# APPLICATION OF NEAR INFRARED SPECTROSCOPY AND CHEMOMETRICS FOR THE ANALYSIS OF NUTRACEUTICALS IN SOUTH AFRICA

**by**

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# DECLARATION

I, Sanette van der Merwe, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

**Signed** **Date**

# ABSTRACT

The high demand of omega-3 fish oil nutraceuticals (dietary supplements) is due to the numerous health benefits contributed by the polyunsaturated fatty acids (PUFA's). The nutraceutical industry is required to follow good manufacturing practice (GMP) standards in order to ensure label claims and prevent adulteration. It is vital that the quality control (QC) procedures will be able to detect adulterated products. It is thus necessary to ensure the analytical techniques are adequate by using validated methods. The qualification or identification of natural fish oils is a difficult task due to overlapping concentration ranges of PUFA's and other similar properties. Gas chromatography (GC) is the prescribed technique in the nutraceutical industry for analysis of omega-3 fatty acids, but it is time-consuming and costly. Near infrared (NIR) spectroscopy is a rapid and cost-effective technique that can replace the prescribed method if proven equivalent, through validation according to pharmaceutical criteria. In this study, NIR spectroscopy in combination with chemometrics was considered as an alternative method to GC, to identify various commercial fish oils and to quantify the PUFA's. Identification methods were developed for nine commercial omega-3 fish oils using spectral libraries. Quantitative NIR methods were developed for arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in fish oils expressed as mg.g$^{-1}$ as well as percentage (%) area using partial least squares (PLS) regression and independent validation by superimposing datasets with mutual properties. Based on the statistics in terms of SEC, $R^2$, SEP and r of the PUFA models, the NIR method was equivalent to the prescribed GC methods and precision results obtained were within the prescribed criteria. NIR spectroscopy and chemometrics can be used for conclusive identification and quantification of omega-3 fish oils, thereby minimizing the risk of adulteration. The method also complied with the prescribed pharmaceutical method validation criteria; therefore, was demonstrated as an alternative method to GC for the nutraceutical industry.

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# DEDICATION

For Rifka (Rita) Borkum

# GLOSSARY

| **Abbreviations** | **Definition** |
|---|---|
| AA | Arachidonic acid |
| ALA | α-Linolenic acid |
| AOTF | Acousto-optical tunable filter |
| API | Active pharmaceutical ingredient |
| BP | British Pharmacopoeia |
| BCI | Brain computer interfaces |
| CAM’s | Complementary and alternative medicine |
| CCD | Charged coupled devices |
| CDC | Centre for Disease Control |
| CGMP | Current good manufacturing practice |
| CLA | Conjugated linoleic acid |
| CMA | Complementary Medicines Australia |
| CV | Cross validation |
| DHA | Docosahexaenoic acid |
| DOH | Department of Health of South Africa |
| DSC | Differential scanning calorimetry |
| DSHEA | Dietary Supplement Health and Education Act |
| ECA | European Compliance Academy |
| EE | Ethyl ester |
| EEG | Electroencephalography |
| ELISA | Enzyme-linked immunosorbent assay |
| *EMA* | *Economic motivated adulteration* |
| EMA | European Medicines Agency |
| EP | European Pharmacopoeia |
| *EPA* | *Environmental Protection Agency* |
| EPA | Eicosapentaenoic acid |

| | |
|---|---|
| FDA | Food and Drug Administration |
| FFA | Free fatty acids |
| FFC | Functional Food Centre |
| FID | Flame ionisation detector |
| fNIRS | Functional near-infrared spectroscopy |
| FT | Fourier transform |
| GC | Gas chromatography |
| GC-MS | Gas chromatography coupled with mass spectroscopy |
| GMP | Good manufacturing practice |
| GOED | Global Organization for EPA and DHA Omega-3's |
| HPLC | High performance liquid chromatography |
| HPTLC | High performance thin layer chromatography |
| HSI | Hyperspectral imaging |
| ICH | International Conference on Harmonisation |
| IFT | Institute of Food Technologists |
| InGaAs | Indium gallium arsenide |
| ISO | International Organization for Standardization |
| LA | Linoleic acid |
| LC-MS | Liquid chromatography coupled with mass spectroscopy |
| LDA | Linear discriminant analysis |
| LED | Light emitting diode |
| MCC | Medicine Control Council |
| MI | Mutual information |
| MIR | Mid-infrared |
| MLR | Multilinear regression |
| MMI | Multivariate mutual information |
| MSC | Multiplicative scatter correction |
| MUFA | Monounsaturated fatty acid |
| MVA | Multivariate analysis |

| | |
|---|---|
| NCHS | National Centre for Health Statistics |
| NIR | Near infrared |
| NIRS | Near infrared spectroscopy |
| NMR | Nuclear magnetic resonance |
| OA | Oleic acid |
| PASG | Pharmaceutical Analytical Sciences Group |
| PAT | Process analytical technology |
| PbS | Lead sulphide |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| PDMS | Polydimethylsiloxane |
| PIC/S | Pharmaceutical Inspection Co-operation Scheme |
| PLS | Partial least squares |
| PLS-DA | Partial least squares discriminant analysis |
| PRESS | Prediction residual error sum of squares |
| PS | Polysaccharides |
| PSSA | Pharmaceutical Society of South Africa |
| PUFA’s | Polyunsaturated fatty acids |
| QC | Quality control |
| QMS | Quality management system |
| r | Correlation coefficient |
| $R^2$ | Coefficient of determination or multiple correlation coefficient |
| RMSEC (SEC) | Root mean square error of calibration (Standard error of calibration) |
| RMSECV (SECV) | Root mean square error of cross validation (Standard error of cross validation) |
| RMSEP (SEP) | Root mean square error of prediction (Standard error of prediction) |
| RTRT | Real time release testing |
| SA | South Africa |
| SEL | Standard error of the laboratory |

| | |
|---|---|
| S-G | Savitzky-Golay |
| SGC | Soft-gel capsules |
| SIMCA | Soft independent modelling of class analogy |
| SNV | Standard normal variate |
| TG | Triglyceride |
| TLC | Thin layer chromatography |
| USA | United States of America |
| USP | United States Pharmacopoeia |
| USPC | United States Pharmacopeial Convention |
| UV-Vis | Ultra violet-visible |

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# CHAPTER ONE
# INTRODUCTION

## 1.1 The nutraceutical industry

The nutraceutical industry is a competitive environment with a wide range of products available on the market. These products, known as dietary or nutritional supplements (Fig. 1.1), contain different combinations of nutritional ingredients. These ingredients may be vitamins, minerals, herbal extracts, plant oils, animal and fish oil extracts or other food supplements such as amino acids, and are collectively referred to as nutraceuticals (Andlauer & Fürst, 2002).

**Figure 1.1: An assortment of dietary supplement capsules (Hennessy, 2014)**

According to analysis of the 2012 *National Health Interview Survey* by the Centre for Disease Control's (CDC's) National Centre for Health Statistics (NCHS), 17.9 % of United States (US) adults used dietary supplements as a complementary health approach (Adams *et al*., 2013). This was double any other complementary health approach e.g. chiropractic and osteopathic manipulation (8.5 %), yoga (8.4 %) and massage therapy (6.8 %), (Hennessy, 2014).

In 2018, *Insight Survey* published a report on the vitamins and supplements landscape of South Africa (SA) in comparison with the global market. The global nutraceutical market was valued at US$ 91.5 billion in 2015 and estimated to grow at an annual rate of 4.71 % up to 2020. The SA nutraceutical market value increased from

R2.9 billion in 2014 to R3.8 billion in 2016 and has grown by annual growth rate of 13.5 % (Insight Survey, 2018).

## 1.2 Polyunsaturated fatty acids in fish oils

Due to the increasing scientific evidence of omega-3 fish oil supplements supporting health issues such as inflammation, heart disease and mental health, the demand in the nutraceutical industry has grown rapidly over recent years. The health benefits of omega-3 fish oils are due to the presence of polyunsaturated fatty acids (PUFA's), especially eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA). (Swanson *et al*., 2012; Liu *et al*., 2013; Hamed *et al*., 2015; Sprague *et al*., 2017).

A survey conducted by the Global Organization for EPA and DHA Omega-3's (GOED) showed that worldwide shipments of omega-3 oil containing EPA and DHA were estimated at 1.25 million metric tons in 2014 (Sprague *et al*., 2016). This high demand of omega-3 fish oil supplements caused numerous products to enter the market, increasing the risk of adulteration (Levin, 2016).

## 1.3 Quality assurance and regulation

Currently, nutraceuticals are regulated similar to pharmaceuticals. Raw material suppliers as well as manufacturers of nutraceuticals are required to follow good manufacturing practice (GMP) standards (United States of America, 2007; South Africa, 2016; South Africa, 2017; PIC/S, 2018). Various products have entered the global and South African market due to the increasing demand of dietary supplements (Gabriels *et al.*, 2012). Not all of these products are produced according to GMP standards and label claims cannot be guaranteed (Hamilton *et al*., 2010; Opperman & Benade, 2011; Ismail *et al.*, 2016), therefore are at risk of adulteration, which may occur in a number of different ways.

Adulteration is the presence of undeclared ingredients that could have an adverse impact on the quality and safety of the product (CMA, 2012). Diluting or substituting a high quality ingredient with an inferior one for economic gain is called economic adulteration (Levin, 2016). The intentional neglect of quality control (QC) tests on materials and products to save costs, are also classified as economic adulteration. Thus, inadequate QC or the use of non-validated methods renders an adulterated product (United States of America, 2016; ECA, 2018).

A study published by *South African Journal of Clinical Nutrition* in 2012 indicated that the SA nutraceutical industry was not appropriately regulated; product claims were not always accurate and could be susceptible to adulteration. A 2004 survey detected that 12 out of 30 (40 %) over-the-counter nutritional supplements contained stimulants such as ephedrine. The study emphasised the importance of screen testing supplements used by athletes for contaminants and adulterants, in particular, steroids and stimulants (Gabriels *et al*., 2012).

In November 2013, the Minister of the Department of Health (DOH) of South Africa published amended regulations to the Medicines and Related Substances Act 101 of 1965 to address regulation of complementary and alternative medicines (CAM's), (South Africa, 2013). The Medicine Control Council (MCC), the regulating body in South Africa, ensures the efficacy, quality and effectiveness of medicines through clinical research, has introduced strategies to address the regulation of complementary and alternative medicines (CAM's), (PSSA, 2014). The changes proposed stricter procedures for registering CAM's, similar to that required for prescription pharmaceutical drugs, and set maximum nutrient levels for vitamin and dietary supplements (South Africa, 2014).

For nutraceutical companies to be competitive it is imperative that the manufacturing is done according to local and international manufacturing standards to provide good quality products to their customers (Ritter *et al*., 2013). A poorly regulated nutraceutical market places great pressure on companies to stay competitive; thus, manufacturing according to international quality standards relate to products that are more expensive. Therefore, the challenge is to manufacture products cost-effectively without compromising product quality.

## 1.4 Quality Control

A quality management system (QMS) (Haleem *et al*., 2015) must be in place to ensure product quality and prevent contamination or adulteration of the product. This QMS prescribes the criteria when and how to perform QC checks on raw material, in-process and finished product samples and are usually performed by the QC laboratory (South Africa, 2017). QC of nutraceuticals ensure that the correct raw materials and grades are used by performing prescribed identification and purity analysis and will also confirm that the in-process and finished products have the correct formulation. Samples are subjected to time-consuming QC analysis that makes use of sophisticated and costly analytical methods such as high-performance liquid chromatography (HPLC) and gas chromatography (GC), (Skoog *et al*., 2004).

These separation techniques identify specific markers and not the material as a whole; therefore, adulteration with other materials can be easily overlooked (Govindaraghavan, 2014; Marieschi *et al*., 2016). Non-destructive techniques such as Fourier transform infrared (FT-IR) spectroscopy have been reported as a screening method for adulterants in nutraceuticals and is a more comprehensive approach by analysing the sample "as is" (Lohumi *et al*., 2015).

In the pharmaceutical and nutraceutical industry GC is the prescribed technique for analysis of omega-3 PUFA’s. (BP, 2011*a*; USP, 2013*a*). It is a time-consuming and costly procedure that involves extensive sample preparation. Expensive certified reference standards, instrument maintenance and solvent waste contribute to the costs. The GC method can be substituted by an alternative method if the performance is proved equivalent to that of the prescribed methods (BP, 2011*a*; USP, 2013*a*; GOED, 2015).

The qualification of natural fish oils is challenging and increases the risk of adulteration due to overlapping concentration ranges of PUFA’s and other similar physical and chemical properties which cannot be used to discriminate between oils from different fish species (Cozzolino *et al*., 2005). Therefore, quantification only is insufficient to eliminate adulteration; and identification to authenticate the oil is essential.

Fish oils are available in different forms and occur naturally in the triglyceride (TG) form, but can be converted to the ethyl ester (EE) form during molecular distillation. This process separates, concentrates and purifies PUFA’s such as omega-3 fatty acids in fish oil (Cermak *et al*., 2012). The QC method must be able to discriminate between the relevant fish oil species, quantify the relevant PUFA’s and distinguish between TG and EE forms to detect adulteration in fish oils.

## 1.5 Near infrared spectroscopy

Near infrared (NIR) spectroscopy is a vibrational spectroscopy technique similar to FT-IR and Raman spectroscopy (Larkin, 2017). It is a rapid and cost-effective technique and can replace traditional analysis methods when efficiently calibrated and validated. NIR spectroscopy is a non-destructive technique and requires very little or no sample preparation (Burns & Ciurczak, 2001).

Although applications range from agriculture, pharmaceutical, food science, medical to forensics (Workman, 2014), this study will focus on the pharmaceutical requirements placed on the nutraceutical industry. Applying NIR spectroscopy in combination with chemometrics, calibration models can be developed for the use of qualitative and quantitative applications (Blanco *et al*., 1998). Further applications are discussed in *Chapter 2.4*.

Chemometrics is defined as “*the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods*” (Hibbert *et al*., 2009). Chemometric models can be applied in the laboratory for routine analysis or in the factory for process control (Berntsson *et al.*, 1997; Huang *et al*., 2008).

Vibrational spectroscopy has been investigated in various studies as a non-destructive method for the analysis of oils. FT-IR in combination with chemometrics was used as a method to classify different vegetable oils (Javidnia *et al*., 2013) and detect adulteration of dietary supplement oils (Ozen *et al*., 2003), as well as characterization of dietary omega-3 fish oil supplements (Plans *et al*., 2015). Portable FT-IR devices were also reported as a rapid screening method to verify label claims of marine oil omega-3 dietary supplements (Karunathilaka *et al*., 2016). Dispersive and FT-NIR techniques were investigated and both were found valuable for the quantification of omega-3 PUFA's in fishmeal and fish oils (Cozzolino *et al*., 2005; Azizian *et al*., 2010; Zhang *et al*., 2013; Cascant *et al*., 2018). FT-Raman was also found to be a useful technique for quantification of PUFA's as well as detection of ethyl esters and oxidation in fish oil capsules (Killeen *et al*., 2017). Other non-destructive spectroscopic techniques, such as nuclear magnetic resonance (NMR) spectroscopy and hyperspectral imaging, were also considered for the analysis of PUFA's in fish and fish products (Xu *et al*., 2015).

## 1.6 Method equivalence

In 2009, the United States Pharmacopeial Convention (USPC) published a *Stimilu* article which stated that *the key to successful implementation of an alternative method in the pharmaceutical industry* (hence nutraceutical industry) *is to prove by comparison that it is either acceptable, equivalent or better than the prescribed compendial method* (Hauck *et al*., 2009). In this study, dispersive NIR spectroscopy in combination with chemometrics was investigated as an alternative method to GC to detect adulteration by the identification of various commercial fish oils in the TG form and quantification of the PUFA's.

In NIR spectroscopy methods the standard error of the laboratory (SEL) is carried over from the reference method to the NIR spectroscopy method, therefore, cannot be *better,* but rather *equivalent* to the reference method (Burns & Ciurczak, 2001). The purpose for this paper was to determine if there were a substantial difference between the alternative and reference method. Therefore, demonstrate *equivalence* through validation to meet pharmaceutical requirements.

## 1.7 Pharmaceutical validation

Guidelines to assist industry to comply with regulatory requirements on method development and validation for NIR spectroscopy were published by the United States Pharmacopoeia (USP, 2013*b*), British Pharmacopoeia (BP, 2011*b*), Pharmaceutical Analytical Sciences Group (PASG, 2002), the European Medicines Agency (EMA, 2014) and the International Conference on Harmonisation (ICH, 2005).

Validation criteria include specificity and robustness for qualitative NIR spectroscopy methods, whereas specificity, linearity, accuracy, precision, range, robustness, and outliers are included for quantitative NIR methods. Pharmaceutical validation requirements are summarised in *Table 3.1 in Chapter 3*.

Due to constant changes in the consumer and regulatory environments (Bannenberg *et al*., 2017), it was necessary to adapt the GC method from an USP (USP, 2013*a*) to a BP (BP, 2011*a*) method to meet requirements. Thus, the challenge was to develop NIR spectroscopy methods validated to pharmaceutical standards by merging the spectra from natural TG and concentrated TG oils to quantify the PUFA's expressed as percentage (%) area and mg.g$^{-1}$.

## 1.8 Multivariate mutual information

The main objective of this study was to superimpose data from two different GC methods applied in the context of multivariate mutual information (MMI) as illustrated in the information diagram (Fig. 1.2).

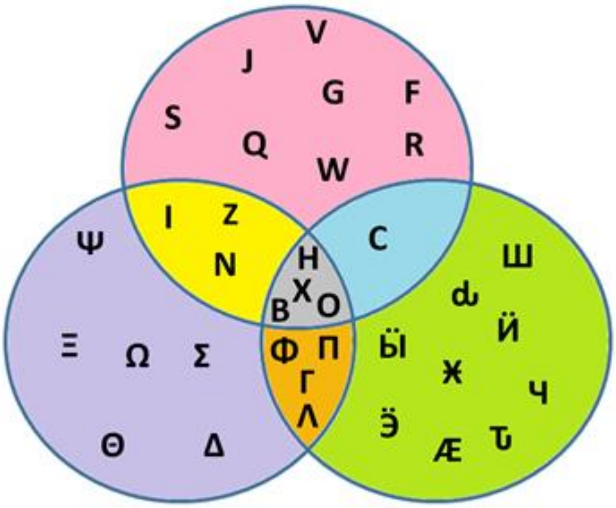


**Figure 1.2: A Venn diagram of information theoretic measures for three variables. The uppercase letter glyphs are shared by the Greek, Latin and Cyrillic alphabets and represents the MMI**

In probability and information theory, the mutual information (MI) of two random variables is a measure of the mutual dependence between the two variables (Srinivasa, 2005). Whereas, multivariate mutual information (MMI) is a measure of the mutual dependence between *more than two* variables and can be illustrated in the context of information diagrams such as a Venn diagram (Venn, 1880) that shows all possible logical relations between a finite collection of different sets. The spectral and reference data sets were viewed in the same context, displaying mutual properties that can be applied to construct predictive models.

## 1.9 Problem statement

QC analysis is vital to manufacturing quality products and building consumer confidence, but is a time-consuming and costly process. GC maintenance costs are high; leading to a high cost of analysis; therefore, the investigation into the use of a more cost-effective and environmentally friendly technique such as NIR spectroscopy is justified. Minimizing QC analysis time can also improve the efficiency of the production process; thus, the application of NIR spectroscopy will contribute to the cost-effectiveness of the manufacturing of nutraceutical products containing omega-3 PUFA’s. Before implementing NIR spectroscopy as an alternative method to GC for the analysis of nutraceuticals such as omega-3 fish oils, the NIR method must demonstrate equivalence through method validation according to pharmaceutical requirements. The NIR method must be able to evolve with the changes in the prescribed reference method to avoid having to initiate a new spectroscopic calibration. The challenge was to develop and validate NIR spectroscopy models according to pharmaceutical criteria by superimposing the spectral and reference data from natural TG and concentrated TG oils to quantify the PUFA’s expressed as % area and $mg.g^{-1}$ applied in the context of MMI.

## 1.10 Research objectives

The aim of the research is to investigate by what means NIR spectroscopy and chemometrics can be applied to the QC of nutraceuticals, specifically omega-3 polyunsaturated fatty acids (n-3 PUFA’s), considering the following research objectives:

1. Implement NIR spectroscopy as a more cost-effective, environmentally friendly and timesaving technique compared to GC for the quality control of nutraceuticals.
2. Demonstrate spectroscopic calibration of qualitative (spectral libraries) and quantitative models for the QC of nutraceuticals to minimise the risk of adulteration.
3. Establish if the NIR spectroscopy method is equivalent to GC through validation to meet the prescribed pharmaceutical criteria.
4. Combine reference data from different methods to extend the calibration range
5. Superimpose the spectra from natural TG and concentrated TG oils to quantify the PUFA’s expressed as % area and $mg.g^{-1}$ applied in the context of MMI.

## 1.11 References

Adams, P.F., Kirzinger, W.K. & Martinez, M.E., 2013. Summary health statistics for the U.S. population: National Health Interview Survey. National Centre for Health Statistics (NCHS). *Vital Health Statistics*, 10(259). https://www.cdc.gov/nchs/data/series/sr_10/sr10_259.pdf [07 June 2018].

Andlauer, W. & Fürst, P., 2002. Nutraceuticals: a piece of history, present status and outlook. *Food Research International*, *35*(2):171-176.

Azizian, H., Kramer, J.K., Ehler, S. & Curtis, J.M., 2010. Rapid quantitation of fish oil fatty acids and their ethyl esters by FT-NIR models. *European Journal of Lipid Science and Technology*, *112*(4):452-462.

Bannenberg, G., Mallon, C., Edwards, H., Yeadon, D., Yan, K., Johnson, H. & Ismail, A., 2017. Omega-3 long-chain polyunsaturated fatty acid content and oxidation state of fish oil supplements in New Zealand. *Scientific Reports*, *7*(1488):1-13.

Berntsson, O., Zackrisson, G. & Östling, G., 1997. Determination of moisture in hard gelatin capsules using near-infrared spectroscopy: applications to at-line process control of pharmaceutics. *Journal of Pharmaceutical and Biomedical Analysis*, 15(7):895-900.

Blanco, M., Coello, J., Iturriaga, H., Maspoch, S. & de la Pezuela, C., 1998, Near-infrared spectroscopy in the pharmaceutical industry, Critical Review, *The Analyst*, 123:125R-150R.

BP *see* British Pharmacopoeia

British Pharmacopoeia Commission, 2011*a*. Composition of fatty acids, Appendix XP1. In *BP 2012*, Great Britain: Stationery Office: A301.

British Pharmacopoeia Commission, 2011*b.* Infrared spectrophotometry, Appendix II A. In *BP 2012*, Great Britain: Stationery Office: A156.

Burns, D.A., & Ciurczak, E.W. (eds) 2001. *Handbook of Near-Infrared Analysis,* (2nd ed). New York: Marcel & Dekker.

Cascant, M.M., Breil, C., Fabiano-Tixier, A.S., Chemat, F., Garrigues, S. & de la Guardia, M., 2018. Determination of fatty acids and lipid classes in salmon oil by near infrared spectroscopy. *Food Chemistry*, 239:865-871.

Cermak, S. C., Evangelista, R. L. & Kenar, J. A., 2012. Distillation of natural fatty acids and their chemical derivatives. In *Distillation - Advances from Modelling to Applications: InTech,* 5:109-140.

CMA *see* Complementary Medicines Australia (formerly the Complementary Health Council of Australia)

Complementary Medicines Australia, 2012. *Guideline for the quality and safety of raw materials used in complementary medicines*, ver. 1. http://www.cmaustralia.org.au/Resources/Documents/Codes%20and%20Guidelines/The%20CHC%20Raw%20Material%20Guidelines%20.pdf [01 December 2017].

Cozzolino, D., Chree, A., Scaife, J.R. & Murray, I., 2005. Usefulness of near-infrared reflectance (NIR) spectroscopy and chemometrics to discriminate fishmeal batches made with different fish species. *Journal of Agricultural and Food Chemistry*, *53(*11):4459-4463.

Cozzolino, D., Murray, A.I., Chree, A. & Scaife, J.R., 2005. Multivariate determination of free fatty acids and moisture in fish oils by partial least-squares regression and near-infrared spectroscopy. *LWT - Food Science and Technology*, *38*(8):821-828.

ECA *see* European Compliance Academy

EMA *see* European Medicines Agency

European Compliance Academy, 2018. *Warning letter due to non-validated Analytical Methods*. https://www.gmp-compliance.org/gmp-news/warning-letter-due-to-non-validated-analytical-methods [16 May 2018].

European Medicines Agency (EMA), 2014. *Guideline on the use of near infrared spectroscopy by the pharmaceutical industry and the data requirements for new submissions and variations*. http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000764.jsp&mid= [01 December 2017].

FDA *see* United States of America Food and Drug Administration

Gabriels, G., Lambert, M. & Smith, P., 2012. Information on nutritional supplement labels: time for legislation? *South African Journal of Clinical Nutrition*, 25(1):22-26.

Global Organization for EPA and DHA Omega-3 (GOED), 2015. *Voluntary Monograph*, ver.5. http://www.goedomega3.com/index.php/files/download/350 [01 December 2017].

GOED *see* Global Organization for EPA and DHA Omega-3

Govindaraghavan, S., 2014. Pharmacopeial HPLC identification methods are not sufficient to detect adulterations in commercial bilberry (*Vaccinium myrtillus*) extracts. Anthocyanin profile provides additional clues. *Fitoterapia*, *99*:124-138.

Haleem, R.M., Salem, M.Y., Fatahallah, F.A. & Abdelfattah, L.E., 2015. Quality in the pharmaceutical industry - A literature review. *Saudi Pharmaceutical Journal*, 23(5):463-469.

Hamed, I., Özogul, F., Özogul, Y. & Regenstein, J. M., 2015. Marine bioactive compounds and their health benefits: a review. *Comprehensive Reviews in Food Science and Food Safety*, 14(4):446-465.

Hamilton, K., Brooks, P., Holmes, M., Cunningham, J. & Russell, F.D., 2010. Evaluation of the composition of omega-3 fatty acids in dietary oil supplements. *Nutrition and Dietetics*, 67(3):182-189.

Hauck, W.W., De Stefano, A.J., Cecil, T.L., Abernethy, D.R., Koch, W.F. & Williams, R.L., 2009. Stimuli to Revision Process: Acceptable, equivalent, or better - Approaches for alternatives to official compendial procedures. *United States Pharmacopeial Forum*, 35(3):772-778.

Hennessy, M., 2014. *Supplements are most popular complementary health approach: Survey*. https://www.nutraingredients-usa.com/Article/2014/04/23/Supplements-are-most-popular-complementary-health-approach-Survey [07 June 2018].

Hibbert, D.B., Minkkinen, P., Faber, N.M. & Wise, B.M., 2009. IUPAC project: A glossary of concepts and terms in chemometrics. *Analytica Chimica Acta*, 642(1-2):3-5.

Huang, H., Yu, H., Xu, H. & Ying, Y., 2008. Near infrared spectroscopy for on/in-line monitoring of quality in foods and beverages: A review. *Journal of Food Engineering*, 87(3):303-313.

ICH *see* International Conference on Harmonisation

Insight Survey, 2018. *South African Vitamins and Supplements Industry Landscape Report 2018*. http://www.insightsurvey.co.za/2017-south-african-vitamins-and-supplements-landscape-report [07 June 2018].

International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use, 2005. Validation of analytical procedures: text and methodology, Q2 (R1), *ICH Harmonised Tripartite Guideline*. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf [14 April 2018].

Ismail, A., Bannenberg, G., Rice, H.B., Schutt, E. & MacKay, D., 2016. Oxidation in EPA- and DHA-rich oils: an overview. *Lipid Technology*, 28(3-4):55-59.

Javidnia, K., Parish, M., Karimi, S. & Hemmateenejad, B., 2013. Discrimination of edible oils and fats by combination of multivariate pattern recognition and FT-IR spectroscopy: A comparative study between different modeling methods. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 104:175-181.

Karunathilaka, S.R., Mossoba, M.M., Chung, J.K., Haile, E.A. & Srigley, C.T., 2016. Rapid prediction of fatty acid content in marine oil omega-3 dietary supplements using a portable Fourier Transform infrared (FTIR) device and partial least-squares regression (PLSR) analysis. *Journal of Agricultural and Food Chemistry*, 65(1):224-233.

Killeen, D.P., Marshall, S.N., Burgess, E.J., Gordon, K.C. & Perry, N.B., 2017. Raman spectroscopy of fish oil capsules: Polyunsaturated fatty acid quantitation plus detection of ethyl esters and oxidation. *Journal of Agricultural and Food Chemistry*, 65(17):3551-3558.

Larkin, P., 2017. *Infrared and Raman spectroscopy: Principles and spectral interpretation*. Waltham, MA: Elsevier.

Levin, M.D., 2016. The new food current good manufacturing practices and their effect on dietary supplement quality: What you need to know. *Integrative Medicine: A Clinician's Journal*, 15(5):22-24.

Liu, J. J., Galfalvy, H. C., Cooper, T. B., Oquendo, M. A., Grunebaum, M. F., Mann, J. J. & Sublette, M. E., 2013. Omega-3 polyunsaturated fatty acid status in major depression with comorbid anxiety disorders. *The Journal of Clinical Psychiatry*, 74(7):732-738.

Lohumi, S., Lee, S., Lee, H. & Cho, B.K., 2015. A review of vibrational spectroscopic techniques for the detection of food authenticity and adulteration. *Trends in Food Science and Technology*, 46(1):85-98.

Marieschi, M., Torelli, A., Beghé, D. & Bruni, R., 2016. Authentication of *Punica granatum L.*: Development of SCAR markers for the detection of 10 fruits potentially used in economically motivated adulteration. *Food Chemistry*, 202:438-444.

Near-infrared spectroscopy in the pharmaceutical industry, Critical Review, *The Analyst*, 123:125R-150R.

NCHS *see* National Centre for Health Statistics

Opperman, M. & Benade, A.S., 2011. Analysis of omega-3 fatty acid content of South African fish oil supplements: Cardiovascular topics. *Cardiovascular Journal of Africa*, 22(6):324-329.

