TUS

Technological University of the Shannon: Midlands Midwest

Ollscoil Teicneolaíochta na Sionainne: Lár Tíre Iarthar Láir

A risk assessment-based control strategy to formulate a potent, efficacious, and non-invasive topical onychomycotic dosage form

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Microbiology (Pharmaceutical Sciences and Pharmaceutical Microbiology).

Based on research carried out under the supervision of Mr. Jim Roche, Dr. Damien Brady, and Dr. Carmel Kealey.

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Declaration

I hereby declare that this thesis, submitted to the TUS: Midlands Midwest for the degree of Doctor of Philosophy, is a result of my own work and has not in the same or altered form, been presented to this institute or any other institute in support of my degree other than which I am now a candidate.

Signature

Date

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Abstract

This project involves the development of a strategy to formulate a novel dosage form with modified coconut oil (MCO) as the active pharmaceutical ingredient to treat onychomycosis caused by *T. rubrum* and *C. albicans*.

The therapeutically effective concentration of the modified coconut oil towards *T. rubrum* has been established by preparing its dilutions in water-soluble and fat-soluble bases separately and testing them against the organism. It was observed that modified coconut oil dilutions were more potent, efficacious, and advantageous than Naloc™ – a commercial dosage form available to treat onychomycosis caused by *T. rubrum*. The water-soluble modified coconut oil formulations were determined to be comparatively more efficient than either fat-soluble compositions or Naloc™ with superior activity at lower concentrations and also within a short period of exposure.

Therefore, developing a risk assessment-based strategy to formulate modified coconut oil into a water-soluble matrix to treat onychomycosis was targeted in this research. The principles of the tripartite guideline of the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use – a collaboration of the regulatory authorities of the United States of America, Japan and Europe also known as ICH Q8 (R2) Pharmaceutical Development (2009) have been employed in this research for the development of dosage forms.

Pre-formulation studies including a combination of spectroscopic and thermal analytical techniques and biological assays, were conducted on MCO to identify critical quality attributes to inform a quality targeted product profile. A matrix of chemical and biological tests had first been designed to characterize the compatibility of various established excipients with MCO. The best-in-class dosage form amongst them was discriminated by evaluating the extent of risk posed by the components of the formulation towards the stability of the dosage form. The stability of the finalised formulation towards induced stress conditions was evaluated for a period of 90 days. The stability was assessed using turbidity measurements, particle size distribution, conductivity and texture analysis. The antimicrobial efficiency of the samples from the ICH-mandated pull point intervals was assessed using microbiological assay and resistance towards microbial contamination conducted as per USP <61>. Based on the data generated from the pull point samples, quality control charts were constructed, and the optimum formulation developed using this risk assessment-based strategy was shown to be stable over 90 days.

Future research will include scaling up production of the prototype formulation to identify critical process parameters in order to improve yield and perform extended stability studies. Dissolution studies could be performed to establish the drug release profile of MCO and the development of chromatographic techniques to quantify the components of MCO will be required to further characterise the formulation.

Presentations

- Dinesh Kapu, Damien Brady, Carmel Kealey, Mr Jim Roche (May 14 and 15th. 2018). Oral presentation on "*A correlation of the physical attributes of a potent anti-onychomycotic dosage form with microbiological performance*" in Eurachem 2018, Dublin Castle, Dublin, Ireland.

- Dinesh Kapu, Dr Damien Brady, Dr Carmel Kealey, Mr Jim Roche (April 13 and 14th. 2016). "*Rapid analytical strategy for the selection of excipients for topical dosage form*" in "Industry meets academia", the international symposium conducted by BASF in collaboration with University College London, UK.

- Dinesh Kapu, Dr Damien Brady, Dr Carmel Kealey, Mr Jim Roche (June 25 and 26th. 2014). "*Developing a physiologically relevant in vitro analytical protocol for infections of the oropharyngeal tract***"** in the fourth International Malaysia-Ireland Symposium. The University of Malaysia. Penang, Malaysia.

- Dinesh Kapu, Dr Patricia Hughes, Dr Damien Brady, (June 13. 2013). "*Antimicrobial Properties of Essential Oils*" in the third International Malaysia-Ireland Symposium. Athlone Institute of Technology. Athlone. Ireland.

List of Abbreviations

AAE	Active Antifungal entity
API	Active pharmaceutical ingredient
AOCS	American Oil Chemists Society
ANDA	Abbreviated new drug application
ATR-FTIR	Attenuated total reflection – Fourier transform infrared
BET	Bacterial endotoxin test
Cd	Cadmium
cm	Centimetres
CTD	Common technical document
CPV	Continuous process verification
Cu	Copper
CMA	Critical material attributes
CMC	Critical micellar concentration
CQA	Critical quality attributes
cGMP	Current good manufacturing practices
DA	Decanoic acid
^{o}C	Degrees Celsius
DEA	Diethanolamine
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetic acid
FAAS	Flame atomic absorption spectrometry
GRAS	Generally regarded as safe
g	Grams
HPLC	High pressure/performance liquid chromatography
HIV	Human Immunodeficiency Virus
HCl	Hydrochloric acid
HLB	Hydrophilic lipophilic balance
ICP-MS	Inductively coupled plasma – mass spectrometry
IR	Infrared

ISO	International standards organisation
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals For Human Use
Ifs	Interfilaments
IPEC	International Pharmaceutical Excipients Council
IND	Investigational novel drug
Fe	Iron
KF	Karl Fischer
kDa	Kilodaltons
kg	Kilograms
Pb	Lead
LAL	Limulus amoebocyte lysate
Mn	Manganese
MHz	Megahertz
m	Metres
Hg	Mercury
µ	Micro
mL	Millilitre
mm	Millimetre
MCO	Modified coconut oil
M	Molar
MEA	Monoethanolamine
nm	Nanometres
NIR	Near-infrared
NCE	Novel chemical entity
NDA	New drug application
NME	Novel molecular entity
Ni	Nickel
NMR	Nuclear magnetic resonance
o/w	Oil-in-water
o/w/o	Oil-in-water-in-oil

OTC	Over-the-counter
Pd	Palladium
ppm	Parts per million
PDE	Permitted daily exposure
KOH	Potassium hydroxide
PEG	Polyethylene glycol
QbT	Quality-by-testing
QbD	Quality-by-design
QOS	Quality overall summary
QTPP	Quality targeted product profile
RH	Relative Humidity
Rh	Rhodium
rpm	Rotations per minute
Ru	Ruthenium
NaOH	Sodium hydroxide
SDA	Sabouraud dextrose agar
SA	Stearic acid
Th	T – helper cells
TA	Texture analysis
TGA	Thermal gravimetric analysis
TEA	Triethanolamine
US FDA	United States Food and Drug Administration
USP	United States Pharmacopoeia
VCO	Virgin coconut oil
w/o	Water-in-oil
w/o/w	Water-in-oil-in-water
W	Watt
cm^{-1}	Wave number
w/v	Weight per volume
w/w	Weight per weight
WHO	World Health Organisation

XRD	X – ray diffraction
Zn	Zinc

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Origin

During the course of a systematic investigation into the nature and extent of antiproliferative activity at various process stages during bench preparation of naturally-derived antimicrobials, a surprisingly potent effect on those fungal species associated with nail-based infections was observed during different modes of antimicrobial testing (such as agar mixing, spreading and disc diffusion).

Scope

Based upon the former two contact (spreading and mixing) methods, growth of the key fungal organism was not observed at any level of concentration with the pathogen being mixed directly with the active compound of interest. Whereas, in the disc diffusion assessment, the zone of inhibition increased with increase in concentration. The results from the above studies informed that maximum antifungal activity was observed when the pathogen was in direct contact with the active antifungal entity. Accordingly, it was decided to further investigate the compound's *in vitro* potential through the topical route of administration which provides maximum/direct contact with the pathogen.

Novelty of this research

In contrast to more traditional approaches for developing a dosage form by testing key attributes and comparing the results with reference-listed drugs to enumerate product quality, the principles of ICH Q8 (R2) have been implemented in this research.

Based on patient-centric requirements for an effective treatment of onychomycosis, a developed dosage form has been optimised, informed by functional characterisation in a bio-relevant environment. A parallel and cross-functional analytical strategy has been developed and implemented throughout the project to ensure this linkage and quality has been maintained consistently.

Results derived from the amalgamated analytical techniques such as physical texture determinations and chemical identification standing alongside microbiological assays, positioned the developed dosage form in challenging an over-the-counter commercial product with an efficiency of nearly three times higher than the latter, while remaining stable over a period of 90 days.

Therefore, the strategy implemented to develop, scrutinise, and optimise the dosage form established upon applications of cross functional analytical techniques, has been presented as foundational technology in this research.

1.0 Anatomical features of a healthy nail

The nail apparatus of the limb phalanges is an association of modified skin materials, containing a nail bed of peri-ungual tissues similar to the dermis (See Figure 1.1). This bed extends from distal parts of the lunula to the hyponychium supporting the nail plate (Lencastre *et al*., 2013). The stratum corneum of skin is modified into a keratinaceous nail plate and the matrix connects the plate and bed (Sangshetti *et al*., 2013). Nail matrix constitutes 15 – 25 % and the nail bed from 75 – 85 % of the tissues below nail plate, where the former is visible in some individuals adjacent to the lunula. The distal and proximal territories of the matrix can be differentiated by the number of cells evolved from different regions or by any surgery to the distal part that can leave scarring (Andre *et al.*, 2013). Unlike skin, dermis of the nail bed is collagenous adhering to the underlying periosteum without any sebaceous glands or follicular appendages and with sweat glands at the distal end. The space between the onychodermal band and hyponychium is the primary site of infection and serves as a potential reservoir for pathogens responsible for infections such as onychomycosis. Subungual keratosis is the major effect observed in such infections (Berker, 2013).

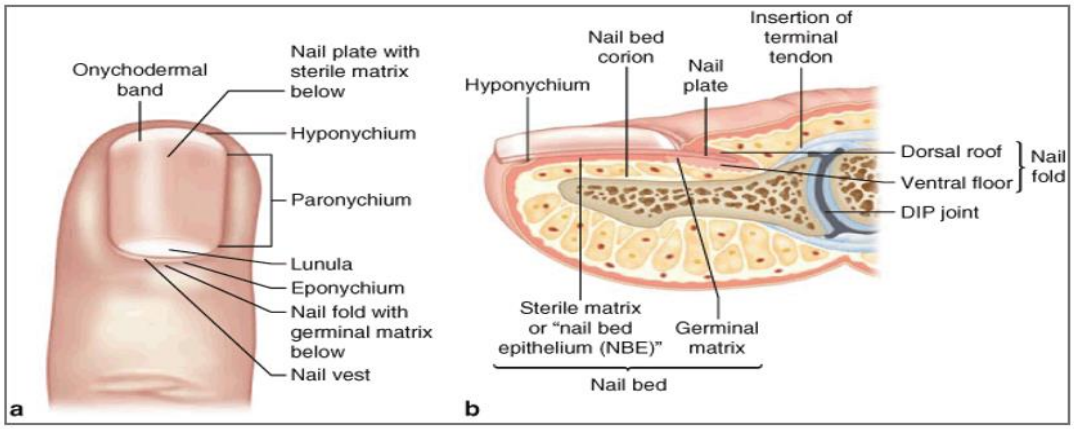


Figure 1. 1| **Anatomy of nail** (a) Nail apparatus explaining proximal nail fold followed by waxy cuticle cementing the junction of fold with nail plate. A white half-moon shaped lunula represents healthy nail. Groove of nail plate into the nail bed is proximal matrix followed by distal matrix. The gap between the nail plate fold and nail bed is the hyponychium. (b) Transverse section of human finger explaining nail bed epithelium and germinal matrix from which nail plate develops (Thomas *et al.,* 2010; Baswan *et al.,* 2016, Gollins & Berker 2021).

Increased surface area due to curvature of lateral and proximal folds of the nail bed provides cushioned support during walking, holding and cutting etc (Berker,

2013). Any changes in the occupancy of these lateral folds lead to pathological invasion, which is generally observed in the toes or after severe trauma has been exerted on the fingers. Along with the proximal nail fold, the cuticle forms a protective layer against microbial or chemical invasion. Repeated intensive manicure or dermatophytic infection can deplete this layer and allow microbial penetration. Any trauma or infection will thin the nail plate and reverses the lunula (Berker & Baran, 2012).

Continuous mitosis in the nail bed develops the nail matrix, in which the protein containing superficial cells differentiate and lose water upon aging. These cells further discard their components into matrix, form layers, and harden into a nail plate made of sulphur-enriched keratin protein (Wang *et al*., 2016). The growth rate of a nail plate is the change in longitudinal length per unit time. A healthy nail plate contains 196 cell layers, which can be differentiated into ventral (near to nail bed), intermediate (above ventral) and dorsal (top layer) based on the distance between nail bed and the last layer of the nail plate (See Figure 1.1). Dorsal layers are tough and dry due to sulphydryl groups being at a higher concentration than other layers, providing strength and preventing nail infections. The intermediate layers are soft and in a state of transition to replace any damaged dorsal layers. The amino acid cysteine is highly concentrated in this region providing disulphide crosslinking. The ventral layers next possess sensory nerves and provide required nutrients for the growth of cells. The ratio of thicknesses of these layers is 3:5:2 (Wang *et al*., 2016).

1.1 Keratin – a functional protein for pathogenesis

The structural component of the nail plate is keratin, a fibrous protein also observed in skin and hair. In humans, keratins are one of the most commonly found proteins along with collagen. Based on the arrangement of peptides in their polymeric chain similar to conventional alpha and beta-pleated sheets of proteins, keratins are classified as α and β types. α – Keratins or soft keratins range from seven to ten nanometres (nm) in diameter and are abundant in hair, hooves, wool, skin and nails of mammalian species. Structural components of α – keratins can be divided into intermediate filaments (IFs) and matrix and with a molecular weight ranging from 40

to 68 kilodaltons (kDa). IFs contain low sulphur proteins and the matrix is made of high sulphur proteins of high-glycine-tyrosine sequences (See Figure 1.3). These sulphur matrices are the preliminary target of infection during the proliferation of fungal pathogens.

IFs and matrix intertwine into right-handed helices. Two such helices form a dimer, which aggregates end to end and staggers side by side through disulphide bonds constructing protofilaments with another dimer (See Figure 1.2). Two such protofilaments bind together into protofibril and four such protofibrils form into a circular or helical protein of seven nanometres (nm) in diameter (Wang *et al*., 2016).

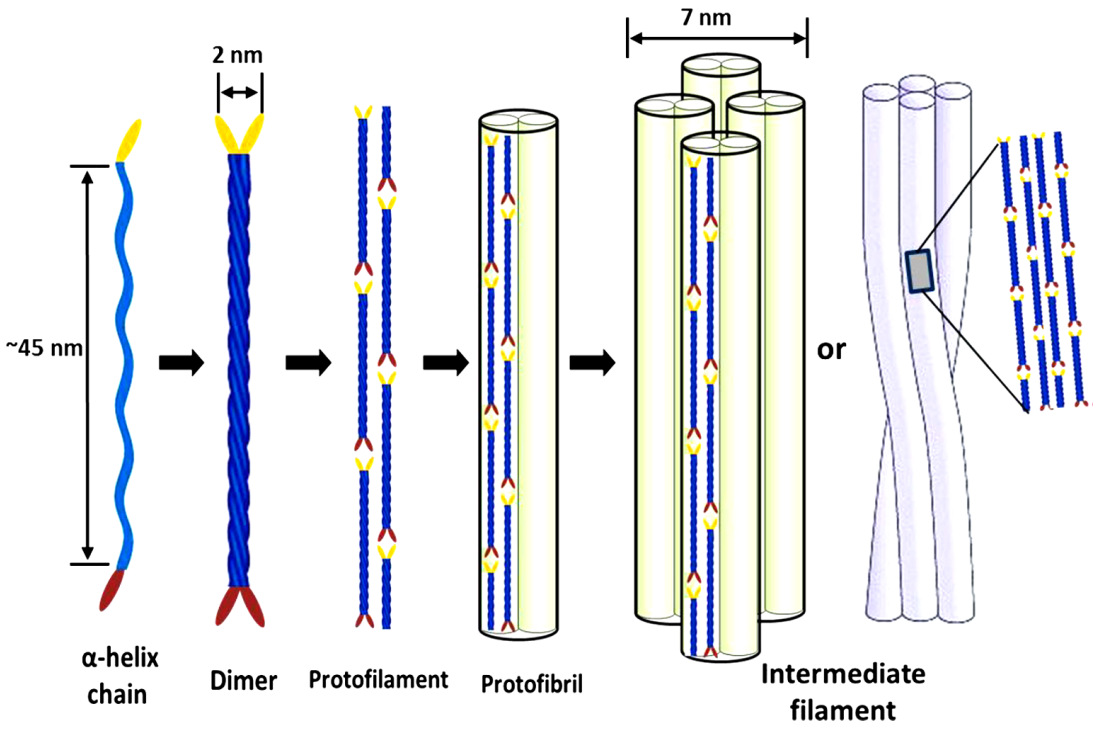


Figure 1. 2| **Formation of α – keratin.** Intermediate filaments form two α – helices of 45 nm length each into a dimer, followed by formation of protofilament, protofibril and intermediate filament (Wang *et al*., 2016).

β – Keratins or hard keratins vary from three to four nm in diameter and are present in scales, cornified skin and provide anti-dehydration protection from the skin of reptiles with a role related to feathers of birds. β – Pleated sheets are laterally packed either in parallel or antiparallel directions with a molecular weight range from 10 – 22 KDa. Intermolecular hydrogen bonds hold the sheets together and the peptide bonds between the amino acid chains maintain the core sheet structure. The

central polypeptide chain folds into four pleated sheets, which are held together by hydrogen bonds. A couple of sheets connected by a horizontal diad, superpose onto each in opposite directions to form a filament of four nanometre (nm) diameter. The terminal peptide chains wind around the sheets to form matrix. Due to these structural arrangements, β – keratins are also called as polymer/polymer-composite crystalline structures embedded into amorphous matrix (Wang *et al*., 2016).

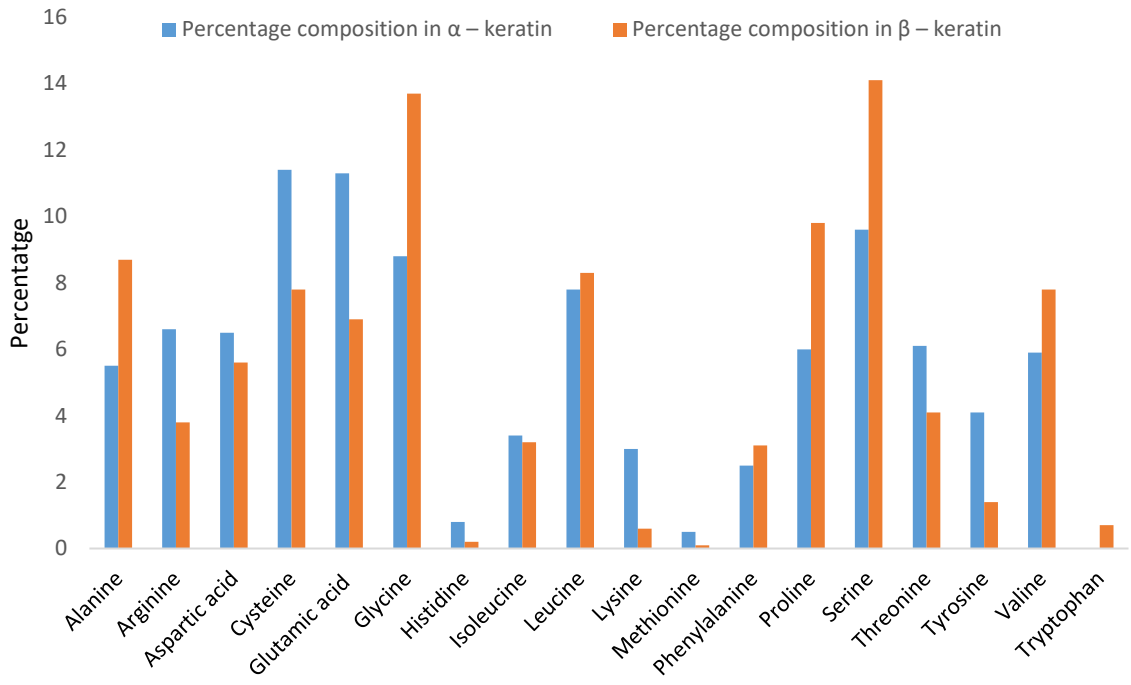


Figure 1. 3| **Comparison of chemical composition of α and β keratins**. Sulphur containing amino acids cysteine and methionine are comparatively high in α – keratins than in β type.

A total of 54 genes code keratin structures in humans making them the largest group among all gene traits. Anthropogenic keratins (α) can be broadly divided into two major categories, type I – acidic and type II – basic or neutral. Nomenclature includes higher case ''K'' followed by a number according to the position observed in two-dimensional polyacrylamide gel electrophoresis. Type-II keratins are from one to nine and type – I start from ten with four subgroups individually in each. Old nomenclature coined type – I as Ha1 to Ha8 as the major locus of investigation was hair samples. Similarly type – II was referred as Hb1 to Hb6 (See Table 1.1) (Gong *et al*., 2012).

Table 1. 1| **Types of keratin observed in healthy adult human nail complex**(de Berker 2013):

Type II keratins	Type I Keratins	Nail fold	Nail bed	Matrix
K1	K10	+	-	+
K5	K14	+	+	-
K6a		-	+	+
K6b	K16	-	+	+
	K17	-	-	+
K81 (Hb1)	K31 (Ha1)	-	-	+
K85 (Hb5)	K32 (Ha2)	-	-	+
K86 (Hb6)	K34 (Ha4)	-	-	+
	K38 (Ha8)	-	-	+

+ = present; - = absent

Owing to the structural integrity of IFs and matrix of fibrils, the Knoop hardness value of a healthy nail will range from 20 – 40 kg/mm^2 and Young's modulus of elasticity being typically 4.3×10^{10} dynes/cm^2 in parallel transection and 2.1×10^{10} dynes/cm^2 in perpendicular transection. Thus, both the structure and composition of keratin support the nail apparatus during regular activities. Onycholysis, a detachment of nail from its bed is the development of a granular layer; this is an expression of keratins of terminal differentiation and loss of the corrugations on the inner surface of the nail plate during infections or disorders such as onychomycosis.

1.2 Onychomycosis: a pressing problem among ungual diseases.

Onychomycosis is a fungal nail condition caused by dermatophytes (organisms that infect topical surfaces), yeasts and non-dermatophytes. It is one of the concurrent diseases of *Tinea pedis* or athlete's foot and a most common disease among immune-compromised individuals. Symptoms include discolouration, surface modifications, hyperkeratosis, slow nail growth and breakdown of nails (Gupta & Simpson 2012). A majority of the British population aged over 60 years and above are observed to have onychomycosis (Ameen *et al*., 2014, Stewart *et al.,* 2020). Increasing age is associated with reduced peripheral-circulation coupled with inactivity, a suboptimal immune status, larger and distorted nail surfaces, diabetic conditions, difficulty in grooming the nails and improper foot hygiene are the factors which cumulatively render the subject more susceptible to infection (Thomas *et al.,* 2010).

Sportspeople, especially athletes, are particularly susceptible and estimated to be 1.5 times more prone to onychomycosis than non-athletes due to increased sweating, severe exposure to mycoses, trauma and previous exposure to dermatophytes that left secondary metabolites such as polyketide synthases and lysin motif (Baran & Hay, 2015; Thomas *et al*., 2010).

One third of diabetics suffer from onychomycosis. Age, gender, peripheral vascular disease, poor glycaemic control, obesity and hypertriglyceridemia are the factors responsible for elevated onychomycotic conditions in diabetic populations (Gupta *et al.*, 1998; Chang *et al*., 2008, Lipner & Scher 2019). High blood sugar levels can promote fungal infection and may ultimately cause severe problems such as amputation if left untreated (Cathcart *et al*., 2009). Cornified skin and nails can disrupt the adjacent healthy tissues and epithelial layers, allowing the growth of infectious pathogens. Debris below the infected nail plate acts as a potential reservoir of several opportunistic pathogens such as *Candida albicans* (*C. albicans*) and aggravates the condition. The prominent treatment of nail evulsion cannot be performed in diabetic populations with onychomycosis due to risk factors such as gangrene, concurrent dermal infections and amputation (Robbins, 2004).

1.2.1 Clinical classification of onychomycosis

Based on the pattern of infection on the ungual region, onychomycosis is divided into five major categories (Figure 1.4) distal and lateral-subungual (a, b) superficial (c) proximal subungual (d) and endonyx - total dystrophic (e) (Agarwal *et al.,* 2021).

In distal subungual onychomycosis, the nail bed, plate and hyponychium are affected. These symptoms are also observed with lateral onychomycosis, but the invasion starts from the *Tinea pedis*. Immune-compromised conditions during athlete's foot weakens the subject and promotes onychomycosis. Superficial white onychomycosis is a rare clinical condition in which the nail develops a white chalky appearance or islands. In severe conditions, the patient suffers from yeast (*Candida sp*) infection due to low immune response along with onychomycosis leading to paronychia (a clinical condition observed at the junction of nail plate and skin of fingers). In proximal onychomycosis, pathogen proliferates from the proximal parts of nail and the frequency of this mode is high in immunosuppressed patients. The nail plate is directly affected without onycholysis or hyperkeratosis in endonyx modes of infection. Complete loss of nail is observed in total dystrophic onychomycosis caused by *Trichophyton rubrum* (*T. rubrum*) along with other *Trichophyton* species, due to the erosion of infected nail plate from the bed (Welsh *et al.,* 2010).

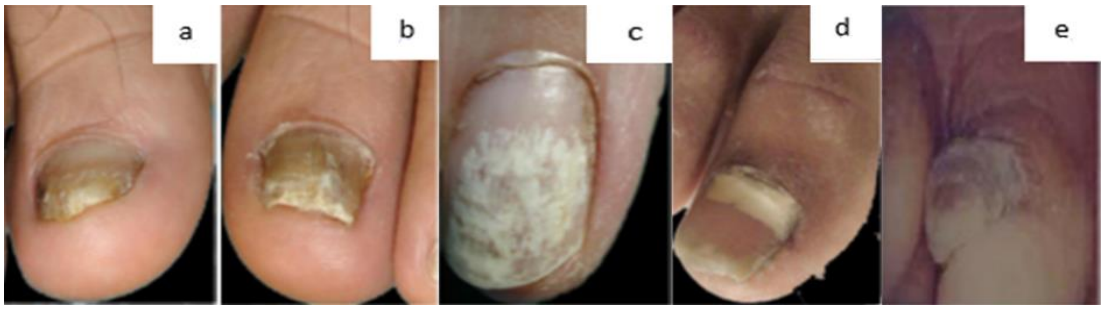


Figure 1. 4| **Appearance of toenails during onychomycosis.** (a) distal subungual – terminal part of the nail is infected (b) lateral subungual – paronychial margins are affected (c) superficial white – upper surface of the nail plate is affected with patchy appearance (d) proximal – lunular region of the nail is infected (e) total dystrophic onychomycosis – nail plate is completely eroded from the nail bed (Agarwal *et al*., 2021).

For an effective treatment, thorough examination of nails is required to diagnose the preliminary cause for the condition, as certain fungal infections such as candidiasis can deplete nail apparatus and resemble onychomycosis.

1.2.2 Diagnosing pathogens of onychomycosis

Preliminary symptoms of onychomycosis are white-yellow or orange-brown streaks or patches on nail. Onycholysis or subungual keratolysis or cornified nail plate are secondary presentations (Scher *et al*., 2007). The diagnostic techniques include direct microscopic examination with potassium hydroxide (KOH) solution; culturing nail clippings and acid-Schiff staining of samples from nail biopsy; immunohistochemistry; restriction fragment length polymorphisms (RFLP) and polymerase chain reactions (Welsh *et al*., 2010, Agarwal *et al.,* 2020).

In chemical mode, the KOH solution containing a mixture of 15% KOH in dimethyl sulphoxide is used. The alkali dissolves the keratin of the specimen and exposes the pathogen. The visibility is high in the presence of dyes such as Parker blue or chlorazol black. The most efficient dye however is Calcofluor white, due to its higher affinity towards polymers such as cellulose and chitin, the common cell wall components (Gong *et al., 2016*). According to Welsh *et al.,* (2010), sample collection includes careful removal of infected or suspected regions after cleaning with 70% ethanol. The accuracy of the test depends on the clinician's ability to adequately collect samples. Thus, collected specimens must be stored in a medium augmented with antibiotics and cycloheximide to avoid contamination. The incubation temperature for diagnostic purposes would be 25 – 27^{o}C for three to four weeks in a sugar rich medium such as Sabouraud dextrose agar.

Histomycology serves as a better option when microscopic examination of KOH solution-treated samples is not successful. It includes treatment of acid-Schiff dye with the debris or samples followed by microscopic evaluation. Morphological features such as hyphae as thread-like materials, pseudohyphae and dots (anthroconidia or yeasts) are examined. However, the precision of identifying the responsible pathogen is very low with this method, as the microscopic studies cannot differentiate the species (Petinataud, 2014). RFLP is a more accurate and discriminating procedure in which the clinician can establish causative species (and the occurrence of disease) based on the ribosomal analysis of the pathogen. Other genetic tools include PCR to diagnose the disease. However, the cost and need of expensive consumables for the procedures limit these methods (Welsh *et al*., 2010).

From the various investigations performed by Welsh *et al.,* (2010) for different clinical groups, it has been determined that the organisms responsible for infection are dermatophytes (90%), *Candida sp.* (6%) and non-dermatophytes or moulds (4%). Those dermatophytes responsible for this infection belong to three major genera: *Trichophyton*, *Microsporum* and *Epidermophyton* species.

1.2.3 Immunology of host towards dermatophytic invasions

Pathogenic fungi that can cross barriers of the host and infect the targeted habitat are regarded as mycoses (Garcia and Blanco, 2000). Innate immunity is the first line of defence in the host towards such microbial penetration, which activates the adaptive immunity (second line of defence) through germ-line encoded receptors. In the innate system, physical barriers such as skin and mucous membranes that have antimicrobial components attached to them synthesised by epithelial and endothelial cells provide defence. Sometimes, commensal microflora also assists this defensive mechanism in impeding colonisation by pathogenic species. The next level includes cell membranes and receptors, humoral factors, phagocytes such as neutrophils, mononuclear leukocytes, dendritic cells and natural killer cells etc. For example, neutrophils are the primary response for *C. albicans* infection. Chemotactic factors generated in response to the pathogenic stimuli include leukotrienes of polypeptides and chemokines of the supergene family of small peptides. Production of antibodies to prevent adherence, toxin neutralisation, antibody opsonisation and antibody-dependent cellular toxicity are mediated in adaptive immunity. T – Helper one (Th1) type cell mediated immunity is common in most of the fungal diseases including mild and acute infections. Th2 type is also observed in those cases with inflammation and allergic reactions (Sangshetti *et al.,* 2013).

The first response of the human body towards the fungal invasion is epidermopoesis at the point of contact and detaching the pathogen from the region including the dermal cells at the site. If the infection persists, Langerhans cells of skin will identify the antigens of pathogen and will be presented to T- lymphocytes through the major histocompatibility complex (MHC) - II. Langerhans cells along with keratocytes have mild phagocytic activity, which further stimulate macrophages and degrade the antigens. Lymphocytes with $CD4^+$ and $CD8^+$ along with T γ/α cells are

recruited to the site (Garcia and Blanco, 2008). Dermatophytes trigger Th1 – type adaptive immune response leading to the production of interleukins (IL-2) and interferons (IFN - γ). The mechanism of stimulation varies based on the habitat of organism prior to infection. Intensity of response towards anthropologically originated pathogens is low when compared to zoological or geological species. From this description of pathogen suppression over T – helper cells, it is evident that a population with HIV as concurrent disease are highly susceptible to onychomycosis. For example, HIV patients with a T- lymphocyte count as low as 400 cells/mm^3 have increased risk of developing onychomycosis. Indeed, proximal subungual onychomycosis has been considered as an indication of HIV (Schlefman 1999).

1.3 *Trichophyton rubrum* (*T. rubrum*)

Those fungi consuming keratin as a major nutrient are categorised as dermatophytes. The primary ecological habitat of these pathogens is soil. These organisms lost sexual reproducibility during their transition to the anthrophilic habitat. *T. rubrum* belongs to *Deuteropmycota* or *Fungi imperfecti* of *Antheroderma* and order *Onygenals* of dermatophytes. The other genera include *Epidermophyton* and *Microsporum* (Blyskal 2009).

T. rubrum is the most prevalent agent responsible for onychomycosis among the dermatophytes (Tejedor, Garcia & Mayordomo 2021). The organism has been categorised as a *very common* skin pathogen according to its ecological habit Keratinised parts of the body such as skin, nail and hair are the locations most commonly invaded by this dermatophyte, as it consumes the keratin of these structures. There are two different strains of *T. rubrum* - (A) down type and (B) granular type. Down type produces a brick-red pigment in the medium featuring on the surface with piriform mitochondria and lateral hyphae; whereas the granular type will not produce any pigment on the medium and will have thin membraned mitochondria, with clavate-shape, multi-septate and a smooth cell wall. *T. rubrum* and related dermatophytes moderate the immune response by releasing enzymes and mannans. The former degrades the invaded tissue for carbon and nitrogen metabolism and mannans supress the proliferation of lymphocytes at the stratum corneal region of the skin delaying the replacement of dead with new cells. The mechanism of Th1 immune response has not been established, but the *in vitro* evidence has proven the decreased proliferation of lymphocytes and mononuclear cells in particular in the presence of mannans. During acute infection conditions, immunoglobulin-G and Immunoglobulin-E are also observed (Tovar, 2010).

1.3.1 Infection mechanism

Hair, hooves, skin and nails are the highly keratinaceous components of mammals. Fungi infecting these regions survive by consuming the keratin from these biomaterials as primary carbon and nitrogen resources, and which are metabolised by degrading the structural keratin with keratinases. These enzymes dissolve the disulphide bonds between the layers, thereby partitioning the components for further metabolism. Hence, keratinases released by such infectious organisms are regarded as virulence factors for corresponding infection (Blyskal 2009). Upon secretion, keratinases trigger an immune response of the infected species leading to inflammation. The extent of inflammation is directly proportional to the number of enzymes secreted, which can be correlated to the severity of infection. Among those keratin-dependent fungal pathogens, *T. rubrum* releases keratinases at higher amounts (Khan and Ahmad 2011; Shapourzadeh *et al.,* 2016).

Environmental aspects such as soil, air, water, humidity and geographical locations are common factors of infection along with transmissions from infected individuals. In particular, areas of the body with open borders or cavities such as the hyponychium, navel and concealed entrances of genitals are comparatively more exposed to the external environment. These openings serve as reservoirs for infectious agents to enter the body. *T. rubrum* adheres for a period of 6 hours in the hyponychium from the point of contact and requires 4 hours to germinate. During this period, *T. rubrum* releases anthroconidia to continue proliferation. Once established, it releases keratinases, metalloproteases and serine proteases along with lipases and ceramides. These compounds disrupt disulphide bonds between the keratin layers and cause inflammation. Although these opportunistic species invade dead or keratinised parts, they are regarded as pathogens because of the effects produced of the de-keratinization; like itching, inflammation and sometimes bleeding. Trichophytin an antigen released by the *Trichopyton* species during infection rapidly stimulates immune response. A completed description of mechanisms of immune suppression of the host by the fungus is not established. However, studies conducted by Petinataud *et al.,* (2014) suggested the localisation of organism to the stratum corneum as one of the reasons. The mycelium develops only

in the outer layers of the infected part. The chances of proliferation into deeper tissues are very low due to the absence of keratin (Johnson 2003).

1.3.2 Virulence factors of *Trichophyton*

Virulence factor/s can be of any chemical origin released by a pathogen during invasion or through procurement of nutrition to proliferate and cause disease in the host. Major fungal virulence factors are proteases, phospholipases, catalases, calcineurin, melanin and lipid signalling molecules (Sangshetti *et al.*, 2013). Among these, proteases are released by *T. rubrum* during its invasion in the host biological system. Proteases are of two types - endo and exoproteases. The former cleaves peptide bonds internal to a polypeptide, whereas the latter breaks bonds at an N- or C- terminal only. A classical hierarchy of proteases is based on the targeted amino acids such as aspartic, cysteine, metallo-serine, threonine and also proteases of unknown activity. The nomenclature includes a capital letter of representative catalytic activity along with a unique number. Subfamilies are further denoted with another capital letter. Families with common origin are termed a clan. For example, S9B represents serine protease of the clan serine exoproteases and subfamily 'B' with analogous number 9 (See Table 1.2) (Monod *et al*., 2002).

Along with the above proteases, *T.rubrum* secretes carboxypeptidase of M14 and S10 families to derive carbon and nitrogen from the cultured medium such as Sabouraud dextrose broth or agar. These enzymes resemble carboxypeptidases of human intestine.

Table 1. 2| **Types of endo and exoproteases of some pathogenic fungi**(Monod *et al*., 2002):

Clan	**Protease (subfamily)**	**Non-dermatophytes (*C. albicans*)**	**Dermatophytes (*T. rubrum*)**
		Endoproteases	
AA	A1	Sap1 – Sap6 Sap8 Sap9, Sap10	-
MA (E)	M36	-	Protein family
SB	S8A		Protein family
		Exoproteases	
SC	S9B	-	DppIV
	S9C	-	DppV

In the investigation performed by Jousson *et al.,* (2004) metalloproteases and serine proteases were extracted from the media cultured with *T. rubrum*. The authors had isolated seven genes responsible for putative serine proteases and determined that the adaptation and secretion of enzymes by the pathogen were a result of the type of nutrient medium used. It has been identified that around twenty genes are responsible for protease secretion from *T. rubrum*. Five of these genes belong to the fungalysins family. For example, skin keratin is composed of K1 and K10 keratins whereas healthy nail plate (which is considered as modified stratum corneum) is composed of hard keratins of Ha and Hb 1 to 4. To digest such hard and high molecular weight keratins of the nail plate, *T. rubrum* secretes proteases of molecular weight ranging from 27 to 44 kDa depending upon the thickness and moisture content of nail plate (Chen *et al*., 2010).

The virulence of keratinases released by the *T. rubrum* was confirmed in the study conducted by Maranhão *et al*., (2007). In their experimentation, the *T. rubrum* was cultured in keratin-rich medium along with glucose medium as negative control. From the results, it was correlated that, the pH of keratin-containing medium changed from 5.5 to 8.3 over a period of seven days, whereas, the glucose medium pH had remained constant. In addition, the enzymes released to metabolise keratin to procure nitrogen and sulphur were determined. From these studies, it was

confirmed that, keratinases were the virulence factors released by the *T. rubrum* that assist in procuring nutrition and trigger an immune response in the host.

Among those non-dermatophytes responsible for onychomycosis, *C. albicans* is the predominant species. The suppression of immune systems by the dermatophytes (*T. rubrum*) also promotes the growth of *C. albicans* as opportunistic pathogen.

1.4 Candidal onychomycosis

Infectious skin conditions of fingers can affect a nail region causing paronychia. In this condition, surrounding dermis of the nail apparatus undergoes inflammation with severe trauma. As discussed in the Section 1.2.1, it is one of the concurrent diseases in immune-compromised individuals. The subject cannot hold minimal weights or perform regular activities. Candidal onychomycosis starts from paronychial conditions extending into the nail bed and affecting the nail matrix supporting the plate. It is also observed in patients suffering from chronic mucocutaneous candidiasis with a high susceptibility among immune compromised populations due to poor cell-mediated immunity. The prevalence of candidal onychomycosis is increasing along with other dermatophyte infections. Among the *Candida* species, *C. albicans* is the main organism associated with HIV and other immune system-related conditions, whereas it acts as an opportunistic pathogen in malnutrition, peripheral vascular diseases like diabetes and aged nail complexes. Candidal onychomycosis is also observed in poor socioeconomic categories due to unhygienic conditions and improper nutrition. The pathogen produces cellular lipases, proteinases and phospholipases to infect the host once established through pleomorphic hyphae (Watamoto *et al*., 2009).

The extent of immune-suppression and virulence of a pathogen determine the severity of the infection. A defective humoral and cell-mediated immunity weakens the host. Along with HIV, these conditions are also observed in patients suffering with DiGeorge syndrome, agammaglobulinemia and thymus dysplasia. Similar to dermatophytes, *C. albicans* release proteinases to disrupt the nail keratin matrix. Digested keratin fragments are used to produce melanin as a defensive mechanism by the pathogen to protect itself from antimicrobials and high energy electromagnetic radiations such as ultraviolet light. Thus, by producing pleomorphic hyphae, proteolytic enzymes and converting keratin to melanin *C. albicans* exhibits multiple infection mechanisms and constructs various routes for proliferation (Watamoto *et al*., 2009).

Primarily, the targeted site of attack is the fingernails. Although *C. albicans* produces proteinases to degrade the nail plate barrier. The potency of these enzymes is comparatively lower than keratinases released by dermatophytes. Hence, it targets thinner nail plates of fingers of hands (particularly in women) than nails of toes of feet (Watamoto *et al*., 2009). Infected nails can be identified with severe swelling along the border of the discoloured nail plate due to trauma. Diagnosis involves microbiological culturing of suspected nail clippings on Sabouraud dextrose media followed by testing and microscopic examination.

1.5 Systemic mode of treatment

Classical antifungal agents are categorised into five major categories (Table 1.3): polyenes; pyrimidine analogues; allylamines; azoles and the echinocandins (Campoy & Adrio 2016). Among the five classes of antifungals, the US FDA has approved nearly nine drug candidates (Monod M, 2019). The predominant treatment in systemic therapy includes oral administration of terbinafine (Lipner & Scher 2019). A desirable strategy includes anticipation of the disease (or prevention of relapse) as the treatment may render the subject vulnerable to *Tenia pedis* (Hay *et al.,* 2015).

Table 1.3| **Antifungals and their respective mode of action** (Campoy & Adrio 2017)

Class	Mode of action
Polyenes Examples: Nystatin, fracidin, rimocodin, endomycin, ascosin, trichomycin, antimycin and amphotericin A and B	Depletes the cell membrane by interfering with the phospholipid membranes.
Pyrimidine analogues Examples: Flucytosine and 5 - fluorouracil	Interferes with synthesis of DNA and RNA.
Azole compounds Examples: Miconazole, ketoconazole, fluconazole and itraconazole	Disrupts the cell membrane and inhibits 14α – methylase enzyme for sterol production
Allylamines Example: Terbinafine	Inhibition of the squalene epoxidase enzyme responsible for sterol production
Echinocandins Examples: Cliofungi, capsofungin, micafungin and anidulafungin	Inhibition of 1,3 – *D* – glycan responsible for cell wall synthesis

Oral administration of miscellaneous antifungal – griseofulvin to treat onychomycosis was the first medication in diabetic population. Severe adverse effects and lipophilic deposition limited the administration of azole antifungals. Hepatotoxicity and multidrug interactions limited azole antifungals. Among the

antifungals available, terbinafine is considered most effective in treating onychomycosis in diabetic populations (Monod M, 2019).

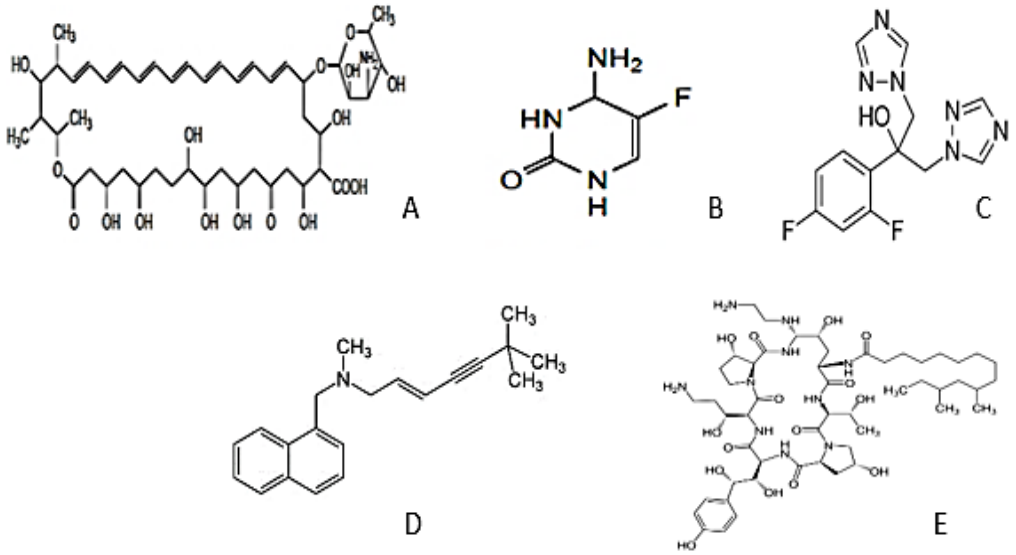


Figure 1. 5a| **Chemical structure of some classical antifungal drugs for systemic use** (A) Amphotericin B of polyenes (B) 5–Fluorouracil of pyrimidine analogues (C) Fluconazole of azole antifungals (D) Terbinafine of allylamines and (E) Capsofungin of echinocandins.

A patient-oriented and evidence-based approach of categorising such therapeutically active moieties to rate individual studies has been developed by the editors of 'United States Family Medicine' and 'Primary Care Journals' known as strength of recommendation taxonomy (See Table 1.4). According to this; 'A' and level of evidence +1 – represents consistent and good quality patient-oriented evidence; 'B' and level of evidence +2 – based on inconsistent or limited quality patient-oriented evidence; and 'C' and level of evidence +3 – indicates consensus, usual practice, opinion, disease-oriented evidence or case series (Maymone *et al*., 2014). The therapeutic agents mentioned in Table 1.4 are used for the systemic treatment of onychomycosis caused by *T. rubrum.*

Table 1. 4| **Antifungals used for systemic treatments of onychomycosis**

Therapeutic agent	**Features**	**Disadvantages and adverse effects**	**Reference**
Griseofulvin **strength of recommendation - C** **level of evidence - 2+***	Weak fungistatic	Low bioavailability Improper penetration Long treatment duration Causes nausea and rashes Contraindicated in pregnant women	Scher *et al*., 1999; Campoy & Adrio 2016
Terbinafine **strength of recommendation - A** **level of evidence - 1+***	Only oral fungicidal antimycotic More potent than griseofulvin	Broad spectrum Prolonged half life Causes nausea, diarrhea, taste disturbance Hepatotoxic	Scher *et al*., 1999
Itraconazole **strength of recommendation - A** **level of evidence - 1+***	Active towards yeast and fungi Minimum inhibitory concentration (MIC) is ten times more than terbinafine	Headache and gastrointestinal upset Asymptomatic liver function abnormalities	Lipner & Scher, 2019

*Maymone *et al*., (2014)

1.6 Topical treatment of onychomycosis

The targeted site of application for the topical treatment of onychomycosis is the nail plate region. In these non-invasive treatments, the dosage form is applied on the nail surface (hydrophilic) to allow the active pharmaceutical ingredient to permeate through the nail plate. Hence a water-soluble active ingredient that can cross the nail plate barrier and be more effective when compared to a non-permeable or lipophilic agent (Agarwal *et al.,* 2020; Agarwal *et al*., 2021).

1.6.1 K101 Nail solutions

The efficacy of a topical dosage form to treat onychomycosis depends on the penetrability of the active ingredient and its ability to remain on the targeted site in order to eradicate the infection. The most common ingredients of over-the-counter (OTC) topical dosage forms towards this organism are urea and lactic acid in a diol solvent such as propylene glycol. This mode of treatment is categorised as a chemical avulsion therapy and such dosage forms are termed as K101 nail solutions. Urea dissolves the bond between infected nail plate and nail bed, and then softens the nail plate thereby inhibiting the growth of the pathogen (Pajaziti & Vasili 2015).

K101 nail solutions are available in the market under several tradenames such as Canespro®, Naloc™, Nalox™, Emtrix© and Kerasal Nail™ etc. In the clinical investigation performed by Faergemann *et al*., (2011) to evaluate the efficacy of K101 formulations (Naloc™) improvements were observed in 91.8% in the participants after eight weeks of treatment. At least some improvements were observed in the physical characteristics of diseased nails after two weeks. Adverse effects were not observed in this study, but in the preliminary analysis performed by Emtestam *et al*., (2012) in a placebo-controlled investigation, 6% of test population had shown some adverse effects. It was explained that drop-wise addition of formulation had leached into the skin and resulted in unwanted effects.

In an efficacy examination of K101 combination in a placebo-controlled treatment of 493 individuals, a majority of the subjects had shown slight improvement in the second week and maximum cure after 23 weeks (Emtestam *et al*., 2012).

Faergemann *et al*., (2014) investigated the mechanism of action of K101 formulations on *T. rubrum* and *C. albicans* by the application of transmission and scanning electron microscopic techniques. By comparing the morphological differences of the organism before and after treatment, the minimum effective concentration was determined to be 50% (v/v) after 60 minutes of exposure. Cells of the pathogen treated with K101 had collapsed with damaged cell walls and the organelles disrupted from the cytoplasm with small membrane vesicles and large vacuoles. By these observations, it was inferred K101 mediates the antimicrobial action through disruption of the cell wall followed by disruption to the cytoplasmic membrane causing cell lysis.

The pH of K101 formulations is approximately 4.0, an optimal value to reduce microbial growth. Invasion of *T. rubrum* is proliferated by the degradation of keratin and release of ammonia which alters the pH of skin to slightly alkaline conditions (~7.4) (Rossi *et al*., 2011). To achieve maximum therapeutic efficacy, the intended ungual formulation should resist this transformation of pH (Faergemann *et al*., 2014).

Prescription dosage forms intended for topical administration sites include antifungal agents like amorolfine which inhibit the enzymes of the ergosterol synthesis pathway (Gupta *et al.,* 2013). It has a lower minimum inhibitory concentration (MIC) of 5.0% w/v, but the disadvantages of this drug include a burning sensation, itching, redness, irritation and pain. Another broad spectrum antifungal agent, ciclopiroxolamine, inhibits cytochrome enzymes by reducing the energy production of the pathogen (Gupta *et al.,* 2013).

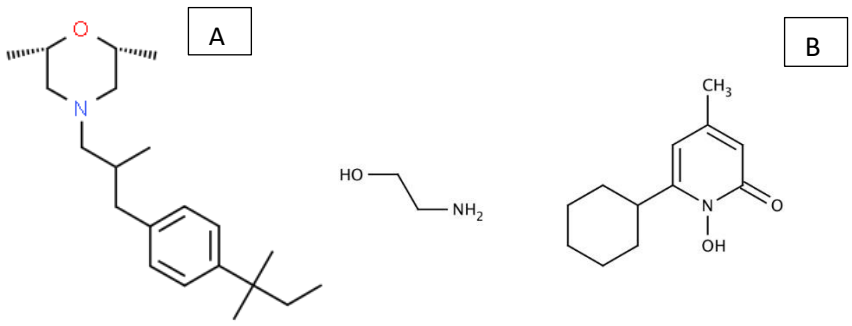


Figure 1. 6b| **Chemical structure of some classical antifungal drugs for topical use** (A) Amorolfine (B) Ciclopiroxolamine

The other topical dosage forms for the treatment of onychomycosis include lacquers, collodions and vaporising agents. Although these dosage forms promise prolonged period of contact, the composition and technique of application may require special care or technical guidance from a professional, particularly in elder populations, as the frequency of infection is high. OTC dosage forms containing urea need a prolonged period of time to cure the infection (Gupta & Paquet, 2013; Murdan, 2016).

1.7 Alternative treatment strategies

Non-chemical modes of treatment for onychomycosis includes laser and photodynamic therapies (PDT). In the former, the infected nail region of the limb is exposed to a beam of laser of a chosen wavelength based on the severity of infection. It serves as a non-invasive method, but with a low success rate and subjective treatment (Mordon *et al.,* 2014; Shen *et al.,* 2020). PDT includes light absorbing material such as a synthetic dye as photosensitizer to generate free radicals to disrupt the cellular membrane of the pathogen, thereby causing cell lysis. Photosensitizers absorb projected electromagnetic radiation and causes electronic transition to higher levels. Excited electrons return to the ground state by releasing the energy as either heat or light or change in the angular momentum. Thus, released energy is utilised by the atoms such as oxygen or nitrogen, which contain lone pair(s) of electron to generate free radicals. These free radicals inactivate the pathogen by demobilising or destructing the cellular membrane. Improved efficiency independent of resistance and photo-inactivation of microorganisms facilitate PDT as an effective therapy amongst novel approaches. Limitations of this treatment include mixed results determined during the treatments of infections caused by more than one type of pathogen, anecdotal settings of operations and regulatory challenges (Agarwal *et al*., 2020; Shen *et al.,* 2020).

1.8 Interferences and enhancers for effective treatment

T. rubrum secretes proteases in higher amounts than the other dermatophytes. This is an adaptive feature of the organism, as the targeted site of infection by this fungus is the nail region of limbs. Nails or hooves are stronger than other keratin containing biomaterials being nearly hundred-fold thicker than skin (Elkeeb *et al.,* 2010). Therefore, to dissolve such robust material the number of enzymes required will be high.

Presentation of the active ingredient at the targeted site of action for an effective output is the key to a successful formulation. The composition of any such formulation should ideally facilitate the passage of the therapeutic moiety to protrude through any barrier and reach the intended region. Permeating nail plate and eradicating the infection for an effective treatment is a challenge in any ungual drug delivery system (Aditya and Paquet, 2013, Agarwal *et al*., 2020).

On average, a healthy nail plate takes 12 to 18 months to grow completely and 4 to 6 months to replace the impaired nail. Therefore, a profound and time-based assessment of nail is a recommended treatment to confirm complete eradication of pathogen and avoid relapse (Scher *et al.,* 2007).

1.9 Antifungal drug resistance – a public health predicament

Although antifungals have been proven potent for most related infections, severe adverse effects and deleterious results from combinations are major disadvantages. However, existing technologies and novel dosage regimes attempt to reduce these unwanted effects. Nevertheless, the development of resistance by the exposed pathogens is a growing concern in the scientific area of antimicrobials (Evans & Leeds 1998; Anon, 2013). Table 1.5 notes the resistance developed by certain pathogens towards antifungals.

Table 1.5| **Various resistant species towards popular antifungals**

Antifungal	Commercialized dosage form	Identified resistant species
Amphotericin B	Fungizone	*Fusarium, Pseudalleshiria, Scedosporium, Aspergillus, Trichosporon* and *Candida*.
Flucytosine	Ancobon	*Candida*, *Cryptococcus* and *Aspergillus*.
Fluconazole	Diflucan	*Candida* and *Cryptococcus*.
Capsofungin	Cancidas	*Candida* and *Aspergillus*
Terbinafine	Lamisil	*Candida* and *Aspergillus*

Resistance towards antifungals is categorised into primary – before the exposure; secondary (acquired) – after the exposure, and, clinical resistance – progression or relapse of an infection by a fungal isolate during laboratory testing. The prevalence of resistance is high among the immune-compromised populations.

Santos *et al.,* (2007) have investigated the influence of prolonged exposure of various antifungals on *T. rubrum* isolated from clinical subjects. It was determined from the ribosomal studies, that the number of genes responsible for antifungal resistance had increased and the chances of developing resistance was high towards azole antifungals and allylamines.

Along with the issue of antifungal resistance, the necessity to produce a novel, rapid acting, potent formulation which is non-invasive and superior to the current

topical treatments many of which require an administration duration of six months, is much sought after.

1.10 Fatty acids as antifungal agents

In the search for a solution for antifungal resistance, science has diversified into various fields from synthetic assembly to extraction of therapeutic natural compounds (Levy & Marshall, 2004). The choice of new alternative therapies include modification of existing compounds and exploring structural motifs to eradicate the encountered difficulties with existing candidate antifungals (Lee, 2008). The identification of new antimicrobial compounds from natural resources such as plants, animals and microorganisms has gained importance and the categories promising effective treatment include proteins, carbohydrates, fatty acids, aromatics and aliphatic unsaturated compounds. The mechanisms of action from naturally derived active moieties include interruption of cell wall biosynthesis or DNA replication and protein synthesis, interfering with cellular mechanisms such as respiration, lipid metabolism, and phosphorylation. Among such compounds, fatty acids are known to have antimicrobial activity (Kabara, 1972). Treatment is expedient in this approach to antimicrobial therapy as patient compliance will be enhanced due to its origin and high biocompatibility, as the contesting moiety is a food derivative (Cheng *et al.,* 2016).

The investigation of antimicrobial potency of free fatty acids was emphasised by Kabara *et al.,* (1972), in their publication - "Fatty acids and derivatives as antimicrobial agents". The derivatives of short, medium and long-chained fatty acids such as alcohols, monoglycerides and aldehydes, along with individual free fatty acids against various microorganisms, were investigated. From the results obtained, medium and short-chained saturated fatty acids have been determined to be more potent towards various oral and dermal fungal pathogens in their monoglyceride configuration (Kabara *et al.,* 1972).

Unsaturated fatty acids have been proven potent against various pathogens in the different fields of applications. For instance, Avis & Bélanger (2001), have extracted *cis*-9-heptadecenoic acid from *Pseudozyma flocculoza* a yeast with biocontrol properties against yellow fungi. This fatty acid has been proven active

towards a broad spectrum of pathogenic fungi, by incorporating itself into the cellular lipid membrane and altering the dynamics of molecular exchange leading to death of the organism.

In the review published by Pohl (2011) it has been reported that fatty acids penetrate into the cell membrane of the organism and substitute themselves with existing lipids in the bilayer, disturb the balance of the membrane and thus causing cell death. Another mechanism of action includes the substitution of *N*-myristoyltransferase enzyme by the analogues of myristic acid (C_{14}) thereby interfering with the protein metabolism of the cell and causing cell death. Inhibition of fatty acid metabolism and topoisomerase enzymes which effect the genotype are the other pathways by which fatty acids mediate antifungal action (Carbelleira, 2008).

Among the possible alternatives for antibiotics, elements extracted through biological sources are highly efficient (See Table 1.6). The biological source of action and minimised side effects promote these novel moieties as potential candidates to treat diseases caused by resistant fungi.

