	LOQ (μg/mL)
Retinyl palmitate	0.9995	115.179	-	0.008	0.023
Retinyl acetate	0.9993	105.591	-	0.028	0.086
β-Carotene	0.9959	-	-	0.058	0.176
α-tocopherol	0.9998	107.49	0.47	0.661	2.002
γ-tocopherol	0.9995	106.29	-	0.136	0.412

U Expanded uncertainty corresponding to a confidence level of approximately 95% (Linsinger, 2005)
* % recovery based on calculation by Trisconi, et al. (2012).

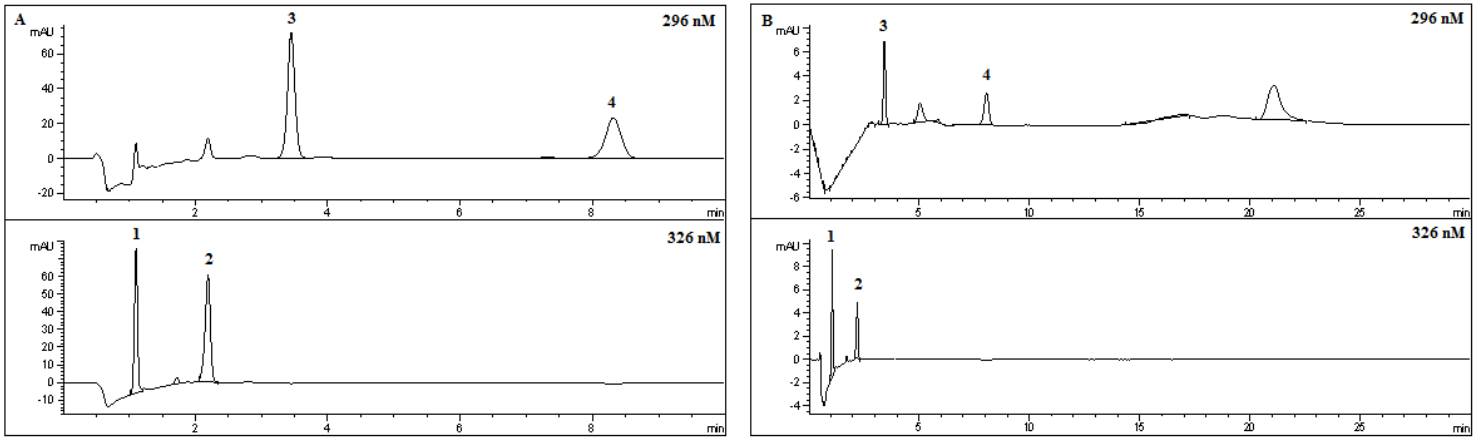

Figure 4.1 HPLC chromatogram of: A. standard mixture and B. sample (1. retinyl palmitate – 1.1 minutes, 2. retinyl acetate – 2.2 minutes, 3. α-tocopherol – 3.5 minutes and 4. γ-tocopherol – 8.3 minutes).

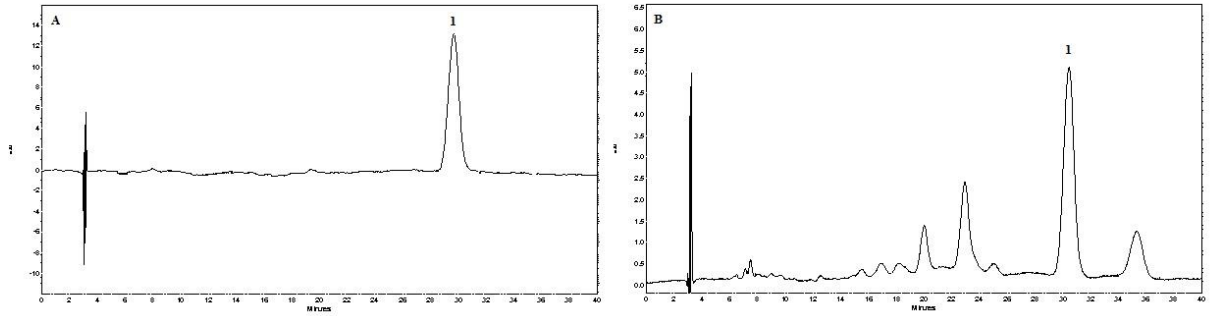

Figure 4.2 HPLC chromatogram of: A. standard mixture and B. sample (1. β-carotene – 30 minutes).

4.2.8 Quality assurance

The accuracy of the method was verified by analysing the certified reference material (ERM-BD600) purchased from the Institute for Reference Materials and Measurements for Certified Reference materials (Geel, Belgium). The expanded uncertainty for α-tocopherol was greater than the absolute differences between the mean of the measured value and the certified value. The mean measured value was, therefore, not significantly different from the certified value; this demonstrates the validity of the method (Linsinger, 2005). Spiking of samples was used to verify the recovery of the vitamins tested, as shown in **Fig. 4.2** (Trisconi, et al., 2012).

4.2.9 Statistical methods

The experimental results were subject to statistical analysis using Excel 2010 and SPSS package v.17.0. A 2-sided unpaired T test at p = 0.05 level of significance (with 95% confidence interval) was used to examine mean differences between meat and vegetable based varieties. The data was further subjected to analysis of variance (ANOVA) at p = 0.05 to examine the differences between brands and replicated (n=3) measurements.

4.3 Results and Discussion

The concentration of vitamin A from pre-formed sources, in the form of retinyl acetate and retinyl palmitate, provitamin A sources, in the form of β-carotene and total carotenoids, and vitamin E, in the form of α-tocopherol and γ-tocopherol, from eight commercial 'ready-to-feed' infant foods are presented in **Table 4.3**.

There was no significant difference between the two varieties of meat and vegetable based infant food products at the 95% significance level for α-tocopherol (p = 0.460), γ-tocopherol (p = 0.685), retinyl palmitate (p = 0.198), retinyl acetate (p = 0.688), β-carotene (p = 0.552) and total carotenoids (p = 0.942). It was thought that there may have been differences seen between meat and vegetable based products, as pre-formed vitamin A is mainly from animal sources and provitamin A from plants; however this does not appear to be the case.

Table 4.3 Concentration of vitamin A and E in commercial 'ready to feed' infant foods in the UK intended for consumption by 7 month + old infants (mean (*n*=3) ± SD).

(µg/g)	Brand A	Brand B	Brand C	Brand D	Mean
Meat Based Products					
Retinyl Palmitate	0.35± 0.06	0.33 ± 0.05	0.39 ± 0.02	0.47 ± 0.09	0.38 ± 0.06
Retinyl Acetate	0.30 ± 0.14	0.46 ± 0.07	0.31 ± 0.03	0.88 ± 0.08	0.49 ± 0.27
β-Carotene	34.28 ± 0.47	10.36 ± 0.42	20.56 ± 1.26	5.72 ± 0.21	14.02 ± 13.68
Total Carotenoids	79.68 ± 0.85	41.41 ± 0.01	38.65 ± 1.40	10.37 ± 0.55	42.52 ± 28.47
α-Tocopherol	4.02 ± 0.82	7.55 ± 0.54	6.66 ± 0.33	8.52 ± 0.51	6.69 ± 1.93
γ-Tocopherol	4.51 ± 0.58	6.30 ± 0.21	6.05 ± 0.13	3.67 ± 0.22	5.13 ± 1.26
Vegetable based products					
Retinyl Palmitate	0.62 ± 0.04	1.94±0.22	0.62 ± 0.03	0.41 ± 0.04	0.89 ± 0.70
Retinyl Acetate	0.25 ± 0.01	0.99 ± 0.05	0.53 ± 0.04	ND	0.59 ± 0.38
β-Carotene	0.18 ± 0.01	11.38 ± 0.71	0.63 ± 0.09	22.21 ± 0.58	8.60 ± 10.45
Total Carotenoids	2.87 ± 0.29	99.47 ± 0.65	20.87 ± 0.44	54.64 ± 0.55	44.46 ± 42.49
α-Tocopherol	5.96 ± 0.18	12.02 ± 0.44	6.45 ± 0.17	7.64 ± 0.25	8.02 ± 2.76
γ-Tocopherol	3.33 ± 0.21	6.16 ± 0.22	5.92 ± 0.14	6.80 ± 0.35	5.55 ± 1.53

ND not detected.

The results of the vitamin A and E content in the selected infant food products were further subjected to both single factor with replication and two factor ANOVA without replication analysis. The calculated F value after ANOVA between brands showed a significant difference between the brands for all forms of vitamin A and E. The calculated *p*-value (retinyl acetate p = 6.58e-30, retinyl palmitate p = 1.5e-18, β-carotene p = 1.93e-15, total carotenoids p = 8.11e-17, α-tocopherol p = 2.15e-41, γ-tocopherol p = 9.09e-13) were significantly lower than the critical *p*-value at 0.05. The calculated F value for the ANOVA within groups (between the replicates), showed no significant difference with *p*-values calculated (retinyl acetate p = 1.00, retinyl palmitate p = 0.16, β-carotene p = 0.68, total carotenoids p = 1.00, α-tocopherol p = 0.086, γ-tocopherol p = 0.99), which indicates the consistency of measurements.

Dietary intake of vitamin A is a combination of both pre-formed and provitamin A sources, however the bioavailability of the different sources of vitamin A vary between the different forms. Pre-formed vitamin A, such as retinyl palmitate has high absorption rates, whereas provitamin A carotenoids have poor absorption rates within the body (Preedy, 2012). Traditionally vitamin A activity has been described in terms of RE, which takes into consideration the different forms bioavailabilities (WHO & FAO, 1967). All forms of vitamin A tested in this study were converted into RE in order to calculate the estimated total daily intake. **Table 4.4** shows conversions factors used to transform the different forms of vitamin A tested into RE. It is important to be aware that RE are based upon certain assumptions regarding absorption and ultimately the quantity that is converted to retinol itself, for example studies have shown β-carotene absorption ranging between 3.6% - 28.1% from various food sources (Tang, 2010), the conversion factor used here is based on the conversion of β-carotene and carotenoids from a mixed diet.

Table 4.4 Conversion factors for the different forms of vitamin A into retinol equivalents (Food Standards Agency, 2002).

Vitamin A	Retinol Equivalents (RE)
1 μg Retinyl palmitate	0.55 μg RE
1 μg Retinyl acetate	0.87 μg RE
1 μg β-carotene	0.17 μg RE
1 μg other carotenoids	0.083 μg RE

RE – retinol equivalents

The vitamin E potential of foods can be expressed as α-TE (Jiang, et al., 2001). In the UK vitamin E is expressed as α-TE on the labelling of infant formula, therefore α-TE are used to represent the vitamin E content of the infant's diet for this particular study. Based on the vitamin E potential, 1 mg of α-tocopherol is equal to 1 mg of α-TE and 1 mg of γ-tocopherol is equal to 0.1 mg of α-TE. The γ-tocopherol content of the selected samples therefore was converted to α-TE in order to represent the true value in calculation of the total daily intake, shown in **Table 4.5**.

It is essential to examine the entire nutrient daily intake when assessing the nutrient quality of complementary foods. To estimate the total daily dietary intake of an infant based on the consumption of commercial 'ready-to-feed' infant foods and infant formula a standardised menu has been derived, as suggested by Zand, et al. (2012) and based on the manufacturers feeding recommendations in **Table 4.5**. The volume of ingested meals was based upon the gastric capacity of an infant (30 g/kg body weight/day) in accordance with the Committee of Medical Aspect of food and nutrition policy (COMA) Weaning and Weaning Diet report (1994). Furthermore the estimated milk consumption used in the standard menu was 600 mL, which is advised by COMA, for infants up to 12 months.

The recommended nutrient intake (RNI) of vitamin A for infants is based upon the amount present in breast milk from well-nourished, healthy mothers and is set at 350 μg/day for a 7-12 month old infant. The Department of Health (DOH) also recommends that regular daily intakes of vitamin A should not exceed 900 μg/day for infants. From the standardised menu used in this study the infant's diet exceeds the recommended upper intake dose by 193% and exceeds the RNI by 497%. This could cause numerous negative effects for the infant. The vitamin A contribution from infant formula (396 μg) already exceeds the RNI by 13%. The form of vitamin A in infant formula will be from pre-formed sources of vitamin A, retinol and retinyl esters (Koletzko, et al., 2005).

Table 4.5 Total daily intake of vitamin A (RE) and vitamin E (α-TE) by an infant aged 6-9 months old[a], based on gastric capacity of an 8 month old infant and a standard feeding regime composed of commercial 'ready to feed' infant food products and infant formula milk.

Meals	Infant Formula		Lunch (meat-based)		Dinner (vegetable-based)		Total daily intake [d(b+c)] (mg/day)	RNI (mg/day)
	100 mL	600 mL [b]	100 g	124.5 g [c]	100 g	124.5 g [c]		
RE (mg)	0.07	0.40	0.54	0.67	0.54	0.68	1.74	0.35
α-TE (mg)	1.41	8.43	0.72	0.90	0.86	1.07	10.40	5.00*

[a] Average weight about 8.3 kg.
[b] Recommended volume of milk intake for a 6-9 month old infant.
[c] The portion size is based on the gastric capacity of an infant aged 6-9 months old (30 g/kg of body weight) to make up for lunch and dinner (30 x 8.3 = 249 g), lunch/dinner = 249/2 = 124.5 g
[d] Daily intake is simply calculated by the sum of milk (b) and non-milk intake (c).
*Actual intakes (AI) recommended by the Institute of Medicine only based on α-tocopherol intakes.
RNI – recommended nutrient intake.

Vitamin A toxicity is related to pre-formed vitamin A content not from provitamin A sources. More research is needed to establish whether subclinical vitamin A toxicity is a concern in certain populations, possibly in infants. However the majority of the food based vitamin A in this study is provided by carotenoids (β-carotene and total carotenoids), which are thought to have less negative effects than the pre-formed vitamin A types: retinyl acetate and retinyl palmitate. This may be partly because carotenoids are not stored in the liver like retinol, but are deposited throughout the body in fat cells, adrenal glands and other fatty tissues in addition to their different absorption and conversion rates. However, when only pre-formed vitamin A intake is considered in the infant's diet, food sources contribute 58.3% of the RNI, this may become a deficiency issue for the infant when milk intake is compromised or when milk intake is reduced after the first year of life. Furthermore when the carotenoid content is removed from the standardised menu the total daily intake is reduced to 600 μg RE, which is below the upper intake recommendation but still higher than the RNI. National diet and nutrition survey data indicate that intakes are also above the RNI (Public Health England, 2014). However due to the lack of knowledge concerning toxicity and bioavailability of the different vitamin A forms in infant's further investigations are required.

Breast milk concentrations of vitamin A vary widely: 150 μg/L – 1100 μg/L of pre-formed vitamin A, dependent on maternal diet and vitamin A status (Thompkinson & Kharb, 2007). Therefore if the infant is being breastfed then the mother's diet is also essential for the infant's nutritional status. If the infant receives human milk with a high vitamin A concentration then vitamin A intakes from pre-formed sources will be higher than those calculated in this study (1100 μg/L = 660 μg/600 mL). At the other end of the spectrum if an infant is consuming human milk low in vitamin A, then deficiency issues may arise (150 μg/L = 90 μg/600 mL).