Ozen, B.F., Weiss, I. & Mauer, L.J., 2003. Dietary supplement oil classification and detection of adulteration using Fourier transform infrared spectroscopy. *Journal of Agricultural and Food chemistry*, 51(20):5871-5876.

Pharmaceutical Analytical Sciences Group (PASG), 2002.PASG, NIR sub-Group: *Guidelines for the development and validation of near-infrared spectroscopy methods in the pharmaceutical industry.* http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.452.9685&rep=rep1&type=pdf [30 November 2017].

Pharmaceutical Inspection Co-operation Scheme (PIC/S), 2018. *Guide to good manufacturing practice for medicinal products*, July. https://www.picscheme.org/en/publications?tri=gmp [22 August 2018].

Pharmaceutical Society of South Africa (PSSA), 2014. *PSSA Perspectives:* Roadmap for the registration of complementary medicines, *South African Pharmaceutical Journal,* 81(1):8-11.

PIC/S *see* Pharmaceutical Inspection Co-operation Scheme

Plans, M., Wenstrup, M.J. & Rodriguez-Saona, L.E., 2015. Application of infrared spectroscopy for characterization of dietary omega-3 oil supplements. *Journal of the American Oil Chemists' Society*, 92(7):957-966.

PSSA *see* Pharmaceutical Society of South Africa

Ritter, J.C.S., Budge, S.M. & Jovica, F., 2013. Quality analysis of commercial fish oil preparations. *Journal of the Science of Food and Agriculture*, 93(8):1935-1939.

Skoog, D.A., West, D.M., Holler, F.J. & Crouch, S., 2004. *Fundamentals of Analytical Chemistry*, 8th edition. Belmont: Brooks-Cole-Thomson.

South Africa. Department of Health, 2013. Medicines and Related Substance Act 101 of 1965, Notice R. 870 of 2013, *Government Gazette*, 581(37032):1-16, November 15.

South Africa. Department of Health, 2014. Medicines and Related Substance Act 101 of 1965, Notice R. 837 of 2014, *Government Gazette*, 593(38133):3-6, October 28.

South Africa. Department of Health, 2016. Foodstuffs, Cosmetics and Disinfectants Act 54 of 1972. Government Gazette, 921(40216), August 19. http://www.sahpra.org.za/documents/590f2f52GG40216_nn921_Cosmetics_for_comment_19August2016.pdf [22 December 2017].

South Africa. Department of Health, 2017. Medicines Control Council (MCC), *Guide to good manufacturing practice for medicines in South Africa*, ver. 6, December 2017. http://www.mccza.com/documents/c2add5ba4.01_SA_Guide_to_GMP_Dec17_v6.pdf [08 January 2018]

Sprague, M., Betancor, M., Dick, J. & Tocher, D., 2017. Nutritional evaluation of seafood, with respect to long-chain omega-3 fatty acids, available to UK consumers. *Proceedings of the Nutrition Society,* 76(OCE2).

Sprague, M., Dick, J.R. & Tocher, D.R., 2016. Impact of sustainable feeds on omega-3 long-chain fatty acid levels in farmed Atlantic salmon, 2006-2015. *Scientific reports*, 6(21892):1-6.

Srinivasa, S., 2005. A review on multivariate mutual information. University of Notre Dame, Notre Dame, ID, 2:1-6.

Swanson, D., Block, R. and Mousa, S. A., 2012. Omega-3 fatty acids EPA and DHA: Health benefits throughout life. *Advances in Nutrition: An International Review Journal,* 3(1):1-7.

United States of America. Food and Drug Administration (FDA), 2007. *Good manufacturing practices (GMP's) for dietary supplements*. https://www.fda.gov/Food/GuidanceRegulation/CGMP/ucm079496.htm [01 December 2017].

United States of America. Food and Drug Administration (FDA), 2016. *Contract manufacturing arrangements for drugs: quality agreements, guidance for industry*, November. https://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm353925.pdf [16 May 2018].

United States Pharmacopoeia (USP), 2013*a*. Fats and fixed oils, omega-3 fatty acids determination and profile, Chemical Test 401. In *USP 35–NF 30*, Rockville, MD: US Pharmacopeia: 167-172.

United States Pharmacopoeia (USP), 2013*b*. Near-Infrared Spectroscopy, General chapter 1119. In *USP 35–NF 30*, Rockville, MD: US Pharmacopeia: 714–720.

USP *see* United States Pharmacopoeia

Venn, J., 1880. I. On the diagrammatic and mechanical representation of propositions and reasonings. *The London, Edinburgh, Dublin Philosophical Magazine and Journal of Science*, 10(59):1-18.

Workman, J. Jr, 2014. *An Introduction to Near Infrared Spectroscopy*, http://www.spectroscopynow.com/ir/details/education/sepspec1881education/An-Introduction-to-Near-Infrared-Spectroscopy.html [08 January 2018].

Xu, J.L., Riccioli, C. & Sun, D.W., 2015. An overview on non-destructive spectroscopic techniques for lipid and lipid oxidation analysis in fish and fish products. *Comprehensive Reviews in Food Science and Food Safety*, 14(4):466-477.

Zhang, Y., Cao, F. & He, Y., 2013. Application of visible and near infrared spectroscopy for rapid and non-invasive quantification of adulterants in fish oil. *Spectroscopy and Spectral Analysis*, 33(6):1532-1536.

# CHAPTER TWO
# LITERATURE REVIEW

*Chapter two is an overview of nutraceuticals with respect to classification, quality assurance, adulteration, as well as a review of NIR spectroscopy calibration, validation and industry applications. Specific emphasis is given to PUFA's to link with the contents of chapter three that include a review of the use of NIR spectroscopy in the analysis of PUFA's. Chapter three was written in article format according to the requirements of The Journal of Near Infrared Spectroscopy (JNIRS). Some information is repeated due to the article format of the thesis.*

## 2.1 Nutraceuticals

During the manufacturing of nutraceutical products, the raw materials, in-process and finished products are subjected to laborious and expensive analytical methods. NIR spectroscopy is a fast, cost-effective and non-destructive technique that can replace traditional QC methods when efficiently calibrated and validated.

### 2.1.1 Classification of nutraceuticals

The term nutraceutical is a combination of the words "*nutrition*" and "*pharmaceutical*" and was coined in 1989 by Stephen L. De Felice, founder and chairman of the Foundation of Innovation Medicine (Hunt, 1994; Kalra, 2003). De Felice defined it as "*any substance that is a food or a part of a food and provides medical or health benefits, including the prevention and treatment of disease*." Such products may range from isolated nutrients, dietary supplements and specific diets to genetically engineered designer foods, herbal products and processed foods such as cereals, soups and beverages (Fardet, 2015).

The term has also been defined as "*a product isolated or purified from foods and generally sold in medicinal forms not usually associated with food and demonstrated to have a physiological benefit or provide protection against chronic disease*" (Chauhan *et al*., 2013). Nutraceuticals are found in both traditional and non-traditional food sources. Natural sources of nutraceuticals are found in traditional foods such as fruits, vegetables, meat and dairy products. Non-traditional nutraceuticals, on the other hand, are foods resulting from agricultural breeding or added nutrients and/or ingredients (Chintale Ashwini *et al*., 2013). Various terms are used to refer to nutraceutical products and may lead to confusion; therefore, it is necessary to review the various terms and their definitions.

Nutrients were defined by the Institute of Food Technologists (IFT) as "*traditional vitamins, minerals, essential fatty acids for which recommended intakes have been established and other components that include phytonutrients or bioactives present in foods for which a physical or physiological effect has been scientifically documented or for which a substantial body of evidence exists for a plausible mechanism, but for which a recommended intake and function have not been definitively established*" (IFT, 1998).

Functional foods were defined by the IFT as *foods and food components that provide a health benefit beyond basic nutrition. Examples may include conventional foods; fortified, enriched or enhanced foods; and dietary supplements. These substances provide essential nutrients often beyond quantities necessary for normal maintenance, growth, and development, and/or other biologically active components that impart health benefits or desirable physiological effects*" (IFT, 1998). The Functional Food Centre (FFC) defined functional foods as "*natural or processed foods that contain known or unknown biologically-active compounds; which in defined amounts provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease"* (Martirosyan, & Singh, 2015).

The term dietary supplement was defined in the Dietary Supplement Health and Education Act (DSHEA) of 1994 *as "a product taken by mouth that contains a dietary ingredient intended to supplement the diet. These ingredients may include vitamins, minerals, herbs or other botanicals, amino acids or substances to supplement the diet by increasing the total dietary intake. These supplements can be concentrates, metabolites, constituents or extracts and may be found in tablets, capsules, softgels, gelcaps, liquids or powders. They also can be in other forms, such as a bar; in this case, information on the label must not represent the product as a conventional food or a sole item of a meal or diet*" (FDA, 1994).

The DSHEA classify dietary supplements separately from whole foods and apply different requirements for benefit claims and supporting scientific documentation (FDA, 1994). The IFT expert panel considered this legal distinction and decided that, from a scientific perspective, dietary supplements should be included in the definition of functional foods. Supplements merely constitute a different delivery vehicle for a bioactive component, and therefore the scientific demonstration of efficacy and safety remains the same (IFT, 1998).

In this study, the term nutraceuticals refers collectively to, ingredients or nutrients such as vitamins and minerals, PUFA's found in plant and fish oils, herbal or other botanical extracts, bio-actives and other dietary supplements.

Nutraceuticals are often classified or grouped together according to their chemical nature (e.g. vitamins, minerals, oils), origin (e.g. plant or animal) or their intended use (e.g. weight loss, joint health, sports nutrition), (Wildman, 2016). Isolated nutraceuticals are produced by various processes such as extraction, concentration and chemical synthesis (Wang & Weller, 2006). These nutraceuticals are used as raw materials in producing different dosage forms such as tablets, capsules, softgels, liquids or powders. Table 2.1 contains examples of nutraceuticals classified by chemical nature/molecular structure (Wildman, 2016).

**Table 2.1: Examples of nutraceuticals classified by chemical nature**

| | |
|---|---|
| **Isoprenoid derivatives** | Carotenoids, saponins, tocotrienols, tocopherols, terpenes |
| **Phenolic substances** | Coumarins, tannins, lignin, anthocyanins, isoflavones, flavonones, flavanols |
| **Fatty acids and lipids** | n-3 PUFA, conjugated linoleic acid (CLA), monounsaturated fatty acid (MUFA), Sphingolipids, lecithin |
| **Carbohydrates and derivatives** | ascorbic acid, oligosaccharides, non-starch polysaccharides (PS) |
| **Amino acid-based substances** | amino acids, capsaicinoids, isothiocyanates, indoles, folate, choline |
| **Microbes** | Probiotics, prebiotics |
| **Minerals** | Calcium, selenium, potassium, copper, zinc |
| **Vitamins** | Retinol, biotin, nicotinamide, riboflavin, pyridoxine, thiamine |

### 2.1.2 Polyunsaturated unsaturated fatty acids

The high demand of fish oil nutraceuticals (dietary supplements) is due to the various health benefits in issues such as inflammation, heart disease and mental health (Ruxton *et al*., 2004; Hamed *et al*., 2015). These benefits of fish oils have been studied extensively and are contributed to the PUFA's, specifically the omega-3, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), (Swanson *et al*., 2012; Sprague *et al*., 2017) as well as their ratio to the omega-6 PUFA arachidonic acid (AA), (Liu *et al*., 2013). Excessive amounts of omega-6 PUFA are found in today's Western diets and a very high omega-6/omega-3 ratio promotes the pathogenesis of many diseases, such as cardiovascular disease, cancer, and inflammatory and autoimmune diseases. Increased levels of omega-3 PUFA (a low omega-6/omega-3 ratio) have suppressive effects and is more desirable in reducing the risk of many of the chronic diseases (Simopoulos, 2002).

At present, fish oil is the main industrial source of EPA and DHA for nutritional supplementation (Vongsvivut, *et al*., 2012) and is extracted from fatty fish species such as tuna, mackerel, trout and salmon (Ruxton *et al*., 2004).

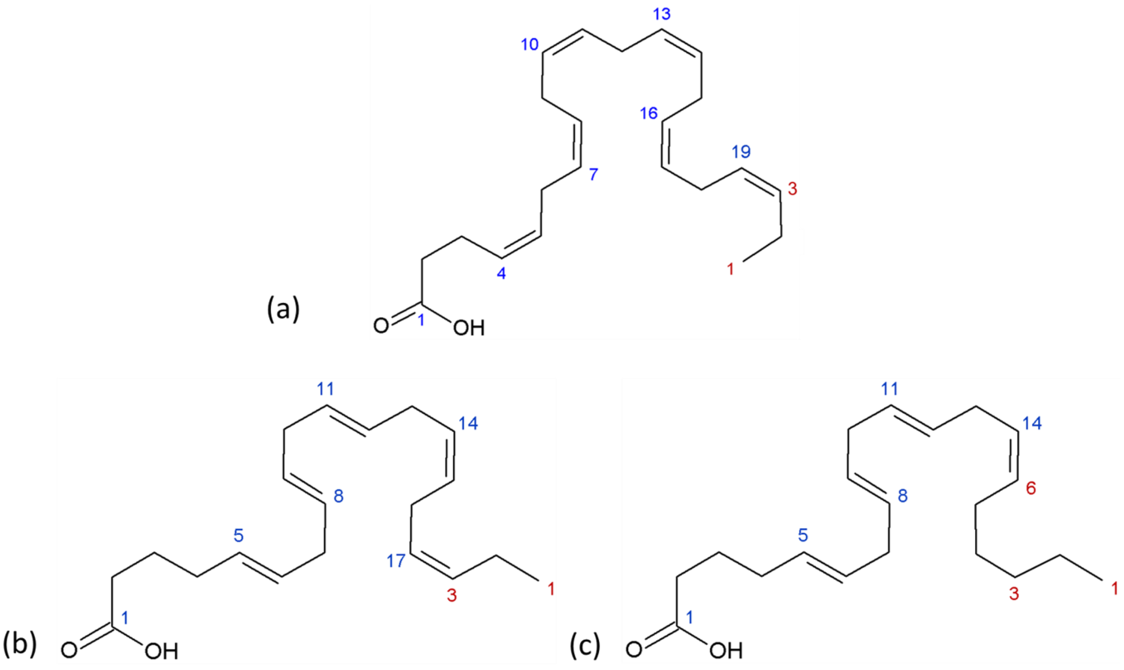


**Figure 2.1: Chemical structure of PUFA'S: (a) docosahexaenoic acid (DHA), C22:6n3, (b) eicosapentaenoic acid (EPA), C20:5n3 and (c) omega-6 PUFA arachidonic acid (AA), C20:4n6**

At the beginning (alpha) of the fatty acid chain, is the carboxylic acid group (-COOH); and the end (omega) of the chain is the methyl group ($-CH_3$). A fatty acid is named using the location of the first double bond, counted from the omega (ω-) or the n- end (fig.2.1), (Ruxton, *et al*., 2004). The most common PUFA's in the nutraceutical industry (Johnson & Bradford, 2014) are listed in Table 2.2.

**Table 2.2: The most common PUFA's in the nutraceutical industry**

| **Fatty Acid** | **Structure** | **Dietary Source** |
|---|---|---|
| | | *Omega-3 Fatty Acids* |
| α-linolenic acid (ALA) | C18:3n3 | Plant oils: linseed oil, kiwifruit oil, chia seed oil, flaxseed oil, canola (rapeseed) oil, soybean, purslane, walnuts |
| Eicosapentaenoic acid (EPA) | C20:5n3 | Oily fish, fish oil, certain seaweeds, human breast milk |
| Docosahexaenoic acid (DHA) | C22:6n3 | Cold water fish, metabolic synthesis from EPA |
| | | *Omega-6 Fatty Acids* |
| Linoleic acid (LA) | C18:3n6 | LA, Corn, peanut, soybean, cottonseed, other plant oils |
| Arachidonic acid (AA) | C20:4n6 | Meat, eggs, dairy products |
| | | *Omega-9 Fatty Acids* |
| Oleic acid (OA) | C18:1n9 | Olive oil, macadamia oil |
| Nervonic acid | C24:1n9 | King salmon, yellow mustard seed, flaxseed |

The PUFA's in fish oils are available in two molecular forms i.e. triglyceride (TG) form and ethyl ester (EE) form. PUFA's naturally occur as triglycerides and are made of three fatty acids attached to a glycerol backbone; whereas, ethyl esters are made of one fatty acid attached to one ethanol molecule (Shahidi, 2005). Generally, EE oils are not found in nature, but TG oils can be converted to the EE form through molecular distillation.

Molecular distillation is a process that separates, concentrates and purifies PUFA's by reacting free fatty acids (FFA) with ethanol in a process called trans-esterification. This process involves removing the glycerol backbone of triglyceride fish oil, resulting in FFA and a free glycerol molecule. An ethanol molecule is then attached to each of the FFA, creating ethyl esters (Cermak *et al*., 2012). The resulting ethyl esters allow for the concentration of the omega-3 long chain fatty acids at lower temperatures. Molecular distillation allows for the selective concentration of EPA and DHA to levels greater than found naturally in fish.

EE concentrates can be converted back to the TG form using enzymes in a process called glycerolysis. Food-grade enzymes such as lipases e.g. *Candida antarctica* (lipase B, Novozym® 435), (Wanasundara & Shahidi, 1998) separate the ethanol molecule from the fatty acid, creating an FFA and a free ethanol molecule. When glycerol is re-introduced to the solution, the enzymes then re-esterify the fatty acids back onto a glycerol backbone, creating triglyceride oil. These oils are commonly referred to as re-esterified (or reformed) triglycerides, which have identical structures to natural triglycerides but with higher concentrations of the desired fatty acids, EPA and DHA (Breivik & Kristinsson, 1997).

While converting concentrated EE fish oil back to its TG form increases manufacturing costs, it improves metabolism and bioavailability. Thus, EPA and DHA were significantly better absorbed in the TG concentrate than they were in the EE concentrate and natural TG fish oil (Dyerberg *et al*., 2010). All lipids containing unsaturated fatty acids oxidize over time and cause the oil to become rancid. In omega-3 fish oils, this degradation usually led to a fishy taste or odor, which is why it is important to manage the oxidation process (Ismail *et al.*, 2016). During a shelf-life study investigating the oxidation rates of TG and EE fish oils, it was found that the rate of oxidation was lower in re-esterified TG than in EE fish oil; thus, TG fish oils have a longer shelf-life than EE oils (Ritter *et al*., 2015).

It is important that analytical techniques used are able to distinguish between TG and EE oils, as well as natural TG and concentrated TG oils. This study aims to achieve discrimination using NIR spectral libraries (*Appendix*).

### 2.1.3 Quality assurance and regulatory requirements

The nutraceutical industry is subjected to regulatory control similar to the pharmaceutical industry. Unlike pharmaceutical products, nutraceuticals are not regulated as strictly in South Africa as in other countries, (Gabriels *et al*., 2012) such as the United States of America (USA), Australia and some European nations. To be able to export nutraceutical products to these countries, the manufacturing facility and processes must comply with the relevant regulatory standards. As mentioned in *Chapter 1.4*, the South African pharmaceutical manufacturers need to conform to GMP as prescribed by the MCC standards in terms of all its regulations (South Africa, 2016; South Africa, 2017; PIC/S, 2018). Certain nutraceuticals are referred to as complementary and alternative medicines (CAM's) and are regulated by the MCC according to the Medicines and Related Substances Act 101/1965 (South Africa, 2013).

### 2.1.4 Good manufacturing practice

GMP is the part of a QMS (Haleem *et al*., 2015) covering the manufacture and testing of active pharmaceutical ingredients (API's), diagnostics, foods, pharmaceutical products, and medical devices. GMP's are guidelines that outline the aspects of production and testing that can affect the quality of a product (South Africa, 2016; South Africa, 2017; PIC/S, 2018). GMP is not a set of prescriptive instructions on how to manufacture products, but a set of principles and procedures that when followed, helps ensure that medicines and related substances are of high quality, safety and efficacy. Most countries require that pharmaceutical and medical device companies must follow GMP procedures, and have created their own GMP guidelines that correspond with their legislation. In the United States GMP is enforced by the US Food and Drug Administration (FDA) and the GMP requirements also need to be followed by dietary supplement manufacturers (FDA, 2007). In South Africa, GMP inspections of nutraceutical manufacturers are performed by the MCC which is a participating authority of the Pharmaceutical Inspection Co-operation Scheme (jointly known as PIC/S), (South Africa, 2016; South Africa, 2017; PIC/S, 2018). The PIC/S aims to develop international standards between countries and pharmaceutical inspection authorities, to provide harmonised and constructive co-operation in the field of GMP (PIC/S, 2018). As a participating authority of PIC/S the MCC requires that manufacturers, importers and exporters of medicines and related substances in South Africa meet the standards laid out in the PIC/S Guide to GMP (South Africa, 2017; PIC/S, 2018).

Thus, internationally, the nutraceutical industry is subjected to the same regulatory control as the pharmaceutical industry. Due to an increasing demand of dietary supplements, numerous products have entered the international market

(Gabriels *et al.*, 2012). However, not all of these products are produced according to GMP standards and label claims cannot be guaranteed; therefore are at risk of adulteration (Hamilton *et al*., 2010; Opperman & Benade, 2011; Ismail *et al.*, 2016).

### 2.1.5 Adulteration of nutraceuticals

The adulteration of nutraceutical ingredients has become a significant issue worldwide (Gil *et al*., 2016; Nounou *et al*., 2018) and it is imperative to your business and the consumer that the QC procedures will be able to identify adulterated products. (Wright, R. 2007; Keservani *et al*., 2014).

According to the Complementary Medicines Australia (CMA) adulteration is "*the presence of undeclared ingredients that impact adversely on the quality and safety of the product*" (CMA, 2012) and can be classified as intentional or unintentional (Everstine *et al*., 2013). Intentional adulteration is usually motivated by economic gain and therefore it is referred to as *economic motivated adulteration (EMA)* (Levin, 2016; Everstine *et al*., 2017). Unintentional adulteration of products occurs with the presence of environmental or microbiological contamination as well as artefacts from the manufacturing process and misidentification of materials due to confusion (Everstine *et al*., 2013). Adulteration in nutraceuticals include contamination with pesticides, herbicides and heavy metals, contamination or adulteration with pharmacologically active medications, and even misidentification of the plant/fish species incorporated into the product (Van Breemen *et al*., 2007).

There was no statutory definition of EMA of nutraceuticals in the United States. However, as part of a 2009 public meeting, the FDA adopted a working definition, defining EMA as the "*fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production, i.e., for economic gain*" (Johnson, 2014). Typical examples of EMA are the addition of chemical compounds to increase the efficacy or to disguise sub-standard material, mixing or mislabelling and withholding information regarding the product (Everstine *et al*., 2013). The intentional neglect of QC tests on materials and products to save costs, are also classified as EMA. Thus, *inadequate QC or the use of non-validated methods renders an adulterated product* (FDA, 2016*a*; ECA, 2018).

The 2007 version of the U.S. GMP guide (FDA, 2007) was applicable to the dietary supplements (*finished goods*) and not the dietary ingredients (*raw materials*), which placed the responsibility of ensuring quality on the dietary supplement manufacturer. In 2016, the GMP guidelines were revised by the FDA to include dietary ingredient manufacturers and economically motivated adulterants (FDA, 2016*a*). Following the FDA, the South African GMP guidelines were also revised to include specifications for nutraceutical raw materials and requires "*specific distinctive tests if a substance is liable to be adulterated/substituted"* (South Africa, 2017).

A drug contract testing laboratory in France was inspected by the U.S. FDA in 2017 and found that *non-validated analytical methods* (ECA, 2018); which were not part of the pharmacopoeia monographs, were used for analysing GMP-liable products and therefore resulted in adulterated products. A warning letter was issued (FDA, 2016*a*), which summarized significant violations of current good manufacturing practice (CGMP) regulations for testing finished pharmaceuticals; "*Because your methods, facilities, or controls for manufacturing, processing, packing, or holding do not conform to CGMP, your drugs are adulterated within the meaning of section 501(a)(2)(B) of the Federal Food, Drug, and Cosmetic Act (FD&C Act), 21 U.S.C. 351(a)(2)(B)."*

Thus, nutraceutical companies, manufacturing *raw material and/or finished goods*, should take responsibility to ensure that their analytical techniques are able to detect adulteration and guarantee authenticity by using validated methods i.e. NIR spectral libraries (*Appendix*).

### 2.1.6 Quality control of nutraceuticals

The South African GMP guidelines are aligned with the international guidelines of PIC/S (PIC/S, 2018) and therefore the MCC accepts the European (EP), British (BP) or United States Pharmacopoeia (USP) "or other relevant pharmacopoeia" (South Africa, 2017). As part of the QMS (Haleem *et al*., 2015) the QC laboratory's responsibility is to perform physical and analytical testing of all incoming raw materials, packaging materials, in-process and finished product samples according to the required specifications. The specifications for all raw materials, intermediates and finished products are controlled and prescribed by the GMP guidelines (South Africa, 2017).

Nutraceutical companies usually manufacture a wide range of products with various formulations and dosage forms. Some formulations are very simple such as fish or plant oils which are encapsulated 'as is' with a small amount of added vitamin E to prevent oxidation (Dieber-Rotheneder *et al*., 1991). Other formulations are very complex and can easily contain up to 30 different ingredients or nutraceuticals. It would be very costly and time-consuming to analyse all the nutraceuticals in such complex formulations and thus analyses are based on regulatory requirements and risk assessments (South Africa, 2017).

The choice of which nutraceuticals to analyse will depend on the QMS (Haleem *et al*., 2015) and by the applicable regulatory authorities. API's or nutraceuticals are usually selected for analysis based on their therapeutic activity, stability, potential risk or are regulated compounds such as vitamin E and ginseng (South Africa, 2016). If certain label claims are made, analysis of the product must be able to substantiate the claim according to regulatory requirements (South Africa, 2017).

The tests and criteria for API's are obtained directly from accepted pharmacopoeias and are performed in-house or outsourced to an accredited laboratory. According to the United States Pharmacopeial Convention (USPC) an alternative method may be implemented in the pharmaceutical/nutraceutical industry to prove by comparison that it is either *acceptable, equivalent or better* than the prescribed compendial method (Hauck *et al*., 2009). Therefore, an alternative method must demonstrate equivalence through validation to meet pharmaceutical requirements.

The methods vary from simple wet chemistry tests to sophisticated instrumental analysis (Morena & Salvado, 2000). Samples are subjected to time-consuming QC analysis that makes use of analytical instruments such as high-performance liquid chromatography (HPLC) and gas chromatography (GC), (Skoog *et al*., 2004). Chromatography techniques identify specific markers and not the material as a whole, therefore adulteration with other materials can go unnoticed. (Wright, 2007; Govindaraghavan, 2014; Marieschi *et al*., 2016).

Both GC and HPLC are time-consuming and require sample preparation using solvents and other costly reagents. Large volumes of mobile phase are used during HPLC analysis to condition the system before and after analysis increasing the amount of chemical waste produced. Separation columns are expensive and need special care. Maintenance costs are high; leading to a high cost of analysis, therefore the investigation into the use of a more cost-effective and environmentally friendly technique such as NIR spectroscopy is justified.

### 2.1.7 Detection of adulteration in nutraceuticals

Compliance with GMP alone is not enough to eliminate adulteration. Nutraceutical companies should take it upon themselves to ensure the specificity of their analytical techniques is adequate by using validated methods. Except for method validation, analytical instruments are also subjected to qualification procedures as prescribed by the USP, General Chapter <1058> on *Analytical Instrument Qualification (AIQ)*. This chapter describes general principles of instrument qualification and a general risk classification of analytical equipment, instruments and systems (USP, 2017). HPLC and high performance thin layer chromatography (HPTLC) are frequently used for the identification of nutraceuticals with the disadvantage of analysing for specific markers (Wright, 2007). Spectroscopy as a screening method for adulterants has been used as a more comprehensive approach by analysing the sample “as is” (Lohumi *et al*., 2015).

Various studies have investigated techniques such as spectroscopy i.e. ultraviolet (UV), NIR, mid-infrared (MIR), visible (Vis) and Raman, isotopic analysis, chromatography i.e. GC and HPLC; electronic nose, polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA) and thermal analysis i.e. differential scanning calorimetry (DSC) for the detection of food and nutraceutical authenticity and adulteration (Reid *et al*., 2006; Lohumi *et al*., 2015; Cozzolino, 2017). Nuclear magnetic resonance (NMR) spectroscopy (Colson *et a*l., 2014), GC coupled with mass spectroscopy (GC-MS) and HPLC coupled with MS (LC-MS) (Vaclavik *et al*., 2014) have been demonstrated as valuable techniques to detect adulteration of natural plant extracts for herbal remedies or nutraceuticals. The use of hyperspectral imaging (HSI) for the product quality and safety control of agricultural food has also been reported (Dale *et al*., 2013).

### 2.1.8 Detection of adulteration in fish oils

GC-FID is the prescribed technique for analysis of omega-3 PUFA's in the nutraceutical industry (BP, 2011*a*; USP, 2013*a*). It is a time-consuming and costly procedure that involves extensive sample preparation. Expensive certified reference standards, instrument maintenance and solvent waste contribute to the costs. The GC method can be substituted by an alternative method if the performance is proved equivalent to that of the prescribed methods (BP, 2011*a*; USP, 2013*a*; GOED, 2015).

The qualification of natural fish oils is challenging and increases the risk of adulteration due to overlapping concentration ranges of PUFA's and other properties which cannot be used to discriminate between oils from different fish species (Cozzolino *et al*., 2005). Therefore, quantification alone is not sufficient to detect adulteration; and identification to authenticate the oil is critical.

Fish oils occur naturally in the triglyceride (TG) form, but can be converted to the ethyl ester (EE) form during molecular distillation. This process separates, concentrates and purifies PUFA's such as omega-3 fatty acids in fish oil (Cermak *et al*., 2012). The QC method must be able to discriminate between the relevant fish oil species, quantify the relevant PUFA's and distinguish between TG and EE forms to detect adulteration in fish oils.