Table 1. 6| **Examples of fatty acids with antifungal properties**

Fatty acid and or their derivatives	**Active towards**	**Source**	**Reference**
Cis-9-Heptadecenoic acid	Pathogenic fungi	*Pseudozyma flocculoza*	Avis *et al*., (2001)
Lauric and caprylic acids and corresponding monoglycerides	Pathogenic fungi	Edible oils	Altieri *et al*., (2009)
Myristic and palmitic acids	*Aspergillus* and *Fusarium*	Edible oils	Carballeira (2008)
Salts of palmitic acid	*Scedosporium apiospermum*		
Hydroxyl octadecanoic acid	Bread moulds	*Lactobacillus*	Black *et al*., (2013)
Medium chain fatty acid esters	*Aspergillus flavus* and *ochraceus*	*Linum usitatissimum*	Abdelillah *et al*., (2013)
3 – Hydroxyl derivatives of decanoic and dodecanoic acids	Yeasts and various fungal species	*Lactobacillus plantarum*	Crowley *et al*., (2013)
Fatty acid methyl esters	Fungal pathogens	Microalgae	Suresh *et al*., (2014)

1.11 Active antifungal entity (AAE)

According to the supervision of Brady and Kealey, some novel antimicrobial applications of modified coconut oil (MCO, here after referred as AAE) have been reported (Hughes, 2014) by the Bioscience Research Institute at Athlone Institute of Technology which is a derivative of virgin coconut oil (VCO). The method of preparation of MCO and components required for the preparation cannot be disclosed according to the institute's Non-Disclosure Agreement. For any further information, please contact Dr. Damien Brady - co-supervisor of this project.

Building on this research, additional efforts towards expanding potential targets for MCO's antimicrobial activity against ungual (*Trichophyton rubrum*) and *Candida albicans*) are now presented in this research.

2.0 Principles of converting active candidate into therapeutic moiety

In this section, the scientific and regulatory principles of designing a dosage form towards the targeted application are described.

2.1 Overcoming dosage form design challenges

Pharmaceutics is the science of developing a potent, efficacious and stable matrix of active pharmaceutical ingredient (API) in combination with suitable excipients to deliver intended performance. An API is a chemical moiety of either synthetic or biological origin with therapeutic effects for the established concentration. Excipients are the non-therapeutic ingredients of formulation, which induce a characteristic appearance, maintaining the organoleptic properties and shelf-life along with affirmative presentation of API at the targeted site. A compatible mixture of such API and excipients with desired medicinal properties is a dosage form (Lachman and Lieberman, 2009).

In topical dosage form development, conventional method, or quality by testing (QbT) employs identification of a problem, designing the formulation as per the reference listed drugs (RLDs) and analysing them periodically from raw materials to the finished product to meet the respective regulatory requirements. Any changes in the development or process shall be reported to the regulatory authorities the entire process shall be repeated with justification for the approval (See Figure 2.1). In topical drug delivery systems, the number of ingredients used and method of manufacture, is more complex. Ascertaining regulatory status for every small change during the process is the major issue in traditional approaches (Jain, 2015). In generic development of topical formulations, efficiency and safety of the manufacturing process or analytical technique or material characteristics is evaluated on qualitative and quantitative reproducibility of a relative RLD. This mode of practice was a self-defeating mechanism when abbreviated new drug applications (ANDA) were developed, as no previous information was available. Sometimes, events such as patent protection and/or undesirable effects of excipients on API with no equivalent

match in RLDs brings the process to an indefinite endpoint (Chang *et al.,* 2013; Chavda, 2016).

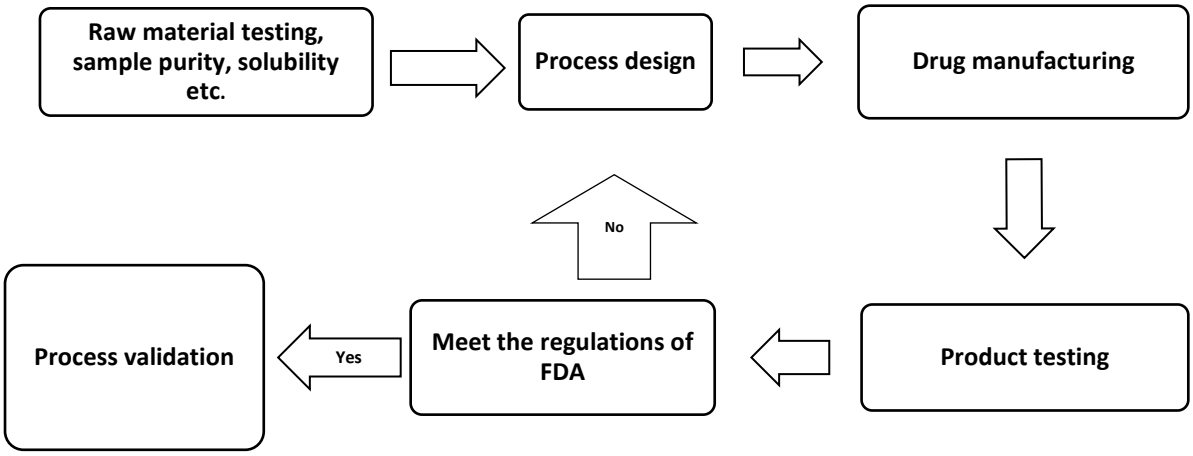


Figure 2. 1| **Scheme of QbT approach** (adapted from: Zhang and Mao, 2016).

In 2004, FDA released the first in time quality guidelines for current Good Manufacturing Practices (cGMP). The oversight of quality of drug candidate and its product in this regime includes two major objectives: (1) regulatory review of drug applications and (2) inspection of facilities for the manufacture in compliance with compendial status. The data determined from these analyses through periodic audits by US FDA is examined with RLD information for the confirmation (Woodcock 2015) During this application, FDA has encountered several issues such as:

- product recalls
- shortages in critical drugs due to outdated equipment
- supplementation have been sent for review after approval
- lack of discrimination in the methods and regulations regarding product type
- available information is extremely limited and primitive which cannot be implemented for upcoming ANDAs
- inspections carried out for subset of representative values which are not statistically significant

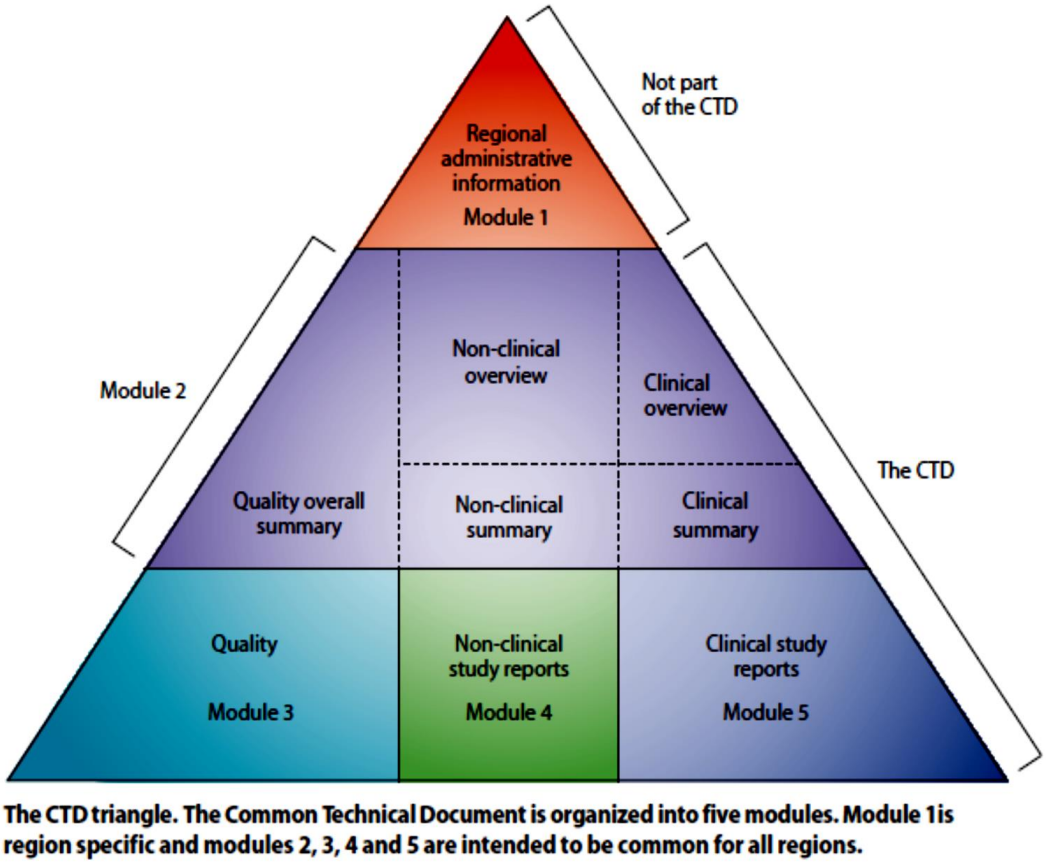
Based on the number of requests submitted by several worldwide concerns regarding development of ANDAs, the quality office of the FDA determined that, the irregularities among the RLD was not replaced by new information in terms of

regulatory concerns towards novel technologies. These lapses between the applications were also due to numerous changes requested by firms in the process as well as material attributes to substantiate official literature. It had been concluded that focus was majorly concentrated on chemistry of the respective compounds used in the product including API. The information about engineering, statistical approach and validation in terms of quality have not been considered as major aspects. International compendial authorities such as FDA, World Health Organisation (WHO) and European Medicines Agencies have been informed for a cumulative approach. This resulted in segregation of these official bodies as 'The International Council for Harmonisation of Technical Requirements for Registration for Pharmaceuticals for Human Use (ICH)'. ICH has framed a novel approach in a Tripartite Guideline known as ICH Q8 to facilitate the development of a dosage form along with its manufacturing process, using a systematic approach to develop a product with quality (Kovacas *et al*., 2021). It is the suitability of either a drug substance or a drug product for its intended use and thus to deliver the intended performance consistently to position it to file a Common Technical Document (CTD) (Figure 2.2) as per ICH Q8 (R2)[1].

This document includes the Quality Overall Summary[2] (QOS) of a developed dosage form, which will provide information to the quality reviewer. General QOS information will include details of the dosage form such as basic information, manufacture, characterization, control, reference standards, container closure system and stability of a drug substance. The description, composition, pharmaceutical development, manufacture, and control of excipients of the dosage form or drug product are also included according to *The Common Technical*

[1] ICH Q8 (R2), 2009. 'Pharmaceutical Development'. International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

Document For The Registration of Pharmaceuticals For Human Use also known as ICH M4Q (R1)[3].



The CTD triangle. The Common Technical Document is organized into five modules. Module 1 is region specific and modules 2, 3, 4 and 5 are intended to be common for all regions.

Figure 2.2| **The modules of a CTD (ICH M4Q (R1)).** Module 2 is quality overall summary of the designed novel chemical entity. It includes description of the dosage form type, application, materials used, container closure system and manufacturing process variables. The description of chemistry of active moiety and its interactions with corresponding excipients should be provided too. Non-clinical information includes data derived from the experiments conducted *in vitro* such as dissolution profiles and incompatibility studies. Whereas drug release profile and dosage form efficacy derived from Phase I, II and III of clinical studies should be submitted under clinical data. The in-vitro-in-vivo correlation determined for the NCE during all the phases should be provided as well.

[3] ICH M4Q (R1) 2002. 'Quality Overall Summary'. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

Elements of ICH Q8 Pharmaceutical Development (R2) (2009) for the pharmaceutical product development of quality by design (QbD) are as follows (Kovacas *et al*., 2021):

2.1.1 Quality target product profile (QTPP) & Critical quality attributes (CQAs)

The QTPP includes information about clinical setting, route of administration, dosage form, dosage strength, container closure system, therapeutic moiety release or delivery and attributes affecting pharmacokinetic characteristics and drug product quality criteria ('sterility', 'purity', 'physical attributes', '*in vitro* release test', 'package integrity', 'stability'. See Table 2.1).

CQAs are physical, chemical, biological or characteristic properties of drug substances, excipients, intermediates (in-process materials) and drug product that affect the QTPP of the dosage form. These can affect product purity, strength, drug release and stability of the dosage form. In this project, CQAs of the active pharmaceutical ingredient (API) have been identified in the pre-formulation studies to construct therapeutically equivalent QTPPs of the dosage form developed.

Table 2. 1| **The desired QTPP and CQA for an onychomycotic application to treat onychomycosis**(Chang *et al*., 2013)

Elements	**Target**	**Justification**	**CQA**
Dosage form	Onychomycotic application	To provide prolonged and direct effect on the infected area	No
Route of administration	Topical	Hypo and paronychia regions of nail plates of fingers	No
Dosage strength	Clinically effective	To have an effective concentration of MCO	No
Dosage design	Oil-in-water emulsion dispersed in aqueous base	To enhance the solubility of drug for the essential action	No
Appearance	Colourless or cloudy	Elegance	No
Identification	For the AAE (MCO)	To meet the standards	Yes
Assay	To quantify the amount of API in a unit dosage form	To provide information for the quality reviewer	Yes
Impurities	Within the limits	For safety	Yes
Homogeneity	Uniform release of API	To have consistent activity and uniformity	No
Physical attributes	Consistency, viscosity, cohesiveness etc.	To facilitate extrusion from the container and application	Yes
In vitro release test	Using Franz apparatus	For appropriate release over the target site of application	Yes
Microbial limits	Within the limits as per USP <61>	To avoid interference with API and enhance stability	Yes
Residual solvents	Within the limits as per USP <467>	For safety	Yes
Container closure system	Appropriate materials	Proper support for the storage and extrusion	No
Package integrity	No failure	Needed for stability and clinical efficacy	No
Stability	Not less than 24 months	Match the shelf-life of reference listed drugs	No

2.1.2 Risk assessment (RA)

This is an ongoing process, which identifies material attributes and process parameters affecting the product CQAs. In general, this methodology is performed in the early stages of the product development based on the compendial literature available and repeated throughout the product cycle to identify risks during a process.

For a novel chemical entity, RA is performed based on the information available from the characterisation and preliminary analysis and compendial data available for relevant chemical moieties.

For example, FDA recommends the identification of CQAs as a crucial step to establish the relationship of the inputs (including raw materials and processing conditions) to quantify the extent of impact on a final dosage form. This identification must be carried out based on the risk evaluation with an intention to minimise it, but not just as a material input of a CQA or modifications in the process parameters. The method of risk evaluation is the risk assessment, which is a qualitative or quantitative examination of variables that are likely to cause harm or have negative impact on product performance. RA has to be performed in the initial stages of product development and the data generated shall be recorded for future implementation or amendments of scale-up. This data is sometimes applicable in continuous process improvement and verification (Tomba *et al*., 2013).

RA has been performed during each procedure that has been applied in this research to identify risks and rank them to minimise them for the transfer of the technology to any later industrial scale/bulk production phases.

2.1.3 Design space

The correlation between process inputs and CQAs to perform a method ensuring the targeted quality of product is called design space (ICH Q8). Factors to be considered are:

- selection of variables for the risk assessment.
- unit operation design space.
- proven acceptable ranges and edge of failure (the limit of acceptance for a change in design space being the components of this element, which play a crucial role in maintaining the quality of the drug substance and final product) are considered for the selection of design space.

Optimising the CQAs based on the risk assessment in a multidimensional strategy to justify the process parameters is carried out in a design space. Within these established proven limits, any modifications in the process or material inputs would not require any legal or compendial approval.

ICH guidelines include a general description of design space as multivariate network for the optimisation. The choice of attributes and other related factors are subjective to the applications. For example, in the dosage form development, proportions of ingredients used is a crucial factor rather than the type of it. Similarly, for the manufacturing process, each unit operation may be considered for a design space or a collective design space can be constructed for a series of events. The operator justifying the scale-up with identified risks can construct a multi-scale design space (Tomba *et al*., 2013).

2.1.4 Control strategy

This is designed to ensure that a product of required quality will be produced consistently based on the type of formulation and process. The elements of control strategy include in-process checks, controls of input materials, intermediates, container closure system, critical process parameters and material attributes[4]. Where

[4] ICH Q8 (R2), 2009. 'Pharmaceutical Development'. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

relevant these have been developed here according to the chemical behaviour of the API in order to draft a quality overall summary form for later submission to ensure quality.

Control strategy is a policy employed in a process to limit the variables included in the design space for optimising the quality. It includes the information established from the CQA and CPP in the initial stages as the preliminary dossier. Any modifications in the process or attributes shall be approved and updated in the control strategy (Tomba *et al*., 2013). The following are the general aspects included in control strategy:

- attributes of input materials
- product specifications
- controls for unit operations
- in-process or real-time testing information
- a monitoring programme for verifying prediction model performance.

2.1.5 Product life-cycle management and continual improvement

This aspect involves monitoring the process performance throughout the product life cycle to ensure that it is working as anticipated to deliver product quality attributes predicted by the design space (and to modify any parameter if necessary) so as to enhance the performance. This method is designated as continuous process validation (CPV) during industrial or bulk production which can be performed by applying QbD tools such as process analytical technology (PAT). Analytical techniques such as near-infrared spectroscopy (NIR) and multivariate data analysis are indispensable PAT tools.

Identifying the variables affecting the process and controlling them according to the risk-based assessment to deliver quality is the principle of QbD. It is a systemic approach with higher precision and accuracy than the trivial QbT or trial and error practice. Attributes of a process or inputs of materials influencing the performance are framed and analysed to establish the design space. This provides territorial

boundaries to the quality-oriented delivery. Crucially, any changes in these limits will not require regulatory approval for the continuation of process (Hubert *et al.,* 2014).

These principles have been applied in this research to formulate MCO into an efficacious dosage form with a QTPP challenging the condition of onychomycosis. In Section 1.3.1, keratin was identified as the major interfering substance in an ungual therapy. The respective physical and chemical attributes of infected nail plate influencing the drug penetration are now discussed in the following section.

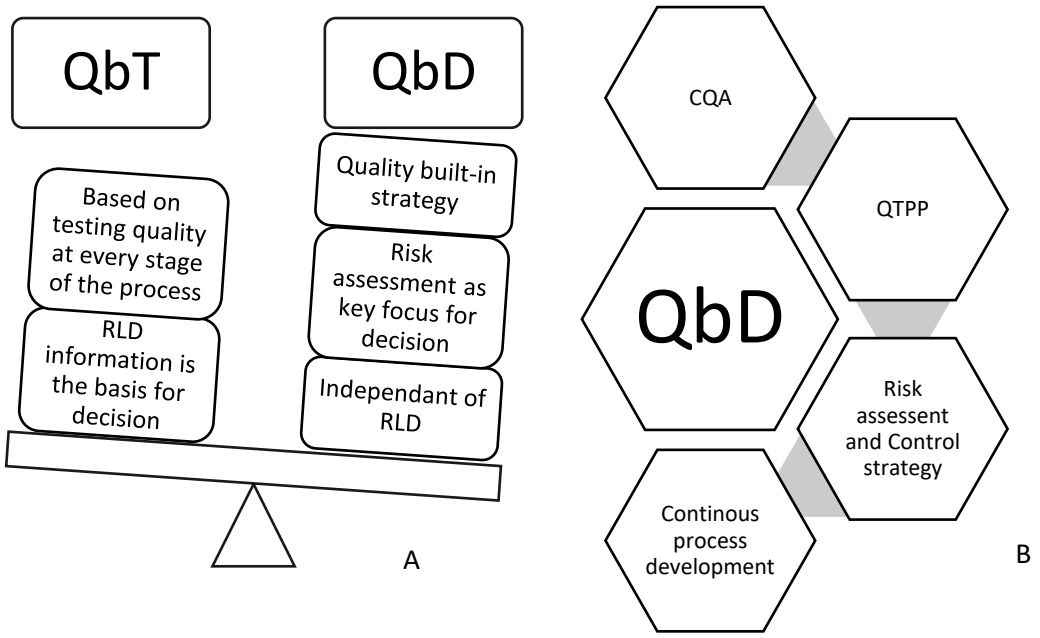


Figure 2.3| **Advantages of QbD over QbT towards pharmaceutical product development.** (A) Trivial QbT approach (B) and schematic approach of QbD as per ICH Q8 (R2), 2009.

2.2 Nail plate as barrier

Although regulatory authorities have approved use of antifungals such as Ciclopirox for topical administration to treat onychomycosis, a nail plate acts as a major barrier to effective drug penetration (Quatrin *et al*., 2020; Yang *et al*., 2020). As discussed earlier in Section 1.1 human nail plate is made of α – keratins interlinked by disulphide bonds. In the polymer complex of keratin, the property of available water content present supports elasto-plasticity. Depending on the environmental and nutritional habits, water content in a healthy nail plate will range from 10 – 25% (Auley *et al.,* 2016). Thickness of the nail plate alters penetration of drug materials during ungual therapy along with the physico-chemical interactions between the active moiety and the nail plate. Based on Fick's law of diffusion, the resistance (R) offered by nail plate towards the penetration of an active component can be expressed as

$$R = \frac{h}{DK} \qquad Equation(2.1)$$

Where, h = thickness of nail plate (m), D = permeant diffusion coefficient (m^2/s) and K = equilibrium constant. According to this, the resistance offered by the nail plate is directly proportional to its thickness, and inversely proportional to the permeant diffusion coefficient at a constant equilibrium. Similarly, for a given active moiety, the permeability coefficient, P (m/s) (reciprocal of resistance) increases with a decrease in the thickness of nail plate and the D factor. Therefore, the amount of distance to be covered by drug molecule to reach the targeted site in a given time will be greater when the thickness of the nail plate is comparatively high (Elsayed 2015).

2.2.1 Molecular weight

At constant conditions of equilibrium and thickness of nail plate, permeability decreases here exponentially with an increase in the molecular weight of the drug candidate. The permeability of a drug molecule at the nail plate is inversely proportional to its molecular weight as mentioned below:

$$\log P = \log\left(\frac{D}{h}\right) + logK - \beta' MW \qquad Equation\ (2.2)$$

β′ = nail plate constant and MW = molecular weight of the drug substance.

This is due to the restricted pore size of the keratin matrix in the nail plate. This problem of diffusivity through the nail pores can be reduced by hydration. At induced relative humidity conditions, the pore size of nail plate increases due to swelling (Chouhan and Saini, 2012). Consequently, the permeability (Log P) of a drug molecule under swelling conditions can be expressed as:

$$\log P = \log\left(\frac{D}{h}\right) + logK - \beta' MW\left(\frac{H_{sat}}{H}\right) \qquad Equation(2.3)$$

H = water volume fraction and H_{sat} = saturation water volume fraction in the nail plate. Therefore, at constant conditions of drug molecular weight and nail plate thickness, permeability increases with increased hydration. However, this mode of action is limited to a certain extent, as the accumulated humidity can aggravate the clinical conditions of infection by favouring the proliferation of the organism (Elsayed 2015).

2.2.2 Influence of keratin polymer

Affinity of a drug molecule towards keratin can be expressed in the terms of its octanol/water ($logK_{o/w}$) distribution coefficient. The chemical constitution of keratin favours the penetration hydrophilic moieties. The linear free energy relationship between keratin affinity and $logK_{o/w}$ can explained as below:

$$logK_{ker/w} = \alpha + \delta\, logK_{\frac{o}{w}} \qquad Equation(2.4)$$

Where, α = log γ, γ = permeant lipophilicity; δ = relative selectivity of keratin matrix and n-octanol. From Equation 2.4 it can be deduced that distribution of a drug molecule across the nail plate barrier is an integral factor of the octanol/water partition coefficient along with relative selectivity of matrix. Other attributes affecting the affinity are pH and water fraction of the keratin.

$$log\ P = \log\left(\frac{Do}{H}\right) + \log\left[(1-H)K_{ker/w} + H\right] - \beta' MW\ \frac{Hsat}{H} \qquad Equation\,(2.5)$$

Where, H_{sat} = saturated water volume fraction. Based on Equation 2.3, Equation 2.5 describes the correlation between the permeability of a drug moiety for given conditions of water volume fraction and molecular weight. The permeability of a

highly lipophilic drug moiety is a challenging process considering the above conditions of keratin affinity.

It also affects the drug activity along with its permeability. In the case of polar or ionised compounds, a majority of active ingredient is braced at the nail plate minimising the potency of the candidate. Therefore, it is required to consider keratin affinity as one of the key parameters to improve efficacy (Saini and Chouhan 2012; Meng *et al.,* 2020).

2.2.3 Ionisation and pH

In general, ionisation is achieved by a reaction with electron donating or withdrawing groups. It is one of the drug pre-treatment strategies to improve solubility in polar media. The average isoelectric point of nail plate proteins ranges from 4.0 – 5.0. The net charge on the plate is positive under a pH lower than the isoelectric point and negative at higher pH. Under normal healthy conditions, the pH of nail plate is 7.4 with no charge. The process of permeability of drug molecules through nail plate in terms of ionisation is influenced by three factors. Firstly, ionised drug candidates have higher penetration capacities through the nail plate. The mobility of the polar moiety of an ionised drug is accelerated by the interactions with aqueous-based keratin complex. At a given pH, oppositely-charged ions to that of a nail plate have enhanced penetration over similarly-charged species. Secondly, permanent ionisation of a drug candidate increases its deposition in the nail plate, thereby diminishing the activity. The hydrophilic gel behaviour of the nail plate entraps the permeant thereby decreasing the efficiency. Thirdly, ion hydration can increase the molecular weight of the permeant and delay the penetration (Silva *et al.,* 2017).

Hence, an ambient pH and ionisation of drug molecule can yield higher permeation with intended performance. The active pharmaceutical ingredient of this research is a modified coconut oil, which is highly lipophilic. Considering the above clinical conditions of nail and the attributes affecting nail permeation during onychomycosis, the following QTPP as mentioned in Table 2.1 earlier is proposed in this research as per the guidelines of ICH Q8 (R2).

2.3 Intended dosage form

From the previous sections, it has been observed that topical formulations are effective when compared to systemic dosage forms in treating onychomycosis, as the targeted product profile is bioequivalent (Elsayeed *et al*., 2016; Agarwal *et al*., 2020).

Elements of a typical QTPP of an onychomycotic application for a therapeutically essential action have been mentioned in Table 2.1. As the route of application is topical with direct application on the infected area and as the drug moiety will not be metabolised upon administration before any action, the impact of clinical activity is high (with dose of active ingredient provided) when compared with a systemic approach. This maintains the dosage strength for a prolonged period. Administration is easy even if the subject is unconscious and the viscosity provides prolonged or desired time of contact based on the regime or directions of use. It is also a CQA which supports the content uniformity and extrusion from the container during application (Chang *et al.,* 2013). As discussed in the Section 2.2 and from the QTPP established an aqueous soluble medium to carry the active ingredient is a critical attribute to deliver intended performance.

Based on the insolubility, chemical moieties can be categorised into lipophilic (soluble in lipids or non-polar compounds), hydrophilic (soluble in water or polar compounds) and amphiphilic (soluble in both polar and non-polar compounds). Emulsions provide an indefinitely stable environment to an insoluble ingredient at the targeted site. These are biphasic dosage forms, in which an insoluble phase is dispersed into continuous medium by the aid of surface-active agents[5]. Major factors influencing the solubility of immiscible liquids are (i) interfacial tension, (ii) surface tension and (iii) chemical interaction between the liquids. Interfacial tension is a result of adhesiveness between two liquids (Lachman and Lieberman, 2009).

[5] The concepts in this sub-section were adopted from "Martin's – Physical Pharmacy and Pharmaceutical Sciences". Author: Patrick. J Sinko, sixth edition (2011). Lippincott Williams, PA, USA.

According to Gibbs free energy concept, interfacial tension influencing miscibility between two liquid components of a system can expressed as:

$$\Delta G = \gamma \,.\, \Delta A \qquad Equation\ (2.6)$$

Where, ΔG = Gibbs free energy; γ = interfacial tension and ΔA = change in surface area. By thermodynamic convention, ΔG is defined as the work required to increase surface area of dispersed globule by ΔA. As per the Young-Laplace law, this function of work in combination of interfacial tension (force per unit length) and surface area can be explained in terms of pressure on the dispersed globule.

$$\Delta P = \gamma \left(\frac{1}{R1} + \frac{1}{R2}\right) \qquad Equation\ (2.7)$$

Equation 2.7 represents pressure experienced by a droplet dispersed into continuous phase under interfacial tension of γ N/m of radii R1 and R2. Assuming that dispersed globules are spherical (due to maximum surface area), the equation can be represented as:

$$\Delta P = \gamma \left(\frac{1}{R}\right) \qquad Equation\ (2.8)$$

Where R is the radius of an assumed sphere.

From Equation 2.8, it can be correlated that internal pressure exerted on the surface of a globule is inversely proportional to its radius at a standard interfacial tension. On the other hand, for a set of uniform-sized globules, pressure is directly proportional to the interfacial tension.

Therefore, to obtain a dispersion of uniformed sized droplets in a continuous medium that supports physical attributes (Table 2.1) of the dosage form, appropriate balance between the two phases is highly essential.

Surface tension is defined as the force acting per unit length along the surface of a fluid, which is a consequence of cohesive attraction among the molecules in the fluid. Interfacial tension is observed between two individual fluids, whereas surface tension is pertained to one type of fluid. Surface and interfacial tension are interconvertible based on the concentrations of the dispersed phase in the medium. The surface tension of a liquid being dispersed into a continuous phase will determine

its solubility. Compounds with higher surface tension are not soluble into lower valued fluids, for example, oil dispersion in water. Similarly, surface tension of a fluid is directly proportional to its viscosity. As the cohesiveness increases, inter molecular distance decreases, which results in low void space and viscous behaviour.

Van der Waal's forces of attraction intermolecular hydrogen bonding, polarity or ionic nature, electrostatic behaviour and temperature are the factors that influence chemical interaction between two immiscible liquids. However, among these, intermolecular hydrogen bonding and temperature have the higher impact on the solubility along with their chemical structure. For example, saturated organic alcohols until pentanol are freely soluble in water, whereas octanol is insoluble due to a longer hydrocarbon chain. Similarly, fatty acids of long-chain hydrocarbons (lauric, myristic and stearic acids) are insoluble in water. Hence, to solubilise an insoluble fatty acid or oil into an aqueous medium, a complex relationship of interfacial and surface tension balance, along with chemical interaction is to be considered. Such moieties that mediate these physical attributes to enhance dispersibility of an internal phase are called surface-active agents.

2.3.1 Surface-active agents[6]

The relation between the interfacial tension and surface area of a dispersed phase in a continuous medium was illustrated in Equation 2.6 in the previous section. Therefore, at constant energy, the surface area of a globule introduced into an insoluble phase is inversely proportional to the interfacial tension of the medium. On the other side, the droplet size of a dispersed medium is directly proportional.

A surface-active agent is a chemical moiety with both hydrophilic and lipophilic terminals that can reduce the surface tension of a liquid or medium when introduced into the opposite phase; for example: salts of fatty acids. The ionic (hydrophilic) region of these soaps interact with hydrophilic components and the fatty chains solubilise the lipophilic component. Thus, in connecting these two opposite phases, the solubility of a dispersed phase is enhanced into continuous phase.

Therefore, the presence of hydrophilic and lipophilic moieties on a compound is the basic structural criterion of a surface-active agent. Based on the type of charge present on the hydrophilic region of these compounds, they are categorised into anionic, cationic and non-ionic surfactants. The amount of surfactant required depends on the concentration of dispersed phase to be solubilised into the medium, as the influence of surfactant on the interfacial tension depends on the chemical interaction between the number of molecules of surfactant and the phases.

Upon introduction, surfactants form an interfacial layer around the dispersed phase by entrapment. The outer end of film will interact with a continuous medium and stabilises the system. Such an entity of entrapped phase with surfactant oriented towards a continuous medium, is called a micelle (Figure 2.4).

[6] The concepts in this sub-section were adopted from "Martin's – Physical Pharmacy and Pharmaceutical Sciences". Author: Patrick. J Sinko, sixth edition (2011). Lippincott Williams, PA, USA.

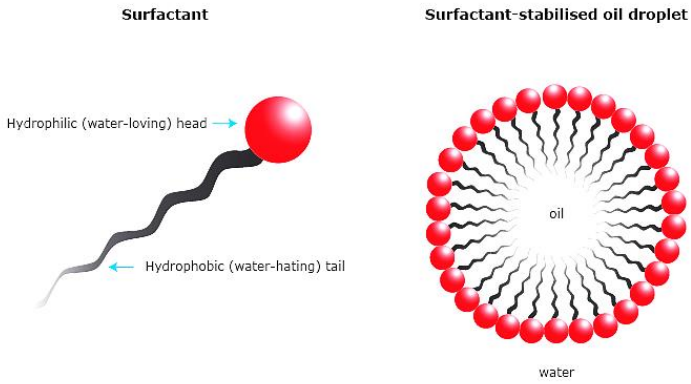


Figure 2.4| **Structure of surfactant solubilising oil into aqueous medium.** In this case, hydrophilic end is regarded as head and lipophilic end as tail. The formation of a micelle can be observed with the tails entrapping an oil globule in an oil-in-water system.

The concentration of surfactant after which there is no more influence on the interfacial tension between the phases of an emulsion is termed the critical micellar concentration. It is very specific for every surfactant due to differences in chemical composition of the compound.

The selection of a surfactant for an emulsion depends on the hydrophilic-lipophilic balance (HLB) of the system. It is defined as the ratio of total water-soluble fraction to that of total oil-soluble. Based on the type of solute dispersed into continuous medium, emulsions are majorly classified into (i) oil-in-water (o/w), (ii) water-in-oil (w/o) and (iii) multiple emulsions. In o/w emulsions, oil is dispersed into a continuous aqueous phase, whereas in w/o emulsions, water is emulsified into oil. Multiple emulsions contain several compartment systems in which, dispersions of water into oil and then into water (water-in-oil-in-water, w/o/w) or oil into water followed by oil again (oil-in-water-in-oil, o/w/o) takes place. In any of these emulsifying conditions, surfactants play a crucial role in stabilising and solubilisation.

2.4 AAE Characterization to identify CQA

In this research infrared (IR) and nuclear magnetic resonance (NMR) spectroscopic analysis were used to establish and interpret functional groups of interest. Differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), density and saponification value determination were used to establish physical properties of a novel AAE. Karl Fischer titration to evaluate water content; atomic absorption spectrometry (AAS) and *Limulus* amoebocyte lysate (LAL) testing to verify the presence any possible toxic impurities were used. This section includes the principles and applications of novel and robust analytical techniques to characterise an AAE and establish CQAs. Similarly, the methodologies of scrutinising incompatible excipients towards an effective dosage form to meet the targeted profile using a comprehensive combination of analytical techniques have been introduced.

2.4.1 IR spectral analysis

Principle: Bonds in molecules vibrate above absolute zero temperature and these vibrations result in absorption at IR frequencies corresponding to the bond's natural frequency. An IR spectrometer records these absorptions in wavenumbers (cm^{-1}). IR spectroscopy is used to identify the chemical structure of an unknown compound by the peaks produced on a spectrum typically from high (4000 cm^{-1}) to low (400 cm^{-1}) frequency (Silverstein *et al*., 2005).

Easy sample preparation and rapid unambiguous peak assignments and availability of results in minimal time with key descriptive information has established IR spectroscopy as an effective fingerprinting technique (Rohman *et al*., 2009). As the measurement involves specificity towards the bonds and their interactions between the atoms, differentiation between similar compounds such as isomers is also prudent through IR spectroscopy. Analysis can be performed ranging from core structure elucidation to minute presence of adulterants. In attenuated total reflectance – Fourier transform IR (ATR-FTIR) spectroscopy, there is no-to-minimal sample preparation, and the results are produced almost instantaneously. Hence, this technique is widely used in testing raw materials and for determination of any contaminants prior to production in the pharmaceutical sector. It is one of the

immediate identification methods for the analysis of chemicals prescribed in European Regulation EC 440/2008[7].

IR spectroscopy serves as an efficient tool to assess many ongoing processes such as reaction mechanisms, mixing, drying and stability assessment during application stress conditions. A sample's spectrum is compared to that of the reference material or established libraries to identify any potential disturbances due to the process or condition being applied. For example, esterification and transesterification of fatty acids and their glycerides are common procedures among food and related industries to produce novel emulsifiers or to improve satiety. The extent of these esterification reactions using either enzymes or metal-catalysed acid/base hydrolysis can be diagnosed using IR spectroscopy (Rajan *et al*., 2006). Malina & Shai (2005) have used ATR-FTIR to investigate the influence of fatty acid chain length on antimicrobial potency. Similarly, Rahman *et al*., (2013) have employed ATR-FTIR in their pre-formulation studies to investigate API and excipient interactions. Rapid analysis of compounds by IR spectroscopy and easy interpretation of data enables the analyst to decide on the next step of the process without proceeding to expensive analytical techniques. In similar controlled drug release studies conducted by Naumann *et al*., (2014) on a novel antifungal compound towards onychomycosis, the ability of the API to penetrate bio-relevant membrane has been qualified initially by IR spectroscopy and then quantified by high performance liquid chromatography.

Considering these applications, IR spectroscopy has been employed in this research as an identification tool to investigate structural properties of API and its interactions with excipients during pre-formulation and stability studies.

2.4.2 Thin-layer chromatography (TLC)

Thin-layer chromatography is an analytical technique to assist in the characterisation of compounds and establish composition. It is an adsorption technique, in the which analyte is separated on a solid stationary phase using a liquid mobile phase. In this mode of chromatography, analyte is dissolved in the mobile phase and run over a

[7] Available at: http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32008R0440

stationary phase onto which it is adsorbed. This chromatography is generally applied to observe retention or elution phenomenon of an analyte for selected stationary and mobile phases (Fontana *et al*., 2009).

2.4.3 Density

The density of a substance is defined as the mass of substance in unit volume (kg/m$^{3)}$ occupied by it at specific temperature. A general procedure for density determination involves measuring the volume and weight of sample using a specific gravity bottle, followed by calculation (USP, <841>). In this project, the density of MCO was determined by using a density-meter with oscillating 'U' tube principle. This is applied to measure the density of liquids, in which the sample is loaded into a U-tube where it is electronically excited to oscillate at its characteristic frequency. The instrument determines the frequency at which the liquid oscillates and measures the density (Klug-santner *et al*., 2012).

2.4.4 Saponification value

A saponification value is defined as the number of milligrams of potassium hydroxide required to neutralize free fatty acids and saponify esters contained in 1.0 g of substance (USP <401>[8]). It indicates an average molecular weight of the fatty acids in an oil and so is useful in the preparation of soaps and in qualitative analysis (Toscano *et al*., 2012).

2.4.5 Differential scanning calorimetry (DSC)

Various techniques are available in order to identify the physicochemical properties and potential incompatibilities of an API. Among them, differential scanning calorimetry (DSC) serves as a multipurpose tool, in which heat flow through a compound over a range of temperature is studied. In a DSC experiment, the rate of heat flow through the sample over the required temperature range is studied in comparison with an empty reference pan (Chadha & Bhandari 2014). These studies can be conducted either in heat flux mode or power compensation mode. In the former, power change in the sample in comparison to standard/reference during the

[8] Fats and Fixed oils / Chemical Tests <401>. United States Pharmacopoeia 37 – National Formulary 32.

temperature changes is being measured in a same thermo couple, whereas power changes when constant temperature difference has been maintained between sample and reference pans in different thermal chambers is measured in the latter (Gaisford S. 2015).

DSC measurements co-operate the heat capacities of sample and reference materials or interpret their physical changes such as melting or heat of fusion (heat required to convert a liquid compound into its vapour state), sublimation (amount of heat consumed by a solid compound in order to convert it into its gaseous/vapour state) glass transition and degradation. In modulated DSC (mDSC) measurements the linear relation between the phase transition or changes in the molecules with respect to temperature is measured. Even though the results appear in sinusoidal shape the modulation programme assists the analyst to determine the linear relationship based on the correction factors provided by the respective manufacturers of the instruments. Choice of experimental parameters also contributes towards the quality of the information generated from the sample. For example, rapid heating rate or technically called as high temperature ramps can fail to quantify any possible slow degradants. Similarly, type of pans selected and the input of sample into these pans hermetically (absence of air) or non-hermetically can provide useful information regarding the sample behaviour. However, the sealing of sampling pans also depends on the basic thermal characteristics of the sample such as volatility and experimental conditions (Gaisford S. 2015).

In pharmaceutical sector well-designed experiments considering above parameters can provide information about the thermal behaviour of the API over the selected temperature range, which might then be applied during the manufacturing process if required, without compromising thermal stability. Incompatibility studies using DSC can be performed by observing the thermal changes of the drug when mixed with respective additives (Wissing *et al*., 2000).

Pharmaceutical operations such as preparation of syrups, hot melt extrusion, drying, coating etc. include endothermic and exothermic reactions. It is important to evaluate the stability of active components and excipients under these thermal procedures to retain their potency. Physico-chemical changes of an analyte of

interest in such processes can be evaluated by the application of DSC under induced thermal conditions and the data generated in these studies can be used to optimise the process (Bond *et al*., 2002).

Fat is generally the synonym of the solid phase of oils, which are liquids at higher temperatures. Unsaturated fatty acids exhibit geometrical isomerism-associated polymorphism based on positioning of multiple bonds in their structure. The crystallisation of such unsaturated fatty acids is influenced by these polymorphic behaviours. Application of X – ray diffraction studies in combination with DSC can inform this polymorphic crystallisation mechanism, which is useful to predict the oxidative and induced stress stability of unsaturated lipids (Sato, 2001; Himawan *et al*., 2006). Piska *et al.,* (2006) have investigated these conversions of liquid samples into solid fats by DSC. The amount of heat consumed by the sample and the onset temperature of this phase change are useful to establish critical identification attributes. The difference in these phase transitions among molecules of similar functional groups such as acids or esters is an outcome of atomic composition. In fats, the variations in number of carbons in a respective fatty acid attached to glycerine in the triacylglycerol, influences the phase transitions and liquid fractions. DSC can be employed to determine these phase changes in correlation with chemical constituency of the lipids. This information is useful to identify any potential impurities or changes in given sample (Tolstorevrov *et al*., 2014).

However, if incompatibilities are identified, DSC analysis alone cannot explain chemical interactions between the components and provide possible solutions to minimise it. Therefore, DSC studies augmented with IR spectroscopy can detail stepwise chemical mechanisms involved in the incompatibility through structural elucidation. For example, the brown pigmentation due to interactions between an amine drug and sugar related excipients – popularly known as Maillard's Reaction, can be demonstrated by the shift in the peaks of free amine groups of reacted material to that of reference material by IR spectroscopy (Bharate *et al*., 2010). With improvements in technology of user interface systems towards monitoring real time data IR spectroscopy has been included as a part of PAT in current modern manufacturing technologies (Gouveia *et al*., 2017).

Saadi *et al.,* (2012) have used reverse-phased high-performance liquid chromatography and proton nuclear magnetic resonance spectroscopy in combination with DSC to study the crystallisation behaviour of various chained fatty acids. The changes in peaks and enthalpy values of the analogue samples determined to discriminate fatty acid glycerol, have been observed to agree with the results of other analytical techniques employed. Adulteration of oils and fats can be identified using DSC, the comparison of phase transition temperatures of neat and test sample provides qualitative information (Verstinge *et al.*, 2014). The normalisation of differences in heats of enthalpies of neat and adulterated samples with their weights is useful to quantify the extent of adulteration (Chaleepa and Ulrich, 2010; Dahimi *et al.*, 2014).

As discussed earlier, application of DSC in compatibility assessment between the API and excipients provides wide range of information which can be further utilised during manufacturing. For example, Bachmaier *et al*., 2021, used DSC as characterisation tool to understand the compatibility between hydroxypropylcellulose and itraconazole to formulate amorphous solid dispersions. The findings from these experiments proven successful combinations of API and excipients with great correlation between *in vitro* and in *vivo* dissolution studies. Therefore, DSC is considered in this research as one of the tools to characterise API and excipient combinations.

2.4.6 Thermogravimetric analysis (TGA)

Experiments perfomed at constant temperature (isothermal) or increasing temperature conditions (ramping) or cooling are used to monitor the alterations in the weight of the sample changed per degree change in the temperature in a given time. It is generally used to characterize the thermolabile fractions of compounds such as entrapped moisture, volatile organic content and residual components. At higher temperatures (usually ≥ 400^{o}C) pyrolysis of polymers and related materials are also studied. Devolatisation, the process of degradation of components resulting in the rheology change due to reactions at elevated temperatures can be observed as a function of mass. Iso-conversional or model free data treatment is useful to calculate the activation energy of compound, which requires several curves of TGA at various rates of heating. Analytical conditions are usually preferred in vaccum or with nitrogen purging to avoid unecessary hazards or collateral damage. However, the parameters of analysis are subjective to the type and properties of sample (Jain *et al.,* 2006).

Lipid oxidation is an undesirable effect observed in essential oils, nutritive compounds and vitamins leading to changes in color, texture, odor and inactivation of functional moiety. This is a free radical reaction initiated under stress conditions. Application of TGA on such compounds under simulated oxidative stress and mild thermal conditions can predict the oxidisability of vulnerable chemical moieties (Coni *et al.,* 2004).

Schanez *et al.,* (2015) used TGA to study the extent of acylation and polymerisation of various fatty acids with chitosan for the production of gel-like drug delivery systems. The onset points and weight variations determined during the temperature changes and degradation studies of the analysis have profiled the individual components of the system. This information has been further utilised to optimise the production of chitosan compounds to maximise the yield. Rezaei *et al.,* (2021) have characterised components of thyme oil encapsulated in cyclodextrin nanosponges with the help of TGA.

2.4.7 Water content

When present, water as bound, free, inherent or occluded forms, can have substantial technical impact over the properties of API. The physical state of a compound may be altered by the presence of water upon a change in the temperature; for example, the crystalline properties of an API can be altered in the presence of water. Similarly, the water present can react with any of the excipients and cause incompatibility. Apart from the influence on physical and chemical characteristics, the presence of moisture can initiate microbial growth thereby degrading the stability or efficacy of an API/dosage form (Kestens *et al*., 2008). Therefore, water content determination is one of the major investigations to be performed in pre-formulation studies. For example: The Institute of Reference Materials and Measurements of the Directorate General-Joint Research Centre of the European Commission prescribes the determination of water content of certified reference materials as one of the major investigation procedures for a product.

The following methods are applied for the determination of water content in a compound:

(i) Loss-on-drying: The compound is dried in an oven at a certain temperature and the difference in the weight is observed at particular time intervals. The water evaporated during drying, creates a difference in the weight of the compound (before and after drying). This procedure is continued until no difference in the weight is observed. Along with water, other volatile components can evaporate; hence, accuracy may be less with this method. This procedure is not suitable for thermolabile compounds.

(ii) Karl Fischer (KF) titration: In this method, determination of water content is performed without the application of heat. Hence, it is suitable for very sensitive compounds such as proteins and biological derivatives. It was developed on the following principle that in the presence of methanol, an organic base (RN) – imidazole, and sulphur dioxide, iodine reacts with water at 1:1 molar ratio.

$$RN\ (base) + SO_2 + CH_3OH \rightarrow [RNH]SO_3CH_3$$

$$H_2O + I_2 + 2\ RN + [RNH]SO_3CH_3 \rightarrow [RNH]SO_4CH_3 + 2[RNH]I$$

KF titration can be performed in two methods: (a) volumetric – this method suits for the determination of water present in higher amount (up to 100%) (b) coulometry – determination of water content in a sample by measuring the amount of current generated during the titration. For the generation of one mole of iodine, 2 × 96,485 Coulombs of current are needed. This method is suitable to determine trace amounts of water up to 10 mg/g.

The solubility of oils is very low in methanol, hence the sample to be examined is solubilised by a co-solvency technique, in which an additional inert solvent such as chloroform, 1-hexanol, or 2-propanol is used to solubilize the analyte. In order to determine a water content in MCO by KF coulometry, 2-propanol has been used in this research.

2.4.8 Nuclear magnetic resonance (NMR) spectroscopy

Negligible sample preparation, requirements of lower quantities of solvents during the analysis, being a non-invasive strategy and easy data acquisition diagnosing the fingerprints of the molecules, present NMR as a leading spectroscopic technique in the characterisation of drug candidates and their products in pharmaceutical sector (Raluca *et al.,* 2015). These technologies utilise a low-field/high-field permanent magnet for the analysis. Adulterants, including those at trace amounts, can now be quantified using bench-top NMR spectrometers in complex organic molecules such as edible oils (Parker *et al.,* 2014).

NMR was listed among official methods of analysis to determine the solid fat content in edible oils by AOCS in 1993. The results obtained for different samples of oils are comparable with free fatty acid number or acid value of the respective samples. Along with fingerprinting of core molecular structure, lipid oxidation values of vulnerable oil samples can be determined by NMR spectroscopy. Proton - ^{1}H NMR provides a detailed information of acyl groups of various mono-, di- and triacyl glycerols in a given oil sample. A low frequency detection at 60 MHz has been proven effective to trace the differences between the lipid's nuclei present in the sample when compared with the popular spectroscopic techniques such as FTIR. This analysis is useful to discriminate adulterated samples from the pure (Almoselhy *et al.,* 2014).

Presence of the long-chain saturated or unsaturated hydrocarbon chains increases the boiling point of oils to higher values such as 190, 220 and 280^{o}C. This thermal stability of such oil samples can be investigated using NMR spectroscopy including the by-products such as aldehydes and ketones formed during degradation (Cordella *et al.,* 2012).

Triacylglycerols are the common ingredients of oils or fats. These are the carboxylic esters of respective fatty acids with the hydroxyl groups of glycerine. Based on the length of carbon chain of the fatty acid bonded with glycerine, they are termed short-, medium- and long-chained esters. Biocatalysts can degrade these triglycerides into mono, diglycerides and free fatty acids. The extent of this transesterification can be determined by ^{1}H NMR spectroscopy. The behaviour of a hydrogen nucleus

connected to a carbon of glycerine molecule will change with its type of attachment, producing a different shift in the spectrum. This enables the identification of type, extent of digestion and any by-products during the transesterification quicker than trivial gas chromatography (Nieva *et al.,* 2015).

Stress conditions could be oxidative, thermal, relative humidity and sometimes in combinations. Implementation of an appropriate analytical tool of constant terms and conditions with respect to sample preparation and method of analysis is a crucial factor in these studies. ^{1}H and ^{13}C NMR techniques serve as robust tools in this strategy as the analysis elucidates the atomic arrangements of components. For example, NMR can assess oxidative stability of olive oil by comparing the stressed samples with alternative vegetable oils (Salces *et al.,* 2011; Skiera *et al.,* 2012).

Dais and Hatzakis (2013) developed a strategy of metabonomics, metabolic profiling and fingerprinting in correlation with data analytics of NMR of the various olive oil samples, to establish quality limits and authenticity. This approach helps to identify any adulterants and to assess the quality of oil before release into the market.

Considering these applications of NMR in fingerprinting and identification of adulterants in a product post-manufacturing, it has been employed in this research to characterise MCO.

2.5 Preliminary toxicological evaluation

Drug applications through topical and ungual routes of administration enter the blood stream of the patient directly through subcutaneous tissue without undergoing hepatic metabolism. Hence, the chances of undesired effects of chemicals or their residues or biological components are high. Some techniques that help to identify potential toxins in the topical administration are detailed in this section.

2.5.1 Pyrogen testing

Pyrogens are usually the structural features or fragments of Gram-negative bacterial cell walls. The lipopolysaccharides (LPS), lipopeptides, peptidoglycans and techoic acids of the cell wall act as endotoxins and can cause fever during bacterial invasion. A rise in the body temperature is mediated by excessive release of inflammatory cytokines by the LPS (Park *et al*., 2005; Tamura *et al*., 2021). In order to identify any pyrogens in a medical product: the rabbit pyrogen test or the *Limulus* amoebocyte lysate (LAL) test are employed. The *in vivo* rabbit pyrogen test is used to identify pyrogens from medical products by injecting samples through the intravenous route to at least three rabbits and measuring the temperature rise by rectal probe. This method is sophisticated, will need appropriate licensing or trained persons to conduct the test and is a time consuming process (Ding & Ho 2001; Tamura *et al., 2021*).

In 1956, Bang, observed that, amoebocytes from the blood of the *Limulus polyphemus* (horseshoe crab) had a coagulation factor, which forms clots when reacted with Gram-negative bacterial endotoxin. This principle was then transformed into a pyrogen investigation test or as a diagnostic tool. The resultant LAL test is a quick, cost-effective and bench-top bacterial endotoxin measurement (BET). The United States Pharmacopoeia has categorised this method as its bacterial endotoxin test for the detection or quantitation of pyrogens from Gram-negative organisms in a product under the physical test <85> (Tamura *et al*., 2021; Akimov *et al.,* 2021).

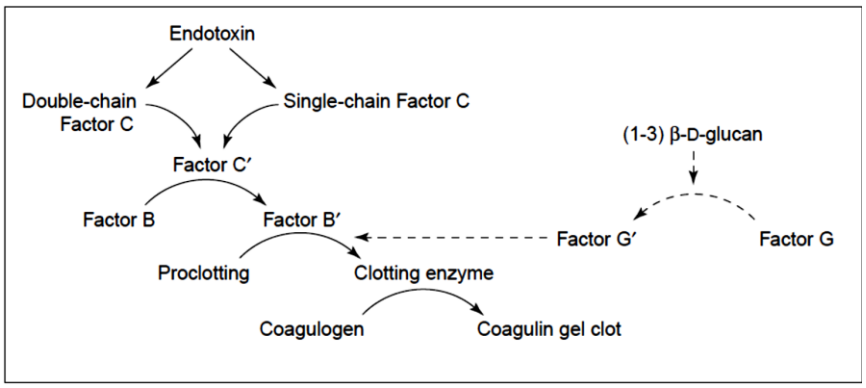


Figure 2.5| **Coagulation cascade applied in LAL gel clot test method** (Ding & Ho 2001).

Factor 'C' in the amoebocytes of the horseshoe crab, which is an endotoxin-sensitive serine protease, initiates the cascade (Figure 2.5). The *β*-D-glucan (one of the cellular components) is the major interfering substance when a test is performed (Figure 2.5). Hence, appropriate measures have to be taken in order to avoid any cross contamination.

LAL testing can be performed by one of the three methods: (i) gel-clot method (limit test and semi-quantitative test) based on gel formation (ii) turbidimetric (kinetic and endpoint) based on the development of turbidity which can be measured by optical density and (iii) chromogenic (kinetic and endpoint) based on the development of colour technique. The sample to be tested is diluted in pyrogen free water or LAL reagent water in order to avoid the interference of contaminants from any external sources. In this research, the gel-clot technique (as it a quick and effective method) has been employed to investigate and affirm the absence of any pyrogenic properties of MCO.

2.5.2 Control of potential elemental impurities

This section details the significance of trace metal analysis during the developmental stages of a drug product. Contamination from trace metals in a dosage form can have deleterious effects over its stability as well as the therapeutic performance. Therefore, quantification of such in accordance with harmonised technical requirements to assure quality and safety is a critical step in dosage form design.

The analysis of metals in a pharmaceutical product is considered as a traceable measurement, as the results are obtained and compared according to standards such as that of the United States Pharmacopeia (USP) or British Pharmacopoeia following CHMP "Guideline on the specification limits for residues of metal catalysts or metal relevant" (2008). The ICH has drafted guidelines for the elemental impurities (Q3D) [9] as an important step in dosage form development with novel chemical entities.

Any elemental impurities are generally unwanted metals that are available in pharmaceutical products in free form, or sometimes added intentionally as a catalyst at some stage of pharmaceutical processing. Other routes of entry into pharmaceutical products include contamination of raw materials, from the machinery equipment that has been used, or due to reaction between the process materials and the production equipment or storage container and closure systems. ICH Q3D has been divided into three categories: (i) the evaluation of toxicity data of potential impurities (ii) to establish permitted daily exposure (PDE) (iii) to set controls to limit the levels of elemental impurities below the PDE (Chawla *et al.,* 2021). The assessment and control strategy of such elemental impurities should be performed as per the guidelines of Quality Risk Management dossier published by The International Council on Harmonisation of Technical Requirements of Pharmaceuticals for Human Use as ICH Q9[10].

Based on the toxicological profiles, ICH Q3D has classified the common metal impurities into four different classes (Table 2.2). The general procedures for the

[9] Prepared as per the draft of ICH – Guideline for Elemental Impurities (Q3D) – 2013. available at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3D/Q3D_Step_4.pdf
[10] ICH Q9, Quality Risk Management (2005). Available at: www.ich.org/products

assessment prescribed by ICH Q3D are (i) identify the impurities present in the pharmaceutical product (qualitative analysis) (ii) quantify the identified impurities (iii) evaluate by comparing the determined amounts of elemental impurities with the standards or PDE (iv) controlling by documentation and implementation of the limits or possible interferences of the elemental impurities observed or determined during pharmaceutical processing.

Table 2. 2| **Elemental impurity classification** (ICH Q3D)

Class	Included elemental impurities	Include in risk assessment
1	arsenic, lead, cadmium, mercury	Yes
2A	vanadium, molybdenum, selenium, and cobalt	Yes
2B	silver, gold, thallium, palladium, platinum, iridium, osmium, rhodium, and ruthenium	Yes, only if intentionally added
3	antimony, barium, lithium, chromium, copper, tin and nickel	Dependent upon route of administration
4	boron, iron, zinc, potassium, calcium, sodium, manganese, magnesium, tungsten and aluminium	No

During the identification process, unless otherwise mentioned in the classes 1 and 2, attention must be paid to the type of manufacturing equipment and the type of chemicals being processed in the equipment, as the possibility of leaching of impurities into the product depends on the nature of chemical interaction between them. After the equipment, the container closure system plays a crucial role in avoiding contamination from the external environment into the product. At the same time, the materials used for the container closure system can also leach into product. The chances for leaching from a container closure system is higher with liquid and semi solid dosage forms than solid dosage forms. Thus, any influential parameters are to be considered with a container closure system for the identification of elemental impurities. These include hydrophilicity/hydrophobicity, ionic content, pH, temperature, contact surface area, container/component composition, terminal sterilization, packing process, component sterilization, migration potential, duration storage and inclusion of metal chelating agents in the formulations (Chawla *et al.,* 2021).

Analysis and evaluation during the assessment should be performed based on the prior knowledge or published literature or data generated from any similar processes or material safety data sheets or analysing each and every component i.e., the raw materials that are used for the drug. After the analysis, the detected limits should be addressed in comparison with regulatory standards.

Using the established PDEs the common permitted concentrations of elements can be achieved for a drug substance under test, which is taken as 10 g or less per day by the Equation 2.9 below:

Concentration (µg/g) = PDE (µg/day)/daily amount of drug product (g/day) (*Equation 2.9)*

For a drug product more than 10 g a day, Equation 2.10 applies:

$$PDE\ (\mu g/day) \geq \Sigma_{k=1}^{N} C_k.M_k \qquad \textit{(Equation 2.10)}$$

Where k = an index for each of N components in the drug.

C_k = concentration of the elemental impurity in component k (µg/g).

M_k = mass of k in the maximum daily intake of the drug product (g).

The equation (i) is applicable for the permitted concentration of the finished product.

The important factors to be considered during the assessment of levels of the potential elemental impurity in the drug product are:

- efficiency of removal of elemental impurities during further processing
- natural abundance of elements
- prior knowledge of elemental impurity concentration factors from specific sources.