It is important to mention that in 2001 the Food and Nutrition Board in the USA introduced a new conversion factor for vitamin A, retinol activity equivalents (RAE) due to changes in knowledge of carotenoid bioconversion, where the RAE of β-carotene was defined as 12 μg instead of 6 μg (Food and Nutrition Board, Institute of Medicine, 2001). Infant formula in the UK, expresses vitamin A content in RE, therefore in this study RE were employed to express the vitamin A content of the infants diet. There is a large debate over the bioavailability of carotenoids as their bioavailability is dependent on numerous factors including type of food matrix, amount of fat ingested and food processing methods. Although carotenoids with provitamin A activity are precursors of vitamin A excess intake has not been associated with

vitamin A toxicity in humans due to carotene conversion being regulated by vitamin A status. However high intakes should still be approached with caution due to human studies conducted in the 1990's in smokers who had been previously exposed to asbestos taking high doses of β-carotene being associated with increased risk of lung cancer (Tanvetyanon & Bepler, 2008). To date there is insufficient scientific evidence to set a tolerable upper intake level for β-carotene and further research is required. Furthermore as the equivalence factors for both retinol and carotenoids are unknown in infants they may not be adequate (Koletzko, et al., 2005).

As vitamin E requirements are based on PUFA intakes it is difficult to set up dietary reference values as the range of PUFA intakes in the UK varies widely therefore acceptable ranges of intake are more acceptable than a fixed level. In addition the relationship between PUFA intake and vitamin E requirements is not linear. Some committees, however, in other countries such as the US have calculated vitamin E requirements from the PUFA content of the diet as 0.4 mg α-TE/g of dietary PUFA, which is also used in the UK for fortification of infant formulas (Department of Health, 1991).

Based upon the UK guidelines for fat intake and vitamin E requirements being 0.4 mg α-TE/g dietary PUFA, for a 7-12 month infant, the recommended daily intake based on PUFA intake at 6% of total energy is 5.625 g/day. Based on this assumption infants aged 7 – 12 months require 2.25 mg/day of α-TE. The US Institute of Medicine recommended an actual intake for infants aged 7-12 months of 5 mg/day of α-tocopherol, rather than α-TE (Food and Nutrition Board, Institute of Medicine, 2000). Taking into consideration that the major contributor of vitamin E is γ-tocopherol in the US diet this does not appear to take into account other forms of the vitamin that may potentially be important for human health. It is also higher than the UK recommendations even for adults (3-4 mg/day).

From the finding of this study, however, it appears that the majority of vitamin E is provided by the infant formula which is in the form of α-tocopheryl acetate, with only a small concentration (18.9%) contributed from commercial 'ready-to-feed' infant foods. It is also apparent from national feeding studies in the US that after one year of life vitamin E intake is inadequate, which may be explained by the reduction in infant formula (Butte, et al., 2010). It is important to highlight that breast milk contains less vitamin E than infant formula, 0.66 mg/100 mL, compared to 1.4 mg/100 ml (Thompkinson & Kharb, 2007). Therefore breastfed infants will be closer to meeting the recommended intakes for infants aged 7 to 12

months. However breast milk concentrations will vary due to the maternal diet and the vitamin E status of the mother. Hence if milk intake is compromised, reduced or breast milk concentrations are low then there may be a cause for concern over the adequacy of the infant's diet in meeting vitamin E requirements.

Moreover, the bioavailability of vitamin E is currently largely unclear with studies ranging from 10 – 79%. This highlights the importance of undertaking further research in order to understand the absorption, metabolism efficacy of the different forms of vitamin E and its antioxidant properties and non-antioxidant properties (Borel, et al., 2013).

The main limitations from calculating the vitamin intake in this manner is that it is unlikely to represent the actual amount of consumption that is ingested and retained by the infant. It does not take into consideration wastage and fails to take into account any contribution from breast milk, snacks or homemade foods.

4.4 Conclusions

The concentration of vitamin A (retinyl acetate, retinyl palmitate and β-carotene) and E (α and γ-tocopherol) was quantitatively determined by HPLC. The estimated total daily intake of vitamin A converted into RE from the consumption of a combination of commercial complementary infant foods and infant formula, based on the standardised menu used in this study, was found to be in excess with respect to the upper intake levels for infants of 900 μg RE/day. It is worth mentioning that the contribution of vitamin A from infant formula alone was found to be 396 μg RE which exceeds the RNI of 350 μg RE for infants from 7 - 12 months of age by 13%. The remaining vitamin A content of the diet was mainly from carotenoids (β-carotene and total carotenoids) which are considered to be less harmful. However further studies into the bioavailability of the different forms of vitamin A in infants and the effects of excess intake are necessary. Pre-formed vitamin A food sources contributed 58.3% of the RNI, therefore if the contribution from milk intake is reduced, deficiency may become an issue and parents should be aware of the impact that the reduction in fortified infant formula use may have on the nutritional composition of an infant's diet.

The total daily intake of vitamin E based upon a diet consisting of complementary ready-to-feed infant foods and infant formula provided a vitamin E intake of 10.4 mg/day of α-TE, which exceeds the recommended adequate intakes of 5 mg/day α-tocopherol set by the US. However 81% of vitamin E was provided from infant formula with a minor contribution from

infant food products, which may become a concern when milk intake is compromised or reduced as it is after the first year of life.

4.5 References

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Chapter 5

Calcium to phosphorus ratio, essential elemental and vitamin D content of infant foods in the UK: possible implications for bone health

5.1 Introduction

Infancy is a time of rapid growth and development during which infants require the correct types and amounts of specific nutrients to ensure optimal growth and development. Typically full term neonates will double their birth weight by 5 months and treble it by the end of the first year of life, in addition to increasing their body length by 25 cm (Gokhale & Kirschner, 2003). During the first year of life bone mineralization and calcium accretion are greatest (Bass & Chan, 2006). It has been suggested that the calcium to phosphorus ratio (Ca:P) is important for bone growth and development during infancy (Sax, 2001). It is believed that bone mass accumulation in infancy is essential for the prevention of poor childhood growth and adult osteoporosis (Bass & Chan, 2006).

The optimal homeostasis of calcium, phosphorus and magnesium is essential for the formation of the structural matrix of bone; with 99% of calcium and 85% of phosphate present in bone as microcrystalline apatite (National Health and Medical Research Council, 2006). The maintenance of the optimal homeostasis of calcium and phosphorus is also dependent on absorption in the intestine, skeletal accretion and re-absorption and excretion in urine, in addition to vitamin D status and dietary intake (Bozzetti & Tagliabue, 2009).

Extremely low calcium intakes of infants have been associated with rickets, even though classically the disease is caused by a nutritional vitamin D deficiency. High phosphorus intakes have been suggested to contribute to hypocalcaemia (low serum calcium levels) and fractures in children (Abrams & Atkinson, 2003), this may in part be due to the actions of parathyroid hormone (PTH) causing resorption of calcium and phosphate from the bone, however further studies are required to evaluate the relationship and mechanisms underlying this proposed effect.

The Ca:P may be an important determinant of calcium absorption and retention due to the regulatory mechanisms which control calcium and phosphorus homeostasis within the body (Bass & Chan, 2006). Animal studies have shown that low Ca:P diets cause low bone densities (Sax, 2001). Common practice is to have a Ca:P molar ratio between 1:1 and 2:1 (Koletzko, et al., 2005). Hypothetically low Ca:P may adversely affect calcium balance which subsequently may increase the risk of bone fracture and osteoporosis. Typical Western diets are abundant in phosphorus however calcium intake may be too low (Kemi, et al., 2006). A high dietary phosphorus intake is suggested to have negative effects on bone through increased PTH secretion, as high serum PTH concentration increases bone resorption and decreases bone formation (Kemi, et al., 2010).

Other food derivatives can also affect the bio-availability of calcium, for example zinc and iron (Hallberg, 1998). Therefore it is important to consider the inter-relation of the nutrients in the diet (Fairweather-Tait & Teucher, 2002).

Formula milk has higher concentrations of calcium and phosphorus but with lower bio-availabilities of both nutrients compared to human milk (Bozzetti & Tagliabue, 2009). In breast milk the Ca:P is approximately 2:1, with similar ratios in infant formulas; however absolute quantities are higher in infant formulas to account for the differing bioavailabilities. Breast milk calcium levels remain constant over the first year; however the phosphorus content decreases over the course of lactation (Bass & Chan, 2006).

In addition, Vitamin D is also important during phases of rapid growth and bone mineralization as in infancy, to ensure optimal calcium balance (Thompkinson & Kharb, 2007). Deficiency of vitamin D in children results in rickets, characterised by skeletal deformity and muscle weakness. Hypovitaminosis D (deficiency of vitamin D) is caused by a combination of inadequate exposure to UVB radiation and dietary supply. There is a limited supply of natural sources of dietary vitamin D, the highest contributors being fatty fish and eggs. Currently in the UK fortification of foods with vitamin D is practised under regulation (EC) no 1925/2006, including breakfast cereals and infant formula products, with mandatory fortification of margarine products (Department of Health, 2011).

Pregnant women, breastfeeding mothers and infants are recommended to use vitamin D supplements, however according to the 2010 Infant Feeding Survey only 14% of infants aged 8-10 months were taking vitamin D supplements along with 33% of mothers taking vitamin D supplements at this age. Infant levels of vitamin D usually decline at the weaning period as

most foods and cow's milk are low in vitamin D. Between 6 months and 3 years infants and toddlers have an increased need for adequate vitamin D levels due to the high rate at which calcium is being laid down in the bone; they are also susceptible to vitamin D deficiency due to restricted exposure to UVB radiation from limited outdoor physical activity in day-care centres, low concentrations present in breast milk and limited intake of vitamin D rich dietary sources (McAndrew, et al., 2012).

The 2008/2009 – 2010/2011 National Diet and Nutrition Survey (NDNS) survey observed an increased risk of vitamin D deficiency in all age groups of the survey, 7.5% in the 1.5 – 3 year old group had serum 25(OH)D levels below 25 nmol/L, a level below which increases the risk of rickets and osteomalacia. Furthermore mean intakes of vitamin D from food sources were well below the recommended nutrient intake (RNI) for the 1.5 – 3 year old age group (Food Standards Agency, 2015).

This primary study investigates the ratio of Ca:P in conjunction with vitamin D and other essential elements (Cu, Fe, K, Mg, Na, Zn) in a range of commercial infant food products (including infant breakfast and snack products and infant ready meals) in the UK. The quantitative data was further evaluated, based on a standardised menu approach, to calculate the total daily intake of the nutrients analysed for an infant aged 7-12 months and intakes were compared against the Recommended Nutrient Intake (RNI), to ascertain the nutritional adequacy. Finally, as the study is the first to include consumption of infant snack products, the level of total calorie intake is also calculated in order to assess estimated energy intake (EEI).

5.2 Materials and methods

5.2.1 Sample collection for essential elemental analysis

A selected number of dairy based commercial infant food products representative of the four leading brands available on the UK market for infants aged 7-12 months, including eight ready to feed infant meals, four infant snacks and one infant breakfast, were obtained from leading supermarkets during June and July 2014. The declared ingredients of all samples and their characteristics are presented in **Table 5.1**. Three independent replicates of each sample were analyzed from different food packages which were purchased from different leading supermarkets in the UK. Samples were stored unopened at room temperature to match the market environment.

Table 5.1 Ingredients and characteristics of commercial infant food samples for essential elemental analysis.

Brand Code	Product name	Ingredients	Nutritional Information (per 100 g)
A	Creamy tomato and leek pasta	Skimmed milk (26%), cooked pasta (durum wheat) (19%), water, carrots (12%), cooked rice (10%), tomatoes (7%), leeks (5%), cheddar cheese (4%), rapeseed oil (1.3%), herbs and spices (rosemary, pepper).	**Energy** 304kj/72kcal, **Protein** 3.0g, **Carbohydrate** 8.9g of which sugars 2.2g, **Fat** 2.6g of which saturates 0.9g, of which linolenic acid (Omega 3) 0.10g, **Fibre** 0.7g, **Sodium** 0.03g.
B	Creamy cauliflower cheese	Baby-grade cauliflower (36%), cooking water, skimmed milk, cheddar cheese (8%), rice, corn starch, parsley.	**Energy** 263kj/63kcal, **Protein** 3.3g, **Carbohydrate** 5.8g of which sugars 1.5g, **Fat** 2.7g of which saturates 1.6g, **Fibre** 1.0g, **Sodium** 0.07g.
C	Cheesy tomato pasta stars	Water, tomato (20%), pasta (18%, water, durum wheat semolina), vegetarian cheddar cheese (8%), cornflour, natural flavouring (contains celery, celeriac), iron sulphate.	**Energy** 285kj/68kcal, **Protein** 2.9g, **Carbohydrate** 8.5g of which sugars 0.7g, **Fat** 2.4g of which saturates 1.8g, **Fibre** 0.3g, **Sodium** 0.1g, **Iron** 0.9mg.
D	Cheesy pie	Organic potatoes (25%), organic vegetable stock (24%), (water and organic vegetables: carrots, parsnips, leeks, onions, Swedes), organic sweet potatoes (12%), organic cheddar cheese (10%), organic tomatoes (8%), organic onions (7%), organic carrots (5%), organic broccoli (4%), organic Swedes (4%), organic mixed herbs (<1%), organic peppercorns (<0.01%).	**Energy** 350kj/84kcal, **Protein** 3.7g, **Carbohydrate** 8.1g of which sugars 2.3g, **Fat** 3.7g of which saturates 2.2g, **Fibre** 1.6g, **Sodium** 0.1g.
E	Pasta carbonara	Water, cooked pasta (durum wheat) (25%), skimmed milk (21%), cooked rice, onions, ham (5%), grated hard cheese (3%), egg yolk, rapeseed oil (1.5%), herbs and spices (parsley, garlic, pepper).	**Energy** 372kj/89kcal, **Protein** 4.1g, **Carbohydrate** 9.3g of which sugars 1.5g, **Fat**, 3.8g of which saturates 1.1g, of which linolenic acid (Omega 3) 0.13g, **Fibre** 0.5g, **Sodium** 0.07g.
F	Broccoli cheese	Baby-grade vegetables (30%) (Carrot, broccoli (8%), onion), potato, skimmed milk, rice (10%), cooking water, cheddar cheese (9%), tapioca starch, black pepper.	**Energy** 338kj/80kcal, **Protein** 3.9g, **Carbohydrate** 9.9g of which sugars 1.7g, **Fat** 2.8g of which saturates 1.7g, **Fibre** 1.3g, **Sodium** 0.08g.
G	Cheesy spaghetti with 5 veggies	Water, vegetables (31% carrot, broccoli, onion, parsnip, peas), spaghetti (14% water, durum wheat semolina, egg white), vegetarian cheddar cheese (8%), cornflour, natural flavouring (contains celery, celeriac), iron sulphate.	**Energy** 333kj/79kcal, **Protein** 3.2g, **Carbohydrate** 9.8g of which sugars 3.9g, **Fat** 2.8g of which saturates 1.8g, **Fibre** 1.0g, **Sodium** 0.1g, **Iron** 1.0mg.
H	Spaghetti Bolognese	Organic tomatoes (37%), organic vegetable stock (19%) (water and organic vegetables: parsnips, carrots, leeks, onions and swedes), organic carrots (11%), organic beef (10%), organic	**Energy** 277kj/66kcal, **Protein** 3.9g, **Carbohydrate** 6.3g of which sugars 2.5g, **Fat** 2.5g of which saturates 1.1g, **Fibre** 1.4g, **Sodium** <0.01g.