The fish oil industry has also been victimized by adulteration and various studies have been conducted to detect adulteration using non-destructive techniques such as MIR or FT-IR (Yoshida & Yoshida, 2003; Rohman & Man, 2009; Dominguez-Vidal *et al*., 2016; Lingzhi *et al*., 2016) and NIR (Cozzolino & Murray, 2012; Kramer *et al*., 2013) in combination with chemometrics. Separation techniques such as GC in combination with chemometrics (Bosque-Sendra *et al*., 2012) and GC-MS (Yi *et al*., 2014) have been used to detect adulterants in TG fish oils.

Some studies used unusual and sophisticated techniques such as $^{1}$H NMR (Tan *et al*., 2017) to study oxidation in fish oils; a high impedance surface electromagnetic bandgap inspired resonator patch, bonded with a polydimethylsiloxane (PDMS) cavity was implemented as a sensor device for detecting adulteration in a fish oil dietary supplement (Yadav & Patel, 2016) and $^{13}$C NMR spectroscopy and thin layer chromatography with flame ionisation detector (TLC-FID), were used to investigate fish oil adulteration of krill oil with ethyl esters and triacylglycerol (Akanbi & Barrow, 2018).

In this study NIR spectroscopy in combination with chemometrics will be investigated as an alternative to the GC to reduce the risk of adulteration of nutraceutical products specifically fish oils containing omega-3 PUFA's.

## 2.2 Near infrared spectroscopy

NIR spectroscopy is a rapid and cost-effective technique and when validated can replace other time consuming and costly procedures (Blanco *et al*., 1998). It requires no, or very little sample preparation, is non-destructive and analysis of a sample is performed within seconds. NIR spectroscopy also belongs to the reagent-free methodologies, thus is an environmentally friendly technique that is an excellent example of the *Environmental Protection Agency's (EPA)* attempt to encourage green chemistry (Anastas & Kirchhoff, 2002). NIR spectroscopy is a versatile technique that can be applied in the laboratory by bench top instruments or in the factory by on-line or in-line instruments. NIR spectroscopy methods can be validated for the use of qualitative and quantitative applications (Burns & Ciurczak, 2001; Metrohm, 2014).

### 2.2.1 Background on NIR spectroscopy

The electromagnetic spectrum is divided into several regions. The NIR region of the electromagnetic spectrum was discovered in 1800 by William Herschel who published his findings titled "Experiments on the refrangibility of the invisible rays of the sun" in the *Philosophical Transactions of the Royal Society* (90:284-292) (Workman, 2014).

Spectroscopy uses electromagnetic radiation to analyse materials by describing the energy transfer between light and matter. Every region represents a specific kind of molecular or atomic transition and is suitable for a specific spectroscopic technique. Gamma rays and X-rays have wavelengths of only a few nm and are very harmful, because they break chemical bonds and ionize molecules. Ultra-violet (UV) radiation covers the region 190 - 350 nm and visible radiation (VIS) the region 350 - 780 nm. Photon absorption in the UV-VIS range transfers electrons from low-energy to high-energy molecule orbitals and returning to the ground state easily proceeds via fluorescence. The NIR spectral region ranges from 780 - 2500 nm (12821 - 4000 cm$^{-1}$)

and lies between the visible and mid-infrared (MIR) regions (Figure 2.2) and corresponds to the frequency of vibrational transitions (Burns & Ciurczak, 2001).

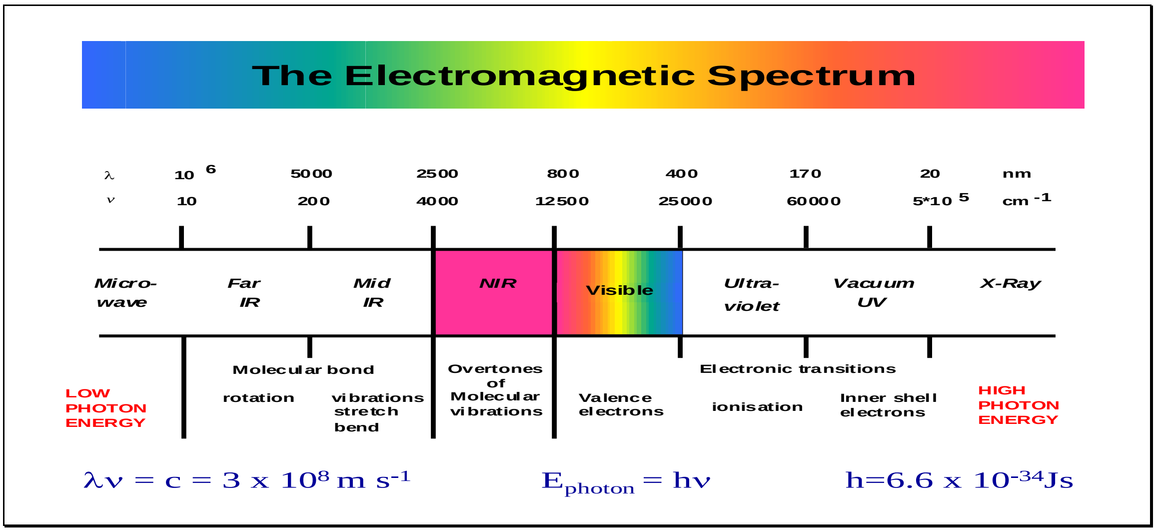


**Figure 2.2: Electromagnetic spectrum and positioning of spectral regions (Murray, 2004)**

A wavenumber is indicated in $cm^{-1}$ and is the reciprocal of the wavelength in nm multiplied by $10^7$. Equation 2.1 shows that the shorter the wavelength, the higher the wavenumber and the higher the energy of the photon. The energy of a single photon is defined as:

$$E = hv = {}^{hc}/_{\lambda} = hc\bar{v}$$

*Thus* $\bar{v} = 1/\lambda$ **Equation 2.1**

*and* $\boldsymbol{\bar{v}[cm^{-1}]} = {}^{\boldsymbol{10^7}}/_{\boldsymbol{\lambda}}\ \boldsymbol{[nm]}$

Where, $\boldsymbol{E}$ = energy, $\boldsymbol{h}$ = Planck constant in $m^2.kg.s^{-1}$, $\boldsymbol{\tilde{v}}$ = frequency of the light in $s^{-1}$, $\boldsymbol{c}$ = speed of light in $m.s^{-1}$, $\boldsymbol{\lambda}$ = wavelength of the light in m and $\boldsymbol{\tilde{v}}$ = wavenumber of the light in $m^{-1}$.

Molecules can absorb infrared light by exciting certain vibrational frequencies. The sample absorbs the frequencies of polychromatic light that corresponds to its molecular vibrational transitions. The frequency of the vibration and thus the energy are a function of the bond strength. Vibrational frequencies are thus the key that contains information about the structure of the investigated compound and is the basis for the application of vibrational spectroscopy. NIR spectroscopy is also classified as a vibrational spectroscopy technique, similar to FT-IR and Raman spectroscopy (Larkin, 2017; Metrohm, 2014).

The predominant molecular overtones and combination vibrations observed in the NIR spectral region is due to the bending and stretching vibrations of the C-H, N-H, O-H and S-H bonds (Burns & Ciurczak, 2001). The low energy transitions result in weak or low molar absorptivity in the NIR region. Thus, NIR spectroscopy is not a very sensitive technique, but the radiation is able to penetrate further into the sample than MIR radiation. Therefore, a larger sample path length or sample thickness can be used with little or no sample preparation resulting in non-destructive analysis (Burns & Ciurczak, 2001).

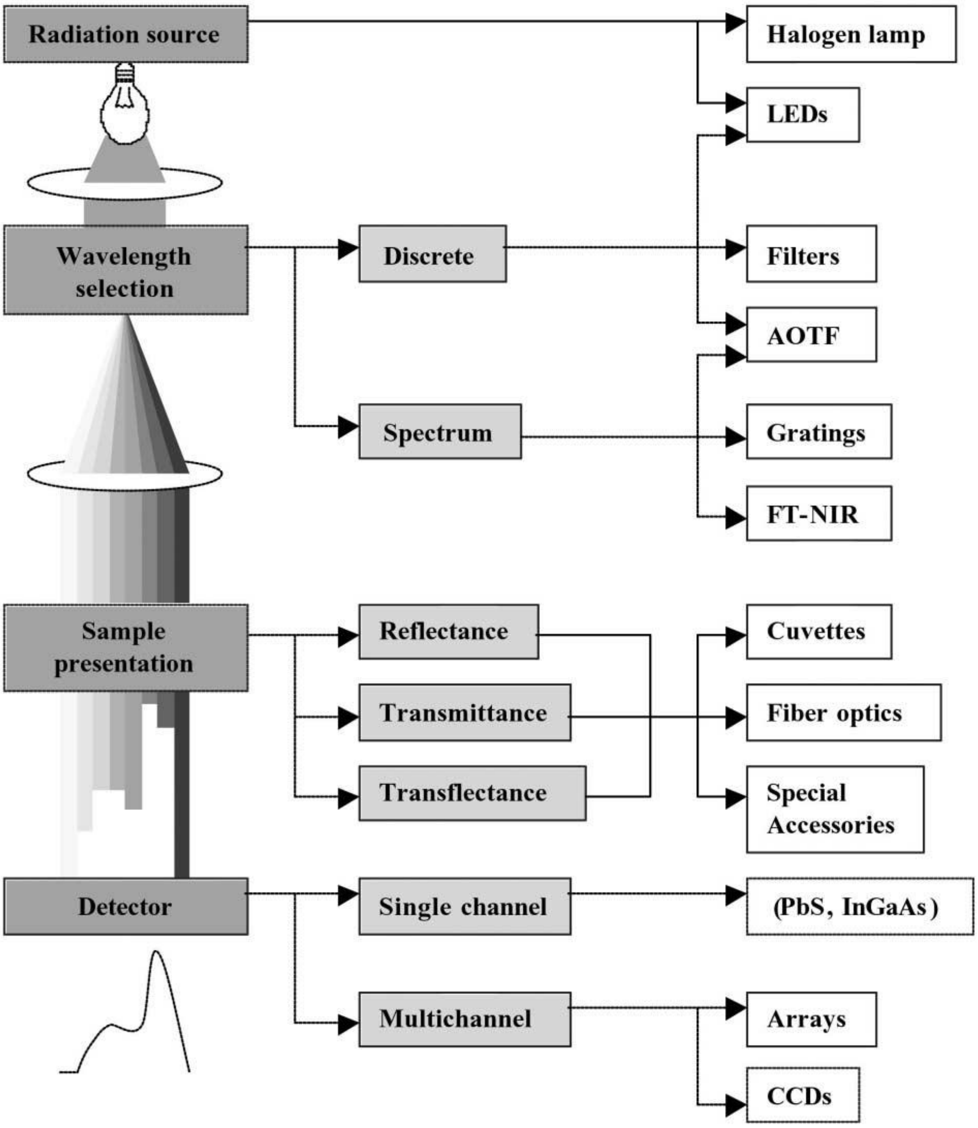


**Figure 2.3: Basic components of a near infrared instrument (Blanco & Villarroya, 2002)**

NIR spectra contain chemical and physical information and can be applied to measure a variety of properties. The chemical information can be used for the qualitative and quantitative analysis as well as process control, whereas properties such as crystalline form, particle size, dissolution and blending can be derived from the physical information (BP, 2011*b*).

### 2.2.2 NIR spectroscopy instrumentation

NIR spectrophotometers can be classified according to their optical configuration, into filter and dispersive (scanning) instruments (fig 2.3). Filter instruments such as light emitting diode (LED) and acousto-optical tunable filter (AOTF) based instruments, apply discrete wavelengths in the NIR region (Pasquini, 2003). Filter based instruments are usually applied when only a few pre-selected wavelengths are measured to monitor specific constituents. The advantage of this equipment is its lower cost compared to scanning instruments, as well as its suitability for use in portable NIR scanners (Blanco & Villarroya, 2002).

Dispersive instruments, based on diffraction gratings and interferometric (Fourier transform) instruments, are the most common scanning NIR instruments (Pasquini, 2003). Grating dispersive instruments split the light radiation spatially by means of an optical interference such as grating monochromator (fig 2.4); whereas, Fourier transform (FT) instruments make use of interferometers and Fourier transformation to recapture intensities of single wavelengths (fig 2.5). Laboratory instruments are usually scanning type instruments due to the fixed high resolution it provides over the entire range or bandwidth. Dispersive instruments display good signal-to-noise ratios and can be used in a variety of applications (Burns & Ciurczak, 2001; Ciurczak *et al*.,2008).

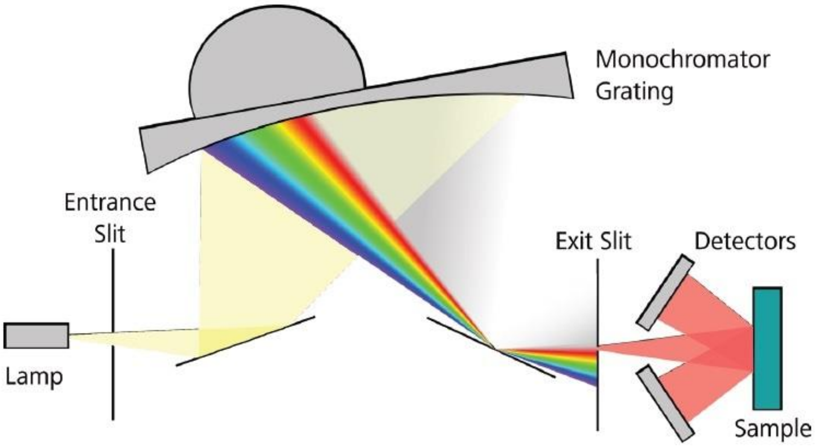


**Figure 2.4: Illustration of the dispersive spectrometer in diffuse reflectance mode (Metrohm, 2017)**

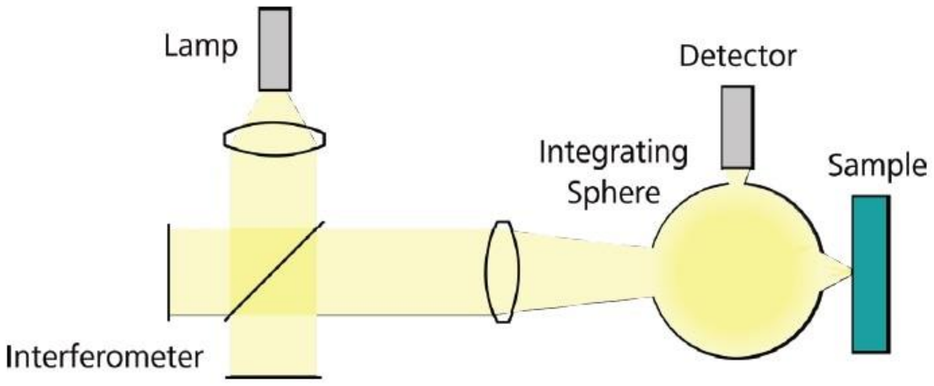


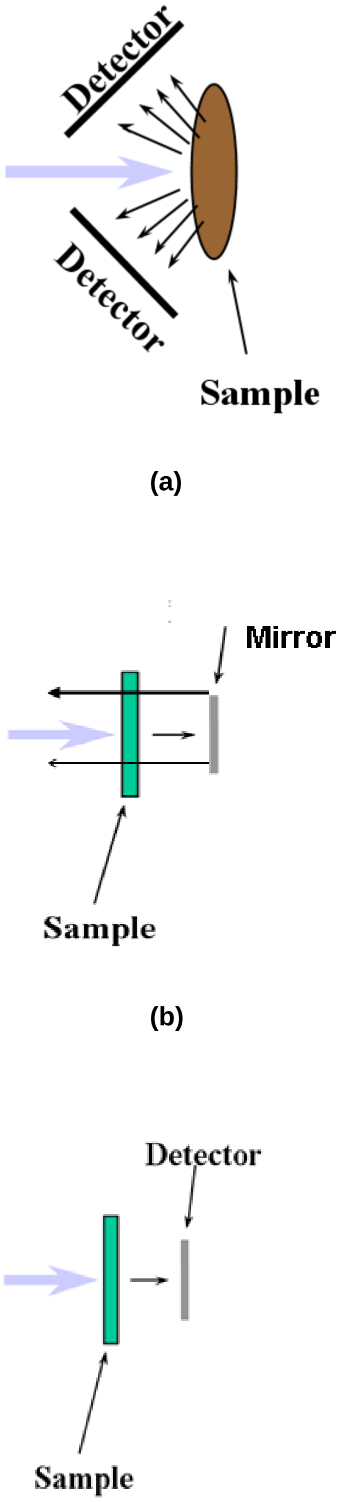
**Figure 2.5: Illustration of an FT spectrometer with Michelson interferometer in reflectance mode (Metrohm, 2017)**

The most frequently employed detectors for the NIR spectral region are based on silicon, PbS and InGaAs photoconductive materials (Pasquini, 2003). In multi-channel detectors, several detection elements are arranged in rows i.e. diode arrays or planes i.e. charged coupled devices (CCDs) in order to record many wavelengths at once, therefore increase the spectral acquisition speed (Blanco & Villarroya, 2002). PbS or InGaAs detectors, in conjunction with high powered radiation sources such as a tungsten coil or a halogen lamp, is used by most of NIR measurement manufacturers and offer a very high signal-to-noise ratio, which to some extent compensates for the lower intensities of NIR absorption bands (Pasquini, 2003). Many researchers have determined equivalent performance of dispersive and FT systems for routine analysis, whereas some demonstrated better predictive capabilities with dispersive systems, and recommend this type of technology for offline use in laboratories (Ciurczak *et al*., 2008).

### 2.2.3 NIR spectroscopy measurement modes

There are many different types of sample accessories available and instruments can operate in different modes depending on the type of sample. NIR absorption by materials is up to 1000 times lower than the absorption resulting from fundamental bands in the MIR region (Pasquini, 2003). This allows intact samples to be used for analysis without prior dilution and has resulted in several different sample presentation modes for NIR measurements.

The most commonly applied measurement modes applied are reflectance, transmittance or transflectance. Unique for NIR spectroscopy is the ability to measure solid samples, which occurs by diffuse reflectance of the radiation from the solid matter (fig 2.6a). However, scattering and absorbance of some radiation by the solid may contribute to inaccuracies.



(a)

(b)

(c)

Figure 2.6: Three different possibilities of sample presentation: a) diffuse reflectance, b) transmittance and c) transflectance (Metrohm, 2014)

Different from MIR spectra which have distinct bands, the NIR spectra have broad overlapping absorption bands. Transmittance NIR spectra are obtained when measuring liquids in quartz cuvettes with an optical path length usually ranging between 0.2 and 50 mm and the radiation penetrates the sample without obstruction (fig 2.6b) (Pasquini, 2003). Radiation that is transmitted through a sample, however, reflected by an object such as a mirror or metal plate, and is transmitted back through the sample is known as transflectance (fig 2.6c). In this mode, the radiation beam passes through the sample twice, thereby doubling the optical path length (Pasquini, 2003). Reflectance measurements are usually applied to solids, powders, slurries and opaque liquids. Transmittance measurements are applied to translucent samples such as liquids, but can also be used in the measurement of solid dosage forms. Transflectance can be used for both liquids and solid suspensions (PASG, 2002).

NIR spectra are very complex due to molecular overtones and combination bands. Therefore, it is very difficult to assign specific spectral features to specific chemical components and data analyses rely on multivariate analysis (MVA) techniques to extract and process the chemical information (Chalmers & Griffiths, 2002). The use of MVA techniques is also called chemometrics and refers to techniques such as principal component analysis (PCA), partial least squares (PLS) regression and multilinear regression (MLR) to calibrate and validate a model (Beebe & Kowalski, 1987; Haaland & Thomas, 1988; Brereton, 2003). As the calibration and validation of measured NIR spectral data is correlated through statistical methods to reference data, NIR spectroscopy is a secondary analytical method (Blanco et al., 1998; Burns & Ciurczak, 2001).

## 2.3 Chemometrics

Chemometrics is defined by the International Chemometrics Society as "*the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods*" (Hibbert *et al*., 2009). Due to the complex nature of NIR spectra with broad overlapping absorption bands, the data analysis and pre-processing are dependent on mathematical transformations which are done using MVA techniques. Chemical information is correlated to spectral information by the application of mathematical algorithms such as MLR, PCA or PLS regression using specialised computer software (Beebe & Kowalski, 1987; Haaland & Thomas, 1988; Brereton, 2003). The process of applying chemometrics to correlate chemical and spectral information to develop qualitative and quantitative methods is called spectroscopic calibration (Mark, 2006).

Various mathematical algorithms can be applied to the raw data at different stages of the calibration process. Raw data can be pre-processed, also referred to as data pre-treatment, to reduce spectral complexity and eliminate interferences and facilitate information extraction from NIR spectral data (Gemperline, 2006; Metrohm, 2014). Classification methods such cluster analysis are called unsupervised methods and are commonly used in the pharmaceutical industry; however, supervised classification methods such as correlation and distance-based methods are preferred (EMA, 2014). Other supervised methods are linear discriminant analysis (LDA), soft independent modelling of class analogy (SIMCA) and partial least squares discriminant analysis (PLS-DA), (Geladi, 2003; Luypaert *et al*., 2007; Roggo *et al*., 2007).

### 2.3.1 Pre-processing methods

Pre-processing is often used to enhance certain spectral features and/or to remove noise from the spectra. Derivatives and multiplicative scatter correction (MSC) are often used to reduce baseline offsets due to physical properties such as particle size and changes in instrumental conditions (Gemperline, 2006).

In some cases, it will be necessary to use the raw NIR spectra and not apply any pre-processing if the physical properties are of interest. Pre-processing will remove the effect of the physical properties and thus it is important to understand the influences of the algorithm on the raw data.

*Second derivative* pre-processing is often applied to improve spectral resolution, correct for spectral offset and slope due to changes in instrument response (FOSS, 2004: Gemperline, 2006; Metrohm, 2015). Calculation of the first derivative with respect to wavelength $(\boldsymbol{w})$ eliminates the offset term $(\boldsymbol{C})$ and the slope term $(\boldsymbol{a})$ becomes a constant. Therefore, it is common practice to take the second derivative with respect to wavelength to eliminate both offset and slope (Eq. 2.2):

$$\frac{\boldsymbol{d^2}}{\boldsymbol{dw^2}}[\boldsymbol{f}(\boldsymbol{w}) + \ (\boldsymbol{a} \cdot \boldsymbol{w} + \boldsymbol{C})] = \boldsymbol{f}'(\boldsymbol{w})$$

**Equation 2.2**

Consequently, if each spectrum $f(w)$; in a data set exhibits a slightly different slope $(\boldsymbol{a})$; then the first derivative spectra will exhibit offset variation. One way to calculate the second derivative is as the second order finite-difference derivative. This method requires two values to be specified: the length of the *segment* (segment size); and the length of the *gap* between segments (gap size). The second derivative calculation begins by identifying three segments at one end of the spectrum, each separated from the other by a gap. Average absorbance values are calculated for the first, second, and third segments (*A, B* and *C*, respectively). The second derivative value computed as *A-2B+C* is assigned to the midpoint of the second segment. Then the whole sequence of three segments and two gaps is shifted one data point and the calculations repeated until a second derivative value has been calculated for all data points in the spectrum (FOSS, 2004: Gemperline, 2006; Metrohm, 2015).

Other pre-processing methods are *N-point smooth, standard normal variate (SNV), baseline correction, Detrend, Savitzky-Golay (S-G) and multiplicative scatter correction (MSC)*, (FOSS, 2004: Gemperline, 2006; Metrohm, 2015).

*N-point smooth* is a boxcar type of smoothing with a specified segment size, which defines the size of the boxcar in nanometers. The average spectral value over the segment is placed in the middle of the segment (FOSS, 2004: Gemperline, 2006; Metrohm, 2015).

*Standard normal variate (SNV)* is a scatter correction method used commonly to normalize spectra when the effective pathlength varies among samples in a data set. When measuring the spectra of granular or powdery samples pathlength variation can occur due to sample presentation that is not perfectly reproducible, or particle size variance between samples (FOSS, 2004: Gemperline, 2006; Metrohm, 2015).

*Baseline correction* is used to change spectral offset by subtracting either a spectral value at a specified wavelength, or a constant value entered manually (FOSS, 2004: Gemperline, 2006; Metrohm, 2015).

*Detrend* is a method that can be used to remove baseline offset, slope, or curvature from a spectrum. This is accomplished by calculating a baseline function as the least squares fit of a polynomial to the sample spectrum, and then subtracting that function from the spectrum (FOSS, 2004: Gemperline, 2006; Metrohm, 2015).

*Savitzky-Golay (S-G)* is a well-known method of smoothing and derivative calculation relies on the least squares fit of a polynomial to a spectral segment. Though both the *S-G and Detrend* methods are based on the least squares fit of polynomial functions, they differ in scope and effect. In *Detrend,* a single polynomial function is fitted to the entire spectrum. In the *S-G* method, a smoothed spectrum or a derivative spectrum of any order can be calculated using coefficients of the data points in a segment that is modelled by a polynomial function fitted to a spectrum (FOSS, 2004: Gemperline, 2006; Metrohm, 2015).

All math pre-treatments discussed so far are based on and applied to individual spectra: they operate on data points in each spectrum, and yield results determined by the unique characteristics of that spectrum. Whereas, *multiplicative scatter correction (MSC)* is set-dependent: it is a scatter correction method based on a related set of spectra.

In *MSC*, the mean spectrum is calculated from all spectra in a defined data set. Then a least squares linear regression is performed on absorbance values of the sample spectrum versus those at corresponding wavelengths in the mean spectrum. This operation yields a linear equation with a defined intercept and slope. The value of the intercept is then subtracted from every data point in the spectrum and finally, each absorbance value in the resulting spectrum is divided by the value for slope. This same set of operations is performed on every spectrum in the data set using the mean spectrum (FOSS, 2004: Gemperline, 2006; Metrohm, 2015).

Pre-treatments like *derivatives, SNV, Detrend and S-G* are applied to individual spectra without any preconception of what the resulting spectrum should look like. By contrast, the *MSC* method calculates a mean spectrum under the assumption that spectra in the data set are distributed normally. Thus, the mean spectrum is the most probable spectrum, i.e. it is the highest probability representation of all spectra in the data set (FOSS, 2004: Gemperline, 2006; Metrohm, 2015).

*MSC* effectively forces individual spectra to behave like the mean spectrum as much as possible. The method strongly depends on the calculated mean spectrum closely resembling the true mean spectrum, which depends on a large sample set. Quantitative method development usually requires that the data sets must span the full range of chemical and spectral variation encountered (FOSS, 2004: Gemperline, 2006; Metrohm, 2015).

Sample sets for qualitative models development may also be large in comparison with quantitative models and often include hundreds of products. However, a given product may contain only a few samples. Therefore, *MSC* may not be as suitable for qualitative analysis as it is for those quantitative applications where elimination of pathlength and slope variation in a single pre-treatment is important (FOSS, 2004: Gemperline, 2006; Metrohm, 2015).

### 2.3.2. Principal component analysis (PCA)

Highly correlated variables are typical in NIR spectra, which means that the measured absorbance's at two or more wavelengths are dependent of each other. This is referred to as multi-collinearity and can cause problems during calibration when using linear regression models (Wold et al., 2001; Brereton, 2003).

Calibrations are developed with regressions of the measured NIR spectral data against the reference data. These variables have to be arranged into matrices to perform chemometrics. There are two types of variables: dependent and independent variables. Independent variables are the measured absorbances and dependent variables are to be predicted (e.g. concentration values). For each sample, the corresponding absorbance values is placed into the absorbance matrix as a column vector (X-values), while the concentration matrix holds the dependent variables (Y-values), namely the known concentrations of the samples or reference values (Wold *et al*., 2001; Brereton, 2003).

It is necessary to reduce the amount of data in the pre-treated spectra to facilitate classification and calibration and address multi-collinearity. PCA is a very popular method for data compression (dimensionality reduction) and is used as an unsupervised pattern recognition technique in qualitative NIR analysis (Wold *et al*., 2001; Brereton, 2003; Metrohm, 2015). However, when it comes to quantitative NIR analysis, the PLS regression algorithm is the most popular linear calibration method used in quantitative NIR data analysis. It simultaneously reduces the amount of data (dimension reduction of the NIR spectra matrix) and does the regression (Beebe & Kowalski, 1987; Haaland & Thomas, 1988).

PCA is performed on data that was pre-processed and is not dependent on results from reference analytical methods. The purpose of PCA is to mathematically decompose the originally correlated variables (absorption values) into a much smaller set of uncorrelated latent variables (principal component factors). Mathematically principal components are vectors. The appropriate set of principal components represents the most common variations in the data set. The first principal component calculated accounts for most of the variance in the data. Each succeeding principal component is orthogonal to the preceding principal component and is calculated to have the highest remaining variance possible. This process is done until only noise remains (Wold *et al*., 2001; Brereton, 2003).

Choosing too many principal components so that one or more of them only represent noise is called *over-fitting*. However, using too few principal components *(under-fitting)* may cause important information to be lost. Both *over-* and *under-fitting* must be avoided, as it has a negative effect on the robustness and prediction performance of the model. An *overfitted* model may show excellent results for samples that are part of the calibration set, but fails to predict properties of samples that are part of an external validation data set (Wold *et al*., 2001; Brereton, 2003).

Each principal component holds information of the whole measured spectrum as it is a linear combination of the original variables. For every original variable a score is obtained that describes the orientation of this variable to the principal component. The orientation of a principal component vector is characterized by loadings. Score plots are used to interpret the NIR data and identify outliers (Wold *et al*., 2001; Brereton, 2003).

### 2.3.2 Qualitative methods

To develop a qualitative NIR spectroscopy method a spectral library database must be constructed. The NIR spectra of the samples are recorded over a period of time incorporating different batches of material to include as much spectral variation as possible into the database. These samples are also tested by the laboratory reference method to ensure the quality and authenticity of each sample captured in the database. The samples in the library that will be used to develop the method are called the training set (Hasley, 1994; Reich, 2005).

The training set is first subjected to data pre-processing methods if applicable where after sample selection algorithms or classification methods such as *correlation in wavelength space* and *maximum distance in wavelength space* are applied to identify outliers and redundant samples (Hasley, 1994; Metrohm, 2015). Outliers are samples whose spectral properties differ significantly from the sample population and should be investigated whether they should be kept or discarded. Redundant samples are samples that do not add spectral variation to the matrix and can be removed to be used for internal validation of the spectral database (Hasley, 1994; Reich, 2005).