2.5.2.1 Atomic Absorption Spectrometry[11]

Relevant techniques available for the trace metal detection are flame atomic absorption spectrometry (FAAS); electro-thermal atomic absorption spectrometry (ET AAS); direct current (DC) or inductive coupled plasma (ICP) with optical emission spectrometry (OES) or mass spectrometry (MS) and atomic fluorescence spectrometry (AFS).

Absorption of electromagnetic radiation by elements required to promote electrons from lower energy to higher energy level is the principle involved in AAS. The wavelength of radiation absorbed for electronic transition is specific for each element and can be used in qualitative analysis. Metals are the common analytes of interest in this technique as electronic excitation is more rapid than for non-metallic elements. Similar to other spectroscopic techniques, in FAAS, the absorption is directly proportional to concentration of analyte in the sample. The sample is excited through vaporisation and hollow cathode lamps are used as the source of light or radiation that contain a filament made of the analyte of interest.

2.5.2.2 Sample pre-treatment

MCO is a derivative of vegetable oil composed of free fatty acids, mono and triglycerides. The solubility of MCO in water is poor due to its composition. In FAAS, medium or matrix of dispersion is mostly aqueous-based, and the atomiser requires a clear solution to spray vaporise the sample for excitation. Hence, an appropriate sample preparation is a crucial step to minimise matrix interference effects and keep the atomiser safe from blockages.

Vegetable or edible oils undergo various processing steps from extraction to the packing prior to market release. Many of these steps include metallic equipment made of various alloys or pure metal like iron (Fe). The most common metallic contaminants of edible or vegetable oils are Fe, Cu, Zn, Ni, Cd, Hg, Mn, Ru, Rh, Pd and

[11] The description under this section has been adopted from the publications of Royal Society of Chemistry. Available at: http://www.liskeard.cornwall.sch.uk/images/Liskeard-Sixth-Form/Atomic-Absorption-Spectrometry.pdf

Pb. Hence, quantifying respective metals for traceability is an important step in assuring quality of oils (Zeiner *et al*., 2005).

The chemical nature of the sample mediates the contamination, for example: water and aqueous solutions are more vulnerable for heavy metal contamination than non-aqueous composites. In the models of latter format, presentation of metal from the sample for analysis in the instrument is a challenging process. Oil or organic samples have problems in direct injection into the atomisers and with the calibration standards. Thus, a dispersion of sample into an appropriate medium is required.

Techniques to compensate matrix interference effects for oil or organo-metallic compounds include extraction, ashing, acid dissolution, dilution with organic solvents, acid digestion and emulsification (Aucelio *et al.,* 2007).

2.5.2.2.1 Solubilisation

Oils are often emulsified into aqueous media to extract metals for analysis. Gunduz *et a.l,* (2015) have investigated various edible oils sold in the Turkey to quantify Pd, Cu, Cr, Ni and Cd. The oil samples have been modified into macro- and micro-emulsions using 0.1 M nitric acid, Triton X114 surfactant and propan – 2 – ol as co-solvent. The limits of detection and quantification for each element have been determined to be better in macroemulsions than the microemulsions.

Acidic emulsions of various vegetable oils have also been prepared using Triton X114 (7%) and nitric acid (10%) in water by Bakircioglu *et al.,* (2014) and cracked at 80 ± 2^{o}C. The acid portion has been recovered and analysed by FAAS and ICP-MS. This method of sample pre-treatment has produced improved results and been claimed to be beneficial and safe with a recovery rate from 99.5 – 105.5%.

Nunes *et al.,* (2011) have carried out multi-elemental determinations in vegetable oils by micro-emulsification of samples prior to analysis by continuum source atomic absorption spectroscopy.

2.5.2.2.2 Acid digestion suitability and selection

Pre-treating oil samples by solubilising organic moieties into an aqueous phase and ionising the trace metals by the application of stronger acids such as perchloric, nitric, sulphuric or hydrochloric acids under pressure and high temperatures, is termed acid digestion. Rationalised mixtures of these acids can also be used to improve efficiency or ionisation. Similar procedure is mentioned in USP – NF, Fats and fixed oils (method <401>) for the analysis of heavy metals in lipids and related matrices[12].

In dry digestion, samples with high organic content are burnt to ash and diluted by acids for analysis, whereas sample is treated with acids to dissolve and avoid loss of volatile matter in a wet process. Altundag & Tuzen (2011) have investigated fruit extracts for the concentrations of trace metals using ICP-OES. Samples have been pre-treated by dry, wet and microwave-assisted digestion of the sample. Among the methods employed, the microwave assisted sample digestion has been proven reliable with good recovery values.

Hydrogen peroxide solutions can be added along with acids to ionise elements of inert samples. In these conditions, open vessel digestion is recommended to avoid explosion due to any gaseous products formed during the digestion (Calle *et al*., 2016).

Along with the above-mentioned methods, other alternatives (See Table 2.3) include application of chelating or complexing agents and resins. These matrix compensation practices have disadvantages such as dilution or dissolution with organic solvents which can cause loss of volatile matter and high noise content. When metallic containers are used, acid digestion can contaminate a sample matrix from vessels used; emulsification will reduce organic content and temperature during the analysis, and storage conditions can alter the globular size and block atomizers.

[12] USP 39 – NF 35, <401>, Fats and Fixed oils. ''Heavy metal analysis''.

Table 2. 3| **Alternative sample pre-treatment methods for trace metal analysis**

Technique	Type of sample	Reference
Complexation *Agent* *N, N*'-bis (4-methoxysalicylidene) ethylenediamine 2-[(*E*)-(8-hydroxy-2-methylquinolin-5-yl) diazenyl] benzoic acid	Edible oils and lipophilic materials	Tokay *et al*., (2016); Yildez *et al*. (2016)
Solid-phase extraction Examples: Poly [2-(4-methoxyphenylamino)-2-oxoethyl methacrylate-co-divinylben zene-co-2-acrylamido-2-methyl-1-propanesulphonic acid] - chelating resin	Viscous samples like honey and sewage sludge	Reid *et al*., (2009)
Co-solvents Examples: kerosene, water and propanol (0.5:0.02:4)	Lubricating oils	Zmozinski *et al., (2010)*
Eutectic mixture Examples: choline chloride, nitric acid and urea	Edible oils	Karimi *et al*., (2015)

2.6 Excipient suitability and selection

Excipients occupy the majority of composition by weight or volume in a dosage form. Early-stage pharmaceutical development can be a time-consuming process to provide a fully comprehensive characterisation for a single batch of raw materials. It is therefore advantageous for a pharmaceutical firm to have a contract or quality agreement with a designated supplier. This step restricts the supplier to modify any steps during subsequent excipient manufacture. Along with quality information, a summary of timelines about the stock or production ledger will be advantageous to continue the dosage form production without interruption. Apart from implementing current good manufacturing practices (cGMP), it is the responsibility of the marketing authorisation holder to ensure the quality of product. This has an indirect relationship with quality of raw materials i.e., excipients (Elder *et al*., 2015). In the USP – NF 2020, under the chapter <1078>, "Manufacturing Practise Guide for Bulk Pharmaceutical Excipients", these requirements were documented. This is framed according to the standards of ISO 9000.

In the review published by K. Zhang *et* al., (2015), impurities generated by excipients were classified into reactive and non-reactive impurities. Reactive impurities include but not limited to peroxides, aldehydes, organic acids, reducing sugars and elemental. Storage conditions, interactions with the API and remaining excipients can produce these reactive materials in the formulation thereby effecting the stability of the dosage form. For example, polymeric excipients such as polysorbates, povidone and crospovidone etc are major sources of peroxide generation upon oxidation. Nonreactive impurities are resultant of manufacturing process, for example residual solvents from the media used during the production.

Appropriate supply chain management such as vendor-managed inventories are now followed by most companies to avoid any deficits in supply. Quality of excipients according to ICH Q8 (R2) is pertained to the critical material attributes (CMA) irrespective of supplier. As long as the CMA of the excipients has been proven robust in terms of pharmaceutical grades intended for the design, Q8 allows its usage for product manufacture. In terms of QbD, the CMA of excipients so established

should confine themselves to the design space preserving the attributes and performance of the product during manufacture and throughout the product life cycle (shelf life). An algorithm of continuous process verification (CPV) should monitor the respective properties to prove the quality assured in the design. An ideal summary of quality of an excipient would include the conditions of procuring it from the manufacturer, a timely and secure transportation, introduction into the dosage form, performance under stress, no, or limited, interactions with container closure systems and good *in vitro* and *in vivo* correlation of drug dissolution profile. To standardise the processing conditions and to harmonise the international pharmacopeial regulations, "The International Pharmaceutical Excipients Council (IPEC)" has been established since 2010. This body includes members from the USA, Europe, Japan, India and China.

A series of analytical examinations to identify the attributes of API and to establish target-oriented agreement with the excipients by screening out possible retarding agents are termed incompatibility studies. In this phase of pre-formulation, any physical or chemical interactions between the API and excipients or among the excipients that affect the stability and targeted profile of the respective dosage form is studied and the compounds that cause these effects are suboptimal and deemed incompatible. Establishing critical process parameters and applying these as characteristic features to perform continuous process verification to maintain overall product quality for a targeted lifecycle are the key objectives of incompatibility studies.

Physical incompatibility is a condition of undesirable change in the physical properties of the drug molecule such as solubility, ionisation etc. The outcomes of these interactions may include changes in colour, odour, appearance, polymorphic state and crystallisation. This affects the elegance of the final dosage form and sometimes may precipitate product recall. Chemical incompatibility includes direct reaction between the drug and excipients affecting the functionality of the API. The active chemical groups of the candidate are affected thereby minimising the therapeutic efficiency. Sometimes, these reactions may favour such changes in pH and water content of the dosage forms as to promote microbial growth (Chada 2014).

Napthoquinones are one of the developmental antimicrobials and anticancer active moieties. In the research of developing Lapachol (a naphthoquinone) into a topical dosage form, DSC has been employed to discriminate incompatible excipients. A disappearance of characteristic peaks of Lapachol when treated with excipients under higher temperature conditions has indicated the incompatibility (Lira *et al.,* 2007).

Harding *et al.,* (2008) proposed microthermal analysis with photothermal micro-spectroscopy as a comprehensive tool for compatibility assessment. In their research, interactions between magnesium stearate and acetylsalicylic acid have been evaluated by this technique. A series of localised thermomechanical analysis localised differential thermal analysis, nanosampling and thermally-assisted particle manipulation in this strategy, allowed the study of interaction between the samples at microscale. Hot-stage microscopy associated with IR spectroscopy has complimented these findings at individual particle levels.

Another adoption of IR spectroscopic studies with chemometric analysis (such as principal component analysis and cluster analysis) has discriminated some incompatibilities of baclofen with chosen excipients. A rotational strategy of principal component analysis with 'Varimax' normalisation and hierarchical agglomeration in cluster analysis provided interpretable results for such an assessment by IR spectroscopic studies (Wesolowski *et al.,* 2013).

Chromatographic techniques such as high-performance liquid chromatography (HPLC) serves as robust tool in non-thermal characterization. HPLC and related chromatographic studies aid the evaluation of synergism between the drug and excipients under humid conditions. This mode is generally not feasible with thermal methods. However, an agonistic tool along with chromatographic technique will provide a more detailed information about the chemical interaction (Liltorp *et al.,* 2011).

Identification and isolation of by-products during the compatibility studies along with the nature of interaction, provides an efficient level of information to

stabilise the formulation. During developmental stages, these studies can inform future treatment methods of dosage form design under anticipated stress conditions. Analytical techniques such as liquid chromatography associated with mass spectroscopy and nuclear magnetic resonance spectroscopy are very helpful in tracing these by-products at nano scale (Dousa *et al.,* 2011).

Laxmi *et al*., (2013) developed antipsoriatic topical gel formulation using natural excipients. Purity of the API was assessed using TLC, later the interactions between API and the selected excipients were studied by comparing the initial chromatograms from the API assay with those of excipient combinations. Along with this, viscometry was also implemented to achieve targeted combinations of API and excipients for an effective treatment.

Lima *et al*., (2018) used DTA to investigate the incompatibilities for finasteride towards topical release. It was explained that, DSC served as a rapid analysis in screening unfriendly excipients. The TGA reports described the degradation profile of individual components in ternary mixture. However, this technique cannot explain the long terms effects of storage under accelerated conditions of humidity. Therefore, stability indicating HPLC method was developed further to evaluate chemical changes would provide stronger information about stability and compatibility.

Kumar and Goindi (2021) developed itraconazole loaded liposomes for topical applications. A full factorial design was implemented to study the relationship between formulation components. A design space was constructed based on the QTPP of the formulation and the performance of formulation components was confirmed through a series of physical analytical techniques such as particle size distribution analysis, x-ray diffraction, rheology and texture analysis. HPLC was used to study the drug release profile and assay. The information determined from the techniques above was correlated to confirm the targeted dosage form combinations. For example, large sized vesicles in the formulation exhibited poor rheology which in turn produced irregular drug release profiles.

V. Agarwal *et al*., (2021) efinaconazole microemulsions to treat onychomycosis. As described in the earlier Chapter 2.3, particle size distribution is a crucial parameter indicating the stability of emulsion. In their research, zetasizer was used along with transmission electron microscopy to evaluate particle size distribution of microemulsions developed. Physical techniques such as viscometry, pH and morphological studies were implemented to scrutinize the excipients. Formulation thus developed using the information from the combination of analytical studies was proven stable for 6 months

Preliminary testing for most of the compatibility assessments include combinations of API with excipient at 1:1 (w/w). Then cumulative mixtures of following ingredients are assessed stepwise in subsequent runs. The information derived from this type of assessment is very particular to the most recent respective excipient only. In a dosage form, a combination of multiple excipients with API is applied to meet the QTPP. Hence, a cumulative information of excipients with API is more appropriate. Some of the individually proven compatible excipients have been scrutinized in cumulative testing due to mutual interactions. Therefore, based on the QTPP and process parameters to be employed in the final dosage form production a series of analytical examinations have to be designed to scrutinize incompatibilities and stabilize quality (Mora *et al.,* 2006).

Considering the above literature with proven applications of combinations of analytical methodologies, a series of testing techniques have been implemented in this research to scrutinise the incompatibilities and achieve the desired formulation. To ensure traceability of quality, excipients manufactured by established suppliers were used in this research and thereby the time required to characterise them individually was also minimised.

2.7 Drug product characterisation towards QTPP

Coalescence, creaming, cracking and Ostwald's ripening are the major problems encountered in stability of emulsions. Coalescence includes segregation of droplets of the dispersed phase into one continuous droplet. This results in separation of phases. An inappropriate selection of surfactant or changes in environmental factors such as pH or temperature can result in separation of phases with differences in opacity. This phenomenon is termed as creaming, and it is an outcome of the particle size profile. In Ostwald's ripening, smaller droplets formed during the dispersion are consumed by larger sized. This results in nucleation of dispersed phase causing instability. Although creaming is reversible, it impacts the quality profile of the dosage form. From the above principles of emulsions, it has been understood that surfactants reduce the interfacial tension by dispersal of the insoluble phase into the continuous medium. Upon dispersion, the intensive property that affects the majority of emulsion attributes is particle size distribution (Roland *et al.,* 2003). Therefore, emulsion instability can be picturised as gravitational separation of the droplets based on size. Stoke's law of sedimentation can explain the above-mentioned negative effects. The rate of settling or velocity of a particle moving in a fluid is directly proportional to its radius (Equation 2.11).

$$F_d = 6\pi\eta r v \qquad \textit{Equation (2.11)}$$

Where F_d (N), is the frictional force experienced by particle of radius r (m), moving in a fluid of viscosity η (p) with a velocity of v (m/s). Therefore, at constant viscous conditions, decrease in globular size of dispersed phase in an emulsion will slow down its velocity to settle down. Thus, it further discourages possible negative effects. For example, a droplet of 0.1 µm moves 100 times slower than 1.0 µm sized. Therefore, a uniform particle size of dispersed medium is the major requisite of a stable emulsion (Mirhosseini *et al.,* 2008).

2.7.1 Significance of particle size distribution

The distribution of particle diameters in a given quantity of sample includes particle sizes around the mean; here the mode (in the terms of particle size distribution) may be a more useful measurand. The average diameter of particles based on the volume

in the sample is called d_v, based on the surface area is d_s, and for particles that sediment, is d_{st} or Stoke's diameter.

Rheological properties of dispersed systems depend on the particle size of the internal phase. Based on the solubiliser used for the emulsification, the diameter of dispersed phase is directed. Solid particles with a coarse size typically used for solubilisation can enhance the hydration capacity of the aqueous phase, resulting in viscous behaviour along with increased shear rate. This is often observed in pipelines in which, the w/o emulsions of moisture and oil supplied are hydrated by the solid particles used for lubrication and blocking the pipes. The rate of hydration is proportional to the size of the particles used (Raman *et al.,* 2016). In semisolid dosage forms containing emulsified systems, rheology of the final product is one of the CQAs for which analysis of particle size distribution (and monitoring it periodically) is a crucial step in dosage form development.

Osborn and Akoh (2004) have evaluated the influence of type of emulsifier used for lipid-based o/w emulsions on oxidative stability. It was determined that, emulsified samples of smaller droplet size were comparatively stable towards oxidation. It was also ascertained that, the emulsifier used was responsible for the particle size of the dispersed phase. In a similar research conducted by Kiokias *et al.,* (2007), a comparison of particle size of oil droplets in the emulsions before and after treatment with oxidative conditions, confirmed the stability of the emulsions. Lipid oxidation also depends on the concentration of unsaturated fatty acids in the sample. As the type and amount of surfactant required will depend on the constitution of an oil phase, the factor of concentration is also related to particle size.

Multiple emulsions are three component distributions, which are indefinitely stable thermodynamically. Physically they are clear and more viscous than macro emulsions. The clear appearance is due to the size of globules dispersed into the system. Usual size of these emulsion droplets would be less than 10 µm whereas it will range from 20 to 100µm in macroemulsions. Hence, determination of the emulsification endpoint based on the particle size profile of dispersed phase is an advantageous method (Ursica *et al.,* 2005).

The CMC is a crucial property of surfactant in stabilising the emulsion for longer periods. Establishing the CMC of novel surfactants is a challenging and time-consuming process. The investigation of particle size distribution by a surfactant can reduce the steps of establishing a CMC. The concentration at which no further reduction of size of the dispersed medium emulsified can be regarded as the CMC. This approach is quicker than trivial surface tension measurements (Mikulcova *et al.,* 2016).

Pickering emulsions are formed by the solid-state particles as surfactants between two immiscible phases, which are quite common in the food industry. The size of dispersed particles and the interaction between the surfactant are crucial factors of stability (Tavernier *et al*., 2016). The particle size distribution of oils in o/w food emulsions affects the consumption rate. Emulsions of lower particle sizes are known to increase the satiety when consumed before a meal, than emulsions with larger particular sizes (Lett *et al.,* 2016).

The opacity of a solution is a result of internalised light scattering over the internal phase droplets. An increase in the diameter of the droplet leads to larger scattering, making the solution more turbid. Therefore, an appropriate average particle size diameter is a crucial quality attribute for the product performance. Stabilisers are added to formulations to maintain the required particle size and monitor stability under stress conditions. The major function of such additives in this respect is to maintain uniformity of the formulation through consistent effort towards particle size stability (Uluata *et al.,* 2016).

The pH of the continuous phase is another crucial factor of emulsion stability. When ionic surfactants are employed for the emulsification, any disturbance in the pH value produces an imbalance between the dipoles and cracking of the emulsion. This event is often observed in milk-related products upon prolonged storage. The influence of pH over the stability of an emulsion can be evaluated by a particle size distribution analysis. Depending on the targeted site of application, desired pH of emulsion is designed in the formulation. Sometimes, it is compensated by the addition of buffers. Studying the changes in particle size of emulsion droplets with

variance in pH can produce reliable results for optimal perfomance (Romero *et al.,* 2009).

As discussed earlier, HLB of surfactant is a critical attribute towards long-term stability of emulsion. During the development of ANDA, the establishment of HLB of novel ingredient is a convoluted process. On the other hand, stable emulsions of such new compounds can be designed by investigating particle size distribution under the influence of a blend of surfactants. These studies can be accelerated by concentrating on the blends which produce a desired droplet size at lower concentrations, as a wide range of emulsifiers are available in modern society. Augmenting these studies with turbidity measurements can produce efficient and reliable results (Orafidiya and Oladimeji, 2002). Similarly, HLB values of novel surfactants can be achieved by comparing the droplet size distribution developed by them with compendial surfactants (Castel *et al.,* 2016). Bai *et al.,* (2017) have discriminated the emulsifying properties of polysaccharide emulsifiers such as gum Arabic, beet pectin and corn fibre gum based on the particle size distribution developed by these candidates over same range of concentrations of internal phase.

Mengual *et al.,* (1999) have studied the stability of emulsions in terms of creaming, cracking, segregation and sedimentation using by then novel instrument Tubiscan MA 1000. It analyses the particle size in a dispersion by measuring the scattering of photons of light when incident on it. However, with advancements in technology, several novel analytical tools are now available in modern science to determine the crucial nature of the particle size distribution of a dispersed phase and evaluate related parameters.

Triglycerides are the most common chemical forms of oils/fats. They are the esters of three fatty acids (identical or different) with hydroxyl groups of one glycerine molecule. Due to environmental changes or induced stress conditions these esters break down into free fatty acids. Thus, formed individual acids, pose a threat to those emulsified oils regarding stability. Free fatty acids promote oxidation of emulsified oils leading to disruption of stability. Analysing the particle size of dispersed medium in a periodic basis can explain the oxidative stability and shelf life of such emulsions (Waraho *et al.,* 2011).

Ionic strength is the total concentration of ions or polar compounds available in a given aqueous solution. In emulsions formed by ionic surfactants, a presence of polyvalent ions can cause deleterious effect by destabilising the interfacial film formed by the surfactant. Effect of such ionic entrants from environmental contamination or adulteration can be evaluated by the particle size distribution analysis (Shao *et al.,* 2017).

Lipids are insoluble components which require emulsification for aqueous related bioprocesses. In the human digestive system, bile salts and pigments from the liver catalyse the digestion process by emulsifying the insoluble food materials into the aqueous medium. From chemical kinetics, it can be derived that catalysis is a surface phenomenon and its rate increases with an increase in surface area. This hypothesis has been experimentally proven by Trujello *et al.,* (2017). Oil samples have been emulsified with beta carotenes and resultant droplet size distribution has been evaluated. The Droplets of smaller size have been digested well in simulated gastric conditions, compared to larger-sized droplets.

Considering these effects over the critical quality attributes of emulsification, a determination of particle size and its distribution has been employed in this research to characterise onychomycotic applications developed with MCO. Thus, established quality attributes have been further utilised in the determination of stability of MCO onychomycotic application samples exposed for stress conditions.

2.7.2 Texture analysis (TA)

Texture profile analysis is mechanical testing of physical attributes such as hardness, elasto-plasticity, thickness, consistency etc. It involves introduction of a probe (usually a solid) into the material of interest to determine those properties as a function of force and time. In the development of topical dosage forms, texture properties such as ability of extrusion from the container, stickiness, or adhesiveness and spreadability on the applied area, are some of the crucial factors in terms of quality and clinical efficacy (Jones *et al.,* 1996).

Surfactants reduce the interfacial tension between two immiscible liquids to improve solubility. Sometimes a blend of surfactants is required to achieve the

required HLB. Selecting surfactants for such combinations require extensive calculation and pre-formulation assessment to identify any potential incompatibilities. Upon finalising a respective mixture, rheological studies provide viscosity of the system, tensiometry analyses the changes in surface tension. These two investigations provide inter-particular intimacies. The strength, dispersibility and consistency can only be evaluated through TA. In these studies, introduction of a suitable probe and evaluating its journey of intrusion and extrusion from the emulsion details the extent of stability achieved by the respective surfactant (Simovic *et al.,* 1999).

Periodontal diseases are majorly due to microbial invasion of acid-forming bacteria on the surface of teeth. These pathogens adhere to the enamel and form a biofilm as a non-invasive defence mechanism to proliferate their life-cycle. Generic modes of treatment involve toothpastes, mouthwashes, systemic antibiotics and surgery in severe conditions. Oral gels adhere to teeth for a targeted period of time under selected conditions to perform intended actions. Incorporation of potent antibiotics towards periodontal pathogens into these gels can mediate effective mode of treatment. However, gel behaviour is crucial factor in these applications, as the oral cavity is hydrated by the saliva and mucilage. Chances of draining by these oral fluids are high. Hence, to investigate drug product stickiness, cohesiveness, adhesiveness and drug release profile under simulated conditions, TA can be employed for most-relevant information (Jones *et al.,* 2000).

In contrast to trivial rheological measurements, texture analysis studies the behaviour of a sample on the applied area. In the former methods, intrinsic behaviour of sample under the induced stress conditions is determined. TA correlates the *in vitro* physical assessment to the conditions *in vivo*, whereas rheology studies internal changes of the materials under simulated *in vivo* conditions *in vitro*. TA is related towards the analysis of physical attributes of samples affecting QTPP of this dosage form or therapeutic ointment, whereas rheology is a CQA identification. Emulgels are one of the emerging topical applications formed by hydrogels incorporated with emulsified systems. Rheological analysis of these products establishes the cross-linking of chemical moieties responsible for gel formation. Whereas, texture analysis

identifies the physical attributes resultant of these cross-linking and emulsified components (Gwartney *et al.,* 2004).

Cosmetics and pharmaceutical emulsions stored in wide-opening container-closure systems encounter severe effects from the environment. It is required to estimate the shelf-life of such formulations before and after opening. The common adverse reaction observed among such cosmetic emulsions is cracking and grittiness due to evaporation of external phase and a disturbance in phase-volume ratios. Thermal methods such as DSC and TGA provide basic information about changes in formulation for a short period. Moreover, these techniques are performed under nitrogen purging for safety reasons, which is a different environment than real exposure. TA of such stressed samples can evaluate the shelf-life more effectively. The force employed by the instrument to penetrate and extrude from the formulation vary with changes in its chemical composition. Spectroscopic analysis such as FTIR or Raman on such samples can identify the respective chemical species responsible for the change (Masmoudi *et al.,* 2005).

In the research conducted by Liu *et al.,* (2006) towards the development of low-fat mayonnaise by incorporating fat mimetics to substitute the high calorie components, the effectiveness of these replacements was decided based on the TA performed on the final products. In support of these assessments, rheology and sensory analysis were also conducted. Rheological studies described the changes in the intraphasial interactions due to addition of mimetics, whereas TA has explained the interphasial interactions. Sensory analysis evaluated the organoleptic properties of modified recipes.

In general, hydrophilic polymers that swell for a respective period and then dissolve, achieve this, based on the design of the modified release formulation. Drug release profile from such dosage forms is studied using dissolution apparatus of compendial methods. However, these experiments cannot explain the physical changes in dosage forms inside the simulated dissolution medium, as only the drug released is quantified. TA can overcome this limitation, by studying the physical changes of a dosage form in the medium and simultaneously recording the drug release profiles. For example, TA instrumentation enables fixation of a tablet in the

medium with a suitable probe. The changes in compositional matrix of a tablet can be investigated by changes observed in the force area over time (Li and Gu, 2007).

Emulsions are gaining higher attention in the market due to increasingly prolonged stability. The opportunity of incorporating numerous components into one matrix by managing the HLB through the internal phases opens the window to multiple applications. Nevertheless, inappropriate combinations or irregularities can diminish the elegance of a product. This principle is of major interest in the pharmaceutical sector to compensate patient compliance. Hence, TA based information is a critical quality report in ascertaining the consumer requirements and maintaining the QTPP (Peng *et al.,* 2010).

Outcome of the texture, elegance and performance of a product are a resultant of cumulative behaviours of individual compounds. In topical applications componential behaviour influences the final texture of the dosage form. For example, stearic acid is a most common thickening and emulsifying agent in topical products. Higher concentrations of stearic acid may yield grittiness and crystalline texture of the product, affecting the spreadability and extrusion from the container. Those individual attributes affected by the changes in the components can be evaluated effectively through TA (Youssef and Barbut, 2009).

Ministration of wounds with ointments by repeated application over prolonged periods is a trivial and primitive treatment mode. More recently, novel strategies define the applications of hydrogels as an effective method of wound healing, as they provide a more enhanced contact period than generic topical antimicrobials. The cohesiveness, adhesiveness, consistency and spreadability of such antimicrobial hydrogels intended for prolonged treatments, can be evaluated effectively through TA. Rheological studies can demonstrate viscous flow of these gels, whereas TA can provide real-time testing information of these products under reflective *in vivo* conditions. Feasibility of implementing biological conditions *in vitro* is more accessible through TA than rheology, as the equipment and tools assisting the study in the former are comparatively simple (Hurler *et al.,* 2011). Similarly, TA can evaluate the physical attributes of nano sponges formed by an emulsion diffusion process. These complexes are effective transporters of lipophilic materials across

hydrophilic barriers such as skin. TA can investigate diffusivity of sponges through skin by evaluating their penetration capacity, strength and cohesiveness (Sharma and Pathak, 2011).

Swelling studies are crucial to characterise novel hydrogel composites. One trivial method is gravimetric, which includes measuring the weight of hydrogel over respective periods after exposure to the water or related buffer. This is both a time and solvent consuming process to establish swelling behaviour of the gels. More relevantly TA serves as an effective method to characterise swelling behaviour by measuring the force required to break the gel structure or permeate the gel. Parameters influencing the swelling behaviour such as pH, temperature and ionic strength can be altered in real-time testing (Lamberti *et al.,* 2013). Bhatia *et al.,* (2013), have developed tamoxifen-loaded lecithin organogels for topical applications wherein TA evaluated the penetration behaviour and adherence of these gels on clinically relevant skin conditions. Similar analysis was conducted by Feng He *et al.,* (2021) to determine the controlled release properties of yak-caesin cold-set gels.

On reviewing such enhanced and beneficiary information provided by TA on the physical characteristics of pharmaceutical products, it has been employed in this study to interrogate and illustrate critical physical attributes of MCO onychomycotic applications.

2.7.3 Role of turbidity in emulsion stability

Dispersion of an insoluble phase into a continuous phase with the aid of emulsifying agents generates cloudiness due to changes in interfacial tension and the formation of droplets. Light scattering through these systems varies with size and dispersion of the internal phase droplets. When exposed to monochromatic light, these dispersions absorb light. The extent of absorption depends on the concentration and wavelength of the light used. Turbidity is a measurement of opaqueness of such dispersions by analysing the amount of light that has passed through the sample. As discussed in section 2.8, the undesirable effects of emulsions such as cracking, creaming, Ostwald ripening and coalescence are resultant of improper interaction between the phases of emulsions or imposition of environmental stress. The

common outcome in all these effects is altered particle size and consistency of the emulsions, which are factors accessible through turbidity (Robins *et al.,* 2002).

Oily wastewater generated in metal and petrol refineries requires pre-treatment to separate toxic oil before being discarding into the environment. A general strategy employed by industries is incorporation of salts such as calcium chloride and/or aluminium chloride into the wastewater. These compounds reduce the interfacial tension between the oil and water of the emulsified wastes and separate them. Examination of changes in particle size diameter and measuring zeta potential are trivial methods to determine the extent of de-emulsifying by the salts. However, turbidity measurements of these treated oily wastewater emulsions periodically simplify the process to study de-emulsification. These studies are comparatively more economical than other alternatives.

Binks and Lumsdon (1991) have studied the stability of o/w emulsions formed by silica gel particles in the presence of various electrolytes. Turbidity measurements performed for emulsion samples in the presence of electrolytes dictated the stability. Reducing the interfacial tension between internal and external phases of the emulsion by the electrolyte resulted in increases in globular size of droplets and decreases in turbidity. Song *et al.,* (2000), constructed calibration curves for changes in turbidity of emulsions stabilised by Tween – 80 surfactants. The slope of curve decreased with increase in creaming of emulsion. Therefore, stability of emulsions is inversely proportional to the turbidity ratio.

Similarly, in the development of surfactants by reacting organic acids with alcohols followed by dehydration, Manabe *et al.,* (2001) have employed turbidity measurements for the endpoint detection. Reaction has been carried out by emulsifying insoluble fatty acids into an aqueous medium containing alcohol. The change in particle size distribution affecting the turbidity of medium is the key principle of the mechanism. Reduction in emulsion turbidity was considered as an indicator of reaction progress, as the reacted acid was converted into an aqueous-soluble surfactant.

Degassing water removes dissolved gaseous components and improves the polarity of water. Oil can be dispersed into such water by the aid of catalytic components such as Teflon. Emulsification of oil into degassed water is mediated through reduction in hydrophobicity of oil by forming intermolecular hydrogen bonding. Turbidimetry serves as a rapid and real-time, analytical platform to study this emulsification. Thus, formed dispersions are stable and free from additives such as surfactants, stabilisers etc. Catalytic agents employed for the emulsification can be removed once an endpoint is determined through turbidimetry. Such emulsions are stable even after the degassed components are introduced, due to strong intermolecular interactions (Pashley, 2003).

Turbidity of an emulsion is an optical illustration of particle size distribution. Pickering emulsions formed by solid materials have a polynomial distribution of particle sizes due to the presence of solid solubilizers along with the micelles of dispersions. A general particle size distribution cannot differentiate these individual components, but turbidity measurements can discriminate based on the settling rate of solid particles in the emulsion. Optical microscopy, augmented with turbidity measurements, help rapid investigation of emulsions, more so than particle size analysis alone (Sacanna *et al.,* 2007).

In development of emulsions with lemon oil using sucrose monopalmitate as surfactant for oral administration, Rao and McClements (2011) have employed various methods of preparation. The effect of heat, variations in homogenisation, phase volume ratio and CMC have been explored in these studies. Particle size distribution and rheology have been employed as tools to evaluate these effects. Results from these measurements have been supported by turbidity values. Coalescence and creaming of the emulsions were determined by an integrated understanding between the results of particle size and turbidity determinations.

Therefore, turbidimetry determines the stability of emulsions by analysing the creaming or coalescence of a dispersed phase by the light scattering behaviour. Hence, it was employed in this research to examine the stability of MCO onychomycotic applications.

2.8 Stability of dosage forms

Identification of any relevant threats and recognition of the need for continuous improvement of a process or cycle of the product according to the information available from CQAs are the basis of efforts to minimise those risks towards an intended QTPP encapsulated within the principle of ICH Quality guidelines.

A risk is "*a combination of the probability of occurrence of harm and the severity of that harm*". Quality risk management is "*a systemic approach to identify, control, communication and review of risks to the quality of the drug product across the product life-cycle*". These principles of risk management are applicable for API, excipients and final drug products. In the pharmaceutical sector, the two major principles of risk management are: (i) evaluation of risk towards quality should be targeted considering the final effect on patient and (ii) that the level of effort, formality and documentation of a quality risk management process should be commensurate to the level of risk. The ideal phases of risk management process are risk assessment, identification, analysis, evaluation, control, communication and review (ICH Q9, 2005).

Humidity, temperature and packaging systems of a drug or its formulation are the physical factors of degradation upon prolonged storage. Biological factors include microbial contamination and water activity. Interactions between the components of the formulation, particle size and shape, pH of the formulation and nature of the solvent present are the chemical factors. In pharmaceutical terms, stability is often referred to as the capacity of a dosage form to withstand these external factors. "The shelf-life is the minimum amount of time taken by the formulation or drug to degrade into toxic components" (ICH 2003). A principal reason to evaluate stability of a dosage form during developmental stages is to assure consistent quality along the intended shelf-life. Apart from degradation of product into toxic compounds, 85% of cases report reduction in product performance due to inadequate stability assessment (Bajaj *et al*., 2012). Therefore, it is necessary to evaluate these phenomena of degradation to ascertain stability of the formulation. Application of statistical tools

and programmed studies under induced stress conditions inform the interaction of the above factors with the formulation (Bajaj *et al*., 2012).

The number of degradants in a post-stressed formulation are categorised into three major classes: reporting, identification and qualification depending on the total daily intake (See Table 2.4) (ICH 2003). Reporting level degradants have a higher impact on the key performance attributes of dosage form such as strength. Identification and qualification levels constitute a further ringfencing of the QOS of the dosage form.

Table 2. 4|**Total amount of degradants allowed for new drug products according to regulatory guideline ICH Q1A**[13]

Type of threshold	Maximum daily dose	Threshold (% of TDI)
Reporting	≤1 g	0.05 to 0.1
Identification	<1 mg	0.1 or 5 µg
	1 – 10 mg	0.5 or 20 µg
	10 mg – 2 g	0.2 or 2 mg
	>2 g	0.1
Qualification	<10 mg	1 or 50 µg
	10 – 100 mg	0.5 or 200 µg
	100 mg – 2 g	0.2 or 3 mg
	>2 g	0.15

Presence of any of the above-mentioned degradants in the product require toxicological justification to substantiate the regulatory status of the product. Change in lower values for high numbered product is very hard to trace. Therefore, sophisticated tools are required to determine these types of degradation at minute concentrations. Any changes observed during clinical trials may recycle the process for additional work. Similarly, during the product development, degradation predicted from theoretical principles is insufficient to substantiate its approval for the market. Hence, a strategic and practical investigation of stability is required to assure quality of the product (Waterman and Adami, 2005).

[13] International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Stability Testing (2003) ''New drug substances and Products''. Available at: https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1B/Step4/Q1A_Guideline.pdf

Table 2.5| **Some possible degradations determined post-stressed conditions**(Carstensen, 2000; Bajaj *et al*., 2012)

Effect	**Reason**	**Example**	**Parameter tested**
Reduction in API performance	Degradation leading to 90% less than label claim	Nitroglyceride tablets	Time elapsed before the drug content no longer exceeds
Microbial instability	Inappropriate packaging system design	Multiuse creams and lotions	Total bioburden after storage
Container-closure system failure	Undesirable effects on storage	Development of cap-seals and rancidity of rubber materials	Container-closure compatibility testing
Modifications in physical performances	Changes in particle size, pH, adherent properties (topical) etc.	Loss of texture integrity, decreased adherence on targeted site of action	Physical assessment of dosage forms post-stressed

2.8.1 Conduct and application of stability studies for topical and related formulations

Bakshi *et al.,* (2001) employed a prescription of ICH guidelines to evaluate the stability of ornidazole under acidic, alkaline, oxidative and photolytic conditions. HPLC was used to separate and quantify degradants during these studies. The procedure developed during chromatographic analysis was validated as per ICH Q2_R1. It has been reported by the authors that, simulating the stress conditions and further validations of the analytical techniques as per ICH suggestions have detailed the drug degradation more precisely than any other non-compendial procedures.

Liposomes are one of the developmental chemical moieties deploying surfactant technology to improve solubility of lipophilic drugs into aqueous based media. In research conducted by Manosroi *et al.,* (2004) topical, liposomal-based amphotericin B formulations were developed and the physical, chemical and biological stability of these formulations under induced stressed conditions of varied temperatures over a period of 90 days was examined. HPLC was employed as the analytical technique to study the composition of the stressed samples. Formulations stored under 30^{o}C were determined to be stable based on visual inspection and degradant quantification through RPLC.

Niosomes are structural analogues of liposomes with ability of enhancing skin permeability of poorly soluble and low-bioavailability drug compounds. Formulations with niosomes for topical treatment is gaining attention. Balakrishnan *et al.,* (2009), have developed niosomal composites of minoxidil for the treatment of androgenetic alopecia. These combinations have been stored under refrigerated conditions to establish the stability profile. Particle size analysis, visual characterization and *in vitro* drug analysis have been employed to investigate the stability of these niosomal formulations.

The ongoing quest for novel components for cosmetic applications is now increasingly concentrated on natural derivatives. Daudt *et al.,* (2015) have investigated the feasibility of polysaccharides of *Pinhao* extracts as cosmetic excipients. Stability data of these components under thermal stress in terms of pH,

viscosity, spreadability, rheology and potential toxic compounds such as phenolics, has been provided to meet the requisites of regulatory standards and potential customers. Normal pilot scale experimental data cannot suffice these requirements due to nature of short-term studies.

Drug degradation studies are one of the important quality control examinations to establish storage conditions as per the guidelines of US FDA and ICH guidelines. Doxorubicin, a natural anthracycline used in the chemotherapy of tumour treatment, produces four types of degradants under stressed conditions. Under alkaline conditions it is highly unstable at room temperature and degrades at 80^{o}C in acidic environment. This information determined from the induced thermal and environmental conditions during such stability evaluations is crucial to determine the type of container-closure system required for a targeted climatic region (Kaushik and Bansal, 2015).

Topical formulations contain numerous excipients to meet the QTPP of an intended treatment. Applying design of experiments in developmental stages is a time-consuming process. However, these methodologies can scrutinise the variables to be examined during stability assessment and minimise the efforts (Sonawane and Gide, 2012). Topical administration is one of the effective treatment modes in ocular therapy. The major disadvantage in this route is short contact period with the cornea, due to continuous secretions from lachrymal glands. Novel strategies to minimise this effect include bio-adhesives that will not obstruct the vision and facilitate prolonged contact periods. However, along with product performance assessment under simulated conditions, stability examination at equivalent RH conditions for the eye's corneal region provides detailed, patient-relevant information about quality (Ibrahim *et al*., 2016).

Crude extracts of *Scutia buxifolia* have been reported to possess topical anti-inflammatory properties in the herbal treatment of ear oedema. In a study of developing these extracts into a gel formulation, stability assessments have been employed to ascertain performance. Viscosity, pH measurements and quantification of active components by HPLC of the stressed samples evaluated the long-term drug-excipient and intra-excipient compatibilities (Boligon *et al*., 2017).

2.8.2 Investigating stability mechanisms

Compliance model is one of the common modes of evaluating shelf-life of a dosage form, in which samples at various time points during stress conditions are collected and analysed. The point at which an out-of-specification potency of the active components or of performance of component is observed, is established as the shelf-life. A single set of data based on the period is a disadvantage, limiting the applications of this method (Egan and Schofield, 2009). Other alternative modes include regression analysis of data generated over respective period of time. Based on the chemical nature of the active component, zero or first order rate of kinetics are applied to predict shelf-life of the dosage forms. The combined effect of relative humidity (RH) and temperature on the degradation mechanism can be predicted by the application of modified Arrhenius equation (Equation 12) (Simon *et al*., 2004).

$$\ln k = \ln A - \frac{E_a}{RT} B(RH) \qquad \textit{Equation (2.12)}$$

Here, k = degradation rate, A = Arrhenius collision energy, E_a = activation energy, R = universal gas constant, T = temperature, B = humidity constant and RH = relative humidity.

Along with the above-mentioned factors, photolysis and photo-oxidation are the undesired effects observed in light sensitive formulations. Molecules ionise by the absorption of light and degrade into unwanted or sometimes toxic compounds. A presence of oxygen aggravates the degradation process due to strong affinity towards electrons. Addition of antioxidants and light-absorbing chelating compounds of citrates are the general practices in stabilising these light sensitive formulations. However, detailed information regarding the mechanism and resistance offered by any vulnerable formulations is required before commercialising the product.

In a solution, the solutes or dispersions move with kinetic energy, which is a result of work done to stabilise these systems. Upon being subjected to stress, molecules alter their movement according to shifts in the equilibrium. This change is not uniform as the type and concentration of the respective moieties alter for a given formulation. Therefore, steady-state kinetics is difficult to derive in such a complex mixture. An isoconversion model can predict the stability of these matrices by

keeping the degradation product/potency constant at the specification limit as time varies. Under historical modes of assessment, the degradant(s) formed during the time frame points is/are quantified and extrapolation of these values with time is used to decide the shelf-life. The isoconversion model produces a straight line when compared with historical or conventional methods, which is usually curved due to discrete changes in degradant with time (Calvino *et al*., 2021).

Methods and limits to determine stability of a developmental dosage form have not been established in any compendial literature. It can be achieved through the examination of CQA towards the QTPP in modern QbD pharmaceutics. A series of such analytical procedures to investigate the stability of dosage form as per pre-determined attributes is often collectively termed a stability-indicating method (SIM). The variables to be considered and their respective outcomes during stress conditions can be anticipated during the development of dosage form and can be implemented further to during stability studies (Blessy *et al*., 2014).

Other modes of analysis include ``stability-specific method'' in relation to the API analysis, ``selective stability-indicating method'' which examines stability of formulation with respect to one type of stress condition such as thermal or relative humidity and ``specific stability-indicating method'' that determines resistance of targeted excipient towards the stress condition (Bakshi and Singh, 2002). In any of the above methods, analytical procedures developed to investigate new drug application (NDA) or (INDA) or investigational new drug (IND) have to be validated as per the guidelines of ICH Q2_R1[14] (1996) (Kaufman *et al*., 2013).

[14] ICH Q2_R1 (1996), ``Validation of Analytical Procedures: Texts and Methodology'', International Council on Harmonisation of Technical Requirements of Pharmaceuticals for Human Use. Available online at: www.ich.org

2.8.3 Statistical testing of stability data

The application of statistical principles to evaluate the process or product performance under validated, induced, or targeted environmental conditions is termed statistical process control (SPC).

ICH Q1A (R2) dictates the assessment of stability under the induced stress conditions as one of the quality attributes of any formulation design. Based on the climatic zone of the targeted area of distribution and storage conditions, stress parameters shall be considered during the evaluation. The stability of the dosage form should be assessed based on the regression analysis of the data generated from the quality control testing of the stressed samples collected from the time points. The limits obtained from optimsation studies should be used as the acceptance criteria.

In the pharmaceutical sector, this SPC includes application of control charts to evaluate the data obtained during the stability examination of finished product. These control charts also called as Shewhart charts or trend charts include statistically calculated upper and lower limits above and below the mean or average line.

Aim

- The growing concern of antifungal resistance; extensive period of treatment by popular K101 formulations and undesired effects of classical fungal candidates during the therapy have provided a window of opportunity towards effective treatment of onychomycosis using novel alternatives. Considering the antimicrobial performance of MCO it has been targeted in this research to develop a novel molecular entity (NME) for the topical treatment of onychomycosis.

Objectives

1. MCO was proven to be potent towards oral bacterial and yeast pathogens. Therefore, initial investigations have been designed to determine the antimicrobial activity of MCO against *T. rubrum* and *C. albicans.*

2. The targeted region of application for the therapy is an infected nail plate which is highly hydrophilic by nature. Therefore, for maximum therapeutic efficacy, development of a hydrophilic novel molecular entity from MCO with antifungal activity for the treatment of onychomycosis has been targeted.

3. The QTPP for effective treatment of onychomycosis requires the penetrability of AAE to cross the nail plate barrier. Antimicrobial assays has been implemented in this research to evaluate the bio-relevant activity of an onychomycotic application containing the NME in the presence of keratin infected by *T. rubrum*.

4. As MCO is a novel antifungal entity, it is required to establish CQAs to identify it in the formulations and discriminate it from its raw materials. Using various analytical techniques experiments have been designed to develop analytical methods in order to analyse the functional performance and assure the quality of the developed dosage form.

5. To develop the dosage form as per the guidelines of ICH Q8 (Quality by Design) so as to position it to submit a common technical document (CTD), this being an industry recognisable signpost likely to realise suitable licensing opportunities.

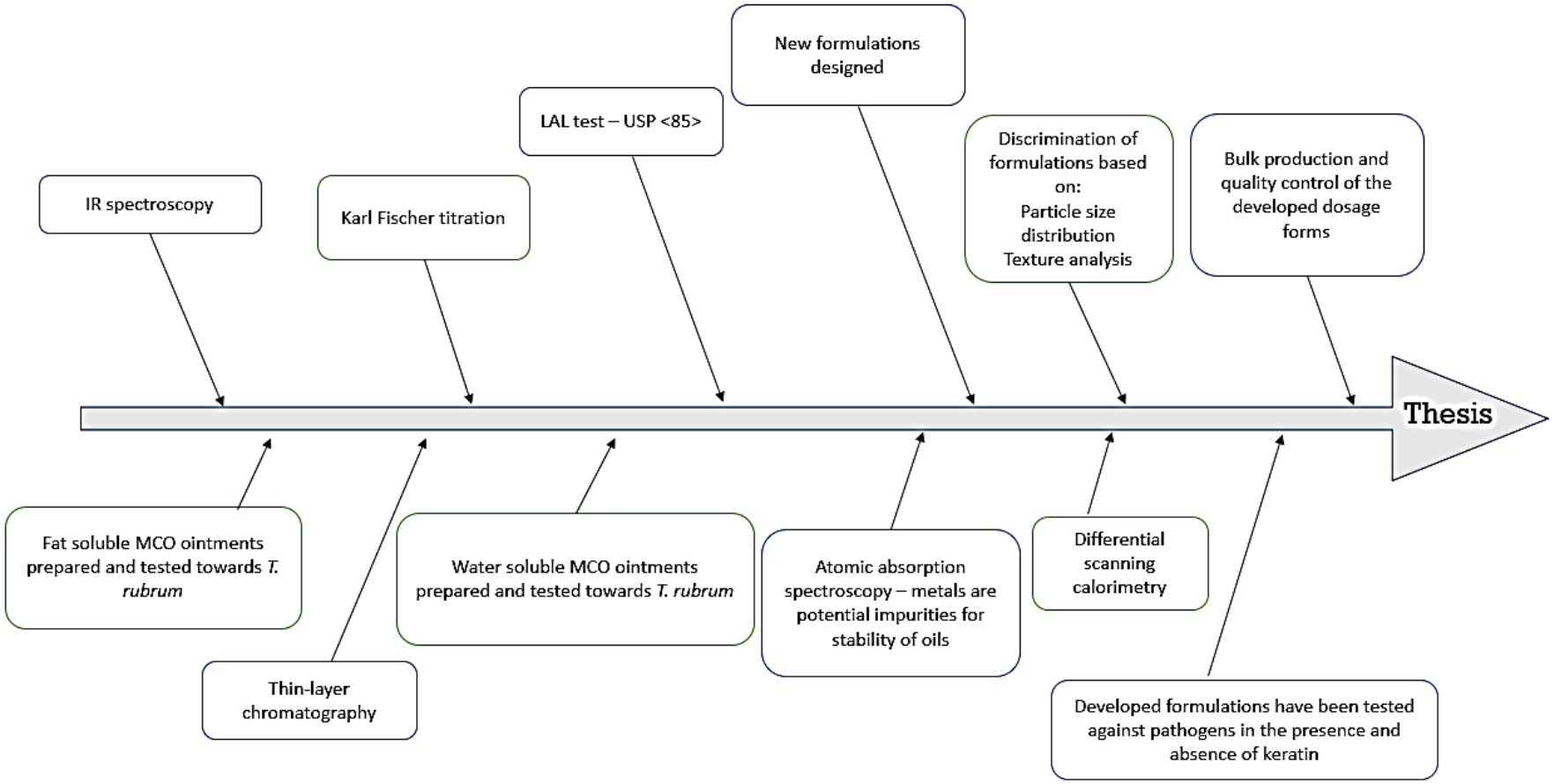


Figure 2.6| **Flow chart explaining the steps performed to achieve desired formulation.**

3.0 Procedures

3.1 Materials

3.1.1 Microbial Cultures

Bacillus subtilis (ATCC 6633), *Candida albicans* (ATCC 10231), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538) and *Trichophyton rubrum* (ATCC 28188) were purchased from Cruinn Diagnostics Ltd., Ireland.

3.1.2 Reagents

Soybean – Casein Digest agar (SCD) (Batch no: 12115-05) and medium, used were bought from Cambio Ltd., England.

Sabouraud dextrose agar (Batch no: 8463.0500) and medium used were purchased from VWR International Ltd., Ireland.

Sterilized Petri plates, tips and inoculating loops were bought from Sarstedt Ltd., Ireland.

Virgin coconut oil (manufactured and distributed by Tiana Fairtrade Organics. Ltd. UK. Batch no: ORG 14197 VCO) was purchased from Holland & Barrett, Athlone, Ireland.

Agar was purchased from VWR International Ltd, with batch no: D/BAM9869-M3 and lot number: 1035.

Naloc™ was purchased from a local pharmacy, it was manufactured and distributed by Mead Health Sales, Ireland.

Petrolatum used for onychomycotic application base was manufactured by Health Point Ltd, Blackpool, which was purchased from Dealz®, Athlone.

Diethanolamine (D8885), triethanolamine (T58300) and monoethanolamine (E9508) were purchased from Sigma Aldrich.

Solvents used: acetone (EC no: 200-662-2, obtained from SciChem, Ireland), acetic acid (ACS grade, Batch no: 00996Dk, from Sigma-Aldrich, Germany), chloroform

(HPLC grade, Lot no: A0344490, from Acros Organics, Ireland), diethyl ether (batch no: 31690, ACS grade, from Sigma-Aldrich, Germany), 2-methoxyethyl ether (Lot no: S21393-224, ACS grade, from Sigma-Aldrich, Germany), methanol (Lot no: SZBB357SV, ACS grade, from Sigma-Aldrich, France), deionised water.

LAL – Gel clot kit was purchased from Charles River Laboratories, Paris, with control standard endotoxin of lot no: EX41362; LAL reagent of lot no: F4501X and reference standard endotoxin of lot no: H0K364. Neat enzyme modified coconut oil (EMCO) sample and 5% w/w EMCO-DEA onychomycotic application (prepared in-house).

Atomic absorption spectrometric standards (1000 ppm) each of Cd, Cu, Ni, Pb and Zn manufactured by VWR were procured from Avantor VWR, Dublin

Hydrolysed keratin (part no:LQ-37) was purchased from Garden of Wisdom, Arizona, United States of America.

Nitric acid (N/2300/PB15) was purchased from Fisher Scientific, Ireland.

Polyethyleneglycol (4000), part no 8.07490 was procured from Sigma – Aldrich, Ireland.

Potassium hydroxide (part no: 221473) and sodium hydroxide (part no:221465) were purchased from Sigma – Aldrich, Ireland.

3.1.3 Equipment

Infrared spectral analysis – Shimadzu®, Affinity - 1; Cat. no: 206-73500-38. Japan.), sodium chloride plates.

Density determination: density-meter (DMA 35 N, density meter from Anton Paar®, Austria – Germany with batch no: 24164).

TA differential scanning calorimeter 2010 (Universal V3.OG TA instrument), TA aluminium hermetic pans (A39710-06), liquid nitrogen.

Acid digester – Mars Orbit microwave digestor kindly supported by Bioclin Research Laboratories (now called as Almac Sciences Ireland).

Atomic absorption spectrometer - Varian AA240FS, serviced and calibrated annually.
Particle size assessment – Malvern Mastersizer 2000, Malvern, PANalytical Products Particular Sciences, Ireland.

Nuclear magnetic resonance spectrometer – Delta 2 NMR spectrometer kindly supported by NUI, Galway for high frequency analysis. Spinsolve Carbon 60 MHz NMR spectrometer kindly supported by Magitrek, Germany.

Thermo gravimetric analyser - Perkin Elmer Pyris 1 TGA Thermogravimetric Analyzer P/N N5370030, kindly supported by Center for Industrial Services and Design, Athlone Institute of Technology.
Texture Analyser – Stable Micro Systems, TA.XT Plus texture analyser, Mason Technology, Dublin.

3.2 Establishment of active concentration of MCO towards *T. rubrum*

The antimicrobial activity of MCO towards *E. Coli, S. mutans, S. pyogenes and C. albicans* has been investigated by Hughes (2015) during the development phases. In order to expand the antimicrobial spectrum of MCO, it was tested towards *T. rubrum* in this research. The methods employed to determine the active concentration of MCO towards *T. rubrum* and developing the respective concentration into dosage form are now discussed in this section.

3.2.1 Reconstitution of *T. rubrum*

Kwikstik® of *T. rubrum* was aseptically inoculated onto sterile Sabouraud dextrose agar plates and these were incubated at 25°C protected from light for a period of 21 days. Ingredients of this agar include: 4% w/v dextrose, 1% w/v peptone, and 2% w/v agar. After the incubation period, a colony was inoculated into each sterile universal containing sterilised Sabouraud dextrose broth and were incubated at 32°C which is equivalent to nail temperature for a period of 21 days for the purpose of testing. The agar plates with established colonies of *T. rubrum* were stored at 4°C protected from light for three months for any further reconstitution or testing.

3.2.2 Investigation of antimicrobial potency of MCO towards *T. rubrum*

The conidia of *T. rubrum* were harvested using 5.0 mL of sterile deionised water to prepare the stock. A one-in-hundred dilution of this stock was prepared and 100 µL of this dilution was spread uniformly on four sterile SDA plates respectively. On these inoculated plates sterile Whatman filter paper grade 40 of twelve millimetres diameter containing 30 µL of MCO was placed. A similar procedure was followed for VCO and Naloc™. The prepared plates were incubated at 32°C and the diameters developed by MCO, VCO and Naloc™ were measured after seven days.

3.3 Identification of critical quality attributes of MCO

In the previous microbiological assay, the activity of MCO was determined superior to a commercial control. Considering these results, the physicochemical properties of MCO that affect the targeted product profile have been investigated using analytical techniques. The methods employed during these studies are now presented in this chapter.

3.3.1 Application of IR spectroscopy as analytical tool to discriminate MCO from VCO

Sodium chloride plates were cleaned with chloroform and dried in a cold current of air. A background scan was performed using these sodium chloride plates and saved. Onto the same plates, a drop of MCO was placed using a sterile Pasteur pipette and the spectrum was recorded from 4000 to 400 cm^{-1}.

A similar procedure was followed throughout this project to determine the IR spectra of VCO and respective MCO onychomycotic application samples. As a part of a systematic control strategy, this method and the peaks determined discriminating MCO from its precursors were used throughout this project to identify MCO in various mixtures with excipients.

3.3.2 Differential scanning calorimetry of MCO to characterise thermal attributes.

After calibrating the instrument using indium as per manufacturer's description, liquid nitrogen was used as coolant in order to reduce the temperature to -25^{o}C for each run. Each MCO sample (hermetically sealed) was run (along with an empty pan as reference) for a ramped heating flow from -25 to 400^{o}C. In order to detect potential incompatibility between the active MCO component(s) and candidate excipients in a timely manner MCO was mixed with each intended excipient cumulatively at equal proportions and the DSC runs were performed under same conditions to those used for neat MCO.

3.3.3 Determination of thermal degradation of MCO.

To evaluate the loss in the mass of MCO with increase in temperature and thereby identify the number of components, thermo-gravimetric analysis was employed in this research. The analysis was carried out from 25 to 600^{o}C under a nitrogen gas purge and the percentage weight loss with increase in the temperature was recorded.