Brand Code	Product name	Ingredients	Nutritional Information (per 100 g)
		broccoli (6%), organic onions (6%), organic spaghetti (5%) (durum wheat and egg whites), organic mushrooms (4%), organic cheddar cheese (2%), organic garlic (<1%), organic mixed herbs (<1%), organic peppercorns (<0.01%).	
S1	Mini cheese crackers	Organic wheat flour (48%), organic rice flour (19%), organic cheese (14%), organic sunflower oil (8%), organic malt extract (6%), organic malted wheat flour (2%), raising agents (<1%) (sodium bicarbonate, ammonium bicarbonate), Thiamin (vitamin B1) (<1%).	**Energy** 1931kj/459kcal, **Protein** 12.1g, **Carbohydrate** 65.7g of which sugars 3.9g, **Fat** 15.8g of which saturates 7.1g, **Fibre** 3.1g, **Sodium** 0.2g, salt equivalent 0.5g, **Thiamine** 1.9mg.
S2	Milk and vanilla cookies	Organic malt extract (27%), organic wheat flour (25%), organic brown rice flour (17%), organic fresh whole milk (12%), organic palm oil (9%), organic wholemeal flour (9%), raising agent (<1%) (Sodium bicarbonate), calcium carbonate (<1%), organic vanilla (<0.1%), thiamine (vitamin B1) (<0.1%).	**Energy** 1471kj/349kcal, **Protein** 6.6g, **Carbohydrate** 55.4g of which sugars 16.1g, **Fat** 10.6g of which saturates 4.9g, **Fibre** 3.2g, **Sodium** 0.2g.
S3	Farley's rusks original	Wheat flour, sugar, vegetable oil, raising agents (ammonium carbonates), calcium carbonate, emulsifier (monoglycerides), niacin, iron, thiamine, riboflavin, vitamin A, vitamin D.	**Energy** 1737kj/411kcal, **Protein** 7.0g, **Carbohydrate** 79.5g of which sugars 29.0g, **Fat** 7.2g of which saturates 3.1g, **Fibre** 2.1g, **Sodium** 0.01g, **Vitamin A** 450ug, **Vitamin D** 10µg, **Thiamine** 0.53mg, **Riboflavin** 0.82mg, **Niacin** 8.8mg, **Calcium** 390mg, **Iron** 7.0mg.
S4	Yogurt (strawberry)	Fromage frais, sugar (8.6%), strawberry puree from concentrate (5%), aronia juice, fructose (1%), modified maize , starch, stabilisers: guar gum, pectin, xanthan gum; flavourings, acidity regulator: lactic acid; vitamin D.	**Energy** 405kj/96kcal, **Protein** 5.3g, **Carbohydrate** 12.6g of which sugars 12.2g, **Fat** 2.3g of which saturates 1.6g, **Fibre** 0.2g, **Sodium** 0.05g, **Calcium** 150mg, **Vitamin D** 1.25µg.
BF	Multigrain breakfast	Fortified milk (demineralised whey powder, skimmed milk powder, vegetable fat (contains soya lecithin), calcium, vitamins (vitamin C, niacin, pantothenic acid, vitamin E, vitamin B, vitamin B6, vitamin A, folic acid, vitamin K1, vitamin D3, biotin, vitamin B12), iron, zinc, copper potassium, milled cereals (wholegrain wheat, rice, wholegrain millet, wholegrain oats. Skimmed milk powder, dietary fibre (GOS, FOS), demineralised whey powder, rice crispies (rice, corn, whey powder).	**Energy** 1826kj/434kcal, **Protein** 15g, **Carbohydrate** 61.9g of which sugars 37.2g, **Fat** 12.9g of which saturates 5.5g, **Fibre** 5.2g, **Sodium** 0.1g, **Vitamin A** 380µg, **Vitamin D3** 7µg, **Vitamin E** 2.7µg, **Vitamin K1**µ, **Vitamin C** 38mg, **Thiamine** 0.9mg, **Niacin** 7.5mg, **Vitamin B6** 0.4mg, **Vitamin B12** 0.7µg, **Folic acid** 120µg, **Biotin** 0.01mg, **Pantothenic acid** 3mg, **Calcium** 459mg, **Iron** 5.6mg, **Zinc** 2.6mg, **Copper** 0.2mg, **Iodide** 104µg.

5.2.1.1 Sample preparation for analysis of essential elements

A microwave accelerated reaction system (CEM MARS 5®, MARS IP, USA with XP-1500 vessels), equipped with standard temperature and pressure control systems, was used to digest all samples. Each ready to feed baby food sample was mixed and homogenised using a domestic blender (Multi-quick Braun 300) and each baby snack was crushed down using a Vorwerk Thermomix TM31 food processor. Three independent replicates of 0.5 g (wet weight) were weighed prior to the addition of 5.0 mL of concentrated nitric acid (70% trace analysis grade, Fisher Scientific) and 0.5 mL of hydrogen peroxide (30% trace analysis grade, VWR international). The samples were then heated for 20 minutes using microwave digestion; operating conditions shown in **Table 5.2**. The digested samples were quantitatively analyzed for 8 essential elements (Ca, Cu, Fe, K, Mg, Na, P and Zn) using an Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) (Perkin Elmer Optima 4300 DV, USA), operating conditions shown in **Table 5.3.**

Table 5.2 CEM MARS 5®, (XP-1500 vessels) microwave digestion conditions[a] for essential elemental analysis.

Microwave Conditions	Nitric acid digestion of semi-solid samples
Sample	0.5 g
Nitric Acid (HNO_3)	5 mL
Hydrogen Peroxide	0.5 mL
Pressure [b]	Max 400 psi
Power	1200 W – 100%
Temperature [c]	Step 1: ramp to 190°C over 20 min. Step 2: Hold at 190°C for a further 5 min; allow to cool at room temperature for 1 hour.

[a] Microwave conditions and digestion procedures were adapted and modified based on the CEM operation manual (674007 version)

[b, c] The electronic temperature and pressure sensors (EST & ESP-1500) were used to control and monitor the conditions inside the vessels to avoid exothermic reaction and over-pressurisation of the digestion vessels.

Table 5.3 ICPOES instrument operating parameters applied for the determination of essential elements.

Parameter	Value
View mode	Variable
View distance	15 mm
Plasma gas flow	15 L/min
Auxiliary gas flow	0.2 L/min
Source equilibration time	15 s
Pump flow rate	1.50 mL/min
Detector	Segmented array charge coupled device
Power	1300 watts
Nebulizer	0.80 min
Sample aspiration rate	1.50 mL/min
Read	Peak area
Number of replicates	3
Background correction	2-point
Read delay	60 s
Rinse delay	30 s

5.2.1.2 Preparation of standards for essential elemental analysis

Eight multi-element calibration solutions were prepared at different concentration levels (5-25,000 µg/L) from 1000 µg/L single element ICP grade standards (Inorganic ventures, US) using high purity nitric acid (70% trace analysis grade, Fisher Scientific) and matched to the sample matrix (10% HNO_3).

A calibration curve, at six different concentrations (min 5 ppb – max 25000 ppb), was obtained using these multi-element standards ($r^2 = 0.9999$).

5.2.1.3 Quality assurance for essential elemental analysis

The accuracy of the analysis was verified by analysing the Certified Reference Material (NCS ZC73009: wheat) and the concentration for each of the samples were typically within the certified range of ± 10% of the certified value shown in **Table 5.4**, demonstrating the validity of the method. Blank samples of ultrapure water were also prepared using the same procedures as the samples. Results from the blank controls were subtracted appropriately.

Table 5.4 Measured results (*mean (n = 5) and RSD), **certified values (mean ± uncertainty) and % recovery for Certified Reference Material (NCS ZC73009: wheat) to determine quality assurance of ICPOES method for essential elemental analysis.

Element	*Measured (mg/kg)	**Certified (mg/kg)	% Recovery
Ca	319.62 (3.07)	340.00±20	94.01
P	1331.38 (0.98)	1540.00±70	86.45
Fe	14.52 (1.32)	18.50±3.1	78.49
Zn	10.92 (2.28)	11.60±0.7	94.14
Mg	377.60 (1.59)	450.00±70	83.91
K	1280.00 (2.35)	1400.00±60	91.43
Na	10.80 (12.45)	17.00±5	63.53
Cu	2.52 (1.77)	2.70±0.2	93.33

*Average of measured Certified Reference Material values (*n*=5) and relative standard deviations (RSD).
**Certified Reference Material values (NCS ZC73009: wheat) ± uncertainty.

5.2.2 Sample collection for vitamin D3 analysis

Due to limited availability of food sources rich in vitamin D, a different range of food samples were selected for the vitamin D analysis, on the basis of their ingredients that are known to be rich in vitamin D, such as cheese, fish and eggs. Four different infant meal products were purchased from leading supermarkets in the UK between June and July 2014. The list of the ingredients of the baby food samples and their characteristics are presented in **Table 5.5**. The sample jars were stored unopened at room temperature, similar to their distribution and market environment. Three independent replicates of each sample were analyzed from different food packages which were purchased from different supermarkets in the UK.

5.2.2.1 Sample preparation for analysis of vitamin D3

The current analytical methods for vitamin D analysis are time consuming, labour intensive, require experienced analysts and have only been validated for a few materials. The official methods available are relatively similar and involve saponification and extraction, clean-up steps and separation using HPLC and detection with diode array, with RSD between 10-15% (Byrdwell, et al., 2008). In this study analysis of vitamin D3 was performed using Vitakit D™ (SciMed Technologies, Canada, USA), which is a competitive enzyme immunoassay kit. The ELISA could detect vitamin D3 between 0.125 – 0.75 IU/mL, where no sample in our analysis fell outside this detectable range and the intra assay RSD for the ELISA was 6.8%.

Table 5.5 Ingredient and characteristics of commercial infant foods for vitamin D3 analysis.

Brand Code	Product Name	Ingredients	Nutritional Information (per 100 g)
VD1	Cheesy fish pie	Cheese sauce (33%, skimmed milk, cornflour, vegetarian cheddar cheese (2%, contains milk)), water, vegetables (27%, cauliflower (10%), broccoli, potato, onion), hake (8%, fish), iron sulphate.	**Energy** 192kJ/45kcal, **Fat** 0.7g, of which saturates 0.4g, **Carbohydrate** 6.2g, of which sugars 1.9g, **Fibre** 0.6g, **Protein** 3.2g, salt 0.08g, **Sodium** 0.04g, **Iron** 1.1mg
VD2	Creamy fish pie meal	Vegetables (52%, peas (12%), potato (10%), carrot (10%), sweetcorn, onion), water, Alaska Pollock (8% fish), cheddar cheese (6%, milk), skimmed milk powder, cornflour, parsley, iron sulphate.	**Energy** 359 kJ/85kcal, **Fat** 2.2g, of which saturates 1.3g, **Carbohydrate** 10.0g, of which sugars 3.6g, **Fibre** 2g, **Protein** 5.4g, **salt** 0.21g, **Sodium** 0.1g, **Iron** 1.0mg, **Calcium** 80mg
VD3	Pasta bake with tuna	Cheese sauce (water, whole milk, cornflour, vegetarian cheddar cheese(contains milk)), vegetables (21%, tomato (11%), sweetcorn, carrot), pasta (14%, water, durum wheat semolina), tuna (8%, fish), iron sulphate.	**Energy** 277 kJ/66kcal, **Fat** 1.3g, of which saturates 0.8g, **Carbohydrate** 9.6g, of which sugars 1.2g, **Fibre** 1.0g, **Protein** 3.4g, **salt** 0.11g, **Sodium** 0.05g, **Iron** 1.6mg
VD4	Egg Custard	Skimmed milk (30%), full cream milk (30%), rice (29%), sugar, water, egg (3%), nutmeg (0.1%).	**Energy** 332 kJ/79kcal, **Fat** 1.3g, of which saturates 0.7g, **Carbohydrate** 13.5g, of which sugars 8g, **Fibre** 0.8g, **Protein** 2.8g, **salt** 0.1g

Each of the food samples were diluted with deionised water to a fat content of 1-3%, then mixed and homogenised using a domestic blender (Multi-quick, Braun 300) and three independent replicates of 1 g (wet weight) were weighed prior to the addition of 0.55 g of potassium hydroxide (laboratory reagent grade, Fisher Scientific) into 15 mL centrifuge tubes. The tubes were gently mixed and left uncapped for 2 minutes in the dark. The tubes were then capped and incubated in the dark for 4 minutes, followed by 2 minutes of vigorous shaking; this step was repeated twice. 2 mL of hexane (HPLC grade, Fisher Scientific) were then added to the tubes, which were then capped and shaken vigorously for another 2 minutes in the dark. Centrifugation at 3500 RCF for 10 minutes at room temperature was then performed. 200 µL of the upper organic phase was transferred to an amber screw cap glass vial.

10 µL of calibrators, extracted samples and controls were pipetted into the ELISA plate accordingly. The plate was shaken for 8 minutes on a plate shaker (180 ± 10 rpm) to evaporate the hexane. 60 µL of assay buffer was added to each well and mixed gently for 30 seconds. A lid was placed over the plate and shaken for 5 minutes (180 ± 10 rpm). 60 µL of anti-vitamin D3 conjugate with HRP diluted in conjugate diluent were added to each well and gently mixed for 20 seconds. The plate was covered and shaken for 10 minutes in the dark (180 ± 10 rpm). A Labtech LT-3000 microplate washer was used to wash the plate 6 times with 380 µL/well of distilled water. After washing the plate was tapped against absorbant paper until no trace of water was visible on the paper. 60 µL of substrate was added to each well and gently mixed for 10 seconds. The plate was then incubated in the dark for 5 minutes. Finally 60 µL of stopping solution (0.2M H_2SO_4) was added to the plate and gently mixed for 10 seconds. A Microplate Reader (Thermo Multiskan Ascent 96 & 384 well) was used to measure the absorbance at 450 nm immediately.

5.2.2.2 Preparation of standards for vitamin D3 analysis

Five different concentrations of vitamin D were supplied with the kit; ranging from 0 – 0.75 IU/mL. A calibration curve was obtained with a correlation coefficient of 0.9814.

5.2.2.3 Quality assurance for vitamin D3 analysis

Two control concentrations were supplied with the VitaKit D; 0.2 and 0.6 IU/mL. Analytically obtained concentrations were typically ± 10%, demonstrating validity of the method.

5.2.3 Estimation of Total Daily Intake

A standardised menu approach has been implemented to estimate the total daily intake of an infant aged 7-12 months, as proposed by Zand, et al. (2012a), taking into consideration the consumption of commercial infant foods tested in this study and commercial infant formula contribution. Using the gastric capacity of an infant (30 g/kg body weight/day) with the average weight of an eight month old infant (8.3 kg), an infant requires 249 g/day from foods. With respect to the elemental analysis, the gastric capacity has been divided by four to allow 25% for breakfast (62.25 g), 50% for lunch and dinner (124.5 g) and a further 25% for snacks (62.25 g), based on the consumption of infant food products described in the sample collection for essential elemental analysis section. The estimated amount for milk consumption has been set to 600 mL as recommended by the manufacturer's labelling of infant formula. The concentrations of elements and vitamin D from infant formula have not been analytically quantified in this study; the values have been calculated based on average concentrations provided by the manufacturer's label from leading brands of infant formula available in the UK. The total daily intake is finally calculated by adding the contribution from infant formula and from the foods analyzed in this study, this value can then be compared against the RNI to ascertain whether infants are meeting recommendations based on the proposed standardised menu.

5.2.4 Estimated Energy Intake

The daily EEI was calculated based on the nutritional labelling information provided by the manufacturer, for infant food products from the sample collection for essential elemental analysis section and commercial infant formula. Taking into consideration the energy contribution from the commercial infant formula (600 mL), 62.25 g for breakfast and snack products and 124.5 g for infant meal products to ascertain whether infants are meeting energy requirements (Estimated Average Requirements, EAR) based on the proposed standardised menu.

5.2.5 Statistical Analysis

The experimental results were subject to statistical analysis using Excel 2010. Means with the standard error of mean of the data are presented.

