

# **The use of apricot oil emulsions for the transdermal delivery of selected statins**

**S Marais**

 [orcid.org/ 0000-0002-1131-1424](https://orcid.org/0000-0002-1131-1424)

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Supervisor: Prof M Gerber

Co-supervisor: Prof J du Plessis

Co-supervisor: Prof LH du Plessis

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Student number: 22819517

*“Our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure. It is our light, not our darkness that most frightens us. We are all meant to shine, as children do. We were born to make manifest the glory of God that is within us. It's not just in some of us; it's in everyone. And as we let our own light shine, we unconsciously give other people permission to do the same. As we are liberated from our own fear, our presence automatically liberates others.”*

*Marianne Williamson*

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## *Abstract*

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Familial hypercholesterolemia can be described as a condition associated with significantly high levels of low-density lipoprotein (LDL) in the plasma, caused by an autosomal-dominant genetic disorder of lipid metabolism. To date, statins are considered as the first-line therapy. However effective, statin side-effects can hinder the reach of LDL target levels due to poor patient compliance. It is proposed that by utilising safe alternative ways of administration, complications with the current dosage form, might be overcome. One such route is transdermal delivery, which poses as a preferred safer alternative to oral administration.

For transdermal delivery, ideal physiochemical properties of the active pharmaceutical ingredient (API) are essential. Upon investigation, it became evident that the statins possessed some ideal properties; however, inadequacies with regard to the log P and aqueous solubility were observed. Hence, the aim was to formulate and investigate oil-in-water (o/w) nano-emulsions (droplet size 20 – 200 nm), containing 2% (w/w) of the respective statin and 8% (w/w) apricot kernel oil, since literature suggests this system can aid in the delivery of molecules, which otherwise would not penetrate the skin. In this study, it is proposed that the use of apricot kernel oil as the oil phase of the nano-emulsions, would act as a chemical penetration enhancer, due to the fatty acids present in this oil, such as oleic and linoleic acid.

Initially, two o/w nano-emulsion formulas were characterised, both containing 2% (w/w) API and different amounts of apricot kernel oil. Thereafter, the optimised formula was utilised to formulate a nano-emulgel. After characterisation, it was apparent that both formulas attained properties considered ideal for transdermal delivery. Firstly, membrane studies were conducted to determine whether API release from the vehicle occurred, the flux values obtained were indicative that release of the four statins occurred from each of the two respective vehicles. Thereafter skin diffusion studies were performed to assess the extent of drug absorption through the skin. Tape stripping was performed after the 12 h extraction of the receptor phase (phosphate buffer solution (PBS):ethanol (9:1 at pH 7.4)) to determine the amount of API within the skin, which is an indication to whether topical or transdermal delivery was achieved. Concentrations of each of the statins within each of the respective formulas were quantified in the receptor phase, as well as the stratum corneum-epidermis (SCE) and epidermis-dermis (ED) respectively. Thus, the aim of transdermal delivery was achieved.

Lastly, *in vitro* cytotoxicity studies were conducted on normal immortalised human keratinocytes (HaCaT) cells, by means of a methylthiazol tetrazolium (MTT) assay and neutral red (NR) assay to determine whether the excipients used in the formulation of nano-emulsions could be considered safe for application on human skin. Subsequently, the half-maximal inhibitory concentration ( $IC_{50}$ ) of the respective statins and excipient could be established. Simvastatin

alone, and within the optimised nano-emulsion, was found to be the most cytotoxic, although the concentrations tested still exceeded the amounts that diffused through the skin, suggesting only a small possibility of side-effects.

**Keywords:** transdermal drug delivery, hypercholesterolemia, statins, nano-emulsion, nano-emulgels, Franz cell diffusion, cytotoxicity.

# Uittreksel

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Familiale hipercholesterolemie kan beskryf word as 'n toestand wat geassosieer word met beduidende hoë vlakke van laedigheidlipoproteïen (LDL) in die plasma, wat veroorsaak word deur 'n outosomale-dominante genetiese versteuring van lipiedmetabolisme. Statiene word tans as die eerstevlak-terapie beskou. Alhoewel dit effektief is, kan statien se nuwe-effekte die bereiking van LDL-doelvlakke verhinder as gevolg van swak pasiëntmeewerkendheid. Die gebruik van veilige alternatiewe maniere van toediening word voorgestel om komplikasies met die huidige doseervorm te oorkom. Een van hierdie maniere is transdermale aflewering wat as 'n verkose, veiliger alternatiewe roete tot orale toediening voorkom.

Ideale fisieschemiese eienskappe van die aktiewe farmaseutiese bestanddeel (AFB) is noodsaaklik vir transdermale aflewering. Gedurende die studie het dit na vore gekom dat statiene sekere ideale eienskappe besit, maar tekortkominge met betrekking tot die water-oktanol partisiekoëffisiënt ( $\log P$ ) en wateroplosbaarheid is egter waargeneem. Die doelwit was dus om olie-in-water (o/w) nano-emulsies (druppelgrootte 20 – 200 nm) wat 2% (m/m) van die betrokke statien en 8% (m/m) appelkoospitolie bevat, te formuleer en te ondersoek, aangesien literatuur voorstel dat hierdie stelsel kan help met die aflewering van molekules wat andersins nie die vel sal penetreer nie. In hierdie studie word daar voorgestel dat die gebruik van appelkoospitolie, as die oliefase van die nano-emulsies, as 'n chemiese penetrasie-bevorderaar sal dien as gevolg van die vetsure, soos oliënsuur en linoliënsuur, wat in die olie teenwoordig is.

Aanvanklik is twee o/w nano-emulsieformules saamgestel wat beide 2% (m/m) AFB en verskillende hoeveelhede appelkoospitolie bevat het. Daarna is die geoptimaliseerde formule gebruik om 'n nano-emuljel te formuleer. Na karakterisering was dit duidelik dat beide formules eienskappe bekom het wat ideaal is vir transdermale aflewering. Eerstens is membraanstudies uitgevoer om te bepaal of vrystelling van die AFB vanuit die medium plaasgevind het. Die vloedwaarde was 'n aanduiding dat die vrylating van die vier statiene plaasgevind het vanuit die twee betrokke mediums. Daarna is veldiffusiestudies uitgevoer om die mate van geneesmiddelabsorpsie deur die vel te evalueer. Kleefbandstropping is uitgevoer na die 12 h onttrekking van die reseptorfase (fosfaatbufferoplossing (FBO):etanol (9:1 by pH 7.4) om die hoeveelheid AFB in die vel te bepaal, wat 'n aanduiding is of topikale of transdermale aflewering bereik is. Konsentrasies van elk van die statiene binne elk van die onderskeie formules is in die reseptorfase, asook in die stratum korneum-epidermis (SKE) en epidermis-dermis (ED) onderskeidelik gekwantifiseer. Dus is die doel van transdermale aflewering bereik.

Laastens is *in vitro* sitotoksisiteitsstudies op normale menslike keratienosieteselle (HaCaT) uitgevoer deur middel van 'n metieliasoltetrasolium (MTT) proef en neutraalrooi (NR) proef, om te bepaal of die bestanddele wat in die formulering van nano-emulsies gebruik word as veilig vir

aanwending op menslike vel beskou kan word. Vervolgens kon die half-maksimale inhibiesiekonsentrasie ( $IK_{50}$ ) van die betrokke statiene en bestanddele bepaal word. Daar is gevind dat simvastatin alleen en binne die geoptimaliseerde nano-emulsie die meeste sitotoksiteit getoon het, alhoewel die konsentrasies wat getoets is, steeds die hoeveelhede wat deur die vel gediffundeer het oorskry, is daar slegs 'n klein moontlikheid dat newe-effekte kan voorkom.

**Sleutelwoorde:** transdermale geneesmiddelaflewering, hipercholesterolemie, statiene, nano-emulsie, nano-emuljel, Franz seldiffusie, sitotoksiteit

# Table of Contents

---

<i>Acknowledgements</i>	i
<i>Abstract</i>	iii
<i>Uittreksel</i>	v
<i>List of Equations</i>	xix
<i>List of Figure</i>	xxi
<i>List of Tables</i>	xxxix
<i>Abbreviations</i>	xxxvii

## *CHAPTER 1:*

### *Introduction, research problem and aims*

1.1	Introduction	1
1.2	Research problem	4
1.3	Aims and objectives	5
	<b>References</b>	<b>6</b>

## *CHAPTER 2:*

### *Formulation and transdermal delivery of nano-emulsions containing the selected statins and apricot kernel oil*

2.1	Introduction	12
2.2	Hypercholesterolemia	14
2.2.1	Treatment of hypercholesterolemia	15
2.2.1.1	Lovastatin	16
2.2.1.2	Mevastatin	17
2.2.1.3	Simvastatin	17
2.2.1.4	Rosuvastatin	18
2.3	Factors influencing the consideration to use alternative routes of administration	19
2.4	Skin	20

<b>2.4.1</b>	The epidermis	21
<b>2.4.1.1</b>	The non-viable epidermis (stratum corneum)	21
<b>2.4.1.2</b>	The viable epidermis	22
<b>2.4.2</b>	The dermis	22
<b>2.4.3</b>	The hypodermis	23
<b>2.5</b>	Transdermal drug delivery	23
<b>2.5.1</b>	Intercellular route	24
<b>2.5.2</b>	Transcellular route	24
<b>2.5.3</b>	Appendageal routes	25
<b>2.6</b>	Physiochemical properties that influence transdermal delivery	25
<b>2.6.1</b>	Aqueous solubility	26
<b>2.6.2</b>	Melting point	26
<b>2.6.3</b>	Molecular mass	26
<b>2.6.4</b>	Partition coefficient	27
<b>2.6.5</b>	Diffusion coefficient	27
<b>2.6.6</b>	Ionisation, pH and pKa	28
<b>2.7</b>	Approaches to successful transdermal delivery	29
<b>2.7.1</b>	Penetration enhancers	30
<b>2.7.1.1</b>	Fatty acid as a component of natural oils	30
<b>2.7.1.2</b>	The relevance of apricot oil use in nano-emulsion	31
<b>2.7.2</b>	Nano-emulsions	31
<b>2.7.2.1</b>	Advantages of nano-emulsions	33
<b>2.7.2.2</b>	Disadvantages with the use of nano-emulsions	34
<b>2.7.2.3</b>	Methods used in the formulation of nano-emulsions	34
<b>2.8</b>	Semi-solid formulation	35
<b>2.8.1</b>	Emulgel	36
<b>2.8.2</b>	Nano-emulgel	36
<b>2.9</b>	Toxicity testing	37
<b>2.10</b>	Conclusion	37
<b>References</b>		39

### **CHAPTER 3:**

#### *Article for the publication in "Die Pharmazie"*

<b>Abstract</b>	57
1. Introduction	57
2. Investigations, results and discussion	58
3. Experimental	60
<b>Acknowledgements</b>	60
<b>Disclaimer</b>	61
<b>References</b>	61
<b>Tables</b>	63
<b>Figures</b>	64

### **CHAPTER 4:**

#### *Article for the publication in the International Journal of Pharmaceutics*

<b>Abstract</b>	68
<b>Graphical Abstract</b>	69
1. Introduction	70
2. Materials and Methods	72
2.1 Materials	72
2.2 Methods	72
2.2.1 Formulation of nano-emulsions and nano-emulgels	72
2.2.2 Analysis of mevastatin, lovastatin, rosuvastatin and simvastatin	74
2.2.3 Standard preparation	74
2.2.4 Physicochemical properties	75
2.2.4.1 Aqueous solubility	75
2.2.4.2 Octanol-buffer distribution coefficient (log D)	75
2.3 Characterisation of pravastatin formulations	76
2.3.1 TEM	76
2.3.2 pH	77
2.3.3 Viscosity	77

2.3.4	Droplet size	77
2.3.5	Zeta-potential	77
2.4	Diffusion experiments	78
2.4.1	Membrane release studies	78
2.4.2	Skin preparation	79
2.4.3	Skin diffusion	79
2.4.4	Tape stripping	79
2.5	Data analysis	80
2.6	Statistical analysis	80
3	Results and Discussion	81
3.1	Formulation of nano-emulsions and nano-emulgels	81
3.2	Physicochemical properties	82
3.2.1	Aqueous solubility	82
3.2.2	Log D	82
3.2.3	Characterisation of semi-solid formulations	82
3.3	Membrane diffusion experiments	84
3.4	Diffusion experiment	85
3.4.1	Diffusion study	85
3.5	Tape stripping	86
3.5.1	Stratum corneum-epidermis	87
3.5.2	Epidermis-dermis	88
3.6	Statistical analysis	88
3.6.1	Membrane release studies	88
3.6.2	Skin diffusion studies	89
3.6.3	Tape stripping	89
4	Conclusion	90
<b>Acknowledgements</b>		92
<b>Conflict of Interest</b>		93
<b>References</b>		94
<b>Tables</b>		102
<b>Figures</b>		107

## *Chapter 5:*

### *Conclusion and future prospects*

<b>References</b>	116
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## *Appendix A:*

### *The validation of a high performance liquid chromatographic assay for the selected statins*

<b>A.1</b>	Purpose of validation	119
<b>A.2</b>	Chromatographic conditions	119
<b>A.3</b>	Preparation of standard and samples	120
<b>A.3.1</b>	Standard preparation	120
<b>A.3.2</b>	Preparation of samples for the analysis of formulations	121
<b>A.3.3</b>	Placebo preparation	122
<b>A.3.4</b>	Sample preparation for diffusion studies	122
<b>A.4</b>	Validation parameters	122
<b>A.4.1</b>	Linearity	122
<b>A.4.1.1</b>	Linear regression analysis	123
<b>A.4.1.2</b>	Lower limit of detection and quantification	128
<b>A.4.2</b>	Accuracy	133
<b>A.4.2.1</b>	Accuracy analysis	133
<b>A.4.3</b>	Precision	137
<b>A.4.3.1</b>	Intra-day precision (repeatability)	138
<b>A.4.3.2</b>	Inter-day precision (reproducibility)	140
<b>A.4.4</b>	Robustness	142
<b>A.4.5</b>	Ruggedness	146
<b>A.4.5.1</b>	Sample stability	147
<b>A.4.5.2</b>	System repeatability	151
<b>A.4.6</b>	Specificity	152
<b>A.5</b>	Conclusion	157
<b>References</b>		158

## ***APPENDIX B:***

### ***The formulation of o/w nano-emulsions separately containing the selected statins and apricot kernel oil***

<b>B.1</b>	Introduction	161
<b>B.2</b>	The purpose and selection of a novel delivery system	162
<b>B.3</b>	Excipients used to formulate a nano-emulsion	162
<b>B.4.1</b>	Statins	163
<b>B.4.2</b>	Apricot kernel oil	163
<b>B.4.2.1</b>	Solubility of the selected statins in apricot kernel oil	164
<b>B.4.3</b>	Emulsifiers	164
<b>B.4.3.1</b>	Span <sup>®</sup> 60 (sorbitol monostearate)	165
<b>B.4.3.2</b>	Tween <sup>®</sup> 80	165
<b>B.4.4</b>	Water	165
<b>B.5</b>	Formulation of nano-emulsions	166
<b>B.5.1</b>	Formulation of pre-formulated o/w nano-emulsions	167
<b>B.5.1.1</b>	Formulation of o/w nano-emulsions	167
<b>B.5.1.2</b>	Formulation method of a nano-emulsion	168
<b>B.5.1.3</b>	Outcome	170
<b>B.6</b>	Characterisation of the pre-formulated nano-emulsions	171
<b>B.6.1</b>	Morphology	171
<b>B.6.2</b>	pH	173
<b>B.6.3</b>	Droplet size and distribution	174
<b>B.6.4</b>	Zeta-potential	177
<b>B.6.5</b>	Viscosity	179
<b>B.6.6</b>	Drug entrapment efficiency	181
<b>B.7</b>	Decision on final formula to be used	182
<b>B.8</b>	Characterisation of chosen optimised nano-emulsion placebo	183
<b>B.9</b>	Conclusion	185
<b>References</b>		187

## *Appendix C:*

### *Formulation and characterisation of a semi-solid dosage form of an o/w nano-emulsion separately containing the selected statins and apricot kernel oil*

<b>C.1</b>	Introduction	192
<b>C.2</b>	Intended purpose of the formulation	193
<b>C.2.1</b>	Semi-solid dosage form selection	193
<b>C.2.2</b>	Gels as a semi-solid dosage form	193
<b>C.2.2.1</b>	Emulgel	194
<b>C.2.2.2</b>	Nano-emulgel	194
<b>C.2.3</b>	Suitable semi-solid dosage form	194
<b>C.3</b>	Excipients used to formulate the nano-emulgels	195
<b>C.3.1</b>	General excipients used for nano-emulgel	195
<b>C.3.2</b>	Excipients used to formulate a nano-emulgel	195
<b>C.3.2.1</b>	Oils (apricot kernel oil)	196
<b>C.3.2.2</b>	Emulsifiers	196
<b>C.3.2.3</b>	Gelling agent	197
<b>C.3.2.4</b>	Water	197
<b>C.4</b>	Formulation of a nano-emulgel	197
<b>C.4.1</b>	Formulation method	197
<b>C.4.2</b>	Formula used for preparation of <b>(NEG1)</b>	198
<b>C.4.3</b>	Formulation method used for <b>(NEG1)</b>	198
<b>C.5</b>	Outcome	199
<b>C.6</b>	Characterisation of the nano-emulgels (semi-solids)	200
<b>C.6.1</b>	Light microscopy	200
<b>C.6.2</b>	pH	202
<b>C.6.3</b>	Droplet size and distribution	202
<b>C.6.4</b>	Zeta-potential	205
<b>C.6.5</b>	Viscosity	207
<b>C.7</b>	Discussion and conclusion	209
<b>References</b>		211

## **APPENDIX D:**

### ***Franz cell diffusion studies of an o/w nano-emulsion and nano-emulgels dosage forms containing the selected statins and apricot kernel oil***

<b>D.1</b>	Introduction	217
<b>D.2</b>	Methods	218
<b>D.2.1</b>	HPLC analysis of the selected statin samples	218
<b>D.2.2</b>	Physicochemical properties of the selected statins	219
<b>D.2.2.1</b>	Solubility in various solvents	219
<b>D.2.2.1.1</b>	Aqueous solubility	219
<b>D.2.2.1.2</b>	Solubility in PBS:ethanol (9:1 at pH 7.4)	220
<b>D.2.2.1.3</b>	Solubility in <i>n</i> -octanol	220
<b>D.2.2.2</b>	Octanol-buffer distribution coefficient	220
<b>D.2.3</b>	<i>In vitro</i> diffusion studies: vertical Franz cell method	221
<b>D.2.3.1</b>	Vertical Franz cell components	222
<b>D.2.3.1.1</b>	Preparation of receptor phase	222
<b>D.2.3.1.2</b>	Test formulations and the preparation of the donor phase	223
<b>D.2.3.2</b>	Membrane release studies	224
<b>D.2.3.3</b>	<i>In vitro</i> skin diffusion	226
<b>D.2.3.3.1</b>	Skin ethics and collection	226
<b>D.2.3.3.2</b>	Preparation of dermatomed skin	227
<b>D.2.3.3.3</b>	Skin diffusion studies	228
<b>D.2.3.3.4</b>	Tape stripping	228
<b>D.2.3.4</b>	Data analysis	229
<b>D.2.3.5</b>	Statistical analysis	229
<b>D.3</b>	Results and discussion	230
<b>D.3.1</b>	Solubility in various solvents	230
<b>D.3.2</b>	Octanol-buffer distribution coefficient	231
<b>D.3.3</b>	Membrane release studies	232
<b>D.3.4</b>	Skin diffusion studies	243
<b>D.3.5</b>	Tape stripping	250

<b>D.3.5.1</b>	Stratum corneum-epidermis concentration	250
<b>D.3.5.2</b>	Epidermis-dermis concentration	256
<b>D.4</b>	Statistical analysis	262
<b>D.4.1</b>	Membrane release studies	263
<b>D.4.2</b>	Skin diffusion	264
<b>D.4.3</b>	Tape stripping	264
<b>D.5</b>	Conclusion	267
<b>References</b>		272