3.3.4 Titrimetric analysis to discriminate MCO from its precursor.

3.3.4.1 Determination of saponification value as per USP <401>.

Into 1000mL volumetric flask 6g of potassium hydroxide was weighed and dissolved in 5mL of water. This was then diluted to mark with absolute alcohol. The solution was allowed to stand for 24 hours before use. The molarity of this solution was standardised using 0.1M HCl before use. The sample of 2.0 gm was placed in a round bottom flask and 25 mL of the alcoholic potassium hydroxide which was previously standardised was added. Anti-bumping beads were included, and the mixture was refluxed for 1 hour with occasional stirring. After the reflux period, to the hot alcoholic mixture, 2 to 3 drops of phenolphthalein indicator were added and titrated against 0.5 M hydrochloric acid until the pink colour of the solution was changed to colourless. A blank value was determined for the alcoholic potassium hydroxide refluxed under the same conditions.

3.3.4.2 Determination of acid number as per USP <401> - Fats and fixed oils.

The method employed for acid number determination of “Fats and Fixed Oil <401>” was adopted to discriminate MCO from VCO by direct titration. 5.61 g of potassium hydroxide was dissolved in 100mL of water and diluted to 1000mL with same solvent to prepare 0.1M KOH. 10.0 g of sample was weighed into conical flask containing 50 mL of equal volumes of ether and alcohol which were previously neutralised with phenolphthalein. To this one mL of phenolphthalein was added and titrated with standardised 0.1 M KOH until a permanent pink colour was obtained in the conical

flask. Same procedure was followed for MCO and VCO samples throughout this project.

3.3.5 NMR Spectroscopy analysis to determine chemical constitution.

NMR spectroscopic analysis was performed at two different frequencies (100 and 60 MHz). The analysis at 100 MHz was kindly supported by National University of Galway, Galway. MCO and VCO samples were dissolved in deuterated chloroform at 1 mg/mL whereas compound A was dissolved in deuterated water. Sample concentration was adjusted as per the requirements accordingly.

Analysis at 60 MHz was kindly supported by Magritek, Germany, 79.6 mg/mL of MCO was prepared in deuterated chloroform and used for the analysis. The compound A was analysed at 11 mg/mL in deuterated water.

3.3.6. LAL Pyrogen test of MCO to evaluate protein contamination.

MCO and 5% w/w MCO-DEA (prepared as mentioned in Section 2.3.2) were tested in this experiment by the LAL gel clot technique. LAL reagent water is the medium in which the sample has to be dispersed to perform the test as it is free from endotoxin as per the certificate of analysis provided by the manufacturer. As MCO was insoluble in LAL reagent water, washings of it prepared by vortex of 0.5 mL MCO with 5 mL of LAL water for 30 seconds (for 5 times with an interval of 30 seconds between each vortex) were used in the test (Arambašić. 2009). Similarly, to maintain consistency of concentration, MCO-DEA onychomycotic application was diluted at a 1:10 ratio with LAL reagent water for the test.

As per the description of the manufacturer, the provided control standard endotoxin (CSE) was reconstituted with LAL reagent water, producing a concentration of 80 endotoxin units per millilitre (EU/mL), referred as 2λ.

Sample of 100 µL was placed in each of 4 pyrogen-free test tubes provided by the manufacturer with the kit. Reconstituted CSE of 100 µL was added to the sample in

the first test tube (2λ) and subjected to vortex mixing for 15 seconds. From this mixture 100 µL was taken and added to the next test tube (with sample) to produce λ concentration of endotoxin. Serial dilutions in duplicate were performed in the same manner until 0.25λ concentration of endotoxin was obtained in the remaining test tubes with sample. LAL reagent was reconstituted with LAL reagent water as per the label claim of manufacturer. The sample and endotoxin mixture of 100 µL were transferred into a test vial provided by the manufacturer and 100 µL of reconstituted LAL reagent was added. This mixture was then incubated at 37 ± 1^{o}C for one hour. After this incubation period, each test vial was inverted 180^{o} to observe the gel. The same procedure was repeated for neat MCO and MCO-DEA onychomycotic application samples in duplicate for every dilution of endotoxin concentration.

3.3.7 Determination of trace metals by atomic absorption spectrometry

The method employed to determine traceable metals in MCO was adopted from USP 40 – NF 35, <401>, Fats and Fixed Oils chapter. 0.5 g of sample was mixed with 6.0 mL of trace metal free nitric acid and 4.0 mL of hydrochloric acid in a cleaned polytetrafluoroethylene flask and sealed as per the manufacturer's procedure. The contents of this flask were digested in a microwave at 80% of power for 15 minutes, 100% of power for five minutes and 80% power for 20 minutes[15]. Digested samples were allowed to cool to room temperature and 4.0 mL of sulphuric acid was added and the same digestion process was repeated. After cooling, the contents of the flasks were transferred carefully to 50 mL volumetric flasks and 1.0 mL each of 10mg/mL magnesium nitrate and 100 mg/mL of ammonium dihydrogen phosphate were added and diluted to the mark with deionised water. Similar procedure was repeated with MCO, VCO and blank (without any sample) and labelled respectively for the analysis.

Standard solutions of Cu, Pb, Ni, Fe, Cd and Zn were prepared from the respective certified standards of 1000 ppm each. Initially a mixed standard stock of 10 ppm was prepared for each element from which 5, 10, 20, 40 and 80 mL were diluted to 100 mL each with deionised water producing 0.5, 1, 2, 4 and 8 ppm of each element. Digested blank stock prepared above was diluted similar to sample was used as blank and standard linear calibration line was constructed using absorbance values determined for each at following conditions as mentioned in Table 3.1.

Table 3. 1| **Instrumental parameters used during the analysis of trace metal determination in MCO, VCO and Blank**

Parameter	Element					
	Cd	**Cu**	**Fe**	**Pb**	**Ni**	**Zn**
Wavelength (nm)	228.8	324.8	248.3	283.5	232	213.9
Slit width (nm)	0.5	0.5	0.2	0.5	0.2	0.5
Lamp current (mA)	6	7	5	5	10	7

[15] Microwave digestion of MCO, VCO and blank samples was kindly supported by Bioclin Research Laboratories (currently known as Almac Sciences Ireland).

3.4 Preparation of MCO dilutions

Fat-soluble dilutions of five different concentrations of MCO were prepared. The required quantities of diluent base and MCO were weighed into dry and clean mortars; this mixture was triturated vigorously with a dry pestle until a uniform texture was observed. A similar method was employed for the preparation of following onychomycotic applications mentioned in Table 3.2.

Table 3. 2| **Ingredients used for the preparation of fat-soluble MCO onychomycotic applications**

MCO dilution %w/w	Yellow petrolatum (Diluent base) for 100 g (g)
5	95
10	90
15	85
20	80
25	75

25% w/w VCO onychomycotic application was prepared similarly for control purpose.

3.4.1 Antimicrobial evaluation of MCO dilutions

1.0 mL of broth containing *T. rubrum* which was cultured for 21 days was transferred aseptically onto sterile Sabouraud dextrose agar plate and was spread uniformly with a sterile spreader. The sample of 1.0 g was placed on a sterile filter paper disc of diameter 25 mm and located on the inoculated agar surface and inverted such that sample, was in contact with the agar. This procedure was repeated for all onychomycotic application samples including controls, in duplicate. The inoculated plates were incubated at 32^{o}C for a period of 21 days. The diameters of the zones of inhibition were measured after 3, 8, 11, 14, 17 and 21 days respectively.

3.4.2 Preparation of prototype candidate

In order to enhance the aqueous solubility of MCO and thereby optimise its activity in hydrophilic medium, it was reacted with diethanolamine to form a water-soluble amide. A stock of 2.0 M diethanolamine (DEA) solution was prepared by dissolving the corresponding volume in deionised water as per the density provided by the manufacturer. MCO and diethanolamine were heated to 80^{o}C and at this isothermal condition, DEA was added to MCO from a burette until a pH of 7 in the MCO and DEA

mixture was realised. The prepared mixture was then dissolved in glycerine (previously heated to 151^{o}C) at different ratios so as to obtain 5, 10, 15, 20 and 25% w/w MCO-DEA glycerine onychomycotic applications. A similar procedure was repeated to prepare 25% w/w virgin coconut oil (VCO) onychomycotic application in glycerine as a negative control.

3.4.2.1 Antimicrobial evaluation of water-soluble MCO Onychomycotic applications

Onychomycotic applications prepared with MCO-DEA mixture were tested by two different methods:

Well-diffusion method: In each plate, two wells were cut with sterile tips for the sample to be located. Into each well 500 mg of sample was placed. The method and amount of inoculum introduced onto the plates were similar to the above agar-surface method as per Section 3.4.1

Disc diffusion method: As per Section 3.4.1

The above procedures were repeated for the samples recorded in Table 3.3 (in duplicate).

Table 3. 3| **The samples tested for the antimicrobial evaluation of water-soluble onychomycotic applications**

Test sample MCO onychomycotic application concentration (% w/w)	**5, 10, 15, 20, 25, 100**
controls	
25% w/w VCO-DEA onychomycotic application	Negative control
Naloc^{TM}	Positive control
Glycerine+DEA mixture	Negative control
Neat MCO	Positive control
Neat VCO	Negative control

Thus, prepared plates were then incubated at 32^{o}C in dark and observations were recorded after 3, 10 and 17 days.

3.5 Designing MCO into target-oriented application.

Considering the observed superior activity of MCO when solubilised into an aqueous matrix, multiple formulations using various compendial excipients, were so designed. The procedure employed to prepare these formulations is described in this section.

The following Table 3.4 describes the excipients selected for the formulations and their applications. As discussed in Section 2.7, excipients selected were procured from registered suppliers.

Table 3. 4| **Excipients and their role in MCO formulation**

Excipient	Role	Characteristic solubility
Stearic acid (SA)	Emulsifier by salt formation	Lipid soluble (L)
Decanoic acid (DA)		
Sodium hydroxide (NaOH)	Emulsifier by salt formation	Aqueous soluble (A)
Polyethylene glycol (PEG)	Emulsifier	
Tween – 20 (T20)		
Monoethanolamine (MEA)	Emulsifier by salt formation	
Diethanolamine (DEA)		
Triethanolamine (TEA)		
Water (dH_2O)		

It was targeted that the fatty acids in the chosen excipients react with base thereby forming surfactant with both hydrophilic and lipophilic ends to emulsify MCO into formulation. The aqueous based materials in the above-mentioned description have been chosen for the presence of hydroxyl groups along with amine group. Hydroxyl groups can form hydrogen bonds with water in the formulation and as well as with the moisture at the targeted region. This phenomenon was targeted to improve stability and performance of the formulation. The aqueous-soluble (NaOH/PEG/T20/MEA/DEA/TEA) and lipid-soluble components (SA/TA) were mixed separately and heated to 80^{o}C. At this isothermal condition, the lipid phase was added into the aqueous phase with constant stirring until a uniform dispersion of internal phase and a pH of 7.4 was determined. A similar procedure was employed during the preparation of onychomycotic applications from A to Z' mentioned in Table 3.5. Placebos were prepared in similar manner with ingredients at highest

concentrations of the corresponding MCO formulations and these are presented in Table 3.6.

Table 3. 5| **Formulations designed with MCO (15% w/w) and the chosen excipients (% w/w) for scouting experiments to scrutinise for incompatibilities**

Formulation	DA	SA	MEA	DEA	TEA	NaOH	Tween-20	PEG	Water
A	2.00	0.00	0.00	0.00	0.00	1.50	3.75	10.00	69.25
B	0.00	2.00	0.00	0.00	0.00	1.32	3.75	10.00	67.93
C	0.00	2.00	0.00	0.00	0.00	1.32	3.75	20.00	62.93
D	0.00	2.00	0.00	0.00	0.00	1.32	3.75	40.00	37.98
E	0.00	4.00	0.00	0.00	0.00	1.60	3.75	10.00	65.65
F	0.00	8.00	0.00	0.00	0.00	2.57	3.75	10.00	62.13
G	4.00	0.00	0.00	0.00	0.00	2.37	3.75	10.00	64.88
H	4.00	0.00	0.00	0.00	0.00	2.37	3.75	20.00	54.88
I	8.00	0.00	0.00	0.00	0.00	3.30	3.75	10.00	59.95
J	0.00	3.75	1.00	0.00	0.00	0.00	0.00	10.00	70.45
K	0.00	7.50	2.00	0.00	0.00	0.00	0.00	10.00	66.70
L	0.00	15.0	3.00	0.00	0.00	0.00	0.00	10.00	56.79
M	0.00	3.75	0.00	1.38	0.00	0.00	0.00	10.00	69.87
N	0.00	7.50	0.00	2.76	0.00	0.00	0.00	10.00	65.24
O	0.00	15.0	0.00	5.53	0.00	0.00	0.00	10.00	54.47
P	0.00	3.50	0.00	0.00	1.96	0.00	0.00	10.00	69.29
Q	0.00	7.50	0.00	0.00	3.93	0.00	0.00	10.00	63.57
R	0.00	15.0	0.00	0.00	7.86	0.00	0.00	10.00	52.14
S	3.75	0.00	1.00	0.00	0.00	0.00	0.00	10.00	69.93
T	7.50	0.00	3.00	0.00	0.00	0.00	0.00	10.00	64.86
U	15.0	0.00	5.00	0.00	0.00	0.00	0.00	10.00	54.72
V	3.75	0.00	0.00	2.29	0.00	0.00	0.00	10.00	68.96
W	7.50	0.00	0.00	4.58	0.00	0.00	0.00	10.00	63.42
X	15.0	0.00	0.00	9.15	0.00	0.00	0.00	10.00	50.85
Y	3.75	0.00	0.00	0.00	3.24	0.00	0.00	10.00	68.01
Z	7.50	0.00	0.00	0.00	6.48	0.00	0.00	10.00	61.02
Z'	15.00	0.00	0.00	0.00	13.0	0.00	0.00	10.00	47.00

Table 3. 6| **Placebos designed without MCO and the chosen excipients (% w/w) for scouting experiments to scrutinise for incompatibilities**

Formulation	DA	SA	MEA	DEA	TEA	NaOH	Tween-20	PEG	Water
PL1	2.00	0.00	0.00	0.00	0.00	1.50	3.75	10.00	69.25
PL2	0.00	2.00	0.00	0.00	0.00	1.32	3.75	40.00	37.98
PL3	0.00	8.00	0.00	0.00	0.00	2.57	3.75	10.00	62.13
PL4	4.00	0.00	0.00	0.00	0.00	2.37	3.75	20.00	54.88
PL5	8.00	0.00	0.00	0.00	0.00	3.30	3.75	10.00	59.95
PL6	0.00	15.0	3.00	0.00	0.00	0.00	0.00	10.00	56.79
PL7	0.00	15.0	0.00	5.53	0.00	0.00	0.00	10.00	54.47
PL8	0.00	15.0	0.00	0.00	7.86	0.00	0.00	10.00	52.14
PL9	15.0	0.00	5.00	0.00	0.00	0.00	0.00	10.00	54.72
PL10	15.0	0.00	0.00	9.15	0.00	0.00	0.00	10.00	50.85
PL11	15.0	0.00	0.00	0.00	13.0	0.00	0.00	10.00	47.00

3.5.1 Determination of antifungal activity of the designed formulations towards targeted profile.

As per section 3.2.1, *T. rubrum* was inoculated onto Sabouraud agar. Prior to plating the agar, sufficient keratin was mixed in such that each agar plate contained 100 mg of keratin. Similar to the Well-diffusion method mentioned in Section 3.4.2.1, 500 mg of each formulation and respective placebo were individually placed in duplicate in wells which were cut using sterile pipette tip in the agar. The zones of inhibition developed by each formulation were measured after 3 days.

3.5.2 Texture analysis to determine the integrity of the dosage form.

4% w/w of each onychomycotic application was prepared with deionised water and then heated to 32°C. This solution was then placed in the respective containers provided by the manufacturer of TA.XT plus texture analyser equipped with a backward extrusion rig and tested (Figure 3.1). The probe (attached with part) for semisolid-material analysis was connected to the arm and the physical properties deemed most informative: consistency, firmness, index of viscosity and cohesiveness were recorded for each onychomycotic application sample using the software provided by the manufacturer. Measurements were recorded at 37, 32 and 27°C for all designed formulations from A to Z' and at 32°C for samples from the indicated pull point of stability studies.

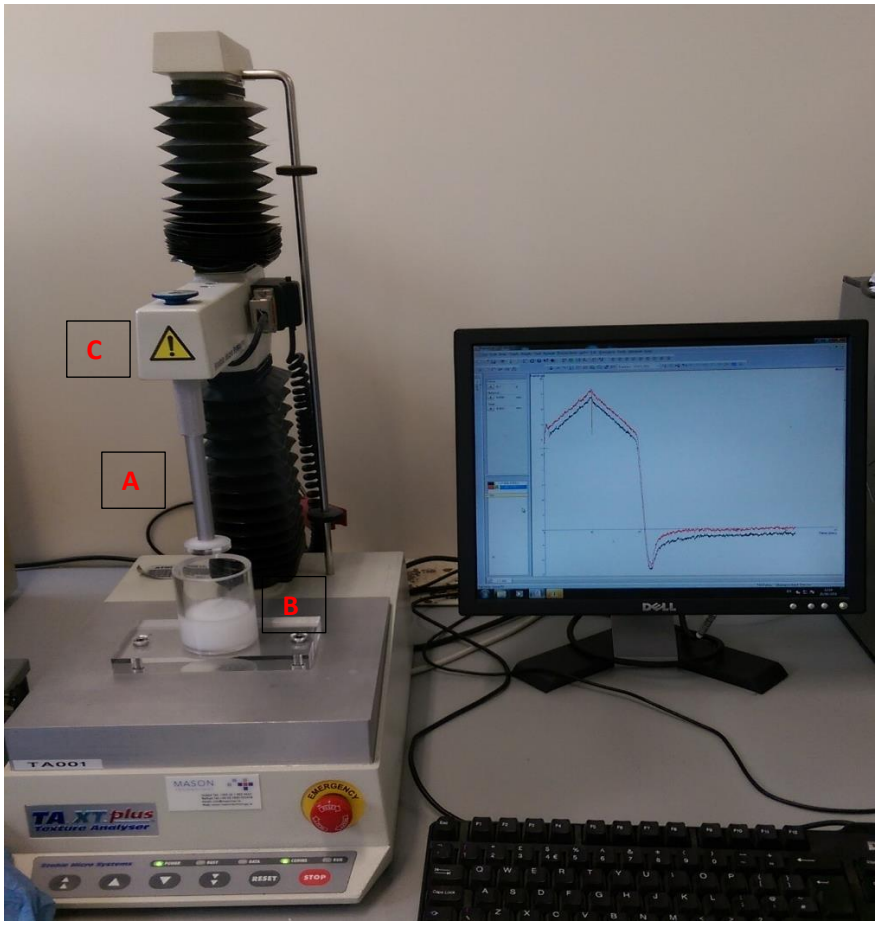


Figure 3.1| **Texture analyser connected with Back Extrusion Rig.** The probe (A) is connected to 5kg load cell (C), sample is placed in specialised container (B) provided by the manufacturer for semisolid dosage form analysis.

3.5.3 Particle size distribution determination of MCO candidates to evaluate extent of emulsification

The size and distribution of droplets of a dispersed phase in the continuous phase determines the duration of functional stability of an emulsion. A 4% w/w dispersion of each onychomycotic application sample with deionised water was analysed to determine distribution of particle size of the internal phase. The prepared sample was introduced into the wet sampler of a Malvern mastersizer 2000 and the distribution was measured at an obscuration of 23 – 28% range.

3.5.4 Optical microscopy

The respective onychomycotic application sample was mixed thoroughly with a clean spatula. A smear of this onychomycotic application was prepared on a clean glass slide. A cover glass was not used above the sample to avoid any external pressure enlarging the globule size of the internal phase. It was then placed under microscope and pictures were captured under 100X magnification against white fluorescent light. A similar procedure was followed for all onychomycotic application samples and placebos.

3.5.5 Determination of chemical compatibility of MCO and its excipients by infrared spectroscopic analysis.

As per Section 3.3.1, sodium chloride plates were cleaned with chloroform and dried in a cold current of air. A background scan was performed using these sodium chloride plates and saved. Onto the same sodium chloride plates, a small portion of onychomycotic application sample was carefully placed (using spatula) and the spectrum was recorded from 400 to 4000 cm^{-1}. A similar procedure was followed for placebos to discriminate the peaks of excipients in the respective formulations.

3.6 “Scale-up” manufacturing of MCO and its formulation for stability studies

As per intellectual property policy of AIT, the ingredients and respective composition of MCO preparation cannot be disclosed in this thesis.

Three lots of MCO of 220 g each were prepared on three different days using identical raw materials. 15 samples of 12 g each of MCO were packed in polypropylene containers and closed with a lid. Each sample was labelled with the manufacturing date and lot number and stored under the artificial stress conditions described under section 2.7.

Three lots of 400g each of MCO onychomycotic application were prepared on three different days as per the composition described under Table 3.7.

Table 3. 7I **Ingredients and their respective composition in MCO onychomycotic application per lot**

Ingredient	Quantity (g)	Application	Solubility
MCO	60 (15.0%)	API	Lipid
Stearic acid	60 (15.0%)	Emulsifier by salt formation	Lipid
Triethanolamine	24 (7.86%)	Emulsifier by salt formation	Aqueous
Polyethylene glycol (Mol Wt: 40000)	40 (10.0%)	Viscosity modifying agent	Aqueous
Water	216 (52.14%)	Continuous phase	Aqueous

Similar to the method mentioned in Section 3.5 lipid soluble components were mixed in a sterile and neat container. The aqueous-based ingredients were mixed in another sterile and neat container. Both components were heated to 80^{o}C and the lipid ingredients were added to the aqueous mixture while stirring with hand blender at 100 rpm speed. Controlled addition of aqueous components was performed to ensure uniform mixing and emulsification. After cooling, 15 g of onychomycotic application was dispensed into each sterile and clean polypropylene extruding container. An identical procedure was replicated for three lots on three alternative days.

3.7 Stress conditions to evaluate the stability.

Artificial stress conditions were induced in a sealed polypropylene container placed in an incubator at 25^{o}C for the period of study. Humidity was provided by saturating the chamber with supersaturated sodium chloride solution. The samples were introduced into the chamber under 75% ± 5% relative humidity and 25^{o} ± 5^{o}C temperature. These conditions were maintained throughout the stability examination period of 90 days. The pull times and the number of samples to be removed per each pull are mentioned in the Table 3.8.

Table 3. 8| **Pull times and the number of samples to be removed for analytical evaluation**

Pull point (T) in days	**Number of samples from freshly opened container**	**Number of samples from opened container on day1 pull point**
T= 1	1, 2, 3	-
T=15	4, 5, 6	1A, 2A, 3A
T=30	7, 8, 9	1B, 2B, 3B
T=60	10, 11, 12	1C, 2C, 3C
T=90	13, 14, 15	1D, 2D, 3D

It was ensured that samples were collected from a freshly opened container as well as samples from those containers opened on the day 1 pull point. The required amount of sample was collected directly into sterile polypropylene tubes for testing. This procedure was repeated for MCO (AAE) batches and the developed formulation. Table 3.9 describes the tests performed for both AAE and finished formulations for the stability analysis.

Table 3. 9| **The analytical tests performed for the samples collected during pull points from AAE and finished product**

Test	MCO (AAE)	MCO onychomycotic application (finished product)	Method
IR spectroscopy	+	+	Section 3.3.1
Appearance	+	+	*
Acid number	+	-	Section 3.3.4.2
USP<61>	-	+	*
Antimicrobial Assay	-	+	Section 3.5.1
Texture analysis	-	+	Section 3.5.2
Particle size distribution	-	+	Section 3.5.3
pH	-	+	Using pH paper
Turbidimetry	-	+	*

*-Method described below, + = Test will be performed, - = test will not be performed.

3.7.1 Appearance assessment

Sample collected from the pull point was examined below fluorescent white light against white background.

Acceptance criteria:

- MCO – colourless liquid.
- MCO onychomycotic application – white semisolid with glossy appearance.

3.7.2 Procedure employed for antimicrobial enumeration USP <61>

Spread plate method for non-sterile products has been employed in this research. One gram of onychomycotic application sample was diluted to 10mL using sterilised phosphate buffered saline at pH of 7.4. One millilitre of sample dispersion was used per plate of organism. The American type culture collection strains of organisms, culturing media and respective incubation conditions are now mentioned in Table 3.10.

Table 3. 10| **The strains used for microbial growth promotion examination of MCO onychomycotic application for stability conditions and the respective media used**

	Growth promotion			**Suitability of counting method in the presence of product**	
Microorganism	**Preparation of test strain**	**Total aerobic microbial count**	**Total yeasts and mold count**	**Total aerobic microbial count**	**Total yeasts and mold count**
Staphylococcus aureus ATCC 6538 *Pseudomonas aeruginosa* ATCC 9027 *Bacillus subtilis* ATCC 6633	Tryptic soy agar/broth 30^{o} – 35^{o} C 18 – 24 hrs	Tryptic soy agar/broth ≤100 cfu 30^{o} – 35^{o} C ≤3 days	N/A	Tryptic soy agar/broth ≤100 cfu 30^{o} – 35^{o} C ≤3 days	N/A
Candida albicans ATCC 10231	Sabouraud dextrose Agar/broth 20^{o} – 25^{o} C 2 – 3 days	Sabouraud dextrose agar/broth ≤100 cfu 30^{o} – 35^{o} C ≤5 days	Sabouraud dextrose Agar/broth ≤100 cfu 20^{o} – 25^{o} C ≤5 days	Sabouraud dextrose agar/broth ≤100 cfu 30^{o} – 35^{o} C ≤5 days	Sabouraud dextrose Agar/broth ≤100 cfu 20^{o} – 25^{o} C ≤5 days

N/A = not applicable.

Respective cultures were reconstituted as per the conditions mentioned above and the dilution factor at which no more than 100 cfu/mL was achieved for each strain. One gram of each sample was dissolved in 10 mL of sterilised phosphate buffer saline. Agar plates were prepared using sterilised agar medium according to the respective organism. Onto each plate one mL of cultured medium containing no more than 100 cfu/mL was added. Onto this inoculated plate one mL of sample prepared above was spread uniformly using sterilised spreader and incubated at respective conditions. Same procedure was repeated for all strains and for all samples pulled at respective time point. A similar procedure was followed using sterilised phosphate buffer saline solution without sample as negative control. After the incubation period the number of cfu/mL grown on each plate were counted and compared with negative control.

3.7.3 Conductivity measurement to evaluate emulsion integrity

Samples were prepared similar to texture analysis in Section 2.5.1. Conductivity meter was calibrated using certified conductivity standard. Using this, conductivity of each sample prepared from each pull point was measured.

3.7.4 Turbidity measurement to determine phase integrity of emulsion

Turbidity meter was calibrated using 1000 nephelometric turbidity unit (NTU) standard. Similar to conductivity measurement, turbidity was measured for all samples prepared as per Section 2.5.1 from each pull point.

4.0 Results from provisional examinations on the origins of antimicrobial efficiency of MCO

During development and optimisation, MCO was tested against a range of microbial pathogens and the respective minimum inhibitory concentrations were established by Hughes (2014). In her research, MCO activity was verified by testing it against *Streptococcus mutans* (*S. mutans*), *Streptococcus pyogenes* (*S. pyogenes*), *Escherichia coli* (*E. coli*) and *Candida albicans* (*C. albicans*). Table 4.1 summarises results obtained during these early studies along with data for the fungus *T. rubrum*, expressed as a measurable zone of inhibition.

Considering the performance of MCO against bacterial and yeast species, the potency of MCO was tested against *T. rubrum* to extend its antimicrobial spectrum.

Table 4. 1| **Antimicrobial activity of MCO towards various Gram (G) negative (-ve), positive (+ve), yeast and fungal pathogens over the various exposure periods. Results submitted under *S. mutans*, *S. pyogenes* and *C. albicans* were presented from the work conducted by Hughes (2014)**

E. coli **(G-ve)**	*S. mutans* **(G+ve)**		*S. pyogenes* **(G+ve)**		*C. albicans* **(yeast)**				*T. rubrum* **(fungus)**
8 hrs	30s	10 min	30s	10 min	30s	10 min	2 hrs	8 hrs	7 days
$<1.0^{\alpha}$	3.4^{α}	4.1^{α}	$>9.9^{\alpha}$	$>9.9^{\alpha}$	0.6^{Δ}	0.8^{Δ}	2.7^{Δ}	3.2^{Δ}	$29^{\beta 1}$ and $20^{\beta 2}$

S = seconds; min = minutes; hrs = hours.

Log reduction in colony-forming units per mL when compared to α - BHI controls; Δ - tryptic soy broth controls. β1 - average zone of inhibition (mm) developed when compared to its precursor virgin coconut oil (VCO) during the disc-diffusion method, similarly β2 – during well diffusion method in Sabouraud dextrose agar growth medium (See Figure 4.1).

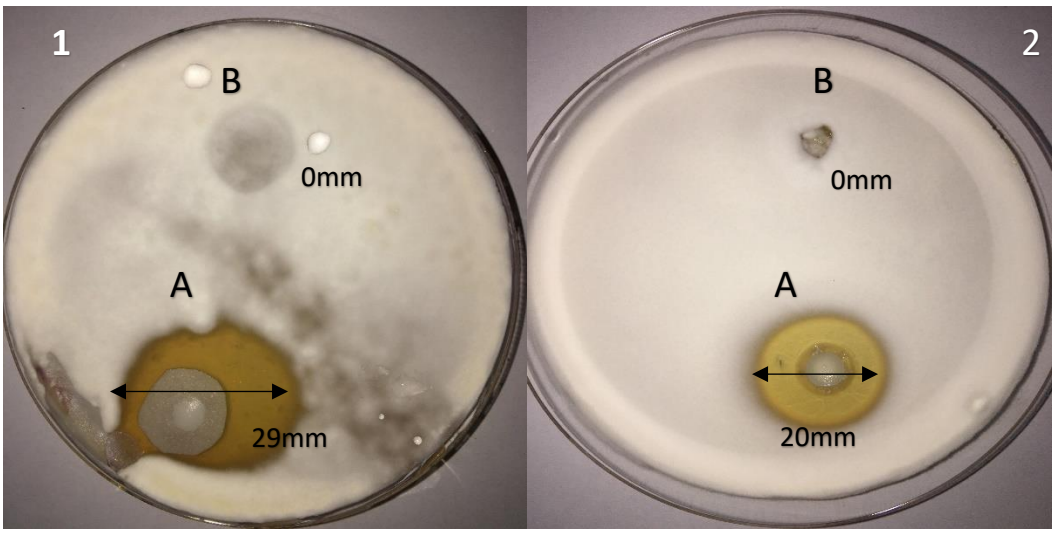


Figure 4. 1| **(1) Disc diffusion of MCO (A) vs VCO control (B) on a plate showing the efficacy of the disc diffusion of the MCO. (2) Well diffusion of MCO (A) & VCO control (B) on a plate showing efficacy of well diffusion.**

From Figure 4.1, It was determined that MCO activity was higher when tested during the disc diffusion method than well diffusion method. The results described in Table 4.2 represent the diameters of zones of inhibition developed by MCO and the corresponding marketed positive controls.

Table 4. 2|**Average diameter of zones of inhibition developed by MCO and respective positive controls when tested by well diffusion method**

Test material	Average diameter of zones of inhibition (mm)	Chemical composition
MCO	52	Mixtures of tri and monoglycerides and free fatty acids
Naloc™	0	Propylene glycol and urea
Boots	10	Olive leaf extract, pentylene glycol and dimethyl isosorbide
Scholl	12	Citric acid and urea

From the above results MCO was determined as a potent antifungal candidate towards *T. rubrum*. The marketed products Boots and Scholl have performed exceptionally low when compared to MCO and there was no inhibition developed by Naloc™. The period and quantity of marketed products used in the test medium was equivalent to the quantity of MCO tested. Therefore, identifying therapeutically effective concentration of MCO in patient-oriented formulation and

developing such novel molecular entity towards effective treatment of onychomycosis has been targeted in this research.

4.1 The investigation of antimicrobial efficiency of MCO towards *T. rubrum*.

Although the broth dilution method serves as an efficient tool to identify the minimum inhibitory concentration of novel candidates, factors such as prolonged incubation time, a non-relevant environment and reduced chances of observing resistance, limit the application of this method towards some pathogens (Balouiri *et al.,* 2016). Therefore, a disc-diffusion method was chosen to identify the effective concentration of MCO towards *T. rubrum*. With this procedure, attributes such as diffusion capacity of MCO, growth prevention over time (21 days) and quantitative comparison with a commercialised control were investigated. In disc-diffusion methodology the diameter of the zone of inhibition produced is directly proportional to the concentration of test material. Therefore, it was used to determine the quantitative activity of MCO towards *T. rubrum* and Naloc™ was used as the positive control. To verify any possible interference of diluent or solvent with the activity of MCO, yellow petrolatum was tested as a negative control. Similarly, to determine the qualitative activity, MCO was tested without dilution at 100%.

Sterilised Whatman filter paper Grade 40 discs were used to assist the uniform diffusion of MCO and its dilutions. It was determined that the diameter of zones of inhibition developed had increased with increase in the concentration of MCO across the period of testing from 3rd to 21st day (Table 4.3 and Figure 4.2 – A to E).

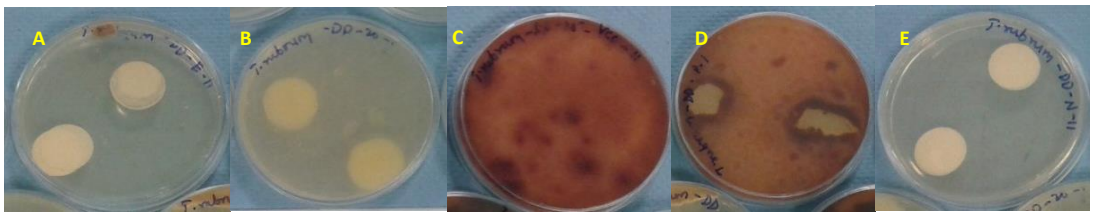


Figure 4.2| **Antifungal activity of various MCO dilutions towards the growth of *T. rubrum* when tested by disc-diffusion after 21 days.** (A) 100% w/w MCO (B) 20% w/w MCO (C) 25% w/w virgin coconut oil (VCO) (D) yellow petrolatum and (E) Naloc™.

MCO dilutions above 20% w/w concentration produced a maximum zone of inhibition like Naloc™ from day 0. Antifungal activity of MCO was due to the area of contact and concentration of the MCO that was in contact with the organism as the yellow petrolatum used for the dilutions of MCO and the VCO used for MCO production did not produce a zone of inhibition during the test (Table 4.3). These results confirmed that MCO and its dilutions above 20% were potent towards *T. rubrum* and even after a period of 21 days.

Table 4. 3| **Antimicrobial activity produced by various MCO dilutions and controls against *T. rubrum* for 21 days when tested by the disc-diffusion method on Sabouraud dextrose agar.**

Sample	**Average (n=2) diameter of zones of inhibition (mm) observed after (days)**					
(% w/w MCO)	3	8	11	14	17	21
5	41.0	40.0	39.6	39.0	38.7	38.7
10	43.0	42.6	41.9	40.0	39.8	39.8
15	43.4	43.0	42.4	41.1	41.1	41.1
20	45.0	45.0	45.0	45.0	45.0	45.0
25	45.0	45.0	45.0	45.0	45.0	45.0
100	45.0	45.0	45.0	45.0	45.0	45.0
Naloc™	45.0	45.0	45.0	45.0	45.0	45.0
100% Petrolatum	0.0	0.0	0.0	0.0	0.0	0.0
25% VCO	0.0	0.0	0.0	0.0	0.0	0.0

4.1.1 Superior activity of MCO in bio-relevant environment

The constructed QTPP towards effective treatment of onychomycosis demands that the active ingredient be in an aqueous matrix to cross the nail plate barrier (Elsayed 2015). Therefore, a prototype formulation was developed with diethanolamine (DEA) and glycerol as water-soluble excipients to investigate the retention of MCO's potency towards the pathogen in an aqueous-based medium.

The medium used for the growth and antimicrobial susceptibility testing of *T. rubrum* was Sabouraud dextrose agar. The organism develops lawn of conidia on the agar surface with mycelium grown inside the medium distinguished by a brick red pigment (McCarthy 2004). To determine the effect of aqueous solubilisation over antimicrobial activity, MCO-DEA complexes and non-emulsified MCO have been tested by well-diffusion method along with DEA-glycerol and VCO-DEA-glycerol mix as negative controls and Naloc^{TM} as positive control.

From Table 4.4 it was determined that the diameters of zones of inhibition developed by the MCO-DEA formulations increased with increasing concentration of MCO-DEA mixture in glycerine indicating the enhanced diffusion of MCO when solubilised into an aqueous medium.

Table 4. 4| **Antimicrobial activity of MCO-DEA formulations during the well-diffusion assay for a period of 17 days*.**

Sample (%w/w MCO-DEA)	Average (n=2) diameter of zones of inhibition (mm) developed after		
	3 days	**10 days**	**17 days**
5	30.4	39.5	41.2
10	40.1	45.0	45.0
15	45.0	45.0	45.0
20	45.1	45.1	45.1
25	45.0	45.0	45.0
100	45.1	45.1	45.1
Neat MCO	30.0	34.8	35.1
NalocTM	26.4	29.8	30.0
Glycerine-DEA (negative control)	0.0	0.0	0.0
25% w/w VCO-DEA in glycerine	0.0	0.0	0.0
Neat VCO	0.0	0.0	0.0

* - the experiment was ended after 17 days as the zones of inhibition were unclear due to microbial efflorescence.

The medium used was a mixture of peptone, sugars and agar dispersed in water. Hence, it acts as a nutrient base with water for the inoculated culture to grow. The agar well-diffusion method described here, involved testing a sample by its ability to permeate through the agar medium thereby inhibiting the growth of the organism along the region permeated or diffused. The MCO developed lesser zone of inhibition when compared to the diameter developed by the MCO in DEA complexes (Tables 4.3 and 4.4), this suggested that efficiency of MCO is superior when solubilised into aqueous medium to in fat-soluble matrix. The negative controls used did not produce any of zone of inhibition during the study, which confirmed the activity determined was due to MCO only. Similar to previous disc-diffusion assay, the precursor of MCO i.e., VCO emulsified with DEA-glycerol didn't exhibit any activity in aqueous conditions. This reassured MCO as a potent antimicrobial compound due to modification.

The aqueous soluble MCO matrices are more potent and advantageous than the petrolatum dilutions and Naloc™ as the minimum inhibitory concentration of MCO towards *T. rubrum* was observed to be 5% w/w (Table 4.3) a much-reduced concentration of API/active component when compared to the – 40% keratolytic agent (urea) in Naloc™. Similarly, Naloc™ developed a zone of inhibition with an average diameter of 30.0 mm after 17 days, which was equivalent to the activity of the 5% w/w MCO-DEA formulation after three days (Table 4.4).

The minimum concentration at which a relatively maximum potential effect observed was 15% w/w MCO-DEA as the zone of inhibition developed was consistent from the initial time of observation (Table 4.4) whereas similar activity had been determined with 20% w/w MCO in petrolatum when tested by disc-diffusion method (Table 4.3). Therefore, 15% w/w MCO concentration has been chosen for the preparation of MCO onychomycotic applications as the targeted region is hydrophilic nail plate.

As detailed in Section 1.9, therapeutic moieties derived from natural resources are one of the novel antimicrobial alternatives to treat diseases caused by antifungal resistance. Among those, fatty acids and their derivatives are one of the leading chemical entities (Pohl *et al*., 2011). Supporting this, the antimicrobial activity of MCO determined towards *T. rubrum* in this research provides an efficient alternative to treat onychomycosis. The activity produced is three times potent than a popular over-the-counter dosage form. Along with fatty acids other novel actives include essential oils from plants such as *Allium* (Pyun & Shin 2006), *Lavendulan* and *Thymus* (Pinto *et al*., 2013), *Oenanthe* (Valente *et al*., 2013), *Distichoselinum* (Tavares *et al*., 2010), *Matricaria* (Jamalian *et al.,* 2012) and *Cinnamomum* (Khan & Ahmad 2011). However, influence of aromatic composition on lipid deposition and toxicity levels of these compounds over extended use have not been completely investigated.

Similarly, several research publications have proven the potency of individual medium chained saturated fatty acids towards pathogenic species such as *Candida* and *Aspergillus* (Pohl *et al*., 2011), *Staphylococcus*, *Streptococcus* and *Propionibacterium* (Sado-Kandem *et al.,* 2009; Desbois & Lawlor 2013), *Mycobacterium bovis* and *tuberculosis* (Carballeria 2008), Listeria (Gutierezze *et al*.,

2008), *E. coli* (in combination with essential oils) (Kim & Rhee 2016). However, the synergistic activity of these antimicrobial fatty acid combinations has not been explored towards *T. rubrum*. MCO is a derivative of saturated oil containing both free form and glycerides of stearic, lauric, decanoic and octanoic acids (Hughes 2015). Therefore, application of such integrated MCO as a potent antifungal candidate in the formulation for an effective treatment of onychomycosis has been targeted in this research.

Phase separation had been observed in the prototype emulsion of MCO developed with DEA and glycerine over period of time. Therefore, a detailed characterisation and compatibility assessment of MCO with selected excipients has been targeted to develop potent, efficacious, and stable dosage form.

4.2 Optimising MCO towards quality-oriented candidate

MCO was formulated into several combinations using compendial excipients and the results of these mixtures in the presence and absence of keratin towards *T. rubrum* are discussed in this section. An overview of the excipients used in the formulations is presented in Table 4.5.

Table 4. 5| **Formulation groups based on the composition**

Group A	Excipients	Group B*	Excipients	Group C*	Excipients
A, G, H, I	SA+NaOH+Tween 20+Water	J, K, L	SA+MEA	S, T, U	DA+MEA
B, C, D, E, F	DA+NaOH+Tween 20+PEG+Water	M, N, O	SA+DEA	V, W, X	DA+DEA
		P, Q, R	SA+TEA	Y, Z, Z'	DA+TEA

NaOH = sodium hydroxide; SA = stearic acid; DA = decanoic acid; MEA = monoethanolamine; DEA = diethanolamine; TEA = triethanolamine; PEG = polyethylene glycol (molecular weight = 35000). * - Along with the mentioned excipients, PEG at 10% w/w and water (quantity sufficient) were fixed in Groups B and C.

As per the established QTPP, the targeted site of application of the intended formulation for an effective treatment of onychomycosis is the infected nail plate. In Chapter 1.0, the composition of the nail plate, matrix, and keratin levels in healthy nails has been discussed (approx. 90% w/w). As the activity of solubilised MCO was determined to be higher in aqueous formulation it was emulsified using stearic or decanoic acid salts formed with NaOH/MEA/DEA/TEA (at various concentrations) into a medium containing the hydrating agent PEG as mentioned in Table 4.5. The performance of these designed formulations towards the pathogen was tested in the presence and absence of keratin along with their placebo controls (Table 4.6) containing the highest concentration of emulsifier. In these simulated conditions, the content of keratin added to the growth medium of each agar-plate was equivalent to the average amount (0.1g) of keratin in a healthy adult human nail plate. This was to supplement the pathogen's nutrient requirements. The performance observed in such conditions was considered significant because the organism would have a stronger and more supportive environment than a usual growth medium.

Table 4. 6| **Compositions of placebo of the formulations mentioned in Table 4.5**

Placebo	Ingredients	Corresponding formulation
PL1	SA+NaOH+Tween 20+Water+PEG	B, C, D, E, F
PL2	SA+NaOH+Tween 20+PEG+Water	
PL3	DA+NaOH+Tween 20+PEG+Water	A, G, H, I
PL4	SA+MEA+PEG+Water	J, K, L
PL5	SA+DEA+PEG+Water	M, N, O
PL6	SA+TEA+PEG+Water	P, Q, R
PL7	DA+MEA+PEG+Water	S, T, U
PL8	DA+DEA+PEG+Water	V, W, X
PL9	DA+TEA+PEG+Water	Y, Z, Z’

During all antimicrobial activity investigations of MCO towards pathogen, the type of cultures used and the growth media for testing were maintained constant. Naloc^{TM} had produced equivalent zones of inhibition with MCO during the initial testing phases (See Section 4.1). Later the activity of Naloc^{TM} was observed to diminish with the progress of examinations as the zones of inhibition developed by it were determined to be minimal to nil even though it was ensured that the expiration date of the Naloc^{TM} used always fell due not less than three months beyond the date of experiment. However, MCO formulation activity had remained constant in both the presence and absence of keratin throughout the project with the same strain.

Among the designed formulations, A and I have shown the highest activity in the presence of keratin, and formulation T has shown the highest activity in the absence of keratin (Figure 4.3). The formulations from C to H have shown nearly equivalent activity in both conditions. S, T and U have not produced any activity in the presence of keratin but were determined to be active in its absence. Formulations J, Q, R, Y, Z and Z’ had produced lower activity in the presence of keratin. Similarly, placebos containing DA as a part of the emulsifying agent had produced equivalent or higher activity than their corresponding formulation.

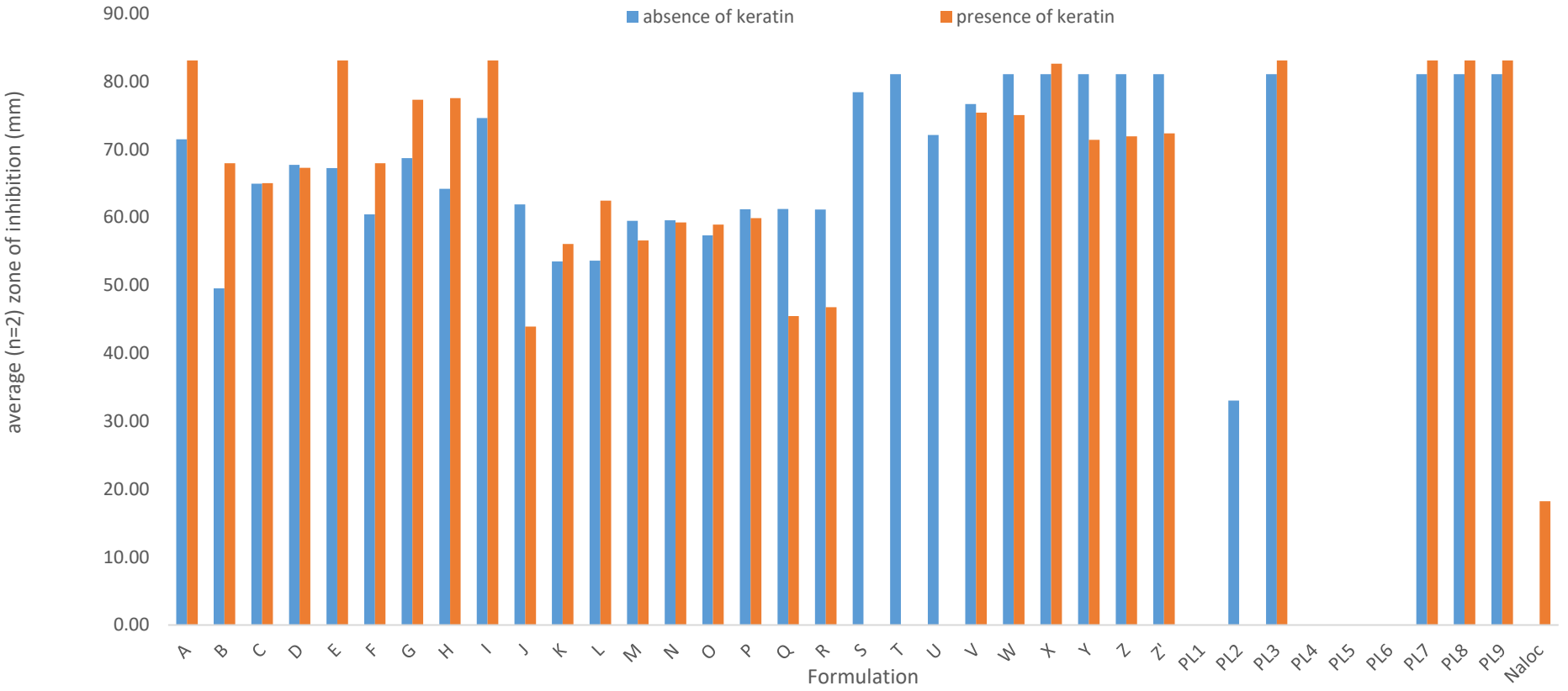


Figure 4.3| **Antimicrobial activity of designed formulations against *T. rubrum* in the presence of keratin on Sabouraud dextrose agar.** Here A to Z' are samples designed using MCO as AAE and SA, DA, MEA, DEA, TEA, NaOH and water as excipients in the respective formulations. PL represent placebos prepared using respective excipients of formulations at highest concentration possible without MCO. Combinations with DA as the emulsifying agent had produced a lower zone of inhibition than the expected synergistic activity of MCO. This observation confirmed the masking effect of DA over the activity of MCO.

Note – In this figure the values graphed above were an average of n = 2 readings per sample. The range between the two readings was not more than 1.2 mm.

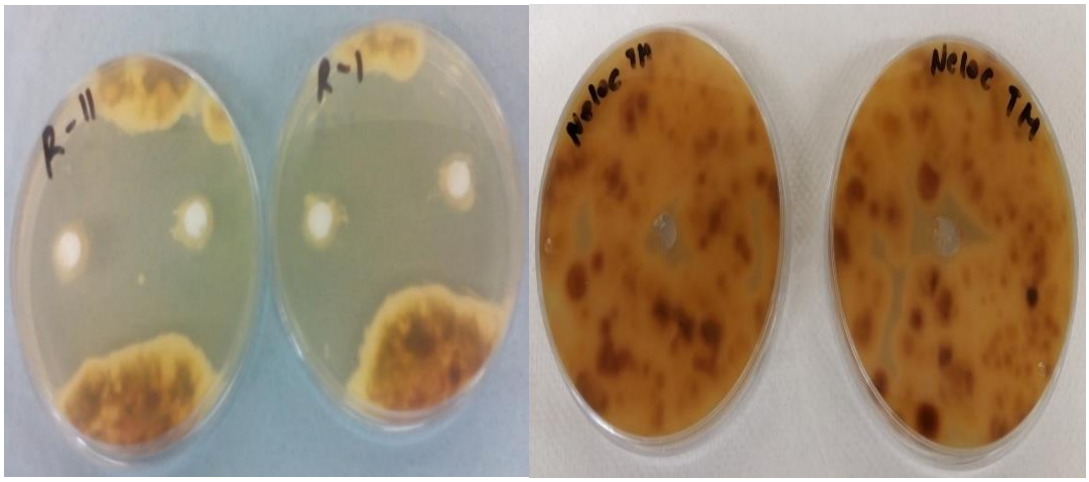


Figure 4. 4| **An example of zones of inhibitions developed by the Formulation R (left-hand side) and no activity observed for Naloc^{TM}** (commercial control – right-hand side) in the presence of keratin.

C. albicans is one of the opportunistic pathogens that can invade the keratin of the infected nail along with *T. rubrum*. Hence, in this research, the potencies of designed formulations had been evaluated towards *C. albicans*. From Figure 4.5, it was determined that all formulations were active towards *C. albicans* in the presence of keratin with the highest performance by G and I. The formulation L had produced lowest activity among all. The activity of placebos was determined like that of *T. rubrum* studies.

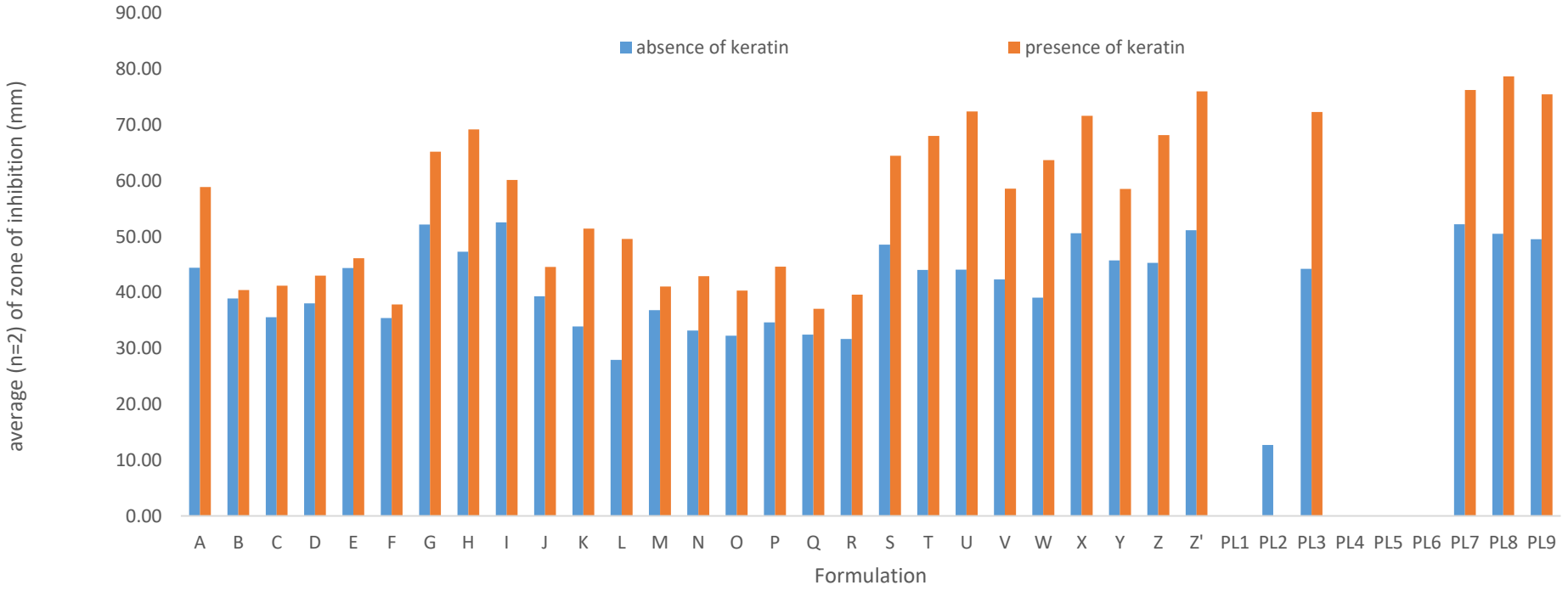


Figure 4. 5| **Antimicrobial activity of designed formulations in the presence and absence of keratin against *C. albicans* on Sabouraud dextrose agar.**

Note – In this figure the values graphed above were an average of n = 2 readings per sample. The range between the two readings was not more than 1.2 mm.

Although all formulations have produced zones of inhibition towards the growth of the organism, potent formulation had to be identified after the physical characterisation. This is because the formulations are emulsions by nature which are not indefinitely stable. Therefore the appropriate formulation with proven potency was finalised based on the physicochemical characterisation along with the antimicrobial activity.

4.3 Characterisation of AAE towards establishing CQA

The physico-chemical attributes of MCO affecting the established quality targeted profile towards the effective treatment of onychomycosis have been evaluated by several analytical techniques. Determinations from these studies and their role in placing MCO as AAE into the formulations are now discussed in this section.

4.3.1 Infrared spectroscopic studies as a quick discriminating tool

To establish an analytical profile thereby identifying MCO to discriminate it from its precursor, a rapid and robust characterisation process is required. As mentioned in Section 2.4.1, IR spectroscopy has been employed in this research to differentiate and realise MCO.

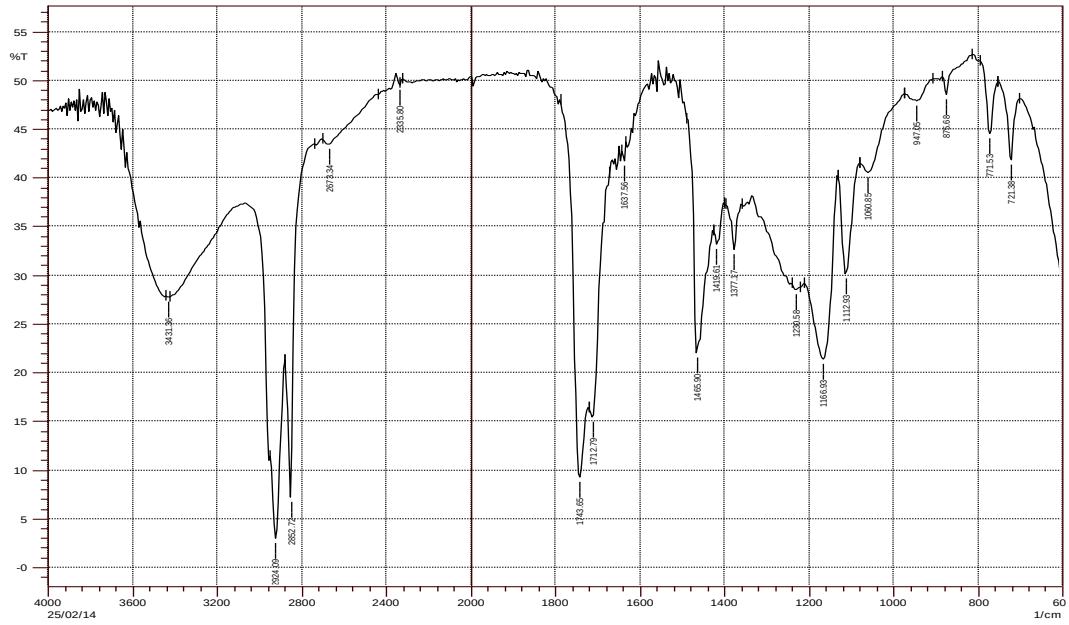


Figure 4.6| **IR spectrum of MCO (thin-film vs air as background, NaCl plates).** Scan was performed from 4000 to 400 cm^{-1}. Peak frequencies were identified using instrument software.

From Figures 4.6 and 4.7, the spectra of MCO and CMCO (MCO prepared by a modified centrifuge method for bulk production) had a broad and robust peak at 3431 cm^{-1} that indicated the presence of an intra-molecular bonded O-H stretch (presumably moisture) in it. The sharp peak at 2924 cm^{-1} was due to the stretching vibration of a saturated C-H bond. The '-C=O' stretching of a triacylglycerol group was observed at 1743 $cm^{-1,}$ and the shoulder peak at 1712 cm^{-1} indicated the presence of

carboxylic acid. Along the fingerprint region of 1500-500 cm^{-1}, bends at 721, 771 and stretching at 1112 and 1166 cm^{-1} indicated the presence of methylene bridges, n-propyl C-H stretching and saturated acyl group stretching. Therefore MCO is a saturated, long-chain fatty acid ester (Rohman & Che Man 2011).