5.3 Results and Discussion

5.3.1 Essential elements

This primary study investigates the Ca:P ratio of an infant's diet based on the consumption of commercial complementary infant foods. The concentration of eight essential elements, in eight different infant food products, four different infant snacks and one infant breakfast product, targeted for infants aged 7-12 months, were determined using ICP-OES. The results obtained are presented as per 100 g of the food samples in **Table 5.6.**

Although the data is insightful, it is important to examine the entire daily nutrient intake when studying the nutrient quality of complementary infant foods in order to ascertain the suitability of these products in relation to dietary recommendations. Therefore the results shown in **Table 5.6** were further analyzed to estimate the total daily intake of a 7-12 month old infant based on a standard feeding regime suggested by Zand, et al. (2012a), which is demonstrated in **Table 5.7**. The total daily intake in **Table 5.7** is based on the formula milk contribution of an infant (600 mL) as well as the gastric capacity of an average 8 month old infant (30 g/kg of body weight) in order to ascertain the nutritional value of these products in relation to the RNI. The gastric capacity of an 8 month old infant, with an average weight of approximately 8.3 kg is estimated to be 249 g per day, which ideally should be divided by three to make up breakfast, lunch and dinner (Zand, et al., 2012b). In this particular study the gastric capacity has been divided by four to allow 25% for breakfast (62.25 g), 50% for lunch and dinner (124.5 g) and a further 25% for snacks (62.25 g).

The calculated Ca:P ratio of the diet was found to be 1.49:1 (**Table 5.7**), which is within the recommended range of 1:1 – 2:1 (weight/weight) by the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) to ensure optimal bone health and development (Koletzko, et al., 2005). However, the estimated total daily intake for calcium and phosphorus was 924 and 618 mg/day respectively (**Table 5.7**); which equates to 176% and 155% above the RNI respectively. It is important to note that the aforementioned is in agreement with previous studies carried out by Skinner, et al. (1997), Butte, et al. (2010) and Melo, et al. (2008). In these studies, the estimated daily intakes of calcium and phosphorus are also shown to be above the RNI. The study herein however demonstrates the highest value for the calcium and phosphorous intake, which could be due to inclusion of infant snack products being investigated for the first time.

Table 5.6 Concentration of essential elements (Ca, Cu, Fe, K, Mg, Na, P and Zn) in commercial infant foods determined by ICP-OES (mean (*n*=3) and SEM, standard error of mean).

	Brands	Ca (mg/100g)		P (mg/100g)		Fe (mg/100g)		Zn (mg/100g)		Mg (mg/100g)		K (mg/100g)		Na (mg/100g)		Cu (mg/100g)	
		n (3)	SEM	*n* (3)	SEM	*n* (3)	SEM	*n* (3)	SEM	*n* (3)	SEM	*n* (3)	SEM	*n* (3)	SEM	*n* (3)	SEM
Meals	**A**	77.88	1.61	58.09	0.97	0.28	0.02	0.35	0.01	11.58	0.09	128.78	1.34	32.67	0.18	0.04	0.02
	B	109.01	0.09	78.16	1.20	0.12	0.01	0.50	0.01	9.05	0.42	86.94	1.24	72.93	1.04	0.02	0.00
	C	68.14	0.75	55.27	0.31	1.26	0.03	0.42	0.00	8.88	0.07	78.01	0.71	97.90	0.89	0.03	0.00
	D	88.09	1.67	67.23	0.76	1.20	0.12	0.42	0.00	13.22	0.22	186.71	2.90	72.53	1.34	0.07	0.01
	E	62.09	1.21	64.91	1.50	0.31	0.00	0.42	0.01	9.21	0.08	76.39	0.35	66.41	0.07	0.05	0.01
	F	87.50	2.24	74.38	2.69	0.16	0.00	0.47	0.01	10.91	0.32	161.61	3.50	76.35	1.68	0.02	0.01
	G	68.79	1.05	54.90	0.73	1.17	0.06	0.37	0.02	9.36	0.07	80.72	0.50	83.43	1.04	0.02	0.00
	H	35.46	0.90	51.98	0.52	0.79	0.02	0.59	0.01	13.10	0.33	237.04	6.12	37.48	0.70	0.12	0.01
	Mean	74.62	7.68	63.12	3.41	0.66	0.18	0.44	0.03	10.66	0.64	129.52	21.35	67.46	7.82	0.04	0.01
Snacks	**S1**	217.30	1.51	247.62	2.05	1.56	0.03	1.74	0.01	38.17	0.33	161.03	1.43	175.49	1.84	0.42	0.00
	S2	187.28	2.78	196.35	1.58	1.19	0.04	0.97	0.01	56.84	0.32	287.37	0.22	171.35	0.59	0.16	0.00
	S3	564.02	3.73	77.72	0.69	9.17	0.29	0.76	0.01	22.37	0.25	147.50	1.72	2.57	0.28	0.11	0.00
	S4	160.82	4.00	109.63	1.59	0.04	0.01	0.61	0.01	11.80	0.17	134.46	2.51	30.30	0.83	0.02	0.00
	Mean	282.35	94.59	157.83	39.04	2.99	2.09	1.02	0.25	32.29	9.81	182.59	35.35	94.93±	45.68	0.18	0.09
	BF	437.25	4.18	322.26	3.32	5.17	0.07	2.15	0.04	56.05	0.54	532.63	5.20	120.21	1.94	0.19	0.00

Table 5.7 Total daily intake of essential elements (Ca, Cu, Fe, K, Mg, Na, P and Zn) by an infant age 7-12 months old [a], based on the gastric capacity of an 8 month old infant and the standard feeding regime composed of commercial infant food products and infant formula milk in relation to the recommended nutrient intakes (RNI).

	Infant Formula		Breakfast		Meals (lunch and dinner)		Snacks		Total Daily Intake [f]	RNI (7-12 months)	% RNI
Element (mg)	100 ml	600 ml [b]	100 g	62.25 g [c]	100 g	124.5 g [d]	100 g	62.25 g [e]	mg/day	mg/day	
Ca	64.25	385.50	437.25	272.19	74.62	92.90	282.35	175.77	926.36	525	176.45
P	40.50	243.00	322.26	200.61	63.12	78.58	157.83	98.25	620.44	400	155.11
Fe	1.05	6.30	5.17	3.22	0.66	0.82	2.99	1.86	12.20	7.8	156.42
Zn	0.67	4.01	2.15	1.34	0.44	0.55	1.02	0.64	6.53	5	130.56
Mg	5.65	33.90	56.05	34.89	10.66	13.28	32.29	20.10	102.17	77.5	131.83
K	77.75	466.50	532.63	331.56	129.52	161.26	182.59	113.66	1072.98	700	153.28
Na	21.75	130.5	122.21	74.83	67.46	83.99	94.93	59.09	348.42	335	104.00
Cu	0.04	0.23	0.19	0.12	0.04	0.06	0.18	0.11	0.51	0.3	170.39

[a] Average weight about 8.3 kg

[b] Recommended volume of milk intake for a 6-9 month old infant

[c],[d],[e] The portion size is based on gastric capacity of an infant aged 6-9 months old (30 g/kg of body weight) divided by 4 to make up for breakfast [c], lunch, dinner [d] and snacks [e] (30 g x 8.3 kg = 249 g/4)

[f] Daily intake calculated by the sum of milk and non-milk intake

Although the concentration of calcium is below the National Institute of Health (NIH) tolerable upper intake level (UL) of 1500 mg/day, and therefore does not pose any risk of exposure in relation to renal insufficiency and vascular and soft tissue calcification (Institute of Medicine, 2011); it still warrants further investigation due to the inhibitory impact on iron and zinc bioavailability.

All of the snacks and breakfast infant food products were higher in concentration than the ready to feed meals in all micronutrients, which is probably due to fortification (with the exception of sodium); furthermore when the total daily intake does not include the breakfast or snack products all essential elements typically are within ± 10% of the RNI. Therefore it is important for parents to select breakfast and snack options that are nutritionally adequate for the infant's diet and to not exceed recommendations when added to the infant's diet. More attention needs to be focused upon infant snacks as national surveys have shown that snacking increases with age and that a higher percentage of 12–18 month-olds snack on 'sugar preserves and confectionary' (63%) compared with 'savoury snacks' (43%) and there is currently limited data available in relation to their nutritional suitability (Hardwick & Sidnell, 2014).

Infant formula alone contributes 73.4% of Ca, 60.8% of P, 80.8% of Fe, 80.2% of Zn, 43.7% of Mg, 66.6% of K, 39.0% of Na and 76.7% of Cu of the RNI; which for most elements is a high percentage, mainly due to formula being fortified. Therefore if infant formula milk intake is compromised or breast milk concentrations are low due to poor maternal nutrition the infant may be at risk of deficiency.

The other important factor to bear in mind is the issue of nutrient interaction and the impact on bioavailability. The consumption of elements therefore cannot be considered in isolation due to their effects on digestion and absorption (Sandström, 2001).

The issue of bioavailability has a high relevance when considering that the intake of all the micronutrients tested herein, based on the standardised menu, is in excess of the RNI (**Table 5.7**), with the exception of sodium, due to sodium being replaced by potassium in many foods following the Food Standards Agency legislation on reduction of salt (Melo, et al., 2008; Scientific Advisory Committee on Nutrition, 2003; Zand, et al., 2012). The later highlights an important issue in relation to micronutrient interactions. For instance, when considering bone health, high levels of magnesium can suppress PTH secretion and disturb calcium homeostasis and increase bone density. In addition, along with increasing intestinal

absorption of calcium and phosphorus, vitamin D also enhances intestinal absorption of magnesium; whereas phosphate and calcium can reduce the absorption of magnesium (Ilich & Kerstetter, 2000).

On the other hand, increasing calcium consumption may negatively affect the absorption of iron, which may impact on the occurrence of iron deficiency anaemia (IDA) (Hallberg, 1998). Between 6-9 months full term infants are at risk of IDA due to inadequate iron stores and therefore require iron from their diet (Domellöf, et al., 2014). Studies have reported lower iron absorption in infants when iron supplements have been given with milk compared to water (Heinrich, et al., 1975) and juice (Abrams, et al., 1996). However Dalton, et al. (1997) found no effect of calcium and phosphorus supplementation on iron status or iron deficiency in full term infants fed iron fortified formula between 6-15 months. It is important to mention that products C, G, S3 and BF show high iron content, which is due to fortification of these products as illustrated in **Table 5.1**. In addition brands D and H also show a high iron content, these products are from an organic product range. The unfortified infant food products only contribute approximately 20% of iron in comparison to their fortified counterparts for the meal intake. This may be important for parents when selecting appropriate meals for infants. Furthermore, although less clear; calcium is believed to also reduce zinc absorption. A reduction in iron and zinc absorption may cause impaired neurophysiological functions (Sandstead, 2000). Excessive iron and zinc intake may also have a counter-effect on copper (Sandström, 2001). Although iron intake in this study is below the NIH UL (40 mg/day), zinc on the other hand is above (5 mg/day). However copper intake from this study is also above recommendations and at present there is no UL set for copper (Trumbo, et al., 2001).

The extent to which the excess intakes observed in this study will affect bioavailability is unknown and knowledge of the mechanisms involved is relatively limited and needs further attention, especially during infancy (Rosado, 2003).

5.3.2 Vitamin D

The concentrations of vitamin D in selected complementary infant foods tested in this study are presented in **Table 5.8.** The total daily intake of vitamin D, again based on the standardised menu proposed by Zand, et al. (2012a) was 9.66 μg/day, which is 138% above RNI set at 7 μg/day for 7-12 month old infants and illustrated in **Table 5.9,** which is below the UL of 38 ug/day set by NIH (Institute of Medicine, 2011). It is important to mention that

120% of the RNI was supplied by the fortified infant formula, with only 18% being provided by weaning foods. In situations where infant formula intake is compromised or reduced, as it does when the infant becomes older, vitamin D intake may become inadequate, as the majority of the vitamin D at 7-12 months is being supplied by the infant formula. Furthermore, food sources are relatively low in vitamin D, therefore may not supply adequate vitamin D that an infant/toddler requires for optimal development and may possibly even cause deficiency.

Table 5.8 Concentration of vitamin D3 in commercial infant foods (mean (*n*=3) and SEM, standard error of mean).

	VD1		**VD2**		**VD3**		**VD4**	
	n (3)	SEM	*n* (3)	SEM	*n* (3)	SEM	*n* (3)	SEM
Vitamin D3 (µg/100 g)	0.496	0.156	0.288	0.055	0.400	0.003	0.855	0.059

In a study by Skinner, et al. (1997) however, estimated dietary daily intake of Vitamin D for infants aged between 6-12 months, was 6.6 ug/day, which is slightly below the RNI. The lower daily intake reported by Skinner, et al. (1997) compared to the result in this particular study may be due to the inclusion of breastfed infants as breast milk is known to be lower in vitamin D compared to fortified infant formula.

The recent NDNS survey highlighted that the mean intake of vitamin D from foods is well below the RNI of toddlers aged 1.5 – 3 years (Food Standards Agency, 2015). In addition, vitamin D concentrations in breast milk are much lower compared to fortified infant formula; therefore if the vitamin D status of the breastfeeding mother is low then the infant may not be receiving an adequate supply of vitamin D. Furthermore, although breastfeeding mothers and infants are recommended to receive supplements of vitamin D national surveys document that the majority are not following recommendations (McAndrew, et al., 2012). This potential reduction in vitamin D after the first year of life, and potentially in breastfed infants, may have a detrimental effect on bone health as current recommendations are based on calcium absorption and bone health. It is also important to mention that vitamin D has also been implemented in the functioning of the immune system, and further knowledge into the role of vitamin D for immune functions need to be further explored (Muehleisen & Gallo, 2013). Prolonged exclusive breastfeeding without vitamin D supplementation also appears to be important (Ahmed, et al., 1995; Mughal, et al., 1999).

Table 5.9 Total daily intake of vitamin D3 by an infant aged 7-12 month[a] old, based on the gastric capacity of an 8 month old and a standard feeding regime composed of commercial infant food products and infant formula milk in relation to the recommended nutrient intake (RNI).

	Infant Formula		Meals		Total Daily Intake [d]	RNI	% RNI
	100 mL	600 mL[b]	100 g	249 g [c]	µg/day	µg/day	
Vitamin D (µg)	1.40	8.40	0.51	1.26	9.66	7.00	137.95

[a] Average weight about 8.3 kg.
[b] Recommended volume of milk intake for a 6-9 month old infant.
[c] The portion size is based on the gastric capacity of an infant aged 6-9 months old (30 g/kg of body weight) to make up for lunch and dinner (30 x 8.3 = 249 g), lunch/dinner = 249/2 = 124.5 g [d] Daily intake is simply calculated by the sum of milk (b) and non-milk intake (c)

5.3.3 Estimated energy intake

The breakfast and snack infant food products appear to be a good source of micronutrients, however it is also important to consider the contributions made on an energy level from these products. Based upon the nutritional labels provided by the manufacturer, the products from the essential elemental analysis section have been assessed for their contribution to energy. For a 7-12 month old infant the EAR for energy is 687 kcal/day based on a diet of mixed feeding (Scientific Advisory Committee on Nutrition, 2011). In **Table 5.1** S1, S2 and S3 are all biscuit based snack products which contribute a 28% higher energy contribution compared to S4 which is a yogurt product. Breakfast and snacks contribute a total portion size of 62.25 g/day each, which equates to 39% and 30% of the EAR for energy for breakfast and snacks, respectively. Similarly, a portion size of 124.5 g of commercial ready to feed meals provides 14% and 600 mL of infant formula provides 59% of the EAR for energy. Based on these observations, the total daily intake for energy will exceed the EAR by 42% which highlights an important issue in relation to excess calorie intake and the risk of obesity. The diet and nutrition survey of infants and young children highlights that at least 75% of boys over 7 months and 78% of girls are above the 50^{th} percentile for weight compared against the UK WHO growth standards (Department of Health, 2013), which highlights that parents must be aware of the energy contribution that infant foods contribute and select products which provide good sources of micronutrients for optimal growth and development without over consumption of macronutrients (Gidding, et al., 2006).