Internal validation will verify that the library model is able to discriminate between the material included in the model and that the spectra used to construct the model are identified correctly (Hasley, 1994; Reich, 2005). External validation is done by an independent set of samples called the validation set. These samples must prove the selectivity and robustness of the library and will assist in establishing the library thresholds (BP, 2011*b;* EMA, 2014).

A library model consisting of numerous materials can be constructed depending on the limitations of the software and user's requirements. The chemometric methods commonly used in the pharmaceutical industry for library validation are either correlation or distance methods (BP, 2011*b*; EMA, 2014).

In *correlation in wavelength space* a mean spectrum for every product in the training set is calculated using all the sample spectra from the training set (Brereton, 2003; FOSS, 2004; Metrohm, 2015). An unknown can be identified by calculating the correlation between the unknown and the mean product spectrum ($\boldsymbol{D_c}$) (Eq. 2.3):

$$\boldsymbol{D_c} = \frac{\sum_i \boldsymbol{x_i y_i}}{\sqrt{\sum_i \boldsymbol{x_i^2} \sum_i \boldsymbol{y_i^2}}}$$

**Equation 2.3**

In *maximum distance in wavelength space* a mean spectrum for every product in the training set is calculated using all the sample spectra from the training set. An unknown can be identified by calculating the maximum distance ($\boldsymbol{D_x}$) between the unknown and the mean product spectrum (Brereton, 2003; FOSS, 2004; Metrohm, 2015). If the distance value is below the library threshold, the unknown is identified as one of the products (Eq. 2.4):

$$\boldsymbol{D_x = max\left[abs\left(\frac{y_i - \overline{x_\iota}}{S_i^d}\right)\right]_{overall\ i}}$$

**Equation 2.4**

Other classification methods available in the Vision® 3.50 software (NIRSystems Inc. Silver Springs, Maryland, USA) are *Mahalanobis distance in principal component space* and *residual variance in principal component space* (Brereton, 2003; FOSS, 2004; Metrohm, 2015). Most of the NIR spectral libraries in this study achieved discrimination by using the *correlation in wavelength space* equation and *maximum distance in wavelength space* equation (*Appendix*).

### 2.3.3 Quantitative methods

Despite the potential of NIR spectroscopy to replace time-consuming and costly techniques; it also has its limitations. Therefore, it is very important to evaluate the type of sample to be analysed and to conduct a feasibility study before embarking on a quantification project. The low molar absorptivity of NIR radiation has led to the disadvantage of low sensitivity, but the advantage of analysis of samples '*as is'*. In NIR spectroscopy, it is usually not suitable to quantify constituents with concentrations lower than 0.1 % depending on the sample matrix (Burns & Ciurczak, 2001).

Due to the nature of the chemometric techniques it is important to ensure that constituents are not inter-correlated (increasing and decreasing together) which can lead to incorrect calibrations. Inter-correlation can be addressed during the feasibility study or in a separate experimental design by varying the concentrations of the components to compensate for this inter-dependence (Hasley, 1998; Reich, 2005).

In quantitative method development the sample spectra to be used for the calibration model is called the calibration set. As with qualitative analysis, all the samples in the calibration set are tested by the laboratory reference method to ensure the identity and concentration of the components for which the calibration equation will be developed. The standard error of the laboratory (SEL) reference method must be estimated as a comparison to the standard error of calibration (SEC) and standard error of prediction (SEP) of the NIR spectroscopy method (BP, 2011*b*; EMA, 2014).

It is important that there is an even distribution of samples across the concentration range and that the data is not centred weighted, although in practice, analytical results tend to have a normal distribution. By using the correct pre-processing methods (e.g. second derivative) and a suitable multivariate technique (e.g. PLS) these problems can be overcome. The process of using multivariate techniques to create a calibration equation is also called regression. The choice of multivariate technique will depend on the type of data and user preference (Hasley, 1998; Reich, 2005).

The multivariate techniques available in the Vision® 3.50 software (NIRSystems Inc. Silver Springs, Maryland, USA) are *multilinear regression (MLR)* and *partial least squares (PLS)* regression (Brereton, 2003; FOSS, 2004; Metrohm, 2015).

*Multilinear regression (MLR)* is the simplest form of multivariate analysis and relies on the user's knowledge to select appropriate wavelengths for calibration where no or little interference from other components in the matrix occur. If only one wavelength is selected it is normal linear regression and only becomes MLR with the selection of two or more wavelengths. MLR is recommended for simple matrices which are made up with only a few components, but are negatively affected multi-collinearity (Hasley, 1998; Reich, 2005).

*Partial least squares (PLS)* regression is a technique based on PCA which can handle complex matrices and is not dependent on finding wavelengths free from interfering compounds. It uses the entire wavelength selection to find the correlation between the spectral and chemical properties, whereas MLR uses only the selected wavelengths (Beebe & Kowalski, 1987; Haaland & Thomas, 1988; Brereton, 2003).

### 2.3.4 Partial least squares (PLS) regression

PLS is a regression method that permits the use of several wavelengths or even the entire spectrum while avoiding the problem of that affects MLR (Brereton, 2003). Unlike traditional least squares methods, PLS does not assume that spectral data are exact and that all errors are in constituent values. Instead, the spectral and constituent data are simultaneously modelled in steps that incrementally account for spectral signal and constituent values (Wold *et al*., 2001; Brereton, 2003).

In each step, part of the spectral data (factor) and a corresponding part of the constituent data is subtracted from the data set, leaving spectral and constituent residuals. With the determination of each factor, the residual information in the calibration data set becomes smaller and smaller. Partial calibrations for each factor (loadings for spectral data and scores for constituent values) are used to calculate the amount of variance modelled for each factor. At the end of the process, they are assembled into one overall calibration equation (Brereton, 2003; FOSS, 2004; Metrohm, 2015).

The calibration models can be tested by internal (cross validation) or external validation using a validation or test set. The validation set must be an independent sample set and is used to obtain statistical information from the predictive ability of the generated calibration model (Hasley, 1998; Reich, 2005).

The accuracy and precision should compare to that of the reference method. The slope and bias must be evaluated for significance, but the coefficient of determination ($R^2$) has the same relevance as the correlation coefficient (r) with normal univariate methods. The root mean square error of calibration (RMSEC or SEC), root mean square error of prediction (RMSEP or SEP) and residuals need to be evaluated (Williams *et al*., 2017).

### *2.3.4.1 Pre-processing calibration data*

While the PLS calibration is performed, spectral and constituent data of the training set are pre-processed and is not the same operations on spectra before calibration (e.g. second derivative). The vector of constituent values is mean centered and scaled to variance of one. The final calibration equation accounts for the mean values and scaling factors (Hasley, 1998; Reich, 2005).

Before calculating spectral loadings, weighting vectors (weights) are calculated to determine how effectively the data at each wavelength explains residual concentration values. The software scales the data so weights are proportional to the product of correlation and variance in the spectral data, with the result that wavelengths with high absorptivity are emphasized (Brereton, 2003; FOSS, 2004; Metrohm, 2015).

### *2.3.4.2 Selecting the number of factors*

Usually the software allows more PLS factors to be calculated than is suitable for use in a final calibration. An important part of PLS calibration is deciding how many factors to use. With too few factors *under-fitting* occurs and with too many factors *over-fitting* which result in an unstable model prone to prediction errors (Hasley, 1998; Reich, 2005).

The optimal number of factors is determined during cross validation (CV) or using the *external* prediction set, if available. The Vision® 3.50 software (NIRSystems Inc. Silver Springs, Maryland, USA) calculates the prediction residual error sum of squares (PRESS) for each factor and recommends the optimum number of factors with the minimum PRESS value. However, this is not necessarily the optimum number of factors to use and must be done cautiously (Hasley, 1998; FOSS, 2004; Metrohm, 2015).

#### 2.3.4.3 Cross validation

Instead of using *external* validation samples to calculate the PRESS values, a CV can be performed using the training set. In CV, samples in the training set are grouped into subsets that contain several or only one sample. During CV, one subset is withheld while a calibration is created with the remaining training samples. This calibration is then used to analyse samples in the subset as unknowns. Finally, the predicted constituent values are subtracted from the reference values, and their differences squared and summed. The first subset is returned to the training set and in turn, the remaining subsets are analysed in the same manner as the first (Hasley, 1998; FOSS, 2004; Metrohm, 2015). Thus, the PRESS value at each factor is an indicator of how well the PLS model performs. The root mean squared error of cross validation (RMSECV or SECV) is another performance indicator calculated during CV and shows the goodness of the calibration (Hasley, 1998; Reich, 2005).

### 2.3.5 Statistical evaluation of calibration equations

Several parameters are calculated during calibration or prediction of the validation set and indicate the quality of the calibration equation, thus showing its usefulness in predicting unknowns (Williams *et al*., 2017).

#### 2.3.5.1 Multiple correlation coefficient

Multiple correlation coefficient ($R^2$), also called *coefficient of determination,* is a measure of how well the spectral data and the constituent values are correlated. When $R^2$ is equal to zero, the spectral response is unrelated to constituent data and a value of one signifies that the constituent values fit spectral data perfectly and all residuals are equal to zero (Hasley, 1998; FOSS, 2004; Metrohm, 2015).

#### 2.3.5.2 Standard error of calibration

Standard error of calibration (SEC) indicates how well the calibration equation can reproduce constituent values of spectra used to create the calibration. SEC is the standard deviation of the differences between the reference values and the NIR values derived in the calibration step (Metrohm, 2014). When the calibration equation is applied to the training set itself, the SEC is calculated from residuals *f* as (Eq. 2.5):

$$SEC = \sqrt{\frac{\sum f_1^2}{N - K - 1}}$$

**Equation 2.5**

Where *N* is the number of samples and *K* number of wavelengths or factors. The SEC should be similar in value to the SEL of the reference method (Hasley, 1998; FOSS, 2004; Metrohm, 2015).

#### *2.3.5.3 Standard error of prediction*

The calculation for SEP is similar in form to that for SEC, except that *f* denotes residuals obtained from the prediction of the samples not used in the calibration, i.e. the *external* validation set and the denominator of the expression does not include *K*. SEP is usually larger than SEC (Hasley, 1998; FOSS, 2004; Metrohm, 2015).

#### *2.3.5.4 The F-statistic*

F-test statistic (F value) is a useful estimate of goodness of fit of spectral and constituent data. It can also be used as a tool for evaluation of how many factors should be used during regression and for determining which samples to eliminate as outliers from the calibration set (Hasley, 1998; Reich, 2005).

#### *2.3.5.5 Slope and bias*

The slope and bias are indicators of the accuracy and linearity of the calibration model. Bias is the average value of residuals calculated from constituent values of a prediction set. A slope close to one and a bias close to zero indicates that the deviations are distributed randomly. A large bias value (either positive or negative) compared to the typical constituent values indicates systematic error (Hasley, 1998; FOSS, 2004; Metrohm, 2015).

### 2.3.6 NIR method validation criteria

To meet regulatory requirements pharmaceutical method validation is required. The United States Pharmacopoeia (USP, 2013*b*), British Pharmacopoeia (BP, 2011*b*), Pharmaceutical Analytical Sciences Group (PASG, 2002), the European Medicines Agency (EMA, 2014) and the International Conference on Harmonisation (ICH, 2005) and the European Medicines Agency (EMA) published guidelines on method development and validation of NIR spectroscopy methods to assist industry to comply with regulatory requirements. Validation criteria for a qualitative method include specificity and robustness; whereas, for quantitative methods specificity, linearity, accuracy, precision, range, robustness, and outliers are included. These criteria are summarised in *Chapter 3 (Table 3.1).*

In NIR spectroscopy methods the standard error of the reference method is carried over to the NIR spectroscopy method, therefore, cannot be better than the reference method, but rather equivalent. The purpose for this paper was to determine if there were a considerable difference between the alternative and compendial method, and thus demonstrate performance equivalence, through validation to meet the criteria as prescribed by the pharmacopoeias and other industry guidelines (Hauck *et al*, 2009).

## 2.4 NIR spectroscopy applications

The first NIR spectroscopy calibrations were developed in 1973 by Phil Williams for the analyses of cereal products using a commercial grain analyser and together with Karl Norris published a book on the commercial use of NIR technology in the agricultural and food Industries (Norris & Williams, 1987). Since then, the field of NIR spectroscopy has become a popular method to perform rapid, non-destructive analyses on a wide variety of matrices across the agriculture and food industries. NIR analysis has been successfully implemented in the pharmaceutical and chemical industries for more than 25 years (Workman, 2014).

### 2.4.1 Agriculture and food applications

The agricultural and food industries have developed numerous applications using NIR spectroscopy as a rapid and non-destructive technique for measuring food quality (Li-Chan *et al*., 2011). The focus has been the prediction of fat, protein and moisture content in grains and feeds for commercial farming product (Cen & He, 2007), but later also included quantification of fatty acids in forages (Foster *et al*., 2006) and the enforcement of legislation concerning the use of animal by-products in animal feeds (Garrido-Varo *et al*., 2005).

The industry has since expanded in predicting not only the chemical properties but also quality attributes such as post-storage quality of fruit (Myburgh, 2003) and glassiness of processed French-fried potatoes (Sadie, 2005). Further applications include the analyses of nutrients in different potato species (Burlingame *et al*., 2009), measurement of carotenoid concentration among maize genotypes (Berardo *et al*., 2004) and quantification of vitamin C in navel oranges (Xia *et al* 2007);

Numerous successful applications have also been reported in the brandy and distillation industry (De Bruyn, 2003) as well as applications in the wine, fruit and dried fruit industries (Van Zyl, 2000) of South Africa. Instrument manufacturers have also specialised in providing instruments which have built-in calibration models for specific applications.

### 2.4.2 Chemical applications

In the chemical industries NIR spectroscopy has been implemented as an application for the analysis of fertiliser (Morris, 2003), plastics and polymers (Bunding Lee & Johnson, 1993; Camacho & Karlsson, 2001) as well as the petrochemical industry for prediction of various properties of petrol and diesel (Macho & Larrechi, 2002; Correia *et al*., 2018). The pulp and paper industries also use NIR spectroscopy for quality control of properties such as lignin content, moisture, resin and component analysis (Antti *et al*., 1996; Schimleck *et al*., 2000).

### 2.4.3 Nutraceutical and pharmaceutical applications

The pharmaceutical industry and hence the nutraceutical industry are strictly regulated and cautiously accepted the application of NIR spectroscopy as an alternative technique to the compendial methods. The key to successful implementation of NIR spectroscopy is to prove that the method is better or equivalent to that of the prescribed method of testing through validation (Hauck *et al*., 2009).

Initial applications focused on raw material testing, but the focus has shifted to the analysis of solid and liquid formulations for product quality and in-process monitoring of manufacturing processes (Reich, 2005). For material inspection, samples are scanned as received and the identity and quality of a material is confirmed using pattern recognition algorithms. NIR spectroscopy, in conjunction with chemometrics, is an in-process test and measurement tool that provides near real-time chemical information for control of chemical production processes and solvent recovery systems as well as drying, blending, and extrusion operations (Blanco *et al*., 1998; Metrohm, 2014). Further applications include the characterisation of API's, excipients, dosage forms, manufacturing intermediates, chemical raw materials and packaging materials. NIR spectroscopy is also applied in determination of chemical values such as the hydroxyl value, iodine value, acid value, water content, determination of degree of hydroxylation and control of solvent content (BP, 2011*b*; USP, 2013*b*).

NIR spectroscopy has been identified as an ideal technique to study intact tablets and capsules. Extensive research has been done on the application of NIR spectroscopy for the analysis of soft-gel capsules (SGC) such as the use of NIR spectroscopy to study storage-induced changes in SGC shell and fill characteristics (Reich, 2004); monitoring of SGC shell/fill interactions and changes of water and glycerol content in SGC shells (Reich & Bold, 2006); and to monitor water uptake and degree of cross-linking in gelatine capsules (Gold, 1998).

Further, it was identified as a suitable technique to detect API's by recording spectra of empty gelatine shells, excipients and liquid fill, after which the spectrum of the API was determined by spectral subtraction (Mohan *et al*., 2000). Another study reported the use of UV-Vis spectrophotometry for the simultaneous determination of fat and water-soluble vitamins in pharmaceutical multi-vitamin formulations in conjunction with NIR spectroscopy for moisture content with the use of a fibre optic probe (Rovira, 1995).

The use of analytical technology in the production environment to monitor processes is referred to as process analytical technology (PAT). PAT is a FDA initiative established (EMA, 2012) to stimulate the pharmaceutical industry by enabling the use of analytical technology not only during drug research and development, but also during manufacturing. Analytical technology is used to monitor processes in real-time, thus the sample is not submitted to the QC laboratory for analysis, but a result is obtained at the production line (Berntsson *et al*., 1997; Huang *et al*., 2008). NIR spectroscopy is not the only, but the most widely used technology in PAT due to its versatility.

Medicinal products must comply with their approved specifications before it can be released into the market. Compliance with release specifications can be demonstrated by performing a complete set of tests on the active substance and/or finished product, according to the approved specifications. Under certain conditions, an alternative strategy to systematic finished product testing is possible. To date this concept has been mainly applied to sterility testing of terminally sterilised products and has become associated with parametric release applications. Recent guidelines adopted in the International Conference on Harmonisation (ICH) context, namely ICH Q8, Q9 and Q10, have made it possible to apply a similar release decision process to tests other than sterility, this approach has been called Real Time Release Testing (RTRT), (EMA, 2012; ICH, 2005).

RTRT is comprised of a combination of process controls which may utilise PAT tools such as NIR and Raman spectroscopy in combination with chemometrics, together with the control of relevant material attributes. Spectral data monitored on-line controlling content of active substance, polymorphism, water content, blending homogeneity, particle properties or film thickness could thereby replace finished product testing such as uniformity of content, tablet strength and drug dissolution (EMA, 2012). The time and cost-savings will significantly improve the efficiency of the production processes if successfully implemented; therefore, it is important to ‘close the loop’ when it comes to PAT and not just replace QC methods with NIR spectroscopy methods (Maes & Van Liederkerke, 2006).

It is evident, that within the pharmaceutical/nutraceutical industry the analysis of intact tablets and capsules with a non-destructive technique such as NIR spectroscopy improves the cost-efficiency of the production processes. However, the research done on pharmaceutical dosage forms are limited to a few components and do not measure the complex matrices of nutraceutical preparations.

In the nutraceutical industry NIR spectroscopy have been applied to monitor quality parameters of herbal plants and extracts such as devil's claw (Gray, 2003) and St John's wort (Rager *et al*., 2002), South African herbal teas such as rooibos and honeybush (Joubert *et al*., 2008) and various other medicinal plants (Laasonen, 2003). Applications for other nutraceuticals which have been developed using NIR spectroscopy include the quantification of vitamin C in food and in pharmaceutical products such as powders and solutions (Yang & Irudayaraj, 2002), detecting and quantifying sunflower oil adulteration in extra virgin olive oils (Downey *et al*., 2002) and fatty acid determination in sunflower oil (Azizian *et al*., 2007).

Numerous studies have been published with the focus on detection of authenticity and adulterants in fish products containing PUFA's such as the determination of free fatty acids in fish oils (Cozzolino *et al*., 2005; Azizian *et al*., 2010; Bekhit *et al*., 2014; Jianhua, 2014; Cai *et al*., 2016), detecting adulteration of fishmeal (Murray *et al*., 2001; Cozzolino *et al*., 2002; Cozzolino *et al*., 2005; Cascant *et al*., 2018) and adulteration of fish oil (Zhang *et al*., 2013). The evaluation of fish quality (Liu *et al*., 2013), the determination of edible oil parameters (Armenta, *et al*., 2007) and process control of an esterification reaction of fish oil (Blanco, *et al*., 2006) are some of the many applications reported in the fish oil industry.

### 2.4.4 Medical applications

The medical industry used NIR technology for brain imaging instruments, known as non-invasive brain computer interfaces (BCI), such as functional near-infrared spectroscopy (fNIRS) in conjunction with another BCI i.e. electroencephalography (EEG), (Hatakenaka *et al*., 2007; Yamada *et al*., 2012; Torricelli *et al*., 2014). EEG and fNIRS are both very informative techniques when used separately, but combined they form a much more powerful technique due to the complementary mutual information (MI), (Fazli *et al*., 2012). The MI is determined by applying an algorithm to the data obtained from fNIRS with data EEG; however, in this study, mutual information (Srinivasa, 2005) is applied as a concept of superimposition (Venn, 1880).

## 2.5 Conclusion

The high demand of supplements caused various products to enter the market, increasing the risk of adulteration. The intentional neglect of QC tests on materials and products to save costs, are classified as economic adulteration and therefore inadequate QC or the use of non-validated methods renders an adulterated product. The SA nutraceutical industry is not appropriately regulated and label claims are often inaccurate. Current QC methods for omega-3 fish oils incorporate costly techniques such as GC, but excellent progress has been made in investigating cost effective alternatives using vibrational spectroscopy. Numerous studies have established that NIR spectroscopy is a viable alternative QC method, but none of the studies referenced employed pharmaceutical validation criteria to prove equivalence. The data sets of above-mentioned studies were evaluated in a relatively short period and did not incorporate long-term variation in omega-3 fish oil products. NIR spectroscopy is a rapid and cost-effective technique which can replace the prescribed method if it is proven to be equivalent, through validation, to the criteria as prescribed by the pharmacopoeias and other industry guidelines.

## 2.6 References

Akanbi, T.O. & Barrow, C.J., 2018. Compositional information useful for authentication of krill oil and the detection of adulterants. *Food Analytical Methods*, 11(1):178-187.

Anastas, P.T. & Kirchhoff, M.M., 2002. Origins, current status, and future challenges of green chemistry. *Accounts of Chemical Research*, 35(9):686-694.

Antti, H., Sjöström, M. & Wallbäcks, L., 1996, Multivariate calibration models using NIR spectroscopy on pulp and paper industrial applications. *Journal of Chemometrics*, 10(5-6):591-603.

Armenta, S., Garrigues, S. & De la Guardia, M., 2007. Determination of edible oil parameters by near infrared spectrometry. *Analytica Chimica Acta*, 596(2):330-337.

Azizian, H., Kramer, J.K. & Mossoba, M.M., 2010. Progression to fatty acid profiling of edible fats and oils using vibrational spectroscopy. In *Applications of Vibrational Spectroscopy in Food Science, Vol II. Analysis of food drink and related materials* (Li-Chan, E.C.Y., Griffiths, P.R. and Chalmers, J.M. (eds), Chichester: Wiley:519-537.

Azizian, H., Kramer, J.K. & Winsborough, S., 2007. Factors influencing the fatty acid determination in fats and oils using Fourier transform near-infrared spectroscopy. *European Journal of Lipid Science and Technology*, 109(9):960-968.

Azizian, H., Kramer, J.K., Ehler, S. & Curtis, J.M., 2010. Rapid quantitation of fish oil fatty acids and their ethyl esters by FT-NIR models. *European Journal of Lipid Science and Technology*, *112*(4):452-462.

Beebe, K.R. & Kowalski, B.R., 1987. An introduction to multivariate calibration and analysis. *Analytical Chemistry*, 59(17):1007A–1017A.

Bekhit, M.Y., Grung, B. & Mjøs, S.A., 2014. Determination of omega-3 fatty acids in fish oil supplements using vibrational spectroscopy and chemometric methods*. Applied Spectroscopy*, 68(10):1190-1200.

Berardo, N., Brenna, O.V., Amato, A., Valoti, P., Pisacane, V. & Motto, M., 2004. Carotenoids concentration among maize genotypes measured by near infrared reflectance spectroscopy (NIRS). *Innovative Food Science and Emerging Technologies*, 5(3):393-398.

Berntsson, O., Zackrisson, G. & Östling, G., 1997. Determination of moisture in hard gelatin capsules using near-infrared spectroscopy: applications to at-line process control of pharmaceutics. *Journal of Pharmaceutical and Biomedical Analysis*, 15(7):895-900.

Blanco, M. and Villarroya, I.N.I.R., 2002. NIR spectroscopy: a rapid-response analytical tool. *TrAC Trends in Analytical Chemistry*, *21*(4):240-250.

Blanco, M., Castillo, M., Peinado, A. & Beneyto, R., 2006. Application of multivariate curve resolution to chemical process control of an esterification reaction monitored by near-infrared spectroscopy. *Applied Spectroscopy*, 60(6):641-647.

Blanco, M., Coello, J., Iturriaga, H., Maspoch, S. & de la Pezuela, C., 1998, Critical review: Near-infrared spectroscopy in the pharmaceutical industry. *The Analyst*, 123:125R-150R.

BP *see* British Pharmacopoeia

Breivik, H. & Kristinsson, B., 1997. Preparation of highly purified concentrates of eicosapentaenoic acid and docosahexaenoic acid. *Journal of the American Oil Chemists' Society*, 74(11):1425-29.

Brereton, R.G., 2003. *Chemometrics: data analysis for the laboratory and chemical plant*. Chichester: John Wiley & Sons.

British Pharmacopoeia Commission, 2011*a*. Composition of fatty acids, Appendix XP1. In *BP 2012*, Great Britain: Stationery Office: A301.

British Pharmacopoeia Commission, 2011*b.* Infrared spectrophotometry, Appendix II A. In *BP 2012*, Great Britain: Stationery Office: A156.

Burlingame, B., Mouillé, B. & Charrondière, R., 2009. Nutrients, bioactive non-nutrients and anti-nutrients in potatoes. *Journal of Food Composition and Analysis*, 22(6):494-502.

Burns, D.A. & Ciurczak, E.W. (eds), 2001. *Handbook of Near-Infrared Analysis,* (2nd ed.). New York: Marcel & Dekker.

Cai, J., Hu, W. & Wang, X., 2016. Near-infrared spectrum detection of fish oil eicosapentaenoic acid content based on combinational filtering. *Transactions of the Chinese Society of Agricultural Engineering*, 32(1):312-317.

Camacho, W. & Karlsson, S., 2001. NIR, DSC and FTIR as quantitative methods for compositional analysis of blends of polymers obtained from recycled mixed plastic waste. *Polymer Engineering and Science*, 41(9):1626-1635.

Cascant, M.M., Breil, C., Fabiano-Tixier, A.S., Chemat, F., Garrigues, S. & de la Guardia, M., 2018. Determination of fatty acids and lipid classes in salmon oil by near infrared spectroscopy. *Food Chemistry*, 239:865-871.

Cermak, S. C., Evangelista, R. L. & Kenar, J. A., 2012. Distillation of natural fatty acids and their chemical derivatives. In *Distillation - Advances from Modelling to Applications: InTech,* 5:109-140.

Chauhan, B., Kumar, G., Kalam, N. & Ansari, S.H., 2013. Current concepts and prospects of herbal nutraceutical: A review. *Journal of Advanced Pharmaceutical Technology and Research*, 4(1):4-8.

Chintale Ashwini, G., Kadam Vaishali, S., Sakhare Ram, S., Birajdar Ganesh, O. & Nalwad Digambar, N., 2013. Role of nutraceuticals in various diseases: a comprehensive review. *International Journal of Research in Pharmacy and Chemistry,* 3:290-299.

Ciurczak, E., 2008. Examination of NIR Spectrometers: Dispersive vs. interferometric types, *American Pharmaceutical Review*, 11(4):3-5.

CMA *see* Complementary Medicines Australia (formerly the Complementary Health Council of Australia)

Colson, K.L., Yuk, J. & Luchsinger, S., 2014. What's in your “natural” supplement? Application of NMR spectroscopy in nutraceuticals. *Planta Medica*, 80(10):23.

Complementary Medicines Australia (CMA), 2012. Guideline for the quality and safety of raw materials used in complementary medicines, ver. 1, January. http://www.cmaustralia.org.au/Resources/Documents/Codes%20and%20Guidelines/The%20CHC%20Raw%20Material%20Guidelines%20.pdf [01 December 2017].

Correia, R.M., Domingos, E., Cáo, V.M., Araujo, B.R., Sena, S., Pinheiro, L.U., Fontes, A.M., Aquino, L.F.M., Ferreira, E.C., Filgueiras, P.R. & Romão, W., 2018. Portable near infrared spectroscopy applied to fuel quality control. *Talanta*, 176:26-33.

Cozzolino, D.(ed.), 2017. Vibrational and Fluorescence Spectroscopy. In *Food Authentication: Management, Analysis and Regulation*. Chichester: John Wiley & Sons:277-298. https://doi.org/10.1002/9781118810224.ch8a

Cozzolino, D., Chree, A., Murray, I. & Scaife, J.R., 2002. The assessment of the chemical composition of fishmeal by near infrared reflectance spectroscopy. *Aquaculture Nutrition*, 8(2):149-155.

Cozzolino, D., Chree, A., Scaife, J.R. & Murray, I., 2005. Usefulness of near-infrared reflectance (NIR) spectroscopy and chemometrics to discriminate fishmeal batches made with different fish species. *Journal of Agricultural and Food Chemistry*, 53(11):4459-4463.

Cozzolino, D., Murray, A.I., Chree, A. & Scaife, J.R., 2005. Multivariate determination of free fatty acids and moisture in fish oils by partial least-squares regression and near-infrared spectroscopy. *LWT - Food Science and Technology*, *38*(8):821-828.

De Bruyn, N., 2003. Future applications of near infrared spectroscopy (NIRS) in the South African brandy and distillation industry. South Africa, Cape Town: Stellenbosch University. (MSc dissertassion)

Dieber-Rotheneder, M., Puhl, H., Waeg, G., Striegl, G. & Esterbauer, H., 1991. Effect of oral supplementation with d-alpha-tocopherol on the vitamin E content of human low density lipoproteins and resistance to oxidation. *Journal of Lipid Research*, 32(8):1325-1332.

Downey, G., McIntyre, P. & Davies, A.N., 2002. Detecting and quantifying sunflower oil adulteration in extra virgin olive oils from the Eastern Mediterranean by visible and near-infrared spectroscopy. *Journal of Agricultural and Food Chemistry*, 50(20):5520-5525.