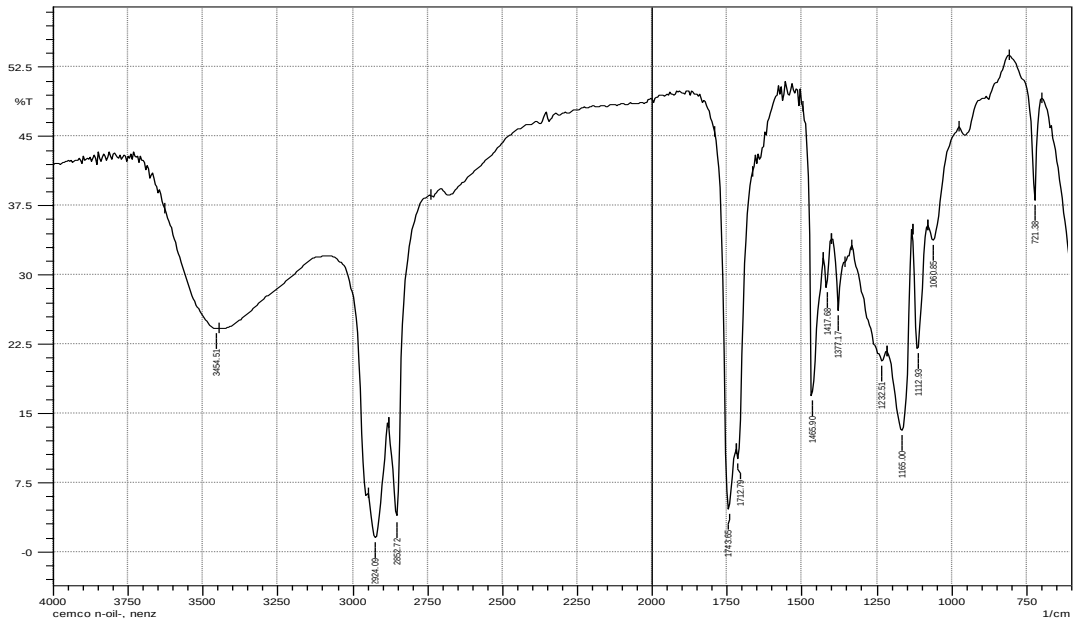


Figure 4.7| **IR spectrum of CMCO (thin-film vs air as background, NaCl plates).** Scan was performed from 4000 to 400 cm^{-1}. Peak frequencies were identified using instrument software. The characteristic peaks of MCO were identified in this sample which confirmed that modified manufacturing procedure did not impact the chemical composition of MCO.

The IR spectrum of virgin coconut oil (Figure 4.8) displayed similar vibrational motions over the same region when compared with MCO; the characteristic difference between MCO and VCO was identified at the region of 1700-1750 cm^{-1}; where MCO had free carboxylic acid stretching at 1701 cm^{-1} (Figures 4.7 and 4.8) a feature absent from the VCO spectrum due to modification. Hence, this specific difference has been used throughout this research to identify MCO from its precursor VCO.

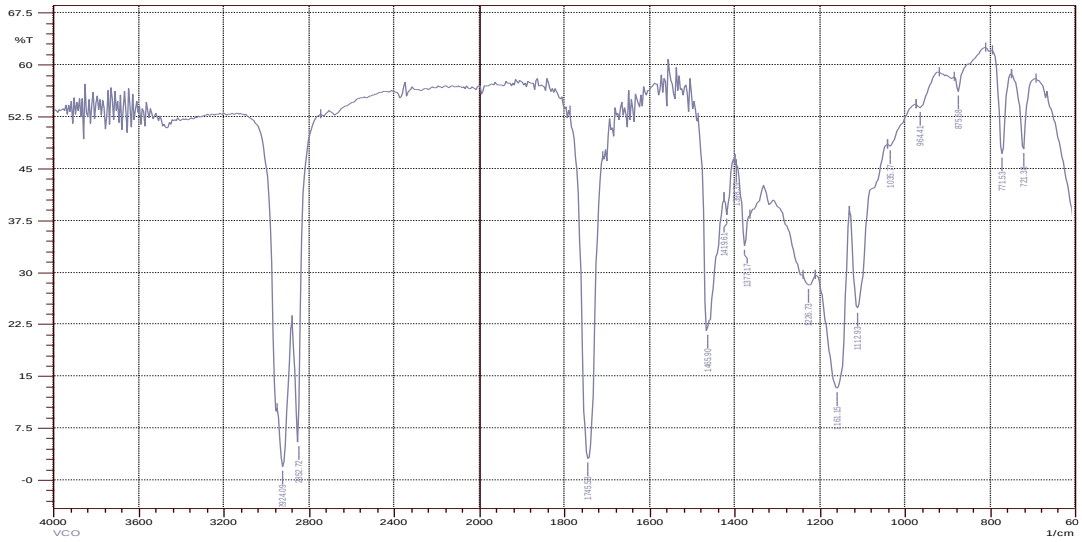


Figure 4.8| **IR spectrum of VCO (thin-film vs air as background, NaCl plates).** Scan was performed from 4000 to 400 cm^{-1}. Peak frequencies were identified using instrument software. Characteristic peaks of MCO at 3400 and 1720 cm^{-1} were not observed in VCO confirming the modification. These peaks were used as critical identification feature for MCO in further studies.

The following properties (Table 4.7) have been established during the earlier stages of this project.

Table 4. 7| **Physico-chemical properties of MCO established as part of CQA identification**.

Attribute	**Technique**	**Value determined**		**Justification**
		MCO	VCO	
Density	Anton Parr® density meter	0.8939g/mL	0.9247g/mL	Useful in mass volume conversion.
Moisture content	KF Titration	0.084% w/w	N/A	To quantify the moisture content, thereby identify if it has any impact over the stability.
Freezing point	DSC	13.04°C	23.48°C	The values determined confirmed the conversion of large molecules of VCO (triglycerides of fatty acids) to smaller molecules of free fatty acids and monoglycerides supporting the observations of IR spectroscopy.
Boiling point		284.52°C	306.18°C	
Saponification value (mg of KOH per g of sample)	USP <401>	211	234	Confirmed that the type of fatty acids in MCO were like that of VCO even after modification. These results prove that modification of VCO yielded change of functional moiety but not the total organic content.
Molecular composition	TLC (R_f)	0.83	0.79	These results support the findings of IR, DSC and saponification value. Higher retention factor of MCO indicated its lower molecular weight constituents when compared with VCO.
Endotoxin test	LAL test USP <85>, end-point method	Free from endotoxin	N/A	Safe to apply in topical formulations, as there won't be undesired effects such as hyperthermia when hepatic metabolism is by-passed.

4.3.2 Thermogravimetric analysis of MCO

A TGA of an MCO sample was carried over a range of 25 to 500°C on a platinum crucible purged under nitrogen gas (for inert atmospheric conditions). Table 4.8 describes the percentage weight loss over a range of temperatures deduced from Figure 4.9.

Table 4. 8| **Percentage weight loss observed for MCO sample during TGA**

Temperature range (°C)	% weight loss
100 – 150	1.070
150 – 200	15.966
200 – 250	10.963
275 – 300	71.968

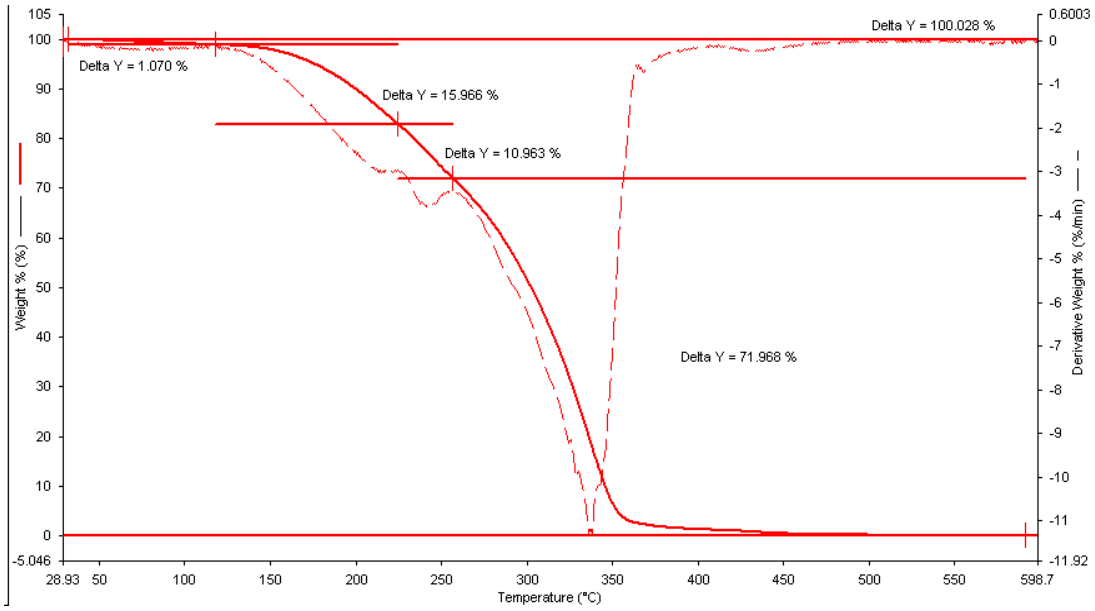


Figure 4.9| **Thermogravimetric curve of MCO over a range of 25 – 600°C purged under nitrogen gas**. The straight line indicates the percentage weight loss of the sample concerning the change in temperature, and the dotted curves are the percentage derivative weight over the time.

The purpose of this experiment was to identify the components of MCO and their respective features. The 1.070% weight loss was due to free or adsorbed moisture content during the analysis. From the Karl Fischer titration experiment, the percentage of moisture (bound) available in the MCO was determined to be 0.084%w/w. Hence, the lost weight mentioned above was due to adsorbed water on the sample, as the temperature range of determination was equivalent to the boiling

point of water. From Figure 4.9 three derived weight loss curves had been observed for MCO which confirmed the presence of three individual components. These results agree with IR spectroscopic studies where peaks most likely attributable to triglycerides, monoglycerides and free fatty acids have been determined. The boiling point of relative compounds increases with an increase in their molecular weights. The maximum exothermic and endothermic peaks of thermograms of MCO during DSC studies match the points of degradation determined in TGA (See Figure 4.10). Hence, it was deduced that the weight loss over the range of 150 – 200^{o}C, i.e., 15.966% was the free fatty acid content. Similarly, 10.963% at a range of 200 – 250^{o}C and 71.968% over the range of 275 – 300^{o}C were most likely attributable to the mono- and tri-glyceride contents of MCO.

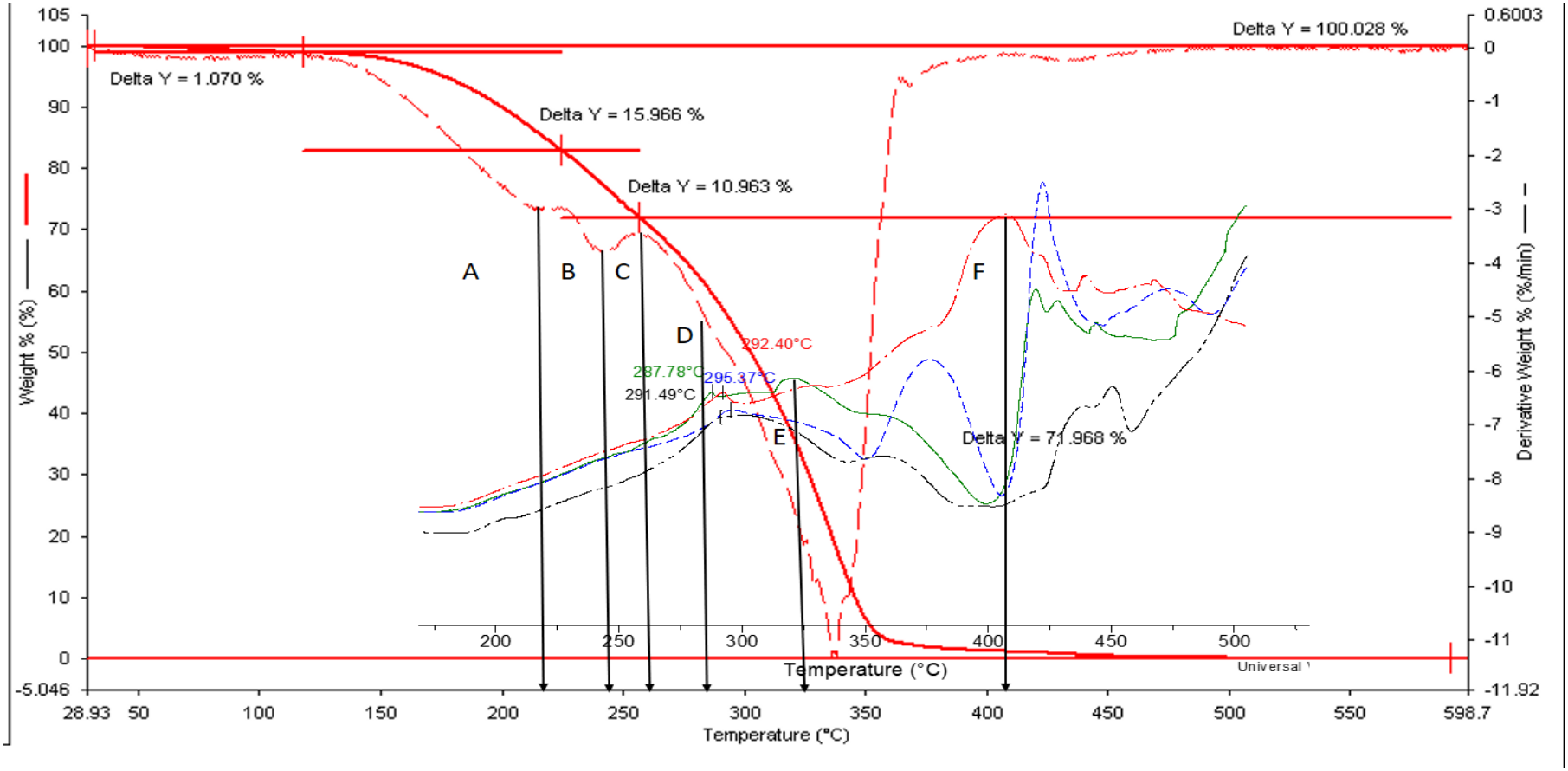


Figure 4.10| **Overlay of thermograms from the DSC analysis and thermogravimetric curves of TGA of MCO sample.** Point A indicates the overlap of phase transition, weight loss of free fatty acids; point B indicates monoglycerides, and points C, D and E indicate triglycerides over the similar temperature range. Point F represents the complete degradation of MCO sample over the similar range in DSC and TGA techniques.

4.3.3 Free fatty acid number determination

The method employed for saponification value determination involved the application of heat and distillation to saponify the oil sample with potassium hydroxide (KOH). In the presence of heat, chances of esterification or transesterification and the loss of volatile matter are high. Therefore, to improve the efficiency and accuracy of the results, “acid number” determination has been performed.

As per the method from USP – NF, <401>, ‘Fats and Fixed Oils’ chapter, an Acid number of MCO sample was determined to be 58.63 mg of KOH per gram, whereas the published value for VCO is 0 to 0.1 mg of KOH per gram. Hence, this property has been considered as a critical quality attribute to assure the modification of VCO into MCO and its presence in onychomycotic applications.

4.3.4 Semi-quantitative analysis of MCO and discrimination of VCO by NMR spectroscopy.

In Section 2.4.8, the application of NMR spectroscopy to determine the structure of organic compounds was detailed. From the IR spectroscopic analysis, it has been confirmed that, the VCO and MCO molecules are made of saturated fatty acid chains. From the literature review, it has been identified that, VCO contains a mixture of fatty acids such as lauric, stearic, myristic and decanoic acids. However, considering the efficacy and sensitivity of NMR it was applied in this research to verify the information gained from IR as well as to explore any traceable impurities from its precursors. The expected shifts of carbon atoms in the VCO and MCO have been detailed with the help of Figure 4.11.

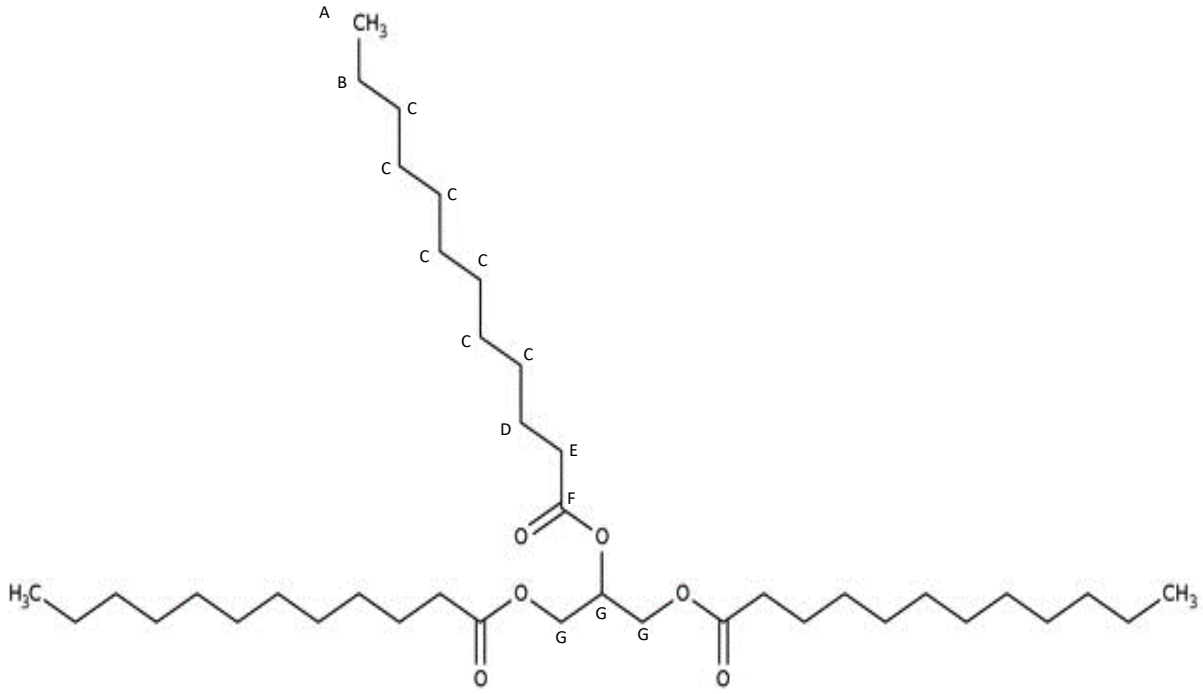


Figure 4.11| **Triglyceride of lauric acid.** Each alphabet represents the type of carbon atom, A – $CH_3(CH_2)$, B – $CH_2(CH_2CH_3)$, C – $CH_2(CH_2CH_2)$, D – $CH_2(CH_2CH_2C{=}0)$, E – $CH_2CH_2(C{=}O)$, F – $C{=}O(CH_2)$ and G – C – O.

A and B type carbon atoms are common among all the free fatty acids and their corresponding esters. C type carbons increase with the carbon chain length, for example: octanoic acid has four methylene bridges in it, these carbon atoms increase

in quantity as the chain of the fatty acid increases until the C_{18} carbon chained stearic acid in both MCO and VCO. As the carbon chain prolongs the D, E and F carbons tend to move towards lower shifts as one end of them is closer to the electron withdrawing group of a carboxylic acid and the electron donating methylene bridges tend to increase on the other end stabilising these carbons. The F type carbon atoms remain same among all the fatty acids and would change in their esters as they are connected to a methylene carbon of the fatty acid and oxygen. The F type carbon atoms appear towards higher shifts in the case of free fatty acids and appear towards comparatively lower and different shifts in tri- and monoglycerides. The chances that G type carbon atoms would appear at different shifts would arise only if the glyceride was formed by mixtures of fatty acids or mono or di or triglycerides. Therefore, B, D, E, F and G type carbons mentioned in the Figure 4.11 change based on the carbon chain length of the triglyceride thereby appearing at different shifts in the NMR spectrum. The following Table 4.9 contains the peaks identified from the NMR spectrum of VCO (Figure 4.12).

Table 4. 9| **Chemical shifts (ppm) determined during ^{13}C–NMR analysis of VCO dissolved in deuterated chloroform**

Chemical Shift (ppm)	Integration	Corresponding Carbon atom
14.21, 22.77, 24.94, 29.20, 29.37	0.6	C in CH_3
29.71*, 32.00, 34.14, 34.31	1.0*, 0.5	-(CH_2)-, methylene bridges between saturated carbons. C – C, the increase in the shift indicates the carbons closer to electron withdrawing groups.
62.19	0.5	C – OH of glycerol of esters
68.94	0.25	C – O carbon of an ester linkage
76.79, 77.12, 77.42	0.8	C – O carbon atoms of ester linkage
173.39	0.25	C = O, carbonyl carbons

The shift at 173 ppm represents the carbonyl carbon present in VCO. The number of peak shifts indicate the number of carbon types present in the molecule. Single carbonyl shift in VCO indicated the presence of one type of carbonyl atom. From the IR spectroscopic analysis, it has been identified that VCO is composed of fatty acid esters, correlating this information to its NMR spectrum, it can be deduced

that the shift at 173.39 ppm is due to carbonyl carbon of triglycerides and its integration of 0.25 indicated that the fatty acid chains have been esterified into triglycerides as shifts over the region of 22 to 34 ppm had an integration of 1.0. The shifts at 76.79, 77.12, 77.42, 62.19 and 68.94 ppm indicated the C – O carbon of an ester linkage in different triglycerides. The integration of 0.8 for the shifts of 76.79, 77.12 and 77.42 confirmed the C – O linkage of ester and glycerol of fatty acid chains of a similar carbon number such as C_{18} (stearic), C_{14} (myristic) and C_{12} (lauric). Similarly, the 62.19 and 68.94 ppm indicated the C – O ester and glycerol linkages of C_{10} (decanoic) and C_8 (octanoic) acids. The integrations of 0.5 and 0.25 confirmed the respective fatty chains based on the composition. The shift at 29.71ppm had the highest integration of 1.0, whereas every other shift was nearly equivalent to 0.5. Based on this, the – (CH_2) – methylene bridges for a total of 6 carbons which is common along the fatty acid chains from C_8 to C_{18} represented the shift at 29.71 ppm, whereas the additional CH_2 bridges in the rest of fatty acids represented from 32.00 to 34.71 ppm. The terminal CH_3 groups of the different fatty acids appeared from 14.21 to 29.37 ppm.

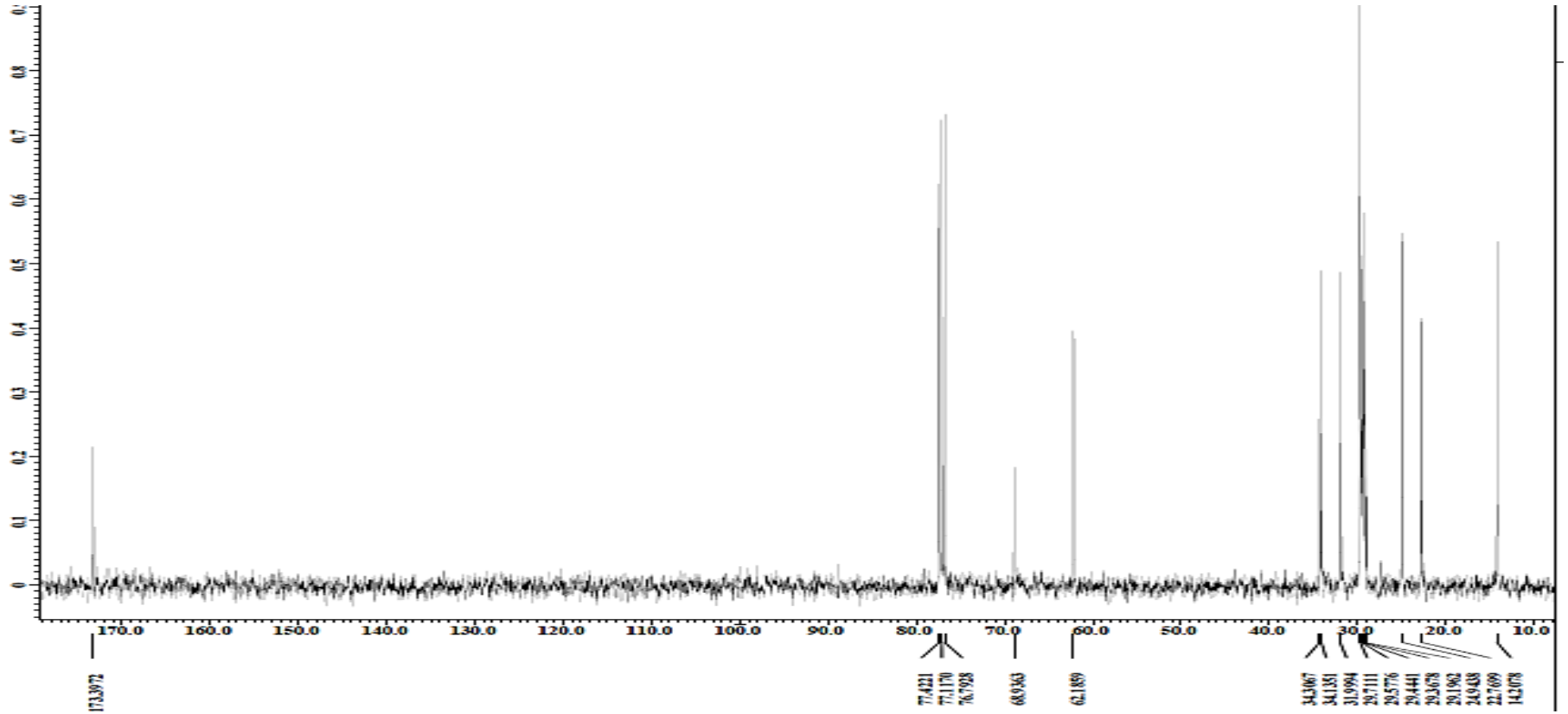


Figure 4.12| **^{13}C-NMR spectrum of VCO dissolved in deuterated chloroform.** The spectrum was recorded at 100 MHz frequency.

From Figure 4.13, the shifts from 173 to 179 ppm represent the carbonyl carbons present in MCO. The three carbonyl shifts indicated three types of carbonyl atoms. From the IR spectroscopic analysis, it has been identified that MCO is composed of mixture of free fatty acids and esters. From the NMR spectrum of MCO, it can be deduced that the carbonyl shifts of free fatty acids at 179.23 ppm, carbonyl atom of monoglycerides at 173.03 ppm and triglyceride carbonyl atom at 173.45 ppm. The integration of carbonyl carbon of triglyceride is higher than the other (See Figure 4.12, Table 4.10) indicating it as major component of MCO and the monoglycerides and free fatty acids at equivalent concentration. The spectrum of MCO appears similar to that of VCO except for the region from 50.0 to 90.0 ppm. The shift at 62.19 in MCO split into three at 61.57, 62.12 and (the parent peak of VCO as it is) 62.18 ppm, the peak at 68.9 of MCO split into 68.4 and 68.9 with an intermediate shift at 65.10. These shifts were due to the carbon atom in C – O linkage of the trans esterified fatty acids and or monoglycerides from triglycerides of VCO to MCO during the modification. The shifts from 76.79 to 77.42 ppm remained same to that of VCO, indicating partial digestion. The breakdown of carbonyl groups over the region of 173 to 179 also affirms the above splits confirming the digestion of VCO into MCO. The shifts for methylene bridges remained nearly same, whereas the CH_3 carbon type count increased in MCO when compared to VCO, confirming the breakdown of triglycerides into respective monoglycerides and free fatty acids.

Table 4. 10| **Chemical shifts (ppm) determined during ^{13}C–NMR analysis of MCO dissolved in deuterated chloroform**

Chemical Shift (ppm)	**Integration**	**Corresponding Carbon atom**
14.21, 22.77, 29.16, 29.20, 29.33	0.5 to 0.6	C in CH_3
29.43, 29.71$^{\beta}$, 32.00	1.0$^{\beta}$, 0.5	-(CH_2)-, methylene bridges between saturated carbons. C – C, the increase in the shift indicates the carbons closer to electron withdrawing groups.
61.58, 62.13, 62.19, 65.10, 65.27, 68.42, 68.94	0.125 to 0.30	C – OH of glycerol of esters
76.79, 77.12, 77.42	0.8	C – O carbon atoms of ester linkage
173.03, 173.45$^{\Delta}$, 179.23	0.25$^{\Delta}$, 0.125	C = O, carbonyl carbons

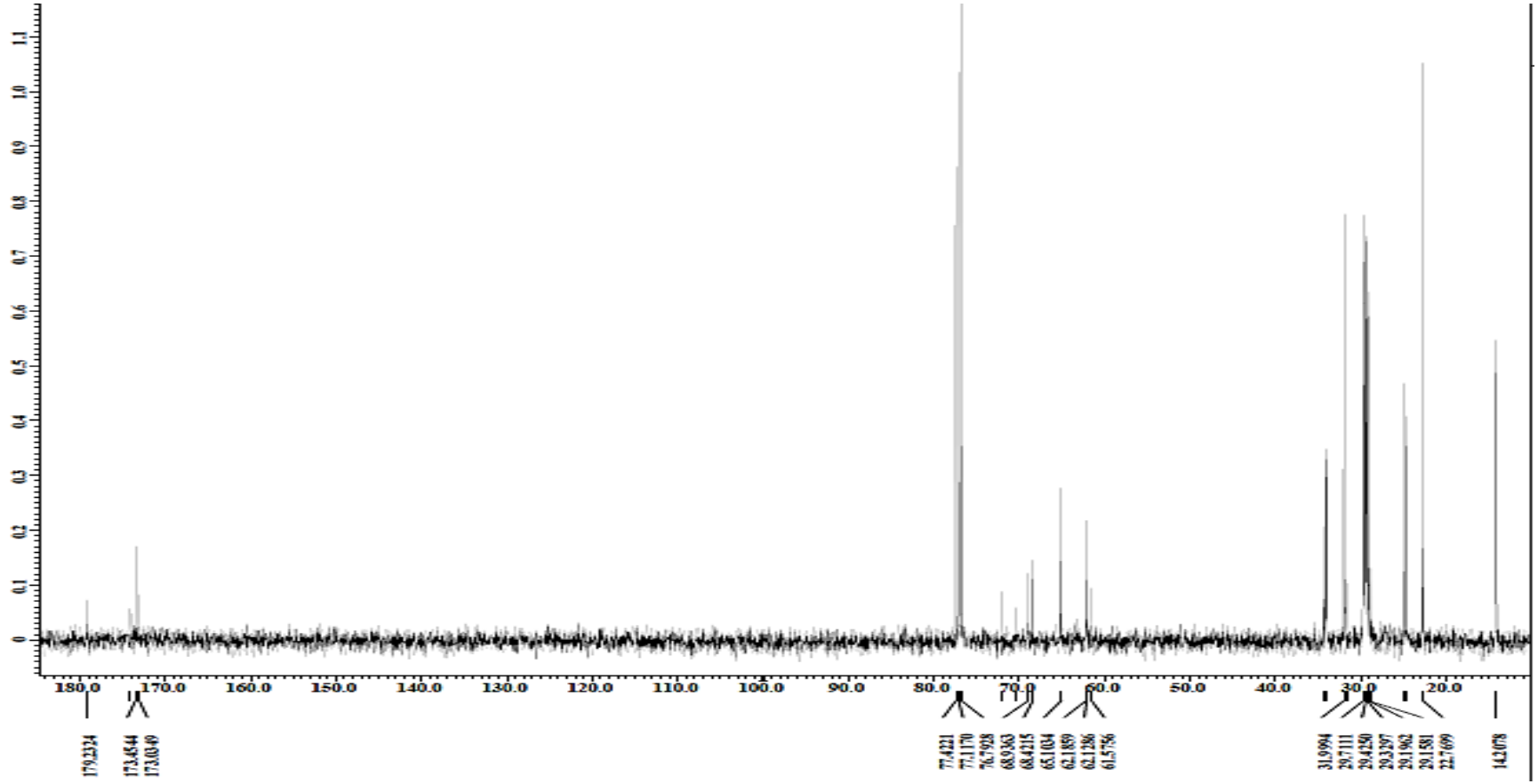


Figure 4.13| **^{13}C – NMR spectrum of MCO dissolved in deuterated chloroform.** Chemical shifts were determined similar to MCO except for the carbonyl carbon atoms. The carbonyl carbon atom from VCO appears to have been split into three majorly confirming the digestion of triglycerides into mono and free fatty acids.

Therefore, NMR spectroscopic studies confirmed the modification of VCO to MCO with critical identification shifts over the region of 173 to 179 ppm and 50 to 90 ppm. These shifts can be used in further studies as critical quality attributes of MCO discriminating it from its precursor.

A comparative spectroscopy studies have been kindly supported by Magritek, Germany using bench-top Spinsolve Carbon NMR spectrometer at 60 MHz. Figure 4.14 represents the NMR spectrum of MCO, the characteristic peaks of C – O of glycerol connected to esterified carbons were not established when compared to Figure 4.13. However, the carbonyl carbons have been established similar to 100 MHz resolution. From the Figure 4.15, the carbon shifts between 20 to 40 ppm have been determined inverted at 135^{o} angle confirming the presence of different types of methylene bridges. As discussed earlier, these shifts have been observed in 100 MHz frequency resolution and the assumption of methylene bridges of different carbon chain fatty acids have been confirmed now with Distortion Enhancement by Polarisation Transfer (DEPT) analysis. Similarly, the results from DEPT analysis at 90^{o} didn't have any peaks inverted to that of 45^{o} (Figure 4.15), these results had confirmed the absence of tertiary carbon atoms due to any substitution or cross linking in the MCO sample.

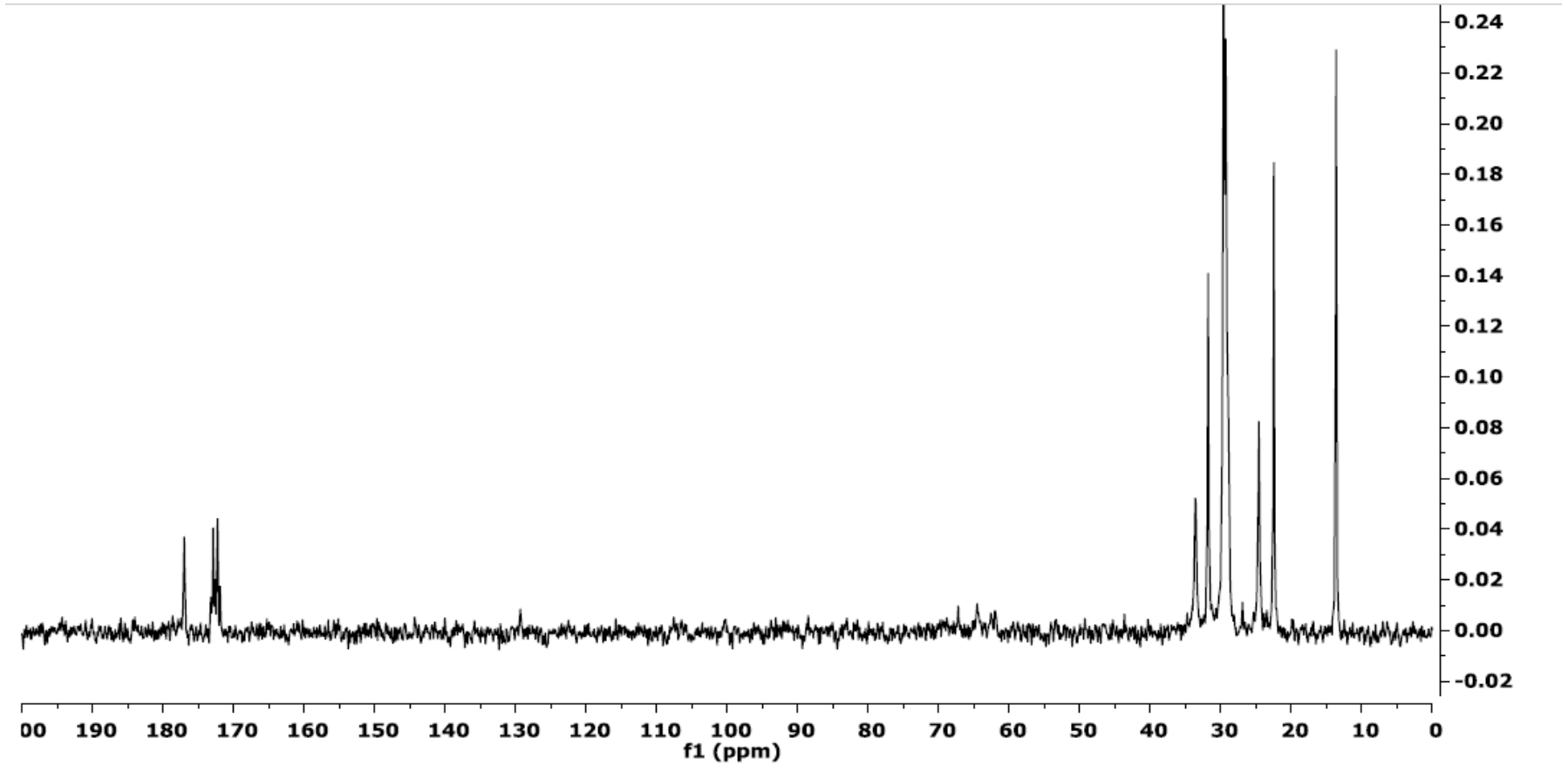


Figure 4.14| ^{13}**C-NMR Spectrum of MCO determined using 60 MHz bench top spectrometer.** MCO sample was prepared by dissolving it in deuterated chloroform for the analysis.

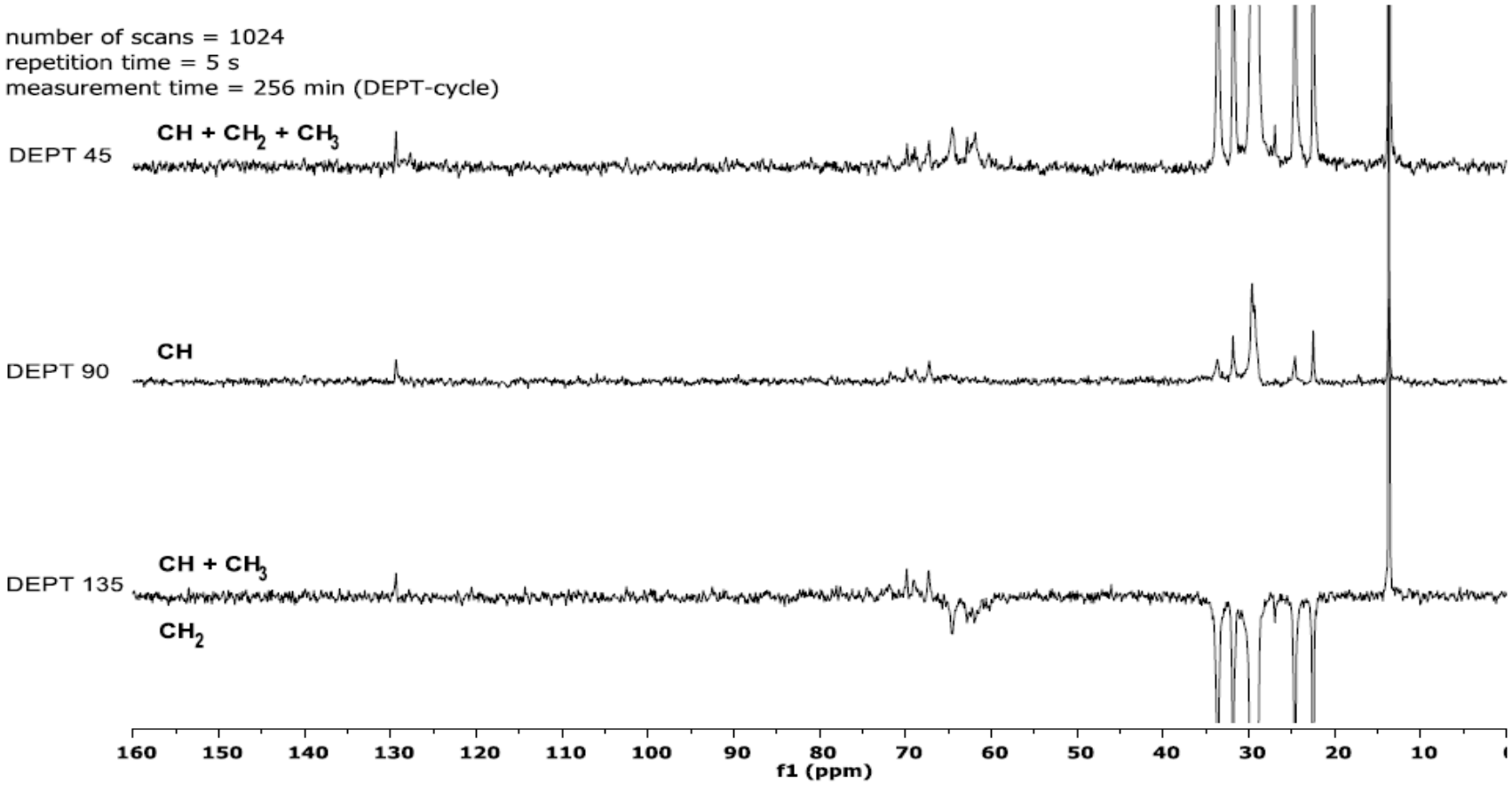


Figure 4.15| **DEPT NMR analysis of MCO. At 45° angle every carbon appears on the positive axis, at 90° angle the tertiary carbon atoms of C – H linkage appears inverted in the negative axis and 135° angle, the secondary carbon atoms of methylene (CH_2) bridges appear inverted.**

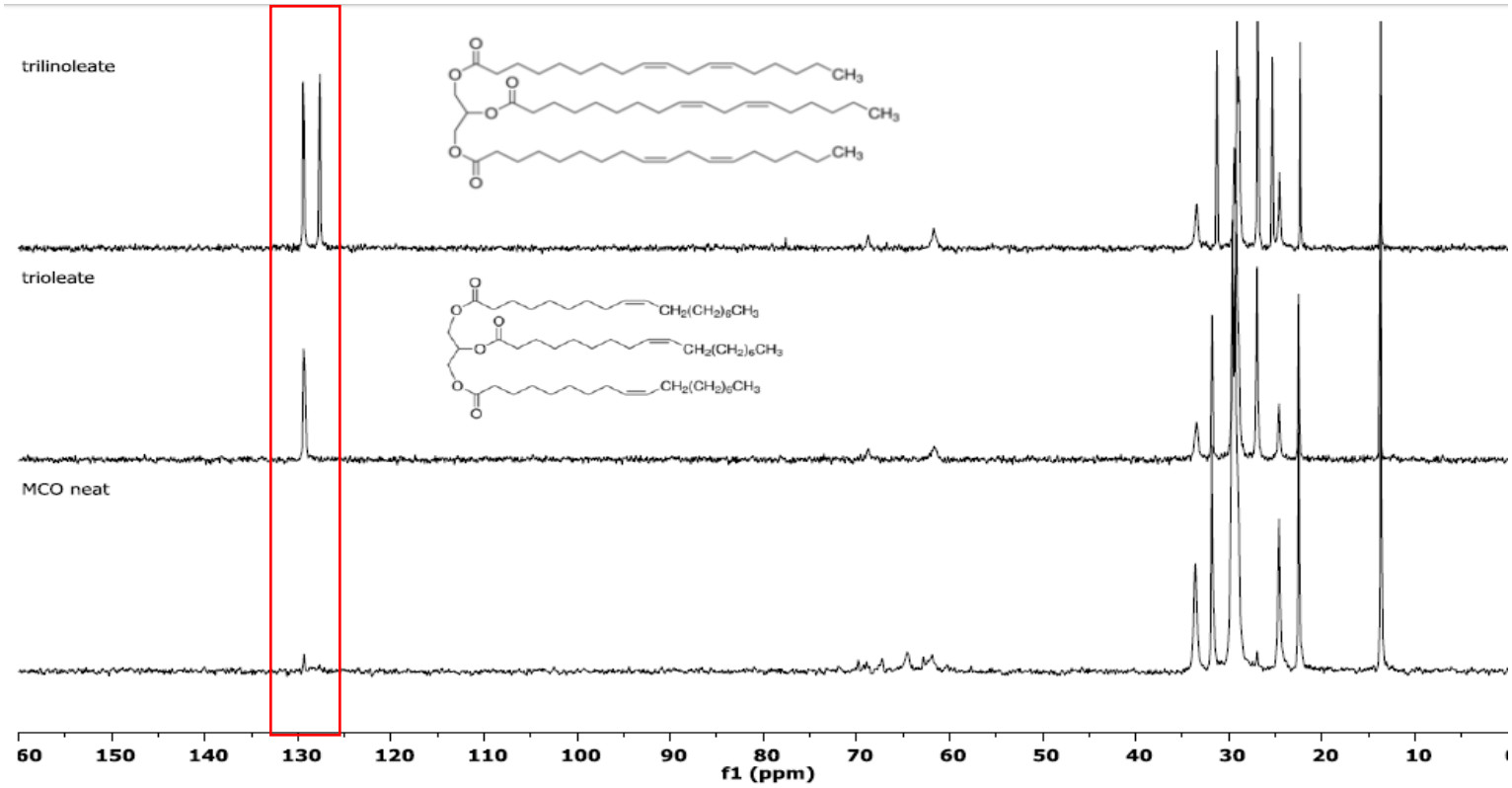


Figure 4.16| **Comparison of ^{13}C-NMR spectra of trilinoleate and trioleate with MCO.** The alkene carbon atoms present in trilinoleate and trioleate which appeared at 130 ppm, were determined to be very minimal to null in MCO.

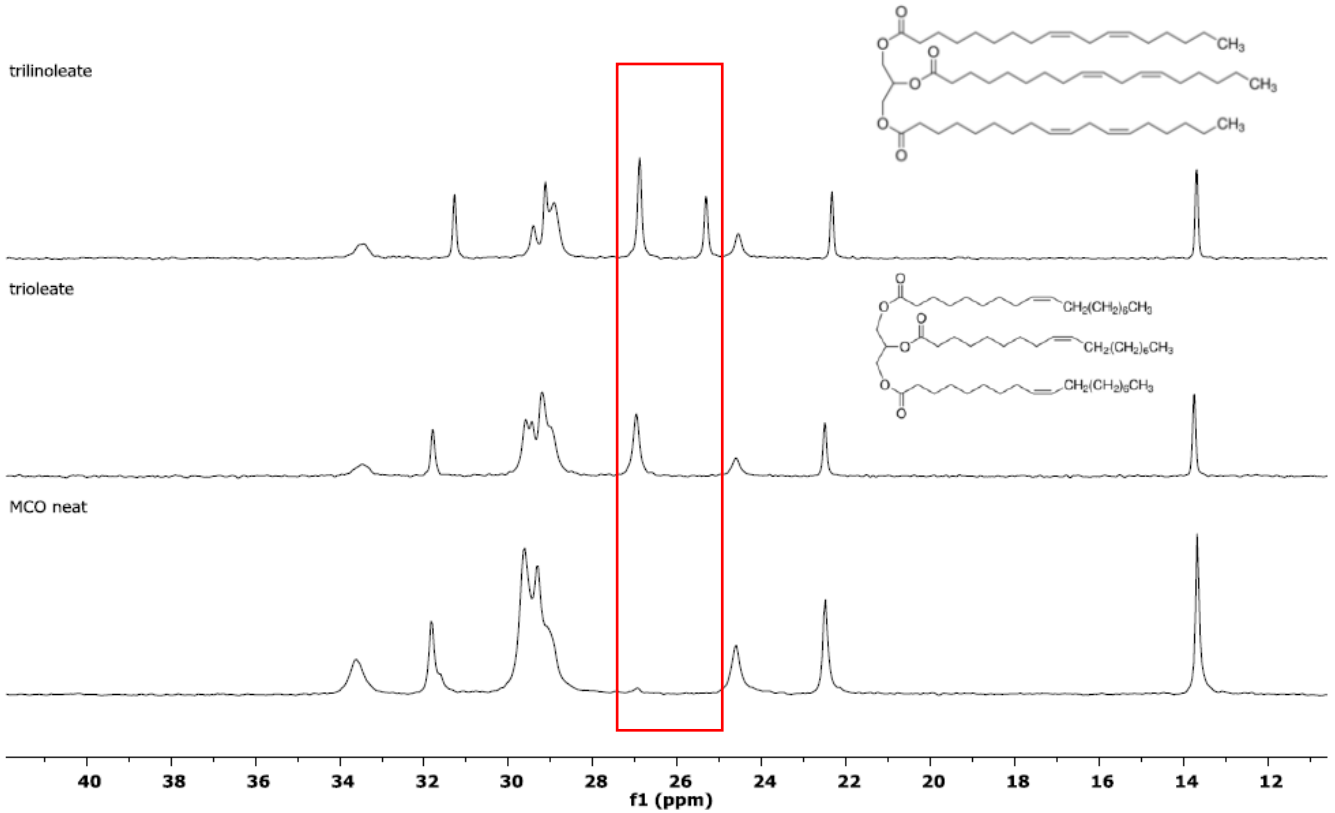


Figure 4.17| **Comparison of ^{13}C-NMR spectra of trilinoleate and trioleate with MCO.** The alkene carbon atoms present in trilinoleate and trioleate which appeared at 34 to 28 ppm were determined to be very minimal to null in MCO.

The Figures 4.16 and 4.17 confirmed MCO as a saturated molecule as the peaks representing alkene carbon atoms were absent in the respective spectrum. These determinations affirm the results obtained during IR spectroscopic analysis. The characteristic alkene carbon shifts present in trilinoleate and trioleate over the regions of 24 to 28 ppm were not observed in MCO. By comparison, an exceedingly small shift has been determined at 130 ppm in the NMR spectrum of MCO. This unsaturation could be due to the residue of raw materials used in the manufacturing process.

Therefore, from the results above, it was concluded that MCO is a saturated compound with a mixture of tri and monoglycerides and free fatty acids.

4.3.5 Investigation of toxicological elements bypassing hepatic metabolism.

4.3.5.1 Limulus amoebocyte lysate (LAL) – Bacterial endotoxin testing (BET) of MCO

In the gel-clot technique, a formation of the gel is considered as positive (+) for the presence of endotoxin and *vice versa* for the absence. In this method, there will be gel formation in the 2λ and λ dilutions, as the LAL reagent procured was sensitive at the corresponding concentrations. Hence, any gel formation below λ concentration confirms pyrogen in the sample. After the incubation period, the MCO had not formed a gel at 0.5 and 0.25λ confirming the absence of pyrogens (Table 4.11 and Figure 4.18).

Table 4. 11| **Results of LAL-BET performed for the detection of pyrogens.** +sign - indicates the formation of clot and the -sign indicates vice versa. Clot formation was not observed in MCO samples below λ dilution.

Dilution concentration of endotoxin	Neat MCO
2λ	+
λ	+
0.5λ	-
0.25λ	-

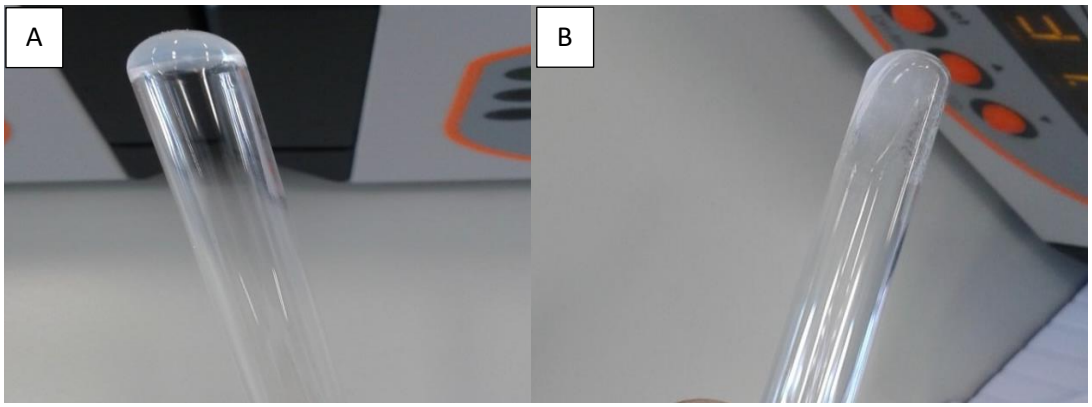


Figure 4.18| **(A) Vial with gel clot – positive result (B) Vial with no clot – negative result.** The manufacturer had provided a positive control, which was tested in the vial of picture A, whereas the sample in picture B is MCO, which was free from endotoxins. Therefore, the raw materials used, and the manufacturing process employed were free from endotoxin and MCO is free from pyrogens for topical administration.

4.3.5.2 Trace metal determination by flame atomic absorption spectroscopy

The principles of USP <401> Fats and Fixed Oils were used to determine the concentration of trace metals in MCO by flame atomic absorption spectrometry. In this context, for the quantification of trace metals, direct calibration and the method of additions have been prescribed. The results obtained during the method development using direct calibration (See Figure 4.19 for calibration plots) for trace metal detection in MCO were as below. This analysis was put in place to establish possible elemental contamination from materials or leaching from contact surfaces.

Table 4. 12| **Absorbance values of various metals determined in Standards for direct calibration method.**

Sample	concentration (ppm)	Cu	Pb	Ni	Fe	Cd	Zn
Standard 1	0.5	0.0546	0.0057	0.0313	0.0239	0.1240	0.1008
Standard 2	1.0	0.1113	0.0105	0.0598	0.0449	0.2224	0.1886
Standard 3	2.0	0.2142	0.0215	0.1213	0.0868	0.4034	0.3191
Standard 4	4.0	0.3942	0.0430	0.2266	0.1694	0.6713	0.4824
Standard 5	8.0	0.6341	0.0830	0.4083	0.3032	0.9509	0.6111

Table 4. 13| **Absorbance values of various metals determined in MCO by direct calibration method.**

Sample	Cu	Pb	Ni	Fe	Cd	Zn
Average (MCO)	0.0010	0.0055	0.0185	0.0138	0.0205	0.0437
Std.Dev	0.0005	0.0012	0.0065	0.0022	0.0053	0.0072
Net	0.0004	-0.0013	-0.0039	-0.0135	-0.0021	-0.0007
Average (Blank)	0.0006	0.0068	0.0224	0.0273	0.0226	0.0444
Std.Dev	0.0009	0.0004	0.0008	0.0010	0.0002	0.0008
Slope	0.0766	0.0103	0.0501	0.0373	0.1083	0.0650
LOD*	0.0376	0.1125	0.0539	0.0908	0.0063	0.0413
LOQ*	0.1141	0.3410	0.1634	0.2752	0.0192	0.1253

*- In this thesis the LOD & LOQ is included for information purposes. It is acknowledged that the procedure followed was compendial and would not normally need formal validation including Pb because of its extreme importance but it is acknowledged that the slope is sub-optimal.

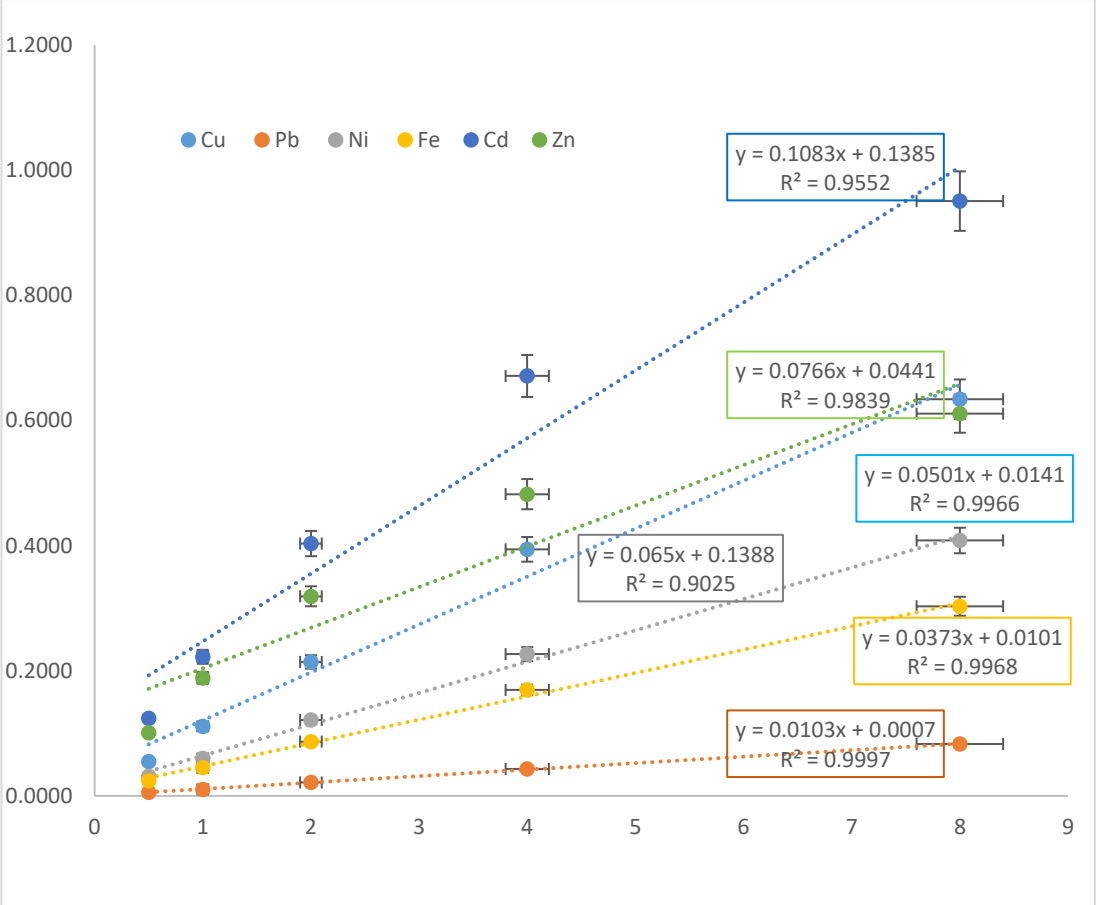


Figure 4.19| **Calibration curves determined for various concentrations of Cu, Pb, Ni, Fe, Cd and Zn by a direct calibration method.** The bars indicate the percentage standard error.

The limit of detection and limit of quantification were carried out as per the ICH Q2 (R1) as below:

$$LOD = (3.3) * \sigma/S$$

$$LOQ = (10) * \sigma/S$$

Where 'σ' is the standard deviation of the blank and '*S*' is slope of the calibration curve.

From the Tables 4.12 and 4.13, the absorbance of difference values, i.e., the net absorbance values of MCO after subtracting the blank values fall below the limit of detection indicating no traceable metals in it.

Therefore, MCO being constituted of proprietary ingredients, and its established manufacturing procedures are free from Cu, Pb, Ni, Fe, Cd and Zn and do

not require any risk assessment process as per ICH Q3D Class 1, 3 and four elemental impurities (See Table 2.2).