### *APPENDIX E:*

#### *Cytotoxicity studies performed on the optimised o/w nano-emulsions containing the selected statins*

<b>E.1</b>	Introduction	280
<b>E.2</b>	Cell culture toxicity studies	281
<b>E.2.1</b>	The selection of an appropriate cell line	281
<b>E.2.2</b>	Concentrations used for exposure	281
<b>E.2.2.1</b>	Treatment	283
<b>E.2.3</b>	Non-assay experimental procedures	284
<b>E.2.3.1</b>	Materials	284
<b>E.3</b>	<i>In vitro</i> toxicity testing	285
<b>E.3.1</b>	Determination of cell viability	285
<b>E.3.2</b>	MTT colorimetric assay	286
<b>E.3.2.1</b>	MTT colorimetric assay results and discussion	288
<b>E.3.2.2</b>	MTT assay results on HaCaT cells	289
<b>E.3.3</b>	Neutral red colorimetric assay	294
<b>E.3.3.1</b>	Neutral red colorimetric assay results and discussion	295
<b>E.3.3.2</b>	Neutral red-assay results on HaCaT cells	297
<b>E.4</b>	Conclusion	301
<b>References</b>		303

## *Appendix F:*

### *Author guidelines: Die Pharmazie*

<b>F.1</b>	Aim	307
<b>F.2</b>	Conditions	307
<b>F.3</b>	Preparation of manuscripts	308

## *Appendix G:*

### *The International Journal of Pharmaceutics: Guide for authors*

<b>G.1</b>	Introduction	312
<b>G.2</b>	Types of paper	312
<b>G.3</b>	Ethics in publishing	312
<b>G.4</b>	Studies in humans and animals	312
<b>G.5</b>	Declaration of interest	313
<b>G.6</b>	Submission declaration and verification	313
<b>G.7</b>	Preprints	314
<b>G.8</b>	Use of inclusive language	314
<b>G.9</b>	Author contributions	314
<b>G.10</b>	Authorship	314
<b>G.11</b>	Changes to authorship	315
<b>G.12</b>	Article transfer service	315
<b>G.13</b>	Copyright	315
<b>G.14</b>	Author rights	316
<b>G.15</b>	Role of the funding source	316
<b>G.16</b>	Funding body agreements and policies	316
<b>G.17</b>	Open access	316
<b>G.18</b>	Elsevier Researcher Academy	318
<b>G.19</b>	Language (usage and editing services)	318
<b>G.20</b>	Submission	318
<b>G.21</b>	Referees	318
<b>G.22</b>	Use of word processing software	319

<b>G.23</b>	Article structure	319
<b>G.23.1</b>	Subdivision-numbered sections	319
<b>G.23.2</b>	Introduction	319
<b>G.23.3</b>	Material and methods	320
<b>G.23.4</b>	Results	320
<b>G.23.5</b>	Discussion	320
<b>G.23.6</b>	Conclusions	320
<b>G.23.7</b>	Appendices	320
<b>G.23.8</b>	Essential title page information	320
<b>G.23.9</b>	Abstract	321
<b>G.23.10</b>	Graphical abstract	321
<b>G.23.11</b>	Keywords	321
<b>G.23.12</b>	Abbreviations	322
<b>G.23.13</b>	Acknowledgements	322
<b>G.23.14</b>	Formatting of funding sources	322
<b>G.23.15</b>	Units	322
<b>G.23.16</b>	Math formulae	322
<b>G.23.17</b>	Footnotes	323
<b>G.23.18</b>	Image manipulation	323
<b>G.24</b>	Electronic artwork	323
<b>G.25</b>	Formats	323
<b>G.25.1</b>	Color artwork	324
<b>G.25.2</b>	Figure captions	324
<b>G.25.3</b>	Tables	325
<b>G.26</b>	References	325
<b>G.26.1</b>	Citation in text	325
<b>G.26.2</b>	Reference links	325
<b>G.26.3</b>	Web references	325
<b>G.26.4</b>	Data references	326
<b>G.26.5</b>	References in a special issue	326

<b>G.26.6</b>	Reference management software	326
<b>G.26.7</b>	Reference formatting	326
<b>G.26.8</b>	Reference style	327
<b>G.27</b>	Video	328
<b>G.28</b>	Data visualization	328
<b>G.29</b>	Supplementary material	329
<b>G.30</b>	Research data	329
<b>G.30.1</b>	Data linking	329
<b>G.30.2</b>	Mendeley Data	330
<b>G.30.3</b>	Data in Brief	330
<b>G.30.4</b>	Data statement	330
<b>G.31</b>	Submission checklist	330
<b>G.32</b>	Online proof correction	331
<b>G.33</b>	Offprints	332
<b>G.34</b>	Author inquiries	332

## *Appendix H*

<b>Language editing certificate</b>	333
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# List of Equations

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## CHAPTER 2:

*Formulation and transdermal delivery of nano-emulsions containing the selected statins and apricot kernel oil*

**Equation 2.1:** %ionised =  $100 / 1 + \text{anti-log}(\text{pKa} - \text{pH})$  28

**Equation 2.2:** %unionised =  $100 - \text{%ionised}$  28

## APPENDIX A:

*The validation of a high performance liquid chromatographic assay for the selected statins*

**Equation A.1:**  $y = mx + c$  123

**Equation A.2:** DL (detection limit) =  $3.3 \times \sigma/S$  128

**Equation A.3:** QL (quantification limit) =  $10 \times \sigma/S$  128

## APPENDIX B:

*The formulation of o/w nano-emulsions separately containing the selected statins and apricot kernel oil*

**Equation B.1:** %EE =  $[(C_t - C_f)/C_f] \times 100$  181

## APPENDIX C:

*Formulation and characterisation of a semi-solid dosage form of an o/w nano-emulsion separately containing the selected statins and apricot kernel oil*

**Equation C.1:** Full scale range (cP) = Spindle coefficient/Spindle speed 208

## APPENDIX D:

*Franz cell diffusion studies of an o/w nano-emulsion and nano-emulgels dosage forms containing the selected statins and apricot kernel oil*

**Equation D.1:**  $\text{Log } D = \frac{\text{Concentration in } n\text{-octanol}}{\text{Concentration in PBS (pH 7.4)}}$  221

**APPENDIX E:**

***Cytotoxicity studies performed on the optimised o/w nano-emulsions containing the selected statins***

<b>Equation E.1:</b>	$C_1V_1 = C_2V_2$	285
<b>Equation E.2:</b>	MTT (mg) = Total volume (ml) x 0.5 mg/ml	287
<b>Equation E.3:</b>	%viable cells = $100 \times (\text{sample abs}) / (\text{control abs})$	288
<b>Equation E.4:</b>	%viable cells = $\frac{(\text{absorbance } 560 \text{ nm} - 630 \text{ nm}) - \text{blank absorbance}}{(\text{Negative control absorbance} - \text{blank absorbance})} \times 100$	288
<b>Equation E.5:</b>	Amount NR solution = Total amount x 10%	295

# List of Figures

---

## CHAPTER 2:

### *Formulation and transdermal delivery of nano-emulsions containing the selected statins and apricot kernel oil*

<b>Figure 2.1:</b>	Chemical structure of lovastatin	16
<b>Figure 2.2:</b>	Chemical structure of mevastatin	17
<b>Figure 2.3:</b>	Chemical structure of simvastatin	18
<b>Figure 2.4:</b>	Chemical structure of rosuvastatin	19
<b>Figure 2.5:</b>	Four major layers of the human skin (adapted from Geerligs (2010:4)).	21
<b>Figure 2.6:</b>	Potential transport pathways for transdermal delivery (adapted from Lane, 2013:13)	24
<b>Figure 2.7:</b>	Representation of a nano-emulsion droplet acting as reservoir for lipophilic API (adapted from Kumar & Divya, 2015:273)	32
<b>Figure 2.8:</b>	Sonication as a high energy method to obtain a nano-emulsion (adapted from Singh et al. (2017:35)).	35

## CHAPTER 3:

### *Article for the publication in "Die Pharmazie"*

<b>Fig 1:</b>	HPLC chromatogram showing specificity data obtained: A) lovastatin, B) mevastatin, C) simvastatin and D) rosuvastatin. In addition for a) placebo solution, b) statin standard solution, following the sample solution of respective statin stressed with 200 µl of c) HCl, d) H <sub>2</sub> O and e) H <sub>2</sub> O <sub>2</sub>	64
<b>Fig 2:</b>	Chromatographic representation of A) lovastatin, B) mevastatin, C) simvastatin and D) rosuvastatin. Chromatograms represents a) standard solution sample of respective statins, b) buffer (receptor phase) extraction sample, c) tape stripping sample of the statin and d) skin sample of the statin.	65

## CHAPTER 4:

### *Article for the publication in the International Journal of Pharmaceutics*

- Fig 1:** Micrographs of oil droplets captured with the TEM: a) **(NEL1)**, b) **(NEM1)**, c) **(NER1)** and d) **(NES1)**, and size thereof. Scale bars for magnification are indicated for each micrograph. 107
- Fig 2:** Box-plot indicating the flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) of: a) the nano-emulsions (**(NEL1)**, **(NEM1)**, **(NES1)** and **(NER1)**) and b) the nano-emulgels (**(NEGL)**, **(NEGM)**, **(NEGS)** and **(NEGR)**) after 6 h. 108
- Fig 3:** Box-plot indicating the amount per area diffused ( $\mu\text{g}/\text{cm}^2$ ) present in the receptor phase of: a) the nano-emulsions (**(NEL1)**, **(NEM1)**, **(NES1)** and **(NER1)**), as well as b) the nano-emulgels (**(NEGL)**, **(NEGM)**, **(NEGS)** and **(NEGR)**) after 12 h 109
- Fig 4:** Box-plot indicating the concentration ( $\mu\text{g}/\text{ml}$ ) present in: a) the SCE with the nano-emulsions (**(NEL1)**, **(NEM1)**, **(NES1)** and **(NER1)**); b) the SCE with the nano-emulgels (**(NEGL)**, **(NEGM)**, **(NEGS)** and **(NEGR)**); c) the ED with the nano-emulsions (**(NEL1)**, **(NEM1)**, **(NES1)** and **(NER1)**), and d) the ED with the nano-emulgels (**(NEGL)**, **(NEGM)**, **(NEGS)** and **(NEGR)**) after tape stripping was performed 110

## APPENDIX A:

### *The validation of a high performance liquid chromatographic assay for the selected statins*

- Figure A.1:** HPLC chromatograms representing a) lovastatin, b) mevastatin, c) rosuvastatin and d) simvastatin standard solution peaks and retention times 120
- Figure A.2:** Linear regression curve of lovastatin standards 124
- Figure A.3:** Linear regression curve of mevastatin standards 124
- Figure A.4:** Linear regression curve of rosuvastatin standards 125
- Figure A.5:** Linear regression curve of simvastatin standards 125
- Figure A.6:** Lovastatin HPLC chromatogram representing the robustness data of a standard solution injected at different test parameters: a) normal conditions of 1.0 ml/min flow rate, 240 nm wavelength and 45% acetonitrile, b) 1.2 ml/min flow rate, 235 nm wavelength and 40% acetonitrile and c) 0.8 ml/min flow rate, 230 nm wavelength and 37% acetonitrile 143

- Figure A.7:** Mevastatin HPLC chromatogram representing the robustness data of a standard solution injected at different test parameters: a) normal conditions of 1.0 ml/min flow rate, 240 nm wavelength and 45% acetonitrile, b) 1.2 ml/min flow rate, 235 nm wavelength and 40% acetonitrile and c) 0.8 ml/min flow rate, 230 nm wavelength and 37% acetonitrile 144
- Figure A.8:** Rosuvastatin HPLC chromatogram representing the robustness data of a standard solution injected at different test parameters: a) normal conditions of 1.0 ml/min flow rate, 240 nm wavelength and 45% acetonitrile, b) 1.2 ml/min flow rate, 235 nm wavelength and 40% acetonitrile and c) 0.8 ml/min flow rate, 230 nm wavelength and 37% acetonitrile 145
- Figure A.9:** Simvastatin HPLC chromatogram representing the robustness data of a standard solution injected at different test parameters: a) normal conditions of 1.0 ml/min flow rate, 240 nm wavelength and 45% acetonitrile, b) 1.2 ml/min flow rate, 235 nm wavelength and 40% acetonitrile and c) 0.8 ml/min flow rate, 230 nm wavelength and 37% acetonitrile 146
- Figure A.10:** HPLC chromatogram showing specificity data obtained for a) a placebo solution, b) lovastatin standard solution, following the sample solution of lovastatin stressed with 200 µl of c) HCl, d) H<sub>2</sub>O and e) H<sub>2</sub>O<sub>2</sub> 153
- Figure A.11:** HPLC chromatogram showing specificity data obtained for a) a placebo solution, b) mevastatin standard solution, following the sample solution of mevastatin stressed with 200 µl of c) HCl, d) H<sub>2</sub>O and e) H<sub>2</sub>O<sub>2</sub> 154
- Figure A.12:** HPLC chromatogram showing specificity data obtained for, a) a placebo solution, b) simvastatin standard solution, following the sample solution of simvastatin stressed with 200 µl of c) HCl, d) H<sub>2</sub>O and e) H<sub>2</sub>O<sub>2</sub> 155
- Figure A.13:** HPLC chromatogram showing specificity data obtained for a) a placebo solution, b) rosuvastatin standard solution, following the sample solution of rosuvastatin stressed with 200 µl of c) HCl, d) H<sub>2</sub>O and e) H<sub>2</sub>O<sub>2</sub> 156

## ***APPENDIX B:***

*The formulation of o/w nano-emulsions separately containing the selected statins and apricot kernel oil*

- Figure B.1:** High-energy emulsification method ultrasonicator (Model UP200St) 166

<b>Figure B.2:</b>	Formulation of nano-emulsion as a diagrammatic representation	169
<b>Figure B.3:</b>	Formulation method of the <b>(NEF1)</b> : a) Tween® 80 and water pre-heated (phase B); b) apricot kernel oil preheated; c) addition of Span® 60 to pre-heated apricot kernel oil (phase A); d) addition of the API to phase A; e) phase A added to phase B (drop wise); f) mixing of phases A and B together; g) sonication 3 min with 1 min intervals	170
<b>Figure B.4:</b>	The formulated dispersions: a) all <b>(NEF1)</b> and <b>(NEF2)</b> dispersions, except b) <b>(NES1)</b> and <b>(NES2)</b>	171
<b>Figure B.5:</b>	Micrographs of oil droplets captured with the TEM: a) <b>(NEL1)</b> , b) <b>(NEL2)</b> , c) <b>(NEM1)</b> , d) <b>(NEM2)</b> , e) <b>(NES1)</b> , f) <b>(NES2)</b> and g) <b>(NER1)</b> and size thereof. Scale bars for magnification are indicated for each micrograph.	172
<b>Figure B.6:</b>	A Mettler Toledo® pH meter with a Mettler Toledo® InLab® 410 electrode	174
<b>Figure B.7:</b>	a) Malvern Zetasizer Nano ZS and b) a clear disposable DTS1070 folded capillary zeta-cell	175
<b>Figure B.8:</b>	Average droplet size measured per droplet radius of a) <b>(NEL1)</b> , b) <b>(NEL2)</b> , c) <b>(NEM1)</b> , d) <b>(NEM2)</b> , e) <b>(NES1)</b> , f) <b>(NES2)</b> and g) <b>(NER1)</b>	176
<b>Figure B.9:</b>	The average zeta-potential (mV): a) <b>(NEL1)</b> , b) <b>(NEL2)</b> , c) <b>(NEM1)</b> , d) <b>(NEM2)</b> , e) <b>(NES1)</b> , f) <b>(NES2)</b> and g) <b>(NER1)</b> .	179
<b>Figure B.10:</b>	A Brookfield Viscometer DV2T LV Ultra connected to a water bath	180
<b>Figure B.11:</b>	a) Average zeta-potential; b) average droplet size measured per droplet radius and c) TEM micrograph of <b>(PNEF1)</b>	184
<b>Figure B.12:</b>	The formulated dispersions a) <b>(PNEF1)</b> , b) <b>(NEL1)</b> , c) <b>(NEM1)</b> , d) <b>(NES1)</b> and e) <b>(NER1)</b>	185

### *APPENDIX C:*

#### *Formulation and characterisation of a semi-solid dosage form of an o/w nano-emulsion separately containing the selected statins and apricot kernel oil*

<b>Figure C.1:</b>	Diagrammatic representation of the formulation process used to obtain <b>(NEG1)</b>	199
<b>Figure C.2:</b>	Mechanical Heidolph RZR 2041 overhead stirrer (Heidolph Instruments GmbH & Co. KG, Germany), used in the formulation of nano-emulgel <b>(NEG1)</b> .	199
<b>Figure C.3:</b>	Nikon Eclipse 50i microscope	201

<b>Figure C.4:</b>	Light microscopy micrographs of: a) <b>(NEGL)</b> , b) <b>(NEGM)</b> , c) <b>(NEGS)</b> and d) <b>(NEGR)</b>	201
<b>Figure C.5:</b>	Average droplet size measured per droplet radius of: a) <b>(NEL1)</b> , b) <b>(NELG)</b> , c) <b>(NEM1)</b> , d) <b>(NEMG)</b> , e) <b>(NES1)</b> , f) <b>(NESG)</b> , g) <b>(NER1)</b> and h) <b>(NERG)</b>	204
<b>Figure C.6:</b>	The average zeta-potential (mV) of a) <b>(NEL1)</b> , b) <b>(NELG)</b> , c) <b>(NEM1)</b> , d) <b>(NEMG)</b> , e) <b>(NES1)</b> , f) <b>(NESG)</b> , g) <b>(NER1)</b> and h) <b>(NERG)</b>	207

#### *APPENDIX D:*

#### *Franz cell diffusion studies of an o/w nano-emulsion and nano-emulgels dosage forms containing the selected statins and apricot kernel oil*

<b>Figure D.1:</b>	Diagrammatic representation of the formulas tested during membrane release studies and skin diffusion studies	223
<b>Figure D.2:</b>	Nano-emulsion formula <b>(NEF1)</b> tested: a) <b>(NEL1)</b> , b) <b>(NEM1)</b> , c) <b>(NES1)</b> and d) <b>(NER1)</b>	223
<b>Figure D.3:</b>	Nano-emulgel formula <b>(NEG1)</b> tested: a) <b>(NEGL)</b> , b) <b>(NEGM)</b> , c) <b>(NEGS)</b> and d) <b>(NEGR)</b>	224
<b>Figure D.4:</b>	Apparatus and materials utilised during membrane release studies in order of use a) Franz cell with donor (top) and receptor compartment (bottom), b) PVDF synthetic membrane, c) Dow Corning® high vacuum grease, d) horseshoe clamp to fasten Franz cell compartments, e) Franz cell after filling the compartments, f) Grant® water bath, g) assembled Franz cells in Franz cell stand, placed on a magnetic stirrer plate within the water bath and h) syringes used for 1 h extractions for 6 h	225
<b>Figure D.5:</b>	a) Dermatome™ (Zimmer TDS, United Kingdom) and b) dermatomed skin samples of ± 400 µm on Whatman® filter paper	227
<b>Figure D.6:</b>	Average cumulative amount per area (µg/cm <sup>2</sup> ) of lovastatin permeated from the <b>(NEL1)</b> through the membrane as a function of time to illustrate the average flux from 1 – 5 h (n = 9)	234
<b>Figure D.7:</b>	Cumulative amount of lovastatin per area (µg/cm <sup>2</sup> ) for each individual Franz cell that permeated through the membrane over 6 h from the <b>(NEL1)</b> (n = 9)	234
<b>Figure D.8:</b>	Average cumulative amount per area (µg/cm <sup>2</sup> ) of lovastatin permeated from the <b>(NEGL)</b> through the membrane as a function of time to	235

	illustrate the average flux from 1 – 5 h (n = 11)	
<b>Figure D.9:</b>	Cumulative amount of lovastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the <b>(NEGL)</b> (n = 11)	235
<b>Figure D.10:</b>	Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of mevastatin permeated from the <b>(NEM1)</b> through the membrane as a function of time to illustrate the average flux from 1 – 5 h (n = 9)	236
<b>Figure D.11:</b>	Cumulative amount of mevastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the <b>(NEM1)</b> (n = 9)	236
<b>Figure D.12:</b>	Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of mevastatin permeated from the <b>(NEGM)</b> through the membrane as a function of time to illustrate the average flux from 1 – 5 h (n = 10)	237
<b>Figure D.13:</b>	Cumulative amount of mevastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the <b>(NEGM)</b> (n = 10)	237
<b>Figure D.14:</b>	Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of rosuvastatin permeated from the <b>(NER1)</b> through the membrane as a function of time to illustrate the average flux from 1 – 5 h (n = 12)	238
<b>Figure D.15:</b>	Cumulative amount of rosuvastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the <b>(NER1)</b> (n = 12)	238
<b>Figure D.16:</b>	Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of rosuvastatin permeated from the <b>(NEGR)</b> through the membrane as a function of time to illustrate the average flux from 1 – 5 h (n = 12)	239
<b>Figure D.17:</b>	Cumulative amount of rosuvastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the <b>(NEGR)</b> (n = 12)	239
<b>Figure D.18:</b>	Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of simvastatin permeated from the <b>(NES1)</b> through the membrane as a function of time to illustrate the average flux from 1 – 5 h (n = 9)	240
<b>Figure D.19:</b>	Cumulative amount of simvastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the <b>(NES1)</b> (n = 9)	240
<b>Figure D.20:</b>	Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of simvastatin permeated	241

from the **(NEGS)** through the membrane as a function of time to illustrate the average flux from 1 – 5 h (n = 8)