Dyerberg, J., Madsen, P., Møller, J.M., Aardestrup, I. & Schmidt, E.B., 2010. Bioavailability of marine n-3 fatty acid formulations. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 83(3):137-141.

ECA *see* European Compliance Academy

EMA *see* European Medicines Agency

European Compliance Academy (ECA), 2018. *Warning letter due to non-validated Analytical Methods*. https://www.gmp-compliance.org/gmp-news/warning-letter-due-to-non-validated-analytical-methods [16 May 2018].

European Medicines Agency (EMA), 2012. *Guideline on real time release testing (formerly guideline on parametric release).* http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/04/WC500125401.pdf [01 December 2017].

European Medicines Agency (EMA), 2014. *Guideline on the use of near infrared spectroscopy by the pharmaceutical industry and the data requirements for new submissions and variations*. http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000764.jsp&mid= [01 December 2017].

Everstine, K., Abt, E., McColl, D., Popping, B., Morrison-Rowe, S., Lane, R.W., Scimeca, J., Winter, C., Ebert, A., Moore, J.C. & Chin, H.B., 2017. Development of a hazard classification scheme for substances used in the fraudulent adulteration of foods. *Journal of Food Protection*, 81(1):31-36.

Everstine, K., Spink, J. and Kennedy, S., 2013. Economically motivated adulteration (EMA) of food: common characteristics of EMA incidents. *Journal of Food Protection*, 76(4):723-735.

Fardet, A., 2015. Complex foods versus functional foods, nutraceuticals and dietary supplements. *Agro Food Industry Hi Tech*, 26(2):20-24.

Fazli, S., Mehnert, J., Steinbrink, J., Curio, G., Villringer, A., Müller, K.R. & Blankertz, B., 2012. Enhanced performance by a hybrid NIRS-EEG brain computer interface. *Neuroimage*, *59*(1):519-529.

FDA *see* Food and Drug Administration of United States of America

Food and Drug Administration (FDA) of United States of America, 1994. Dietary Supplement Health and Education Act (DSHEA). Public Law 103-417. 108 Stat 4325, *Government Publishing Office*, United States, 25 October 1994. https://www.gpo.gov/fdsys/pkg/STATUTE-108/pdf/STATUTE-108-Pg4325.pdf [20 August 2018].

Food and Drug Administration (FDA) of United States of America, 2007. Good manufacturing practices (GMP's) for dietary supplements. https://www.fda.gov/Food/GuidanceRegulation/CGMP/ucm079496.htm [01 December 2017].

Food and Drug Administration (FDA) of United States of America, 2016*a*. Contract manufacturing arrangements for drugs: quality agreements, guidance for industry, November. https://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm353925.pdf [16 May 2018].

Food and Drug Administration (FDA) of United States of America, 2016*b*. Warning letter: Quali Controle & Qual-Controle-C.E.Bac 3/5/18, https://www.fda.gov/ICECI/EnforcementActions/WarningLetters/ucm599876.htm [16 May 2018].

FOSS, 2004. Vision manual – Theory. Silver Spring, MD. FOSS NIRSystems, Inc.:1-36

Gabriels, G., Lambert, M. & Smith, P., 2012. Information on nutritional supplement labels: time for legislation? *South African Journal of Clinical Nutrition*, *25*(1):22-26.

Garrido-Varo, A., Pérez-Marn, M.D., Guerrero, J.E., Gmez-Cabrera, A., de la Haba, M.J., Bautista, J., Soldado, A., Vicente, F., Martnez, A., de la Roza-Delgado, B. & Termes, S., 2005. Near infrared spectroscopy for enforcement of European legislation concerning the use of animal by-products in animal feeds. *Biotechnologie, Agronomie, Société et Environnement*, 9(1):3-9.

Geladi, P., 2003. Chemometrics in spectroscopy, Part 1. Classical chemometrics. Spectrochimica Acta Part B. *Atomic Spectroscopy*, 58(5):767-782.

Gemperline, P. (ed.), 2006. *Practical guide to chemometrics,* (2nd ed.). Boca Raton, FL: CRC press:77-86.

Global Organization for EPA and DHA Omega-3, 2015. Voluntary Monograph, ver.5, November. http://goedomega3.com/index.php/goed-monograph [01 December 2017].

GOED *see* Global Organization for EPA and DHA Omega-3

Gold, T. B.,1998. Studies on the chemistry of crosslinking of gelatin capsules. United States, Kentucky: *University of Kentucky*. (Ph.D. dissertation).

Govindaraghavan, S., 2014. Pharmacopeial HPLC identification methods are not sufficient to detect adulterations in commercial Bilberry (*Vaccinium myrtillus*) extracts. Anthocyanin profile provides additional clues. *Fitoterapia*, *99*:124-138.

Gray, B.R., 2003. Retention of the harpagoside content in dried *Harpagophytum procumbens* (Devil's claw) root through controlled drying and the application of near infrared spectroscopy (NIRS) as rapid method of determination. South Africa, Cape Town: Stellenbosch University. (MSc dissertassion)

Haaland, D.M. & Thomas, E.V., 1988. Partial least-squares methods for spectral analyses. 1. Relation to other quantitative calibration methods and the extraction of qualitative information. *Analytical Chemistry*, 60(11):1193-1202.

Haleem, R.M., Salem, M.Y., Fatahallah, F.A. & Abdelfattah, L.E., 2015. Quality in the pharmaceutical industry - A literature review. *Saudi Pharmaceutical Journal*, 23(5):463-469.

Hamed, I., Özogul, F., Özogul, Y. & Regenstein, J. M., 2015. Marine bioactive compounds and their health benefits: a review. *Comprehensive Reviews in Food Science and Food Safety*, 14(4):446-465.

Hamilton, K., Brooks, P., Holmes, M., Cunningham, J. & Russell, F.D., 2010. Evaluation of the composition of omega-3 fatty acids in dietary oil supplements. *Nutrition and Dietetics*, 67(3):182-189.

Hasley, S.A., 1994. *Qualitative NIR calibration protocol*. Silver Spring, MD: NIRSystems Inc.

Hasley, S.A., 1998. *Quantitative NIR calibration protocol*. Silver Spring, MD: NIRSystems Inc.

Hatakenaka, M., Miyai, I., Mihara, M., Sakoda, S. and Kubota, K., 2007. Frontal regions involved in learning of motor skill - a functional NIRS study. *Neuroimage*, 34(1):109-116.

Hauck, W.W., De Stefano, A.J., Cecil, T.L., Abernethy, D.R., Koch, W.F. & Williams, R.L., 2009. Stimuli to Revision Process: Acceptable, equivalent, or better - Approaches for alternatives to official compendial procedures. *United States Pharmacopeial Forum*, 35(3):772-778.

Hibbert, D.B., Minkkinen, P., Faber, N.M. & Wise, B.M., 2009. IUPAC project: A glossary of concepts and terms in chemometrics. *Analytica Chimica Acta*, 642(1-2):3-5.

Huang, H., Yu, H., Xu, H. & Ying, Y., 2008. Near infrared spectroscopy for on/in-line monitoring of quality in foods and beverages: A review. *Journal of Food Engineering*, 87(3):303-313.

Hunt, J.R., 1994. Nutritional products for specific health benefits—Foods, pharmaceuticals, or something in between? *Journal of the American Dietetic Association*, 94(2):151-153.

ICH *see* International Conference on Harmonisation

IFT *see* Institute of Food Technologists

Institute of Food Technologists (IFT), 1998. Functional Foods: Their role in disease prevention and health promotion, *Food Technology Magazine*, 52(11):63-70.

International Conference on Harmonisation (ICH) of technical requirements for registration of pharmaceuticals for human use, 2005. Validation of analytical procedures: text and methodology, Q2 (R1), ICH Harmonised Tripartite Guideline. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf [14 April 2018].

Ismail, A., Bannenberg, G., Rice, H.B., Schutt, E. & MacKay, D., 2016. Oxidation in EPA- and DHA-rich oils: an overview. *Lipid Technology*, 28(3-4):55-59.

Jianhua, C.A.I., 2014. Near-infrared spectrum detection of fish oil DHA content based on empirical mode decomposition and independent component analysis. *Journal of Food and Nutrition Research*, 2(2):62-68.

Johnson, M. & Bradford, C., 2014. Omega-3, Omega-6 and Omega-9 Fatty acids: Implications for cardiovascular and other diseases. *Journal of Glycomics and Lipidomics,* 4(123):2153-0637.

Johnson, R, 2014. Food fraud and “economically motivated adulteration” of food and food ingredients. CRS report, 7-5700, R43358, 10 January 2014. Congressional Research Service, Washington, DC. http://foodfraud.msu.edu/wp-content/uploads/2014/01/CRS-Food-Fraud-and-EMA-2014-R43358.pdf [27 June 2018].

Joubert, E., Gelderblom, W.C.A., Louw, A. & de Beer, D., 2008. South African herbal teas: Aspalathus linearis, Cyclopia spp. and Athrixia phylicoides—A review. *Journal of Ethnopharmacology*, 119(3):376-412.

Kalra, E.K., 2003. Nutraceutical-definition and introduction. *The American Association of Pharmaceutical Scientists Journal*, 5(3):27-28.

Keservani, R.K., Sharma, A.K., Ahmad, F. & Baig, M.E., 2014. Nutraceutical and functional food regulations in India. In *Nutraceutical and Functional Food Regulations in the United States and Around the World,* (2nd ed.). San Diego, CA: Academic Press:327-342.

Kramer, K., Tseng, C.H. & Ma, K., 2013. Measuring the Levels of EPA and DHA in Nutraceutical Oils. In *Omega-6/3 Fatty Acids*. Humana Press, Totowa, NJ:365-384.

Laasonen, M., 2003. Near Infrared spectroscopy, a quality control tool for the different steps in the manufacture of herbal medicinal products. Finland, Helsinki: *University of Helsinki*. (Ph.D. dissertation).

Larkin, P. J., 2017. *Infrared and Raman spectroscopy: Principles and spectral interpretation*, (2nd ed.). Waltham, MA: Elsevier.

Bunding Lee, K.A. & Johnson, S.C., 1993. Comparison of Mid-IR with NIR in polymer analysis. *Applied Spectroscopy Reviews*, 28(3):231-284.

Levin, M.D., 2016. The new food current good manufacturing practices and their effect on dietary supplement quality: What you need to know. *Integrative Medicine: A Clinician's Journal*, 15(5):22-24.

Li-Chan, E., Chalmers, J. & Griffiths, P. (eds.), 2011. *Applications of vibrational spectroscopy in food science*. Chichester: John Wiley & Sons.

Lingzhi, X., Fei, G., Zengling, Y., Lujia, H. & Xian, L., 2016. Discriminant analysis of terrestrial animal fat and oil adulteration in fish oil by infrared spectroscopy. *International Journal of Agricultural and Biological Engineering*, 9(3):179-185.

Liu, D., Zeng, X.A. & Sun, D.W., 2013. NIR spectroscopy and imaging techniques for evaluation of fish quality - a review. *Applied Spectroscopy Reviews*, 48(8):609-628.

Liu, J. J., Galfalvy, H. C., Cooper, T. B., Oquendo, M. A., Grunebaum, M. F., Mann, J. J. & Sublette, M. E., 2013. Omega-3 polyunsaturated fatty acid status in major depression with comorbid anxiety disorders. *The Journal of Clinical Psychiatry*, 74(7):732-738.

Lohumi, S., Lee, S., Lee, H. & Cho, B.K., 2015. A review of vibrational spectroscopic techniques for the detection of food authenticity and adulteration. *Trends in Food Science and Technology*, 46(1):85-98.

Luypaert, J., Massart, D.L. & Vander Heyden, Y., 2007, Near-infrared spectroscopy applications in pharmaceutical analysis. *Talanta*, 72(3):865-883.

Macho, S. & Larrechi, M.S. 2002, Near-infrared spectroscopy and multivariate calibration for the quantitative determination of certain properties in the petrochemical industry. *Trends in Analytical Chemistry*, 21(12):799-806.

Maes, I. & Van Liedekerke, B., 2006. The need for a broader perspective if process analytical technology implementation is to be successful in the pharmaceutical sector. *Journal of Pharmaceutical Innovation*, 1(1):19-21.

Marieschi, M., Torelli, A., Beghé, D. & Bruni, R., 2016. Authentication of *Punica granatum L.*: Development of SCAR markers for the detection of 10 fruits potentially used in economically motivated adulteration. *Food Chemistry*, 202:438-444.

Mark, H. 2000. Quantitative spectroscopic calibration. In *Encyclopedia of Analytical Chemistry*, Meyers, R.A. (ed). Chichester: John Wiley & Sons:13587.

Martirosyan, D.M. and Singh, J., 2015. A new definition of functional food by FFC: what makes a new definition unique? *Functional Foods in Health and Disease*, 5(6):209-223.

Metrohm, 2014. *NIR Spectroscopy: A guide to near-infrared spectroscopic analysis of industrial manufacturing processes*. Herisau, Switzerland: Metrohm.

Metrohm, 2015. *NIRS Vision manual – Theory*. Herisau, Switzerland: Metrohm Ltd.:5-39.

Metrohm, 2017. *A Technology Comparison of Near Infrared Spectroscopy*, November 21. https://www.azom.com/article.aspx?ArticleID=14371 [23 August 2018].

Mohan, G., Ibechem, J. & Severdia, A., 2000. Application of near infrared (NIR) spectroscopy for Iidentification of active in liquid filled soft gelatin capsules. *American Association of Pharmaceutical Scientists Journal*, 2(S1), Annual Meeting Abstracts.

Morris, W.R., 2003. The analysis of fertiliser by means of near infrared (NIR) spectroscopy. South Africa, Cape Town: Stellenbosch University. (MSc dissertassion)

Murray, I., 2004. Scattered information: philosophy and practice of near infrared spectroscopy. In *Near Infrared Spectroscopy: Proceedings of the 11th International Conference*. Chichester: NIR Publications:1-12.

Murray, I., Aucott, L.S. & Pike, I.H., 2001. Use of discriminant analysis on visible and near infrared reflectance spectra to detect adulteration of fishmeal with meat and bone meal. *Journal of Near Infrared Spectroscopy*, 9(4):297-311.

Myburgh, L., 2003. Prediction of post-storage quality in canning apricots and peaches using near infrared spectroscopy (NIRS) and chemometrics. South Africa, Cape Town: Stellenbosch University. (MSc dissertassion)

Norris, K. H. & Williams, P (eds.), 1987. *Near-infrared technology in the agricultural and food industries*, St. Paul, MN. American Association of Cereal Chemists.

Nounou, M.I., Ko, Y., Helal, N.A. & Boltz, J.F., 2018. Adulteration and counterfeiting of online nutraceutical formulations in the United States: time for intervention?. *Journal of Dietary Supplements*, *15*(5):789-804.

Opperman, M. & Benade, A.S., 2011. Analysis of omega-3 fatty acid content of South African fish oil supplements: Cardiovascular topics. *Cardiovascular Journal of Africa*, 22(6):324-329.

PASG *see* Pharmaceutical Analytical Sciences Group

Pasquini, C., 2003. Near infrared spectroscopy: fundamentals, practical aspects and analytical applications. *Journal of the Brazilian Chemistry Society*, 14(2):198-219.

Pharmaceutical Analytical Sciences Group (PASG), 2002.PASG, NIR sub-Group: Guidelines for the development and validation of near-infrared spectroscopy methods in the pharmaceutical industry. http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.452.9685&rep=rep1&type=pdf [30 November 2017].

Pharmaceutical Inspection Co-operation Scheme (PIC/S), 2018. Guide to good manufacturing practice for medicinal products, July. https://www.picscheme.org/en/publications?tri=gmp [22 August 2018].

PIC/S *see* Pharmacuetical Inspection Co-orperation Scheme

Rager, I., Roos, G., Schmidt, P.C. & Kovar, K., 2002. Rapid quantification of constituents in St. John's wort extracts by NIR spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis*, 28(3-4):439-446.

Reich, G. & Bold, S., 2006. Non-destructive NIR monitoring of softgel shell/fill interactions. *American Association of Pharmaceutical Scientists Journal*, 8(S1), Annual Meeting Abstracts.

Reich, G., 2004. NIR spectroscopy – An alternative approach to assess storage-induced dissolution changes of hard and soft gelatin capsule shells. *American Association of Pharmaceutical Scientists Journal,* 6(S1), Annual Meeting Abstracts.

Reich, G., 2005. Near-infrared spectroscopy and imaging: Basic principles and pharmaceutical applications. *Advanced Drug Delivery Reviews*, 57(8):1109-1143.

Reid, L.M., O'donnell, C.P. & Downey, G., 2006. Recent technological advances for the determination of food authenticity. *Trends in Food Science and Technology*, 17(7):344-353.

Ritter, J.C.S., Budge, S.M., Jovica, F. & Reid, A.J.M., 2015. Oxidation rates of triacylglycerol and ethyl ester fish oils. *Journal of the American Oil Chemists' Society*, 92(4):561-569.

Rovira I Garcia, E., 1995. Analisi de control de preparats farmaceutics mitjancant les tecniques espectrofotometriques UV-visible i IR proper. Spain: *Universitat Autonoma de Barcelona*. (Ph.D. dissertation).

Ruxton, C.H.S., Reed, S.C., Simpson, M.J.A. & Millington, K.J., 2004. The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. *Journal of Human Nutrition and Dietetics*, 17(5):449-459.

Sadie, L., 2005. Characterisation of glassiness in commercially processed French fried potatoes. South Africa, Cape Town: Stellenbosch University. (MSc dissertassion)

Schimleck, L.R., Raymond, C.A., Beadle, C.L., Downes, G.M., Kube, P.D. & French, J., 2000. Applications of NIR spectroscopy to forest research. *Appita Journal: Journal of the Technical Association of the Australian and New Zealand Pulp and Paper Industry*, 53:458-464.

Shahidi, F. (ed.), 2005. *Bailey's Industrial Oil and Fat Products,* (6th ed.). Hoboken, NJ: John Wiley & Sons.

Simopoulos, A.P., 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedicine & Pharmacotherapy*, *56*(8):365-379.

Skoog, D.A., West, D.M., Holler, F.J. & Crouch, S., 2004. *Fundamentals of Analytical Chemistry,* (8th ed.). Belmont, CA: Thomson-Brooks/Cole.

South Africa. Department of Health, 2008. Medicines and Related Substance Act 101 of 1965. *Government Gazette*, 519(31387), July 14. http://www.mccza.com/documents/b335d1c0Act_101_of_1965_published_2003.pdf [22 December 2017].

South Africa. Department of Health, 2013. Medicines and Related Substance Act 101 of 1965, Notice R. 870 of 2013, *Government Gazette*, 581(37032):1-16, November 15.

South Africa. Department of Health, 2014. Medicines and Related Substance Act 101 of 1965, Notice R. 837 of 2014, *Government Gazette*, 593(38133):3-6, October 28.

South Africa. Department of Health, 2016. Foodstuffs, Cosmetics and Disinfectants Act 54 of 1972. Government Gazette, 921(40216), August 19. http://www.sahpra.org.za/documents/590f2f52GG40216_nn921_Cosmetics_for_comment_19August2016.pdf [22 December 2017].

South Africa. Department of Health, 2017. Medicines Control Council (MCC), Guide to good manufacturing practice for medicines in South Africa, ver. 6, December 2017. http://www.mccza.com/documents/c2add5ba4.01_SA_Guide_to_GMP_Dec17_v6.pdf [08 January 2018].

Sprague, M., Betancor, M., Dick, J. & Tocher, D., 2017. Nutritional evaluation of seafood, with respect to long-chain omega-3 fatty acids, available to UK consumers. *Proceedings of the Nutrition Society,* 76(OCE2).

Srinivasa, S., 2005. A review on multivariate mutual information. University of Notre Dame, Notre Dame, ID, 2:1-6.

Swanson, D., Block, R. & Mousa, S. A., 2012. Omega-3 fatty acids EPA and DHA: health benefits throughout life. *Advances in Nutrition,* 3(1):1-7.

Torricelli, A., Contini, D., Pifferi, A., Caffini, M., Re, R., Zucchelli, L. & Spinelli, L., 2014. Time domain functional NIRS imaging for human brain mapping. *Neuroimage*, 85:28-50.

United States Pharmacopoeia, 2013*a*. Fats and fixed oils, omega-3 fatty acids determination and profile, Chemical Test 401. In *USP 35–NF 30*, Rockville, MD: US Pharmacopeia: 167-172.

United States Pharmacopoeia, 2013*b*. Near-Infrared Spectroscopy, General chapter 1119. In *USP 35–NF 30*, Rockville, MD: US Pharmacopeia: 714-720.

United States Pharmacopoeia, 2017. Analytical Instrument Qualification, General chapter 1058. *In USP 40 (1st supplement)*. Rockville, MD: US Pharmacopeia.

USP *see* United States Pharmacopoeia

Van Breemen, R.B., Fong, H.H. & Farnsworth, N.R., 2007. The role of quality assurance and standardization in the safety of botanical dietary supplements. *Chemical Research in Toxicology*, 20(4):577-582.

Van Zyl, A., 2000. The application of Fourier transform near infrared (FT-NIR) spectroscopy in the wine, fruit and dried fruit industries of South Africa. South Africa, Cape Town: Stellenbosch University. (MSc dissertassion)

Venn, J., 1880. I. On the diagrammatic and mechanical representation of propositions and reasonings. *The London, Edinburgh, Dublin Philosophical Magazine and Journal of Science*, 10(59):1-18.

Vongsvivut, J., Heraud, P., Zhang, W., Kralovec, J.A., McNaughton, D. & Barrow, C.J., 2012. Quantitative determination of fatty acid compositions in micro-encapsulated fish-oil supplements using Fourier transform infrared (FTIR) spectroscopy. *Food Chemistry*, 135(2):603-609.

Wanasundara, U.N. & Shahidi, F., 1998. Lipase-assisted concentration of n-3 polyunsaturated fatty acids in acylglycerols from marine oils. *Journal of the American Oil Chemists' Society*, 75(8): 945-951.

Wang, L. & Weller, C.L., 2006. Recent advances in extraction of nutraceuticals from plants. *Trends in Food Science and Technology*, 17(6):300-312.

Wildman, R.E. (ed.), 2016. *Handbook of nutraceuticals and functional foods,* (2nd ed.). Boca Raton, FL: CRC press:8-20.

Williams, P., Dardenne, P. and Flinn, P., 2017. Tutorial: Items to be included in a report on a near infrared spectroscopy project, *Journal of Near Infrared Spectroscopy,* 25(2):85.

Wold, S., Sjöström, M. and Eriksson, L., 2001. PLS-regression: a basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems*, 58(2):109-130.

Workman, J. Jr, 2014. An Introduction to Near Infrared Spectroscopy, http://www.spectroscopynow.com/ir/details/education/sepspec1881education/An-Introduction-to-Near-Infrared-Spectroscopy.html [08 January 2018].

Wright, R. 2007. The state of nutraceutical testing, Nutraceutical World. http://www.thefreelibrary.com/The+state+of+nutraceutical+testing%3A+even+if+final+GMPs+for+dietary...-a0164927867 [29 September 2011].

Xia, J., Li, X., Li, P., Ma, Q. & Ding, X., 2007. Application of wavelet transform in the prediction of navel orange vitamin C content by near-infrared spectroscopy. *Agricultural Sciences in China*, 6(9):1067-1073.

Yamada, T., Umeyama, S. & Matsuda, K., 2012. Separation of fNIRS signals into functional and systemic components based on differences in hemodynamic modalities. *PloS ONE*, *7*(11):e50271.

Yang, H. & Irudayaraj, J., 2002. Rapid determination of vitamin C by NIR, MIR and FT-Raman techniques*. Journal of Pharmacy and Pharmacology*, 54(9):1247-1255.

Yoshida, S. & Yoshida, H., 2003. Nondestructive analyses of unsaturated fatty acid species in dietary oils by attenuated total reflectance with Fourier transform IR spectroscopy. *Biopolymers*, 70(4):604-613.

Zhang, Y., Cao, F. & He, Y., 2013. Application of visible and near infrared spectroscopy for rapid and non-invasive quantification of adulterants in fish oil. *Spectroscopy and Spectral Analysis*, 33(6):1532-1536.

# CHAPTER THREE

# RESEARCH RESULTS

## Enhancing near infrared spectroscopy models to identify omega-3 fish oils used in the nutraceutical industry by means of calibration range extension

This chapter was written in article format according the requirements of *The Journal of Near Infrared Spectroscopy (JNIRS)* and comprises the following sections: abstract, introduction, methods and materials, results and discussion, conclusion and references (referencing was done numerically in order of appearance). Some information may be repeated due to the article format of the thesis. Manuscript submitted to *JNIRS*: 19 December 2017. First published on-line: 23 August 2018.
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## 3.1 ABSTRACT

The high demand of omega-3 fish oil nutraceuticals (dietary supplements) is due to the numerous health benefits contributed by the polyunsaturated fatty acids (PUFA's). The nutraceutical industry is required to follow good manufacturing practice (GMP) standards in order to ensure label claims and prevent adulteration. It is vital that the quality control (QC) procedures will be able to detect adulterated products. It is thus necessary to ensure the analytical techniques are adequate by using validated methods. The qualification or identification of natural fish oils is a difficult task due to overlapping concentration ranges of PUFA's and other similar properties. Gas chromatography (GC) is the prescribed technique in the nutraceutical industry for analysis of omega-3 fatty acids, but it is time-consuming and costly. Near infrared (NIR) spectroscopy is a rapid and cost-effective technique which can replace the prescribed method if it is proven to be equivalent, through validation, to the criteria as prescribed by the pharmacopoeias and other industry guidelines. In this study, NIR spectroscopy in combination with chemometrics, was considered as an alternative method to GC, to identify various commercial fish oils and to quantify the PUFA's. Identification methods were developed for nine commercial omega-3 fish oils by using spectral libraries. Quantitative NIR methods were developed for arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in fish oils expressed as mg.g$^{-1}$ as well as % area using partial least squares (PLS) regression and independent validation by superimposing datasets with mutual properties. Based on the statistics in terms of SEC, R$^2$, SEP and r of the PUFA models, the NIR method was equivalent to the prescribed GC methods and precision results obtained were also within the prescribed criteria. NIR spectroscopy and chemometrics can be used for conclusive identification and quantification of omega-3 fish oils, thereby minimizing the risk of adulteration. The method also complied with the prescribed pharmaceutical method validation criteria; therefore, was proven as an alternative method to GC for the nutraceutical industry.

## 3.2. KEYWORDS

*adulteration, calibration models, chemometrics, fatty acids, fish oils, near infrared spectroscopy, nutraceuticals, omega-3, validation, quality control.*

## 3.3 INTRODUCTION

### 3.3.1 Omega-3 fish oils

The demand of omega-3 fish oil in the nutraceutical industry has grown rapidly over recent years, due to the increasing scientific evidence supporting the health issues such as inflammation, heart disease and mental development.[1] The health benefits of omega-3 fish oils have been studied extensively and are contributed to the polyunsaturated fatty acids (PUFA's), specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA),[2,3] as well as their ratio to the omega-6 PUFA arachidonic acid (AA).[4] According to a survey conducted by the Global Organization for EPA and DHA Omega-3's (GOED), global shipments of omega-3 oil containing EPA and DHA were estimated at 1.25 million metric tons in 2014.[5] Due to this high demand of omega-3 oil supplements containing EPA and DHA, numerous products from various sources[6] have flooded the markets. Not all products are manufactured according to good manufacturing practice (GMP) standards and the EPA and DHA label claims cannot be guaranteed.[7-9]

### 3.3.2 Regulation

The nutraceutical industry has often been scrutinized for the lack of regulation,[10] and it is imperative to the well-being of the consumer and the industry that the quality control (QC) procedures are properly implemented according to accepted standards such as the GOED Voluntary Monograph.[11] Most countries require that pharmaceutical companies must follow GMP procedures and have created their own guidelines that correspond with their legislation. In the United States (US) GMP is enforced by the US Food and Drug Administration (FDA) and the requirements need to be followed by dietary supplement (nutraceutical) manufacturers as well.[12] In South Africa, GMP inspections are performed by the Medicines Control Council (MCC).[13]

Adulteration is a significant issue for the nutraceutical industry and it is crucial that the QC procedures will be able to detect adulterated products.[14] According to the Complementary Health Council of Australia, adulteration is the presence of undeclared ingredients that impact adversely on the quality and safety of the product.[15] Diluting a high priced ingredient or replacing it with an inferior quality ingredient for economic gain is called economic adulteration.[14] Intentionally neglecting to perform required QC tests on materials received or manufactured finished products to save costs, are also classified as economic adulteration.[14] The 2007 review of the U.S. GMP guide was applied to the dietary supplements (*finished goods*) and not the dietary ingredients (raw materials), which rested the responsibility of ensuring quality on the dietary supplement manufacturer. A major revision of the GMP was implemented by the FDA in 2016 to include dietary ingredient manufacturers and economically motivated adulterants.[16] South African GMP were also revised to include specifications for nutraceutical raw materials and requires *specific distinctive tests if a substance is liable to be adulterated/substituted.*[13] Thus, nutraceutical companies, manufacturing raw material and/or finished goods, should take responsibility to ensure that their analytical techniques are able to detect adulteration and guarantee authenticity by using validated methods.

In 2017 the U.S. FDA inspected a drug contract testing laboratory in France and found that *non-validated analytical methods*,[17] which were not part of the pharmacopoeia monographs, were used for analysing GMP-liable products and therefore resulted in adulterated products. A warning letter was issued[18] which summarized significant violations of current good manufacturing practice (CGMP) regulations for testing finished pharmaceuticals; "*Because your methods, facilities, or controls for manufacturing, processing, packing, or holding do not conform to CGMP, your drugs are adulterated within the meaning of section 501(a)(2)(B) of the Federal Food, Drug, and Cosmetic Act (FD&C Act), 21 U.S.C. 351(a)(2)(B).*"

### 3.3.3 Quality Control

High performance liquid chromatography (HPLC), gas chromatography (GC) and thin layer chromatography (TLC) are commonly used for the identification of nutraceuticals. These techniques usually analyse for specific markers and not the material as a whole, therefore adulteration or contamination with other materials can be easily overlooked.[19-20] Non-destructive techniques such as Fourier transform infrared (FT-IR) spectroscopy as a screening method for adulterants in nutraceuticals,[21] have also been reported and is a more comprehensive approach by analysing the sample "as is".