Table 4. 14| **Published PDE of the tested elements when administered through dermal route** (Bouvier *et al.,* 2018)

Class	Included Elemental Impurities	PDE (µg/day)
1	Pb	5
1	Cd	5
3	Cu	3000
3	Ni	110
4	Fe, Zn	N/A

In the review submitted by Elsayed (2015), it was mentioned that the antifungal molecules that are sublimable can reach the deeper tissue layers below the infected nail plate through the pores formed during fungal proliferation and mycotic lesions and are thus likely to enter the blood stream. Similarly, metals such as iron and zinc in their ionic forms act as cofactors for the enzymes of the pathogen to continue its metabolism over the site of infection. Considering the above-mentioned possibilities, MCO was screened for the presence of any such toxic impurities as the effect of them could be higher based on the severity of infection.

4.4 Excipient considerations: Incompatibility studies of AAE with excipients towards targeted profile

Excipients are an integral part of every formulation and therefore careful selection and decisions on nature and proportional quantity have significant roles in the development programme. Furthermore, the safety and tolerability of excipients so selected need to be carefully considered.

A particularly crucial component of QbD – informed product development according to the ICH Q8 (R2) guideline is to develop the intended dosage form with a quality built-in strategy as per the patient-oriented treatment. From the QTPP, it was established that a topical o/w emulsion system as the intended dosage form design was the optimal route. Similarly, physical attributes such as particle size distribution, consistency, viscosity and cohesiveness were so identified as elements of the QTPP affecting the content uniformity, the penetration of AAE at the targeted site and formulation stability.

As mentioned earlier (Section 2.3), emulsifiers are the moieties with hydrophilic and lipophilic fractions facilitating the interactions between the dispersed and continuous phases for a uniform distribution. In this research, long-chain fatty acid (LCFA) based emulsifiers have been chosen to reduce potential incompatibility with MCO. LCFAs have been neutralised with inorganic and organic basic compounds to incorporate sufficient hydrophilicity. Thus, designed emulsifiers have been used for the emulsification in the presence of any viscosity-enhancing agent to improve and maintain the thixotrophy. However, with potential excipients whether compendial or indeed novel, it is required to examine the compatibility to assess and de-risk the long-term stability of the formulation.

Traditionally acacia-based preparation of o/w emulsions constitute the ratio of oil:water: emulsifier as 4:2:1 for fixed oils. As MCO is a novel entity, the compatible ratio of emulsifier to that of water as a continuous phase has been established through scouting experiments of various formulations including the above-mentioned excipients at various combinations (Table 3.5 and 3.6). From the preliminary investigation of antimicrobial potency, it was determined that MCO was

active and efficient from the time of application at 15% w/w concentration. Hence, this percentage was maintained constant in all the formulations.

Table 4. 15| **Compendial excipients chosen for the emulsification of MCO and their role in the formulation**

Excipient	Function
Stearic acid (SA)	LCFA for emulsifier preparation
Decanoic acid (DA)	LCFA for emulsifier preparation
Sodium hydroxide (NaOH)	Inorganic base for emulsifier preparation
Monoethanolamine (MEA)	Organic base for emulsifier preparation
Diethanolamine (DEA)	Organic base for emulsifier preparation
Triethanolamine (TEA)	Organic base for emulsifier preparation
Tween 20	Non-ionic surfactant
Polyethylene glycol (PEG MW:35000)	Viscosity modifying agent
Water	Continuous phase

A particle size distribution in a dispersed system is a result of interactions between the emulsifier and constituent phases. Therefore, selection and optimising an appropriate emulsifier for the emulsification in terms of qualitative and quantitative interactions is a crucial step to improve the performance as well as stability of the system. Similarly, a viscosity-enhancing agent has been also chosen to improve and maintain the thixotrophy of the emulsion during storage and upon application onto the targeted site of action. Hydration of nail plate increases the pore size of the keratin plate thereby enhancing the permeation of some active molecules towards the targeted site. Hence, an ideal hydrating agent with proven viscosity-maintenance properties was chosen in this research. Physical examination has been carried out using DSC, pH, texture analysis, optical microscopy and particle size distribution analysis, whereas IR spectroscopy was used to study the chemical compatibility. Microbiological well diffusion assays have been implemented to evaluate the therapeutic performance of the formulations.

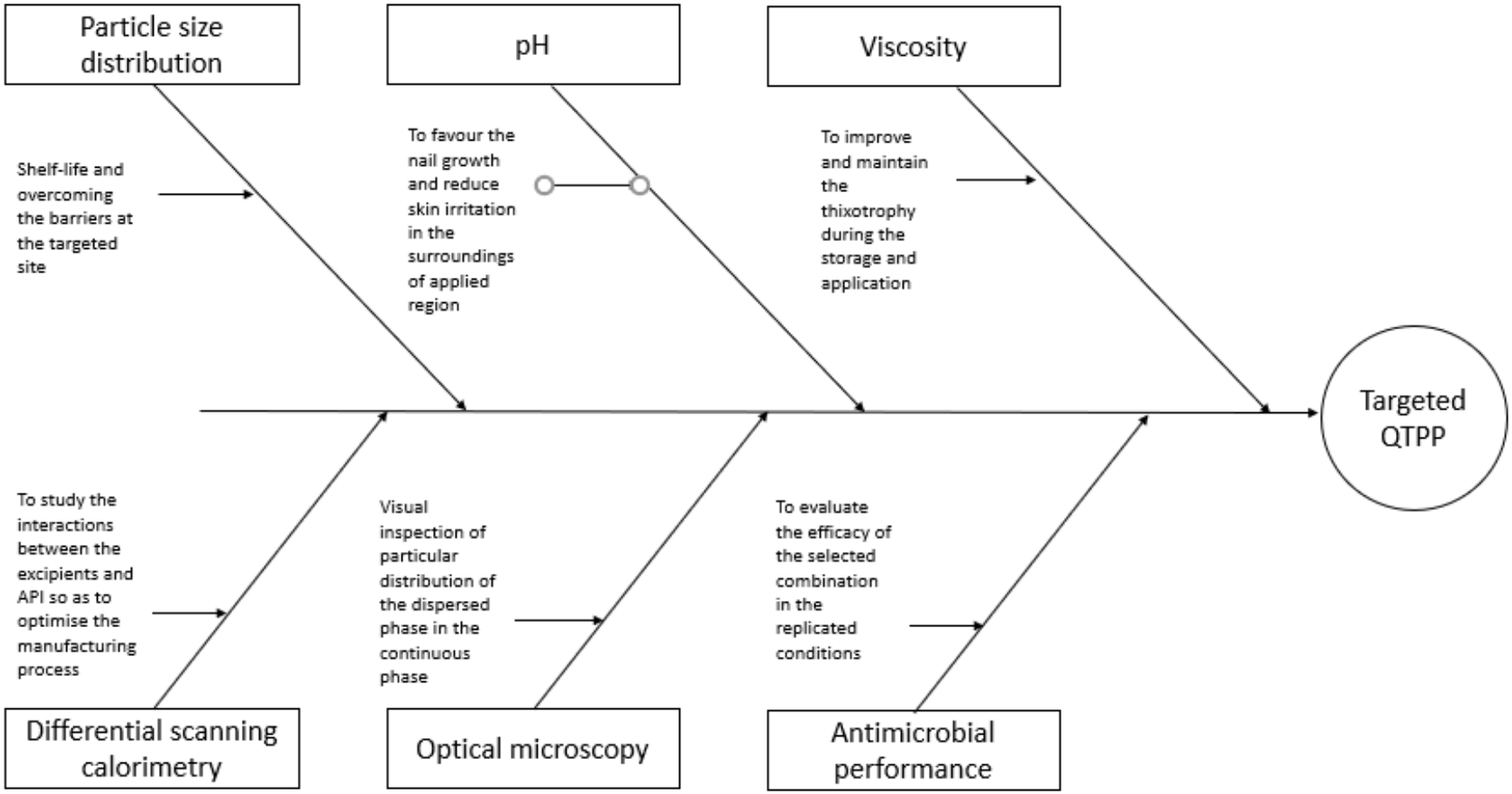


Figure 4.20| **Pictorial representation of strategy employed to eliminate the incompatible excipients towards stable and effective formulation.**

4.4.1 Application of DSC to evaluate thermal compatibility of AAE with excipients.

To emulsify MCO into the aqueous medium a variety of excipients have been chosen. The following Table 4.16 describes the physical properties of excipients in the formulations.

Table 4. 16|**Excipients chosen and the physical properties provided by their manufacturer.**

Name	**Freezing point and boiling point provided by the manufacturer (°C)**
Decanoic acid (DA)	31.6, 268.7
Stearic acid (SA)	69.3, 361
Monoethanolamine (MEA)	10.3, 170
Diethanolamine (DEA)	28, 271.1
Triethanolamine (TEA)	21.60, 335.40
Sodium hydroxide (NaOH)	318, 1388
Polyethylene glycol (PEG) (MW 35000)	65, 250
Tween 20	100
Water	0, 100
MCO	13.04, 284.52

The chosen excipients were well established and are in use in topical formulations for considerable period. In order to avoid time-consuming toxicological assessment, established excipients had been used in this project. Thus, selected excipients had been exposed to thermal conditions in combinations similar to MCO characterisation to study the interactions.

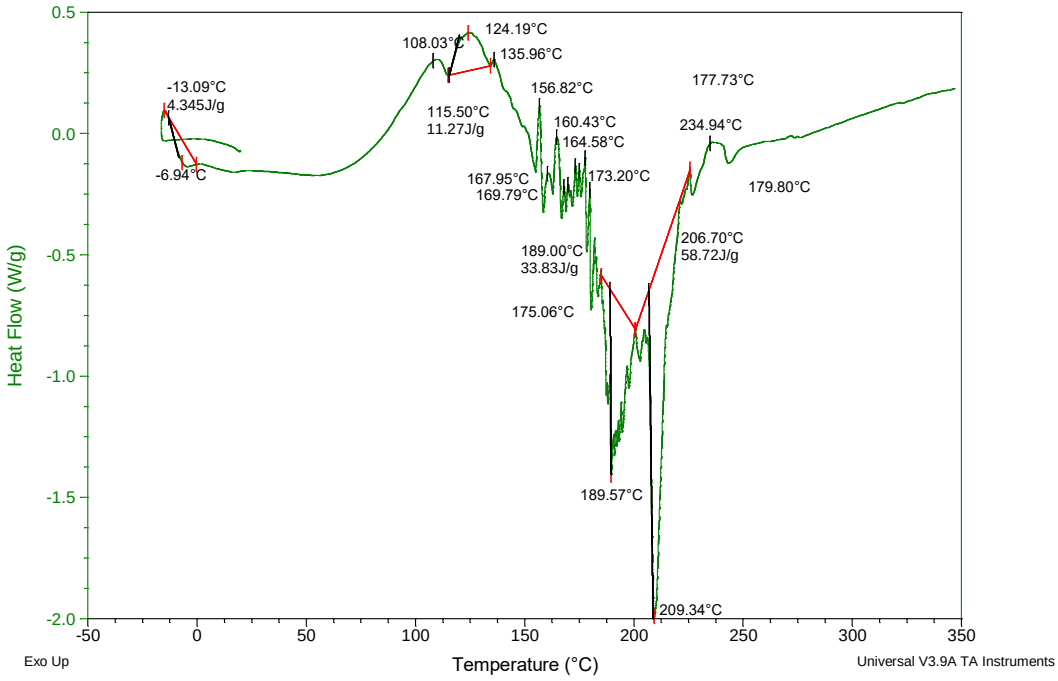


Figure 4.21| **Thermogram of equal proportions of MCO, water, NaOH and Tween20 over a range of -50 to 350^{o}C purged under nitrogen.**

From the IR spectroscopy and acid number experiments, free fatty acids had been determined in MCO. Therefore, a self-emulsifying system has been targeted to solubilise MCO. To achieve this, surfactant formation has been designed by the reaction between the determined free fatty acids of MCO and sodium hydroxide (NaOH). Tween 20 was chosen as secondary surfactant to emulsify additional insoluble triglycerides. From Figure 4.21, various degradation points from 100 to 250^{o}C were determined, indicating fractionation of components and instability. The characteristic peaks of MCO had not been observed in the region of freezing point. Therefore, the emulsifier formed by the combination of sodium hydroxide and MCO and its affinity with Tween 80, water and MCO was determined to be unstable under thermal conditions.

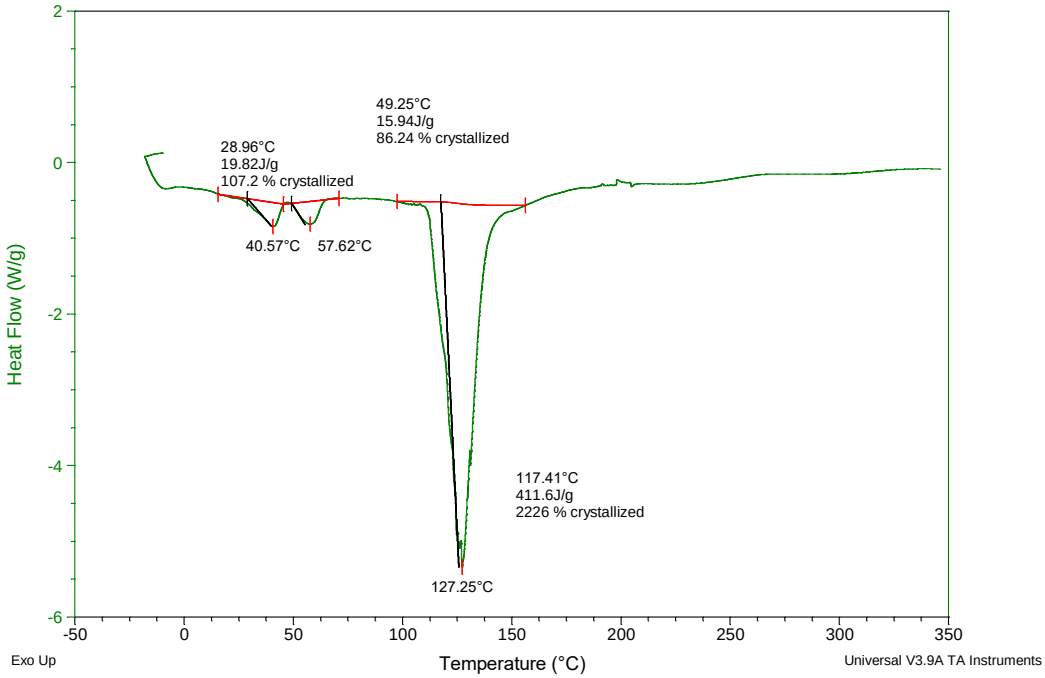


Figure 4.22| **Thermogram of equal proportions of MCO, SA, MEA, PEG and water over a range of -50 to 350°C purged under nitrogen.**

This run was conducted to evaluate the compatibility of MCO with LCFA and MEA. An endothermic peak onset at 28.96°C, a maximum of 40.57°C, additional onset at 49.25°C, maximum at 57.62°C and a final onset at 117.41°C, and maximum at 127.25°C were determined. The former peaks indicated the melting point of DEA and PEG while the latter represented the evaporation of water from the mixture. The characteristic endo and exotherms of MCO and SA had not been observed representing low interaction with the components. These results indicated the incompatibility of MEA with MCO due to separation.

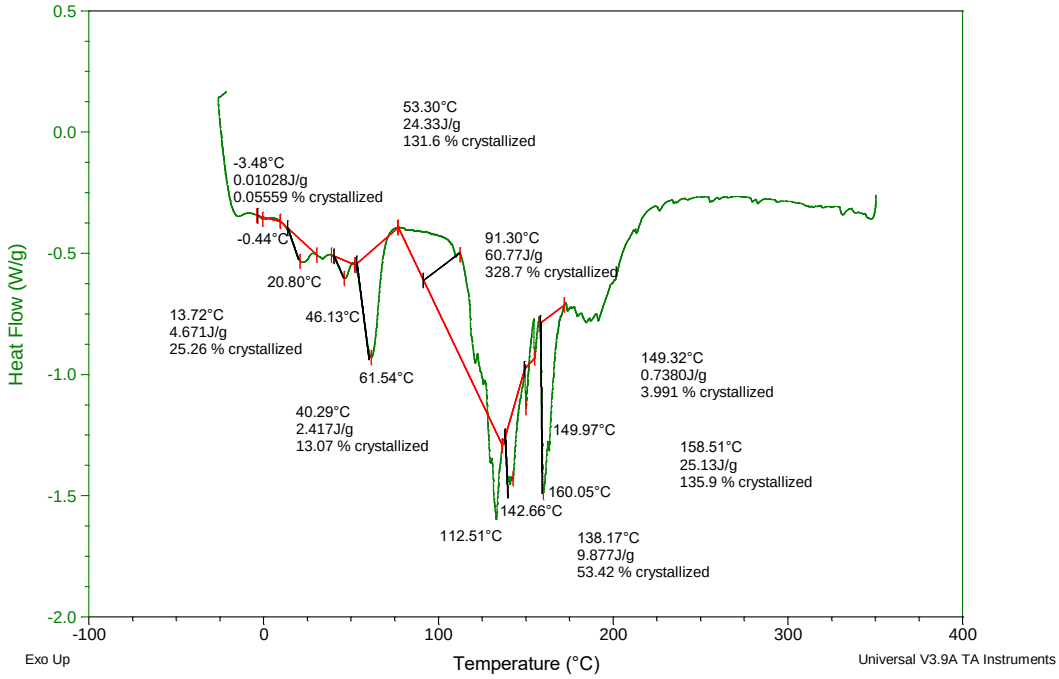


Figure 4.23| **Thermogram of equal proportions of MCO, SA, DEA, PEG and water over a range of -50 to 350°C purged under nitrogen.**

DEA is the functional analogue of MEA with one more hydroxyl group and increased polarity. It was used in this research as an organic basic compound to form emulsifier with LCFA. The hypothesis was to have increased interaction with the hydrophilic groups to stabilise the system. From Figure 4.23, attributes of MCO were determined over the region of 10 to 20°C with an onset at 13.72°C and peak maximum at 20.80°C. Similarly, endothermic peak onset at 40.29°C and maximum at 46.13°C indicated the shift in the melting point of DEA. Peak onset at 53.30°C and maximum at 61.40°C represented the depression in the freezing point of PEG. Water was determined to be evaporated at 112.51°C followed by series of degradation of components at higher temperature. The interaction between the components of this mixture was observed to be compatible below 100°C, whereas phases had separated and degraded at higher temperatures above it. Therefore, it was understood the combination of MCO, SA, DEA, PEG and water was compatible at lower temperatures and unstable at more elevated conditions.

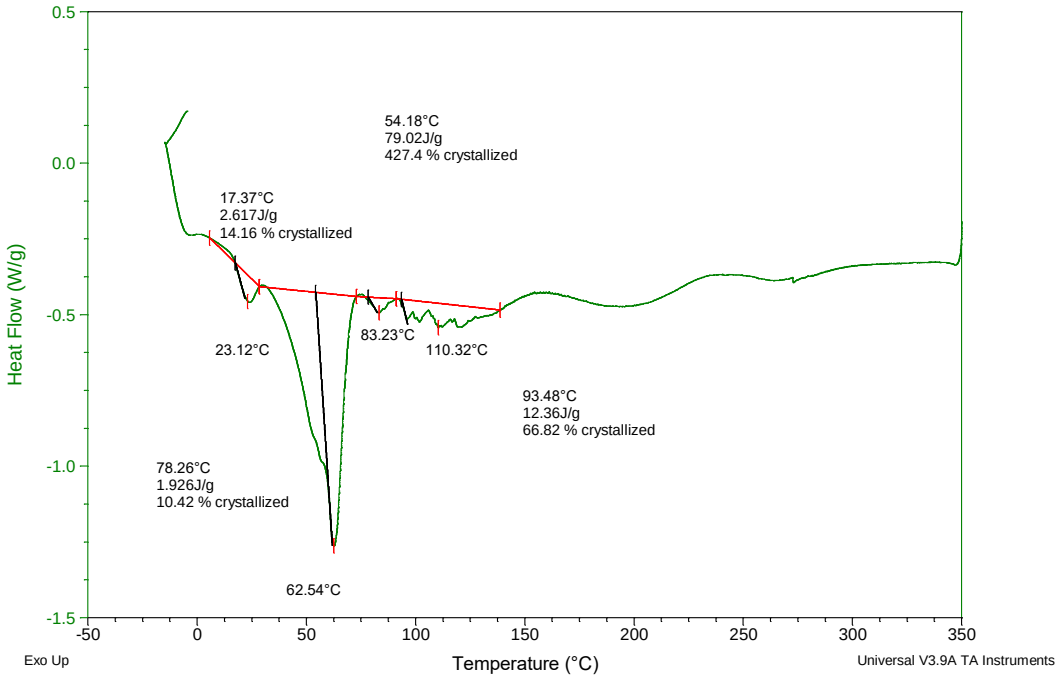


Figure 4.24| **Thermogram of equal proportions of MCO, SA, TEA, PEG and water over a range of -50 to 350^{o}C purged under nitrogen.**

Triethanolamine is a popular excipient in many topical dosage forms as emulsifier by salt formation. It is highly viscous and with larger number of hydroxyl groups than both DEA and TEA. Upon salt formation with LCFA, it forms a quaternary ammonium salt with a highly polar hydrophilic group and nonpolar, long-chained lipophilic group. From Figure 4.24, endothermic peaks at 17.37^{o}C confirmed the fingerprint of MCO and at 23.32^{o}C represented elevated melting point of TEA. Similarly, peaks at 62.54^{o}C and 83.23^{o}C indicated the interactions of PEG with other components. Water had evaporated at 110.32^{o}C without any further degradation peaks at elevated temperature conditions. Shifts in the peaks from fingerprint regions of the respective components and absence of degradation of the components strongly suggested the compatibility and thermal stability of this MCO, SA, TEA, PEG and water combination.

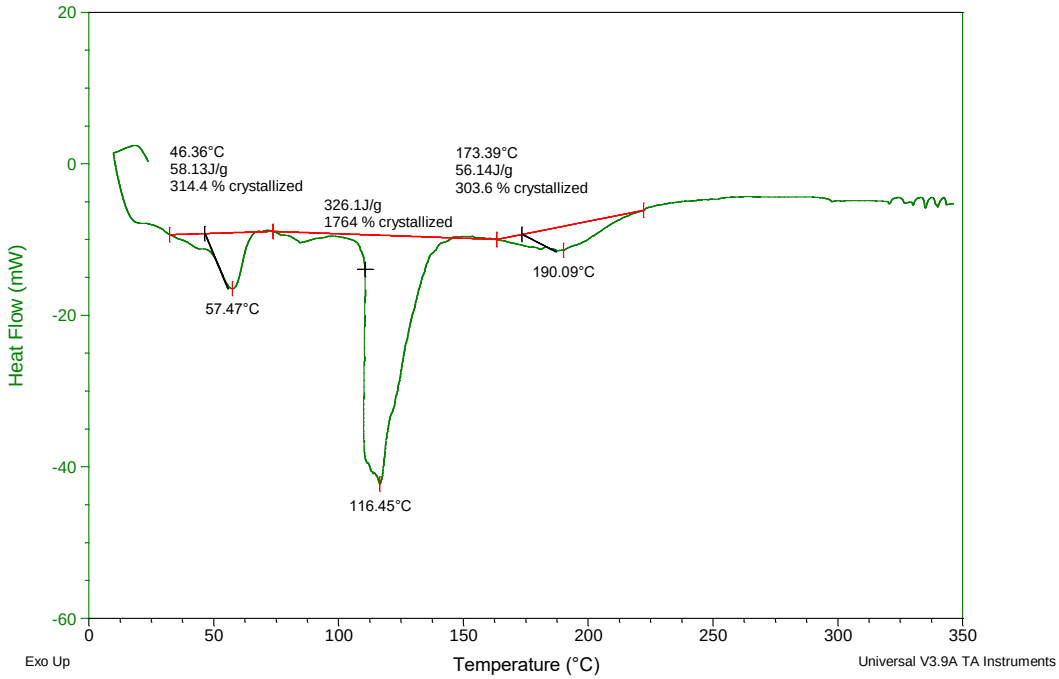


Figure 4.25| **Thermogram of equal proportions of MCO, DA, MEA, PEG and water over a range of -50 to 350°C purged under nitrogen.**

Decanoic or capric acid is widely used in pharmaceutical industry to esterify some drugs and improve their lipophilic character. Considering this property of DA, it was used in this research to solubilise MCO by forming fatty acid chain of the emulsifier. Similar to SA, DA was also treated with MEA and other respective components to determine its compatibility. In the thermogram (Figure 4.25), peaks at 57.47°C, 116.45°C and 190.09°C were determined for elevated melting point of DA, evaporation of water and MEA. The fingerprints of MCO and PEG were not observed, which indicated poor interaction between the components of the mixture. Therefore, it was confirmed that the combination of MCO, MEA and PEG was unstable and incompatible.

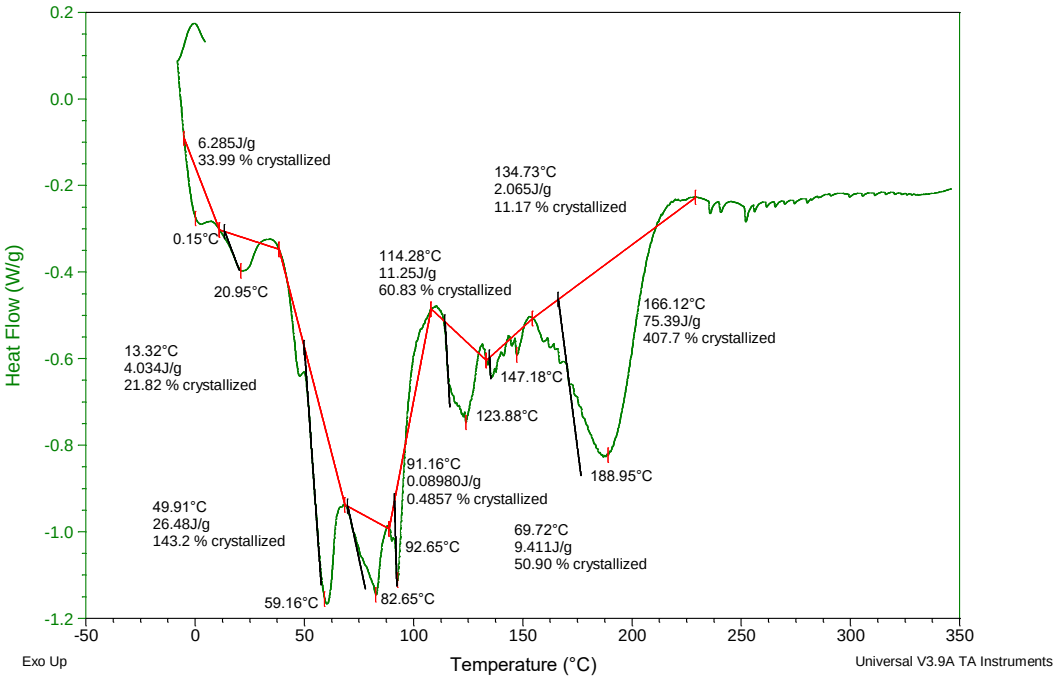


Figure 4.26| **Thermogram of equal proportions of MCO, DA, DEA, PEG and water over a range of -50 to 350ºC purged under nitrogen.**

From Figure 4.26, the peak at 20.95ºC confirmed the presence of MCO. The melting point of DEA was elevated to 59.16ºC and DA was at 82.65ºC. PEG was determined to be melted at 92.65ºC. The amount of water added was observed to have evaporated at 123.88ºC followed by serial degradation of components. From this it was concluded that the interaction between the constituents was poor as the components the respective endo and exotherms were observed individually. Therefore, the combination of MCO, DA, DEA, PEG and water is unstable under thermal conditions.

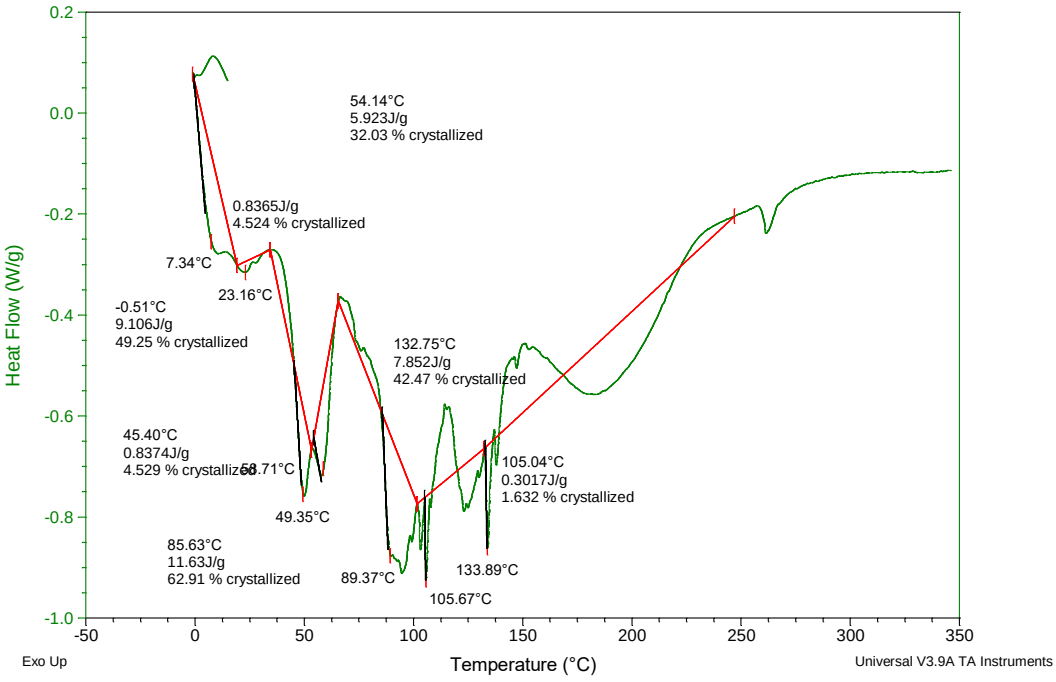


Figure 4.27| **Thermogram of equal proportions of MCO, DA, TEA, PEG and water over a range of -50 to 350^{o}C purged under nitrogen.**

In this experiment, the interactions between DA and TEA were studied as emulsifier formed by the reaction between LCFA and organic base to solubilise MCO into aqueous base. The fingerprint of MCO was traced at 23.16^{o}C, TEA melted at 49.35^{o}C and DA at 53.71^{o}C (Figure 4.27). The melting point of PEG was elevated to 89.37^{o}C and fractioned into several components further. Water was determined to be evaporated at 133.69^{o}C indicating colligative interaction. This combination was determined to be unstable as peaks of components appeared individually with high drift from their original positions.

From the law of colligative properties, a presence of impurity or solute in a solvent leads to distinct depression in freezing point or elevation in the boiling point, with its magnitude proportional to the mole fraction of the solute or impurity. However, in the above discussed cases, there was an elevation in the boiling point of the components from their neat values, but no peaks were determined describing the expected interaction between LCFA and organic or inorganic base compounds. Therefore, from the thermal investigation, combination of MCO, SA, TEA and PEG has

been determined sufficiently stable due to low shifts in the peaks and higher thermal stability.

4.4.2 Application of IR spectroscopy to investigate the chemical interactions between the components of designed formulations.

MCO was targeted to be emulsified by the salt formed as result of reaction between LCFA and organic or inorganic base. IR spectroscopy was applied to evaluate the extent of the interaction and chemical compatibility of thus formed emulsifier with MCO. The respective spectra determined during these examinations are now presented in this section.

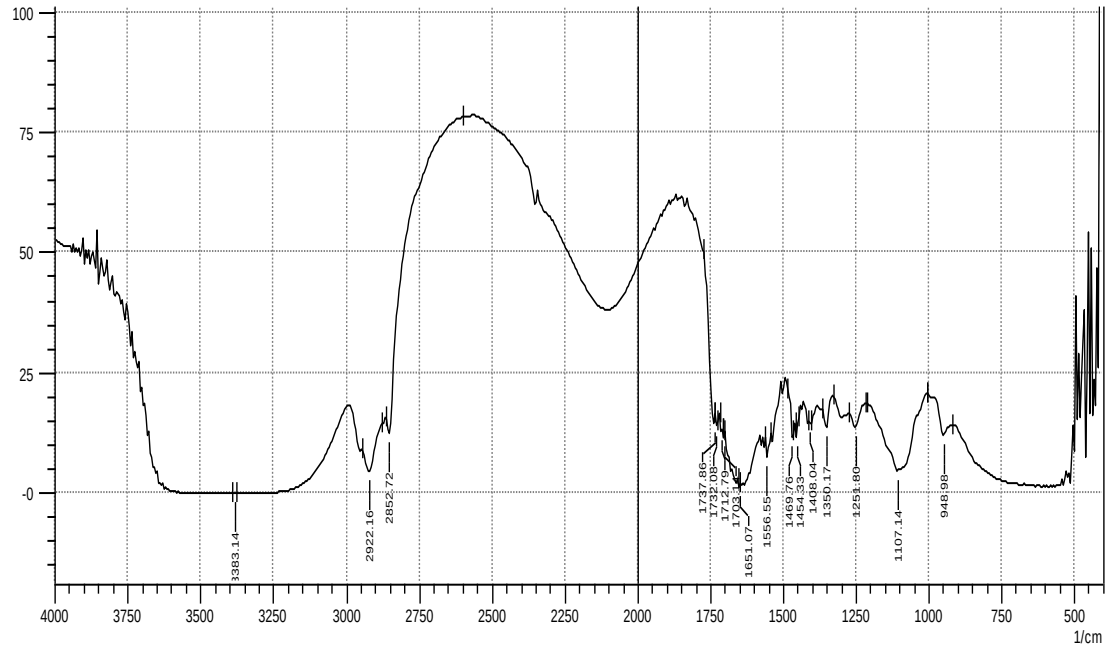


Figure 4.28| **IR spectrum of combination of MCO, Tween 20, NaOH, SA, PEG and water versus air as background (Combinations A, G, H and I).**

From the Figure 4.28, it was determined that formulation A was a combination of saturated ester with a mixture of monoglycerides and triglycerides from MCO. Peaks of carboxylate ions indicated the ionisation of LCFA during the reaction with NaOH. The broad peak of an O – H group at the 3383 cm^{-1} confirmed the presence of free water and incompatibility of PEG in the formulation, as alcohols

produce sharp peaks over the same region. Hence, it was observed that, PEG was incompatible in the mixture of SA, NaOH, MCO, Tween 20 and water.

Table 4. 17| **Interpretation of IR spectrum of combination A, G, H and I**

Peak frequency (cm^{-1})	**Intensity**	**Corresponding functional group/Fundamental vibration**
948	m	Symmetric C – C bend of alkanes
1107	m	Symmetric C – C bend of alkanes
1251	m	Symmetric C – C bend of alkanes
1350	m	Symmetric stretch of carboxylate ion
1408, 1454, 1469	m	Symmetric stretch of acyl group of saturated esters
1651	s	Symmetric carbonyl stretch of saturated amide
1703, 1712	s	Symmetric stretch of carbonyl group of carboxylic acid
1732	s	Symmetric stretch of carbonyl group of saturated monoesters
1737	s	Symmetric stretch of carbonyl group of saturated tri-ester
2852, 2922	s	Symmetric stretch of hydrocarbon of group of alkanes
3383	S, br	Asymmetric stretch of hydroxyl group of water

w = weak; m = medium; s = strong and br = broad.

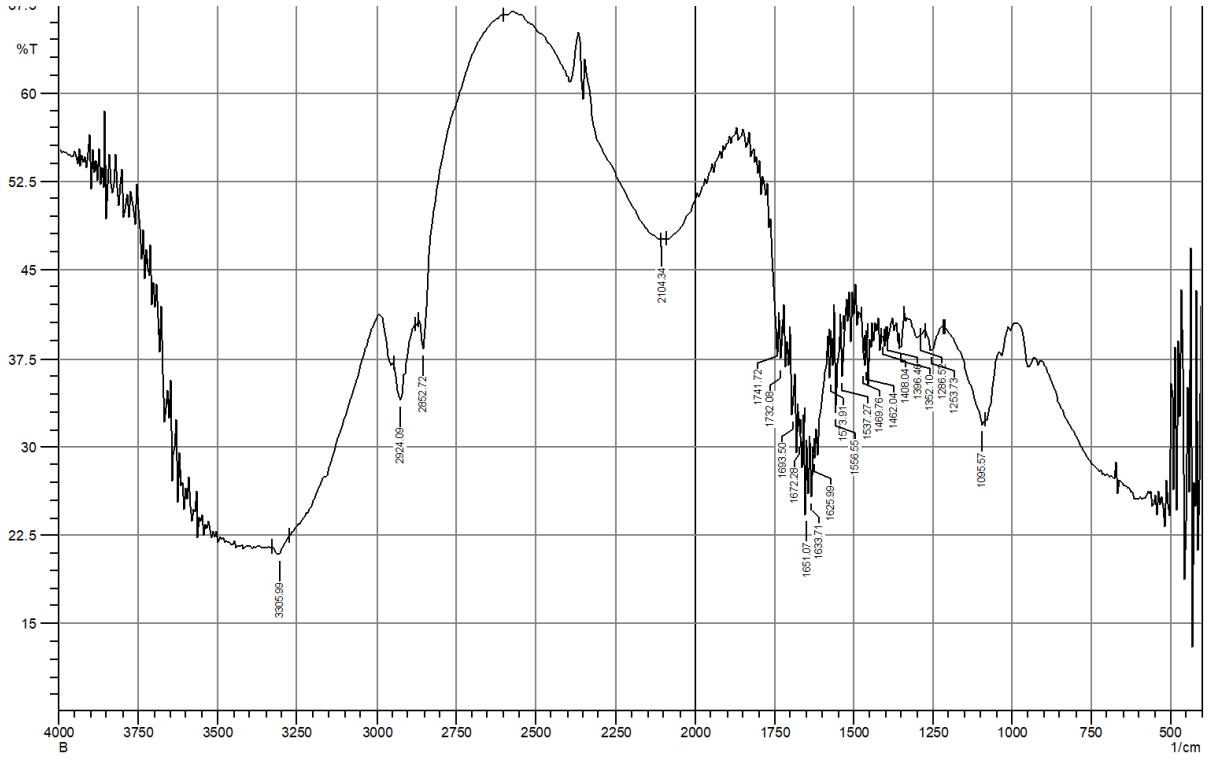


Figure 4.29| **Representative IR spectrum of formulations B, C, D, E, F recorded versus air as background.**

B, C, D, E and F formulations had SA with increasing concentration from 2 to 8% w/w. The compositions of the rest of the ingredients were similar to that of A, G, H and I formulations. These experiments had been conducted to evaluate the compatibility of SA in similar environment to that of A to I formulations. From the Figure 4.29, it was deduced that B, C, D, E and F formulations had traceable fingerprints of every excipient. Similarly, the amides formed by the reaction between free fatty acids of MCO and SA used were determined from 1620 to 1700 cm^{-1}. However, the characteristic peaks of MCO were overlapped by the SA related carboxylate ions and Tween 20 molecules. Similarly, sharp shoulder peaks over the range 3250 to 3750 cm^{-1} indicated free range O-H groups due to improper ionisation of sodium hydroxide with SA (Table 4.18). Hence it was concluded that SA was more compatible with MCO and other excipients except NaOH.

Table 4. 18| **Interpretation of IR spectrum of Figure 4.29**

Peak frequency (cm^{-1})	**Intensity**	**Corresponding functional group/Fundamental vibration**
948	m	Symmetric C – C bend of alkanes
1253, 1286	m	Asymmetric C – C bend of alkanes
1362, 1396	m	Symmetric stretch of carboxylate ion
1408, 1462, 1469	m	Symmetric stretch of acyl group of saturated esters
1537, 1573	m	Stretch of C – O groups of Tween 20
1625, 1633, 1661, 1672, 1693	s	Stretch of carbonyl groups of primary amides
1732	m	Stretch of carbonyl groups of saturated monoglycerides
1741	m	Stretch of carbonyl groups of saturated triglycerides
2852	s	Stretch of hydrocarbon of group of alkanes
2924	s	Stretch of hydrocarbon of group of alkanes
3304	S, br	Stretch of hydroxyl group of water

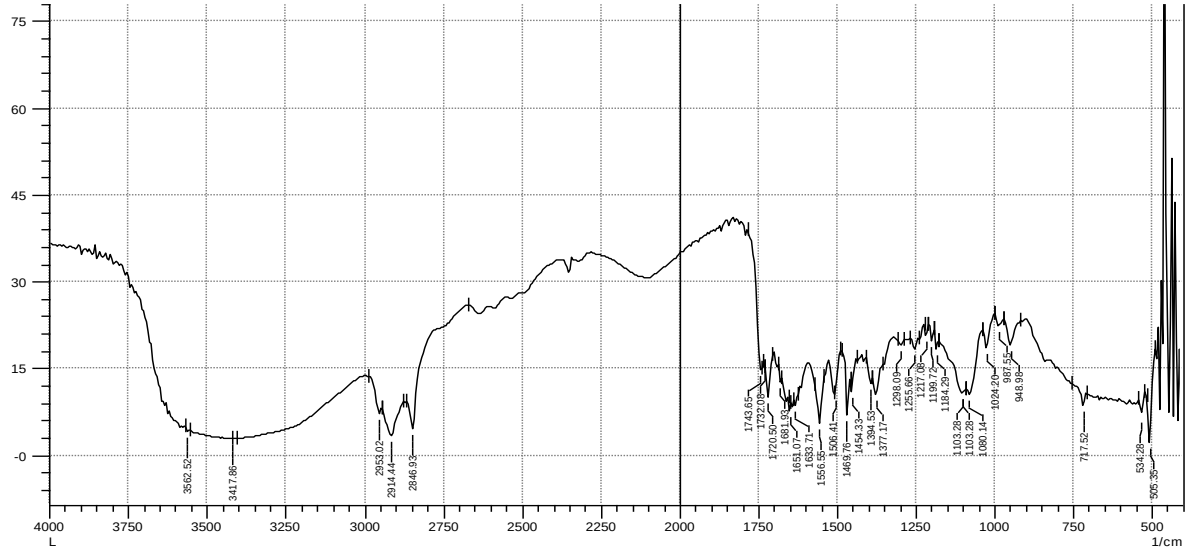


Figure 4.30| **Representative IR spectrum of J, K and L formulations recorded versus air as background.**

Formulations J, K and L were designed with SA as LCFA and MEA as organic base to form emulsifier by salt formation. From the Figure 4.30, the fingerprints of MCO were determined at 1741, 1732 and 1720 cm^{-1} confirming the tri- and monoglycerides and free fatty acids. Peaks below 1720 until 1633 cm^{-1} have proven the formation of amide groups of various fatty acids upon reaction with MEA including DA. The following Table 4.19 describes the interpretation of the peaks of the spectrum.

Table 4. 19| **Interpretation of IR spectrum of Formulations J, K and L**

Peak frequency (cm^{-1})	**Intensity**	**Corresponding functional group/Fundamental vibration**
987	m	Symmetric C – H bend of alkanes
1024, 1080	m	Asymmetric C – H bend of alkanes
1103, 1184, 1199	s	Asymmetric C – H bend of alkanes, alcohols and saturated esters
1217, 1256, 1288	m	Bends of aliphatic primary amines
1377, 1394	m	C – H bends of saturated esters
1454, 1469, 1506, 1556	s	Bends of carboxylate ions and dimers of carboxylic acids
1603, 1661, 1681	s	Carbonyl stretch of amides of various fatty acids with MEA
1720	s	Carbonyl stretch of free fatty acids
1732	s	Carbonyl stretch of monoglycerides
1743	s	Carbonyl stretch of triglycerides
2845, 2915	s	Stretch of saturated C – H bonds
3417	s	O – H stretch of water
3562	s	N – H stretch of amine

Presence of MEA has been confirmed through the identification of peaks over the range of 1200 – 1300 cm^{-1} in the fingerprint region and 3562 cm^{-1} stretch. This affirmed that the reaction between the SA and MEA was incomplete. MCO was identified in its native form without any deviations. This indicated that the interaction of MEA with MCO was unstable.

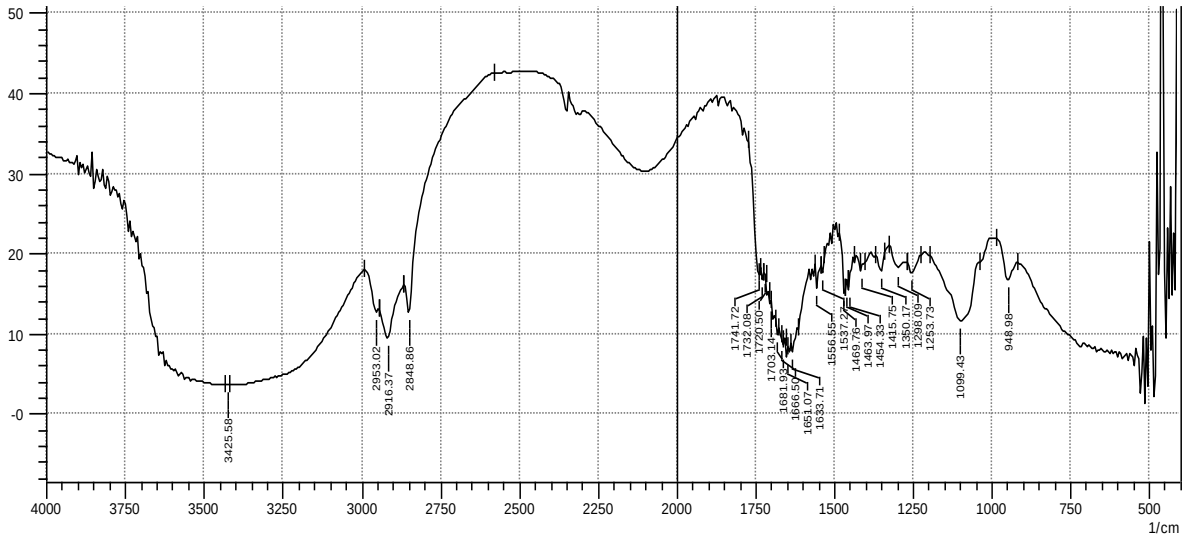


Figure 4.31| **Representative IR spectrum of M, N and O formulations recorded versus air as background. M, N and O formulations were compositional replicates of J, K and L expect for MEA being replaced with DEA.**

The stretches at 1714, 1732 and 1737 cm^{-1} in the Figure 4.31 confirmed the presence of MCO in a stable environment. The increase in the shift of peaks from 1288 to 1298 cm^{-1} indicated the presence of DEA. As no stretches of N – H were observed during the 3200 – 3600 cm^{-1} the extent of salt formation towards emulsifier was comparatively higher than MEA. The peaks over the range of 948 to 1556 cm^{-1} confirmed the presence of long-chained saturated aliphatic groups of SA and esters of MCO. Ionised form or carboxylate ions were determined at 1350 – 1600 cm^{-1} (Table 4.20). But, the intensity of amide groups formed had dominated the MCO functional groups indicating incompatibility.

Table 4. 20| **Interpretation of IR spectrum of M, N and O formulations**

Peak frequency (cm^{-1})	Intensity	Corresponding functional group/Fundamental vibration
948	m	Symmetric C – H bend of alkanes
1099	s	Asymmetric C – H bend of alkanes
1253, 1298	m	Bends of aliphatic secondary amines
1350	m	C – H bends of saturated esters
1417, 1456, 1463	m	Bends of carboxylate ions and dimers of carboxylic acids
1539, 1556		
1633, 1651, 1681, 1703	s	Carbonyl group of amides of various fatty acids with DEA
1714	m	Carbonyl group of free fatty acids
1732	m	Carbonyl group of monoglycerides
1737	m	Carbonyl group of triglycerides
2848, 2918, 2953	s	Stretch of saturated C – H bonds
3402	s, br	O – H stretch of water and PEG

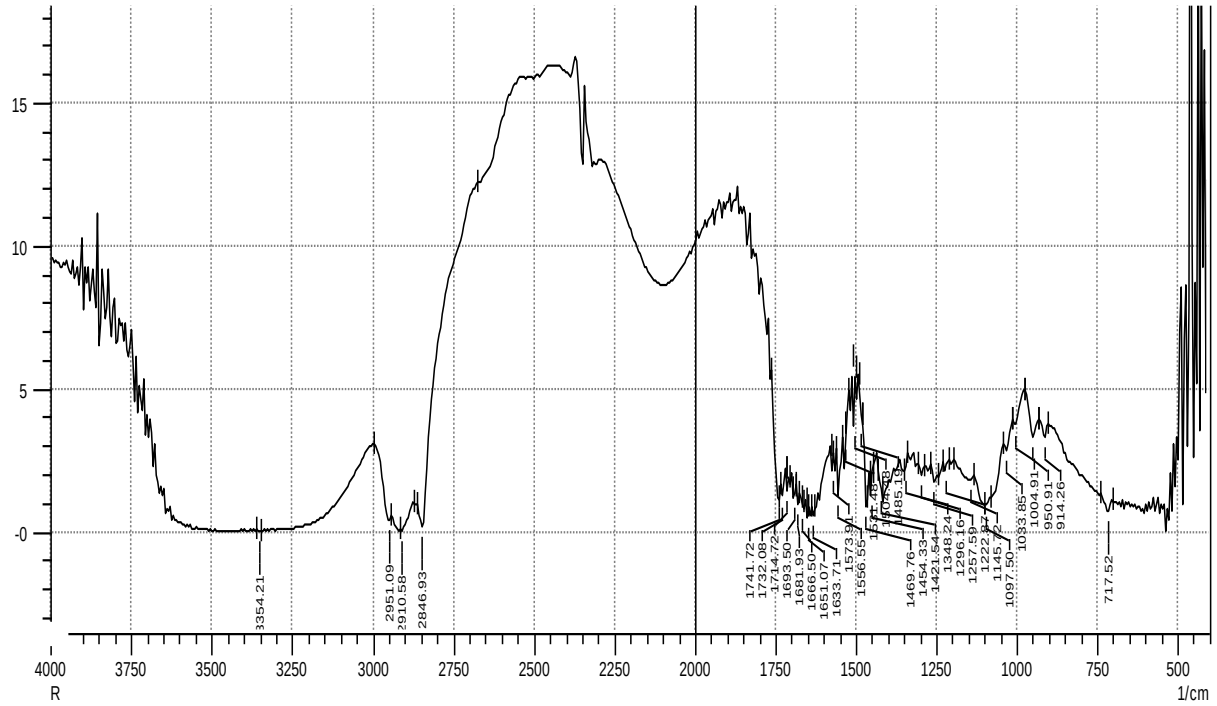


Figure 4.32| **Representative IR spectrum of P, Q and R formulations recorded versus air as background.**

In these combinations, DEA was replaced by TEA. Salt formed by the reaction between TEA and SA was the intended emulsifier. Similar to previous SA formulations stretch and bends characteristic of saturated alkane, esters and carboxylate ions were determined over the region of 1000 to 1500 cm^{-1}. MCO was observed to be stable and in its native form in the composition with fingerprint peaks at 1714, 1732 and 1744 cm^{-1} representing free-fatty acids, mono and triglycerides. The traces of free triethanolamine were comparatively low when compared previous mixtures indicating the compatibility of TEA with the emulsifying system. Formation of amides of different fatty acids in MCO and the tertiary ammonium ion group of TEA was determined from the range of 1633 to 1683 cm^{-1} (Table 4.21). Thus, it was determined that SA and TEA were compatible with MCO in the presence of PEG and water as continuous phase.

Table 4. 21| **Interpretation of IR spectrum of formulations P, Q and R**

Peak frequency (cm^{-1})	Intensity	Corresponding functional group/Fundamental vibration
914, 950	m	Symmetric C – H bend of alkanes
1004, 1033, 1097, 1145	m	Asymmetric C – H bend of alkanes
1222, 1257, 1298	m	Bends of aliphatic tertiary amines
1348	m	C – H bends of saturated esters
1421, 1454, 1469, 1485, 1504, 1531, 1568	m	Bends of carboxylate ions and dimers of carboxylic acids
1633, 1651, 1666	s	Carbonyl group of amides of various fatty acids with TEA
1681, 1693	s	Tertiary amide formation between stearic acid and TEA
1714	s	Carbonyl group of free fatty acids of MCO
1732	s	Carbonyl group of monoglycerides of MCO
1742	s	Carbonyl group of triglycerides of MCO
2848, 2910, 2961	s	Stretch of saturated C – H bonds of stearic acid and MCO
3354	s, br	O – H stretch of water and PEG

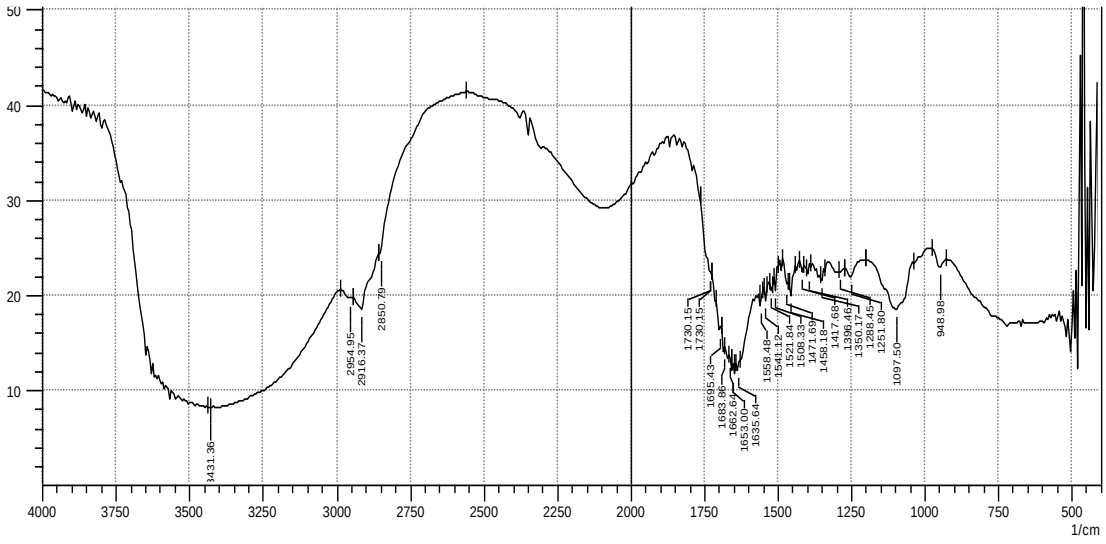


Figure 4.33| **Representative IR spectrum of S, T and U formulations recorded versus air as background.**

These formulations were designed with DA as LCFA and MEA as organic base for emulsifying salt formation. Characteristic peaks of MCO over the range of 1710 to 1750 cm^{-1} were determined indicating the free-fatty acids, mono- and triglycerides (Table 4.22). Characteristics of saturated esters, carboxylate ions and alkanes were determined over the region of 1000 to 1600 cm^{-1}. This indicated the structural stability of DA and respective esters of MCO in the formulation. However, the traces

of free MEA and overlapping of characteristic MCO groups, indicated the incompatibility of DA and MEA mixture with MCO.

Table 4. 22| **Interpretation of peaks determined in the IR spectra of S, T and U formulations**

Peak frequency (cm^{-1})	Intensity	Corresponding functional group/Fundamental vibration
948	m	Symmetric C – H bend of alkanes
1097	m	Asymmetric C – H bend of alkanes
1251, 1288	m	Bends of aliphatic primary amines
1350, 1396	m	C – H bends of saturated esters
1417, 1508, 1521, 1541, 1558	m	Bends of carboxylate ions and dimers of carboxylic acids
1634, 1653, 1662, 1683, 1695	s	Carbonyl stretch of amides of various fatty acids with MEA
1730	s	Carbonyl stretch of monoglyceride of MCO
2850, 2918, 2954	s	Stretch of saturated C – H bonds
3343	s, br	O – H stretch of water and PEG

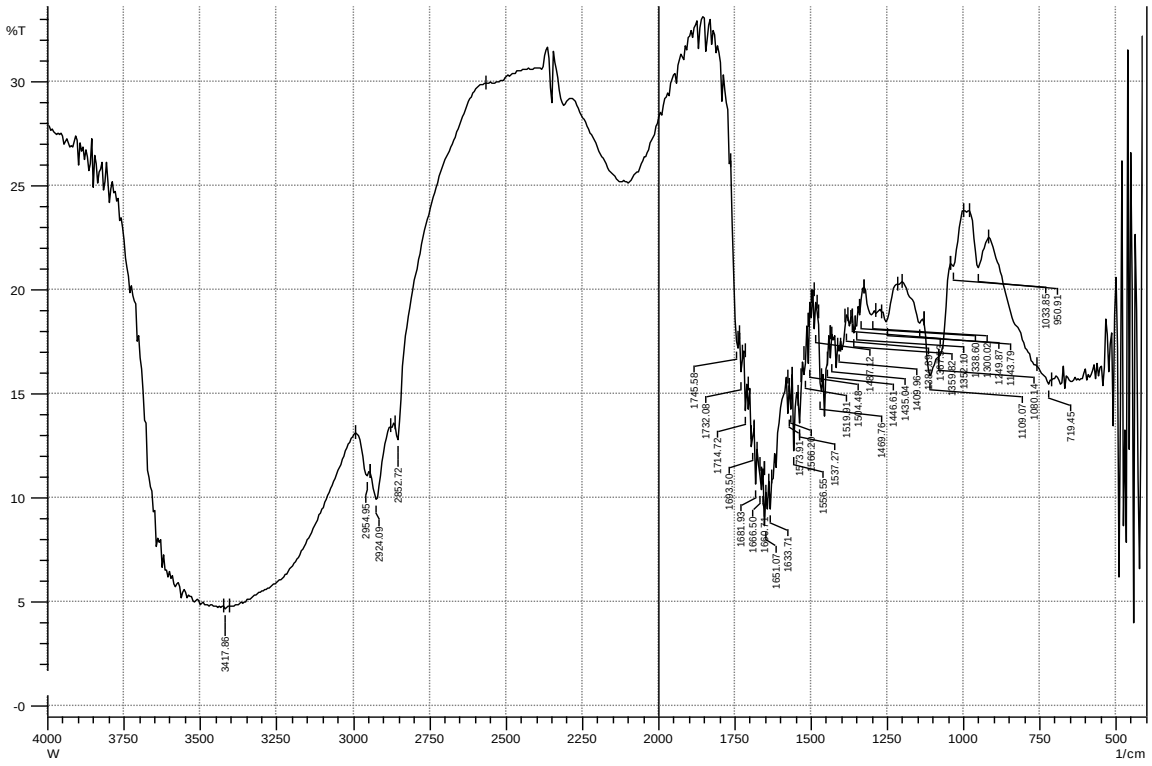


Figure 4.34| **Representative IR spectrum of V, W and X formulations recorded versus air as background.**

V, W and X formulations included similar excipients except, MEA was replaced with DEA. It was determined in the previous experiments of M, N and O that, DEA is

incompatible with MCO, but in this run it was retested to evaluate the compatibility in the presence of DA. MCO finger print peaks were determined in the respective regions of 1700 to 1750 cm^{-1} indicating the free-fatty acids, mono and triglycerides. The presence of saturate alkane or long-chain hydrocarbon groups of MCO and DA was confirmed in the respective regions including their esters and corresponding ions (Table 4.23). However, the MCO peaks were overlapped by the carbonyl groups of amides formed by the reaction between DA and DEA indicating incompatibility. Therefore, DEA was incompatible with MCO.