<b>Figure D.21:</b>	Cumulative amount of simvastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the <b>(NEGS)</b> (n = 8)	241
<b>Figure D.22:</b>	Box-plot indicating the flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) of: a) the nano-emulsions ( <b>(NEL1)</b> , <b>(NEM1)</b> , <b>(NES1)</b> and <b>(NER1)</b> ) and b) the nano-emulgels ( <b>(NEGL)</b> , <b>(NEGM)</b> , <b>(NEGS)</b> and <b>(NEGR)</b> ) after 6 h.	242
<b>Figure D.23:</b>	Lovastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for <b>(NEL1)</b> (n = 8)	244
<b>Figure D.24:</b>	Lovastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for <b>(NEGL)</b> (n = 7)	244
<b>Figure D.25:</b>	Mevastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for <b>(NEM1)</b> (n = 8)	245
<b>Figure D.26:</b>	Mevastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for <b>(NEGM)</b> (n = 8)	245
<b>Figure D.27:</b>	Rosuvastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for <b>(NER1)</b> (n = 8)	246
<b>Figure D.28:</b>	Rosuvastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for <b>(NEGR)</b> (n = 7)	246
<b>Figure D.29:</b>	Simvastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for <b>(NES1)</b> (n = 7)	247
<b>Figure D.30:</b>	Simvastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for <b>(NEGS)</b> (n = 8)	247
<b>Figure D.31:</b>	Box-plot indicating the amount per area diffused ( $\mu\text{g}/\text{cm}^2$ ) present in the receptor phase of: a) the nano-emulsions ( <b>(NEL1)</b> , <b>(NEM1)</b> , <b>(NES1)</b> and <b>(NER1)</b> n = 8, except <b>(NES1)</b> was n = 7) and b) the nano-emulgels ( <b>(NEGL)</b> , <b>(NEGM)</b> , <b>(NEGS)</b> and <b>(NEGR)</b> ) (n = 8, except <b>(NEGR)</b> and <b>(NEGL)</b> were n = 7) after 12 h.	248
<b>Figure D.32:</b>	Lovastatin concentration ( $\mu\text{g}/\text{ml}$ ) from <b>(NEL1)</b> in the SCE after tape stripping (n = 8)	251
<b>Figure D.33:</b>	Lovastatin concentration ( $\mu\text{g}/\text{ml}$ ) from <b>(NEGL)</b> in the SCE after tape stripping (n = 7)	251
<b>Figure D.34:</b>	Mevastatin concentration ( $\mu\text{g}/\text{ml}$ ) from <b>(NEM1)</b> in the SCE after tape	252

	stripping (n = 8)	
<b>Figure D.35:</b>	Mevastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NEGM)</b> in the SCE after tape stripping (n = 8)	252
<b>Figure D.36:</b>	Simvastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NES1)</b> in the SCE after tape stripping (n = 7)	253
<b>Figure D.37:</b>	Simvastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NEGS)</b> in the SCE after tape stripping (n = 8)	253
<b>Figure D.38:</b>	Simvastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NER1)</b> in the SCE after tape stripping (n = 8)	254
<b>Figure D.39:</b>	Rosuvastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NEGR)</b> in the SCE after tape stripping (n = 7)	254
<b>Figure D.40:</b>	Box-plot indicating the concentration ( $\mu\text{g/ml}$ ) present in the SCE of the nano-emulsions ( <b>(NEL1)</b> , <b>(NEM1)</b> , <b>(NES1)</b> and <b>(NER1)</b> ) after tape stripping was performed (n = 8, except <b>(NES1)</b> was n = 7).	256
<b>Figure D.41:</b>	Box-plot indicating the concentration ( $\mu\text{g/ml}$ ) present in the SCE of the nano-emulgels ( <b>(NEGL)</b> , <b>(NEGM)</b> , <b>(NEGS)</b> and <b>(NEGR)</b> ) after tape stripping was performed (n = 8, except <b>(NEGR)</b> and <b>(NEGL)</b> were n = 7).	256
<b>Figure D.42:</b>	Lovastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NEL1)</b> in the ED after tape stripping (n = 8)	257
<b>Figure D.43:</b>	Lovastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NEGL)</b> in the ED after tape stripping (n = 7)	257
<b>Figure D.44:</b>	Mevastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NEM1)</b> in the ED after tape stripping (n = 8)	258
<b>Figure D.45:</b>	Mevastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NEGM)</b> in the ED after tape stripping (n = 8)	258
<b>Figure D.46:</b>	Simvastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NES1)</b> in the ED after tape stripping (n = 7)	259
<b>Figure D.47:</b>	Simvastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NEGS)</b> in the ED after tape stripping (n = 8)	259
<b>Figure D.48:</b>	Rosuvastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NER1)</b> in the ED after tape stripping (n = 8)	260
<b>Figure D.49:</b>	Rosuvastatin concentration ( $\mu\text{g/ml}$ ) from <b>(NEGR)</b> in the ED after tape	260

stripping (n = 7)

- Figure D.50:** Box-plot indicating the concentration ( $\mu\text{g/ml}$ ) present in the ED of the nano-emulsions ((**NEL1**), (**NEM1**), (**NES1**) and (**NER1**)) after tape stripping was performed (n = 8, except (**NES1**) was n = 7). 261
- Figure D.51:** Box-plot indicating the concentration ( $\mu\text{g/ml}$ ) present in the ED of the nano-emulgels ((**NEGL**), (**NEGM**), (**NEGS**) and (**NEGR**)) after tape stripping was performed (n = 8, except (**NEGR**) and (**NEGL**) were n = 7). 262

### *APPENDIX E:*

#### *Cytotoxicity studies performed on the optimised o/w nano-emulsions containing the selected statins*

- Figure E.1:** Diagrammatic representation of the treatment groups and concentrations utilised 282
- Figure E.2:** Diagrammatic representation of the stock solution of each respective treatment group added to wells 283
- Figure E.3:** Cell counting on one side of a haemocytometer (adapted from BioTek, 2014:1) 286
- Figure E.4:** Example of a 96-well plate, 2 h after adding the MTT solution and prior to aspiration and addition of DMSO 287
- Figure E.5:** SpectraMax<sup>®</sup> Paradigm<sup>®</sup> Multi-Mode Microplate reader (Molecular Devices, California, USA) to measure absorbance 288
- Figure E.6:** The three 96-well plates after the addition of DMSO: a) (**NEF1**) dispersions, b) excipients used in dispersions (**ExS**) and (**PNEF1**) and c) APIs alone (**AS**) (note that each sample was added from highest to lowest concentration) 289
- Figure E.7:** The %cell viability after treatment with the five respective concentrations of (**NEL1**), (**NEM1**), (**NER1**), (**NES1**) and (**PNEF1**), determined with MTT 290
- Figure E.8:** The %cell viability after treatment with the five respective concentrations of excipients (**ExS**) determined with MTT 291
- Figure E.9:** The %cell viability after treatment with the five respective concentrations of the APIs alone (**AS**) determined with MTT 292
- Figure E.10:** The three 96-well plates after the addition of Neutral Red Assay Solubilisation Solution: a) (**NEF1**) dispersions, b) excipients used in 295

dispersions (**ExS**) and (**PNEF1**), and c) APIs alone (**AS**)

**Figure E.11:** The %cell viability after treatment with the five respective concentrations of (**NEL1**), (**NEM1**), (**NER1**), (**NES1**) and (**PNEF1**) determined with NR 297

**Figure E.12:** The %cell viability after treatment with the five respective concentrations of excipients (**ExS**) determined with NR 298

**Figure E.13:** The %cell viability after treatment with the five respective concentrations of the APIs alone (**AS**) determined with NR 299

# *List of Tables*

---

## *CHAPTER 1:*

### *Formulation and transdermal delivery of nano-emulsions containing the selected statins and apricot kernel oil*

<b>Table 1.1:</b>	The physiochemical properties of lovastatin, mevastatin, simvastatin and rosuvastatin compared to the ideal physiochemical properties for transdermal delivery	3
-------------------	--	---

## *CHAPTER 2:*

### *Formulation and transdermal delivery of nano-emulsions containing the selected statins and apricot kernel oil*

<b>Table 2.1:</b>	Physiochemical characteristics of the selected statins	25
<b>Table 2.2:</b>	Number of hydrogen bonds and acceptors possessed by the selected statins	28
<b>Table 2.3:</b>	The %unionised species at pH 5 and pH 7	29

## *CHAPTER 3:*

### *Article for the publication in "Die Pharmazie"*

<b>Table 1:</b>	Validation parameters obtained for the four statins	63
-----------------	---	----

## *CHAPTER 4:*

### *Article for the publication in the International Journal of Pharmaceutics*

<b>Table 1:</b>	Ingredients used during the formulation of the nano-emulsions and the nano-emulgels	102
<b>Table 2:</b>	Lowest limit of detection (LOD) and lowest limit of quantification (LOQ) of statins	103
<b>Table 3:</b>	Summary of the characteristics of the nano-emulsions and the nano-emulgels	104
<b>Table 4:</b>	Results obtained from TEM performed on the nano-emulsions	105
<b>Table 5:</b>	The concentration ( $\mu\text{g/ml}$ ) of the selected statins within the respective formulas that diffused through the skin after 12 h	106

## *APPENDIX A:*

### *The validation of a high performance liquid chromatographic assay for the selected statins*

<b>Table A.1:</b>	Nano-emulsion (o/w) standard formula	121
<b>Table A.2:</b>	Nano-emulgel standard formula	121
<b>Table A.3:</b>	Linearity results of lovastatin	126
<b>Table A.4:</b>	Linearity results of mevastatin	126
<b>Table A.5:</b>	Linearity results of rosuvastatin	127
<b>Table A.6:</b>	Linearity results of simvastatin	127
<b>Table A.7:</b>	Results obtained from injecting diluted sample of lovastatin at different injection volumes	129
<b>Table A.8:</b>	Statistical analysis of lovastatin	129
<b>Table A.9:</b>	Results obtained from injecting diluted sample of mevastatin at different injection volumes	130
<b>Table A.10:</b>	Statistical analysis of mevastatin	130
<b>Table A.11:</b>	Results obtained from injecting diluted sample of simvastatin at different injection volumes	131
<b>Table A.12:</b>	Statistical analysis of simvastatin	131
<b>Table A.13:</b>	Results obtained from injecting diluted sample of rosuvastatin at different injection volumes	132
<b>Table A.14:</b>	Statistical analysis of rosuvastatin	132
<b>Table A.15:</b>	The lower limit of detection and quantification (LLOD and LLOQ) of the selected statins as determined by the linear curves procedure	133
<b>Table A.16:</b>	Accuracy results of lovastatin	134
<b>Table A.17:</b>	Statistical analysis results of lovastatin	134
<b>Table A.18:</b>	Accuracy results of mevastatin	135
<b>Table A.19:</b>	Statistical analysis results of mevastatin	135
<b>Table A.20:</b>	Accuracy results of simvastatin	136
<b>Table A.21:</b>	Statistical analysis results of simvastatin	136
<b>Table A.22:</b>	Accuracy results of rosuvastatin	137
<b>Table A.23:</b>	Statistical analysis results of rosuvastatin	137

<b>Table A.24:</b>	Repeatability results of mevastatin	138
<b>Table A.25:</b>	Repeatability results of lovastatin	139
<b>Table A.26:</b>	Repeatability results of simvastatin	139
<b>Table A.27:</b>	Repeatability results of rosuvastatin	140
<b>Table A.28:</b>	Reproducibility results of mevastatin	141
<b>Table A.29:</b>	Reproducibility results of lovastatin	141
<b>Table A.30:</b>	Reproducibility results of simvastatin	141
<b>Table A.31:</b>	Reproducibility results of rosuvastatin	142
<b>Table A.32:</b>	Robustness data for lovastatin	143
<b>Table A.33:</b>	Robustness data for mevastatin	143
<b>Table A.34:</b>	Robustness data for rosuvastatin	144
<b>Table A.35:</b>	Robustness data for simvastatin	145
<b>Table A.36:</b>	Results of sample stability of lovastatin	147
<b>Table A.37:</b>	Results of sample stability of mevastatin	148
<b>Table A.38:</b>	Results of sample stability of simvastatin	149
<b>Table A.39:</b>	Results of sample stability of rosuvastatin	150
<b>Table A.40:</b>	Results of system repeatability of mevastatin	151
<b>Table A.41:</b>	Results of system repeatability of lovastatin	151
<b>Table A.42:</b>	Results of system repeatability of simvastatin	152
<b>Table A.43:</b>	Results of system repeatability of rosuvastatin	152
<b>Table A.44:</b>	Specificity data for lovastatin	154
<b>Table A.45:</b>	Specificity data for mevastatin	154
<b>Table A.46:</b>	Specificity data for simvastatin	155
<b>Table A.47:</b>	Specificity data for rosuvastatin	156

#### ***APPENDIX B:***

#### ***The formulation of o/w nano-emulsions separately containing the selected statins and apricot kernel oil***

<b>Table B.1:</b>	The excipient used in the formulation of o/w nano-emulsions with their function, supplier and batch number	163
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<b>Table B.2:</b>	Formula for <b>(NEF1)</b> dispersions (50 ml)	168
<b>Table B.3:</b>	Formula for <b>(NEF2)</b> dispersions (50 ml)	168
<b>Table B.4:</b>	The average pH values of the respective nano-emulsions	174
<b>Table B.5:</b>	Average droplet size and Pdl of <b>(NEF1)</b> and <b>(NEF2)</b> dispersions	177
<b>Table B.6:</b>	The comparison of zeta-potential average between <b>(NEF1)</b> and <b>(NEF2)</b>	178
<b>Table B.7:</b>	Average viscosity (cP) and torque (%) measurements of the <b>(NEF1)</b> and <b>(NEF2)</b> dispersions	181
<b>Table B.8:</b>	The entrapment efficacy (%EE) as calculated for <b>(NEF1)</b> and <b>(NEF2)</b> dispersions	182
<b>Table B.9:</b>	Summary of the characteristics of the <b>(NEF1)</b> and <b>(NEF2)</b> dispersions	183
<b>Table B.10:</b>	Characterisation summary of <b>(PNEF1)</b>	184

### *APPENDIX C:*

#### *Formulation and characterisation of a semi-solid dosage form of an o/w nano-emulsion separately containing the selected statins and apricot kernel oil*

<b>Table C.1:</b>	The excipients utilised for the formulation of <b>(NEG1)</b> in conjunction with batch numbers, suppliers and function	196
<b>Table C.2:</b>	Formula used to formulate <b>(NEG1)</b> (100 ml)	198
<b>Table C.3:</b>	The average pH values of the respective nano-emulsions and nano-emulgels	202
<b>Table C.4:</b>	Average droplet size and Pdl of <b>(NEF1)</b> and <b>(NEG1)</b> dispersions	203
<b>Table C.5:</b>	The comparison of zeta-potential average between <b>(NEF1)</b> and <b>(NEG1)</b> dispersions	206
<b>Table C.6:</b>	Settings used on Rheocalc-T 1.2.19 to measure the viscosity of the respective nano-emulgels	208
<b>Table C.7:</b>	Average viscosity (cP) and torque (%) measurements of <b>(NEF1)</b> dispersions and <b>(NEG1)</b> formulations	209
<b>Table C.8:</b>	Summary of the characteristics of the <b>(NEF1)</b> dispersions and <b>(NEG1)</b> formulations	210

## *APPENDIX D:*

### *Franz cell diffusion studies of an o/w nano-emulsion and nano-emulgels dosage forms containing the selected statins and apricot kernel oil*

<b>Table D.1:</b>	The chromatographic conditions used during the analysis of samples obtained from the receptor phase to determine the concentration of the selected statin	219
<b>Table D.2:</b>	Solubility (mg/ml) of the selected statins in PBS (pH 7.4), PBS:ethanol (9:1 at pH 7.4) and <i>n</i> -octanol	231
<b>Table D.3:</b>	Experimentally determined log D value of statins	232
<b>Table D.4:</b>	The average %released, the average and median flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) for each of the formulas after a 6 h membrane release study	233
<b>Table D.5:</b>	Transdermal data for all the formulas containing different statins after the 12 h skin diffusion study	243
<b>Table D.6:</b>	The average concentration of the selected statins present in the SCE and the ED collected by means of tape stripping after the 12 h skin diffusion studies	250
<b>Table D.7:</b>	Tukey's HSD-test performed on the <b>(NEF1s)</b>	263
<b>Table D.8:</b>	Tukey's HSD-test performed on the <b>(NEG1s)</b>	263
<b>Table D.9:</b>	Tukey's HSD-test performed on the <b>(NEF1s)</b>	264
<b>Table D.10:</b>	Tukey's HSD-test performed on the <b>(NEG1s)</b>	264
<b>Table D.11:</b>	P-values obtained from the one-way ANOVA of all the combinations for the formula type and the skin layer during tape stripping	265
<b>Table D.12:</b>	Tukey's HSD-test performed on the <b>(NEF1s)</b> and the SCE	265
<b>Table D.13:</b>	Tukey's HSD-test performed on the <b>(NEF1s)</b> and the ED	265
<b>Table D.14:</b>	Tukey's HSD-test performed on the <b>(NEG1s)</b> and the SCE	266
<b>Table D.15:</b>	Tukey's HSD-test performed on the <b>(NEG1s)</b> and the ED	266
<b>Table D.16:</b>	P-values obtained from t-tests performed to compare all the formulas in terms of SCE (group 1) and ED (group 2), respectively	266
<b>Table D.17:</b>	P-values obtained from t-tests performed to compare the specific skin layer (SCE or ED) in terms of the <b>(NEF1s)</b> (group 1) and the <b>(NEG1s)</b> (group 2)	267

## *APPENDIX E:*

### *Cytotoxicity studies performed on the optimised o/w nano-emulsions containing the selected statins*

<b>Table E.1:</b>	Reagents utilised during the <i>in vitro</i> cytotoxicity studies	284
<b>Table E.2:</b>	Calculating the amount of MTT solution needed for the intended plates	286
<b>Table E.3:</b>	%Cell viability used to classify treatment cytotoxicity in this study	288
<b>Table E.4:</b>	The %cell viability of HaCaT cells after treatment with the <b>(NEF1)</b> dispersions (nano-emulsions) determined with the MTT method	290
<b>Table E.5:</b>	The %cell viability of HaCaT cells after treatment with the excipients <b>(ExS)</b> used in the dispersions (nano-emulsions) determined with MTT	291
<b>Table E.6:</b>	The %cell viability of HaCaT cells after treatment with the selected statins <b>((LS), (MS), (RS) and (SS))</b> alone <b>(AS)</b> determined with MTT	292
<b>Table E.7:</b>	IC <sub>50</sub> values obtained from MTT-assay of dispersions	293
<b>Table E.8:</b>	IC <sub>50</sub> values obtained from MTT-assay of excipients alone <b>(ExS)</b>	293
<b>Table E.9:</b>	IC <sub>50</sub> values obtained from MTT-assay of the APIs alone <b>(AS)</b>	294
<b>Table E.10:</b>	Calculating the amount of NR solution needed for the intended plates	295
<b>Table E.11:</b>	The %cell viability of HaCaT cells after treatment with the <b>(NEF1)</b> dispersions (nano-emulsions) determined with NR	297
<b>Table E.12:</b>	The %cell viability of HaCaT cells after treatment with the excipients <b>(ExS)</b> used in the dispersions (nano-emulsions) determined with NR	298
<b>Table E.13:</b>	The %cell viability of HaCaT cells after treatment with the selected statins <b>((LS), (MS), (RS) and (SS))</b> alone <b>(AS)</b> determined with NR	299
<b>Table E.14:</b>	IC <sub>50</sub> values obtained from NR-assay of dispersions	300
<b>Table E.15:</b>	IC <sub>50</sub> values obtained from NR-assay of excipients alone <b>(ExS)</b>	300
<b>Table E.16:</b>	IC <sub>50</sub> values obtained from NR-assay of the APIs alone <b>(AS)</b>	301