The qualification (identification) of natural fish oils is a difficult task due to overlapping concentration ranges of PUFA's and other similar physical and chemical properties which cannot be used to distinguish between oils from different fish species,[11] and increases the risk of adulteration, e.g. substituting an expensive oil with a low-cost oil. Therefore, quantification alone is not sufficient to eliminate adulteration; identification to authenticate the oil is also required.[22] To further complicate matters, fish oils are also available in different forms. Fish oils naturally occur in the triglyceride (TG) form and can be converted to the ethyl ester (EE) form during molecular distillation. This is a process that separates, concentrates and purifies PUFA's such as omega-3 fatty acids in fish oil.[23] To detect adulteration in fish oils, the method must be able to discriminate between the relevant fish oils species, quantify the relevant PUFA's and distinguish between TG and EE forms.

GC is the prescribed technique in the pharmaceutical and nutraceutical industry for analysis of omega-3 fatty acids.[24,25] The GC analysis is a time-consuming and costly procedure due to extensive sample preparation and extractions. Instrument maintenance, certified reference standards and solvent waste all contribute to the costs. The GC method can be replaced by an alternative method if the performance was proved to be equivalent to that of the prescribed GC methods.[11,24,25]

Near infrared (NIR) spectroscopy is a rapid and cost-effective technique which, when accurate calibration models have been developed and independently validated (for each of the components of interest), can replace the prescribed method.[26] Various studies have been conducted investigating vibrational spectroscopy as a method for analysis of oils. FT-IR in combination with chemometrics has been identified as a classification method for different vegetable oils[27] and detecting adulteration of dietary supplement oils,[28] as well as characterization of dietary omega-3 fish oil supplements.[29] Portable FT-IR devices were also reported as a screening tool to verify label declarations of marine oil omega-3 dietary supplements.[30] Dispersive and FT-NIR techniques have been investigated and found to be useful for the quantification of omega-3 PUFA's in fish meal and fish oils.[31-34] FT-Raman was also found to be a valuable technique for quantification of PUFA's plus detection of ethyl esters and oxidation in fish oil capsules.[35] The potential of other non-destructive spectroscopic techniques for the analysis of PUFA's in fish and fish products were also considered, such as nuclear magnetic resonance (NMR) spectroscopy and hyperspectral imaging.[36]

### 3.3.4 Acceptable, equivalent or better

In this study, NIR spectroscopy and chemometrics were investigated as an alternative method to GC to detect adulteration by the identification of various commercial fish oils in the TG form and quantification of the PUFA's. According to a *Stimilu* article published by the United States Pharmacopeial Convention, Inc. (USPC)[37], the key to successful implementation of an alternative method/procedure in the pharmaceutical industry (hence nutraceutical industry) is to prove by comparison that it is either *acceptable, equivalent or better* than the prescribed compendial method. Depending on the desired purpose of the comparison, four options are available to determine if the alternative method can replace the compendial method. Option 1 applies if two methods both meet the minimum performance requirements, they are regarded as equivalent, thus *acceptable*, without direct comparison between the two methods. The other options apply direct comparison between the alternative and compendial method by *method-performance studies*. Option 2 determines if the alternative method is *equivalent or better* in performance, with direct comparison of validation criteria and *similar* results to the compendial method. Option 3 determines if the alternative method and the compendial method agree by numerical result, *results equivalence*, or by decision that is based on some numerical result, *decision equivalence*.[37]

In NIR spectroscopy methods the standard error of the reference method, also known as the standard error of the laboratory (SEL), is carried over to the NIR spectroscopy method, therefore, cannot be *better* than the reference method,[38] but rather *equivalent*. The purpose for this paper was to determine if there were a considerable difference between the alternative and compendial method, and thus demonstrate *performance equivalence* (option 2), through validation to meet the criteria as prescribed by the pharmacopoeias and other industry guidelines.

### 3.3.5 Pharmaceutical validation

The United States Pharmacopoeia (USP),[39] British Pharmacopoeia (BP),[40] Pharmaceutical Analytical Sciences Group (PASG),[41] the European Medicines Agency (EMA)[42] and the International Conference on Harmonisation (ICH)[43] published guidelines on method development and validation of methods such as NIR spectroscopy to assist industry to comply with regulatory requirements. Validation criteria for qualitative NIR spectroscopy methods include specificity and robustness; whereas for quantitative NIR methods specificity, linearity, accuracy, precision, range, robustness, and outliers are included (Table 3.1).

Due to constant changes in the regulatory and consumer environments, it was required to adapt the GC method from an USP[25] based method to a BP[24] based method in order to meet the requirements.[44] Thus, the challenge was to develop NIR spectroscopy models validated to pharmaceutical standards and merging the spectra from natural TG and concentrated TG oils to quantify the PUFA's expressed as % area and mg.g$^{-1}$.

**Table 3.1: Pharmaceutical Validation Criteria for NIR spectroscopy methods**

| | |
|---|---|
| **Specificity** | Relative discriminatory power of the method that could be demonstrated by the use of spectral libraries. |
| **Linearity** | Correlation of results calculated from NIR spectroscopy responses within the used algorithms to reference method results distributed throughout the defined range of the calibration model. |
| **Accuracy** | Correlation of prediction results with the reference data, indicated by the standard error of prediction (SEP). The SEP should be in close agreement with the error of the reference method, expressed as the standard error of laboratory (SEL). |
| **Precision** | Closeness of agreement between a series of measurements under the prescribed conditions and may be considered at two levels, repeatability and intermediate precision (within-laboratory reproducibility). |
| **Repeatability** | Repeatability is achieved by replicate measurements of the same sample with or without variation in sample positioning. |
| **Intermediate precision** | Intermediate precision (within-laboratory reproducibility) is achieved by replicate measurements by different analysts, different days of measurements. |
| **Range** | The range of analyte reference values defines the range of the NIR spectroscopy method and quantitation limits of the method. |
| **Robustness** | Includes the effect of variation in temperature, humidity, sample handling and the influence of instrument changes. These must be covered during data acquisition as part of the development of the method. |
| **Outliers** | A result outside the calibration range indicate that further testing is required by an appropriate analytical method to determine if the analyte content is within the specifications. If it does, the spectrum needs to be incorporated in the calibration. |

### 3.3.6 Mutual properties

The aim of this study was to superimpose data from two different GC methods as illustrated in the information diagram (Figure 3.1) applied in the context of multivariate mutual information (MMI).[45] In probability theory and information theory, the mutual information (MI) of two random variables is a measure of the mutual dependence between the two variables. Whereas, multivariate mutual information (MMI) is a measure of the mutual dependence between more than two variables and can be illustrated in the context of information diagrams such as a Venn diagram that shows all possible logical relations between a finite collection of different sets.[46] The data sets were viewed in the same context, displaying mutual properties which can be applied to construct predictive models.

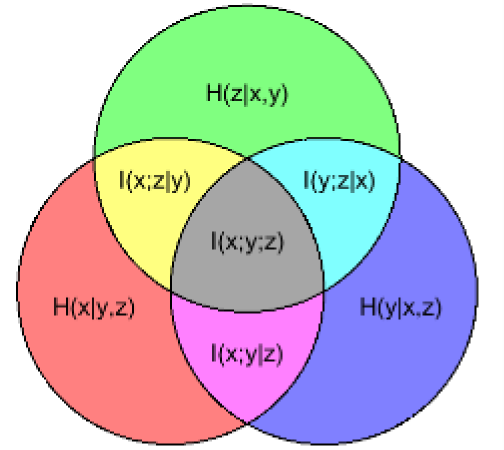

**Figure 3.1: Venn diagram (https://en.wikipedia.org/wiki/Venn_diagram) of information theoretic measures for three variables x, y, and z, represented by the lower left, lower right, and upper circles, respectively. The multivariate mutual information (MMI) is represented by grey region. The overlap of information between the sample groups was viewed in the same context as MMI, displaying mutual properties which can be applied to construct predictive models, where x = group 1, y = group 2 and z = group 3.**

## 3.4. MATERIALS AND METHODS

### 3.4.1 Samples

A total of 178 samples comprising nine different commercial omega-3 fish oils, from different batches and suppliers/manufacturers (Table 3.2) were captured. The number of samples of each type of fish oil was not equal (Tables 3.3 and 3.4) and yielded an uneven distribution of data.

The samples comprised of 3 groups: the first group consisted of natural TG oils with relatively low PUFA content analysed by the USP[25] based method and expressed as % area, the second group contained concentrated TG oils with relatively high PUFA content analysed by the BP[24] based method and expressed as mg.g$^{-1}$ and the third group consisted of both natural and concentrated TG oils, from group 1 and 2, which were analysed with both USP and BP based methods.

The samples were stored at ambient temperature in 150 mL amber glass bottles with a screw cap and blanketed with nitrogen to prevent oxidation. The TG fish oils had limited concentration ranges (Table 3.2); with a high PUFA content for the concentrated oils and low PUFA content for the non-concentrated (natural) oils. No artificial/laboratory samples were prepared, and all samples were used “as is”.

**Table 3.2: Concentration ranges of PUFA's in the 9 different fish oils used for method development expressed as mg.g$^{-1}$ and % area as per manufacturers specifications**

| **Non-concentrated oils** | **AA (mg.g$^{-1}$)** | **EPA (mg.g$^{-1}$)** | **DHA (mg.g$^{-1}$)** | **EPA (%)** | **DHA (%)** |
|---|---|---|---|---|---|
| Cod Liver Oil | - | 63 min | 52 min | - | - |
| Fish 18 (EPA):12(DHA) | - | 160 min | 104 min | 18 min | 12 min |
| Omega 3 Fish Oil | - | - | - | 18 min | 12 min |
| Fish Liver Oil (Mackerel) | - | - | - | 7 - 13 | 8 - 15 |
| Salmon Oil | - | 103 min | 67 min | 8 min | 12 min |
| **Concentrated oils** | **AA (mg.g$^{-1}$)** | **EPA (mg.g$^{-1}$)** | **DHA (mg.g$^{-1}$)** | **EPA (%)** | **DHA (%)** |
| Fish Oil EPA/DHA 23:7 | - | - | - | 23 min | 7 min |
| Incromega DHA 500 TG | 15 - 25 | 70 - 90 | 500 - 600 | 6.5 - 15 | 55 – 65 |
| Incromega TG0525 | 18 - 22 | 60 - 75 | 220 - 264 | 5.5 - 20 | 25 - 40 |
| Omevital 10/50 TG | - | 100 – 140 | 490 min | 17 max | 50 min |
| **Final Combined Ranges** | **15 - 25 (mg.g$^{-1}$)** | **60 -160 (mg.g$^{-1}$)** | **52 - 600 (mg.g$^{-1}$)** | **5.5 - 20 (%)** | **7 - 65 (%)** |

**Table 3.3: Fish oils used in the development of the mg.g$^{-1}$ TG quantification models for AA, DHA and EPA TG were initially developed with 5 different fish oils of which 2 were natural and 3 concentrated oils. These models were later extended to include 4 additional fish oils of which 3 were natural oils and 1 concentrated oil. The total number of samples (N) used in the initial calibration model ($N_i$) and the extended calibration model ($N_e$) for AA, EPA and DHA respectively**

| **TG quantification models** | **mg.g$^{-1}$ AA** | | **mg.g$^{-1}$ EPA** | | **mg.g$^{-1}$ DHA** | | |
|---|---|---|---|---|---|---|---|
| **Fish Oil Product** | **$N_i = 62$** | **$N_e = 91$** | **$N_i = 88$** | **$N_e = 166$** | **$N_i = 89$** | **$N_e = 166$** | **Type of Oil** |
| Cod Liver Oil | 0 | 3 | 2 | 10 | 2 | 11 | natural |
| Omega 3 Fish Oil | 0 | 0 | 0 | 0 | 0 | 0 | natural |
| Fish Oil EPA/DHA 23:7 | 0 | 0 | 0 | 3 | 0 | 4 | concentrated |
| Incromega DHA 500 TG | 39 | 52 | 63 | 89 | 63 | 84 | concentrated |
| Incromega TG0525 | 23 | 26 | 23 | 31 | 23 | 31 | concentrated |
| Fish Liver (Mackerel) Oil | 0 | 0 | 0 | 2 | 0 | 2 | natural |
| Fish Oil 18:12 EPA/DHA | 0 | 1 | 0 | 14 | 1 | 12 | natural |
| Salmon | 0 | 9 | 0 | 15 | 0 | 14 | natural |
| Omevital 10/50 TG | 0 | 0 | 0 | 2 | 0 | 8 | concentrated |

### 3.4.2 Reference Methods

The reference analyses were performed by two different prescribed GC methods for the determination of the PUFA's composition in oils. The obtained results were used for both identification and quantification. For results to be expressed in (mg.g$^{-1}$) a method based on the BP (Appendix XP1) was used.[24] For results to be expressed in % area, a method based on the USP (Volume 35, Chemical Test no. 401, Fats and Fixed Oils) was used.[25] All the samples used in this study complied with product

**Table 3.4: Fish oils used in the development of the % TG quantification models for DHA and EPA were initially developed with 3 different natural fish oils. These models were updated to include 4 additional fish oils of which 1 was a natural oil and 3 concentrated oils. The total number of samples (N) used in the initial calibration model ($N_i$) and the extended calibration model ($N_e$) for EPA and DHA respectively**

| *TG quantification models* | *% EPA* | | *% DHA* | | |
|---|---|---|---|---|---|
| ***Fish Oil Product*** | ***$N_i$ = 74*** | ***$N_e$ = 178*** | ***$N_i$ = 74*** | ***$N_e$ = 169*** | ***Type of Oil*** |
| *Fish Liver (Mackerel) Oil* | *19* | *20* | *19* | *20* | *natural* |
| *Fish Oil 18:12 EPA/DHA* | *12* | *13* | *12* | *11* | *natural* |
| *Salmon* | *43* | *43* | *43* | *39* | *natural* |
| *Omega 3 Fish Oil* | *0* | *5* | *0* | *5* | *natural* |
| *Fish Oil EPA/DHA 23:7* | *0* | *9* | *0* | *9* | *concentrated* |
| *Incromega DHA 500 TG* | *0* | *65* | *0* | *65* | *concentrated* |
| *Incromega TG0525* | *0* | *23* | *0* | *20* | *concentrated* |

specifications by the prescribed reference methods as mentioned above. GC analysis was performed on a model 6890 with flame ionization detector (FID) and 6890 auto-sampler (Agilent Technologies, Santa Clara, California, USA) using a 30-meter capillary column, USP phase G16 (i.e. Polyethylene Glycol Compound 20M, ZB Wax, Carbowax 20M, BPX70 or equivalent).

### 3.4.3 NIR spectral data acquisition

Data acquisition was performed in transmittance on a dispersive NIR spectrophotometer model 6500 with transport module (NIRSystems Inc. Silver Springs, Maryland, USA) using the full wavelength region of 400-2500 nm at an average of 32 scans (2 nm intervals) with a standard 1 mm path length quartz cuvette. Samples were captured “as is” over a 10-year period under normal day-to-day laboratory conditions. Samples from group 1 were captured over the full study period, whereas group 2 and 3 were captured in the last 4 years of the study. In order to capture as much variation in the spectral matrix as possible and ensure robustness, sample scans were repeated on at least two different days using different cuvettes by different analysts. At least 6 different supplier/manufacturers batches for each fish oil were captured to develop and validate spectral libraries. Subsequent batches were captured to develop quantitative models. Data analysis was performed with Vision® 3.50 software (NIRSystems Inc. Silver Springs, Maryland, USA).

### 3.4.4 Qualification by NIR spectroscopy

Qualification/identification methods were developed for the 9 different commercial omega-3 fish oils using the Vision® 3.50 software (NIRSystems Inc. Silver Springs, Maryland, USA). At least three different supplier/manufacturers batches were used for each of the training and external validation sets. Sample selection for all of the libraries was performed using maximum distance in wavelength space applying 2nd derivative (gap-segment) pre-treatment (0,10) to correct for spectral offset. Spectral libraries were developed using correlation in wavelength space, applying 2nd derivative (gap-segment) pre-treatment (0,10), (*Appendix).*

### 3.4.5 Quantification by NIR spectroscopy

Quantitative methods were developed for AA, DHA and EPA in TG fish oils expressed as $mg.g^{-1}$ as well as DHA and EPA expressed as % area. For each constituent, approximately a third of the 178 samples were allocated to the external validation set and two thirds to the calibration set. The concentration range and type of fish oil were taken into consideration to ensure that the concentration (Y-space) and spectral (X-space) variance were representative of the different commercial fish oils (Tables 3.3 and 3.4). Each constituent had a separate calibration and independent (external) validation set. The validation set covered the full range of samples but did not exceed that of the calibration set. The constituent data was evaluated for inter-correlations between AA, DHA and EPA using Vision® 3.50 (Table 3.5).

**Table 3.5: Inter-correlations between the constituents as indicated in Vision® 3.50: AA, DHA and EPA expressed as $mg.g^{-1}$ and % area**

| *Constituents* | *AA $mg.g^{-1}$* | *DHA $mg.g^{-1}$* | *EPA $mg.g^{-1}$* | *DHA %* | *EPA %* |
|---|---|---|---|---|---|
| ***AA*** | *1.0000* | *0.2880* | *-0.5501* | *-* | *-* |
| ***DHA*** | *0.2880* | *1.0000* | *-0.6517* | *1.0000* | *-0.6274* |
| ***EPA*** | *-0.5501* | *-0.6517* | *1.0000* | *-0.6274* | *1.0000* |

*Sample selection* was performed using the Mahalanobis Distance in the principal component space equation with 99% cumulative variance (1100-2500 nm), applying 2nd derivative (gap-segment) pre-processing (0,10). The 2nd derivative (gap-segment) pre-processing corrected for spectral offset and slope due to changes in instrument response. An outlier threshold was set at a match value of 3 and finding 25% redundant spectra.

*Regression development* was performed using partial least squares (PLS) regression (1100-2500 nm), applying 2nd derivative (gap-segment) pre-processing (0,10). PLS regression was used to eliminate co-linearity (high correlation between wavelength terms) that is difficult to overcome using classical methods such as multi-linear regression (MLR). High residual samples were investigated and removed if it were a spectral (X-space) and/or reference (Y-space) outlier.

*Cross validation (CV)* was performed on the calibration samples using k-fold[47] CV grouped in subsets of 4 (k = 4). The purpose of CV is to define a *validation test set* in the training phase, to limit overfitting and give an indication on how the model will predict to an *independent/external validation set*. The resultant coefficient of determination ($R^2$), prediction residual error sum of squares (PRESS) and root mean squared error of cross validation (RMSECV or SECV) were used as indicators of how well the PLS model performed with the selected number of factors. The SECV and $R^2$ decreased with more factors, therefore, the number of factors were carefully chosen to avoid under or overfitting. The PRESS value is usually at a minimum with the optimal number of factors.

*External or independent validation* was performed with an independent set of spectra referred to as the external validation set. These spectra were not included in the calibration set. Several equations for each constituent were developed during regression model development and each evaluated for performance using the predict function in the software. The equation with the lowest prediction errors was selected for validation according to pharmaceutical criteria. All chemometric functions were performed using Vision® 3.50 software (NIRSystems Inc. Silver Springs, Maryland, USA).

*BP based method (mg.g$^{-1}$):* The quantification models for AA, DHA and EPA TG were initially developed with 5 different fish oils of which 2 were natural and 3 concentrated oils. The total of number of spectra used were 62, 89 and 88 for AA, DHA and EPA, respectively. These models were later updated to include 4 additional fish oils of which 3 were natural oils and 1 concentrated oil, bringing the total number of spectra to 91, 166 and 166 for AA, DHA and EPA, respectively (Table 3.3).

*USP based method (% area):* The initial quantification models for DHA and EPA TG were developed with 3 different natural fish oils. A total of 74 spectra were obtained of which 62 were used in the calibration sets and 12 in the validation sets. These models were also updated to include 4 additional fish oils of which 1 was a natural oil and 3 concentrated oils, bringing the total number of spectra to 169 for DHA and 178 for EPA (Table 3.4).

## 3.5 RESULTS AND DISCUSSION

In general, most studies published involving NIR spectroscopy and chemometrics, were developed in a relatively short time frame and with few proven applications in industry.[48] The calibration models in this study were exceptional, as they were developed over an extended period of 10 years, therefore a proven application in the nutraceutical industry which was validated according to pharmaceutical criteria. Models were developed and updated periodically to add authenticated fish oil samples, which failed library identification or fell outside the validated range.

This study aimed to reduce the risk of adulteration of nutraceutical products made from natural and concentrated fish oils. It was thus necessary to develop NIR spectroscopy models to identify various commercial fish oils in the TG form and to quantify the PUFA's as % area and mg.g$^{-1}$. The chemical composition and the corresponding NIR spectrum of the fish oil is modified when converted from TG to EE form and finally to a concentrated TG form (Figures 3.2 and 3.3).

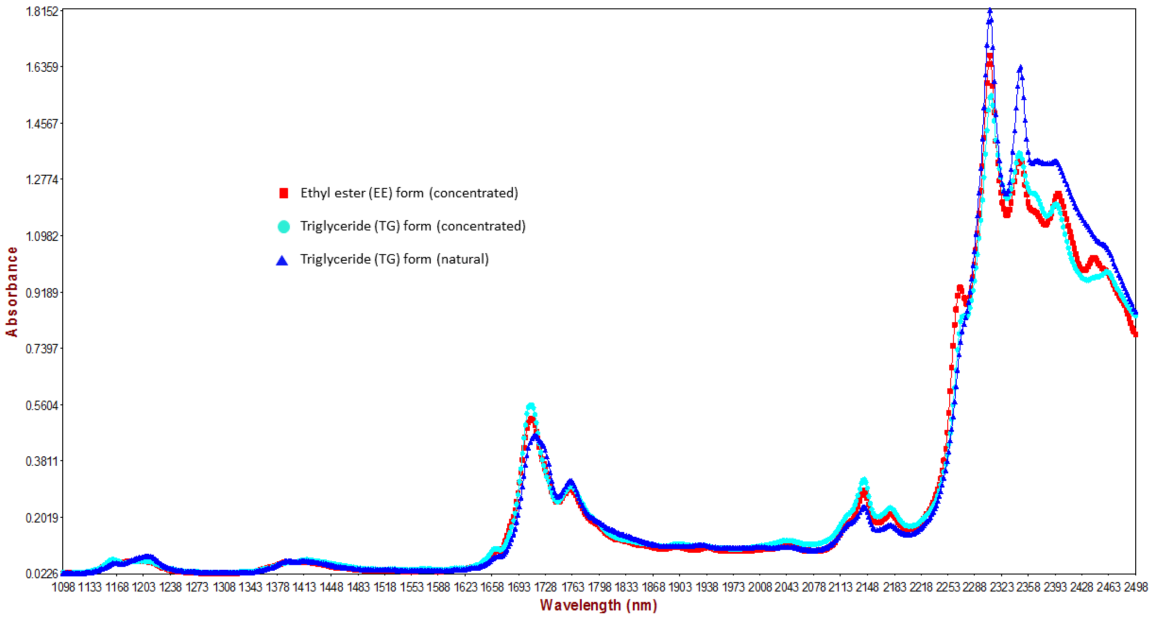

**Figure 3.2: Typical NIR spectra of omega-3 fish oils: ethyl ester (EE) and triglyceride (TG) forms in the region 400 - 2500 nm**

Due to constant changes in the regulatory and consumer environments, it was required to adapt the GC method to meet the requirements during the course of this study.[39,44] One such requirement was that the PUFA's content, which was usually expressed in % area, also had to be expressed in mg.g$^{-1}$.[11] Another requirement was to quantify AA as well and not only DHA and EPA, as initially required. The two different GC methods as prescribed by the USP[25] and BP,[24] differed significantly in terms of sample and standard preparation. The USP based method required the sample to be esterified to the methyl ester and the BP based method to the ethyl ester with the addition of an internal standard. Therefore, results expressed as % area (USP method) could not simply be converted to mg.g$^{-1}$ (BP method). The implication was that batches in storage had to be re-tested to obtain results expressed in mg.g$^{-1}$. Retesting numerous batches by GC using the BP method would have been a costly and time-consuming process and therefore it was necessary to investigate an alternative method such as NIR spectroscopy.

Thus, the challenge was to develop models validated to pharmaceutical standards by merging the spectra from natural TG and concentrated TG forms as well as superimposing reference data from two different GC methods, i.e. BP and USP methods.

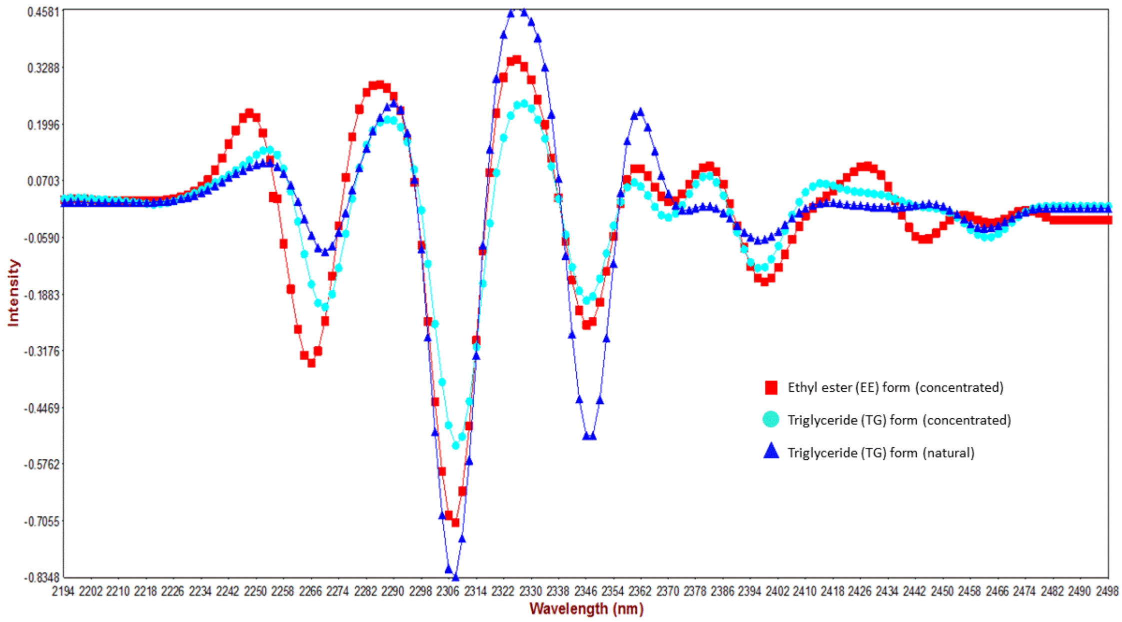

**Figure 3.3: Typical 2nd derivative (gap-segment) pre-treatment NIR spectra of omega-3 fish oils: ethyl ester (EE) and triglyceride (TG) form with slight differences in the region between 2200 – 2500 nm due to C-H combination bands**

### 3.5.1 Qualification using spectral libraries

Identification of natural fish oils using conventional methods was a complicated task due to overlapping concentration ranges of the omega-3 fatty acids and other similar physical and chemical properties which cannot be used to distinguish between different fish oils.[31] Identification methods (classification) such as principal component analysis (PCA) and cluster analysis are called unsupervised methods. Supervised classification methods such as correlation-based methods and distance-based methods are preferred for identification purposes.[42]

Spectral libraries were developed to discriminate between the different omega-3 fish oils as well as the TG and EE forms and hence prove the selectivity of the quantification method (*see Appendix*).

Spectral libraries must be monitored and maintained to ensure ongoing method performance by including new or different variations of samples. Samples failing the library identification must be analysed using the primary reference method such as GC and if authentic, can be included in the library.

### 3.5.2 Quantification using PLS regression

Quantification of omega-3 fatty acids were further complicated by the limited concentration ranges which resulted in an uneven spread of data over the concentration range. In order to extend the calibration ranges, the data from 5 types of concentrated oils were combined with 4 types of natural oils (Table 3.2). Samples were used “as is” and were not mixed to prepare laboratory samples. The constituent data was evaluated for inter-correlations between AA, DHA and EPA using Vision® 3.50 software and found to be acceptable (Table 3.5).

The PUFA concentrations obtained by GC for the natural oils were relatively low (8 - 15% for DHA and 7 - 13% for EPA) and expressed as % area; whereas the concentrated oils were relatively high (220 - 600 mg.g$^{-1}$ for DHA and 60 - 140 mg.g$^{-1}$ for EPA) and expressed as mg.g$^{-1}$. Thus, it was required to obtain results (expressed as % area) from the concentrated oils to cover the high PUFA concentrations for the % area model. Similarly, it was required to obtain results (expressed as mg.g$^{-1}$) from the natural oils to cover the low PUFA concentrations for the mg.g$^{-1}$ model. Therefore, the data sets were superimposed in the same manner as the MMI concept (Figure 3.1). The EPA/DHA ratio for natural oils was typically 18:12, thus EPA high and DHA low. The EPA/DHA ratio of concentrated oils was modified through molecular distillation. Therefore, some samples of *Incromega DHA 500 TG* (EPA/DHA ratio of 6.5:55), *Incromega TG0525* (EPA/DHA ratio of 5:25) and *Omevital 10/50 TG* (EPA/DHA ratio of 10:50) were analysed by the USP method to incorporate the concentration shift for the % area model. Similarly, some samples of *Cod Liver Oil* (EPA/DHA ratio of 8:10), *Fish 18(EPA):12(DHA)* and *Salmon Oil* (EPA/DHA ratio of 12:8) were analysed by the BP method to incorporate the concentration shift for the mg.g$^{-1}$ model. Based on the statistics terms SEC, $R^2$, SEP and r of the omega-3 fatty acids, models generally showed improvement with the updated calibration models (Tables 3.6 and 3.7).