Table 4. 23| **Interpretation of peaks determined in the IR spectra of S, T and U formulations**

Peak frequency (cm^{-1})	**Intensity**	**Corresponding functional group/Fundamental vibration**
950	m	Symmetric C – H bend of alkanes
1033, 1080, 1097, 1143	m	Asymmetric C – H bend of alkanes
1249	m	Bends of aliphatic tertiary amines
1300, 1338, 1352, 1359, 1367, 1384	m	C – H bends of saturated esters
1409, 1435, 1446, 1487, 1504, 1519, 1537, 1556, 1568, 1573	m	Bends of carboxylate ions and dimers of carboxylic acids
1633, 1651, 1689, 1666, 1681, 1693	s	Carbonyl group of amides of various fatty acids with DEA
1714	s	Carbonyl group of free fatty acids
1732	s	Carbonyl group of monoglycerides
1749	s	Carbonyl group of triglycerides
2862, 2924	s	Stretch of saturated C – H bonds
3417	s, br	O – H stretch of water and PEG

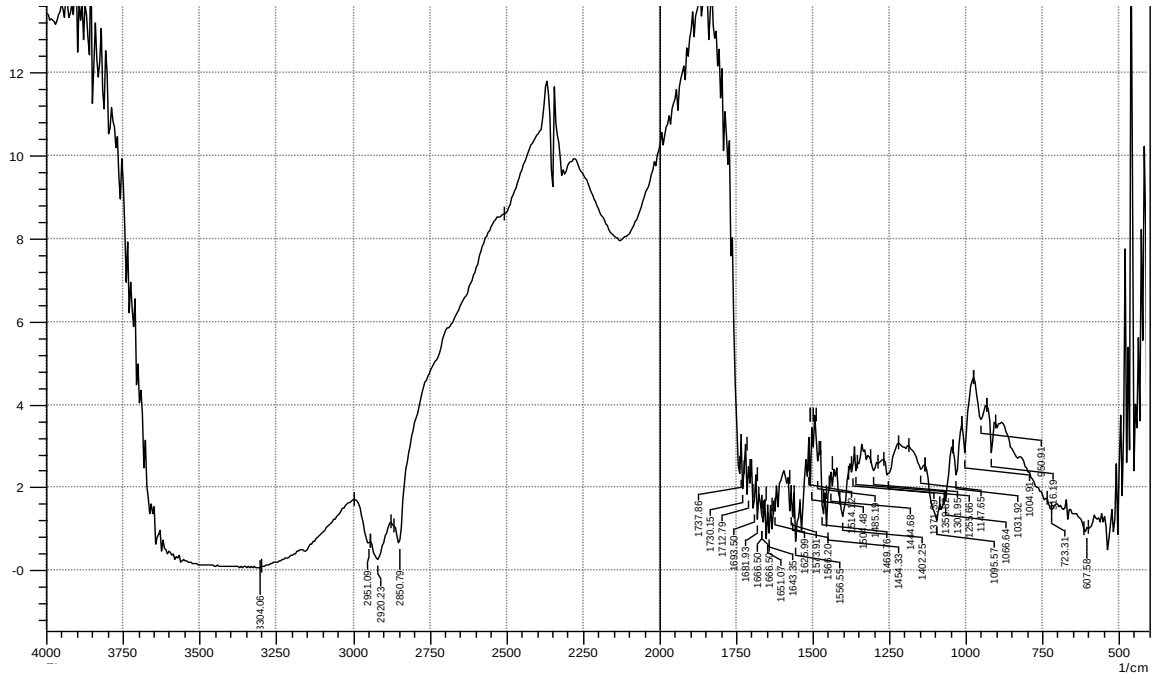


Figure 4.35| **Representative IR spectrum of Y, Z and Z' formulations recorded versus air as background.**

In the previous experiments, SA and TEA were identified compatible with MCO in the formulation. In this study, TEA compatibility with MCO in the presence of DA was evaluated. Characteristic peaks of MCO and corresponding excipients have been determined similar to other formulations (Figure 4.35). At the same time, overlapping of carbonyl groups of amides formed with DA by TEA was also determined indicating the instability of DA-TEA emulsifier with MCO.

The purpose for the application of IR spectroscopy in these experiments was to visualise the interactions between the excipients and AAE. Although, peak formation and shifts in the characteristic peaks indicated the chemistry of the system, the information obtained was qualitative. The concentration of emulsifier also determines the stability of dispersed system, hence physical characterisation employing particle size distribution analysis and texture analysis was performed to scrutinize incompatibilities and select a best-in-class formulation approach.

4.4.3 Particle size distribution analysis for the quantitative evaluation of emulsifier content over the stability

Based on the principles of Mie's theorem, particle size distribution analysis has been carried out using a laser diffraction system. In order to qualify an onychomycotic application, the diameter of distribution less than 90 percent, modal particle size and number of modes observed were considered. The significance of these parameters over the stability or performance of onychomycotic application is described below in the Table 4.24 and Figure 4.36a.

Table 4. 24| **Significance of parameters selected for the qualification of onychomycotic applications**

Parameter	Impact	Outcome	Optimal result
D90	Sedimentation	Creaming and cracking of emulsion	Larger sized D90 value is considered as failure
Average particle size D [4,3]	Creaming	Irregular distribution of dispersed phase and decreased efficiency	Smaller sized average particle size is considered as pass
Number of Modes	Cracking	Reduced shelf life	Single mode is considered as pass

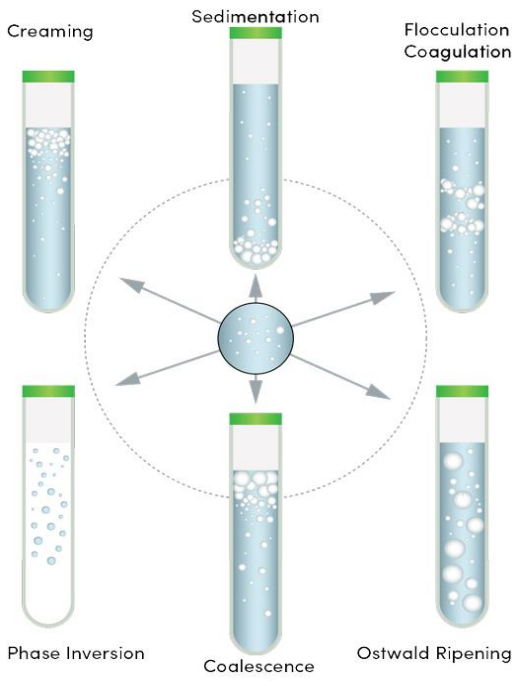


Figure 4.36a| **Pictorial representation of effect of particle size distribution over the stability of emulsion.**

D90 of a particle size distribution dictates the population focus on the sedimentation of dispersed phase in the emulsion. If this value is high, it indicates that the population is likely to sediment faster as majority of the globules dispersed are larger and can be influenced by centrifugal or gravitational force during storage or transportation of the dosage form.

Average particle size indicates the extent of emulsification by the emulsifier chosen for the dosage form. For a stable emulsion, the distribution and the mode are same indicating normal distribution of the dispersed phase (See Figure 4.36b). Inappropriate distribution leads to coalescence of the globules and creaming of emulsion and causes content irregularities in the matrix. Two or more modes indicate different particle sizes among which larger particles are likely to sediment followed by the next larger size. This results in cracking of the emulsion and a catastrophically reduced shelf life.

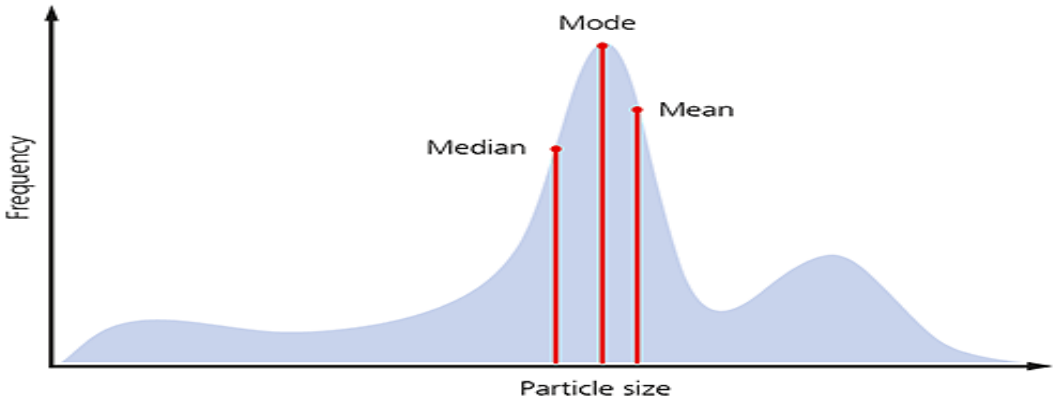


Figure 4.37b| **Pictorial representation of mean, median and mode of a particle size distribution of a sample analysed through laser diffraction technique.**

In the Table 4.25, the determined values of above factors of the formulations A to Z' are now represented.

Table 4. 25| **Parameters derived from the particle size distribution of onychomycotic applications prepared.** Number of modes is considered as preliminary criteria to discriminate stable formulations.

Sample	D90 (µm)	Average particle size (µm) or D [4,3]	Maximum mode diameter (µm)	Modes	Acceptance pass/fail	Possible risk
A	55.08	24.68	32.08	2	Fail	Reduced shelf life
B	2600.9	380.59	2763.17	3	Fail	Reduced shelf life
C	1292.29	572.76	408.22	1	Pass	Stable
D	2154.22	1175.94	1677.94	3	Fail	Reduced shelf life
E	1508.34	308.83	2033.02	2	Fail	Reduced shelf life
F	146.29	59.63	45.63	2	Fail	Reduced shelf life
G	118.32	62.49	73.78	1	Pass	Stable
H	85.52	38.05	49.58	2	Fail	Reduced shelf life
I	577.56	216.49	376.96	2	Fail	Reduced shelf life
J	64.83	35.26	36.24	1	Pass	Stable
K	54.89	36.64	31.15	1	Pass	Stable
L	52.36	30.06	33.17	1	Pass	Stable
M	62.25	28.64	33.25	1	Pass	Stable
N	118.21	58.72	71.83	1	Pass	Stable
O	49.92	18.93	37.97	2	Fail	Reduced shelf life
P	94.84	154.82	2638.23	2	Fail	Reduced shelf life
Q	57.77	35.02	33.38	1	Pass	Stable
R	68.37	25.3	45.45	1	Pass	Stable
S	33.7	16.62	133.6	3	Fail	Reduced shelf life
T	173.88	135.25	2270.27	5	Fail	Reduced shelf life
U	51.68	25.16	143.8	5	Fail	Reduced shelf life
V	45.34	17.59	33.57	3	Fail	Reduced shelf life
W	9.34	7.68	30	2	Fail	Reduced shelf life
X	106.13	74.31	297.09	4	Fail	Reduced shelf life
Y	83.78	71.1	1024.05	4	Fail	Reduced shelf life
Z	56.84	243.11	2722.77	2	Fail	Reduced shelf life
Z'	145.82	176.64	2053.67	3	Fail	Reduced shelf life

From the Table 4.24, it was determined that majority of the formulations had more than one mode which confirmed the presence of more than one particle size in the formulation. As the targeted QTPP demanded the solubilisation of MCO into aqueous medium, more than one mode in the distribution was regarded as an incompatible combination. From this preliminary discrimination, the proven stable formulae were C, G, J, K, L, M, N, Q and R. The compositions of respective ingredients are detailed below in Table 4.26.

Table 4. 26| **Compositions of the proven formulae**

Formulation	MCO	DA	SA	MEA	DEA	TEA	NaOH	Tween-20	PEG	Water
C	15	0.00	2.00	0.00	0.00	0.00	1.32	3.75	20.00	62.93
G	15	4.00	0.00	0.00	0.00	0.00	2.37	3.75	10.00	64.88
J	15	0.00	3.75	1.00	0.00	0.00	0.00	0.00	10.00	70.45
K	15	0.00	7.50	2.00	0.00	0.00	0.00	0.00	10.00	66.70
L	15	0.00	15.00	3.00	0.00	0.00	0.00	0.00	10.00	56.79
M	15	0.00	3.75	0.00	1.38	0.00	0.00	0.00	10.00	69.87
N	15	0.00	7.50	0.00	2.76	0.00	0.00	0.00	10.00	65.24
Q	15	0.00	7.50	0.00	0.00	3.93	0.00	0.00	10.00	63.57
R	15	0.00	15.00	0.00	0.00	7.86	0.00	0.00	10.00	52.14

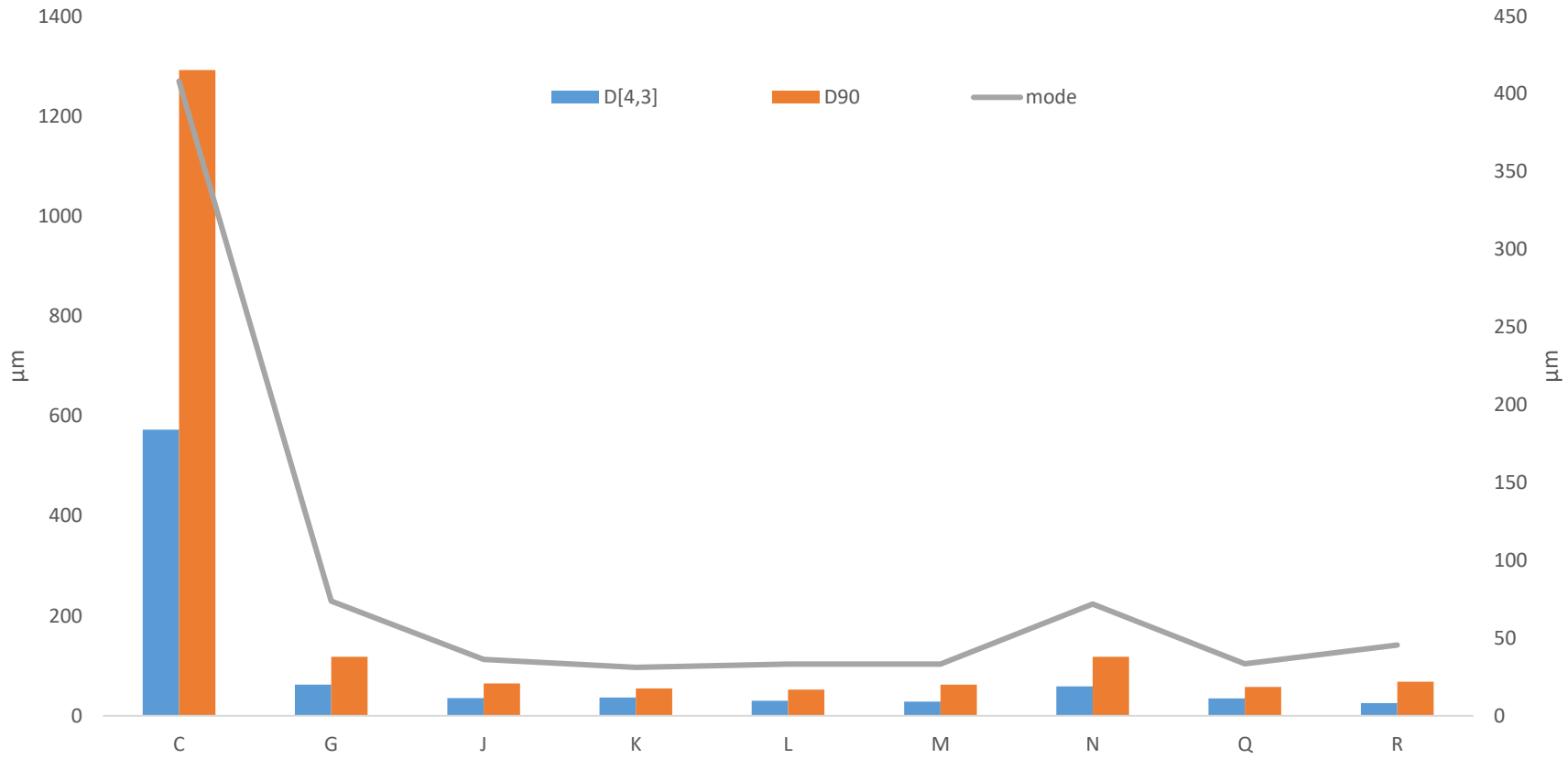


Figure 4.38| **Pictorial representation of particle size distributions of the proven C, G, J, K, L, M, N, Q and R.**

From the Table 4.26, SA was determined as most common excipient among all the proven formulations except for G. This observation confirmed the compatibility assessment results obtained in the IR spectroscopic and DSC analysis. Although G produced a single mode of distribution, from the IR spectroscopy, poor chemical compatibility was observed. This statement could be confirmed by the larger particle size produced by these formulations. Among the others, formulation R had been determined to be the lowest value for average particle size by volume with 90 percent of the particles less than 68.37 µm. In this project emulsifier was formed by reacting the amine group of ethanolamines and carboxyl group of fatty acid. This reaction was proven from the IR spectroscopic studies. However, the compatibility between the excipients and MCO was proven to increase with the increase in hydroxyl groups from monoethanolamine to triethanolamine. The increase in hydroxyl groups has increased the hydrogen bond formation between amines and the continuous phase thereby stabilising emulsion. This was also clear from the data that the particle size of MCO decreased with the increase in hydroxyl groups of ethanol amine (See Figure 4.39).

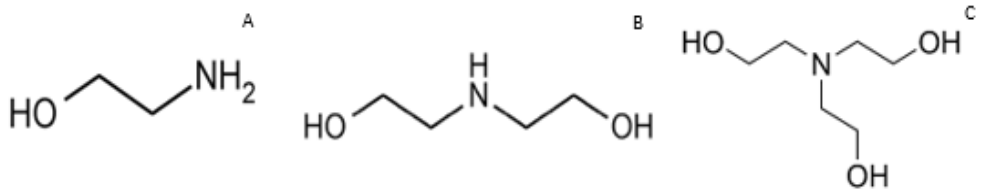


Figure 4.39|**(A) monoethanolamine with one hydroxyl group; (B) diethanolamine with two hydroxyl groups; (C) triethanolamine with three hydroxyl groups.**

Although formulation K, had lower D90 value, but the average particle size number is comparatively higher than R (See Figure 4.37). If the average particle size of a formulation is higher than D90, the chances of unequal distribution of particles are high. Therefore, R is considerably more stable than all other formulations.

4.4.3.1 Optical microscopic examination of formulations

Particle size distribution is an empirical method of evaluating the extent of internal phase dispersion in the continuous phase. By the visual examination actual distribution of the internal phase in the continuous phase can be observed in a given area. Uniformity in the continuous phase cannot be evaluated only through particle size dispersion as the technique includes measurement of particle size distribution in the sample but not its mode of dispersion in the matrix. For example, D90 values dictate the percentile of particles less than a number but not their occupation in a given quantity of samples. If uniform dispersion is not achieved chances of nucleation and Ostwald's ripening through Brownian movement towards creaming are high. Hence, in microscopic examination it is feasible to observe the dispersion of internal phase for smeared sample in the visible light.

In this research, all formulations prepared have been smeared onto a glass slide and covered with a coverslip and examined. Microscopic images captured at 100x magnification of the smears are presented in Figure 4.40.

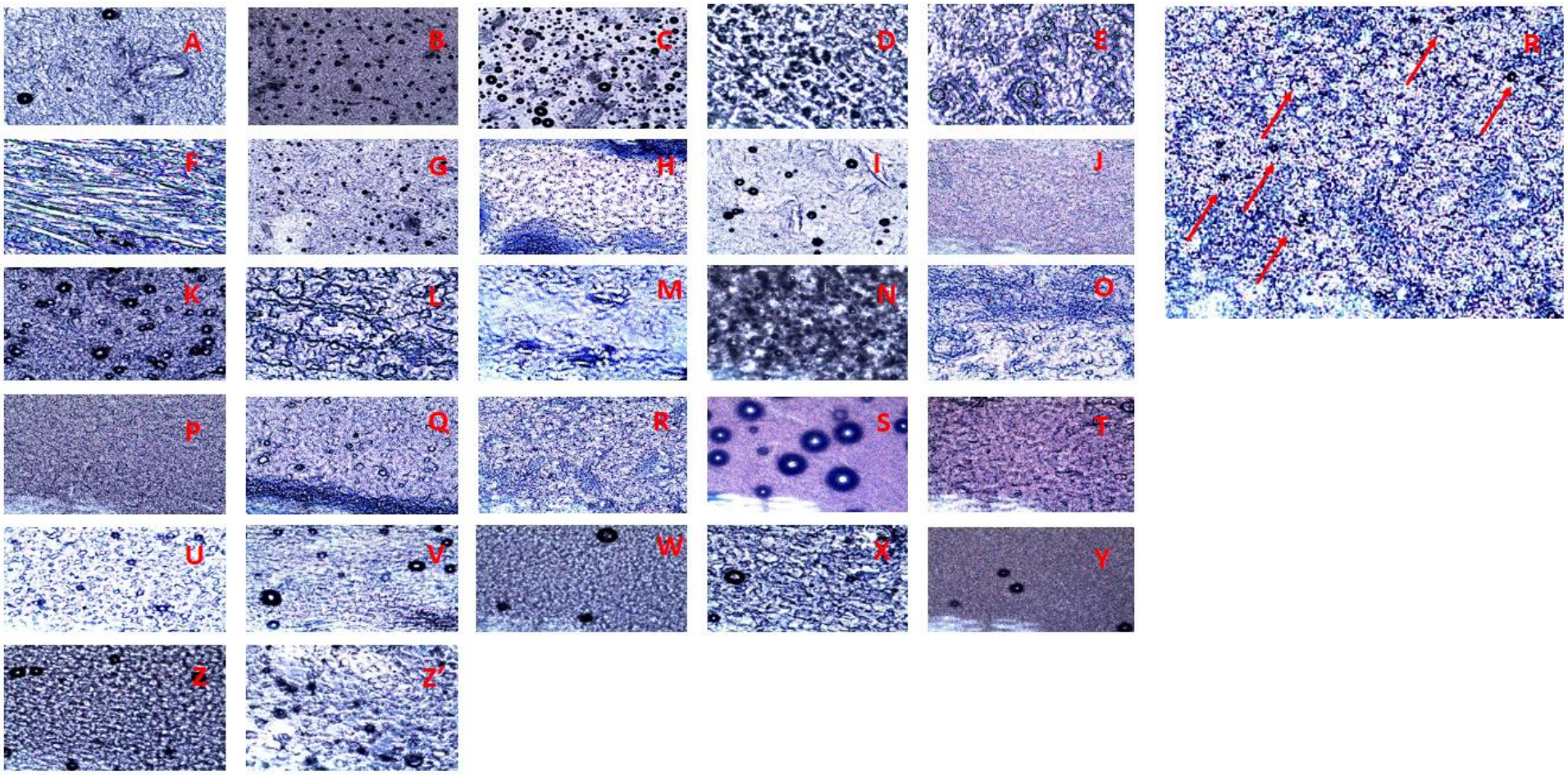


Figure 4.40| **Optical microscopic pictures of Formulations A to Z' under visible light at 100X magnification.** To the left is the enlarged image of formulation R under the same microscopic conditions.

Formulations A to Z were analysed under light microscopy as detailed in Section 2. Certain parameters were considered and evaluated as detailed below.

i. extent of dispersion of internal phase in the continuous phase

all formulations were thoroughly mixed prior to smear on the glass-slide. Amount of sample and position in the container from which it was collected were standardised. Formulations A, T, J have no dispersion of internal phase which proved improper dispersion of internal phase into continuous phase. If this condition persists in a formulation, it will eventually affect the stability of emulsion due to agglomeration of similar sized particles in the container for storage. This can lead to creaming and sometimes breaking or cracking of emulsion under gravity. Hence, these formulations were considered unstable.

ii. frequency of similar droplet size in the smear

During the laser diffraction studies, particle size of internal phase over a range of size was studied. Equal distribution of internal phase particles over the applied region of nail plate indicates content uniformity. Disturbances in this property can affect the performance of formulation as every extrusion of onychomycotic application from container cannot provide similar concentration of AAE. Therefore, scattered droplet size distribution is an unstable property or CQA affecting QTPP, which in this study are C, K, V and W formulations.

iii. involvement or overlap of matrix over the internal phase

one of the most undesired effect in emulsions is phase inversion. If the phase proportions or components are incompatible or internal phase less dense than the continuous phase, phase inversion happens leading to separation of layers. Stable emulsions have balanced interactions between dispersed phase and medium stabilised by the emulsifying agent. Improper emulsification or incompatible emulsifier are the major reasons for phase inversion of an emulsion apart from temperature and microbial contamination. In the formulations E, F, H, J, L, M, O, P and Q this phenomenon was observed (Figure 4.39).

Formulation R had overcome the above-mentioned consequences and stood as best-in-class combination.

4.4.4 Discrimination of the stable emulsion by physical characterisation

Achievement of an Intended particle size distribution alone cannot control the stability of emulsions. Rheological properties such as index of viscosity, cohesiveness, consistency and firmness can influence the long-term performance of the emulsion for its anticipated shelf-life. The correlation of the above-mentioned factors towards the targeted QTPP is mentioned in the Table 4.27.

Table 4. 27| **Impact of rheological attributes of emulsion over its performance**

Attribute	Part of formulation responsible	Overall effect
Viscosity and Cohesiveness	Polyethylene glycol (MW 35000) and its interaction with water in the continuous phase	To minimize the Brownian movement thereby reducing the chances of creaming with maintenance of thixotrophy and stability of the emulsion
Consistency and Firmness	Overall distribution of dispersed phase and multicomponent matrix comprising the complete interaction of AAE with excipients with focus on LCFA and base for emulsifier in reducing the surface tension	Release of AAE at the site of application and extrusion of onychomycotic application from the container closure system

In this research, a texture analyser instrument has been employed to examine the above attributes of the formulations. This equipment utilises a series of probes inserting one into the formulation presented in a proprietary vessel. The instrument measures the positive force required to enter the formulation (degree of firmness) and the area under the curve produced in this process is termed the consistency. Similarly, after penetrating up to the certain depth designed by the test configuration the probe starts a return to its initial position in reverse direction. The negative force determined during this movement is called the cohesiveness and the area under the

curve generated during this work is the index of viscosity of the formulation (Figure 4.40).

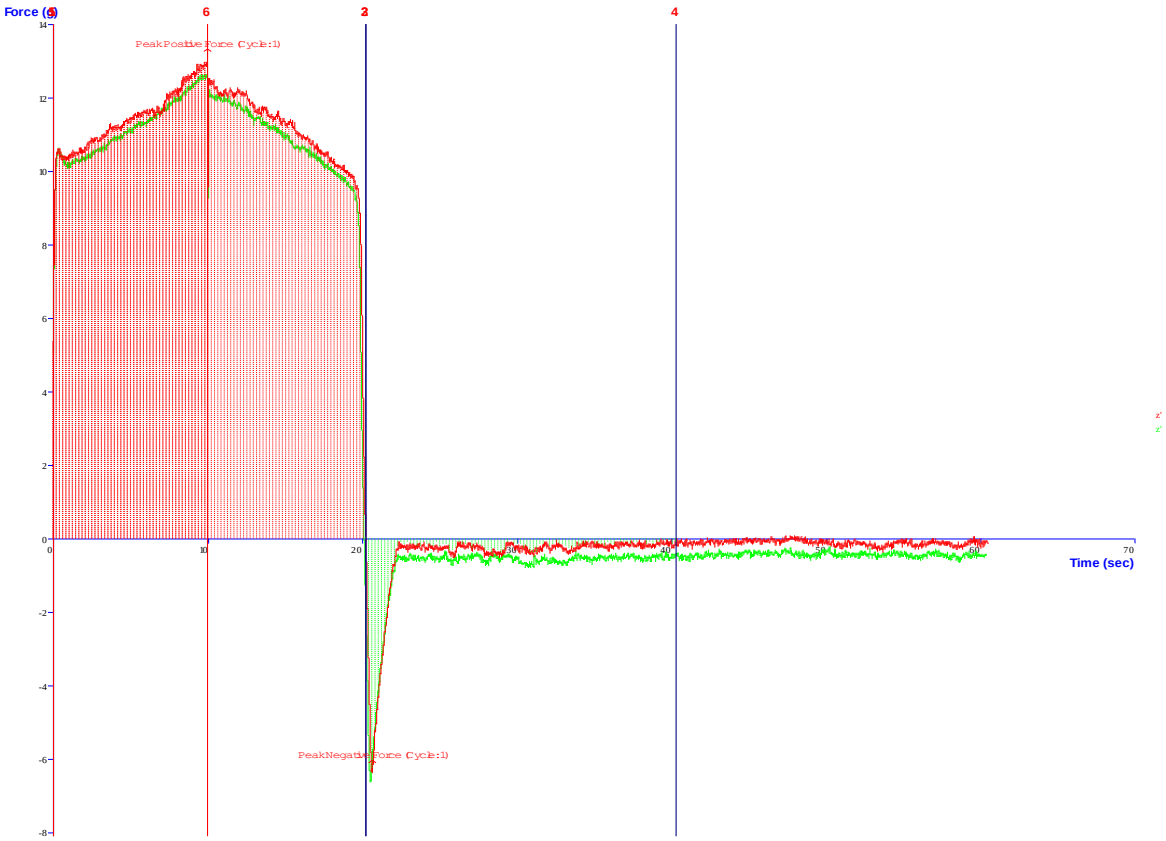


Figure 4.41| **Sample curve explaining the measurement made by texture analyser.** The values above x-axis are considered positive force (firmness) and positive area (consistency) and vice-versa for the values below the x-axis (cohesiveness and index of viscosity).

The ambient storage temperature of onychomycotic application was targeted at 25^{o}C whereas the temperature at the site of application was 32^{o}C (infected nail regions). Upon application, the onychomycotic application is intended to penetrate the tissues beneath the nail plate to reach the pathogen where the temperature would be 37^{o}C. Therefore, the physical attributes were tested at the above mentioned three temperatures using the texture analyser. To ensure robustness, a portion of the same sample was heated to the respective three temperatures in increasing order individually just before the analysis. The results obtained from these experiments were tabulated as below.

Risk assessment is the key approach of QbD in the development of a novel formulation (Simoes *et al.,* 2018). Although formulating the API into desired dosage

form based on the targeted QTPP is the ultimate goal of QbD, risk assessment at each step of this process is the key essence to build quality into the dosage form. A certain amount of risk is present at each step or with each ingredient in the formulation, but managing it is the ultimate goal of ICH Q9 (Guilfoyle *et al*., 2013). Several tools are available to identify, manage and minimise such risk in pharmaceutical operations. These include, but are not limited to, Ishikawa diagrams, Risk ranking and filtering, Hazard operability analysis (HAZOP), Failure mode effect analysis and Fault tree analysis (Herrera *et al*., 2015). In this research, the perceived percentage of risk had been related to the physical attributes affecting stability of the formulation and ranking has been assigned based on the maximum effect from the corresponding excipients towards de-stabilising the formulation quality. The following Table 4.28 describes the percentage risk assigned to each physical attribute defined as per the QTPP.

Table 4. 28| **List of attributes and their influence on the final QTPP as per compatibility test informed risk assessment**

Attribute	Role in the QTPP	Assigned risk (%)	Justification
Particle size distribution	Release of AAE at the targeted site of action and physical stability during the storage	50	Improper distribution can produce creaming or breaking of emulsion
Index of viscosity	Performance of formulation during the stability	20	Minimizes Brownian movement and prevention of microbial contamination
Firmness	Integrity of emulsifying agent with the system	10	Reducing the interference of atmospheric components such as humidity
Consistency	Factor of compatibility explaining the interaction among the ingredients	10	Shelf-life and AAE uniform release
Cohesiveness	Interaction of viscosity modifying agent with the components of formulation	10	Thixotrophy of the formulation decides the extrudability and integrity.

The risk has been distributed equally between internal and external phases. As particle size distribution is the intrinsic property of internal phase, it has been assigned highest risk towards quality. As mentioned in the earlier chapters, viscosity of emulsion supports the integrity and stability of emulsion in combination with firmness, consistency, and cohesiveness. Therefore, the risk has been assigned to these properties at the respective proportions.

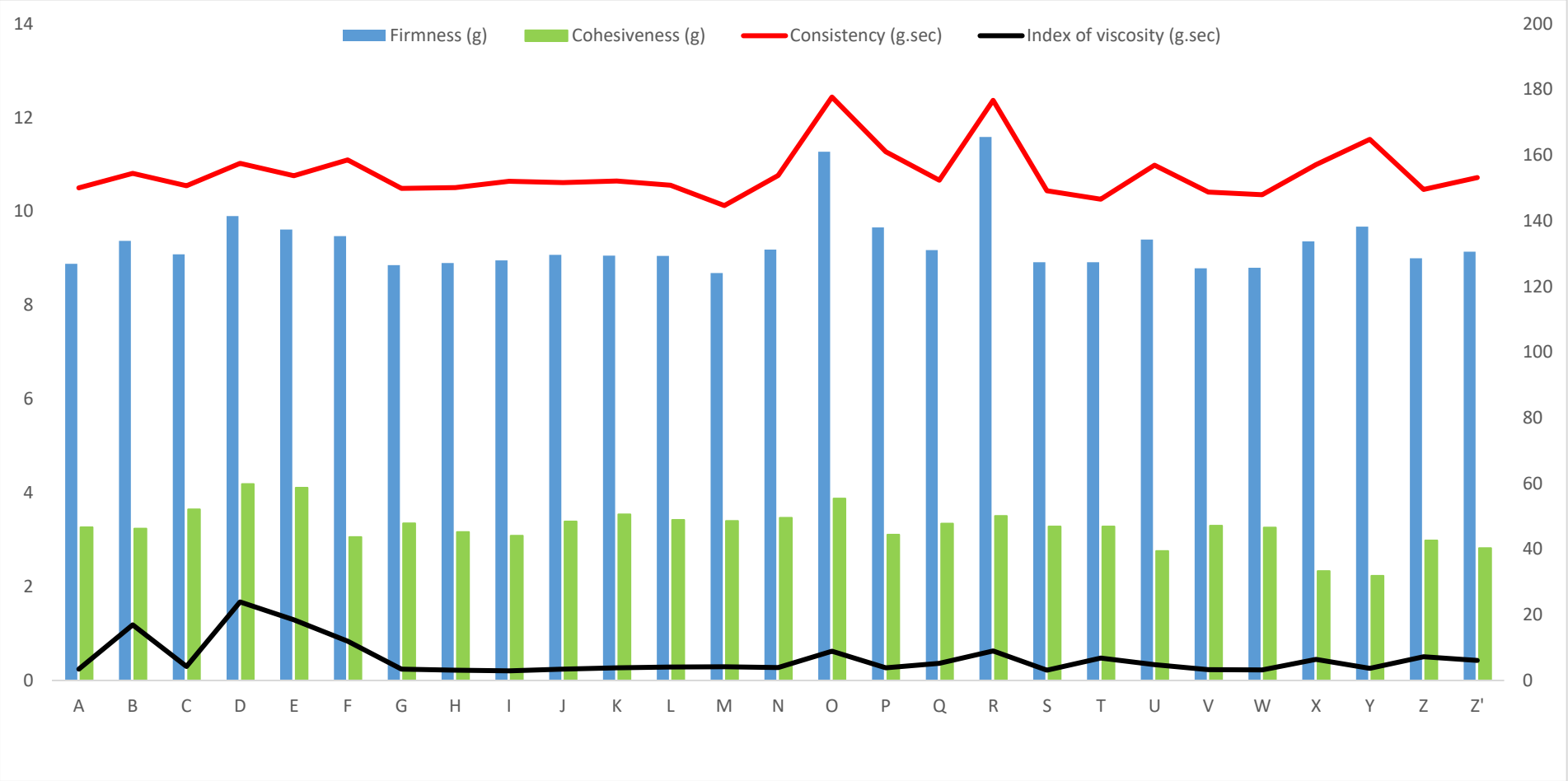


Figure 4.42| **Values of physical attributes determined at 25°C for the formulations using a texture analyser.** The higher the value of the attribute, the greater the contribution or effect. The secondary plot represents the values plotted for index of viscosity and consistency.

Figure 4.41 provides evidence that formulations R and O had the highest firmness when compared to other formulations. Similarly, formulation M had the lowest firmness. Among all, D was highly cohesive comparatively, this presumably was due to ionic attraction between the LCFA and NaOH along with Tween 80 in the formulation. However, extreme cohesiveness could reduce the sample flexibility during the application. Index of viscosity was observed high in the formulation R, which supported the apparent constructive compatibility of its excipient mix. Therefore, it was deduced that Brownian movement could be low and chances of separation or segregation of similar sized molecule were also low with high stability of the formulation. Uniform distribution of excipients and dispersed phase in the continuous phase indicate the consistency of an emulsion. This attribute was determined high for R and O among all the tested formulations (See Table 4.29).

Table 4. 29| **Interpretation of results of the formulations tested at 25^{o}C**

Property	**Formulation showing**		**Analysis**
	Max	**Min**	
Firmness	R, O	M	Extrusion characteristics are high for R and O whereas leakage from container could be observed in M
Consistency	R, O	M	Distribution of components was uniform in R and O whereas an irregular pattern was determined in M. Release of AAE may not be consistent in M and related formulations at similar attributes
Cohesiveness	E	Y	An optimal cohesiveness indicates stability and spreadability of the formulation with suitable thixotrophy. Highly cohesive molecules tend to undergo structural retardation and improper application at the targeted site
Index of viscosity	D	H	Higher viscosity requires increased shear rate and friction to stabilize the formulation. At this condition, chances of degradation are high due application of heat or pressure. Hence, an optimal formulation is value is required to identify the stable formulation.

Max = maximum value determined; Min = minimum value determined

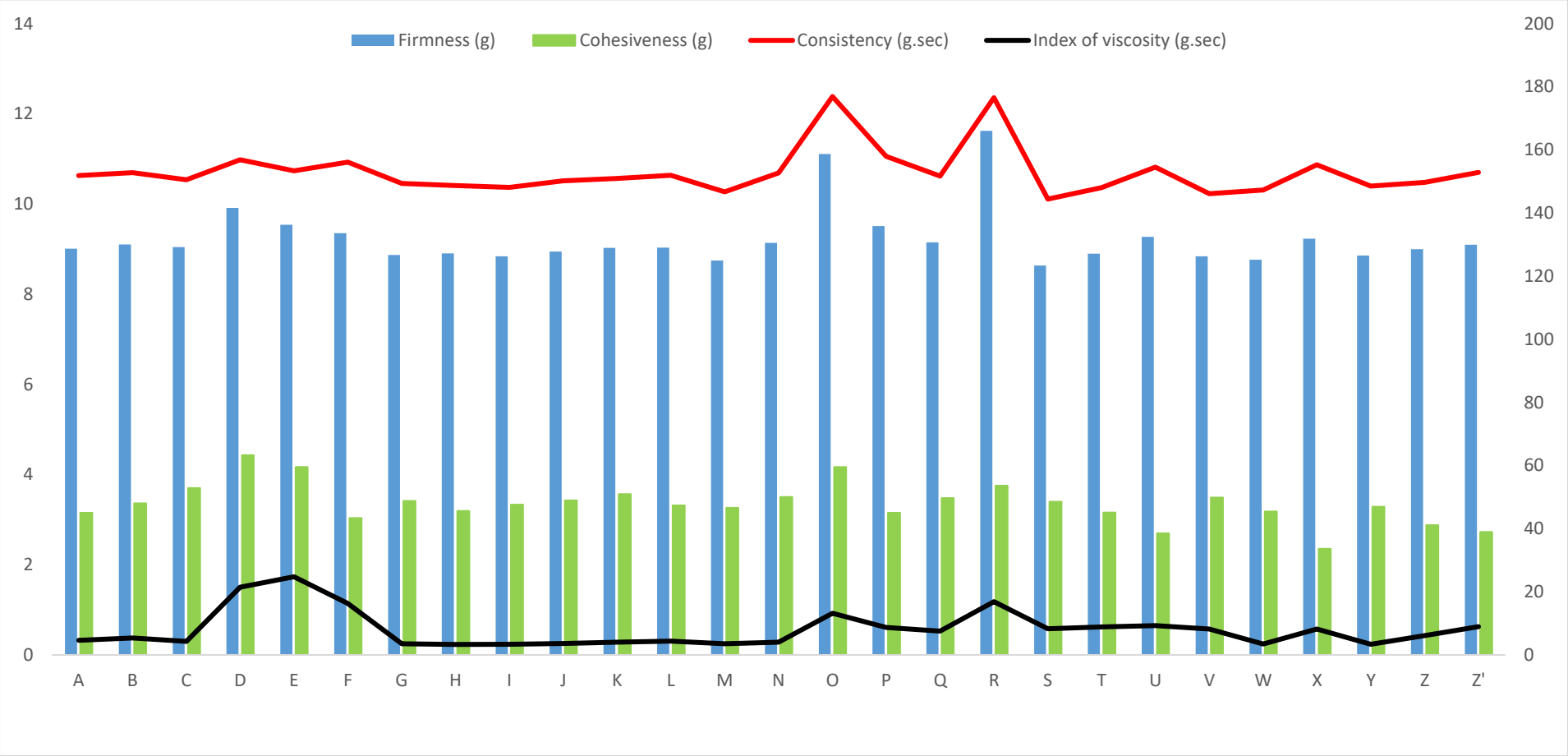


Figure 4.43| **Values of physical attributes determined at 32°C for the formulations using texture analyser.** The higher the value of the attribute, the greater the contribution or effect. The secondary represents the values plotted for index of viscosity and consistency.

In the previous study, the physical attributes of designed formulations under ambient storage conditions were evaluated. As mentioned earlier, the temperature at targeted infected nail region is 32^{o}C. Therefore, effect of this change in temperature from storage conditions to clinical environment were evaluated and the results determined were presented in Figure 4.42.

It was determined that the formulations remained stable with respect to firmness except for a small increase in value at 25^{o}C. This could be due to slow evaporation of water during the heating process. Nevertheless, there was no significant impact on the formulation, affirming that the respective combinations were stable at 32^{o}C.

The intention of different ratios of emulsifying agents was to evaluate the best-in-class combination with MCO. From Figure 4.43 it was evident that, with increase in the emulsifier concentration, stability of emulsion towards physical attributes had increased with a trend for similar LCFA and base used for the salts. However, the interaction of emulsifier between the phases was determined superior in the formulations O and R supporting the results determined during the IR, DSC and particle size distribution studies.

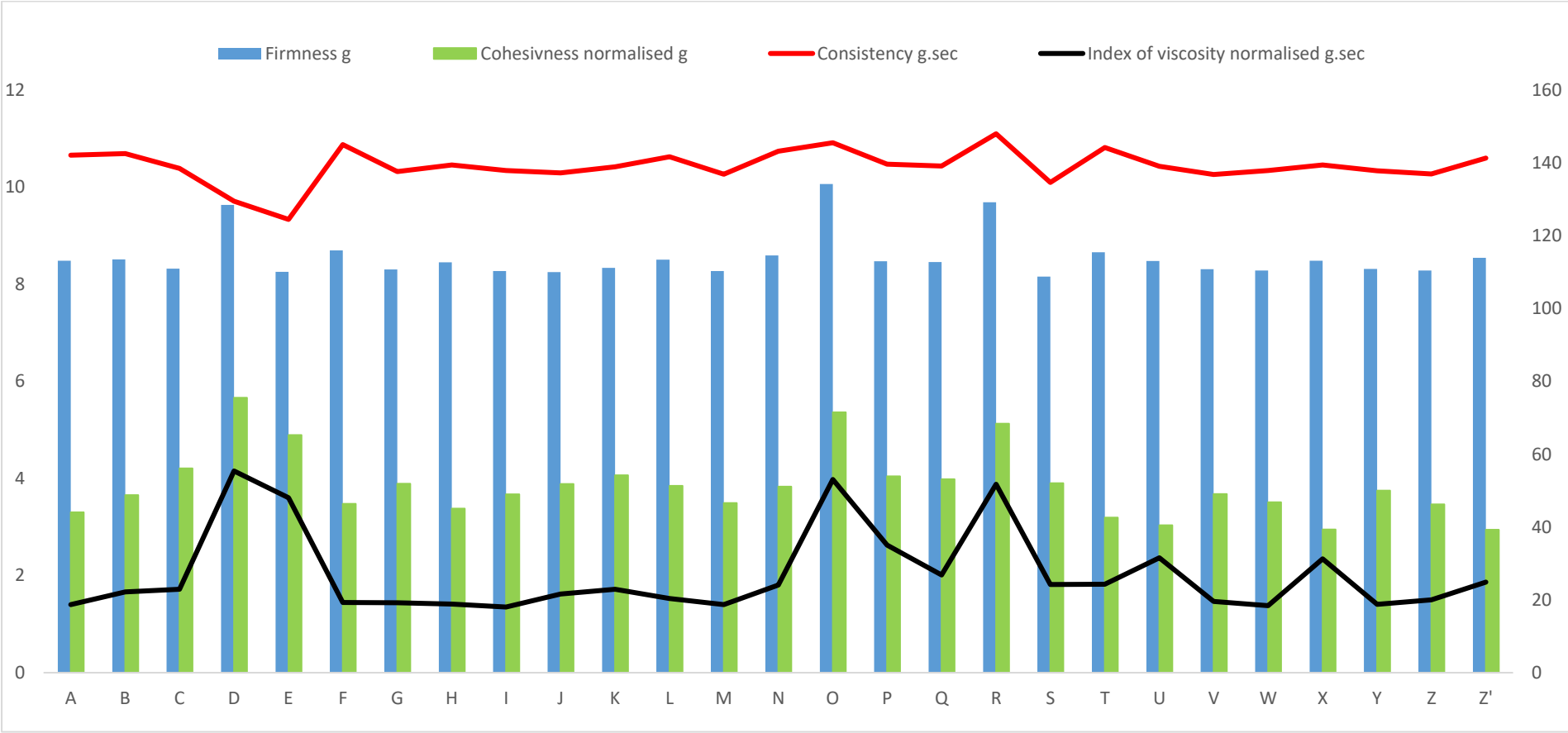


Figure 4.44| **Values of physical attributes determined at 37°C for the formulations using texture analyser.** The higher the value of the attribute, greater the contribution or effect. The secondary axis represents the values plotted for index of viscosity and consistency.

All formulations can be conveniently categorised into three groups based on the type of base used for the emulsifying salt formation. These are recorded in Table 4.30.

Table 4. 30| **Grouped formulations based on the composition**

Group A		**Group B (PEG and water were constant)**		**Group C (PEG and water were constant)**	
A	SA	J	SA+MEA	S	DA+MEA
B	DA	K		T	
C	NaOH	L		U	
D	Tween 20	M	SA+DEA	V	DA+DEA
E	Water	N		W	
F	MCO	O		X	
G		P	SA+TEA	Y	DA+TEA
H		Q		Z	
I		R		Z'	

Group A comprised of stearic acid or decanoic acid in the formulation as long-chain fatty acid and sodium hydroxide as common base to form the emulsifying salt. This group had increasing concentrations of stearic acid or decanoic acid in the respective formulations towards a stable mixture. However, from the DSC and IR spectroscopy it was determined that inorganic base compounds were incompatible with MCO for emulsification. These findings were reconfirmed in the particle size distribution analysis as well. During the physical characterisation studies, formulations with combinations of Group A excipients were determined with poor performance in terms of attributes. The trend of attributes was not sequential with the concentrations of long-chain fatty acids or inorganic basic compounds.

Group B had stearic acid as long-chain fatty acid with monoethanolamine, diethanolamine and triethanolamine as the bases for emulsifying salts individually in the respective formulations. The concentration of long-chain fatty acid was doubled for every successive formulation within the subgroup until the base used was changed for it. This was carried out in common with Group C except for decanoic acid was used in the place of stearic acid as long-chain fatty acid. The purpose of this was to have the long-chain fatty acid in the emulsified salt at one-fourth, one-half and equal concentrations with respect to MCO.

From the equation 2.8 in section 2.3, it was correlated that the pressure inside a globule dispersed was directly proportional to surface tension of the system. Similarly, from the equation 2.6, the free energy of a molecule is directly proportional to the surface area and surface tension of product. Similarly, with the increase in the concentration of emulsifier the size of dispersed internal phase decreases. Given these principles, the globular size of dispersed internal phase should decrease with increase in the concentration of emulsifier formed with long-chain fatty acid and the respective base.

From the Figure 4.40, it was examined that the trend of the attributes with the surfactant was irregular for Group A formulations. Whereas in Group B, the trend was optimal in the formulation containing TEA and SA; yet in Group C a completely opposite trend was determined. Hence, formulations containing SA and TEA were determined to be stable under the induced conditions. However, among them formulation R was considered towards the targeted QTPP due to lower particle size (See Figure 4.44).

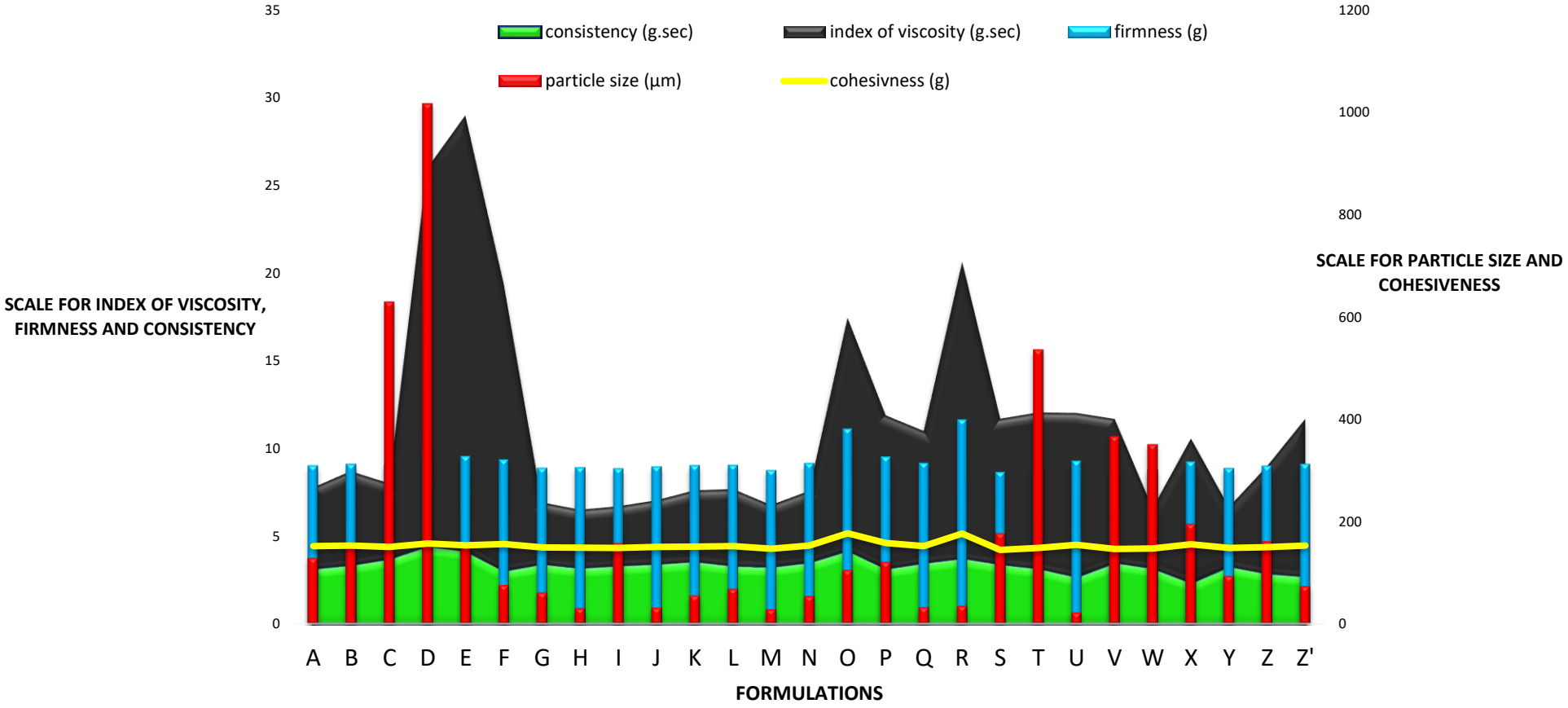


Figure 4.45|**Graphical representation of physical attributes determined for various formulations through particle size distribution and texture analyser.**

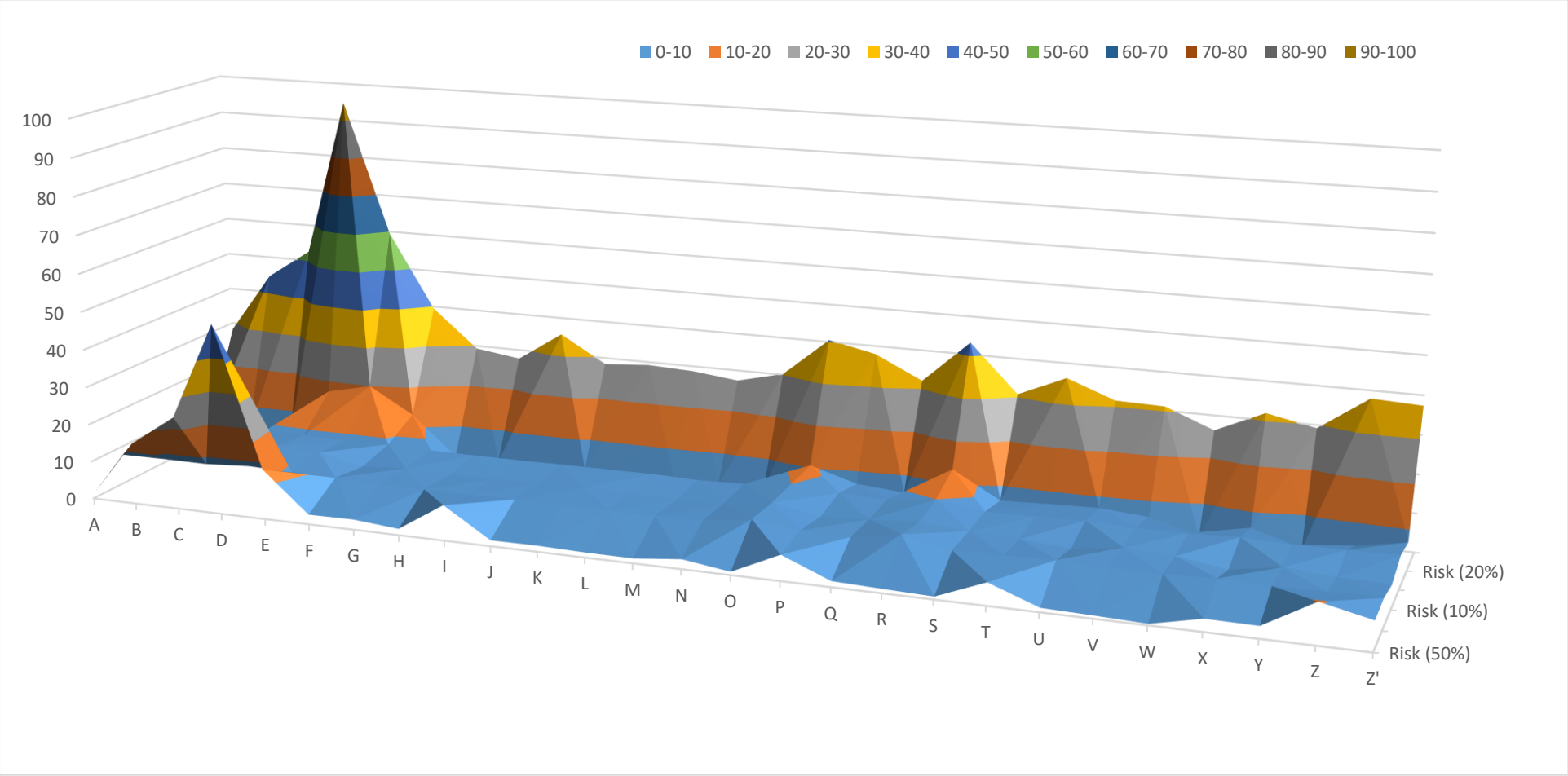


Figure 4.46| **Graphical representation of response surface analysis of cumulative data from the physical characterisation of formulations A to Z' based on the risk assessment.**

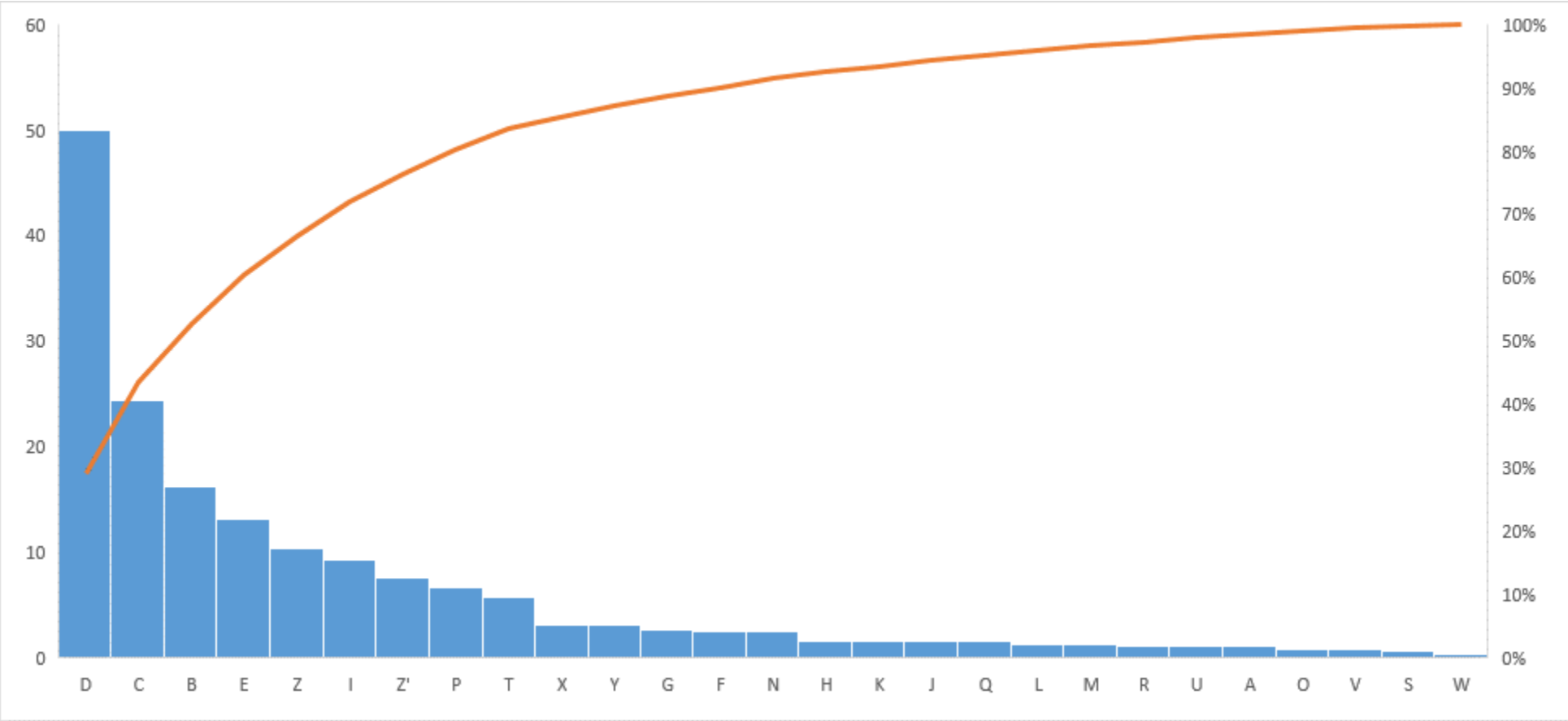


Figure 4.47| **Pareto chart of risk analysis of cumulative data from the physical characterisation of formulations A to Z'.**

Formulations with lowest risk less than 10% were determined to be G, F, N, H, K, J, Q, L, M, R, U, A, O, V, S and W. The reason for selecting less than 10% was to have majority of population within the two standard deviations of the bell-shaped distribution curve. Some of the mentioned formulations were discussed to be unstable during the individual analysis. For example: formulation A was determined to be unstable due to the incorporation of inorganic base to the emulsifying salt. Similarly, formulations from Group C were categorised to be unstable due to an irregular or abnormal trend of physical attributes with the concentration of emulsifying agent.