# *Abbreviations*

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%EE	Entrapment efficiency
%RSD	Percentage relative standard deviation
ACN	Acetonitrile
AFB	Aktiewe farmaseutiese bestanddele
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
Apo B	Apolipoprotein B
APVMA	Australian Pesticides and Veterinary Medicines Authority
AS	APIs (statins) alone
ALT	Alanine aminotransferase
ATL	Analytical Technology Laboratory
BT474A	Human ductal carcinoma cells
CHD	Coronary heart disease
C <sub>max</sub>	Peak concentration
CO <sub>2</sub>	Carbon dioxide
CSUR	Competitive Support for Unrated Researchers
CVD	Cardiovascular disease
CYP	Cytochrome P (Hepatic enzyme)
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ED	Epidermis-dermis
EDTA	Trypsin-Versene®

ExS	Excipients alone
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FC	Franz cells
FH	Familial hypercholesterolemia
H+	Hydrogen ions
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HaCaT	Human keratinocytes / menslike keratinosiete
HCl	Hydrochloric acid
HeFH	Heterozygous familial hypercholesterolemia
HeLa cells	Human cervix cancer cells
HEp-2	Human epithelial type 2 carcinoma cells - HeLa contaminant
HoFH	Homozygous familial hypercholesterolemia
HLB	Hydrophilic-lipophilic balance
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HPLC	High performance liquid chromatographic
HREC	Health Research Ethics Committee
HRTEM	High-resolution transmission electron microscopy
HSD	Honestly significant difference
IC <sub>50</sub>	The half-maximal inhibitory concentration
ICH	International Conference of Harmonisation
IQR	Interquartile range
KB	Sub line of the ubiquitous keratin-forming tumour cell line HeLa

KH <sub>2</sub> PO <sub>4</sub>	Potassium dihydrogen orthophosphate
LAMB	Laboratory for Applied Molecular Biology
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LDLR	Lipoprotein receptor gene
LLOD	Lowest limit of detection
LLOQ	Lowest limit of quantification
LOQ	Limit of quantification
LOD	Limit of detection
Log D	Octanol-buffer distribution coefficient
Log P	Octanol-water partition coefficient
LS	Lovastatin
MCF-7	Breast cancer cells
MS	Mevastatin
MTT	Methylthiazol tetrazolium
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NEAA	Non-Essential Amino Acid
NEF1	O/w nano-emulsion with a Tween <sup>®</sup> 80:Span <sup>®</sup> 60 ratio of 1:1
NEF1	O/w nano-emulsion with a 10.78% (w/w) oil and a Tween <sup>®</sup> 80:Span <sup>®</sup> 60 ratio of 1:2
NEG1	nano-emulgel
NEGL	Lovastatin nano-emulgel
NEGM	Mevastatin nano-emulgel

NEGR	Rosuvastatin nano-emulgel
NEGS	Simvastatin nano-emulge
NEL1	2% lovastatin in nano-emulsion formula 1
NEL2	2% lovastatin in nano-emulsion formula 2
NEM1	2% mevastatin in nano-emulsion formula 1
NEM2	2% mevastatin in nano-emulsion formula 2
NER1	2% rosuvastatin in nano-emulsion formula 1
NES1	2% simvastatin in nano-emulsion formula 1
NES2	2% simvastatin in nano-emulsion formula 2
NR	Neutral Red
NRF	National Research Foundation
NRS	Neutral Red Solution
NWU	North-West University
OH-	Hydroxide ions
o/w	Oil-in-water
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffer solution
PCS	Photon correlation spectroscopy
PCSK9	Proprotein convertase subtilisin/kexin type 9
PdI	Polydispersity index
Pen/Strep	Penicillin/Streptomycin
PIT	Phase inversion temperature
PNEF1	Optimised o/w nano-emulsion placebo

PNEG	Placebo nano-emulgel
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
R <sup>2</sup>	coefficient of determination
RS	Rosuvastatin
SCE	Stratum corneum-epidermis
SD	Standard deviation
SS	Simvastatin
TAM	Thermal activity monitor
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
UNODC	United Nations Office on Drugs and Crime
USP	United States Pharmacopeia
UV	Ultra violet
VLDL	Very low density protein
w/o	Water-in-oil
w/w	Weight per weight

# CHAPTER 1:

## *Introduction, research problem and aims*

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### 1.1 Introduction

Hypercholesterolemia, a condition affecting roughly 1 in 250 individuals globally, is characterised by increased levels of total serum cholesterol or low density lipoprotein (LDL) cholesterol resulting in an increased risk for atherosclerotic cardiovascular disease (CVD) (Nordestgaard *et al.*, 2013:3481-3482; Watts *et al.*, 2015:69). Gupta *et al.* (2017:382) stated that the incidence of hypercholesterolemia varies in between 1 in 125 to 1 in 450 in urban (non-rural) populations. Various guidelines agree that the primary target when treating hypercholesterolemia is LDL cholesterol and that treatment can improve the outcomes of patients (Last *et al.*, 2011:551). Reduction in serum cholesterol levels can be achieved with lifestyle and dietary changes, as well as with drug therapy (Istvan & Deisenhofer, 2001:1160). Currently, treatment of hypercholesterolemia is based on five leading classes of drug therapy, namely: statins, fibric acid- and bile acid binding resins, nicotinic acid and cholesterol absorption inhibitors (Hasani-Ranjbar *et al.*, 2010:2935; Rohilla *et al.*, 2012:16). The American College of Cardiology suggests that statins, as a class, offer many benefits due to clinical evidence and are recommended as first-line treatment (Schaiff *et al.*, 2008:40). During this study the focus will be on four statins, namely simvastatin, rosuvastatin, lovastatin and mevastatin.

Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, commonly known as statins, are the most frequently prescribed medication for hypercholesterolemia (Hobbs *et al.*, 1992:445). The selective inhibition of the HMG-CoA reductase enzyme primarily causes a reduction in hepatic cholesterol concentrations and lowers the cholesterol biosynthesis, but additionally, the increase in the clearance of LDL-cholesterol particles from the blood will occur due to the enhanced expression in the LDL-receptors (Bilheimer *et al.*, 1983:4124; McFarland *et al.*, 2014:20608).

Statins are generally well tolerated (Black, 2002:40), however, the most severe adverse effect is rhabdomyolysis (Das *et al.*, 2015:244), which occurs after the progression of myopathy (Furberg & Pitt, 2001:206; Staffa *et al.*, 2002:540). The aforementioned can be minimised by managing factors such as the dose administered, as well as using combination therapy (Ballantyne *et al.*, 2003:553). In addition, although the prevalence of statin-associated liver disease is low, it is of critical importance, since drug-induced hepatotoxicity can mimic any type of hepatobiliary disease, from chronic liver disease with cirrhosis to fulminant liver failure (Law & Rudnicka, 2006:58C). The mechanism by which statins can lead to adverse liver effects are not entirely understood, but elevated levels of alanine aminotransferase (ALT) can be used as a possible

indicator. Trial evidence showed that 16 out of 17 cases, where elevated ALT presented as a result of statin therapy, resolved once treatment was stopped. The tendency of elevated enzymes will more likely occur when there are drug-interactions, pre-existing hepatic disorders or when the highest dose of statin is administered (Bays, 2005:14C). The monitoring of hepatotoxicity (to prevent serious liver disease) by means of ALT tests has been unsuccessful, mainly because of its poor predictive value (Tolman, 2002:1374). Therefore, by exploring other routes of administration, the adverse effects experienced with regard to the liver might be avoided.

Apart from liver adverse effects, gastrointestinal adverse effects after oral administration of statins have also been noted. Flatulence tends to be the most common statin-induced gastrointestinal effect, although nausea, vomiting, constipation and abdominal cramps can also occur. The prevalence of these symptoms tends to increase where risk factors such as alcohol consumption, higher lovastatin doses, diabetes mellitus and  $\beta$ -blocker administration are involved. The presence of these symptoms can lead to poor patient compliance or even discontinuation of therapy (Mancini *et al.*, 2013:1557). The tendency to cause hepatotoxicity, as well as gastrointestinal effects, varies between the different statins (Mancini *et al.*, 2013:1563).

Currently, statins are administered orally as a daily dose. Metabolism of the statins is largely carried out by the cytochrome P450 (CYP450) family of enzymes, although the metabolism of rosuvastatin, pitavastatin and pravastatin via this pathway is less significant (Bottorff & Hansten, 2000:2275; Feidt *et al.*, 2010:1589). As a result of this, the risk for statin-induced myopathy increases with hepatic dysfunction (Kaspera *et al.*, 2010:2; Maron *et al.*, 2000:208) and creates increased probability for drug interactions (Sica & Gehr, 2002:48; Muscari *et al.*, 2002:115). Due to the problems and limitations with the oral administration of these compounds, the transdermal route of administration will be focused on, since first-pass metabolism is avoided when utilising the skin for administration (Geethu *et al.*, 2014:1809) therefore, lower doses can be used with possible reduced side effects (Cho *et al.*, 2009:230).

The surface area of the human adult skin is about 2 m<sup>2</sup> (Hadgraft, 2001:1; Khan *et al.*, 2012:1), and comprises roughly 16% of the total body mass, making it the largest organ in the body (Venus *et al.*, 2010:469). This organ comprises of four major layers, more specifically the stratum corneum, epidermis, dermis and the hypodermis (Pathan & Setty, 2009:174).

The stratum corneum is a non-viable, lipophilic complex structure, which is extremely impermeable (Shah, 1994:20) and is considered to be the rate-limiting obstacle for transdermal delivery of APIs (active pharmaceutical ingredients) (Barry, 1983:7, 17). Due to the impermeable nature of this layer, it acts as the protective barrier for the body, which is due to the layer's composition, widely described as a "brick" and "mortar" structure, with the 10 –

15 layers of corneocytes acting as the “bricks” and lipid bilayers as the “mortar.” The lipid bilayer consists of cholesterol, ceramides, free fatty acids and triglycerides (Benson, 2005:24). The movement of an API through the skin will be controlled and regulated by these lipids, acting as drug flux regulators (Williams, 2003:10).

After an API passes through the stratum corneum, the high fraction of water present in the viable epidermis causes a more significant barrier towards lipophilic substances, as these compounds have a much higher affinity for this non-polar aqueous environment (Scheuplein & Blank, 1971:702). Thus, certain physiochemical characteristics are very important to enable the API to reach the target area by means of transdermal delivery (Alkilani *et al.*, 2015:442). The ideal physiochemical properties that an API should possess for transdermal delivery, together with physiochemical properties of the statins intended for this study, are listed in Table 1.1.

**Table 1.1:** The physiochemical properties of lovastatin, mevastatin, simvastatin and rosuvastatin compared to the ideal physiochemical properties for transdermal delivery

Properties	Rosuvastatin	Simvastatin	Lovastatin	Mevastatin	Ideal properties
<b>Molecular weight (Da)</b>	481.538 <sup>a</sup>	418.600 <sup>b</sup>	404.540 <sup>c</sup>	390.520 <sup>d</sup>	< 500.000 <sup>m</sup>
<b>Aqueous solubility (mg/ml)</b>	7.800 <sup>e</sup>	0.030 <sup>f</sup>	0.004 <sup>c</sup>	0.032 <sup>g</sup>	> 1.000 <sup>m</sup>
<b>Log D at pH 7.4</b>	-0.30 <sup>h</sup>	1.60 <sup>h</sup>	1.18 <sup>i</sup>	No data	1.00 < 3.00 <sup>j</sup>
<b>Melting point (°C)</b>	122 <sup>a</sup>	135 – 138 <sup>k</sup>	172 – 175 <sup>l</sup>	No data	< 200 <sup>m</sup>
<b>Log P</b>	0.42 <sup>h</sup>	4.42 <sup>h</sup>	4.07 <sup>h</sup>	3.57 <sup>h</sup>	1.00 < 3.00 <sup>m</sup>

a) Tabassum *et al.*, 2017:105; b) Bhagat & Sakhare, 2014:1051 ; c) O’Neil, 2006:968; d) (O’Neil, 2001:1098; e) Crestor, 2009; f) O’Neil, 2006:1472; g) Drugbank, 2005; h) Wong *et al.*, 2008:245; i) Joshi *et al.*, 1999:270; j) Subedi *et al.*, 2010:339; k) Oh *et al.*, 2006:1010; l) Desai *et al.*, 2015:33; m) Naik *et al.*, 2000:319.

It is clear from Table 1.1 that the statins possess the ideal properties for transdermal delivery in terms of molecular mass and melting point. The log P values correlate with literature, as rosuvastatin is regarded as more hydrophilic statin, whilst mevastatin, lovastatin and simvastatin are more lipophilic (Kim *et al.*, 2011:295). Consequently, it can be stated that only rosuvastatin possesses an ideal log P value (1.0 < 3.0), as stated by Naik *et al.* (2000:319). In addition, the poor aqueous solubility of these APIs (with the exception of rosuvastatin) poses a problem for successful transdermal delivery, thus, an appropriate carrier system (e.g. nano-emulsions, high solubilisation capacity for both lipophilic and hydrophilic APIs (Tsai *et al.*, 2014:1)), needs to be selected to overcome the inadequacies of statins in an attempt to facilitate successful transdermal delivery.

Nano-emulsions are systems with nanometric droplets, ranging from 50 – 200 nm, characterised as transparent and kinetically stable (Tadros *et al.*, 2004:303). These systems can lead to enhanced penetration (through the skin), as well as increased solubilisation and bioavailability of API as a result of its small particle size (Sutradhar & Amin, 2013:99). Therefore, nano-emulsions are beneficial for poor aqueous soluble statins. When formulating these systems, water or oil droplets are delicately dispersed into the opposite phase, and by means of an appropriate surfactant and co-surfactant, the system can be stabilised. Systems can then be classified as either oil-in-water (o/w) or water-in-oil (w/o) emulsions (Gurpret & Kumar, 2018:781; Sutradhar & Amin, 2013:99). The selection of the components (e.g. surfactants, oil phase) needed to form stable nano-emulsions is essential, in particular the oil phase, as wrongful selection of an oil phase can effect both *in vivo* and *in vitro* performance of the delivery system. Literature suggests that fatty acid ester can be more advantageous than fatty alcohols for example, due to the flexibility of these lipids in terms of tailored globule size and exceptional solubilisation capacity (Pawar & Babu, 2014:449). Apricot kernel oil was selected to serve as the oil phase during formulation of nano-emulsions.

Analysis of apricot oil has shown that it contains high amounts of oleic acid, as well linoleic acid (Wang, 2012:1746). This is desirable firstly, because these are C<sub>18</sub>-fatty acids (Williams & Barry, 2012:132) that are also present in the human skin, which lowers the probability of skin irritation (Büyüktimkin *et al.*, 1997:433; Vermaak *et al.*, 2011:922) and secondly, increased skin permeability has been observed with these fatty acids (Cižinauskas *et al.*, 2017:1). In addition to the solubilisation capacity of the oil phase, the delivery of both lipophilic and hydrophilic drugs can be enhanced with the use of natural oils as penetration enhancers, because of the fatty acids present in natural oils, which causes denaturation of proteins present in skin (Williams & Barry, 2012:132). These penetration enhancers alter the barrier of the skin, thus increasing skin permeability and allowing drugs to reach the systemic circulation after penetration takes place (Alexander *et al.*, 2012:26; Chauhan, 2017:1).

## 1.2 Research problem

Statins are currently administered as an oral daily dose, which can lead to undesired gastrointestinal and hepatic effects due to first-pass metabolism. There are very few drugs suitable for transdermal drug delivery because even though this type of delivery has numerous advantages over oral delivery, the barrier function of the skin poses many restrictions. This barrier is mainly caused by the complex organised structure of the stratum corneum (Cho *et al.*, 2009:230). The utilisation of natural oil nano-emulsions to enhance the systemic delivery of statins should therefore be investigated and compared to nano-emulgels, since statins do not possess the ideal physicochemical properties for transdermal drug delivery.

### 1.3 Aims and objectives

The main aim of this study is to investigate the potential transdermal delivery of the selected statins (simvastatin, rosuvastatin, lovastatin and mevastatin) by formulating nano-emulsions using apricot oil, each containing the selected statins separately. Subsequently, the transdermal delivery of the statins from the nano-emulsions will be compared to that of the semi-solid formulation or nano-emulgel. Additionally, the potential toxicity of the nano-emulsion with and without the API will be determined.

The objectives of this study are:

- To develop and validate an analytic method, i.e. high performance liquid chromatography (HPLC) to determine the concentrations of the selected statins.
- To determine the aqueous solubility, as well as octanol-buffer distribution coefficient (log D) of the selected statins.
- To formulate both the nano-emulsions and the semisolid nano-emulgel containing the selected statins (separately) and the natural oil (apricot oil) as the oil phase and as penetration enhancer.
- To characterise both the nano-emulsions and the nano-emulgels with regard to viscosity, droplet size, zeta-potential, pH, visual examination, morphology and entrapment efficacy.
- To determine the release of the selected statins from both the nano-emulsions and the nano-emulgels by using membrane diffusion studies.
- To perform both Franz cell skin diffusion studies and tape stripping to determine the transdermal and topical delivery of statins, respectively.
- To assess the cytotoxic effects of the statins (separately), the surfactant(s) used in the nano-emulsion, the nano-emulsion and the combination thereof using *in vitro* cell cultures, specifically with the use of normal human dermal fibroblasts (84BR) and pre-malignant human immortalised keratinocytes (HaCaT) cell lines.

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# CHAPTER 2:

## *Formulation and transdermal delivery of nano-emulsions containing the selected statins and apricot kernel oil*

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### 2.1 Introduction

Cholesterol (in conjunction with cholesterol metabolites and immediate biosynthetic precursors of cholesterol) forms an essential element in cellular membrane physiology and several other areas of the human body (e.g. calcium metabolism, dietary nutrient uptake, reproductive biology and stress response) (Liao & Laufs, 2005:89; Tabas, 2002:583). The risk of excessive amounts of cholesterol (e.g. familial hypercholesterolemia (FH)) however poses a crucial risk for CVD (Liao & Laufs, 2005:89).

FH is a dominantly inherited genetic disorder in lipid metabolism, which is characterised by significant or severe elevation in plasma levels of LDL cholesterol (Besseling *et al.*, 2015:1030; Robinson, 2013:139). FH can be divided into heterozygous FH (HeFH) and homozygous FH (HoFH), which has a prevalence of 1 in 200 – 500, and 1 in 160 000 – 300 000, respectively (Al-Rasadi & Al-Waili, 2017:447). The difference between HeFH and HoFH can be described in terms of the rate of LDL removal from the plasma, since with HeFH and HoFH, LDL will be removed at 2/3 and 1/3 of the normal state, respectively. Consequently, the elevation of LDL will be 2 to 3-fold in the case of HeFH and 6 to 8-fold in HoFH state (Parihar *et al.*, 2012:644). HeFH can therefore be seen as a milder manifestation of FH, whereas HoFH is a more severe form (Najam & Ray, 2015:25). Although in most cases FH is associated with mutations in the LDL receptor (LDLR) gene, other genes such as apolipoprotein B (Apo B) and proprotein convertase subtilisin/kexin type 9 (PCSK9) have also been classified as causative genes (De Castro-Orós *et al.*, 2010: 54). It is estimated that patients with FH have 3 – 13 times higher risk of premature atherosclerotic CVD compared to patients with normal plasma concentrations of LDL (Korneva, 2017:1). Hence, early diagnosis and treatment is essential to mitigate the excess risk of premature atherosclerotic CVD (Bouhairie & Goldberg, 2015:169), which is primarily done with HMG-CoA reductase inhibitors, or statins (Schaiff *et al.*, 2008:42), as two thirds of the body's cholesterol is synthesised in the liver, rendering hepatic inhibition of cholesterol biosynthesis as the target choice of treatment (Liao & Laufs, 2005:89).