It is important to note that one of the limitations associated with this study are that it is unlikely to represent the actual amount of consumption that is ingested and retained by the infant as it does not take into consideration wastage and fails to take into account any contribution from breast milk or homemade foods.

5.4 Conclusions

This primary study investigates the ratio of Ca:P in conjunction with vitamin D and other essential elements (Cu, Fe, K, Mg, Na, Zn) in a range of commercial infant food products in the UK. In addition, as this study is the first to include consumption of infant snack products, the level of total calorie intake is also calculated in order to assess total daily energy intake.

The Ca:P ratio of the infant's diet based on the standard feeding regime used in this study equates to 1.49:1, which is within the recommended range of 1.1 – 2:1 recommended by

ESPGHAN. However the actual total daily intakes of calcium and phosphorus were 176% and 155% above the RNI respectively. The implication of excess intake of micronutrients warrants further investigation for long term health effects.

The total dietary intake of vitamin D3 was determined to be 9.61 μg/day, which is 137% above the RNI. However 120% is contributed from the fortified infant formula. As weaning foods are typically low in vitamin D unless they are fortified and breast milk concentrations are typically low, vitamin D deficiency may arise when infant formula consumption is reduced, which is the case after the first year of life.

Finally the estimated energy intake, from consumption of the products tested herein, is estimated to contribute to a high calorie intake, which poses a possible impact on obesity.

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Chapter 6

Microencapsulation of docosahexaenoic acid (DHA) for potential use in ready to feed infant foods

6.1 Introduction

During infancy long chain polyunsaturated fatty acids (LCPUFA) are thought to be a specific dietary requirement due to the rapid rate of physiological and immunological development (Lapillonne, et al., 2014). Docosahexaenoic acid (DHA) 22:6 *n*-3 is the most abundant *n*-3 fatty acid in the central nervous system and is specifically concentrated within membrane lipids of the grey matter within the brain and the visual elements of the retina. Some intervention and epidemiological studies have associated low plasma and blood cell lipid DHA concentrations with increased risk of poor visual and neural development in infants (Bouwstra, et al., 2003; Dunstan, et al., 2006; Helland, et al., 2003; Hibbeln, et al., 2007; Innis & Friesen, 2008; Innis, et al., 2001; Oken, et al., 2005; Uauy & Danqour, 2006; Williams, et al., 2001).

In the Western diet the consumption of *n*-3 LCPUFA, eicosapentaenoic acid (EPA) 20:5 *n*-3 and DHA, is below recommended levels. Furthermore conversion in the body from the precursor α-linoleic acid (18:3 *n*-3) does not appear to overcome this deficiency (Vongsvivut, et al., 2012). A preliminary study to evaluate the total daily intake of essential fatty acids in an infant's diet based on the consumption of fortified infant formula and commercial 'ready-to-feed' complementary infant foods in the UK indicates that infants are not meeting the requirements for LCPUFA, in particular DHA (Loughrill & Zand, 2016). This may have implications for brain and visual development, as well as inflammatory and immune functions. Furthermore due to there being limited food sources rich in DHA, infant food products may need to be fortified to meet current legislative requirements (Innis, 2008).

Fish oil is a rich source of *n*-3 LCPUFA, especially DHA and EPA, and is currently the main commercial source for nutritional supplements and food fortification of these LCPUFA (Vongsvivut, et al., 2012). However LCPUFA are highly susceptible to oxidative deterioration during food processing and storage, which limits their use in food products due to off-flavour and odour production. In addition, the primary oxidation products, hydroperoxides, are considered to be toxic (Kagami, et al., 2003). Microencapsulation can be

used as a method to protect the LCPUFA against oxidation so as to enable their successful incorporation into food products (Vongsvivut, et al., 2012).

Encapsulation is a rapidly developing area of technology with great potential in the food industry. The process involves small particles of core material being packaged within a wall material to form microparticles, the wall is designed to protect the core from deterioration and release it under desired conditions (Kagami, et al., 2003). In particular for fish oils encapsulation can also offer improved handling properties of the oil, allow easy storage and mask the taste or odour of the core to offer improved palatability of the product (Botrel, et al., 2014). Spray drying is a common technique used to encapsulate oils in the food industry (Jafari, et al., 2008). Furthermore microencapsulation of materials that are susceptible to oxidation has been shown to significantly hinder oxidation (Kagami, et al., 2003).

Fish oils have been encapsulated using a range of technologies in the literature, including fluidized bed coating, spray dried emulsions and single and multi-core complex coacervation (Vongsvivut, et al., 2012). Spray drying is the most commonly used technology in the food industry due to its low cost and ready availability of equipment (Aghbashlo, et al., 2012) and has therefore been used in this study for the microencapsulation of fish oil.

Recently nano-emulsions have attracted pronounced attention in various industrial fields (Jafari, et al., 2008). The core material in the nano-size range will be encapsulated into the matrix of micron-size powder particles. Jafari, et al. (2008) have shown microfluidization to be an efficient emulsification technique to form fish oil encapsulated powders; this technique will be employed for the production of lipid nano-emulsions followed by spray drying to form the encapsulated powder.

Many of the spray dried fish oil formulations on the market and in the literature use water soluble polymers as wall materials, which are not compatible with aqueous based food products, as the fish oil would be released from the microparticles within the food product prior to consumption. Hydroxy propyl methyl cellulose acetate succinate (HPMCAS) is a water insoluble polymer, which is available in three commercially available grades (L, M and H grades) which differ in their acetyl and succinoyl content and vary in their pH solubility. It was originally developed as an enteric polymer for aqueous dispersion coating. The enteric coating prevents dissolution of the core in acidic pH **(**Sarode, et al., 2014). In addition to the polymer being water insoluble to prevent fish oil release in aqueous based food products, HPMCAS also offers encapsulation of the fish oil under certain pH conditions. In particular

to this study, infant foods are typically within the pH range of 3-4; therefore the fish oil will remain encapsulated in the food product and be released within the intestine for absorption after consumption. Therefore HPMCAS has been employed as the polymer for coating in this study.

The objectives of this study are to:

a) Develop a microencapsulated fish oil product using HPMCAS as the wall material which can be used for the fortification of DHA in infant food products.

b) Explore two methodologies for the microencapsulation of fish oil; 1) microfluidization to form nano-emulsions which will be subsequently spray dried in HPMCAS and 2) directly spray dried fish oil in HPMCAS; both products from 1) and 2) will be referred to in this study as encapsulated powder.

c) Undertake an evaluation of the two methodologies used for the production of spray dried encapsulated powders, including DHA content, solid yield % and DHA encapsulation efficiency, to determine the most appropriate method of production.

d) Characterize the microencapsulated fish oil powder produced by the most appropriate method identified in c), using various techniques; including moisture content, water activity, encapsulation efficiency, particle size distribution (PSD), scanning electron microscopy (SEM), X ray powder diffraction (XRPD), Fourier transform infrared (FTIR), differential scanning calorimetry (DSC) and thermal gravimetric analysis (TGA). In addition the stability of the encapsulated DHA will be compared to its un-encapsulated fish oil counterpart. Finally pH dependent release of DHA will be assessed under simulated gastric and intestinal dissolution conditions.

6.2 Materials and Methods

Fish oil (Baby's DHA) was purchased from Nordics Naturals (Watsonville, California); containing 9 kcal, 1 g of fat, 210 mg of omega 3 fatty acids, 70 mg of EPA and 97 mg of DHA per mL of fish oil. The polymer HPMCAS (AS-LG) was supplied by Shin-Etsu Chemical Co., Ltd (Tokyo, Japan). Tristearin and Poloxamer 188 solution (Pluronic F68) were purchased from Sigma Aldrich (Bellefonte, PA). Acetone, methanol and hexane (HPLC grade) were purchased from Fisher UK. All other general chemicals used in this study were of analytical grade.

6.2.1 Preparation and spray drying of solid lipid nanoparticles

The lipid (tristearin) phase (1 g) was fully melted at 75°C and dispersed with heated fish oil (2 mL). The aqueous surfactant solution was prepared by adding surfactant (Pluronic F68, 0.5 g) to 50 mL pre-heated deionised water (75°C). A pre-emulsion was formed by mixing the hot lipid phase with the aqueous surfactant solution and held at 75°C using an IKA® T25 digital Ultra Turrax® (Germany) at high speed for 5 min. The crude dispersion was processed through a Micro DeBEE (B.E.E. International Inc. Easton, MA) high pressure homogenizer at 75°C and 15,000 psi for 10 minutes. The Micro DeBEE was heated (75°C) prior to homogenization. The emulsion was left to cool down at room temperature whilst stirring. The produced solid lipid nanoparticles (SLN) were stored at 5°C prior to spray drying.

The polymer (HPMCAS, 2.5g) was dissolved in 200 mL of deionised water and the pH was adjusted to 6.5 using sodium hydroxide, which was subsequently filtered to remove any remaining particulates. 14.5 mL of the fish oil SLN was added to the polymer solution to make feed solutions. SLN encapsulation in HPMCAS was achieved by using a Buchi mini spray dryer (B-290, Switzerland). Operating parameters were: inlet temperature, 160°C; feed rate, 7%; aspirator, 85%; compressed air, 40%. Yields of the spray-drying process were 40-45%.

The solid yield was calculated as the ratio of the encapsulated powder weight collected after every spray-drying experiment to the initial amount of solids in the sprayed dispersion volume (Martinez et al., 2015).

The solid yield % was calculated as follows:

Collected encapsulated powder weight (g)/ initial amount of solids (g) x 100

6.2.2 Spray drying of DHA-HPMCAS microparticles

5.0 g of the polymer (HPMCAS) was added to 250 mL of acetone/methanol (5:1, v/v) whilst stirring until completely dissolved. Fish oil (1.5 g) was then progressively added to the polymer solution whilst stirring to make feed solutions. DHA/HPMCAS microparticles were produced using a Buchi mini spray dryer (B-290, Switzerland). Operating parameters were: inlet temperature, 78°C; feed rate, 17%; aspirator, 85%; compressed air, 40%. Yields of the spray-drying process were between 50 – 60%.

6.2.3 Fatty acid composition of encapsulated powders

The two encapsulated powders produced by the different methodologies were analyzed by ILS laboratories (Derbyshire, UK) for fatty acid composition. Briefly, after the removal of the extracting solvent, the extracted fat is saponified with methanolic sodium hydroxide, to form soaps with the liberated fatty acids. Upon acidification, the free acids undergo an acid catalysed esterification reaction to form methyl esters of the respective fatty acids. These methyl esters are finally extracted into hexane and the fatty acid profile is determined using capillary gas chromatography using a flame ionisation detector. The DHA encapsulation efficiency was calculated as follows:

DHA in encapsulated powder (g)/ DHA added initially (g) x 100

6.2.4 Characterisation of the encapsulated powder

Following the evaluation of the two approaches to produce fish oil encapsulated powders based on solid yield %, DHA encapsulation efficiency and DHA content, the most appropriate methodology was selected and the selected encapsulated powder characterised using the following techniques.

6.2.4.1 Moisture Content

The moisture content of the encapsulated powder was determined using two methods, oven drying and TGA.

6.2.4.2 Oven Drying

Two grams of the generated encapsulated powder was dried in a Thermo Scientific Heraeus® oven (Germany) at 70°C for 24 hours (Aghbashlo et al., 2012). Weight measurements were performed using an AAA, 250 L balance (Mettler Toledo, China) with a precision of ± 0.0001 g.

The moisture content was calculated as follows:

$$\frac{\text{Wet weight of sample (g)} - \text{Weight of sample after drying (g)}}{\text{Wet weight of sample (g)}} \times 100$$

6.2.4.3 Thermal Gravimetric Analysis

The moisture content and physicochemical properties of the polymer (HPMCAS) and encapsulated powder samples were analyzed using TA Instruments' TGA Q5000 (UK). A sample weight of 2.7 ± 0.6 mg was heated from ambient temperature to 600 °C in aluminium pans at 10 °C/min, under nitrogen atmosphere at a flow rate of 25 mL/min. Results were analyzed using TA universal analysis software.

6.2.4.4 Water Activity

The water activity of the encapsulated powder was measured using an Aqua Lab Dew Point water activity meter 4TE (USA).

6.2.4.5 Encapsulation Efficiency

The amount of un-encapsulated oil was immediately measured to calculate the encapsulation efficiency after production by spray drying. Hexane (15 mL) was added to 1.5 g of the encapsulated powder, followed by shaking of the mixture for 2 minutes. The suspension was then filtered through a Whatman no.1 filter paper, the residue was rinsed three times with 20 mL hexane. The filtrate solution containing the extracted oil was then transferred to a Fistreem vacuum oven (UK) until evaporation of the solvent, finally for complete evaporation the residue was dried for 1 hour in a Thermo Scientific Heraeus® oven (Germany) at 70°C. The amount of surface oil was calculated by the difference in initial and final weights of slurry container (Aghbashlo, et al., 2012; Carneiro, et al., 2013).

The encapsulation efficiency was calculated as follows:

TO-SO / TO x 100

TO: Total oil, SO: Surface oil

In this study no total oil analysis was performed assuming that all the initial oil was retained in the powder due to it being non-volatile, furthermore depositions of the unbound oil on the dryer wall and degradation of the oil during spray drying was ignored.

6.2.4.6 Scanning Electron Microscopy

The encapsulated powder was sputter coated with chromium using a K575X Turbo-Pumped Chromium Sputter Coater (Quorum Technologies Ltd, UK). The morphological features were observed using a Hitachi SU 830 cold-cathode Field Emission Gun Scanning Electron Microscope (Japan) with an accelerated voltage of 1 kV and magnification of x1000-20,000.

6.2.4.7 Particle Size Distribution

The particle size distribution of the encapsulated material was measured using a Mastersizer 2000 (Malvern instruments) and results were analyzed using Mastersizer 2000 analysis software.

6.2.4.8 X ray Powder Diffraction

The crystalline state of HPMCAS and the encapsulated powder was evaluated using a D8 Advance X-ray Diffractometer (Bruker, Germany) using Cu Kα radiation at 40 kV and 40 mA. Samples were analyzed at angles from 3 to 40^{o} in 2θ with an increment of 0.03^{o} and a counting time of 0.5 seconds per step. Data was collected using DIFFRAC plus XRD Commander Version 2.6.1 software (Bruker-AXS) (ICDD, 2008).

6.2.4.9 Fourier Transform Infrared

FTIR spectra of fish oil, HPMCAS and the produced encapsulated powder were obtained on a Spectrum Two FTIR spectrophotometer (Perkin Elmer, UK) coupled with an attenuated total reflectance (ATR) accessory (Perkin Elmer, UK). The crystal was washed with iso-propanol between each sampling. The background spectrum was obtained by measuring the empty chamber. A resolution of 4 cm^{-1} was used and the ATR spectra were averaged on 10 scans. The scanning range was 450 to 4000 cm^{-1}. Results were analyzed using Perkin Elmer spectrum analysis software.

6.2.4.10 Differential Scanning Calorimetry

The thermophysical properties of the fish oil, HPMCAS and the encapsulated powder were studied using TA Instruments' Q2000 DSC (UK) equipped with a refrigerated cooling system under nitrogen atmosphere (50 mL/min) in hermetic aluminium pans with a single pin-hole in the lid. 2.1 ± 0.5 mg of HPMCAS and encapsulated powder and 6.6 ± 0.7 mg of fish oil were cooled and held isothermal for 2 minutes at -90°C and heated to 100°C for fish oil and 200°C for HPMCAS and the encapsulated powder at 10°C/min. Results were analyzed using TA universal analysis software.