**Table 3.6: Regression statistics after performing partial least squares (PLS) regression and applying 2nd derivative (gap-segment) pre-processing. Internal validation was performed by cross validation with groups of four**

| Parameter | AA ($mg.g^{-1}$) Initial | AA ($mg.g^{-1}$) Extended | EPA ($mg.g^{-1}$) Initial | EPA ($mg.g^{-1}$) Extended | DHA ($mg.g^{-1}$) Initial | DHA ($mg.g^{-1}$) Extended | EPA (%) Initial | EPA (%) Extended | DHA (%) Initial | DHA (%) Extended |
|---|---|---|---|---|---|---|---|---|---|---|
| Calibration Set (N) | 39 | 69 | 59 | 127 | 66 | 131 | 62 | 137 | 62 | 132 |
| SEC | 0.64 $mg.g^{-1}$ | 0.41 $mg.g^{-1}$ | 2.45 $mg.g^{-1}$ | 3.42 $mg.g^{-1}$ | 3.93 $mg.g^{-1}$ | 4.16 $mg.g^{-1}$ | 0.92 % | 0.65 % | 1.06 % | 0.65 % |
| No. of factors | 5 | 11 | 6 | 10 | 9 | 10 | 4 | 6 | 5 | 9 |
| $R^2$ | 0.90 | 0.97 | 0.93 | 0.99 | 0.99 | 0.99 | 0.93 | 0.99 | 0.74 | 0.99 |
| PRESS | 56 | 60 | 946 | 2152 | 12647 | 2511 | 61.7 | 45.5 | 90 | 109 |
| F-value | 40 | 128 | 87 | 1110 | 6853 | 19544 | 143 | 749.8 | 22 | 16618 |
| MSECV | 1.38 $mg.g^{-1}$ | 1.08 $mg.g^{-1}$ | 4.64 $mg.g^{-1}$ | 4.79 $mg.g^{-1}$ | 16.07 $mg.g^{-1}$ | 5.09 $mg.g^{-1}$ | 1.16 % | 0.66 % | 1.40 % | 0.91 % |
| Data range | 15.7 - 22.7 $mg.g^{-1}$ | 10.8 - 22.7 $mg.g^{-1}$ | 56.7 - 114.7 $mg.g^{-1}$ | 56.0 – 200.4 $mg.g^{-1}$ | 80.0 - 527.0 $mg.g^{-1}$ | 70.5 - 550.0 $mg.g^{-1}$ | 7.3 - 23.4 % | 6.6 - 23.4 % | 8.4 - 17.1 % | 8.1 - 60.1 % |

**Table 3.7: Prediction results of independent validation sets of fish oil samples analysed in transmittance on a NIRSystems 6500 by using the predict function in Vision® 3.50**

| Parameter | AA ($mg.g^{-1}$) Initial | AA ($mg.g^{-1}$) Extended | DHA ($mg.g^{-1}$) Initial | DHA ($mg.g^{-1}$) Extended | EPA ($mg.g^{-1}$) Initial | EPA ($mg.g^{-1}$) Extended | DHA (%) Initial | DHA (%) Extended | EPA (%) Initial | EPA (%) Extended |
|---|---|---|---|---|---|---|---|---|---|---|
| Validation set (N) | 23 | 22 | 23 | 35 | 29 | 39 | 12 | 37 | 12 | 41 |
| SEL | 0.28 $mg.g^{-1}$ | | 3.22 $mg.g^{-1}$ | | 1.20 $mg.g^{-1}$ | | 0.47% | | 0.61 % | |
| SEP | 0.69 $mg.g^{-1}$ | 0.51 $mg.g^{-1}$ | 4.51 $mg.g^{-1}$ | 3.29 $mg.g^{-1}$ | 2.40 $mg.g^{-1}$ | 4.07 $mg.g^{-1}$ | 0.93 % | 0.68 % | 1.11 % | 0.71 % |
| Correlation (r) | 0.93 | 0.97 | 0.99 | 0.99 | 0.96 | 0.99 | 0.94 | 0.99 | 0.96 | 0.99 |
| Slope | 0.986 | 1.04 | 1.0031 | 0.998 | 1.003 | 0.9909 | 1.0324 | 0.9957 | 1.0314 | 1.0223 |
| Slope Error | 0.0833 | 0.0574 | 0.0068 | 0.003 | 0.0481 | 0.0193 | 0.1141 | 0.0052 | 0.0930 | 0.0235 |
| Bias | 0.3027 | -0.5828 | -1.8393 | 1.6143 | -0.2132 | -0.9455 | -0.049 | 0.2071 | -0.5466 | -0.205 |
| Bias Error | 1.584 | 1.1136 | 2.9143 | 1.168 | 3.3907 | 1.726 | 1.344 | 0.2117 | 1.3042 | 0.2781 |

The coefficients of determination ($R^2$), obtained during regression development were close to 1 which indicated good correlations between the spectral and chemical information. Loadings, weights and regression coefficients were evaluated for each constituent by the Vision® 3.50 software obtained during PLS regression. The regression results of EPA % area of the extended model is displayed in Figures 3.4 to 3.6.

The PRESS value as indicated in the PRESS plot (Figure 3.7) was obtained during CV and was a useful tool in selecting the number of factors. The model improved as the PRESS value decreased and the F-value increased. The F value (or F-test statistic) was also used to evaluate how many factors to select for each equation.

The SECV results obtained by CV were greater than the SEC and indicated the goodness of the calibration. The aim was to obtain the smallest SECV possible without over-fitting the model. Therefore, the number of factors were carefully chosen and equations with more and less factors were also evaluated. Samples with high residuals were investigated and removed if found to be a spectral (X-space) and/or reference (Y-space) outlier. According to a tutorial published earlier,[48] in practice most successful applications used 6 to 8 factors. More than 12 factors indicate uncertainty in the calculation. The updated models used more factors than the initial models, due to a greater variety of fish oil samples, all the equations used less than 12 factors (Table 3.6) and could be reduced in future with further variable selection.

The standard error of prediction (SEP) of the PUFA's were higher than the standard error of calibration (SEC), but less than 1.3 to 1.5 times the SEC.[49,50] The correlation coefficient (r) obtained during the predictions were close to 1 which indicated a good correlation between the reference and predicted values.

The slope and bias of the equations were evaluated for significance by the following criteria: for the slope (1 – slope < slope error) and the bias (0 – bias < bias error). For all the PUFA models, the bias results were less than 0.6 times the SEC[49] and the slope (1.00 ± 0.15).[48] The bias results indicated that the deviations were distributed randomly and had a low systematic error.

*Results based on BP method*: The SEC values for AA, DHA and EPA were 0.64, 3.93 and 2.45 mg.g$^{-1}$ (initial calibration); 0.41, 4.16 and 3.4 mg.g$^{-1}$ (extended calibration) respectively; $R^2$ values were 0.90, 0.99 and 0.93 (initial calibration); 0.97, 0.99 and 0.99 (extended calibration); SEP values were 0.69, 4.51 and 2.40 mg.g$^{-1}$ (initial calibration); 0.51, 3.29 and 4.07 mg.g$^{-1}$ (extended calibration); r values were 0.93, 0.99 and 0.96 (initial calibration); 0.97, 0.99 and 0.99 (extended calibration).

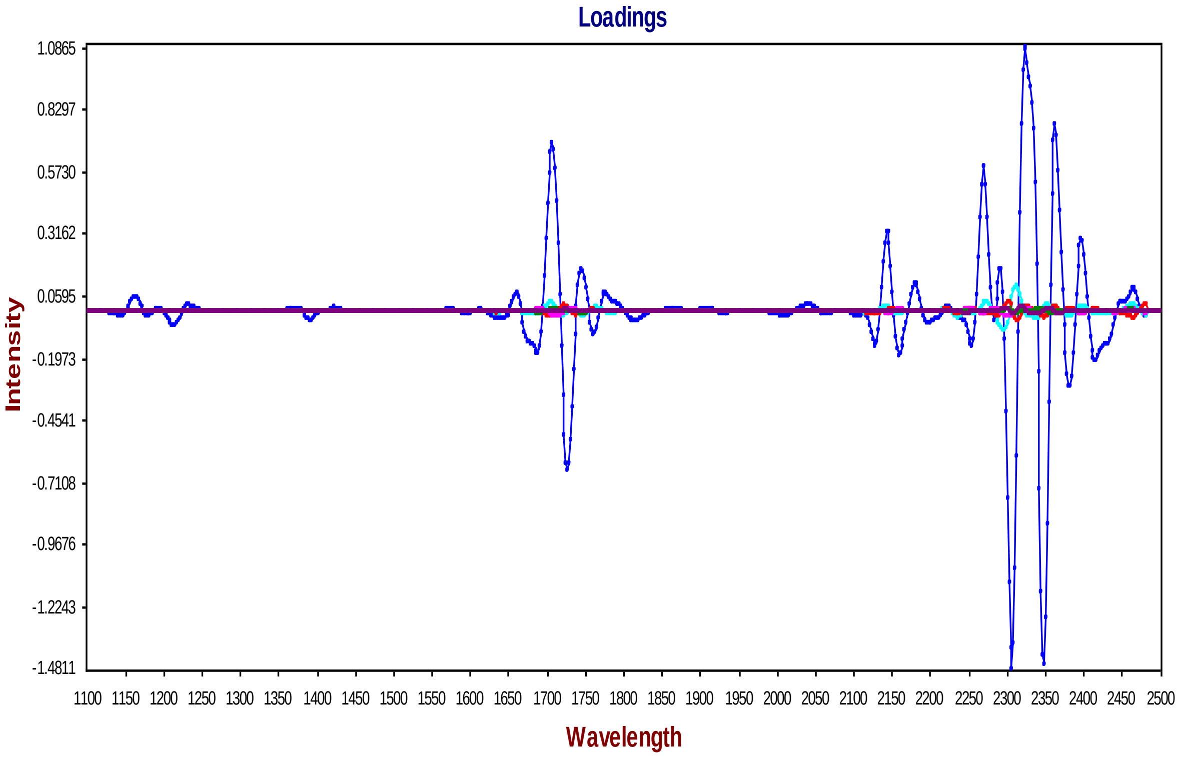

**Figure 3.4: Loadings of EPA % area extended model as displayed by the Vision® 3.50 software obtained during PLS regression with the greatest contribution to the variance was observed in the spectral regions 1650 – 1800 nm and 2100 – 2400 nm**

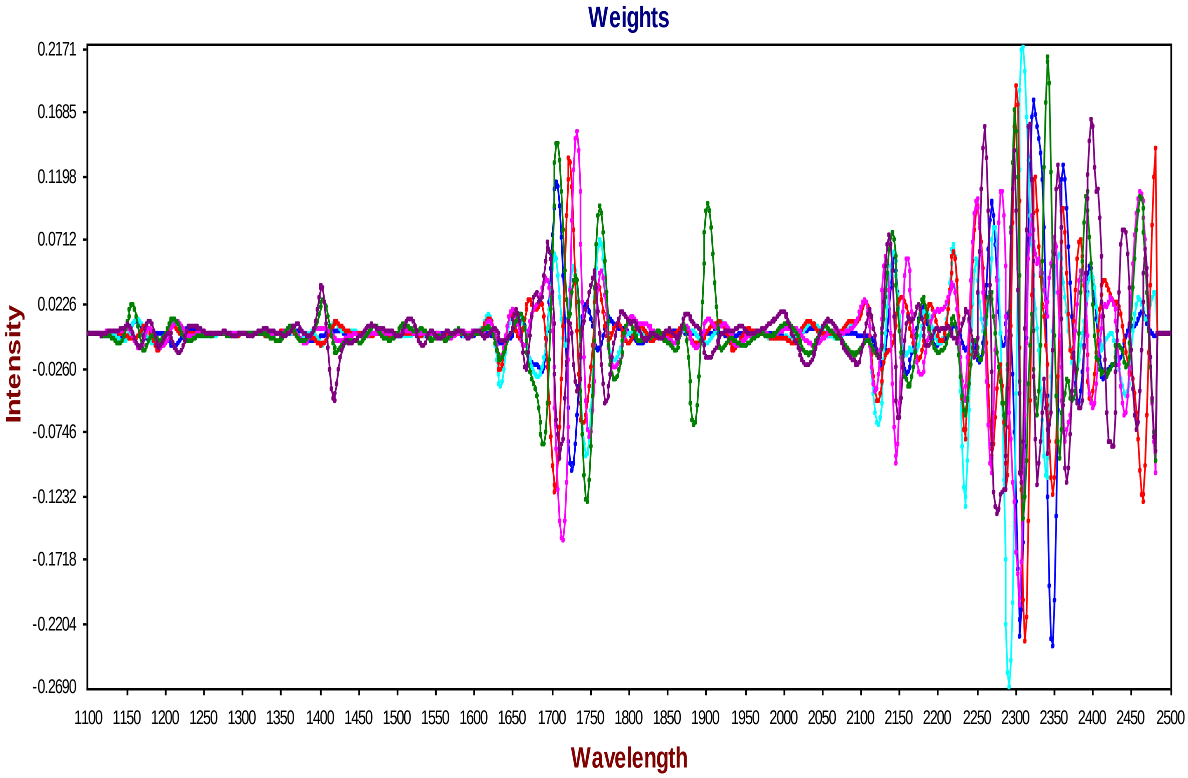

**Figure 3.5: Weights of EPA % area extended model as displayed by the Vision® 3.50 software obtained during PLS regression with the greatest contribution to the variance was observed in the spectral regions 1650 – 1800 nm and 2100 – 2400 nm and lesser contributions at 1150 – 1250 nm, 1400 – 1450 nm and 1800 – 2100 nm**

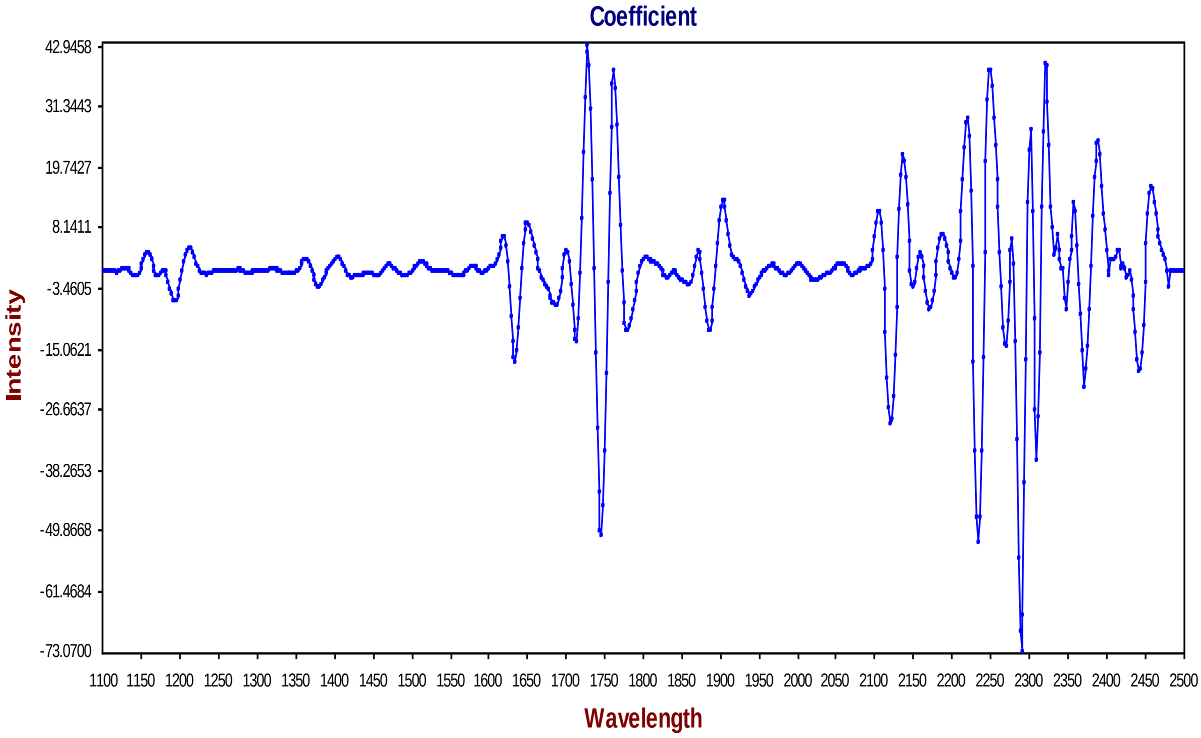

**Figure 3.6: Regression coefficient of EPA % area extended model as displayed by the Vision® 3.50 software obtained during PLS regression and displayed the same spectral regions as observed in the loadings and weight plots which are attributed to the fundamental C-H stretch and their corresponding combination bands**

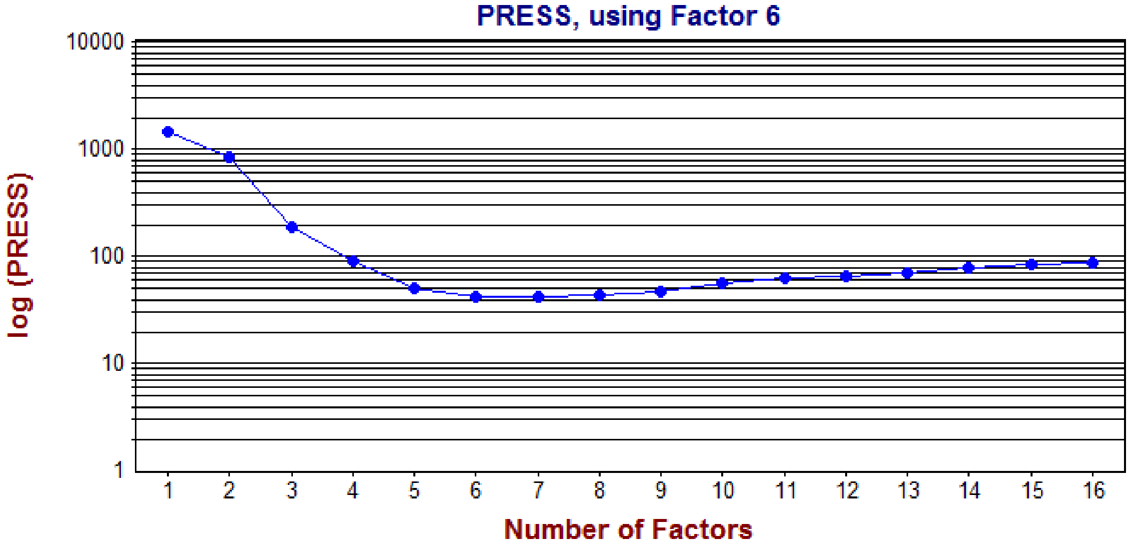

**Figure 3.7: The PRESS plot of the EPA % area extended model displayed by the Vision® 3.50 software indicates the number of factors with the lowest prediction residual error sum of squares (PRESS) value**

*Results based on USP method:* The SEC values for EPA and DHA were 0.92 and 1.06 % (initial calibration) and 0.65 and 0.65 % (extended calibration) respectively; $R^2$ values were 0.93 and 0.74 (initial calibration) and 0.99 and 0.99 (extended calibration); SEP values were 1.11 and 0.93 % (initial calibration) and 0.71 and 0.68 % (extended calibration); r values were 0.96 and 0.94 (initial calibration) and 0.99 and 0.99 (extended calibration).

*Accuracy and linearity:* All equations had high correlation coefficients (r) which indicated good linearity. The slope and bias of all the equations were evaluated for significance and complied with the criteria. Agreement between the SEP and SEL indicated acceptable accuracy of the NIR method, as indicated by the NIR (predicted) vs reference (actual) data plots (Figures 3.8 to 3.12). Ideally, the calibration curve should have an even distribution of samples across the concentration range. In reality, it is not always possible, as seen with the DHA plots which display clustering of the samples (Figures 3.9 and 3.11). The SEP was less than 1.3 times the SEC for all the models and less than 2 times the SEL (except for the initial AA and the extended EPA models);[50] thus the accuracy compared well with the SEL of the prescribed reference method and could be improved to SEP < 1.4 times SEL (Table 3.8). The SEL was determined during the validation of the GC methods.

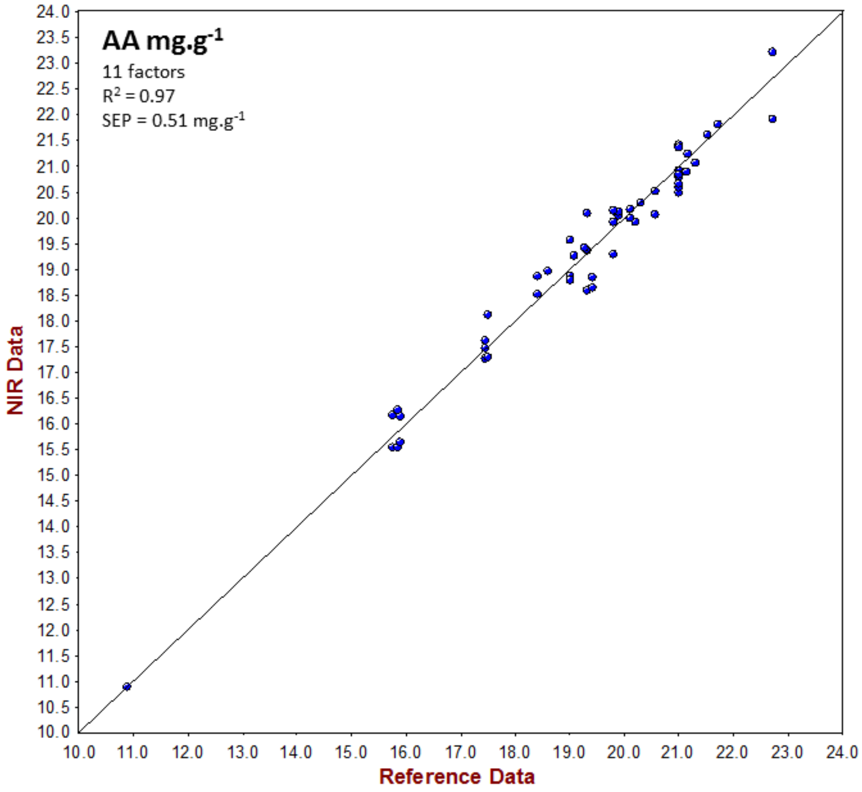

**Figure 3.8: NIR data (predicted) and reference data (actual) plots displaying the concentration distribution of AA (mg.g$^{-1}$)**

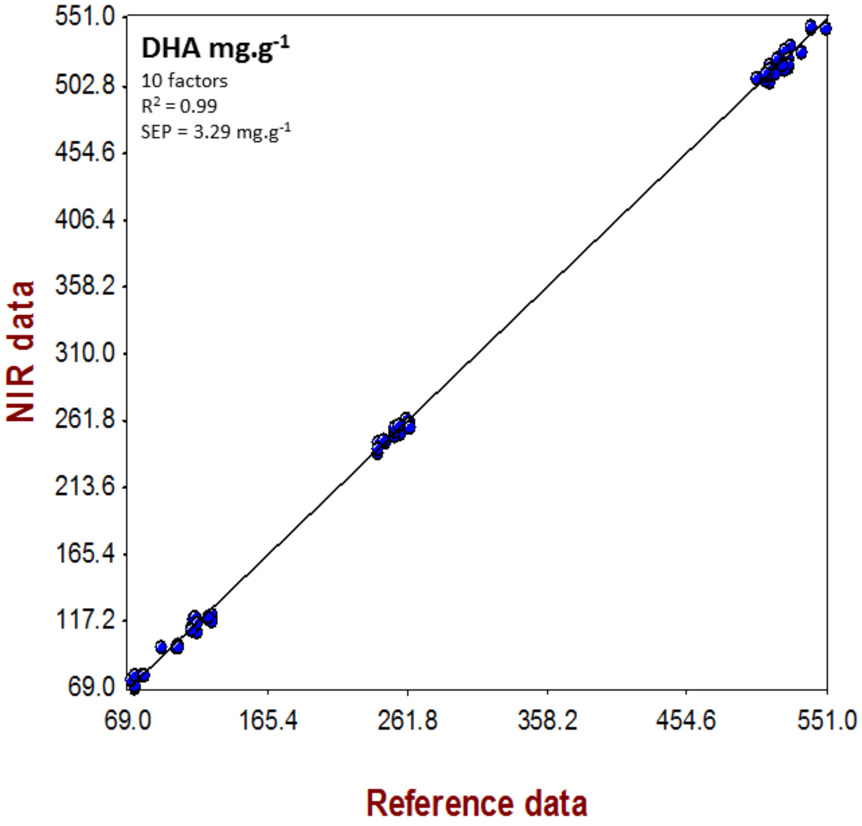

**Figure 3.9: NIR data (predicted) and reference data (actual) plots displaying the concentration distribution of DHA (mg.g$^{-1}$)**

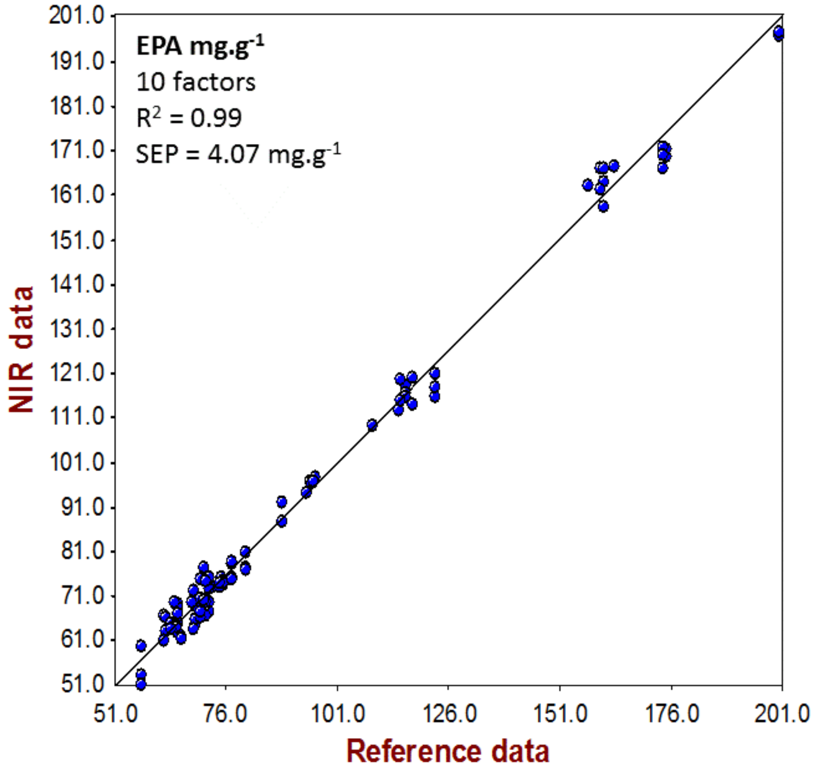

**Figure 3.10: NIR data (predicted) and reference data (actual) plots displaying the concentration distribution of EPA (mg.g$^{-1}$)**

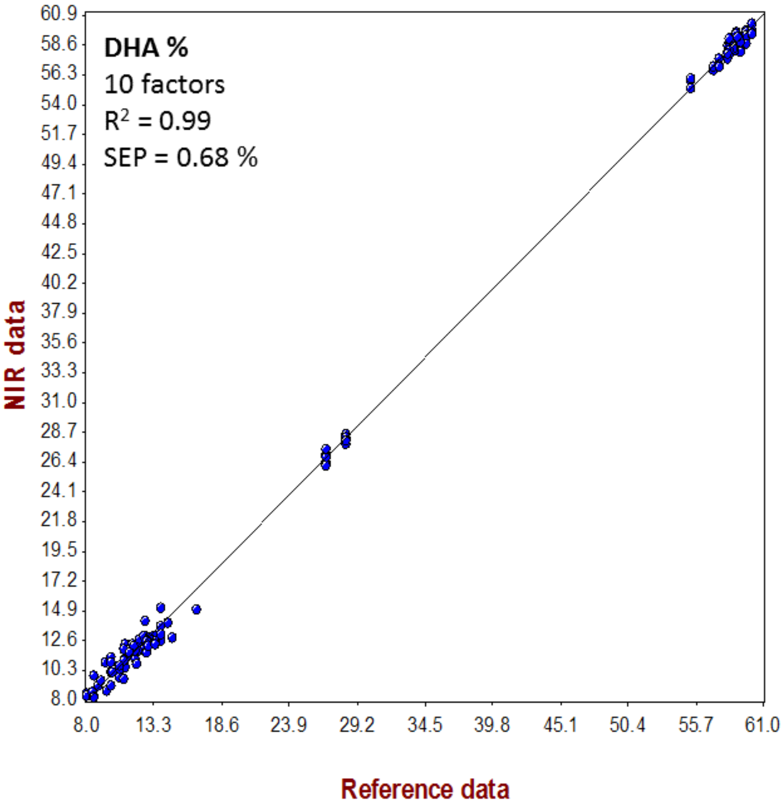

**Figure 3.11: NIR data (predicted) and reference data (actual) plots displaying the concentration distribution of DHA (%)**

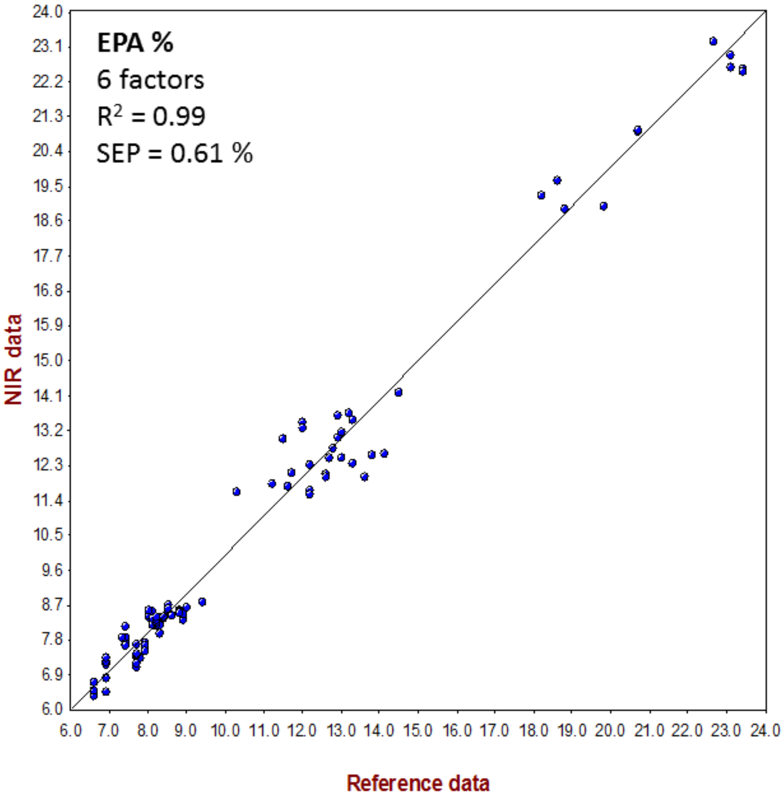

**Figure 3.12: NIR data (predicted) and reference data (actual) plots displaying the concentration distribution of EPA (%)**

**Table 3.8. External validation results of independent validation sets of fish oil samples performed according to prescribed pharmaceutical validation criteria for NIR spectroscopy methods. Ratio of deviation to performance (RPD) was included, but not required for pharmaceutical validation**

| *Parameter* | *AA (mg.g$^{-1}$)* | *EPA (mg.g$^{-1}$)* | *DHA (mg.g$^{-1}$)* | *EPA %* | *DHA %* |
|---|---|---|---|---|---|
| ***Specificity*** | *Identification/ Qualification Models for the different fish oils used* | | | | |
| ***Linearity & Accuracy*** | *Correlation, slope & bias complies (Table 7)* | | | | |
| ***Accuracy SEP <SEL×2*** | *SEP = 0.51 mg.g$^{-1}$* | *SEP = 4.07 mg.g$^{-1}$* | *SEP = 3.29 mg.g$^{-1}$* | *SEP = 0.71 %* | *SEP = 0.68 %* |
| ***Repeatability (RSD < 5%)*** | *RSD = 2.2 %* | *RSD = 0.7 %* | *RSD = 0.6 %* | *RSD = 1.8 %* | *RSD = 3.5 %* |
| ***Intermediate precision (RSD < 5%)*** | *RSD = 2.0 %* | *RSD = 0.8 %* | *RSD = 0.4 %* | *RSD = 1.5 %* | *RSD = 2.6 %* |
| ***Range*** | *10.8 - 22.7 mg.g$^{-1}$* | *56.0 – 200.4 mg.g$^{-1}$* | *70.5 - 550.0 mg.g$^{-1}$* | *6.6 – 23.4 %* | *8.1. – 60.1 %* |
| ***Mean*** | *19.6 mg.g$^{-1}$* | *88.4 mg.g$^{-1}$* | *358.2 mg.g$^{-1}$* | *30.0 %* | *11.5 %* |
| ***Standard Deviation*** | *2.03 mg.g$^{-1}$* | *33.73 mg.g$^{-1}$* | *187.21 mg.g$^{-1}$* | *21.88 %* | *4.66 %* |
| ***RPD*** | *3.98* | *8.29* | *56.90* | *32.18* | *6.57* |
| ***Robustness*** | *Data captured over extensive period incorporating variations in operator, temperature and hardware.* | | | | |
| ***Outliers*** | *Methods setup with upper and lower limits to indicate when samples fall outside calibration range.* | | | | |

*The concentration ranges* of the extended (mg.g$^{-1}$) models were increased from 15.7 - 22.7 to 10.8 - 22.7 (mg.g$^{-1}$) for AA, from 80.0 - 527.0 to 70.45 - 550.0 (mg.g$^{-1}$) for DHA and from 56.7 - 114.7 to 56.0 - 200.4 (mg.g$^{-1}$) for EPA. The concentration ranges of the extended (% area) models were increased from 8.4 - 17.1 % to 8.1 - 60.1 % for DHA and 7.3 - 23.4 % to 6.6 - 23.4 % for EPA (Table 3.6).