These lipid lowering drugs are not only the most prescribed drug in the world, but in addition, considered to be the most efficient agent utilised for lowering LDL cholesterol and preventing cardiovascular events (Schaiff *et al.*, 2008:42). Although these agents perform their pharmacological action primarily by decreasing cholesterol synthesis, further reduction of LDL

(20 – 55%) and triglycerides (8 – 30%) occur due to up-regulating of the LDL receptor gene. Lastly, inhibition of the synthesis of Apo B100 and triglyceride-rich lipoproteins (as well as secretion of last mentioned) causes a reduction in atherogenic lipoproteins (Amly & Karaman, 2015:135).

Although statins caused a revolution in the treatment of hypercholesterolemia 30 years ago (Seidah, 2017:1), pharmacological efficacy and benefits of statins is limited due to poor patient compliance (Al-Foraih & Somerset, 2017:36). Yu *et al.* (2018:6) found lower adherence could be associated with side effects of statins. The most common statin associated side effect is on the liver, which includes clinically significant acute liver injury, asymptomatic elevation in aminotransferases (presenting as transaminitis), autoimmune hepatitis, and fulminant hepatic failure. It is proposed that although the risk of statin induced liver injury is low, cases of idiosyncratic liver injury due to statin use can be severe, hence necessitating the stressing of attention to the adverse effects (Jose *et al.*, 2014:355). The incidence of statin associated hepatotoxicity can however be increased in cases, for example, where maximum doses are used, used in combination with drugs utilising the P450 enzymatic pathway and in the elderly or patients with significant renal or hepatic dysfunction (Karahalil *et al.*, 2017:255). In addition, these factors can also increase the incidence of myopathy (or statin induced muscle toxicity) (Gillett & Norrell, 2011:713). Other adverse effects can occur due to intolerance to the statin dose required to reduce CVD risk, which includes gastrointestinal effect (e.g. nausea), rash and headache (Banach *et al.*, 2015:2).

Because of the limitations with oral administration of statins, along with the numerous advancements in the field of pharmaceutical formulation, transdermal delivery of statins can be posed as an alternative to the conventional pharmaceutical dosage form (Muntha, 2014:83). The consideration of transdermal delivery is due to the many advantages this route poses, which includes avoidance of first-pass metabolism (hence, aiding drugs with low bioavailability), reducing side effects of the drug and increasing patient compliance (Muntha, 2014:83). Consequently, the possibility arises that limitations posed by statins can be overcome by transdermal delivery, as this route is intended for systemic circulation drug delivery (Williams, 2013:676).

As the skin is easy to access and prevalent, it has become an attractive area of delivery (Schoellhammer *et al.*, 2014:394); however, the thin, tough and relatively impermeable layer posed by the stratum corneum provides a rate-limiting step in transdermal diffusion (Ali *et al.*, 2015:103). Due to the barrier property of the skin, a variety of techniques have been developed (both passive and active) to enhance penetration, ranging from formulation optimisation and occlusion to physical and chemical methods (Vitorino *et al.*, 2015:7). As literature suggests, selection of the vehicle contributing to efficient drug delivery and absorption is essential

(Foldvari, 2000:417) and additionally poses that the small droplet size can provide a better possibility of transporting compounds in a controlled manner (Tsai *et al.*, 2014:1), thus the possibility of utilising nano-emulsions arises.

Due to the advantageous characteristics, nano-emulsions pose as a delivery system for various routes (e.g. increased solubilisation capacity for lipophilic drugs, improved bioavailability of API) (Jaiswal *et al.*, 2015:123) in conjunction with the advantages specifically aimed at transdermal delivery (e.g. excipients acting as penetration enhancers, possibly increasing drug flux through the skin) (Barakat *et al.*, 2011:1), an o/w nano-emulsion was selected as the delivery system for the purpose of this study.

## **2.2 Hypercholesterolemia**

The lipid sterol found in the body's cell membranes, better known as cholesterol, has several functions in the human body, one of them being the sustainment and the building of cell membranes by the enhancement of the membrane fluidity. Therefore, cholesterol plays a crucial part in cell wall integrity (Tiwari & Pathak, 2011:983). Cholesterol performs an essential part in a healthy human heart (Ma & Shieh, 2006:46) and acts as a precursor to corticosteroids and sex hormones, as it functions as a regulator in homeostasis (Griffiths & Wang, 2009:15). Endogenous cholesterol produced by cells is sufficient to fulfil the required functions in the body, hence any dietary intake of saturated fats (meats, poultry, dairy, etc.) can cause a dramatic elevation in plasma cholesterol, which includes remnant lipoproteins (products of partially catabolised chylomicrons and very-low-density lipoprotein (VLDL)) and LDL. Factors such as gender, age, smoking, alcohol consumption and physical inactivity (Iversen *et al.*, 2009:140) as well as pre-existing conditions, such as insulin resistance and obesity, can lead to additional exacerbation in plasma cholesterol. At or before reproductive years, this degree of elevation still holds no immediate danger; cells have multiple protection mechanisms and adverse reactions in tissues take years to develop. However, post reproductive age, the consequence of continuous elevated plasma cholesterol will take its toll (Tabas, 2002:588). An elevated level especially of the LDL causes a considerable increase in the risk of atherosclerosis. The preferred level of total cholesterol should be under 200 mg/dl (Tiwari & Pathak, 2011:983). Increase in the total cholesterol levels is associated with an autosomal dominant disorder, namely FH (Austin *et al.*, 2004:407; Hutter *et al.*, 2004:430).

FH is characterised by the significant increase of LDL cholesterol, due to the hypocatabolism of LDL cholesterol. FH is an inherited autosomal dominant disorder with several gene mutations implicated in the pathogenesis. These mutations vary in different populations resulting in a diversity of symptoms and severity between countries. This disorder is mainly due to various mutations in the LDL receptor gene, which lead to a phenotype of FH (Nemati & Astaneh,

2010:1079). However, mutation in gain-of-function PCDK9, as well as gene-encoding Apo B can occur, which causes a similar phenotype (Hutter *et al.*, 2004:431; Nemati & Astaneh, 2010:1079; Nordestgaard *et al.*, 2013:3481-3482; Watts *et al.*, 2015:69).

Consequently, this widespread genetic disorder results in a lifelong increase in LDL and a significant increase in the risk of coronary heart disease (CHD). Research suggests that worldwide as many as 34 million individuals are affected by FH and that every minute a new individual is born with this condition (Goldberg & Gidding, 2016, 1054).

### **2.2.1 Treatment of hypercholesterolemia**

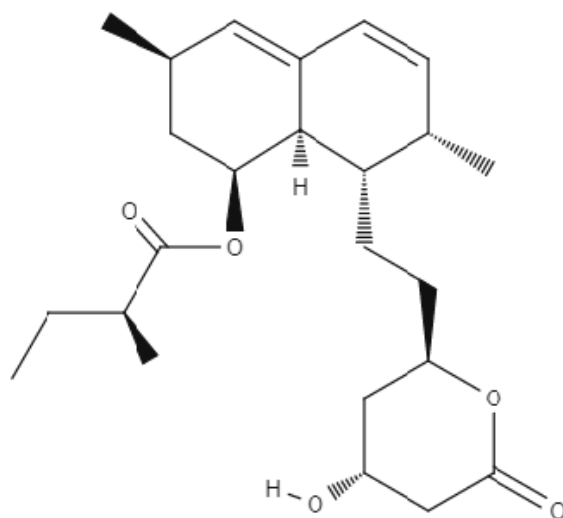
The initial treatment of FH is performed by means of lifestyle changes and statins (Goldberg & Gidding, 2016:1055). CVD is considered to be the leading cause of global deaths (with mortality rates of 235 per 100 000), and in the majority of patients it is accredited to atherosclerosis. Despite the involvement of a variety of factors in the development of atherosclerosis, high plasma cholesterol is proposed to have a primary contributing role (Davies *et al.*, 2016:13). HMG-CoA reductase inhibitors, commonly known as statins, are the most frequently prescribed therapy in the case of hypercholesterolemia, as indicated by clinical trial evidence (Hobbs *et al.*, 1992:445), as statin therapy causes a reduction in development and regression in coronary atherosclerosis and may be the result of the reduction in the core of atherosclerotic plaque (Christians *et al.*, 1998:2; Smilde *et al.*, 2001:577; Vaughan *et al.*, 2000:2). The treatment of hypercholesterolemia has been revolutionised by statins, as these agents are most effective in reducing plasma cholesterol. The competitive inhibition of the principal enzyme involved in the synthesis of endogenous cholesterol, attributes to the advantageous effect of statins (Stancu & Sima, 2001:379), along with the increased clearance of LDL-cholesterol particles from the blood due to the enhanced expression in the LDL-receptors (Bilheimer *et al.*, 1983:4124). As this enzyme, namely mevalonate (a product of HMG-CoA reductase reaction), serves as a precursor for both cholesterol and various other non-steroidal isoprenoid compounds, it can be stated that statins exhibit pleiotropic effects or performs a dual function (Bilheimer *et al.*, 1983:4124; Stancu & Sima, 2001:379). This additional effect is observed with these compounds due to an inhibition in the hepatic synthesis of Apo B100, as well as a decrease in not only the synthesis, but also the secretion of triglyceride-rich lipoproteins (Ginsberg *et al.*, 1987:1696; Grundy, 1998:2B).

Adverse effects are quite rare as statins are generally well tolerated (Black, 2002:40), however the most severe of these effects are rhabdomyolysis, which occurs with progression of myopathy. Rhabdomyolysis increases with the use of cerivastatin, hence the removal for clinical use in 2001 (Furberg & Pitt, 2001:206; Staffa *et al.*, 2002:540). Despite the rare occurrence of adverse effects, its frequency can further be minimised by managing factors such

as combination therapy and the dose of statin administered (Ballantyne *et al.*, 2003:553). Although statins prove to have one common effect, by blocking HMG-CoA reductase, there are differences in their pharmacokinetic profiles (Bonetti *et al.*, 2003:225), chemical structure and lipid-modifying efficacy (Schacter, 2004:117).

### 2.2.1.1 Lovastatin

Lovastatin, derived from fungal metabolites (Syed & Ponnusamy, 2018:62), has a chemical formula of  $C_{24}H_{36}O_5$  resulting in a molecular weight of 404.54 g/mol (O'Neil, 2006:968). The chemical structure of statins determines their aqueous solubility, as determined by the side groups on the rings. Lovastatin (butanoic acid, 2-methyl-, (1S,3R,7S,8S,8aR)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]ethyl]-1-naphthalenyl ester) is a relatively lipophilic compound with an elimination half-life of between 1 and 3 h. Due to the lipophilicity of this compound, it becomes more susceptible for metabolism via the CYP450 system. Although lovastatin provided a significant improvement in the treatment of dyslipidaemias, the limitation of optimal clinical activity and lack of patent protection lead to the semi synthesis of novel compounds, i.e. simvastatin (Sirtori, 2014:5).

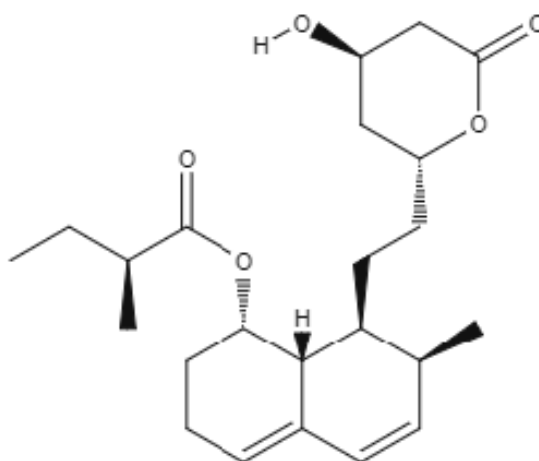


**Figure 2.1:** Chemical structure of lovastatin

Due to the lactone ring of lovastatin, it is only transformed into a biological active (with open acid) form in the body. Hence, lovastatin is administered as a prodrug (due to lactone ring) and this characteristic is indicative of higher log D values, as open ring structure statins tend to have smaller log D values. The higher log D value enables this compound to distribute to tissue non-specifically, by crossing lipid layers by means of passive diffusion (Shitara & Sugiyama, 2006:73-76).

### 2.2.1.2 Mevastatin

Mevastatin (compactin) has attracted much attention over the past few decades. Mevastatin, belonging to the polypeptide class of statins, was produced using *Penicillium citrinum* in a submerged fungal fermentation of glucose medium (Mahesh *et al.*, 2012:001), independently in Japan as well as England in 1967. It was reported three years later that lovastatin (monacolin K or mevinolin) was produced from *Monascus ruber*. In the years following these discoveries, mevastatin and lovastatin have been used to obtain different active compounds (i.e. pravastatin and simvastatin) by means of biotransformation, after direct addition to cultures in fermentation broth (Syed & Ponnusamy, 2018:62).



**Figure 2.2:** Chemical structure of mevastatin

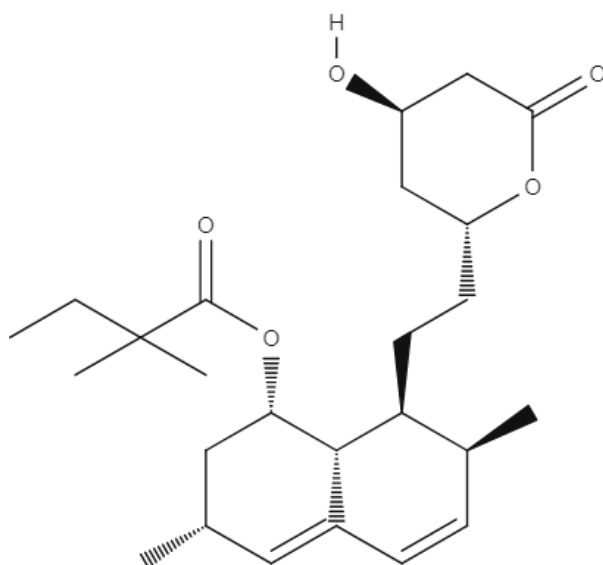
Mevastatin (2S-methyl-(1S,2,3,7S,8S,8aR)-hexahydro-7-methyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]ethyl]-1-naphthalenyl ester-butanoic acid) has a chemical formula of  $C_{23}H_{34}O_5$  and molecular weight of 390.51 g/mol (O'Neil, 2001:1098). While lovastatin and simvastatin are converted in the liver to their active forms, mevastatin presents as active in its parent form (Syed & Ponnusamy, 2018:62).

### 2.2.1.3 Simvastatin

Simvastatin is a fungal-derived statin, with a partially reduced naphthalene ring structure (involved in the binding with HMG-CoA reductase enzyme). This lipophilic statin undergoes extensive hepatic first-pass metabolism, hence exhibiting low system bioavailability (Shuhaili *et al.*, 2017:2).

The appearance of simvastatin, due to the limitations of lovastatin, brought about the discovery that all available molecules (mevastatin, lovastatin and simvastatin), had closed lactone ring configuration of the HMG-CoA analogue moiety. This closed ring results in inactive pro-drug molecules requiring reopening in the liver or gastrointestinal tract. Due to the reliable nature of simvastatin, it led to the development of other novel agents with open ring structures, i.e. rosuvastatin (Sirtori, 2014:5).

Statins present with different potencies for extrahepatic HMG-CoA reductase inhibition, due to the difference in their tissue permeability and metabolism. Consequently, variations in peripheral side effects observed between different statins may be explained by variations in tissue permeability and metabolism. Simvastatin (lipophilic) is for instance more likely to enter endothelial cells, than less lipophilic statins (i.e. rosuvastatin) by passive diffusion (Liao & Laufs, 2005:91).



**Figure 2.3:** Chemical structure of simvastatin

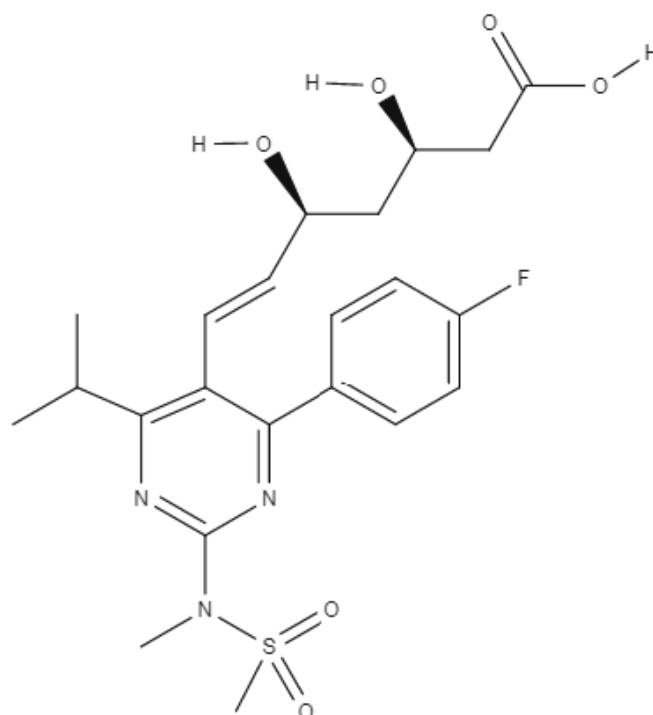
Simvastatin ammonium salt ( $\beta R, \delta R, 1S, 2S, 6R, 8S, 8aR$ )-8-(2,2-dimethyl-1-oxobutoxy)-1,2,6,7,8,8a-hexahydro- $\beta, \delta$ -dihydroxy-2,6-dimethyl-1-naphthaleneheptanoic acid ammonium salt) was utilised in this study. The chemical formula of this compound is  $C_{25}H_{40}O_6 NH_3$ , resulting in a molecular weight of 453.61 g/mol (Toronto Research Chemicals, 2014:2).

#### 2.2.1.4 Rosuvastatin

Rosuvastatin has a pyrimidine ring structure with additional hydrogen binding interactions. Due to the polar interaction between HMG-CoA reductase enzyme and the methane sulphonamide group and the aforementioned structural properties, rosuvastatin proves to be the most efficient statin to reduce the HMG-CoA reductase enzyme. The methane sulphonamide group in

conjunction with the polar hydroxyl group is indicative that rosuvastatin is more hydrophilic than the other selected statins (Shuhaili *et al.*, 2017:2).

Therefore, rosuvastatin (third-generation synthetic statin) is much more potent than the selected second-generation statins. Although rosuvastatin has some physiochemical properties similar to that of pravastatin (second-generation), it presents with an increased half-life and lipophilicity (Liao & Laufs, 2005:91).



**Figure 2.4:** Chemical structure of rosuvastatin

For the purpose of this study, rosuvastatin calcium salt ((3R,5S,6E)-7-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-[methyl(methylsulfonyl)amino]-5-pyrimidinyl]-3,5-dihydroxy-6-heptenoic acid, hemicalcium salt), with a chemical formula of  $C_{22}H_{28}FN_3O_6S \cdot \frac{1}{2}Ca$  and a molecular weight of 501.6 g/mol, was used (Cayman chemicals, 2016:4).

### 2.3 Factors influencing the consideration to use alternative routes of administration

Currently statins are administered orally as a daily dose. After administration of these compounds, rapid absorption takes place and because of the significant amount of statin that binds to plasma proteins, the amount of active pharmacological unbound statin is relatively low, with the exception of pravastatin (Corsini *et al.*, 1999:418). Thereafter, metabolism of the statins is mainly carried out by the CYP450 family of enzymes, although the metabolism of rosuvastatin, pitavastatin and pravastatin via this pathway is less significant (Bottorff & Hansten, 2000:2275). Elimination of these compounds primarily occurs via the bile, after the aforementioned metabolism, thus the risk for statin-induced myopathy increases with hepatic

dysfunction (Kaspera *et al.*, 2010:2; Maron *et al.*, 2000:208). Consequently, the oral route poses limitations regarding low plasma concentration, hepatic first-pass metabolism and in addition gastrointestinal factors and patient compliance (Kaestli *et al.*, 2008:269).