6.2.4.11 Stability Testing

For the stability tests, a comparison between the loss of DHA from un-encapsulated fish oil and microencapsulated fish oil were assessed. The encapsulated powder and un-encapsulated fish oil were sealed in brown glass containers and stored either at room temperature or at 40°C in a Thermo Scientific Heraeus® oven (Germany), in order to accelerate oxidation, for one week. After one week all samples were analyzed for DHA content.

6.2.4.12 Dissolution

The dissolution was carried out at 37°C by adding 2 g of the encapsulated powder into 500 mL of acidic dissolution medium (0.1 mol/L HCl) to mimic the stomach environment for 2 hours or phosphate buffer (PBS pH 6.8) to mimic the intestinal environment until completely dissolved, both stages were agitated using a IKA Werke magnetic hot plate stirrer (Radley Discovery Technologies, Germany). The time required for the encapsulated powder to completely dissolve in the intestinal environment was recorded. After dissolution an aliquot of the dissolution media was centrifuged at 3000 rpm for 5 minutes to remove any encapsulated material and the supernatant was analyzed for DHA content.

6.3 Results and Discussion

6.3.1 Methods of Encapsulation

Two different production methodologies were employed for the production of fish oil spray dried encapsulated powders. Firstly, SLN of encapsulated fish oil were subsequently spray dried in HPMCAS and secondly solid dispersions of fish oil and polymer (HPMCAS) were directly spray dried. Following production by spray drying solid yield % was calculated and the encapsulated powders were analyzed for DHA content in order to determine the DHA encapsulation efficiency (**Table 6.2**). **Table 6.1** illustrates the fatty acid composition of the two formulations. **Table 6.2** shows that the concentration of DHA is 3.5 times greater in the directly spray dried production method compared to the spray dried nano-emulsion technique. Due to these results the encapsulated powder produced by the directly spray dried production method was taken forward for further characterisation due to the fact that less of the microencapsulated material would need to be added to the final food product for fortification. In addition the process offered higher yield production and a much greater DHA encapsulation efficiency (**Table 6.2**). Finally the directly spray dried method was less time consuming and more cost effective.

Table 6.1 Comparison of the fatty acid composition of fish oil encapsulated powders produced by different encapsulation methodologies.

Fatty Acid	Spray dried (g/100 g sample)	Spray dried nano-emulsion (g/100 g sample)
Saturated fatty acids		
Myristic acid	0.83	0.39
Palmitic acid	2.13	1.43
Stearic acid	0.44	1.75
Arachic acid	0.01	0.02
Unsaturated fatty acids		
Palmitoleic acid	1.85	0.67
Petroselaidic acid	0.06	0.03
Oleic acid	4.02	1.34
Linoleic acid	0.48	0.16
Gamma linolenic acid	0.05	0.01
Linolenic acid	0.44	0.14
Dihomo-linolenic acid	0.02	0.01
Arachidonic acid	0.21	0.06
EPA	1.64	0.50
DPA	0.19	0.06
DHA	2.09	0.60
Total saturated acids	3.41	3.61
Total unsaturated acids	11.03	3.58
Mono-unsaturates (cis)	5.87	2.01
Mono-unsaturates (trans)	0.06	0.03
Poly-unsaturates (cis)	5.10	1.54
Total trans acids	0.06	0.03
Total omega 3	4.36	1.30
Total omega 6	0.74	0.24
Total omega 9	4.02	1.34

Table 6.2 DHA content, solid yield % and DHA encapsulation efficiency (EE) of encapsulated powders produced by different methodologies.

Production Method	DHA (g/100g)	Solid yield %	DHA EE%
Directly spray dried	2.09	54	93
Spray dried nano-emulsion	0.60	42	38

6.3.2 Characterisation of the encapsulated powder

6.3.2.1 Moisture content, water activity and encapsulation efficiency

The encapsulated powder produced by the selected production method of directly spray dried fish oil in HPMCAS was characterised according to the methods explained previously in **Section 6.2.4.**

The moisture content of the encapsulated powder was determined to be 1.11% using the oven drying method and 1.39% using TGA which is under the minimum specification of 3-4% for most dried powders used in the food industry. The moisture content of the polymer HPMCAS determined by TGA was higher at 2.19%. Moisture content is well known to have a significant effect on the lipid stability. It has been observed that low water contents are usually associated with low water activities, which might prevent lipid oxidation (Klaypradit & Huang, 2008). The water activity (aw) of the encapsulated powder was found to be 0.324, which again is within the ideal range of 0.2–0.4 for storage stability in lipid oxidation as reported elsewhere (Rockland & Beuchat, 1987; Rükold, et al., 2001).

During the spray drying process of the oil, the encapsulation efficiency is an important outcome, which can be determined indirectly by extracting the un-encapsulated oil. The surface oil or un-encapsulated oil is the amount of oil that is non-encapsulated. This can determine the quality of the microparticles as the surface oil is prone to oxidation, which may subsequently lead to the development of off flavours and affect the quality and shelf life of the product (Drusch & Berg, 2008). Encapsulation efficiency is dependent on many factors, including the nature of the encapsulated material, composition of the blend of coating material, homogeneity of dried slurry and spray drying parameters (Roccia, et al., 2014). The percentage of surface oil of the encapsulated powder was determined to be 12.4%.

Another important parameter to consider is the ratio of wall material to core in the encapsulated powder, where it is generally accepted that a 2-4 (w/w) wall material to core ratio should be suitable for most applications. A ratio below 2 could lead to an unacceptable increase in surface oil, while a ratio greater than 4 could result in a powder with very low oil content, which is not desirable for food applications (Gallarado, et al., 2013). The wall material to core ratio in this particular study was (5 g HPMCAS: 1.5 g fish oil) 3.33:1, which is within the recommended range.

6.3.2.2 Scanning Electron Microscopy

Fig. 6.1 illustrates the SEM images of the encapsulated powder, showing no apparent cracks or pores, which are necessary to protect the core material from oxygen and undesired release of oil droplets to the surface of particles, which is an advantage, since it suggests that capsules have lower permeability to gases, increasing protection and retention of the fish oil. In addition, the encapsulated powder appears wrinkled, with concave surfaces and in a variety of sizes which is typical of powders produced by spray drying. This type of morphology has also been observed by Kolinowski, et al. (2004) and Davidov-Pardo, et al. (2008) when encapsulating fish oils. The formation of hollow particles can be explained by the formation of a vacuole inside the particles, immediately after the crust development. This crust inflates when the particle temperature exceeds the local ambient boiling point and the vapour pressure within the vacuole rises above the local ambient pressure (Nijdam & Langrish, 2006). The lack of pores on the encapsulated powder surface was related to the good encapsulation efficiency obtained in the experimental design and the low surface oil content was a consequence of a continuous wall surface (Roccia, et al., 2014).

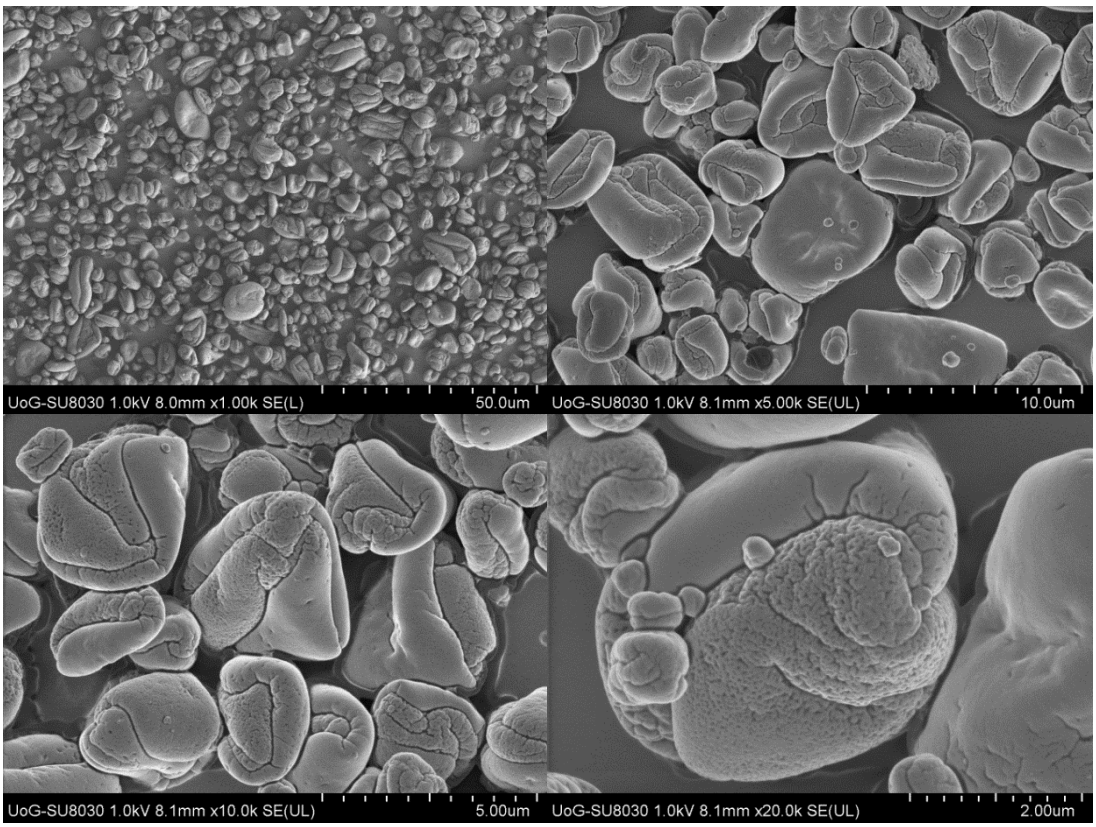

Figure 6.1 SEM micrographs of fish oil encapsulated powder using varying magnifications (x1k – 20k).

3.2.3 Particle Size Distribution

Fig. 6.2 shows the PSD of the encapsulated powder measured through the light scattering properties of the product. The particle size varied from d(0.1) 3.366 to d(0.9) 18.204 μm, with a median particle size of d(0.5) 7.435 μm. The span value observed was 1.996, a relative span less than 2 is normally considered as a narrow distribution in spray drying (Gottlieb & Schwartzbach, 2004). A high span value would indicate a wide size distribution and a high polydispersity (Dubey & Parikh, 2004).

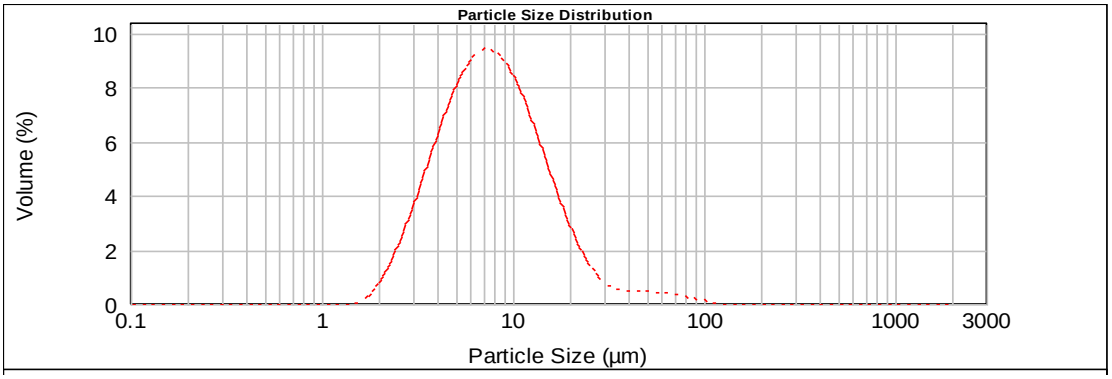

Figure 6.2 PSD of fish oil encapsulated powder (range: d(0.1) 3.366 – d(0.9) 18.204 μM; median d(0.5) 7.435 μM).

6.3.2.4 X ray Powder Diffraction

XRPD was applied to identify the degree of crystallinity in the polymer (HPMCAS) and encapsulated powder. In general, a crystalline material presents sharp peaks while amorphous products provide a broader peak pattern (Caparino, et al., 2012). As shown in **Fig. 6.3**, the polymer HPMCAS and the encapsulated powder exhibited an amorphous structure with minimum organisation based on the occurrence of large diffuse peaks. It is known that amorphous solids are in general more soluble and more hygroscopic (Botrel, et al., 2014). An amorphous nature dictates higher dissolution profiles as the amorphous form has a higher degree of disorder (higher free energy) (Al-Obaidi & Buckton, 2009).

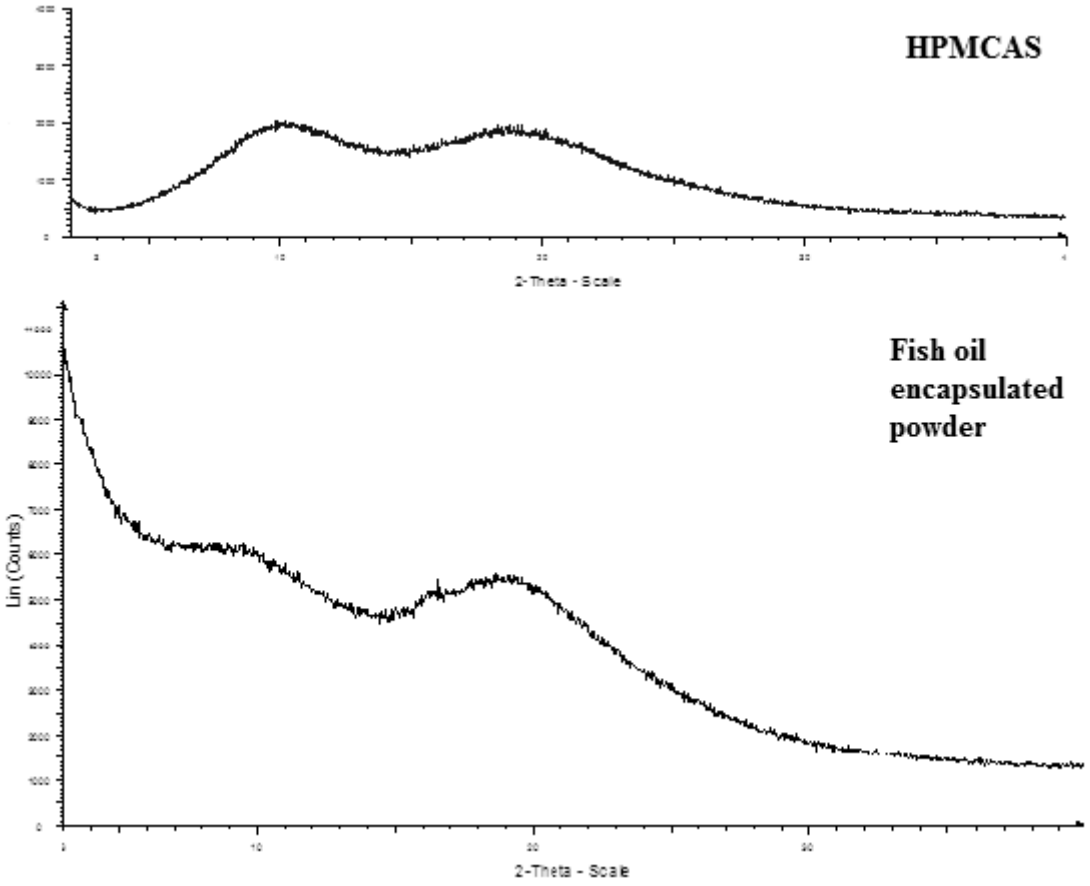

Figure 6.3 XRPD spectra of HPMCAS and fish oil encapsulated powder.

6.3.2.5 Fourier Transform Infrared

FTIR was used to explore fish oil-polymer interactions in the matrix of the encapsulated powder. IR spectra of fish oil, HPMCAS and the produced encapsulated powder are shown in **Fig. 6.4**.