Therefore, a final decision could not be made based only on the physico-chemical analysis and risk assessment. In this research, contact based bioassays were implemented and the designed formulations were tested both *in vitro* and simulated *in vivo* conditions to optimise a final formulation.

4.4.5 Examining designed formulations for the antimicrobial performance to select best-in-class combination

As per the established QTPP, the targeted site of application of the intended formulation for an effective treatment of onychomycosis is the infected nail plate. In the Chapter 1.0, the composition of a healthy nail plate and matrix were confirmed as keratin (up to 90%).

Therefore, the performance of the designed formulations towards the pathogen was tested in the presence and absence of keratin. In simulated conditions, the content of keratin added into the growth medium of each agar-plate was equivalent to the average amount of keratin in a healthy, adult human nail plate. This is to supplement the pathogen excess of nutrient along with medium in order to facilitate its healthier growth. The performance observed in such conditions was considered significant, because the organism would have a stronger potential derived from the supporting environment than on usual growth medium. In order to overcome this enhanced pathogenic virulence, the formulation is challenged to more highly effective.

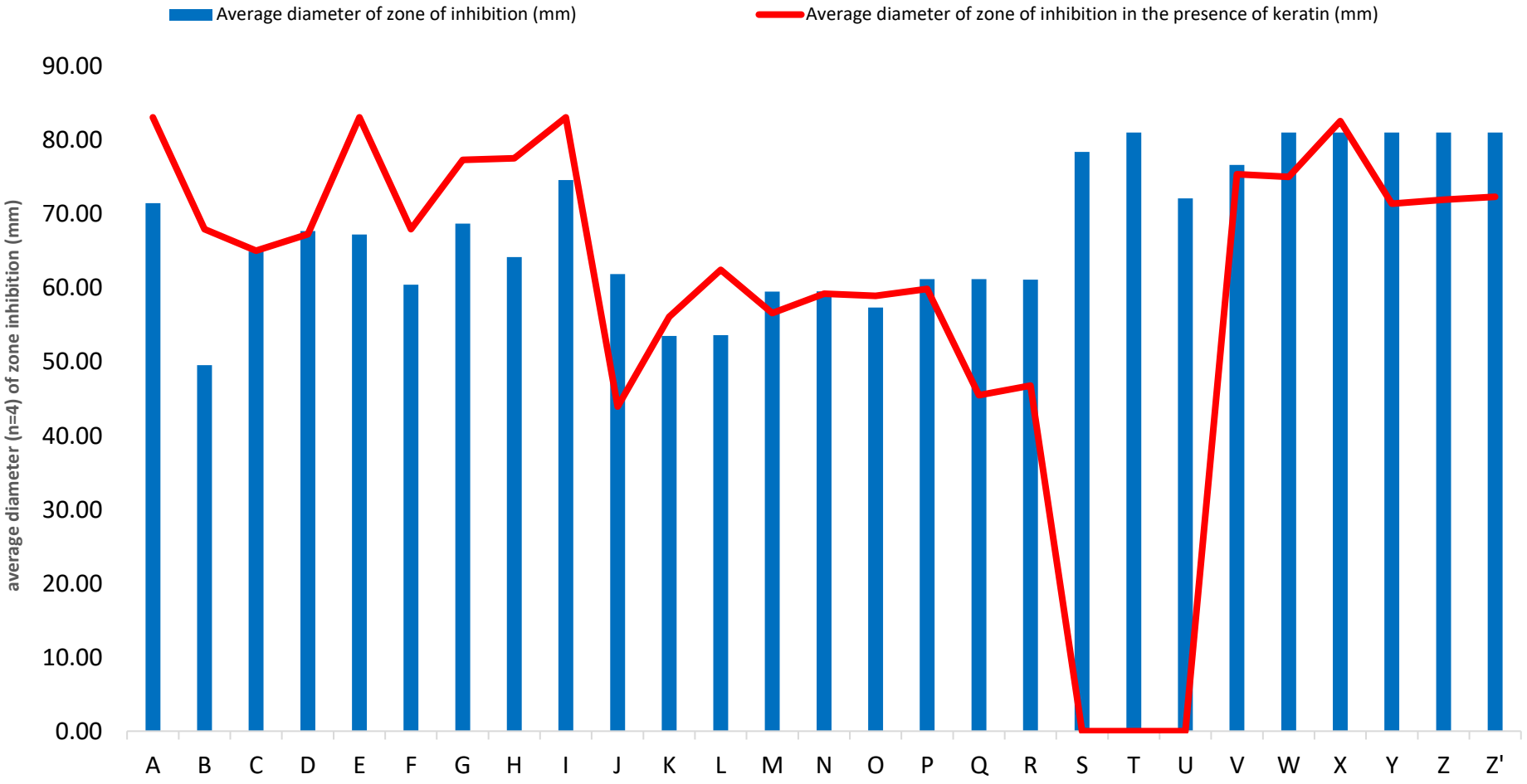


Figure 4.48| **Antimicrobial performance determined of designed formulations in the presence of keratin on Sabouraud Dextrose agar.** Note – In this figure The values graphed above were an average of n = 4 readings per sample. The coefficient of variation (%RSD) of each formulation's readings were determined as ≤5.0%.

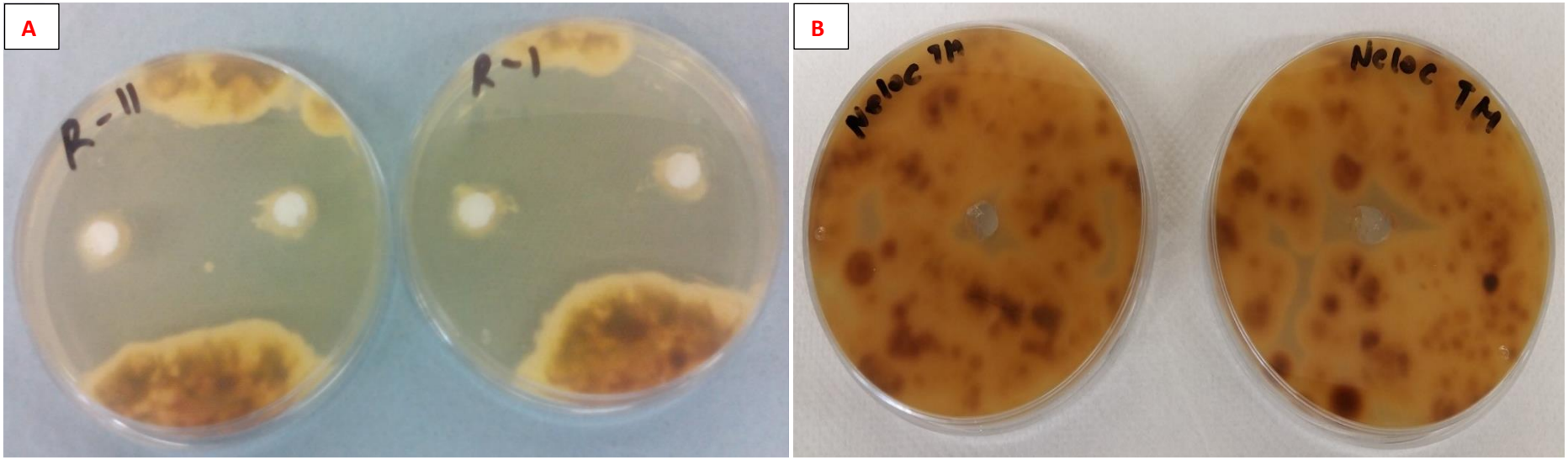


Figure 4.49| **Images of agar-plates containing the zones of inhibition developed by the Formulation R** (A) and no activity observed for Naloc^{TM} (B – commecrcial control) in the presence of keratin. The commercial control (postive control) employed in this research belongs to K101 formulation category with urea, NaOH, lactic acid and propylene glycol as the ingredients. During all the microbiological experiments with the pathogen, the strain used was maintianed constant including the growth media for testing. Postive control had produced zones of inhibtion during the initial testing phases (See Section 4.1) with diameters at delayed time points than MCO. But, the activity of Naloc^{TM} had been observed diminished with the progress of the project as the zones of inhibition were determined to be minimal to nill. This could be due to the development of resistance by the strain used over the span of project, as it was ensured that expiration date of the control used was always due atleast three months from the date of experiment. However, MCO formulation activity had remained constant in both presence and absence of keratin throughout the project with the same strain.

Table 4. 31| **Interpretation of antimicrobial activity shown by the formulations in the presence of keratin**

Formulation	Activity of the formulation in the presence of keratin
G	+
F	+
N	N/A
H	+
K	+
J	-
Q	-
L	+
M	-
R	-
U	--
A	+
O	+
V	-
S	--
W	-

+ = more than in the absence of keratin; - = slightly less than in the absence of keratin; -- = no activity in the presence of keratin; N/A = activity remained same. The formulations mentioned in the Table 3.38 were arranged at decending order of risk percentage from the residual analysis (See Figure 3.42).

From the Figures 4.46 and Table 4.32 it was determined that formulations at low risk were inactive towards the organism, eg. U and S. formulation W was observed less active in the presence of keratin with corresponding lowest risk. Formulations G, F, H, K, L, A and O had produced higher activity in the presence of keratin but did not meet the targeted QTPP in terms of physical and chemical interactions. Therefore, a matrix of virtual control space has been constructed with respect to the risk and antimicrobial activity in order to identify potential formulation. Although design of experiments is considered as crucial part of Qbd, due to time constraints it is included into future work.

Table 4. 32| **Matrix for the identification of optimal formulation as per QTPP**

Formulation	Thermal compatibility	Chemical compatibility	Particle size distribution	Firmness	Cohesiveness	Consistency	Index of viscosity	Antimicrobial activity	Overall rank
G	---	--	-	+	+	-	-	+	-5
F	---	--	-	+	+	-	+	+	-4
N	+	+	+	-	-	-	-	N/A	-1
H	---	-	+	-	-	-	-	+	-5
K	+	+	--	-	-	-	+	+	-1
J	+	+	--	-	-	-	+	-	-3
Q	+	+	+	-	-	-	-	-	-2
L	+	+	-	-	-	-	+	+	0
M	+	+	+	-	-	-	-	-	-1
R	++	++	++	+++	+++	+	++	-	11
U	+	+	+++	-	-	--	-	--	-3
A	---	-	-	-	-	--	--	+	-10
O	-	+	-	++	+	++	+	+	6
V	+	+	--	---	--	+	+	-	-4
S	+	+	--	-	-	-	-	--	-6
W	+	+	--	---	-	-	---	-	-6

In the Table 4.31 symbols were denoted as follows: --- = very poor; -- = poor; - = moderately poor; +++ = best; ++ = better; + = good. The highest grade was awarded to the formulation with best perfromance in the particular category and vice versa for the lowest grade.

The thermal stability was evaluated from DSC, chemical compatibility from IR spectroscopy and the remaining CQAs from the physical characterisation. Among all formulations, R scored the highest rank with superior physico-chemical interaction and optimal stability. Although the activity was observed slightly low in presence of keratin, it was accepted as the other potent combinations were not stable in terms of their physical and chemical attributes.

Although the physical performance was rated based on the risk-ranking and filtering process management, failure of antimicrobial performance mode effects satisfies measurment of the desired action (failure is referred as poor to low performance in this case). In the Ishikawa diagram (Figure 4.20) of the targeted formulation, therapeutic use of the dosage form is one of the key CQAs in the aimed QTPP and any factors manipulating this shall be considered at high risk (Pallagi *et al*., 2018). As discussed earlier, risk is inevitable, but the goal of QbD is to minimise it at each stage and manage product life-cylce with quality (Su *et al*., 2019). Therefore a risk-estimation matrix should be designed with variables of each component of the formulation inter-twined to filter the possible effects and achieve the targeted quality (Grangeia *et al*., 2020). Therefore, always a battery of tests in a constructed design space produce reliable results than just few priliminary investigations. The data generated from such tests will be helpful in future investigations of unexpected events during the product lifecycle (Guilfoyle *et al*., 2013).

Even though the bioassays represent the activity at the targeted site, physical performance of the formulation is also necessary to reach the intended area as per the QTPP. For example: formulation W was determined at lowest risk in terms of physical attributes, but the microbiological performance was poor. Similarly formulations G and F had produced higher efficacy in the bioassay studies, but a poor performance in terms of physical attibutes and chemical compatibility scored them low rank and high risk than R. Hence a synergisitic agreement among the excipients in combination with AAE is crucial towards the targeted performance of the dosage form. Thus, it was determined that stearic acid, triethanolamine, polyethyleneglycol and water, in combination with MCO in formulation R were optimal and with the

advantage of durable stability facilitated by best-in-class excipients for the desired quality targeted product profile.

4.5 Bulk manufacturing of formulation in order to define commercial feasibility

The optimisation of the formulations was carried out using preliminary experiments at lower scale of 30 g with the selected excipients. At industrial scale it will be performed at a larger mass. Therefore it is necessary to optimise the parameters required at larger production levels in order to obtain a commercially feasible protocol for the formulation.

As part of developing feasibility, MCO as AAE and its proven formulation R at pilot scale were investigated in this research. According to the Intellectual Property policy of AIT, the manufacturing method and parameters for the production of MCO were not included in this report. The containers used for the production of MCO were made of borosil glass and stainless steel. From the acid number analysis it was determined that MCO had free fatty acids at higher quantities than VCO. This indirectly provided the fact that, MCO is slightly more polar than VCO.

The average yield for MCO at a production scale of 10 times the established protocol for three batches was determined to be 95%. The loses were observed during the sample transfer and residues remaining in the containers used for the manufacturing. One of the reasons for the residual left-over in the container may be the polarity and affinity of MCO towards the container closure system. Non-stick surfaces such as polytetrafluoroethylene might increase the yield of MCO. This is one of the factors considered for future studies.

A further objective of this feasibility was to study the stability of the formulation under the induced stress conditions over a prolonged period. For this purpose, based on the number of tests that had to be conducted to examine the stability, optimal amount of onychomycotic application sample required was calculated to be 20 g. Therefore, three batches of 15 samples each were designed for stability studies. In total, 300 g of onychomycotic application was theoretically

required for one batch. Considering the factors such as residues in the containers and minimal errors of transfer into containers i.e. standard error during the manual dispensing of onychomycotic application from the production container into storage systems, 400g of onychomycotic application per one batch had been targeted.

Accordingly, three batches were so produced on three different days (See Table 4.33) with fresh raw materials to induce any possible variability as per the documented protocol. However the conditions of manufacturing were maintained constant as per the protocol without any deviations. The documentation was carried out in the presence of a competent individual who was aware of the procedure to ensure good manufacturing practices.

Table 4. 33| **Yield values determined for three batches of the onychomycotic applications**

Batch	Time of production	Raw materials used	Percentage yield for 400 g batch
I	Week one of the schedule	Not less than one week old	98
II	Day 8 from the schedule	Produced on the week one of the scheule	98
III	Day 15 from the schedule	Produced on the day 15 of the schedule	99

The loss was determined by weighing the equipment parts that were in contact with the formulation in the production line. The highest residue was determined to be in the glass container used for the production followed by the shaft of the overhead hand mixer. The hydrophilic and metallic properties of the equipment were the reasons for the residues as the continous phase or major component of the formulation was deionised and sterile water. The lowest residue, comparitively among all equipment was determined in the sterile syringe used to dispense the formulation into container closure system as the components were made of non-polar materials such as polypropylene and rubber.

From the above observations it was determined that, for the production of onychomycotic application, high shear blender made of non-polar material and

dispensing through non-polar suction operated injection system would improve the yield.

4.6 Stability studies to evaluate the interference of atmospheric conditions with the performance of the formulation

As discussed in Section 2.9, stability of the dosage form is the ultimate character governing quality. ICH Q1 dictates the assessment of stability under induced stress conditions as one of the quality attributes of any formulation design. Based on the climatic zone of the targeted area of distribution and storage conditions, stress parameters shall be considered during the evaluation. In this research 25^{o}C and 75% relative humidity (RH) were selected to investigate the stability of the formulation. Batches of AAE and onychomycotic application R produced in the scale-up protocol were tested for stability in this investigation.

Formulation R was examined for stability over a 90 day incubation period under induced stress conditions in a three batch experiment with 15 samples per batch. Samples were collected on 1, 15, 30, 60 and 90 days incubation. To derive maximum relevance to actual use, samples were collected from a container opened on day 1 sample from an unopened container for that particular point (See Table 4.34).

Table 4. 34| **Samples were collected from the containers of the batches of 15 containers each as below**

Time point (days)	3 Samples was collected from each batch	Sample collected from container opened on day 1 from each batch
1	L1, L2, L3	-
15	L4, L5, L6	L1-A, L2-A, L3-A
30	L7, L8, L9	L1-B, L2-B, L3-B
60	L10, L11, L12	L1-C, L2-C, L3-C
90	L13, L14, L15	L1-D, L2-D, L3-D

L = licensce number issued to the individual product in the batch.

As MCO is a NME, its stability was evaluated by the IR spectrocopic analysis to study any structural changes under the elevated temperature and humidity

conditions; compositional stability was tested by free fatty acid number determination and the microbial stability was evaluated by the USP <61>.

In the section 2.8.1 the effect of temperature over the stability of the dosage form was explained by Arrhenius equation and first order rate of kinetics. Based on the ingredients of the formulation R, the anticipated effect of induced temperature and humidity over the stability were explained as below.

Table 4. 35| **Ingredients of the formulation and the anticipated effect by the stress conditions**

Ingredient	Anticipated effect
Salt of SA and TEA	The salt was formed by the amide bond formation between fatty acid group of SA and amine group of TEA by the loss of a water molecule. Therefore, induced humidity might hydrolyse this bond under the temperature and destabilise the emulsion.
PEG	Polyols such as PEG will interact with hydrophilic moieties through hydrogen bond formation. The visco-elasticity of these moieties is always a result of extent of hydrogen bonds formed. Under elevated humidity, condensation might reduce the viscosity due to dilution.
Water	As mentioned above, along with disruption of hydrogen bonds, stressed temperature might evaporate the content upon prolonged storage. This can cause the emulsion to break leading to instability.

Considering the above possible effects and the responsible factors towards the degradation of the product a battery of tests were designed based on the attributes established during the formulation optimisation. These were described in the Figure 4.50.

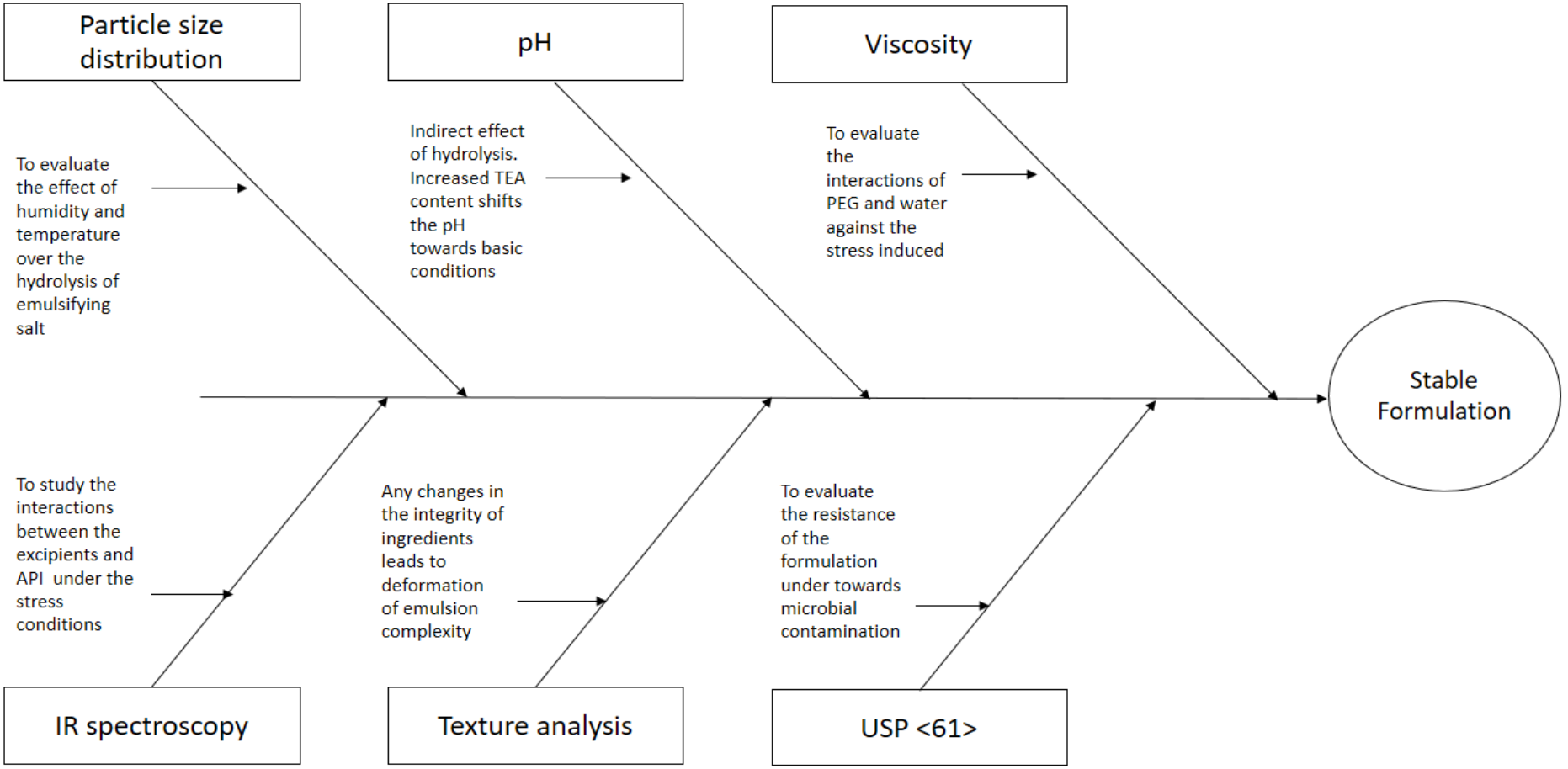


Figure 4.50| **Battery of tests designed to evaluate the stability of pilot batches of onychomycotic applications under induced stress conditions.**
The test series contained a combination of physical, chemical and microbiological techniques with data determined during the optimisation as reference points.

4.6.1 Evaluation of stability

ICH Q1(E) (2003) prescribes the application of regression analysis on the data collected from the testing of the stability samples. In this method, it describes the relation between the chosen attribute of the API/finished dosage form and the stressed conditions as a function of time. A linear regression analysis of data collected from each stability pull point versus time would provide quantitative information regarding retest date or shelf life of the API/finished product.

Similarly, based on the data available from the initial optimisation studies of the formulation development, a process average was determined and the respective limits were established based on the standard deviation of the data. Using these, quality control charts were constructed for the attributes measured on the formulation samples from the various pull points.

4.6.1.1 Stability of MCO

As described under Section 3.8, the stabilty of MCO (AAE) was also evaluated along with the onychomycotic application under similar induced stress conditions. Three batches of MCO containing 15 samples per batch were manufactured and packed in polypropylene extrusion containers individually. All packed batches were stored under 25^{o}C and 75% RH conditions for a period of 90 days and samples were collected as per Table 4.34.

The evaluation was carried by testing the pulled samples using IR spectroscopy and acid number. The characteristic peaks of MCO at the region of 1700 to 1740 cm^{-1} differentiating it from VCO was used a qualitative test and the acid number determination was used for quantitative analysis of stability. Samples pulled were examined against a bright background for the appearance.

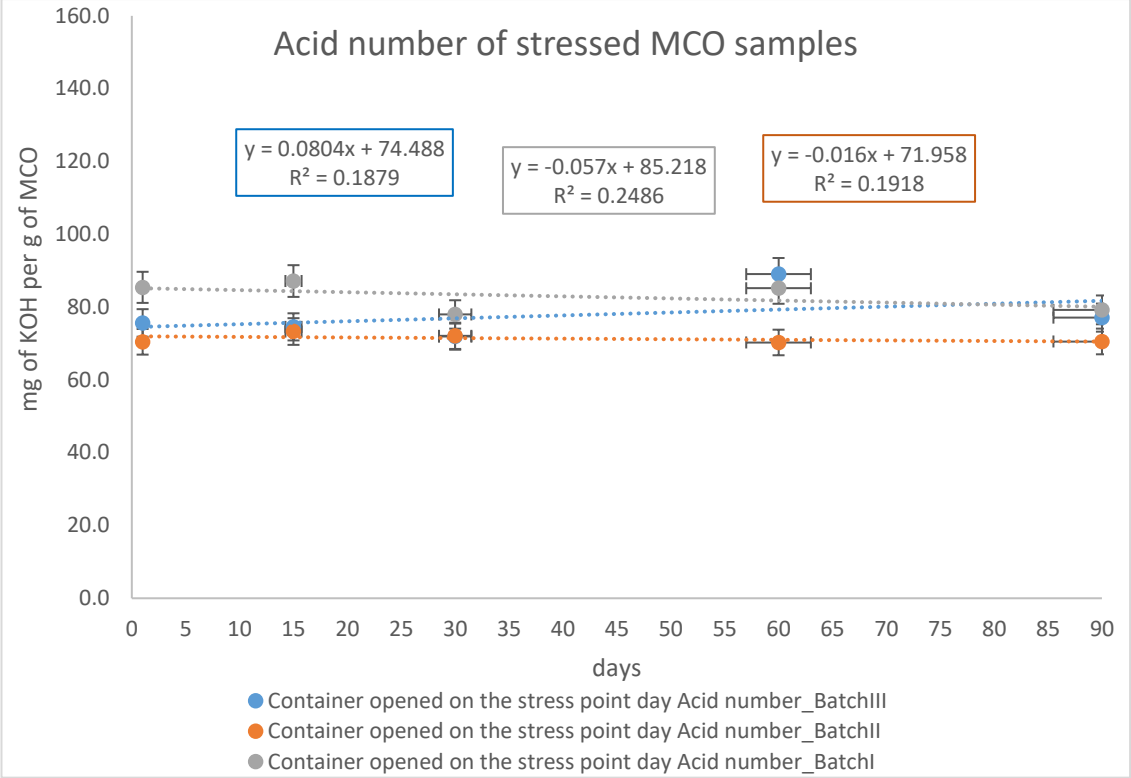


Figure 4.51| **Acid number values of the MCO samples collected over different time points from unopened containers under induced stress conditions.** Orange colour line indicates the linear expression of the data of Batch II, whereas grey and blue represent Batches I and III respectively.

The R^2 values determined for the three batches of MCO 0.2486, 0.1919 and 0.1879 indicated that the dependent variable i.e., acid number which is also the integrity of the MCO composition in this study, did not vary significantly with induced temperature and humidity factors over the period of 90 days of the study. From the Figure 4.50, the regression analysis of the data from each batch produced slopes nearly equivalent to zero for the three batches. This indicated that, the line generated is parallel to x – axis i.e., duration of stability study, which in return described that the composition of MCO was stable over a period of 90 days.

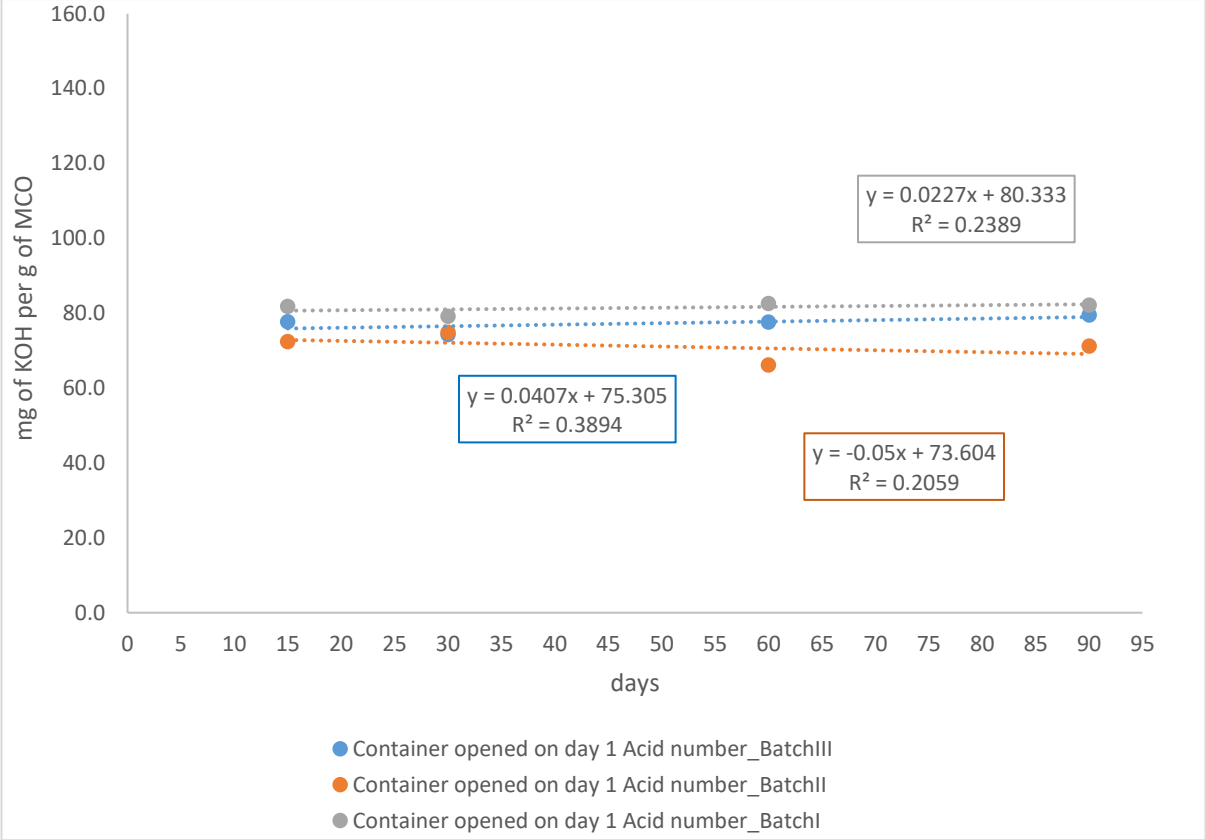


Figure 4.52| **Acid number values of the MCO samples collected from containers opened on day1 over different time points under induced stress conditions.** Orange colour line indcates the linear expression of the data of Batch II, whereas grey and blue represent Batches I and III respectively.

In order to evaluate the influence of atmospheric factors on MCO while opening the containers, samples were collected at each time point from containers opened on day 1 along with unopened containers at the respective time point. The linear regression plots of these values were described in Figure 4.51. The slopes determined for each linear regression plot of batches was determined nearly zero. Similar to the data determined from the samples collected from freshly opened container, MCO was determined stable for a period of 90 days even after opened on day 1.

IR spectoscopic studies were also conducted on same samples prior to Acid Number analysis. The method described under Section 3.3.1 was employed to identify any possible impurities or development of unsaturation or leachables from the storage containers.

Table 4. 36| **Results from the IR spectroscopic analysis, appearance testing and USP <61> enumeration testing of samples collected at time points of stability study**

Parameter	**Wavenumber range (cm^{-1})**	**Batch – I**	**Batch – II**	**Batch – III**
Triacylglycerol	1730 – 1740	Yes	Yes	Yes
Free fatty acid	1700 – 1720	Yes	Yes	Yes
Unsaturation	600 – 800	No	No	No
Unspecified impurities*	400 – 4000	No	No	No
Appearance				
Clear and colourless	N/A	Yes	Yes	Yes
Visible particles	N/A	No	No	No
USP<61>				
TAMC	≤2	Yes	Yes	Yes
TYMC		Yes	Yes	Yes

*-Any atypical peaks identified differentiating the composition from previous studies.

As discussed earlier, MCO is a mixture of saturated fatty acids and their corresponding glycerides. The solubility of MCO was determined high in lipophilic solvents such as methanol (KF titration). Considering these lipophilic properties, any interaction with the container closure system leading to leachables was hypothesised as the ultimate undersired effect under the induced stress conditions during stability evaluation period.

However, the IR spectroscopic and Acid number value determinations affirmed that the chemical composition of MCO remained unaffected due to induced stress conditions. Therefore, MCO was determined stable for a period of 90 days under 25^{o}C and 75% RH.

4.6.1.2 Stability assessment of onychomycotic application

As described in Chapter 2.7.1, particle size distribution in an emulsion plays significant role on the dispersion of internal phase. A surfactant used during the emulsification process is the crucial excipient regulating the extent of distribution. Emulsifier formed by reaction between stearic acid and triethanolamine excipient was used in the onychomycotic application to solubilise MCO into the dispersed medium. Influence of induced stress conditions on the emulsification capabilities of such surfactant was evaluated in this study.

Table 4. 37| **The effect of stress conditions on each excipeint.**

Parmeter	Excipient responsible	Relative humidity	Temperature
Particle size distribution	SA+TEA	+++	-
Cohesiveness	PEG+Water	++	+
Firmness	SA+TEA+PEG	+	++
Consistency	SA+TEA	++	+
Index of viscosity	PEG	++	+
Turbidity	SA+TEA	+++	-
Potency	AAE+SA+TEA	++	+

(+++)-critical – effect is high and process to be stopped immediately, (++)-major – effect is medium and the process to be moniterd, (+)-minor – effect is minimal and process is under control and (-)- no effect

As described in Table 4.37 above each and every excipient contribute towards the emulsion stability opposing the exerting pressure from the external stress conditions. Therefore the samples pulled from each stress point were tested for the parameters described in the Table 4.38 and regression analysis of the data generated was also conducted. Slope with any negative or positive value above and below one was considered significant change in the trend of the formulation over the period of stability studies. Similarly the coeffecient of determination value of 0.985 was considered as statistically significant to describe the variation of samples over the period of stability versus the applied stress conditions. The Table 4.37 below summarises the slope values and regression coeffecients of data of the stability samples for each parameter.

Table 4. 38| **Slope and regression coeffecient values of the data generated for three of onychomycotic applications over the stability period.**

Parameter	Freshly opened container on the day of pull point			Sample collected at each pull point from the container opened on day 1		
			Slope of regression line			
Batch	**I**	**II**	**III**	**I**	**II**	**III**
Turbidity	0.0	0.0	0.0	0.2	0.3	0.2
Firmness	0.0	0.0	0.0	0.0	0.0	0.0
Cohesiveness	0.0	0.0	0.0	0.0	0.0	0.0
Index of viscosity	0.0	0.0	0.0	0.0	0.0	0.0
Consistency	0.0	0.0	0.0	0.0	0.0	0.0
Particle size	0.0	0.0	0.1	0.0	0.0	0.0
Potency	0.0	0.0	0.0	0.0	0.0	0.0
			Regression coefficient			
Batch	**I**	**II**	**III**	**I**	**II**	**III**
Turbidity	0.005	0.001	0.005	0.234	0.155	0.147
Firmness	0.390	0.007	0.617	0.646	0.090	0.964
Cohesiveness	0.466	0.595	0.848	0.858	0.373	0.643
Index of viscosity	0.754	0.009	0.537	0.537	0.725	0.281
Consistency	0.391	0.053	0.519	0.764	0.423	0.000
Particle size	0.061	0.115	0.491	0.366	0.195	0.288
Potency	0.097	0.344	0.510	0.035	0.276	0.570

The values described in the Table 4.38 didn't meet the specification criteria described earlier confirming that there was no significant effect of stress conditions over the physical parameters of the onychomycotic application over a period of 90 days of storage. The quality of these parameters was tested by comparing the data with initial optimal studies. The average value determined from the process optimisation studies is used as process mean and the standard deviation of the respective data was used to set the limits. The figures from 4.52 to 4.55 describe the QC charts of physical parameters of the stability samples (pulled from 25^{o}C/75% RH storage conditions) in comparison with process average.

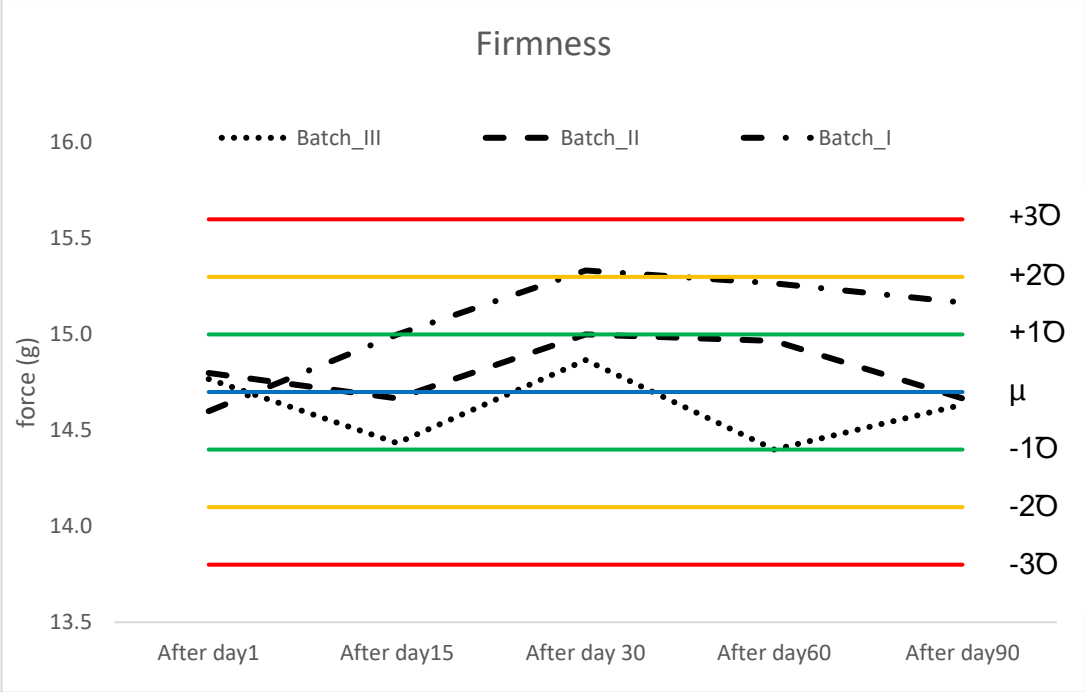


Figure 4.53| **QC chart of Firmness of stability samples (pulled from 25ºC/75% RH storage conditions) versus the process average.** All the three batches were within the maximum limits, with Batch I values slightly towards the second warning limit. The red line above preformulaiton process average (blue line) is calculated by adding three times standard deviation to the process average. Similarly the orange and green above blue line are two and one time the standard deviation added to process average. Similarly, the lines below process average was constructed by substracting three, two and one time standard deviation from the process average.

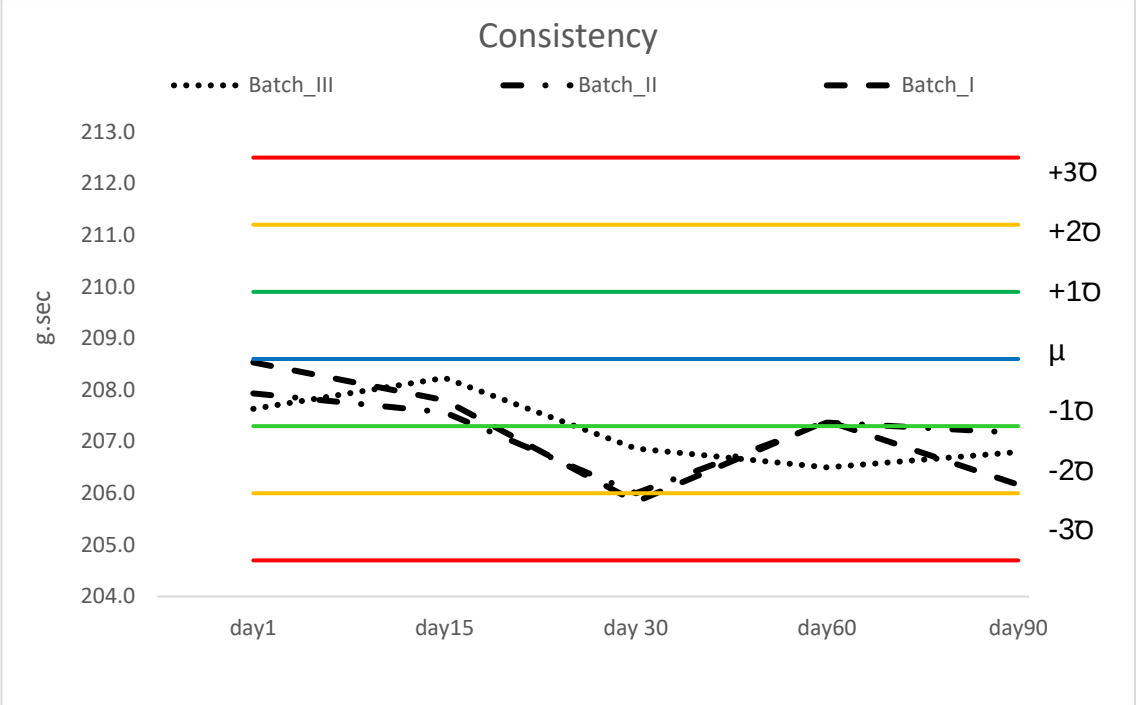


Figure 4.54| **QC chart of Consistency of stability samples (pulled from 25°C/75% RH storage conditions) versus the preformulation process average.** All the three batches were within the maximum limits, with Batch I values slightly towards the second warning limit.

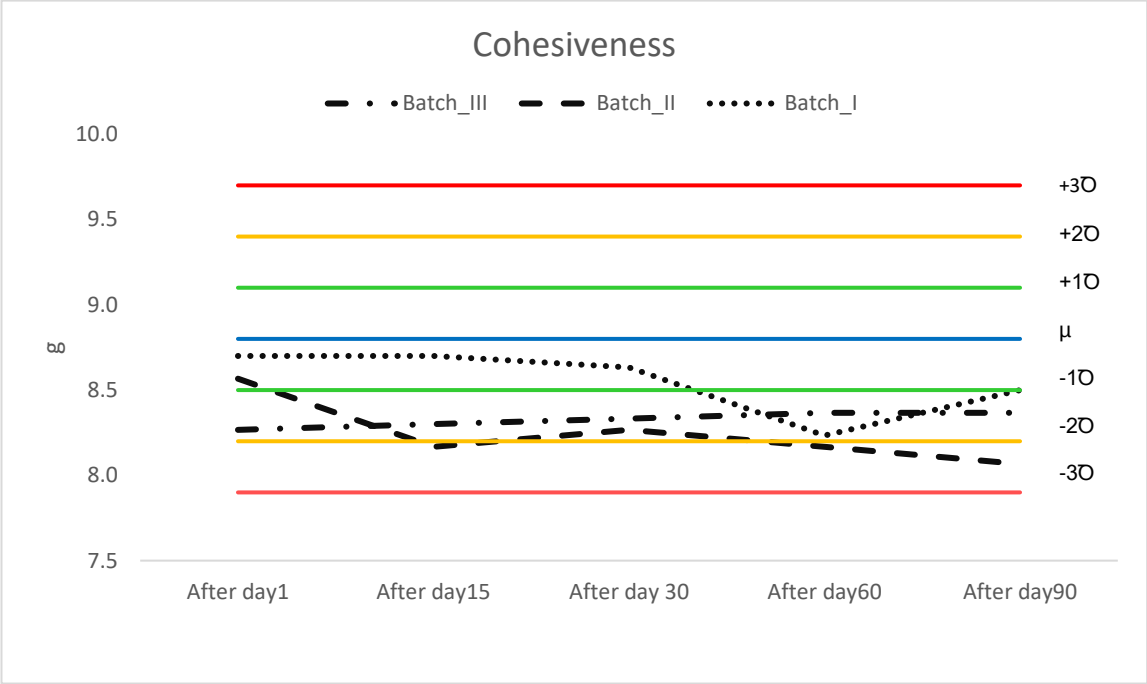


Figure 4.55| **QC chart of Cohesiveness of stability samples (pulled from 25°C/75% RH storage conditions) versus the preformulation process average.** All the three batches were within the maximum limits, with Batches I & III values slightly towards the second warning limit.

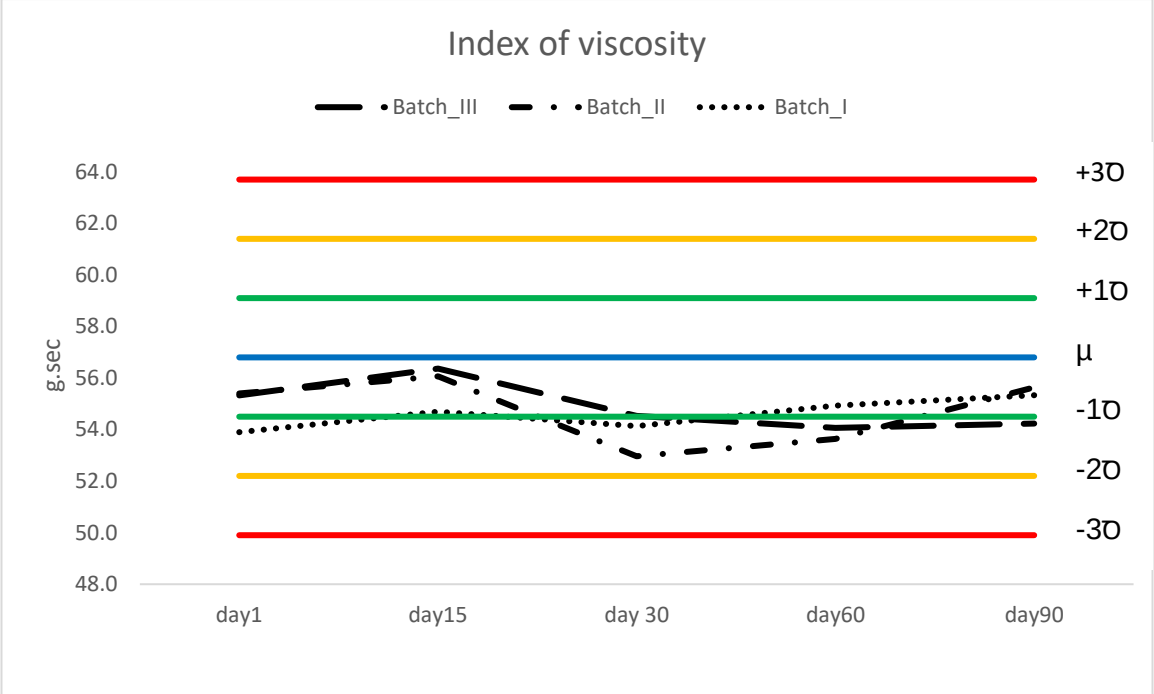


Figure 4.56| **QC chart of Index of viscosity of stability samples (pulled from 25°C/75% RH storage conditions) versus the preformulation process average.**

The data from all batches was determined within the limits of standard deviation. A common trend observed among them was that, data started deviating towards the second warning limit (orange line) after 30 days of stability study. However the deviation was within the limit of alerting to verify the process before the next phase of production.

From the Table 4.38, the influence of each stress condition over the respective physical property was understood. From figures 4.52 to 4.55 it can be inferred that firmness of the onychomycotic application had increased and the values determined were above the process average after a period of 30 days. For the other parameters, the values were determined below the process average. Firmness of onychomycotic application in this sense represents the ability of the onychomycotic application to resist the positive force exerted by the probe (downward movement towards the surface of onychomycotic application) of a texture analyser during the measurement. This ability of resisting the force was accounted for the SA+TEA+PEG excipients of the onychomycotic application. SA+TEA form the emulsifier with HLB scale towards solubilising the aqueous insoluble materials. Similarly PEG is a polyol whose role is to retain the moisture by maintaining appropriate hydrogen bonds with water in the

onychomycotic application. A slight increase in the firmness indicated that onychomycotic application in the container lost minute amount of water thereby became slightly stiff opposing the force of probe exerted on it during the testing. But, the values were within the limits inidcating stable environment. This deficit could be be addressed through judicious choice of packaging container.

The data generated from the cohesivness measurements followed a similar trend but below the process average. Cohesivness of onychomycotic application was measured by determining the amount of force required by the texture analyser to move the probe away from the onychomycotic application after complete contact. It is a result of synergistic interaction of all aqueous soluble materials as the designed formulation is an o/w emulsion. The decreased trend indicated reduced cohesivness due to loss of slight amount of water supporting the observation in the case of Firmness.

Consistency and index of viscosity have similar trend in terms of stability. Both of them observed below the process average but within the limits. Consistency is the positive work done by the probe to penetrate into the onychomycotic application sample whereas the index of viscosity is the negative work (data represented after normalisation) while moving out. Unlike cohesivness and firmness, these parameters are directly related to each other. The higher the consistency i.e., uniform distribution of phases in the given amount of sample, the higher the viscosity (inverse of index of viscosity).

Table 4. 39| **IR spectroscopic analysis and pH measurements of samples collected from timepoints.**

Parameter	**Wavenumber range (cm^{-1})**	**Batch – I**	**Batch – II**	**Batch – III**
Triacylglycerol	1730 – 1740	Yes	Yes	Yes
Free fatty acid	1700 – 1720	Yes	Yes	Yes
Amide (SA+TEA)	1640 – 1670	Yes	Yes	Yes
Unsaturation	600 – 800	No	No	No
Unspecified impurities*	400 – 4000	No	No	No
Appearance				
White in color with glossy texture	N/A	Yes	Yes	Yes
pH				
7.0 – 8.0	N/A	Yes	Yes	Yes
USP<61>				
TAMC	≤2	Yes	Yes	Yes
TYMC		Yes	Yes	Yes

Along with physical attributes, other paramters tested (Table 4.39) didn't provide any out of specification or expectation results. Therefore MCO onychomycotic applications were observed slightly away from the process average after 30 days but were within the limits and were stable for a period of 90 days under 25^{o}C and 75% RH.

5.0 Key project outcomes

MCO is a novel chemical entity with proven antimicrobial properties towards oral pathogens belonging to *Streptococcal* and *Candidal* species (Hughes P. 2015). In this research, its activity towards *T. rubrum* has been evaluated in order to develop a potent, efficacious, and non-invasive dosage form to treat onychomycosis.

The potency of MCO was tested across a wide range of concentrations from 5% to 100% w/w with dilutions prepared in both fat- and water-soluble diluents. The activity of MCO was assessed in comparison with a commercial OTC K101 formulation, Naloc^{TM}. On interrogation using a well-diffusion assay, it was determined that MCO at 15% w/w concentration inhibited the growth of the pathogen in 3 days whereas Naloc^{TM} produced equivalent inhibition but only in 21 days. Therefore, 15% w/w of MCO was confirmed as the effective concentration.

Along with antimicrobial activity assessment, well-diffusion assays provide penetration capacity of test molecules through aqueous-based media. As discussed earlier in Section 2.2, penetration through nail plate is a critical challenge for a therapeutic moiety to deliver maximum anti-onychomycotic effect. Therefore, formulating MCO into an aqueous-based medium at 15% w/w concentration was key focus for systematic investigation in this research.

Informed by the required QTPP (Table 2.1) for an MCO formulation intended to treat onychomycosis, the CQAs of MCO affirming successful manufacture of a quality product were identified using IR spectrometry, NMR spectrometry, acid value determinations (USP <401>), differential scanning calorimetry, thermogravimetric analysis, and density. The conformation determined from this array of tests is that MCO is a saturated molecule containing long chained - monoglycerides, -triglycerides and -fatty acids. This has also reconfirmed that MCO is lipid soluble in nature and requires additional agency to solubilise it into aqueous medium. Similarly, atomic absorption spectrometry conducted to identify and quantify trace metals as listed in ICH Q3D and a *Limulus Amoebocyte Lysate* based – bacterial endotoxin test – have confirmed that MCO and its manufacturing process is free from potential trace metals

and pyrogens. This has eliminated the preliminary toxicological risk to formulate MCO into a feasible dosage form.

As per ICH Q8 guidelines, identification of QTPP based on the clinical aspects of onychomycosis narrowed the type of dosage form required for product development of a viable treatment. In Table 2.1, the QTPP elements of a successful dosage form for effective treatment has also identified the necessary characterisation techniques required. For example, IR spectrometric analysis to identify MCO and its interactions with corresponding excipients was used throughout this project as a part of control strategy. Similarly, acid number (determined as per USP<401>) was identified as a CQA of MCO and included into the control strategy to monitor the quality of MCO during AAE production.

Risk assessment played a crucial role in identifying and confirming a stable configuration of excipients and their respective ratios with MCO. A matrix constituting the amount of risk posed by each property of emulsion towards its stability and the contribution of each excipient towards those properties, were used to design a miniature space to confirm the stable excipient combinations for MCO formulations. An extensive battery of experiments including particle size distribution characterisation, texture analysis and antimicrobial assessment were conducted and the MCO and excipient combinations that would destabilise this design space were considered incompatible.

The formulation ultimately finalised through such process has also demonstrated efficient performance in advanced *in vitro* antimicrobial assays induced with keratin in the nutritional medium. This has reconfirmed that application of ICH Q8 guidelines, especially when combined with novel bio-alternative determinations, can streamline the formulation design process. However, to support the hypothesis of quality-by-design, stability of the thus-finalised dosage form was evaluated by subjecting three batches of it to induced stress conditions of 25^{o}C and 65% relative humidity over a period of 90 days. Samples pulled over the respective time points were tested for the properties within a projected stable design space. For this aspect, statistical process control principles provided valuable proof of the

robustness of the formulation and supported the utility of the characterisation strategies adopted.

The information gained through the characterisation steps of MCO as AAE and subsequently when combined with excipients in formulation as a part of preformulation studies, helped to establish the limits to study the quality of MCO and its formulation under induced stress conditions. These limits help to examine and inform the life-cycle of the MCO formulation in future studies and assist in root-cause analysis to investigate any unexpected or out-of-specification or out-of-trend results (if necessary). For example, when compared with the information from pre-formulation and optimisation studies, the data generated from the analysis of pulled samples from the stress conditions have proven that MCO (at 15%w/W), when optimally formulated in combination with stearic acid (15% w/w), triethanolamine (7.86% w/w), polyethyleneglycol (10%w/w) and water (52.14% w/w), is stable over a period of 90 days. This holds promise for future provision of targeted delivery of the active principle using a cost-effective delivery system.

Therefore, the application of ICH Q8 guidelines to develop the MCO formulation has streamlined the process and navigated this project to a certain extent within the timeframe available to obtain non-clinical information of Module 2 in order to align and position this novel product as a viable and promising scientific treatment and licensable opportunity for an inadequately met medical need.

6.0 Conclusion

In this research, antimicrobial activity of MCO towards *T. rubrum* and *C. albicans* has been established and was determined to be superior at 15% w/w concentration in a water soluble environment towards *T. rubrum* and *C. albicans* than Naloc^{TM}, an OTC dosage form in the market. The CQA of MCO were established using IR, NMR and thermal analytical techniques. Atomic absorption spectrometry and LAL – Pyrogen testing studies confirmed that MCO is free from trace metals and endotoxins. Based on the guidelines of ICH Q8 (R2), a QTPP was established to design MCO into a potent dosage form. Twenty-seven formulations were developed using established excipients. A risk assessment-based particle size distribution analysis, texture analysis and bioassays were used to discriminate as best-in-class dosage form. Scale-up manufacturing has been conducted for MCO and the proven dosage form at three batches each with 15 samples per batch. All the batches were subjected to induced stress conditions of 25^{o}C and 75% RH for a period of 90 days. Samples were pulled at regular time intervals and were tested for physical, chemical and biological attributes as per the methods developed and compared with the results obtained during the optimisation studies. It was determined that, MCO and its onychomycotic application were stable for a period of 90 days at 25^{o}C and 65% RH.

7.0 Future Work

Recommended future work for AAC and the onychomycotic application formulation has been described below in the Table 7.1

Table 7. 1| **Future work and the corresponding techniques to be implemented**

For Active antimicrobial entity			
Property	**Analytical technique**	**CQA/QTPP**	**Justification**
Indentification and quantification of individual components	Gas chromatograhy / Liquid chromatography	CQA	To quantify the components of the complex
Purification of components	Liquid chromatography	CQA	To increase the concentration of respective active component
Assay	Gas chromatography / Liquid chromatography	CQA	To ensure quality of the complex maintained consistently
Residual solvents	Gas Chromatography	CQA	To ensure manufacturing process is free from any toxic solvents
For onychomycotic application			
Assay	Gas chromatograhy / Liquid chromatography	QTPP	To ensure quality and potency of the formulation maintained consistently
***In vitro* dissolution studies**	Franz's cell/Keratin layer*	QTPP	To determine the release profile of complex in the presence of keratin
Stability studies	ICH Q1	QTPP	To evaluate the stability of the formulation over prolonged period (atleast ≥24 months)

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