As statins are metabolised by the liver, alternative routes of administration could be beneficial in elderly patients because of the pharmacokinetic changes that can occur with age. These changes could elevate the risk of side effects due the increase of drug concentration in elderly patients. In addition, elderly patients are often polymedicated, which could increase the risk of drug interactions as result of CYP450 metabolism, which is the main catalyst for the majority of statins and numerous other drugs (Szadkowska *et al.*, 2010:116). However, it is essential to determine whether structural changes in the skin due to age will influence transdermal delivery. Studies have found that although hydrophilic compounds are affected by changes in the structure of the skin, the permeation of lipophilic compounds are unchanged (Singh & Morris, 2011:5).

Hepatotoxicity is a common concern when referring to statins. It can occur when statins are used in conjunction with other lipid lowering drugs or drug utilising the same enzymatic pathways (CYP450 enzymes), used in high doses or when used in elderly patients or those with considerable hepatic and/or renal dysfunction (Karahalil *et al.*, 2017:255). Although statin-associated clinically important drug-induced liver injury is rare, liver abnormalities observed with statins can include (Thapar *et al.*, 2013:605):

- mild asymptomatic elevation of serum alanine aminotransferase;
- cholestatic or mixed hepatitis, associated with the development of jaundice;
- hepatitis and clinical symptoms of liver disease.

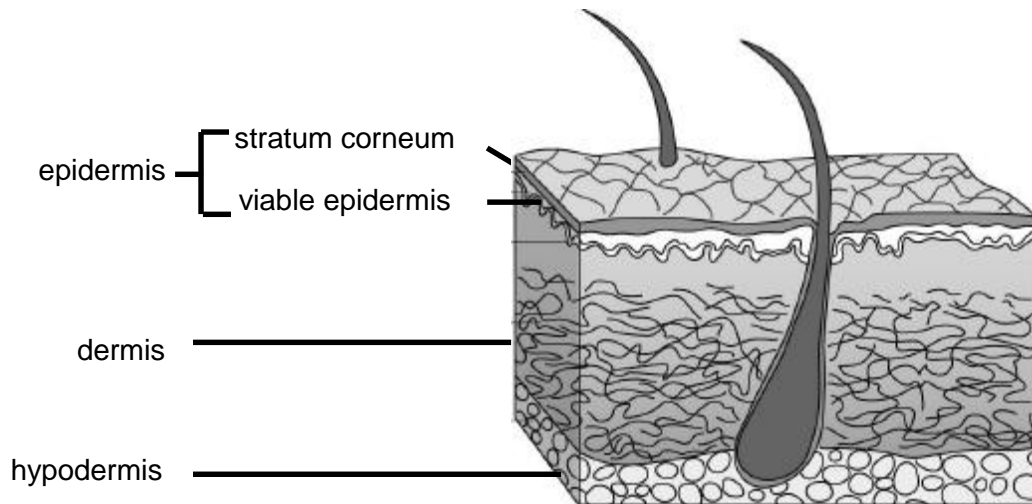
In this context, it can be proposed that transdermal delivery will decrease drug-induced hepatotoxicity due to the avoidance of hepatic first-pass metabolism (Caon *et al.*, 2015:464). Lastly, although rare, gastrointestinal side-effects (i.e. abdominal pain, diarrhoea, nausea, dyspepsia, etc.) have been associated with statins (Kiortsis *et al.*, 2007:8), therefore, by incorporating statins into a transdermal delivery system, gastrointestinal side-effects could be reduced (N'Da, 2014:20781).

## **2.4 Skin**

The surface area of the human adult skin is about 2 m<sup>2</sup> (Hadgraft, 2001:1; Rastogi & Yadav, 2012:162) with a weight comprising roughly 16% of the total body weight, making it the largest organ in the body (Venus *et al.*, 2010:469) and receiving one third of the circulating blood supply (Rastogi & Yadav, 2012:162). This organ is comprised of four major layers, the non-viable

stratum corneum, viable epidermis, viable dermis and the hypodermis (Pathan & Setty, 2009:174).

This skin has numerous functions, the most crucial of these being defending the body by acting as an effective permeation barrier (Folvari 2000:417; Ng & Lau, 2015:1), thus protecting the body from the outside environment and external materials (Hadgraft 2001:1). This barrier also provides protection to the body with regard to water loss (Hadgraft 2001:1).



**Figure 2.5:** Four major layers of the human skin (adapted from Geerligs (2010:4)).

### 2.4.1 The epidermis

The epidermis in the absence of the stratum corneum is considered the viable epidermis (Rastogi & Yadav, 2012:162). The epidermal layer consists of several types of epidermal cells (melanocytes, keratinocytes and Langerhans cells) (Williams, 2013:678) and is composed of four separate layers: the stratum basale, stratum spinosum, stratum granulosum and the stratum corneum (Ng & Lau, 2015:4; Venus *et al.*, 2011:471; Williams, 2013:678). These four layers can also be divided into two layers, known as the non-viable and viable epidermis (Kute & Saudagar, 2013:272). The non-viable layer being the stratum corneum and the viable composed of the stratum basale, stratum spinosum and the stratum granulosum (Asbill & Michniak, 2000:36).

#### 2.4.1.1 The non-viable epidermis (stratum corneum)

The level of hydration in this lipophilic layer will influence the width of the 10 – 15 layers of corneocytes of which this layer is comprised (Benson, 2005:24). These corneocytes primarily contain insoluble bundles of keratins (Asbill & Michniak, 2000:36). Vertical swelling of the corneocytes occurs when this layer is exposed to moisture, thus opening this dense layer (Barry, 2001b:969). This multi-layered membrane consists of proteins and lipids, arranged in an

interlocking impermeable complex structure (Shah, 1994:20). Due to all the characteristics of this layer resulting in diffusional resistance, it is considered a rate-limiting obstacle for transdermal delivery of APIs (Barry, 1983:7, 17).

The “brick” and “mortar” composition in the stratum corneum poses the most crucial barrier for APIs to cross (Benson, 2005:24; El Maghraby *et al.*, 2008:204). When referring to “brick” and “mortar” concept, the dead keratinised cells represent the “brick”, whereas the “mortar” represents the intercellular matrix composed of long chain ceramides, triglycerides, free fatty acids, cholesterol sulphate, sterol esters and cholesterol that surrounds the cells (Bala *et al.*, 2014:1809). Disruption and bypassing of this brick and mortar domain is the main goal when using permeation enhancement techniques (Barry, 2006:5). In addition, another essential part of the stratum corneum is water, which acts as a plasticiser, providing flexibility and preventing cracking (Ali *et al.*, 2015:105).

The barrier function of the stratum corneum is further contributed by interstitial neutral lipids with saturated and unsaturated fatty acids present. The unsaturated chains are dominant, and in contrast with other biological membranes, no phospholipids are present (Barry, 2006:4-5; Ghosh & Pfister, 1997:6; Hadgraft & Finnin, 2006:362; Norlén, 2008:64; Roy, 1997:141).

#### **2.4.1.2 The viable epidermis**

When considering the epidermis without the stratum corneum, it is referred to as the viable epidermis (Jepps *et al.*, 2013:154; Ng & Lau, 2015:7). Diffusion through the first layer leads to the viable epidermis. The second layer comprises several layers of keratinocytes at different phases of differentiation (Foldvari, 2000:418). These also provide a protection function, as upon injury, keratinocytes will stimulate the immune function at the site of infection by secreting cytokines and chemokines; additionally, a protective cover will be formed at wound sites due to migration of keratinocytes (Schoellhammer *et al.*, 2014:294). When drug diffusion takes place across this layer, the high fraction of water present in this layer causes a more significant barrier towards lipophilic substances, as these compounds have a much higher affinity for this non-polar aqueous environment (Scheuplein & Blank, 1971:702).

#### **2.4.2 The dermis**

Located between the viable epidermis and the hypodermis, is the dermis, with a thickness of roughly 3 – 5 mm (Jepps *et al.*, 2013:677; Kute & Saudagar, 2013:372) consisting of extensive microvasculature networks (i.e. smaller blood vessels, hair follicles and sweat glands) (Bala *et al.*, 2014:1809). This layer is composed primarily of elastin and collagen, which is responsible for providing the function of elasticity and strength to the skin (Ng & Lau, 2015:4).

Mucopolysaccharide gel that surrounds the elastin and collagen is responsible for creating a hydrophilic environment as found in this layer (Jepps *et al.*, 2013:677; Williams, 2013:677).

As stated, lymphatic vessels, nerve endings, blood vessels, hair follicles and sebaceous and sweat glands are found in the dermis. The appendageal route in skin permeation is possible via the sweat glands and hair follicles (Barry, 1983:7-8; El Maghraby *et al.*, 2008:204). The blood supply in this layer is crucial for the systemic absorption of substances when applied to the skin (Singh, 1999:597). Most of APIs that cross the stratum corneum will be cleared within minutes as a result of the abundance of capillaries located in the dermal papillary layer (Barry, 2001a:102).

### **2.4.3 The hypodermis**

The hypodermis or subcutaneous tissue is considered the third layer of the skin (Ali *et al.*, 2015:105). This layer consists of adipose or fatty tissue and is mainly composed of lipocytes (Venus *et al.*, 2010). Primarily, this layer is responsible for protecting the body against shock of a physical nature (by acting as a cushion), as well as acting as a thermal barrier in the case of temperature changes (Ali *et al.*, 2015:105; Williams, 2013:677; Yagi & Yonei, 2018:51). Additionally, due to fat storage in the adipose cells, this layer also plays a role in the storage of energy (Yagi & Yonei, 2018:51). Although variation occurs from person to person and depending on the body region (Yagi & Yonei, 2018:51), the average thickness of the hypodermis is considered to be 4 – 9 mm (Ali *et al.*, 2015:105).

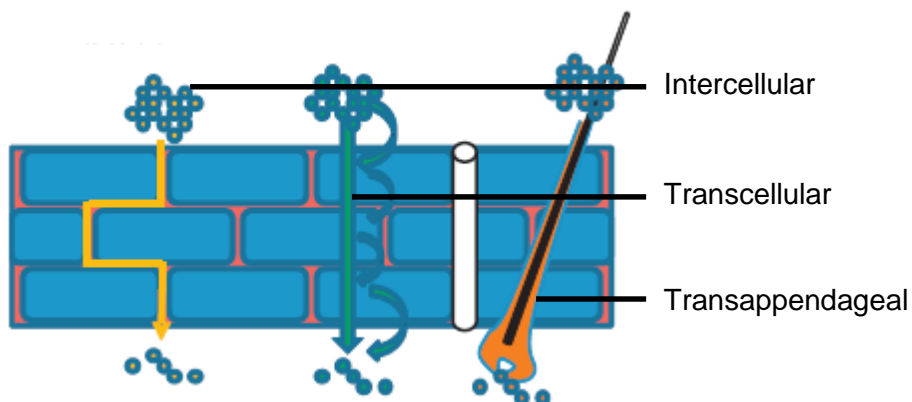
## **2.5 Transdermal drug delivery**

Non-invasive delivery of medications or APIs through the surface of the skin can be used to describe transdermal drug delivery. A homemade medicinal preparation intended for transdermal delivery dating back to the early 20<sup>th</sup> century was mustard plasters, utilised for severe chest congestion; consequently, transdermal delivery is not a modern concept. Over the past few decades, a number of patches for transdermal delivery (e.g. nicotine and analgesic patches) revolutionised this industry (Bajaj *et al.*, 2011:39). Patches are known as first-generation transdermal delivery systems, although not all first-generation systems imply the use of a patch. First-generation transdermal delivery also includes gels and topical formulations (Prausnitz & Langer, 2008:1263).

Transdermal delivery systems pose as an attractive substitute to the conventional delivery systems, as it can reduce or even possibly evade the restrictions that come with the use of oral (as stated in Section 2.3) and parenteral delivery (Mudhinge *et al.*, 2011:130). Accurate and prolonged delivery of drugs, steady-state profiles, decrease in possible peak-associated side effects and the guarantee of therapeutic concentrations above the minimum, are just some of

the advantages of these delivery systems (Magnusson *et al.*, 2001:222). Furthermore, the avoidance of the hepatic first-pass effect suggests another advantage, as this for some substances indicates multiple administrations may not be needed (Kalluri & Banga, 2011:82).

As indicated before, the percutaneous delivery of substances is very challenging because of the barrier posed by the stratum corneum (Lopez *et al.*, 2011:933). The three main pathways in which transdermal delivery can be achieved through diffusion is via the intercellular, transcellular and the appendageal permeation (Alexander *et al.*, 2012:28).



**Figure 2.6:** Potential transport pathways for transdermal delivery (adapted from Lane, 2013:13).

### 2.5.1 Intercellular route

The lipid matrix present within the stratum corneum is utilised when using this route (Morrow *et al.*, 2007:38; Williams, 2003:38). This route is seen as a complex pathway for the API, as the lipid matrix poses difficulty with regard to diffusion and partitioning (Morrow *et al.*, 2007:38; Ng & Lau, 2015:9). In order for dissolution and diffusion of API to take place through this non-polar lipid matrix, it would be more appropriate to make use of non-polar APIs (Geethu *et al.*, 2014:1813).

For API permeation across the lipid stratum corneum, this particular pathway is considered the most important. Modification of the ordered structure and manipulation of the solubility of the lipid domain as techniques to enhance penetration is therefore the primary focus (Benson, 2005:24; El Maghraby *et al.*, 2008:205).

### 2.5.2 Transcellular route

This pathway moves across the keratinocytes of the stratum corneum and is considered a polar route. An important aqueous environment is provided by the cellular components through which the solute diffuses. Polar lipids that connect to the intercellular multiple bilayers are connected to these keratin-filled cells (Williams, 2003:33). Numerous obstacles are faced by a molecule

that crosses the stratum corneum by means of this route (Williams, 2003:33), mainly because this route involves a variety of partitioning and diffusion stages (Morrow *et al.*, 2007:38). This route may be regarded as a favourable route for hydrophilic APIs.

### 2.5.3 Appendageal routes

Diffusion into the epidermis and direct permeation into the dermis can be accomplished with this route via the hair follicles and the aqueous pathway of the sweat glands (Ranade & Hollinger, 2004:214). This route provides the primary gateway to the sub-epidermal layer of the skin for ions and polar molecules (Moghimi *et al.*, 1999:516). These appendages operate as diffusional shunts for the transport of certain APIs across the skin (Barry, 2006:4; Robinson *et al.*, 1997:61).

## 2.6 Physiochemical properties that influence transdermal delivery

Candidates suitable for transdermal drug delivery can be determined by comparing the physiochemical properties of the intended API to that of the properties considered optimal for transdermal delivery (Liu *et al.*, 2016:437). The properties essential to consider for transdermal delivery includes, a log P value around 1 – 3, molecular weight of  $\leq 500$  Da, balanced lipophilicity, adequate solubility in both water ( $> 1$  mg/ml) and oil, and a melting point of  $< 200$  °C (Liu *et al.*, 2016:437, Naik *et al.*, 2000:319). The relevance of considering these physiochemical parameters lies in the important role these parameters play in the prediction of drug skin permeability (Liu *et al.*, 2016:437). Table 2.1 displays some of these physiochemical characteristics for each of the selected statins. These characteristics will be thoroughly discussed in the sections that follow.

**Table 2.1:** Physiochemical characteristics of the selected statins

Properties	Rosuvastatin	Simvastatin	Lovastatin	Mevastatin	Ideal properties
<b>Molecular weight (Da)</b>	481.538 <sup>a</sup>	418.600 <sup>b</sup>	404.540 <sup>c</sup>	390.520 <sup>d</sup>	$< 500.000^m$
<b>Aqueous solubility (mg/ml)</b>	7.800 <sup>e</sup>	0.030 <sup>f</sup>	0.004 <sup>c</sup>	0.032 <sup>g</sup>	$> 1.0000^m$
<b>Log D at pH 7.4</b>	-0.30 <sup>h</sup>	1.60 <sup>h</sup>	1.18 <sup>i</sup>	No data	$1.00 < 3.00^j$
<b>Melting point (°C)</b>	122 <sup>a</sup>	135 – 138 <sup>k</sup>	172 – 175 <sup>l</sup>	No data	$< 200^m$
<b>Log P</b>	0.42 <sup>h</sup>	4.42 <sup>h</sup>	4.07 <sup>h</sup>	3.57 <sup>h</sup>	$1.00 < 3.00^m$

a) Tabassum *et al.*, 2017:105; b) Bhagat & Sakhare, 2014:1051 ; c) O'Neil, 2006:968c; d) O'Neil, 2001:1098; e) Crestor, 2009; f) O'Neil, 2006:1472; g) Drugbank, 2005d; h) Wong *et al.*, 2008:245; i) Joshi *et al.*, 1999:270; j) Subedi *et al.*, 2010:339; k) Oh *et al.*, 2006:1010; l) Desai *et al.*, 2015:33; m) Naik *et al.*, 2000:319.

### **2.6.1 Aqueous solubility**

The ability of a liquid, solid or gaseous chemical substance (solute) to dissolve in a solvent (liquid, solid or gaseous) in order to form a homogenous solution is referred to as solubility (Savjani *et al.*, 2012:1). This value suggests the extent of how well an API will dissolve in the solvent and as a result, be absorbed through the skin (Steele & Austin, 2009:24). The solubility of an API in a medium is highly dependent on the solvent used, and is measured as the concentration where further saturation of the solution will not increase the concentration (Savjani *et al.*, 2012:1). The bioavailability of an API can be established through solubility and it is suggested that an API with an aqueous solubility exceeding 1 mg/ml is ideal for topical or transdermal delivery (Naik *et al.*, 2000:319). Poor aqueous solubility of an API can have an undesirable effect on permeation (Kulkarni & Nagarsenker, 2008:466). The lipophilic nature of an API will also greatly influence the solubility, as seen in the case of lovastatin, mevastatin and simvastatin. However, rosuvastatin is considered a more hydrophilic statin, which can be correlated when comparing the aqueous solubility of rosuvastatin with the other selected statins (Kim *et al.*, 2011:295).

### **2.6.2 Melting point**

Studies have shown that a lower melting point will result in higher extent of skin permeation (Mohammadi-Samani *et al.*, 2014:117) and the ideal melting point for transdermal delivery is considered to be less than 200 °C (Naik *et al.*, 2000:319). A definite correlation can also be drawn between melting point and aqueous solubility, as an API with a lower melting point will present with better aqueous solubility (Williams, 2003:37). The melting point of the selected statins ranges between 122 – 175 °C (Desai *et al.*, 2015:33; Oh *et al.*, 2006:1010; Tabassum *et al.*, 2017:105), therefore within the ideal criteria for transdermal delivery.

### **2.6.3 Molecular mass**

The size and shape of an API molecule is an essential characteristic to consider in attempting transdermal delivery (Williams, 2003:36). Increased permeation is observed with smaller molecules (Carpentieri-Rodrigues *et al.*, 2007:951). Furthermore, molecular mass and size has an influential effect on diffusivity of the API, therefore a correlation can be suggested between transdermal flux and molecular weight (Barry, 2002:513; Williams 2003:36). The number of drugs that are commercially available for transdermal administration is limited (Prausnitz *et al.*, 2004:116) due to the barrier function of the skin prohibiting the penetration of large molecules (Williams, 2013:680). It is proposed that the ideal molecular mass of an API should be smaller than 500 g/mol, or 500 Da, to enable successful transdermal delivery (Naik *et al.*, 2000:319; Williams, 2013:675). The selected statins fall within this suggested criterion as their molecular

masses range between 390.520 – 481.538 g/mol (Bhagat & Sakhare, 2014:1051; O’Neil, 2006:968; O’Neil, 2001:1098; Tabassum *et al.*, 2017:105).