Pure HPMCAS shows a strong absorption at 1738 cm^{-1} (C=O stretch), which is the same in the encapsulated powder spectrum. The absorption at ~3485 cm^{-1} shows broad O-H stretching.

A band at 3011 cm^{-1} in the fish oil is related to C-H stretch (cis-alkene HC=CH) which specifically represents unsaturated fatty acids and triplet bands present within 3000-2800 cm^{-1} which are a feature of the C-H stretching modes of the methyl and methylene backbones of lipids. A sharp band a 1744 cm^{-1} is assignable to C=O stretches of ester functional groups from lipids and fatty acids. C-O stretching in the fish oil was observed at

1147 cm^{-1}. Additional bands relevant to lipids are those at 1458 cm^{-1}, 1147 cm^{-1} and 1097 cm^{-1} assigned to asymmetrical deformation scissor from methylene (-CH2), CH2 out-of-plane deformation modes and C-O-C symmetrical stretches, respectively; these are mainly from triglycerides and cholesterol esters (Vongsvivut, et al., 2012).

The FTIR spectrum of the encapsulated powder was approximately equal to the virtual mixture of initial component spectrums (fish oil and polymer HPMCAS). The nature of the peaks did not vary for the combined fish oil and polymer HPMCAS with the generated encapsulated powder, indicating the absence of any strong chemical interaction between the fish oil and wall material.

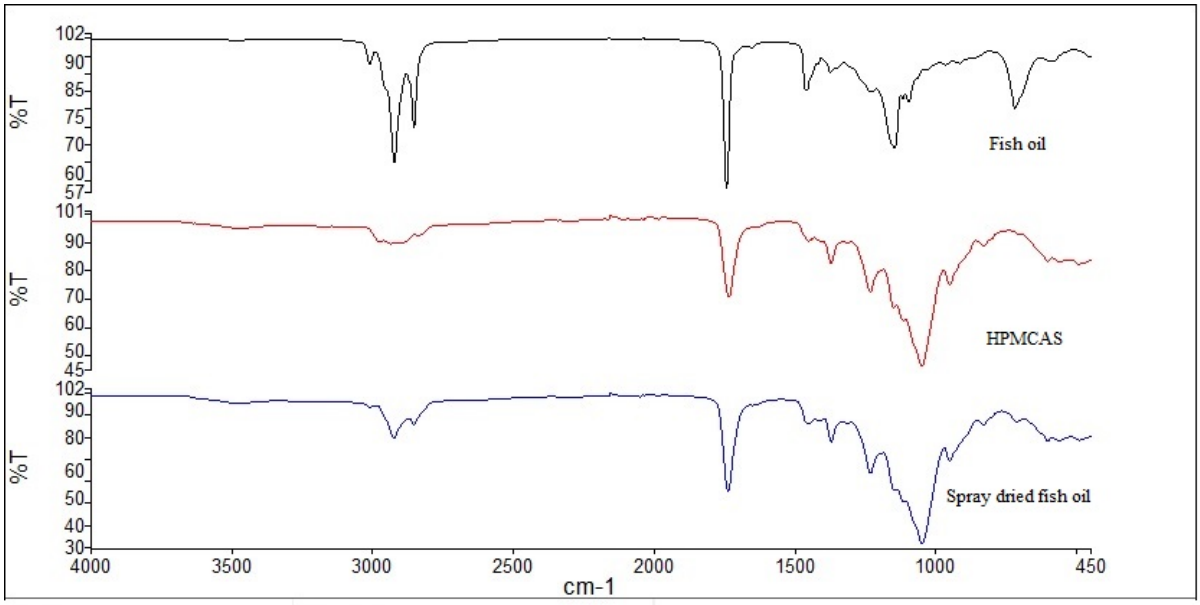

Figure 6.4 FTIR spectra of fish oil, HPMCAS and corresponding spray dried encapsulated powder.

6.3.2.6 Thermal Analysis: DSC and TGA

DSC provides information about the physical behaviour of materials by detecting processes such as melting, solid-solid transitions, dehydration and glass transitions, which is useful for understanding the physical nature of solids i.e. crystalline or amorphous, and how it is influenced by formulation and/or processing methods. Here, DSC was employed to investigate the presence of fish oil in the encapsulated powder after spray-drying. A recent article demonstrates that HPMCAS undergoes cross-linking esterification between the pendent carboxyl and hydroxyl groups above 200°C (Li, et al., 2013). For this reason the HPMCAS and the encapsulated powder were heated to below 200°C. The DSC results (**Fig.**

6.5) show HPMCAS to undergo dehydration (removal of moisture) and a glass transition process at 68 ± 1°C and 133 ± 2°C respectively (**Table 6.3**). The fish oil undergoes two melting processes when heated between -90 and 100°C with peak temperatures at -51 ± 1°C and -15 ± 1°C. The encapsulated powder product exhibits the same processes detected in both starting materials (**Fig. 6.5 and Table 6.3**). This indicates the presence of both the fish oil and HPMCAS in their initial forms and confirms that no chemical reaction(s) or change in physical state occurs when these materials are combined and spray dried. The glass transition detected in DSC analysis supports the findings from XRPD studies (**Section 6.3.2.4**) in that the HPMCAS and the encapsulated powder product are amorphous. The results presented in **Table 6.3** show that the glass transition temperature (T_g) of the product (124 ± 1°C) is ~10°C lower than the pure HPMCAS (133 ± 2°C). The lower T_g detected for the product is due to the presence of the fish oil which plasticizes the HPMCAS.

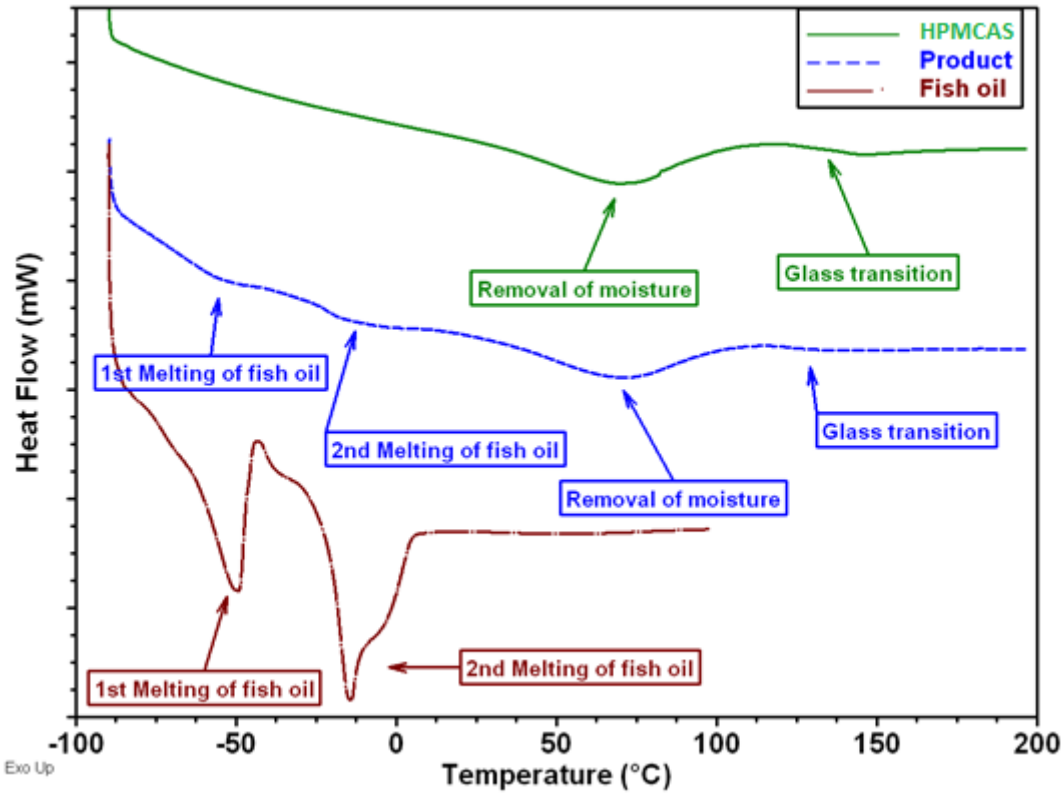

Figure 6.5 Overlay of the DSC data observed for HPMCAS, fish oil and encapsulated powder product heated from -90 to 100°C for fish oil and 200°C for HPMCAS and encapsulated powder product at 10°C/min.

Table 6.3 Mean temperatures at the peak maxima and energies associated with the processes detected in DSC analysis.

Sample	Melting process				Dehydration process		Glass transition	
	1		2					
	°C	ΔH (J/g)	°C	ΔH (J/g)	°C	ΔH (J/g)	°C	ΔC_p (J/(g.°C))
Fish oil	-51 ± 1	43 ± 3	-15 ± 1	54 ± 5	-	-	-	-
HPMCAS	-	-	-	-	68 ± 1	53 ± 4	133 ± 2	0.11 ± 0.02
Product	-56 ± 1	4 ± 1	-14 ± 1	6 ± 1	67 ± 1	42 ± 2	124 ± 1	0.08 ± 0.01

The TGA results for HPMCAS and the encapsulated powder product are presented in **Fig. 6.6** and show that both the polymer and encapsulated powder product undergo the same three processes when heated from ambient temperature to 600°C with a total percentage weight loss in each sample of 96 ± 1 %. This demonstrates that no chemical changes of the starting materials occurred during the formulation. The 1st weight loss process (below 100°C) is a dehydration process in which 2.2 ± 0.1 % of moisture is removed for HPMCAS and 1.4 ± 0.1 % for the product, indicating lower moisture content in the encapsulated powder product. The 2nd weight loss (277 ± 2°C) is likely to be the removal of water molecules as a result of the cross-linking esterification reaction of the HPMCAS reported above 200°C (Li, et al., 2013). Following this process the samples decompose (3rd weight loss process). The peak temperature of this decomposition process is higher for the encapsulated powder product (372 ± 1°C) than the HPMCAS (362 ± 1°C). The higher decomposition temperature detected for the encapsulated powder product is possibly the result of the fish oil acting as a protective layer around the HPMCAS. As such greater amount of heat is required to reach the temperature at which the HPMCAS decomposes.

Thermal analysis results demonstrate that the starting materials did not undergo changes in their physical state and no chemical reaction(s) occurred during the formulation of the encapsulated powder. In addition, the increase in decomposition temperature observed for the encapsulated powder product suggests the fish oil forms a protective layer around the HPMCAS, indicating good encapsulation as inferred from the FTIR analysis (**Section 6.3.2.5**).

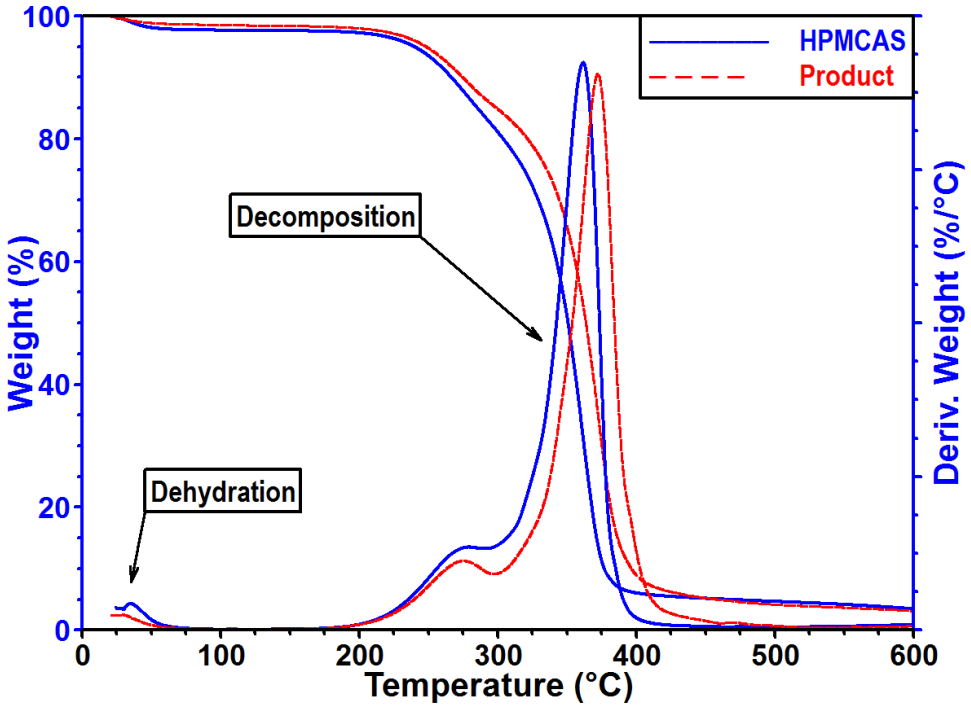

Figure 6.6 TGA curve overlay of HPMCAS and encapsulated powder product heated from ambient temperature to 600°C at 10°C/min.

6.3.2.7 DHA Stability Testing

For the stability tests, a comparison between the loss of DHA from the microencapsulated fish oil was assessed, along with the corresponding un-encapsulated fish oil over one week at 40°C. There was a 0.29 mg and 0.58 mg reduction in DHA in the microencapsulated fish oil and un-encapsulated fish oil respectively. This indicates that encapsulating the fish oil protects DHA against degradation. Further investigations into the oxidation, such as the primary and secondary oxidation products, of the microencapsulated product are warranted.

6.3.2.8 Dissolution

HPMCAS is an enteric coating polymer, meaning that it is insoluble in acidic gastric fluid but will dissolve in the small intestine, due to the pH change. The pH dependent dissolution of the encapsulated powder was evaluated in order to determine the controlled pH release of the encapsulated DHA. As expected there was no DHA release detected in the simulated gastric fluid (0.1 mol/L HCl) after 2 hours. However the encapsulated powder dissolved within 37 minutes in the simulated intestinal fluid (PBS pH 6.8) where 88% DHA release was detected when analyzed. These results indicate that the encapsulated powder will remain intact within

the food product and after ingestion through the stomach and release DHA in the intestine at the site of absorption.

6.4 Conclusions

A directly spray dried fish oil technique has proven beneficial as a production method over a spray dried nano-emulsion of fish oil in terms of DHA content, DHA encapsulation efficiency and solid yield %. The use of the encapsulated powder produced would offer protection against degradation as well as masking the odour and taste to offer palatability of the product. The encapsulated powder may therefore offer a source of DHA with the potential to be incorporated into infant foods to meet recommendations (Food and Agriculture Organisation, 2010). Further work into the storage stability and the effects of the incorporation of the encapsulated powder into the food product are required.

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Chapter 7

General Discussion

Growth and development are absolutely dependent on an adequate supply of energy and nutrients which are sufficient to match the variable needs of the infant as growth progresses, with any limitation in nutrient supply likely to constrain the pace and pattern of development (Scientific Advisory Committee on Nutrition, 2011). Observational studies have raised a cause for concern over later health consequences implicated by deficient or excessive nutrient intake during infancy (Caroli, et al., 2012). Pregnancy, lactation and infancy are specific times in life when essential nutrients are indispensable and where most requirements are different to any other life stages in relation to supporting rapid growth and development (Bryhn, 2006). Feeding surveys show that an increasing number of mothers feed their infants with industrially processed commercial infant foods and in recent years the baby food market and the range of products has grown significantly (Pandelova, et al., 2012).

There is currently no clear and complete analytical nutritional data available for ready to feed complementary infant foods in the UK. The nutritional database in the UK, McCance and Widdowson contains limited data on the composition of complementary infant foods, in addition the analytical techniques and nutrient data contained within the databases may now be outdated. Furthermore the European Food Safety Authority has highlighted that nutrient intake data after 6 months of age is insufficient in European countries (Caroli, et al., 2012).