*Precision* results obtained for the repeatability for the (mg.g$^{-1}$) extended models in terms of RSD were 2.2 %, 0.7 % and 0.6 % for AA, EPA and DHA respectively and the intermediate precision (within-laboratory reproducibility) 2.0 %, 0.8 % and 0.4 % respectively. Results obtained for the repeatability for the (% area) extended models in terms of RSD were 1.8 % and 3.5 % for EPA and DHA respectively and the intermediate precision 1.5 % and 2.6 % respectively (Table 3.8). The repeatability was performed by scanning 10 replicates without re-filling the cuvette and the intermediate precision by 10 replicates with re-filling using different cuvettes. A relative standard deviation (RSD) of less than 5% is acceptable for spectroscopic methods.[24]

*Ratio of deviation to performance (RPD)*, which is the ratio of standard deviation (SD) of the reference data (validation set) to the SEP, was used to assess the performance (prediction) of the model and is not a requirement for pharmaceutical validation. RPD values ranging from 3.1 to 4.9 are adequate for screening purposes, from 5.0 to 6.4 for QC, from 6.5 to 8.0 for process control and values over 8.1 are suitable for any type of application.[51]

RPD results of the (mg.g$^{-1}$) extended models were 4.0, 56.9 and 8.3 for AA, EPA and DHA respectively. RPD results obtained for the (% area) extended models were 32.2 and 6.6 for EPA and DHA respectively (Table 8). According to the criteria listed by Sohn et al.,[51] all the equations were adequate for QC and process control purposes except for AA, which had an RPD of 4.0. Both EPA models had an RPD > 8.1 and were thus suitable for any type of application.

All the models satisfied the required validation criteria, except for the initial AA (% area) and the extended EPA (mg.g$^{-1}$) models which had higher SEP values than desired, but were accepted on the grounds of the RPD values.

### 3.5.3 Comparison to similar applications

Previous studies have found dispersive NIR and FT-NIR spectroscopy techniques to be useful for the quantification of omega-3 PUFA's in fish meal and oils. The results from this study compares well with the results reported below.

Cozzolino et al. [31] performed PLS regression on 160 fish oil samples scanned on a dispersive NIR spectrophotometer (1100-2500 nm) and obtained a $R^2$ of 0.96 and SECV of 0.59 (g.kg$^{-1}$) expressed as free fatty acids (FFA) during CV and a r of 0.98 and SEP of 0.50 (g.kg$^{-1}$) FFA during prediction, but not individual constituents of AA, EPA and DHA.

Azizian et al.[32] also performed PLS regression on commercial fish oil samples and the corresponding ethyl esters (EE) on a FT-NIR with selected wavelength regions and obtained a $R^2$ and SECV of 0.97 (3.23 %) for EPA and 0.98 (2.57 %) for DHA with the commercial fish oils; and for the EE oils obtained a $R^2$ and SECV of 0.99 (1.10 %) for EPA and 0.99 (0.97 %) for DHA respectively during CV, but no independent or external validation was performed.

Cascant et al.[34] determined fatty acids in 50 salmon oil samples with a FT-NIR spectrophotometer using selected wavelength regions. Results were reported as PUFA's and not individual constituents of AA, EPA and DHA. An external validation set was used, and the results were as follows for PUFA's: $R^2$ Cal, RMSECV, $R^2$ Pred and RMSEP were 0.97, 0.35 %, 0.91 and 0.76 % with a RPD of 2.41.

Although these papers reported different aspects, the common fact was that NIR spectroscopy could be implemented as an alternative technique to GC for the analysis of PUFA's in fish oils with adequate accuracy and precision. This study however, simultaneously evaluated numerous commercial oils in different forms and reported results for AA, EPA and DHA in mg.g$^{-1}$ and % area (EPA and DHA only) by independent (external) validation to pharmaceutical industry standards. Three data sets from two different reference methods were superimposed in a similar fashion to the concept of MMI. The combination of different reference methods is not common practice in NIR spectroscopy. However, both methods used the same GC technique and acceptable prediction models were developed.

## 3.6 CONCLUSION

NIR spectroscopy and chemometrics can be used for conclusive identification and quantification of 9 different omega-3 fish oils (concentrated and natural) thereby minimizing the risk of adulteration. By superimposing the data of the two different GC methods, the calibration ranges were extended to include higher and lower values not previously validated by GC; therefore, samples previously analysed by the USP method (% area) were re-tested by NIR spectroscopy to yield results by BP method ($mg.g^{-1}$); for new samples both BP and USP results can be obtained simultaneously using the NIR calibration models. Using NIR spectroscopy as an alternative method to GC, enables a significant time and cost saving on analysis of omega-3 fish oils.

This method can be applied to various stages of manufacturing such as QC, stability testing, in-process monitoring as well as release of raw material and finished products. In the laboratory (*off-line*) a bench top/laboratory instrument can be used for day-to-day routine analysis and stability studies of fish oil for identification and quantification purposes. In-process monitoring can be applied where the NIR instrument is close to production line (*at-line*), interfaced directly to the process using fibre optics and a stainless-steel probe (*in-line*) or the NIR instrument is interfaced indirectly to the process using a sample-loop (*on-line*). Raw material inspection and release can be executed by using an off-line or at-line approach saving time in sampling. Final product release can be performed on-line or off-line on intact solid dosages such as gelatine capsules containing omega-3 fish oils.

The NIR spectroscopy method complied with the prescribed pharmaceutical method validation criteria; therefore, was proven as an alternative method for the strictly regulated nutraceutical industry.

## 3.7 REFERENCES

1. I. Hamed, F. Özogul, Y. Özogul, and J. M. Regenstein, "Marine bioactive compounds and their health benefits: a review," *Comprehensive Reviews in Food Science and Food Safety* **14(4)**, 446 (2015).

2. D. Swanson, R. Block, and S. A. Mousa, "Omega-3 fatty acids EPA and DHA: health benefits throughout life," *Advances in Nutrition: An International Review Journal* **3(1)**, 1 (2012).

3. M. Sprague, M. Betancor, J. Dick, and D. Tocher, "Nutritional evaluation of seafood, with respect to long-chain omega-3 fatty acids, available to UK consumers," *Proceedings of the Nutrition Society* 2017; **76(OCE2)**, E38. doi:10.1017/S0029665117000945

4. J. J. Liu et al., "Omega-3 polyunsaturated fatty acid status in major depression with comorbid anxiety disorders," *The Journal of Clinical Psychiatry* **74(7)**, 732 (2013).

5. M. Sprague, J. R. Dick, and D. R. Tocher, "Impact of sustainable feeds on omega-3 long-chain fatty acid levels in farmed Atlantic salmon, 2006–2015," *Scientific Reports* **6**, 21892 (2016).

6. G. Lenihan-Geels and K. S. Bishop, "Alternative origins for omega-3 fatty acids in the diet," in *Omega-3 Fatty Acids*, Ed by M. Hegde, A. Zanwar and S. Adekar. Springer, Cham, p. 475 (2016).

7. A. Ismail, G. Bannenberg, H. B. Rice, E. Schutt, and D. MacKay, "Oxidation in EPA-and DHA-rich oils: an overview," *Lipid Technology* **28(3-4)**, 55 (2016).

8. M. Opperman and A. S. Benade, "Analysis of omega-3 fatty acid content of South African fish oil supplements: Cardiovascular topics," *Cardiovascular Journal of Africa* **22(6)**, 324 (2011).

9. K. Hamilton, P. Brooks, M. Holmes, J. Cunningham, and F. D. Russell, "Evaluation of the composition of omega-3 fatty acids in dietary oil supplements," *Nutrition & Dietetics* **67(3)**, 182 (2010).

10. J. C. S. Ritter, S. M. Budge, and F. Jovica, "Quality analysis of commercial fish oil preparations," *Journal of the Science of Food and Agriculture* **93(8)**, 1935 (2013).

11. Global Organization for EPA and DHA Omega-3 (GOED), "Voluntary Monograph," ver.5 (2015). (Retrieved on 01/12/2017) http://www.goedomega3.com/index.php/files/download/350

12. United States of America Food and Drug Administration (FDA), "Good manufacturing practices (GMP's) for dietary supplements," (2007). (Retrieved on 01/12/2017) https://www.fda.gov/Food/GuidanceRegulation/CGMP/ucm079496.htm

13. South African Department of Health, Medicines Control Council, "Guide to good manufacturing practice for medicines in South Africa," ver. 5 (2010). http://www.mccza.com/documents/16b9955c4.01SAGuidetoGMPJun10v5.pdf (Retrieved on 01/12/2017)

14. M. D. Levin, "The new food current good manufacturing practices and their effect on dietary supplement quality: What You Need to Know," *Integrative Medicine* **15(5)** (2016).

**15.** Complementary Health Council of Australia, "Guideline for the quality and safety of raw materials used in complementary medicines," ver. 1 (2012). (Retrieved on 01/12/2017) http://www.cmaustralia.org.au/Resources/Documents/Codes%20and%20Guidelines/The%20CHC%20Raw%20Material%20Guidelines%20.pdf

**16.** United States of America Food and Drug Administration (FDA), "Warning letter: Quali Controle & Qual-Controle-C.E.Bac 3/5/18," (2016). (Retrieved on 16/05/2018) https://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm353925.pdf

**17.** European Compliance Academy (ECA), “Warning letter due to non-validated Analytical Methods,” (2018). (Retrieved on 16/05/2018) https://www.gmp-compliance.org/gmp-news/warning-letter-due-to-non-validated-analytical-methods

**18.** United States of America Food and Drug Administration (FDA), " Contract manufacturing arrangements for drugs: quality agreements, guidance for industry," (2016). (Retrieved on 16/05/2018) https://www.fda.gov/ICECI/EnforcementActions/WarningLetters/ucm599876.htm

**19.** S. Govindaraghavan, "Pharmacopeial HPLC identification methods are not sufficient to detect adulterations in commercial bilberry (Vaccinium myrtillus) extracts. Anthocyanin profile provides additional clues," *Fitoterapia* **99**, 124 (2014).

**20.** M. Marieschi, A. Torelli, D. Beghé, and R. Bruni, "Authentication of Punica granatum L.: Development of SCAR markers for the detection of 10 fruits potentially used in economically motivated adulteration," *Food Chemistry* **202**, 438 (2016).

**21.** S. Lohumi, S. Lee, H. Lee, and B.-K. Cho, "A review of vibrational spectroscopic techniques for the detection of food authenticity and adulteration," *Trends in Food Science & Technology* **46(1)**, 85 (2015).

**22.** D. Cozzolino, A. Chree, J. Scaife, and I. Murray, "Usefulness of near-infrared reflectance (NIR) spectroscopy and chemometrics to discriminate fishmeal batches made with different fish species," *Journal of Agricultural and Food Chemistry* **53(11)**, 4459 (2005).

**23.** S. C. Cermak, R. L. Evangelista, and J. A. Kenar, "Distillation of natural fatty acids and their chemical derivatives," in *Distillation - Advances from Modeling to Applications,* Ed by Dr. S. Zereshki. InTech, http://www.intechopen.com/books/distillation-advancesfrom-modeling-to-applications/distillation-of-natural-fatty-acids-and-their-chemical-derivative (2012, accessed 12 May 2017).

**24.** British Pharmacopoeia (BP), "Composition of fatty acids, Appendix XP1," A301 (2012).

**25.** United States Pharmacopoeia (USP), "Fats and fixed oils, omega-3 fatty acids determination and profile," Chemical Test 401 **35**, 167 (2012).

**26.** M. Blanco, J. Coello, H. Iturriaga, S. Maspoch, and C. De La Pezuela, "Near-infrared spectroscopy in the pharmaceutical industry," *Analyst, London Society of Public Analysts Then Royal Society of Chemistry* **123**, 135R (1998).

**27.** K. Javidnia, M. Parish, S. Karimi, and B. Hemmateenejad, "Discrimination of edible oils and fats by combination of multivariate pattern recognition and FT-IR spectroscopy: A comparative study between different modelling methods," *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **104**, 175 (2013).

**28.** B. F. Ozen, I. Weiss, and L. J. Mauer, "Dietary supplement oil classification and detection of adulteration using Fourier transform infrared spectroscopy," *Journal of Agricultural and Food Chemistry* **51(20)**, 5871 (2003).

**29.** M. Plans, M. J. Wenstrup, and L. E. Rodriguez-Saona, "Application of infrared spectroscopy for characterization of dietary omega-3 oil supplements," *Journal of the American Oil Chemists' Society* **92(7)**, 957 (2015).

**30.** S. R. Karunathilaka, M. M. Mossoba, J. K. Chung, E. A. Haile, and C. T. Srigley, "Rapid prediction of fatty acid content in marine oil omega-3 dietary supplements using a portable Fourier Transform infrared (FTIR) device and partial least-squares regression (PLSR) analysis," *Journal of Agricultural and Food Chemistry* **65(1)**, 224 (2016).

**31.** D. Cozzolino, a. I. Murray, A. Chree, and J. Scaife, "Multivariate determination of free fatty acids and moisture in fish oils by partial least-squares regression and near-infrared spectroscopy," *LWT-Food Science and Technology* **38(8)**, 821 (2005).

**32.** H. Azizian, J. K. Kramer, S. Ehler, and J. M. Curtis, "Rapid quantitation of fish oil fatty acids and their ethyl esters by FT-NIR models," *European Journal of Lipid Science and Technology* **112(4)**, 452 (2010).

**33.** Y. Zhang, F. Cao, and Y. He, "Application of visible and near infrared spectroscopy for rapid and non-invasive quantification of adulterants in fish oil," *Spectroscopy and Spectral Analysis* **33(6)**, 1532 (2013).

**34.** M. M. Cascant, C. Breil, A. S. Fabiano-Tixier, F. Chemat, S. Garrigues, and M. de la Guardia, "Determination of fatty acids and lipid classes in salmon oil by near infrared spectroscopy," *Food Chemistry* **239**, 865(2018).

**35.** D. P. Killeen, S. N. Marshall, E. J. Burgess, K. C. Gordon, and N. B. Perry, "Raman spectroscopy of fish oil capsules: Polyunsaturated fatty acid quantitation plus detection of ethyl esters and oxidation," *Journal of Agricultural and Food Chemistry* **65(17)**, 3551 (2017).

**36.** J. L. Xu, C. Riccioli, and D. W. Sun, "An overview on non-destructive spectroscopic techniques for lipid and lipid oxidation analysis in fish and fish products," *Comprehensive Reviews in Food Science and Food Safety* **14(4)**, 466 (2015).

**37.** W.W. Hauck, A.J. De Stefano, T.L. Cecil, D.R. Abernethy, W.F. Koch and R.L. Williams. “Stimuli to revision Process: Acceptable, equivalent, or better - Approaches for alternatives to official compendial procedures.” *Pharmacopeial Forum* **35(3)**, 772 (2009).

**38.** D.A. Burns, & E.W. Ciurczak (eds). “Handbook of Near-Infrared Analysis,” (2nd edition), *Practical Spectroscopy Series* **27**, New York: Marcel and Dekker (2001).

**39.** United States Pharmacopoeia (USP), "Near-infrared spectrophotometry," General chapter 1119 **35**, 714 (2012).

**40.** British Pharmacopoeia, "Infrared spectrophotometry,” Appendix II A, A156 (2012).

**41.** Pharmaceutical Analytical Sciences Group (PASG), NIR sub-Group, "Guidelines for the development and validation of near-infrared spectroscopy methods in the pharmaceutical industry," PASG (2002). (Retrieved on 30/11/2017) http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.452.9685&rep=rep1&type=pdf

**42.** European Medicines Agency (EMA), “Guideline on the use of near infrared spectroscopy by the pharmaceutical industry and the data requirements for new submissions and variations," (2014). Retrieved on 01/12/2017 http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000764.jsp&mid=

**43.** International Conference on Harmonisation (ICH) of technical requirements for registration of pharmaceuticals for human use, “Validation of analytical procedures: text and methodology, Q2(R1),” *ICH Harmonised Tripartite Guideline* (2005). Retrieved on 14/04/2018 http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf

**44.** G. Bannenberg et al., "Omega-3 long-chain polyunsaturated fatty acid content and oxidation state of fish oil supplements in New Zealand," *Scientific Reports* **7** (2017).

**45.** S. Srinivasa, "A review on multivariate mutual information," *Univ. of Notre Dame, Notre Dame, Indiana* **2**, 1, (2005, accessed 17 May 2018). https://pdfs.semanticscholar.org/1fb7/d5ea2212fd36f7ad83ef2630f69609b0dbba.pdf,

**46.** J. Venn, “I. On the diagrammatic and mechanical representation of propositions and reasonings”, *Philosophical Magazine Series 5* **10(59)**, 1 (1880), DOI: http://dx.doi.org/10.1080/14786448008626877

**47.** J. D. Rodriguez, A. Perez, and J. A. Lozano, "Sensitivity analysis of k-fold cross validation in prediction error estimation," *IEEE Transactions on Pattern Analysis and Machine Intelligence* **32(3)**, 569 (2010).

**48.** P. Williams, P. Dardenne, and P. Flinn, "Tutorial: Items to be included in a report on a near infrared spectroscopy project," *Journal of Near Infrared Spectroscopy* **25(2)**, 85 (2017).

**49.** S.A. Halsey, "Quantitative NIR calibration protocol," NIRSystems Inc., Silver Spring, Maryland (1998).

**50.** A. Garrido-Varo, D. Pérez-Marín, J. Bautista-Cruz, and J. E. Guerrero-Ginel, "Near infrared spectroscopy for quantification of animal-origin fats in fat blends," *Journal of Near Infrared Spectroscopy* **16(3)**, 281 (2008).

**51.** M. Sohn, Y. Kim, L. L. Vines, and S. W. Kays, "Near infrared analysis of lipid classes in processed cereal products," *Journal of Near Infrared Spectroscopy* **17(3)**, 127 (2009).

# CHAPTER FOUR
# GENERAL DISCUSSION AND CONCLUSIONS

QC analysis is imperative to manufacturing quality products and generating consumer confidence but is often a costly and time-consuming process. Maintenance costs of analytical equipment such as GC are high, leading to a high cost of analysis. The investigation into the use of a more cost-effective and environmentally friendly technique such as NIR spectroscopy was justified. Minimizing QC analysis time improved the efficiency of the production, thus, the application of NIR spectroscopy lowered the production costs of nutraceutical products containing omega-3 PUFA's.

Before implementing NIR spectroscopy as an alternative QC method to GC for the analysis of omega-3 fish oils, the NIR method had to demonstrate equivalence through method validation according to pharmaceutical requirements. The NIR method also adapted with the changes in the prescribed reference methods and thus avoided having to initiate a new spectroscopic calibration. Therefore, the challenge to develop and validate NIR spectroscopy models according to pharmaceutical criteria by superimposing the spectral and reference data from natural TG and concentrated TG oils to quantify the PUFA's expressed as % area and mg.g$^{-1}$ applied in the context of MMI, was successful.

Considering the research objectives, the aim of the research was to investigate to what extend NIR spectroscopy and chemometrics can be applied to the QC of n-3 PUFA's. Using NIR spectroscopy as an alternative method to GC, enables a significant time and cost saving on analysis of omega-3 fish oils. Sample preparation was performed without the use of solvents thereby confirming to be an environmentally friendly technique.

NIR spectroscopy and chemometrics can be used for conclusive identification (spectral libraries) and quantification of nine different omega-3 fish oils (concentrated and natural) thereby minimizing the risk of adulteration. The NIR spectroscopy method complied with the prescribed pharmaceutical method validation criteria; therefore, was proven as an alternative method for the strictly regulated nutraceutical industry.

By superimposing the data of the two different GC methods, the calibration ranges were extended to include higher and lower values not previously validated by GC; therefore, samples previously analysed by the USP method (% area) were re-tested by NIR spectroscopy to yield results by BP method (mg.g$^{-1}$); for new samples both BP and USP results can be obtained simultaneously using the NIR calibration models.

RPD values ranging from 3.1 to 4.9 are adequate for screening purposes, from 5.0 to 6.4 for QC, from 6.5 to 8.0 for process control and values over 8.1 are suitable for any type of application. RPD results of the (mg.g$^{-1}$) extended models were 4.0, 56.9 and 8.3 for AA, EPA and DHA respectively. RPD results obtained for the extended models were adequate for QC and process control purposes except for AA, which had an RPD of 4.0. Therefore, this method can be applied to different stages of the manufacturing process such as QC, stability testing, in-process monitoring as well as release of raw material and finished products incorporating the concept of process analytical technology (PAT).

Bench top/laboratory instruments can be used in the laboratory (*off-line*) for day-to-day routine analysis and stability studies of fish oil for both identification and quantification purposes. In-process monitoring can be applied *at-line, in-line* or *on-line*: where the NIR instrument is close to production line (*at-line*) or interfaced directly to the process using fibre optics and a stainless-steel probe (*in-line*) or the NIR instrument is interfaced indirectly to the process using a sample-loop (*on-line*). Raw material inspection and release can also be performed by using an *off-line* or *at-line* approach saving time in sampling. Final product release can be achieved *on-line* or *off-line* on intact solid dosages.

In conclusion, NIR spectroscopy has enormous potential for improving the monitoring and control of industrial processes and can be applied to other nutraceuticals, in various forms, analysed by different analytical techniques. The key to successful implementation of NIR spectroscopy in the nutraceutical and/or pharmaceutical industry is to prove that the methods are equivalent to the prescribed methods through validation to meet the criteria as prescribed by the pharmacopoeias and other industry guidelines.

# APPENDIX

## Validation of spectral libraries for identification/ qualification of fish oils

Six spectral libraries as summarised in Table A1 were developed for the different fish oils (TG and EE) using different supplier/manufacturers batches for the training and external validation sets. Sample selection for all of the libraries was performed using *maximum distance in wavelength space* applying 2$^{nd}$ derivative (gap-segment) pre-processing (0,10) to correct for spectral offset. Most spectral libraries were developed using *correlation in wavelength space* and one library using *Mahalanobis distance in PCA space* with a cumulative variance (CV) of 95%, applying 2$^{nd}$ derivative (gap-segment) pre-processing (0,10). The libraries for *Cod liver, Fish 18/12, Salmon* and *Mackerel oil* contained only one library product each due to ambiguity that could not be eliminated with a further qualification step. For these four libraries it was required to cross-check (challenge) the library with the other oils not included in the library to prove selectivity. Qualification steps for the *Incromega* libraries were successfully implemented to overcome ambiguity between library products. The Vision® 3.50. software offers a 3-D histogram with bars representing the different library products which will indicate misidentified samples or conflicting products.  Figure A1 displays the 3-D histogram of library *Incromega_lib_b* with no misidentified samples or conflicting products.

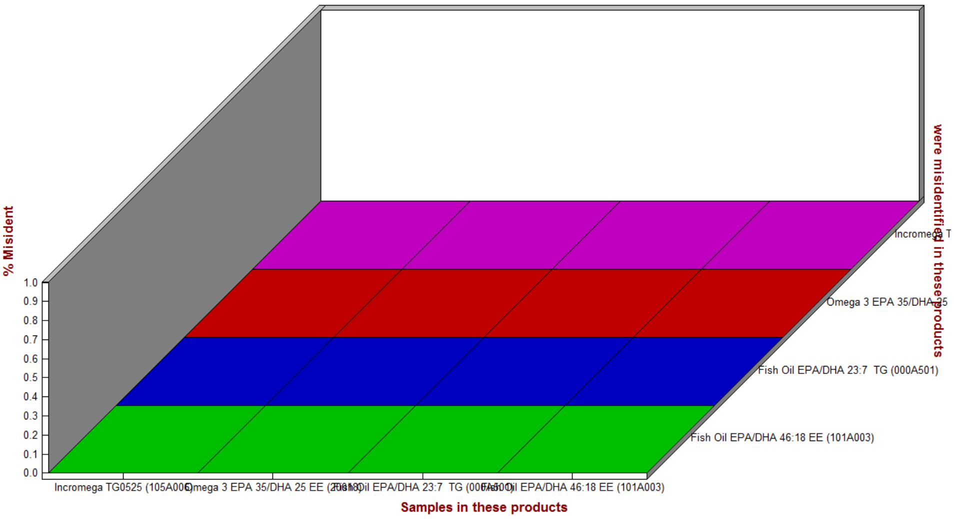

**Figure A1: The 3-D histogram with bars representing the different library products with no misidentified samples or conflicting products for library Incromega_lib_b displayed by Vision® 3.50**

## Table A1: Identification/ Qualification Models (spectral libraries) for the different fish oils (TG and EE) developed using Vision® 3.50

| Library name | ID method | Spectral region (nm) | Pre-treatment | Library threshold | Fish oil product | No. of Samples | Training Set | Acceptance Set | Rejected Set | Qualification method | Spectral region (nm) | Pre-treatment | Qualification threshold |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cod_Liver_lib | Correlation | 1100-2500 | 2nd derivative (10,0) | 0.9980 | Cod Liver Oil | 66 | 49 | 17 | 0 | - | - | - | - |
| Fish_18-12_lib | Correlation | 1100-2500 | 2nd derivative (10,0) | 0.9998 | Fish 18/12 | 21 | 16 | 5 | 0 | - | - | - | - |
| Salmon_oil_lib | Correlation | 1100-2500 | 2nd derivative (10,0) | 0.9990 | Salmon Oil | 77 | 58 | 19 | 0 | - | - | - | - |
| Mackerel_lib | Correlation | 400-2500 | 2nd derivative (10,0) | 0.9990 | Mackerel Oil | 30 | 22 | 8 | 0 | - | - | - | - |
| Incromega_lib_A | Mahalanobis Distance in PCA space, Cumulative Variance (CV) =95% | 1100-2500 | 2nd derivative (10,0) | 0.6000 | Incromega E3322 | 24 | 19 | 5 | 0 | Correlation | 400-2500 | 2nd derivative (10,0) | 0.9998 |
| | | | | | Incromega Trio EE | 9 | 7 | 2 | 0 | Correlation | 400-2500 | 2nd derivative (10,0) | 0.9999 |
| | | | | | Incromega TG DHA500 | 30 | 23 | 7 | 0 | - | - | - | - |
| Incromega_lib_B | Correlation | 400-2500 | 2nd derivative (10,0) | 0.9992 | Incromega TG0525 | 21 | 16 | 5 | 0 | Maximum distance | 1100-2500 | 2nd derivative (10,0) | 6.000 |
| | | | | | Omega 3 EE EPA/DHA 35:25 | 21 | 18 | 3 | 0 | - | - | - | - |
| | | | | | Fish Oil TG EPA/DHA 23:7 | 9 | 9 | 0 | 0 | Maximum distance | 1100-2500 | 2nd derivative (20,0) | 6.000 |
| | | | | | Fish Oil EE EPA/DHA 46:18 | 10 | 8 | 2 | 0 | - | - | - | - |