#### **2.6.4 Partition coefficient**

According to Fick’s first law, a high partition coefficient (K) is indicative of an increased flux (J). APIs with a high partition coefficient are more lipophilic and will therefore partition into the stratum corneum effortlessly, but partitioning into the aqueous tissue will be prone to difficulties (Barry, 2007:578). The partition coefficient or log P of an API is indicative of how distribution molecules between both lipophilic and hydrophilic phases will take place (Williams, 2013:676). Thus, to permeate the “brick and mortar” structure of the stratum corneum, it is essential that a drug is equipped with both lipophilic and hydrophilic properties (Naik *et al.*, 2000:319; Williams, 2003:37). For an API to possess both these properties, thus being relatively soluble in both oil and water, the ideal log P should fall between 1 and 3 (Subedi *et al.*, 2010:339; Wiedersberg & Guy, 2014:150; Williams, 2003:36). From the log P values of the selected statins, as displayed in Table 2.1, it is clear that none of the statins had a log P value considered optimal for transdermal delivery. Lovastatin, mevastatin and simvastatin have log P values above 3 (indicating high lipophilicity), whereas rosuvastatin has a log P value of under 1 (indicative of a more hydrophilic compound) (Kim *et al.*, 2011:295). Rosuvastatin is therefore too hydrophilic and could have difficulty in permeating into the stratum corneum; the other three statins, which are too lipophilic, could have difficulty leaving the stratum corneum and permeating into the underlying layers.

#### **2.6.5 Diffusion coefficient**

Movement of API molecules from a higher to a lower concentration can be defined as diffusion (Williams, 2003:27). The degree of effortlessness by which an API can move through a certain area or tissue is indicated by the diffusion coefficient of the specific API (Hadgraft & Wolf, 1993:163; Williams, 2003:27; Williams, 2013:676); it is expressed as area (cm<sup>2</sup>) per time. Where movement of an API across and through the skin is concerned, it can be described by means of passive diffusions as defined by Fick’s diffusion laws (Williams, 2013:675). The complex structure of the stratum corneum limits diffusion, thus the correct selection of delivery vehicle is essential as it will determine the diffusion speed (Barry, 2002:512). The amount of hydrogen bonding groups present on an API’s structure will also affect diffusivity as well as bioavailability. This impact comes because of interactions that occur between these hydrogen bonds and the lipid polar head groups (Thomas & Finnin, 2004:699; Williams, 2013:680). Ashford (2013:324) proposed for ideal absorption to occur, a molecule should not possess more than five hydrogen bond donors and no more than 10 hydrogen bond acceptors. Table 2.2 displays the number of hydrogen bond donors and acceptors.

**Table 2.2:** Number of hydrogen bonds and acceptors possessed by the selected statins

Statin	Lovastatin	Mevastatin	Simvastatin	Rosuvastatin
Hydrogen bond donors	1 <sup>a</sup>	1 <sup>b</sup>	1 <sup>c</sup>	4 <sup>d</sup>
Hydrogen bond acceptors	5 <sup>a</sup>	5 <sup>b</sup>	5 <sup>c</sup>	20 <sup>d</sup>

a) Pubchem, 2005a; b) Pubchem, 2005b; c) Pubchem, 2005c; d) Pubchem, 2005d

When observing Table 2.2, it can be proposed that since lovastatin, mevastatin and simvastatin have 1 hydrogen donor bond and 5 acceptor bonds, satisfactory absorption results can be obtained. While rosuvastatin possesses an ideal amount of hydrogen donors, the amount of hydrogen acceptors exceeds 10, which is not considered ideal.

### 2.6.6 Ionisation, pH and pKa

The pH of the skin is described as ranging between 4 – 7 (Ng & Lau, 2015:8; Williams, 2013:678), consequently pH value under 3 or above 9 could be harmful to the skin and affect permeability (Naik *et al.*, 2000:319). The dissociation rates of weak bases and acids can be affected by pH depending on the pKa or pKb of the respective API. Both pH and ionisation of the API will affect an effective membrane gradient (Barry, 2002:511).

For APIs intended for topical or transdermal delivery, the degree of ionisation at an appropriate pH has substantial value (Nair *et al.*, 2013:425). The relevance of ionisation is due to the lipophilic barrier posed by the skin (Williams, 2003:38). It is proposed that effective permeation and diffusion in transdermal and topical delivery can be achieved when the API is unionised (Li *et al.*, 2012:985; Williams, 2003:38), as dictated by the pH-partition hypothesis, and although crossing the skin barrier by electrolytes is possible, it occurs with difficulty (Smith, 1990:27; Wiechers, 1989:190). The actual concentration of ionised and unionised species will be determined by the pH of the delivery system and ionisation constant of the weak acid or base (Wiechers, 1989:190). It is known that an ionised API presents with high aqueous solubility and low permeation, whereas an unionised API presents with opposite characteristics (low aqueous solubility, high permeation ability) (Williams, 2003:38-39). Therefore, it is essential to determine the state of ionisation of an API within the formulation, as this could predict the diffusivity of the formulation. The determination was done by utilising an altered form of the Henderson-Hasselbalch equation (Equation 2.1 -2.2).

$$\% \text{ionised} = 100 / 1 + \text{anti-log} (\text{pKa} - \text{pH}) \quad \text{Equation 2.1}$$

Hence, the %unionised could be determined as follows:

$$\% \text{unionised} = 100 - \% \text{ionised} \quad \text{Equation 2.2}$$

The pKa values of the selected statin are as follows:

- Lovastatin: 13.49 (Wong *et al.*, 2008:245)
- Mevastatin: 13.49 (Wong *et al.*, 2008:245)
- Rosuvastatin calcium: 4.25 (Wong *et al.*, 2008:245)
- Simvastatin (hydroxyl acid/ammonium salt form): 4.31 (Chen *et al.*, 2005:539).

By means of the aforementioned pKa values for of the respective statins, the %unionised species at pH 5 and 7 could be determined by utilising Equation 2.1 followed by Equation 2.2. The results obtained were as follows:

**Table 2.3:** The %unionised species at pH 5 and pH 7

<b>Selected statins</b>	<b>pH 5</b>	<b>pH 7</b>
<b>Lovastatin</b>	99.999%	99.999%
<b>Mevastatin</b>	99.999%	99.999%
<b>Rosuvastatin calcium</b>	15.098%	0.178%
<b>Simvastatin ammonium</b>	16.956%	0.204%

From Table 2.3, it can be concluded that both rosuvastatin and simvastatin may have very limited diffusivity through the skin, due to the small %unionised species, which is indicative of rosuvastatin and simvastatin being more ionised. Whereas lovastatin and mevastatin presented as greatly unionised, this is more favourable for diffusivity through the skin. It can be proposed that the limitation of rosuvastatin and simvastatin can possibly be overcome by formulation of o/w nano-emulsions by means of a natural oil, acting as a penetration enhancer.

## **2.7 Approaches to successful transdermal delivery**

In modern therapy, optimising drug delivery through the skin is essential (Barry, 2001:101). The most important objective when considering this method of delivery is to overcome the effective and protective barrier posed by the stratum corneum, as it affects both dermal and transdermal delivery (Neubert, 2011:1). Compared to conventional ointment and creams, a variety of concepts has been proposed, particularly for compounds with limited skin penetration due to particle size and lipophilic nature to overcome this barrier and enhance drug delivery (Vogt *et al.*, 2016:3). One such concepts are by implementing nanotechnology, i.e. nanoparticles. By utilising these delivery systems, drug concentration within the formulation can be increased and, consequently, drug flux. Nano-emulsions are one of the commonly used nanoparticle systems (Goyal *et al.*, 2016:78; Patzelt *et al.*, 2017:174). In addition, penetration-enhancing techniques have been studied by the scientific community for enhanced transdermal delivery (Goyal *et al.*,

2016:78). For the purpose of this study, nano-emulsions and penetration enhancers will be investigated further.

### **2.7.1 Penetration enhancers**

The enhanced level of drug penetration through the skin barrier with the incorporation of penetration enhancers, otherwise known as promoters or accelerants, is believed to be the result of a combination of factors. Penetration enhancers can increase the fluidity of the intercellular lipid lamellae, through interaction with the skin components, and can either induce swelling of the stratum corneum (through hydration) and/or extract some component structures of the skin (Cho *et al.*, 2009:230), thus causing an advantageous increase in API partitioning and absorption (Trommer & Neubert, 2006:108). The properties associated with an ideal penetration enhancer are, for example, that the substance should be non-toxic and have a satisfactory feel on the skin. In addition, their activity as enhancers should be exerted promptly, although no pharmacological activity should be present (Pathan & Setty, 2009:175; Williams & Barry, 2012:129). Penetration enhancers can be divided into different types or classes, as the approach to promote the penetration of an API differs. Wang *et al.* (2003:1612) stated that penetration enhancers can be classified as either chemical or physical. Physical enhancers comprise electroporation, iontophoresis (Mathur *et al.*, 2010:174) and temperature (Asbill & Michniak, 2000:37). According to Naik *et al.* (2000:321), substances that chemically enhance the permeability of the stratum corneum include pyrrolidones, amines, esters, amides, sulphoxides, alcohols, polyalcohols, surfactant, fatty acids, etc. For the purpose of this study, fatty acids will be used as penetration enhancers in the formulations.

#### **2.7.1.1 Fatty acid as a component of natural oils**

Fatty acids as a group have a variety of characteristics and are ideal penetration enhancers due to their non-toxic nature (Van Zyl *et al.*, 2016:188). The increase in skin permeation for a broad diversity drugs with different polarities has been exhibited with the use of fatty acids, thus explaining the substantial attention that has been attracted by these substances. The suitability of fatty acid for use as penetration enhancers can be affected by structural differences (Cho *et al.*, 2009:232). The mechanisms by which fatty acids enhance the movement of an API across the stratum corneum is based on the interaction with the lipids in this layer leading to disruption of the barrier characteristics. In addition, interaction with cellular proteins and increased partitioning of the drug into the stratum corneum is facilitated (Wang *et al.*, 2003:1612). Fatty acids are found in natural seed oils and can be divided into saturated acids (i.e. stearic and palmitic) and unsaturated acids (i.e. linoleic and oleic) (Vermaak *et al.*, 2011:922); saturated fatty acids are commonly less effective than unsaturated acids (Trommer & Neubert, 2006:114; Wang *et al.*, 2003:1612). The chemical structure of fatty acids consists of an aliphatic

hydrocarbon chain and carboxyl group at the end of the chain (Babu *et al.*, 2006:144). The chain can contain 14 – 22 carbons with 1 – 3 double bonds (Sharma & Kundu, 2006:984). The amount of double bonds and configuration of these bonds is indicative of the efficacy of an unsaturated acid. Unsaturated fatty acid with a *cis*-configuration has a higher capability of causing a disturbance in the lipid packing order with the bilayers, thus making them more effective penetration enhancers than those with a *trans*-configuration (Cho *et al.*, 2009:232; Trommer & Neubert, 2006:114). Longer chain fatty acids have proved to enhance percutaneous drug absorption and unsaturated C<sub>18</sub> chain acids near to ideal (Cho *et al.*, 2009:232; Williams & Barry, 2012:132). Fatty acids have the advantage of consisting of components that are endogenous to the skin, thus explaining their popular use in topical pharmaceuticals as well as cosmetic products (Kim *et al.*, 2008:373)

### **2.7.1.2 The relevance of apricot oil use in nano-emulsion**

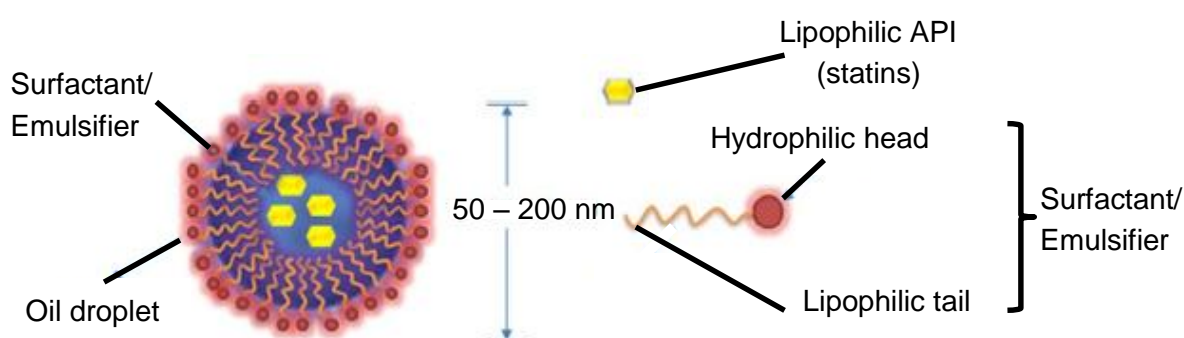
The use of the term apricot signifies the collective of four different species as well as one interspecific hybrid that occurs naturally. The cultivated apricot (*Prunus armeniaca* Linn.) and the Siberian apricot (*Prunus sibirica*) represent two of the five species (Hormaza, 2002:321). Apricot kernel oil is extracted from the pits and has proven to contain a considerable amount of fatty acids, i.e. linoleic and oleic acids, which in their unsaturated form is considered to be of high pharmaceutical significance. The ratio of saturated to unsaturated fatty acid in apricot oil is roughly 13.7% saturated and 86.0% unsaturated (Gupta *et al.*, 2012:366) and the major fatty acids is represented by oleic (58.3 – 73.4%) and linoleic (18.8 – 31.7%) (Alpaslan & Hayta, 2006:470). As mentioned before, fatty acids, especially linoleic acid (a C<sub>18</sub> unsaturated fatty acid), are endogenous to the skin (Kim *et al.*, 2008:373) and as a result, it is considered to have a low probability of inducing skin irritation (Büyüktimkin *et al.*, 1997:433; Menon, 2002:S9; Vermaak *et al.*, 2011:922). In skin care, apricot oil can be used as a non-greasy emollient (moisturiser) where it is appropriate for mature and tired skin (Athar & Nasir, 2005:42). Consequently, apricot oil has been incorporated in a variety of cosmetic products, baby oil, lip balm, moisturising creams and facial scrubs, etc. (Gupta *et al.*, 2012:366). In this study, apricot oil will serve a dual purpose, both as the oil phase in the nano-emulsions and as a penetration enhancer.

### **2.7.2 Nano-emulsions**

The science of nano-sized particles, namely nanotechnology, gained the spotlight rapidly in a variety of areas, including the pharmaceutical sector. In this sector specifically, the advantage of better pharmacological action and more accurate drug design with this technology has presented the possibility of multiple new discoveries. The most beneficent dosage form to come from the use of this technology is nano-emulsions, which presents the prospect to design

delivery systems with enhanced bioavailability, accurate dosing and minimum side effects, therefore an enormous improvement with regard to conventional emulsion systems (Sutradhar & Amin, 2013:97). In comparison with conventional emulsions, nano-emulsions are referred to as systems with a droplet size that fall within the nanometre range. This droplet size range averages from 50 – 200 nm (Tadros *et al.*, 2004:303). In these systems, these droplets (oil or water) are dispersed into the opposite phase and stabilised with the use of an appropriate surfactant (Sutradhar & Amin, 2013:97). The formation of nano-emulsions necessitates energy input, which can be obtained with the use of the chemical potential of the components or generated from mechanical devices (i.e. high-pressure homogenisers, ultrasound generators and high shear stirring) (Uson *et al.*, 2004:415).

The oil droplets in nano-emulsions serve as a reservoir for hydrophobic drugs (Chen *et al.*, 2011:354); therefore, a system both with hydrophilic and lipophilic attributes is created with the use of two-phased dispersed nano-emulsions (Devarajan & Ravichandran, 2011:2). These characteristics can be utilised to enable the delivery of APIs to the stratum corneum (Gaur *et al.*, 2014:37), especially with the formulation of lipophilic drug for transdermal delivery, because of the advantageous high-drug loading capacity, enhanced skin permeation and host tolerance (Chen *et al.*, 2011:354).



**Figure 2.7:** Representation of a nano-emulsion droplet acting as reservoir for lipophilic API (adapted from Kumar & Divya, 2015:273).

Emulsions can be formulated into either o/w or w/o emulsions, depending on the API intended for use. For this study, o/w emulsions will be formulated, thus water will serve as the continuous phase and the oil as the dispersed phase (Chime *et al.*, 2014:77; Kela & Kaur, 2013:9203; Mason *et al.*, 2006:R636). Formulation of o/w nano-emulsions should enhance the absorption as well as the bioavailability of the poor water-soluble drug (Nanjwade *et al.*, 2013:336), which is preferred in the case of the selected lipophilic statins. Research has shown that the most commonly applied oil molecules in the formulation of nano-emulsions include unsaturated and saturated fatty acids, fatty acid esters and soybean oil (Chen *et al.*, 2011:357), which will be dispersed in the water phase as droplets that fall within the nanometre range (Abolmaali *et al.*,

2011:140). When comparing conventional emulsions to nano-emulsions, the distinction can be drawn regarding the droplet size. However, when examining the comparison more closely the aforementioned distinction seems arbitrary, as the small droplet size leads to a considerable reduction in gravity force consequently, the Brownian motion may be adequate to retain sufficient stability against creaming or sedimentation for longer periods (Abolmaali *et al.*, 2011:140; Kela & Kaur, 2013:9203; Mason *et al.*, 2006:R661; Tadros *et al.*, 2004:303).

### **2.7.2.1 Advantages of nano-emulsions**

The kinetic stability of nano-emulsions is due to the droplet size that is obtained within the nanometric range. Consequently, long-standing stability can be achieved with the use of these delivery systems as no apparent flocculation or coalescence is present, thus the system remains dispersed and surface variations are prevented (Tadros *et al.*, 2004:303). The kinetic stability also causes a reduction in the surfactant concentration that is needed in formulation, which is favourable from an industrial viewpoint in numerous areas (Anton *et al.*, 2007:44; Solè *et al.*, 2012:133; Tadros *et al.*, 2004:304). These systems have the capacity to adequately stabilise and dissolve lipophilic APIs, consequently, extended release and activity can be possible due to API molecules being trapped in the inner phase (oil phase) of the nano-emulsion (Abolmaali *et al.*, 2011:147; Chen *et al.*, 2011:357).

The efficient delivery of active ingredient through the skin can be achieved with the use of nano-emulsions because of the versatile characteristics of this delivery system. Firstly, the large surface area and the nanometric droplet size of the emulsion allow rapid and enhanced penetration of APIs. Delivery of the API is further promoted due to the presence of both an oil and water phase in the system. Secondly, the water phase (hydrophilic) acts by hydrating the stratum corneum, thus influencing percutaneous uptake, and the oil phase (lipophilic) in turn interacts with the lipophilic stratum corneum, enabling dissolution and partitioning of the API into the lipids (Gaur *et al.*, 2014:37). In addition to the small droplet size, uniform accumulation of droplets is achieved, when applied topically. The low interfacial tension of o/w droplets in conjunction with the low surface tension of the entire system, can contribute to an improvement where spreading, wetting and penetration is concerned (Tadros *et al.*, 2004:304). With application, the skin feel can be described as aesthetic and pleasant mainly due to the fluid and transparent nature of this system, which is favourable where patient use is concerned. Other factors that could produce an improvement in patient compliance are that nano-emulsions can be administered by the patient, and use can be eliminated at any time. By using these delivery systems, common gastrointestinal effects associated with oral delivery are avoided; hence, these systems create the possibility, that drugs with severe side-effects profiles can be formulated for convenient use by patients (Prakash & Thiagarajam, 2011:5).