Therefore as commercial weaning foods are increasingly being incorporated into an infant diet and nutritional databases lack nutrient content for these particular products, their nutritional adequacy for the rapidly developing infant needs to be assessed. Furthermore the effect of processing on nutrients in these foods also requires attention.

The aims were to evaluate the nutritional suitability of infant food products currently available on the UK market, according to the most up to date recommendations and recent relevant legislation and to explore the microencapsulation of essential fatty acids to optimise the nutrient content of ‘ready-to-feed’ infant food products.

New protocols were developed for the quantitative analysis of certain key nutrients including essential fatty acids, fat soluble vitamins (A, D and E) and essential elements (Ca, Cu, Fe, K, Mg, Na, P and Zn) in commercially prepared infant foods in the UK and estimated daily intakes based on consumption of these products were compared against current dietary recommendations for infants. The Ca:P ratio was also determined in a range of commercially prepared infant foods currently sold in the UK and compared to recommendations in relation to bone health. In addition the effects of commonly practiced re-heating treatments used by parents on essential fatty acids in manufactured infant formula milks were examined. Finally microencapsulation of essential fatty acids was explored in order to improve the nutritional quality of infant food products. The key highlights of each of the analysis (Chapters 3, 4, 5 and 6) are summarised below.

7.1 Highlights of the essential fatty acid analysis (Chapter 3)

The results of the analysis of essential fatty acids indicate that an infant's diet based on the standardized menu, composed of daily consumption of commercial 'ready-to-feed' infant foods and formula milk products does not meet recommendations for LCPUFA (DHA and AA). This provides opportunities and scope for product optimization to improve the nutritive value of commercial infant food products in relation to LCPUFA. DHA may be of more importance due to the differences in conversion as further steps are required for the biological synthesis of DHA, in addition the higher concentrations of LA (precursor of AA) were observed in the infants diet, therefore this may lead to higher concentrations of AA being formed in the body.

With respect to the precursor essential fatty acids (LA and ALA), the estimated total daily intake of LA was found to be higher than the recommendations, whereas the ALA total daily intake was found to be lower than recommendations set by the US; which increases the LA:ALA ratio. This increase may have some effect on allergy development; however, due to insufficient conversion of the essential fatty acid precursors (LA and ALA) to LCPUFA in infants it may not have a significant effect.

Furthermore the major contributor of essential fatty acids in the infant's diet has been identified as infant formula milk. This highlights an important issue in relation to situations where consumption of formula milk is comprised. In addition, the study highlights that the impact of commonly practiced re-heating treatments used by parents for the preparation of infant formula milks introduces significant changes in certain fatty acids (LA and ALA), and

therefore future assessment of the oxidation products following these methods of preparation is suggested.

Finally, in relation to the transparency of the nutritional information declared on the labels by the manufacturers, infant formula milks were all within the limits of EU regulations although there was a degree of significant variation between the quantitative values analyzed in this study and the declared values on the labels. It is also important to be aware that only certain brands of UK infant formulas add LCPUFA. This is important as conversion from essential fatty acid precursors (ALA and LA) to LCPUFA in infants are thought to be insufficient so they are required in their pre-formed sources to meet the requirements of the infant. Therefore parents need to be aware that if the formula that their infant receives does not fortify with LCPUFA the infant will need to receive adequate amounts of pre-formed sources from the foods that they consume, which have been observed to be low from commercial complementary infant foods tested in this study. This may lead to complementary infant foods needing to be fortified with LCPUFA, especially DHA to meet infant's recommendations for optimal development.

7.2 Highlights of the vitamin A and E analysis (Chapter 4)

The concentration of vitamin A (retinyl acetate, retinyl palmitate and β-carotene) and E (α and γ-tocopherol) in commercial 'ready to feed; infant foods was quantitatively determined by HPLC. The estimated total daily intake of vitamin A converted into RE from the consumption of a combination of commercial 'ready to feed' infant foods and infant formula, based on the standardised menu approach, was found to be in excess of the upper intake levels set for infants of 900 μg RE/day. It is worth mentioning here that contribution of vitamin A from infant formula alone was found to be 396 μg RE which exceeds the RNI of 350 μg RE for infant's aged 7-12 months by 13%. The remaining vitamin A content of the diet was mainly from carotenoids (β-carotene and total carotenoids) which are considered to be less harmful. However further studies into the bioavailability of the different forms of vitamin A in infants and the effect of excess intake is necessary. Pre-formed vitamin A food sources contributed 58.3% of the RNI, therefore if the contribution from milk intake is reduced, deficiency may become an issue and parents should be aware of the impact that the reduction in fortified infant formula use may have on the nutritional composition of an infant's diet.

The total daily intake of vitamin E based upon a diet consisting of complementary ready to feed infant foods and infant formula provided a vitamin E intake of 10.4 mg/day of α-TE, which is higher than the recommended adequate intakes of 5 mg/day α-tocopherol set by the US. However 81% of vitamin E was provided from infant formula with a minor contribution from infant food products, which may become a concern when milk intake is compromised or reduced as it is after the first year of life.

7.3 Highlights of the essential elemental and vitamin D analysis (Chapter 5)

This primary study investigated the ratio of Ca:P in conjunction with vitamin D and other essential elements (Cu, Fe, K, Mg, Na, Zn) in a range of commercial infant food products in the UK. In addition, as this study is the first to include consumption of infant snack products, the level of total calorie intake is also calculated in order to assess total daily energy intake in relation to obesity.

The Ca:P ratio of the infant's diet based on the standard feeding regime used in this study equates to 1.49:1, which is within the recommended range of 1.1 – 2:1 recommended by ESPGHAN. However the actual daily intakes of calcium and phosphorus were 176% and 155% above the RNI respectively. The implication of excess intake of micronutrients warrants further investigation for long term health effects.

This investigation is the first to our knowledge to include infant snack products. However when the snack products were removed from the total daily intake the majority of essential elemental intakes met recommendations, indicating that parents need to select snack options which do not exceed requirements.

The total dietary intake of vitamin D3 was determined to be 9.61 μg/day, which is 137% above the RNI. However 120% is contributed from fortified infant formula. As weaning foods are typically low in vitamin D unless they are fortified and breast milk concentrations are typically low, vitamin D deficiency may arise when infant formula consumption is reduced, which is the case after the first year of life.

Finally the estimated total energy intake, from consumption of the products tested herein, is estimated to contribute to a high calorie intake with a possible impact on obesity.

7.4 Highlights of the microencapsulation of fish oil (Chapter 6)

A directly spray dried fish oil technique has proven beneficial as a production method over a spray dried nano-emulsion of encapsulated fish oil. The encapsulated powders produced by the desired method offer a source of DHA that has the potential to be incorporated into infant foods to increase their dietary DHA consumption, which at this time is not meeting dietary recommendations as indicated in **Chapter 3**. Further work into the storage stability and the effects of the incorporation of the encapsulated powder into the food product are required. The use of the encapsulated powder produced would offer protection against degradation as well as masking the odour and taste to offer palatability of the product.

7.5 Implications of the research

Over recent decades the modern lifestyle dynamic has led to an increased parental reliance on commercially marketed complementary infant foods in the UK, which has been highlighted by the Diet and Nutrition Survey of Infants and Young Children. The current nutritional labelling formats for ready to eat complementary foods are a duplicate of the legislative requirements for manufacturing ready meals intended for the general population, the implication of this is that a number of important nutrients maybe limiting or excessive, which will affect their nutritional quality and suitability as an infant food. Furthermore nutritional databases, such as McCance and Widdowson provide limited data on the composition of these types of food products. The European Food Safety Authority has highlighted that nutrient intake data after six months of life is currently inadequate and insufficient and urgently needs to be addressed.

The nutritional evaluation of commercial infant foods undertaken herein has highlighted that these food products are not nutrient dense and that the main dietary contributor is the fortified infant formula. In situations where infant formula is reduced or compromised, these types of food products are unlikely to meet an infant's dietary requirements. This provides opportunities and scope for product optimisation to improve the nutritive value of infant food products. Furthermore it would be beneficial if commercially marketed infant foods provided nutritional information of micronutrients on the labelling of products to enable parents to be able to make informed choices about the foods they feed their infants. Food manufacturers may believe that this would be detrimental to profits. However providing food products that are nutrient dense may actually act as a selling point and be beneficial for sales. Due to infants having a small gastric capacity, it is essential that they are receiving nutrient dense

foods during the weaning stage to satisfy dietary requirements for optimal growth and development and prevent poor health in later life.

7.5 References

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Chapter 8

Overall conclusions and future work

The diet and nutrition survey of infants and young children highlights that at least 75% of boys over 7 months and 78% of girls are above the 50th percentile for weight compared against the UK WHO growth standards, which may in part be due to the overconsumption of macronutrients such as calories, fats and carbohydrates (Department of Health, 2013). However overconsumption of food does not necessarily mean that the diet is providing excess micronutrients (vitamins and minerals). Infant foods need to be micronutrient rich and nutrient dense (amount of nutrient/100 kcal of food) in order for the food to be of a high nutritional quality. Therefore infant foods must provide substantial amounts of micronutrients with relatively fewer calories; in comparison to empty calorie foods where micronutrient levels are low and could potentially lead to deficiencies of certain micronutrients. High nutritional quality products are necessary to provide the infant with the appropriate levels of macro and micronutrients for optimal development.

The study conducted so far indicates that total daily intakes of vitamin A and E exceed recommendations. It is, however, important to note that fortified infant formulas were found to be the major contributor. The aforementioned highlights the importance of nutrient dense foods in situations where consumption of infant formula/breast milk is reduced or compromised. On the other hand, over consumption of certain nutrients, including vitamin A and E, during the first year of life should also be an issue for concern, as currently there is not enough attention paid to the impact of over intake of these particular nutrients following consumption of fortified formula.

The investigation into the essential elemental content of dairy based commercial infant food products also highlighted that total daily intakes exceed recommendations. This investigation is the first to our knowledge to include infant snack products. However, when the snack products were removed from the total daily intake the majority of intakes met recommendations. In addition the calorie contribution from these products also exceeded recommendations, indicating that parents need to select snack options which do not exceed requirements, as this may have implications for obesity. Similarly vitamin D intakes exceeded recommendations and infant formula was identified as the main contributor, which may lead to deficiency issues when infant formula is reduced or compromised. According to

the results from the most recent National Diet and Nutrition Survey there is evidence for an increased risk of vitamin D deficiency in all age and sex groups. In addition to 27% of preschool children aged between 1.5-3 years mean dietary intakes of vitamin D being below the RNI (Public Health England, 2014).

The essential fatty acid content of the infant's diet highlighted that pre-formed LCPUFA DHA and AA are below recommendations set by the US. DHA and AA are important for visual and brain development with numerous other potential health advantages being suggested. DHA may be of more significance as the metabolism of fatty acids requires further steps in the endogenous conversion to DHA in comparison to AA. In addition the high intake of LA in the diet means that AA may be produced endogenously within the body from its precursor (LA). Therefore the microencapsulation of fish oil was explored as a potential source of DHA that could be added to complementary infant foods to improve their nutritional quality.

There is scope for further investigations into the following areas:

8.1 Future work in relation to the current infant food market

a) Assessment of oxidation products and isomers of fatty acids after i) home processing methods used by parents to re-heat infant formula and 'ready to feed' infant foods, ii) industrial processing methods for the production of infant formula and 'ready to feed' infant foods. This information would be useful for future product development to implement the least detrimental conditions during production.

b) Investigate the effects of heating on other heat sensitive nutrients such as vitamin C, thiamine (B1), pantothenic acid (B5), pyridoxine (B6) and folate (B9) in infant foods.

c) Nutritional assessment of commercial 'ready to feed' infant food products and infant formula milk over the shelf life duration.

d) Complete assessment of non-mandatory labelled nutrients in a wide range of infant food products, including infant breakfast, snack and meal products.

e) Future studies are warranted with focus on the bioavailability of excess nutrient intakes in infants.

8.2 Future work in relation to new product development

a) Dissolution profiles of the pH dependent release of DHA from the microencapsulated fish oil.

b) Assessment of the stability of DHA in the microencapsulated fish oil over typical shelf life duration and conditions.

b) Analysis of the microencapsulated fish oil incorporated into an infant food product, i) influence on other nutrients within the food matrix, ii) effects of food processing on the DHA content and iii) stability of DHA within the food product during shelf life.

d) Analysis of the oxidation products produced during degradation of DHA from the microencapsulated fish oil.

e) Investigation into the types of packaging, optimal processing and storage conditions of a food product fortified with the microencapsulated fish oil.

f) Assessment of the nutritional profile of the microencapsulated fish oil.

g) Examine the issue of palatability and the organoleptic properties of the fortified infant food product.

g) Assess the bioavailability of DHA after ingestion of a food product fortified with the microencapsulated fish oil.

h) Future work into the production of new infant food products, including selection of ingredients, processing conditions and packaging. To offer an infant food that is nutritionally superior.

8.3 References

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Addendum

Conferences/ Seminars

1. Nutrition and Nurture in Infancy and Childhood Conference, 10-13 June 2013. The impact of early infant feeding on the development of atopic disease (Grange over Sands, UK)

2. Faculty Research Symposium, 13 June 2013. The impact of early infant feeding on the development of atopic disease (UoG, Medway Campus).

3. 3rd International Conference and Exhibition on Food Processing and Technology, 21-23 July 2014. Determination of essential polyunsaturated fatty acids in complementary infant foods in the UK by RP-HPLC-CAD (Las Vegas, USA).

4. 3rd International Conference and Exhibition on Food Processing and Technology, 21-23 July 2014. Impact of heating on fatty acid content of UK infant formula milk products by HPLC-CAD (Las Vegas, USA).

5. Research Seminars, 18 March 2015. Fatty acid content of complementary infant foods in the UK and the impact of re-heating on the fatty acid content of infant formula using HPLC-CAD – A novel approach (UoG, Medway Campus).

6. 5th Euro-Global Summit and Expo on Food and Beverages, 16-18 June 2015. Improving DHA content of infant foods in the UK (Alicante, Spain).

7. Faculty Research Symposium, 16 June 2015. Microencapsulation of docosahexaenoic acid (DHA) for the potential use in ready to feed infant foods (UoG, Medway Campus).

Research Publications/Manuscripts

1. Loughrill, E., & Zand, N. (2016). An investigation into the fatty acid content of selected fish-based commercial infant foods in the UK and the impact of commonly practiced re-heating treatments used by parents for the preparation of infant formula milks. *Food Chemistry*, 197, 783-789.

2. Emma Loughrill, Pesila Govinden and Nazanin Zand (2016). Vitamin A and E content of commercial infant foods in the UK: a cause for concern? *Food Chemistry* (In Press; accepted for publication, FOODCHEM-D-15-01457).

3. Emma Loughrill and Nazanin Zand (2016). Calcium to Phosphorus ratio and vitamin D content of infant foods in the UK: possible implications for bone health. *British Journal of Nutrition* (Under review, BJN-RA-15-0822).

4. Emma Loughrill, Sharon Thompson, Dennis Douromis, Samuel Owusu-Ware, Martin J. Snowden and Nazanin Zand (2016). Microencapsulation of docosahexaenoic acid (DHA) for the potential use in ready to feed infant foods. *Food Research International* (Submitted, FOODRES-D-15-02694).

Book Chapters/Review Articles

1. Miguel de la Guardia and Salvador Garrigues (2015). *Handbook of Mineral Elements in Food.* John Wiley and Sons.

2. Emma Loughrill, Laurence Harbige, Babur Z. Chowdhry and Nazanin Zand (2016). Dietary and Nutritional Factors in the Development of Allergy in Infants and Children. *Comprehensive Reviews in Food Science and Food Safety (to be submitted)*.