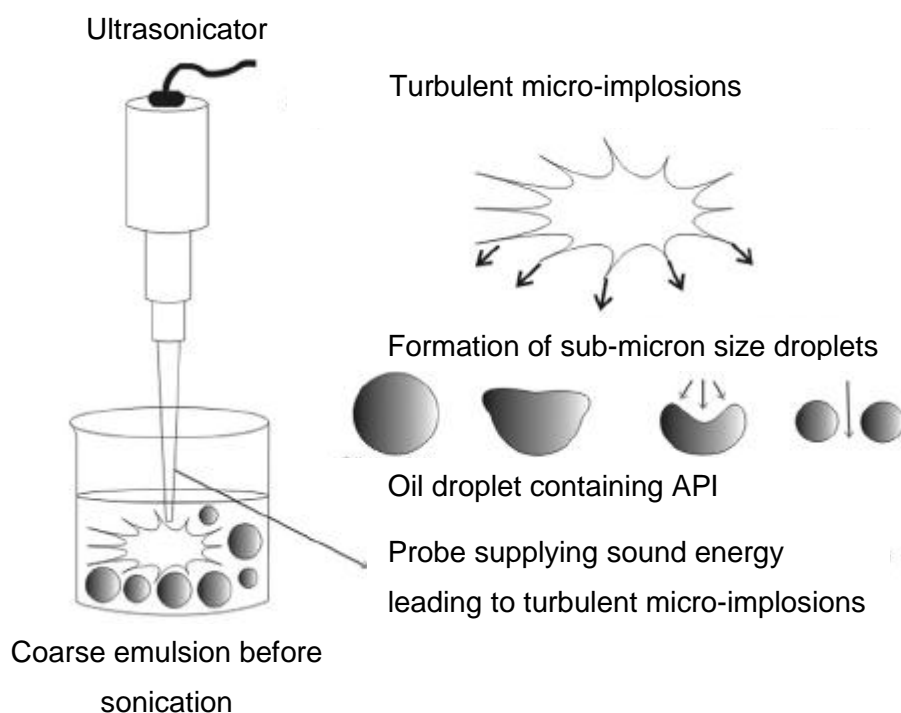
### **2.7.2.2 Disadvantages with the use of nano-emulsions**

Overcoming surface tension by means of adequate disruption force and breaking droplets into the nanometric range can only be achieved by high-energy emulsification methods. Despite the fact that low-energy methods do exist, these methods are not feasible in an industrial viewpoint, because of the high quantity surfactant that is required. High-energy methods come with their own challenges, as mechanical devices used in industrial scale manufacturing, such as high pressure homogenisers, are expensive to obtain and have particularly high energy usage (Kela & Kaur, 2013:9204; Lovelyn & Attama, 2011:635). Aside from the complications with the manufacturing of nano-emulsions, these delivery systems have characteristically low viscosity and consequently, difficulties with the delivery of an API could arise (Ali *et al.*, 2014:1128). In addition to the disadvantages mentioned before, it is known that formulation of a nano-emulsions often require the utilisation of surfactants in high concentrations (Azeem *et al.*, 2009:69), therefore surfactants used during formulation of nano-emulsions should be selected with care, as these compounds can lead to skin reactions (e.g. irritation and inflammation). It is of utmost importance to establish whether the selected surfactant will elicit toxicity towards the skin, as the toxicity between surfactants varies significantly (Lémery *et al.*, 2015:166).

### **2.7.2.3 Methods used in the formulation of nano-emulsions**

When formulating a nano-emulsion there are requirements that must be considered and controlled, i.e. dispersed phase molecules must be insoluble in the continuous phase, component selection particularly regarding surfactants and a surplus of surfactant should preferably be present in the continuous phase. The fourth requirement is that a source of energy or shear is needed to enable rupturing of the droplets into the nano scale (Mason *et al.*, 2006:R645). High- and low-energy emulsification methods can both be utilised to form stable emulsions (Chime *et al.*, 2014:79). High-energy emulsification can be achieved by means of high-pressure homogenisers and ultrasonicators (Prakash & Thiagarajam, 2011:2). With high-pressure homogenisers, nanometric droplet size is achieved through collective forces, such as intense turbulence, cavitation and hydraulic shear (Lovelyn & Attama, 2011:627). This method, however, comes with possible disadvantages, such as component deterioration, poor efficacy and heat generation; this method is also only appropriate in the case of o/w nano-emulsions with an oil phase of less than 20% (Kela & Kaur, 2013:9204). When referring to sonication, the method of emulsification is based on the application of ultrasound energy to cause agitation of particles in the sample. The means by which a nano-emulsion is formed by sonication can be described as a two-step mechanism. Interfacial waves are produced by the acoustic field, which creates instability that ultimately results in the movement of the oil phase as droplets into the water phase. Additionally, the presence of low frequency ultrasound initiates the formation and collapse of microbubbles, due to fluxes in pressure. With each collapse, localised

turbulence is created at an intensely high level. Finally, these turbulent micro-implosions enable the formation of droplets within the sub-micron size (Sutradhar & Amin, 2013:101). Where low-energy is concerned, emulsification or formation of the nano-emulsions is based on the utilisation of inherent energy of the system (Chime *et al.*, 2014:92; Kela & Kaur, 2013:9203; Lovelyn & Attama, 2011:627). The formation of small droplets is influenced by phase behaviour of the selected materials. The three methods that can be classified as low-energy procedures are solvent displacement, phase inversion temperature (PIT) and self-nano-emulsification (Chime *et al.*, 2014:92; Kela & Kaur, 2013:9203). The latter will be applied for the purpose of this study in conjunction with ultrasonication to obtain droplets within the nano scale.



**Figure 2.8** Sonication as a high energy method to obtain a nano-emulsion (adapted from Singh *et al.* (2017:35)).

## 2.8 Semi-solid formulation

The dermal or transdermal administration of nano-emulsions can be improved by transforming these delivery systems into a semi-solid dosage form, which is designed in an approach to deliver nano-emulsions in its intact form over the barrier of the skin (Rai *et al.*, 2018:215). Hence, semi-solid formulations of nano-emulsions will act as a carrier, by increasing contact between the API and the skin (Gupta & Garg, 2002:144), and additionally, easing application to skin due to the increase in viscosity (Mahalingam *et al.*, 2008:267; Pund *et al.*, 2015:152; Williams, 2013:689). Allen *et al.* (2011b:272) stated that a diverse range of semi-solid dosage forms can be formulated (i.e. ointment, creams, and gels), but for the purpose of the study, the gel formulation will be investigated.

Gels can be defined, as semi-solid preparations comprised of a liquid phase within a three-dimensional polymeric matrix, which is formed by the addition of a gelling agent that creates cross-linkage. This group of semi-solids can be divided into hydrophobic and hydrophilic gels. Hydrophobic gels generally consist of a liquid paraffin base, whereas hydrophilic gels consist of a water base (Bora *et al.*, 2014:3595). Gels pose numerous advantages, i.e. uncomplicated formulation, elegance (non-greasy), long-term stability and effortless application (Nabi *et al.*, 2014:41). Despite the advantages of this semi-solid dosage form, disadvantages, such as variations in rheological properties due to environmental factor (i.e. temperature and humidity), sealing of drug within the gel matrix due to covalent bonds present in some gels (Nabi *et al.*, 2014:41) and most importantly the difficulty associated with of incorporating lipophilic APIs into gels (Hyma *et al.*, 2014:2), can result in the consideration of other more suitable semi-solid dosage forms.

### **2.8.1 Emulgel**

The limitation posed by gels for the successful delivery of lipophilic drugs, can be overcome by formulation of an emulgel and in addition, the advantages of gels are still attained (Panwar *et al.*, 2011:333). Emulgels are formed by incorporating a gelling agent within the water phase of an emulsion, hence, emulgels can be viewed as a combined dosage form, consisting of a gel and an emulsion (Panwar *et al.*, 2011:334). Other advantageous characteristics with utilisation of an emulgel, as stated by Yadav *et al.* (2017:15), include:

- improved patient compliance;
- averting hepatic first-pass metabolism;
- enhanced stability and loading capacity,
- low preparation cost, etc.

Although the advantages of emulgels proves to overcome limitation associated with the use of gels, disadvantages of emulgels, such as inadequate permeation of certain APIs through the skin barrier due to large droplet size, pose the most significant limitation for the use of emulgels for transdermal drug delivery (Baibhav *et al.*, 2011:66; Yadav *et al.*, 2017:15). Consequently, nano-emulgels can be formulated, as Jivani *et al.* (2018:1) stated that these delivery systems could result in increased skin permeation.

### **2.8.2 Nano-emulgel**

Limitations or disadvantages associated with nano-emulsions and hydrogel can be overcome by formulation of a nano-emulgel (Sengupta & Chatterjee, 2017:355). In addition, nano-emulgels will increase skin permeability, when compared to emulgels, due to the droplets within the

nanometre range, hence overcoming the most significant limitation of emulgels (Sengupta & Chatterjee, 2017:353). The advantages associated with nano-emulsions (i.e. nano-metric droplets) and gels (i.e. non-greasy nature and spreadability) will be preserved and improved by utilising as nano-emulgels (Basera *et al.*, 2015:1873; Eid *et al.*, 2014:1) and in addition, controlled release can be achieved (Jivani *et al.*, 2018:3; Panwar *et al.*, 2011:337).

Dhawan *et al.* (2014:60) stated that effective permeation can be achieved by means of a nano-emulgel, without the incorporation of chemical enhancers, as the components of the nano-emulsion can act as penetration enhancer (i.e. natural oil). Subsequently, a nano-emulgel will be formulated for the purpose of this study, by utilising the o/w nano-emulsion formulated for each of the selected statins, as this delivery system presents as the most advantageous semi-solid for the transdermal delivery of the selected statins.

## **2.9 Toxicity testing**

Cytotoxicity tests are used as biological evaluation and screening tests that enable the observation of the effects of medical devices on tissue cells. These tests are preferred pilot project tests, as they serve as an essential indicator of toxicity of a medical device and prove advantageous due to the fast, simple and highly sensitive nature of these tests. In addition, the use of animals can be avoided (Li *et al.*, 2015:617). The relevance of these tests on nano-emulsions and semi-solid formulation intended for transdermal or topical use can be explained by the fact the solvents and surfactants used during formulation can influence the cytotoxicity of the preparation (Nemes *et al.*, 2018:1). It should however be stated that both surfactant and other formulation excipients could contribute to toxicity or skin irritation (Lémery *et al.*, 2015:166). Although a variety of cell lines are available, it is suggested that a cell line should be selected that is homologous with the concerned human tissue, hence, 84BR and HaCaT will be appropriate when performing cytotoxicity tests pertaining to the skin (Wiegand & Hiplet, 2009:74).

## **2.10 Conclusion**

FH is an inherited disorder, characterised by the presence of high levels of LDL cholesterol, resulting in an increased risk of early-onset CVD in both men and women. In past years, the prevalence was considered as 1 in 500 persons, although current genetic studies suggest the prevalence to be 1 in 200 - 250. It can also be stated that the statistics differs significantly between populations, as amongst South Africans and French Canadians the prevalence can reach a high of 1 in 100 (Bouhairie & Goldberg, 2015:170). Due to the possible premature onset of CVD in persons with FH, early diagnosis and treatment of FH is essential to limit the occurrence of CVD (Bouhairie & Goldberg, 2015:169). Although statins are considered the first-line or primary therapy for FH (Schaiff *et al.*, 2008:42), side-effects associated with the use of

statins (e.g. hepatotoxicity (Jose *et al.*, 2014:355) and gastrointestinal (Banach *et al.*, 2015:2)) can influence the pharmacological efficacy, because of poor patient compliance (Al-Foraih & Somerset, 2017:36). Consequently, the transdermal delivery of the selected statins can be proposed as an alternative to overcome limitations associated with the oral administration. Transdermal delivery of the selected statins can be advantageous, as avoidance of first-pass metabolism is possible when utilising this route, thus creating the possibility of reduced side effects and better patient compliance (Muntha, 2014:83). Although the skin as a route of administration offers many advantages, the main limitation is posed by the stratum corneum, which is an excellent natural barrier limiting the number of APIs that are able to cross it in amounts adequate to reach as therapeutics plasma concentration (Vitorino *et al.*, 2015:1); this necessitates the selection of an appropriate delivery system to aid in the transdermal delivery of APIs with inadequate physiochemical properties, such as the statins. As mevastatin, lovastatin and simvastatin are more lipophilic statins ( $\log P > 3$ ) and rosuvastatin is more hydrophilic ( $\log P < 1$ ), nano-emulsions pose as potential delivery systems due to their high hydrophilic and lipophilic drug loading capacity. The small droplet size associated with these systems, along with the components (e.g. apricot kernel oil used as oil phase) acting as penetration enhancers, further ascribes to the decision to utilise nano-emulsions for the purpose of this study (Tsai *et al.*, 2014:1). However, semi-solid or nano-emulgels will also be formulated to overcome some of the disadvantages of nano-emulsions (e.g. low viscosity) and subsequently be compared to nano-emulsions.

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## *CHAPTER 3:*

### *Article for the publication in “Die Pharmazie”*

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Chapter 3 was written in article format for publication in “Die Pharmazie”; UK English and Arial 10 were used. This Chapter was justified for the ease of reading. The complete authors’ guidelines are presented in Appendix F.

1 Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag X6001,  
2 Potchefstroom, 2520, South Africa

3 **Development and validation of a method for the determination of lovastatin, mevastatin,**  
4 **rosuvastatin and simvastatin with HPLC by means of gradient elution**

5 **Authors:** Suzanne Marais, Jan L. du Preez, Lissinda H. du Plessis, Jeanetta du Plessis and Minja  
6 Gerber\*

7 \* Corresponding author: Centre of Excellence for Pharmaceutical Sciences (Pharmacem™), North-  
8 West University, Private Bag X6001, Potchefstroom, 2520, South Africa. Tel.: +2718 299 2328; Fax:  
9 +2787 231 5432. E-mail address: [Minja.Gerber@nwu.ac.za](mailto:Minja.Gerber@nwu.ac.za)

10 **Abstract**

11 A novel HPLC method with UV detection was developed and validated to be utilised during *in vitro*  
12 skin penetration studies to identify and quantify lovastatin, mevastatin, rosuvastatin and simvastatin.  
13 A Venusil XBP C18 (2), 150 x 4.6 mm, 5 µm column (Agela Technologies, Newark, DE) was used  
14 with gradient elution (start at 45% acetonitrile and increase linearly to 90% after 1 min; hold at 90%  
15 until 6 min and then re-equilibrate at start conditions), and the mobile phase consisted of **(A)** Milli-Q  
16 water and 0.1% orthophosphoric acid, and **(B)** HPLC grade acetonitrile. The flow rate was set at 1  
17 ml/min, 240 nm UV detection and an injection volume of 10 µl. Linearity was obtained over a range of  
18 0.50 to 200.00 µg/ml and correlation coefficients ranging from 0.998 to 1.000 were obtained. Average  
19 recovery ranged between 95.9 and 100.6%. The LOD and LOQ values obtained from the slope of a  
20 calibration curve and the standard deviation of the response ranged between 0.0138 and 0.0860  
21 µg/ml and 0.0419 and 0.2615 µg/ml respectively, where lovastatin and simvastatin could be detected  
22 at a concentration similar to the other statins, but could only be quantified at a higher concentration  
23 than the remaining statins. The specificity of the method was proved as accurate and quantification of  
24 statins was found, even within the incorporation of other compounds.

25 **1. Introduction**

26 Cholesterol is an essential requirement in a healthy human heart [Ma and Shieh, 2006] and adequate  
27 amount is produced by cells (endogenous cholesterol) to perform its function in the body.  
28 Consequently, elevation in plasma cholesterol can occur with additional dietary intake of cholesterol,  
29 along with other factors, i.e. age, gender and physical inactivity [Iversen et al. 2009] and pre-existing  
30 conditions (i.e. insulin resistance). Familial hypercholesterolemia (FH) is an inherited autosomal  
31 dominant disorder with several gene mutations implicated in the pathogenesis. These mutations vary  
32 in different populations, resulting in a diversity of symptoms and severity between countries [Nemati  
33 and Astaneh, 2010]. This widespread genetic disorder results in a lifelong increase in low-density  
34 lipoprotein (LDL) and a significant increase in the risk of coronary heart disease (CHD). Research  
35 suggests that worldwide as many as 34 million individuals are affected by FH and that every minute a  
36 new individual is born with this condition [Goldberg and Gidding, 2016]. Early diagnosis and  
37 treatment of FH is essential to mitigate the excess risk of premature atherosclerotic cardiovascular  
38 disease [Bouhairie and Goldberg, 2015], which is primarily done with 3-hydroxy-3-methylglutaryl-

39 coenzyme A (HMG-CoA) reductase inhibitors or statins. These agents perform their pharmacological  
40 action primarily by decreasing cholesterol synthesis and further reduction of LDL (20-55%) and  
41 triglycerides (8-30%) occur due to up regulating of the LDL receptor gene. Lastly, inhibition of the  
42 synthesis of apolipoprotein B100 and triglyceride-rich lipoproteins (as well as secretion of last  
43 mentioned) causes a reduction in atherogenic lipoproteins [Amly and Karaman, 2015].

44 Although these lipid lowering agents are generally well tolerated [Black, 2002], side effects such as  
45 hepatotoxicity [Law and Rudnicka, 2006] and gastrointestinal effects (e.g. nausea, vomiting and  
46 abdominal cramps) [Mancini et al. 2013] have been reported with the use of statins. Yu et al. (2018)  
47 found lower adherence can be associated with side-effects of statins, which results in limited  
48 pharmacological efficacy and benefits of statins [Al-Foraih and Somerset, 2017]. As metabolism of  
49 the statins (with the exception of rosuvastatin [Etemad, 2004]) is largely carried out by the cytochrome  
50 P450 (CYP450) family of enzymes, an increased probability for drug interactions can occur when co-  
51 administered with other drugs utilising the same enzymatic pathway [Sica and Gehr, 2002; Muscari et  
52 al. 2002]. These interactions can result in an increased risk of side-effects [Causevic-ramosevac and  
53 Semiz, 2013].

54 Due to the problems and limitations associated with the oral administration of these compounds, the  
55 purpose of this study was to attempt the transdermal delivery of statins to overcome limitations  
56 experienced with oral administration, such as low plasma concentration, hepatic first-pass metabolism  
57 and in addition, gastrointestinal factors and poor patient compliance [Kaestli et al. 2008]. Although  
58 literature suggests the use of HPLC methods for the analysis of statins, *in vitro* skin permeation  
59 studies will require the method to be sensitive enough to detect extremely low drug concentrations.  
60 Consequently, a new method was developed, as no methods for the purpose of transdermal delivery  
61 of lovastatin, mevastatin, rosuvastatin and simvastatin was available in literature.

## 62 **2. Investigations, results and discussion**

63 The validation of this method was done by means of linearity, accuracy, precision (both inter-day and  
64 intra-day), ruggedness (sample stability and system repeatability), specificity, LOD (lowest limit of  
65 detection) and LOQ (lowest limit of quantification). Table 1 represents the validation parameter  
66 obtained during the validation of this method.

67 The linearity of the selected statins was done by preparing a stock solution ( $\pm 20$  mg in 100 ml  
68 methanol (200  $\mu\text{g/ml}$ )) for each of the statins. This stock solution was then utilised to prepare dilution  
69 1 ( $\pm 20$   $\mu\text{g/ml}$ ) and dilution 2 ( $\pm 2$   $\mu\text{g/ml}$ ). The stock solution along with the dilutions were then  
70 transferred into HPLC vials and analysed (in duplicate) at injection volume 2.5  $\mu\text{l}$ , 5.0  $\mu\text{l}$ , 7.5  $\mu\text{l}$  and  
71 10.0  $\mu\text{l}$ . By using this method, different concentration ranges were obtained (Table 1). The  
72 regression equations obtained from plotting peak area versus statin concentration are displayed in  
73 Table 1.

74 When observing the correlation coefficient ( $R^2$ ) displayed in Table 1, it was concluded that an  
75 excellent correlation between the peak area and the statins concentration was obtained within the  
76 tested concentration range. The recovery of the experiments was used to determine the accuracy of

77 the proposed method. Lovastatin reached an average recovery of 99.7% (%RSD = 0.3), mevastatin  
78 98.6% (%RSD = 0.2), rosuvastatin 95.9% (%RSD = 0.3) and simvastatin 100.6% (%RSD = 0.4). The  
79 percentage recovery is required to be within 98 to 102%, as this percentage is seen as a ratio  
80 between the estimated results and the obtained results, thus serving as an indicator of the accuracy  
81 of the methods (APVMA, 2004:5). Shabir (2005:9) also stated that 90-100% could serve as  
82 acceptable recovery range for APIs over a target concentration range of 80-100%. In addition,  
83 according to UNDOC (2009:12) recovery is not necessitated to be 100%, but rather to be consistent  
84 as displayed by the %RSD values. Therefore, it can be concluded that the method was accurate as  
85 recovery of the selected statins fell within the required limits. Calculations of LOD and LOQ were  
86 based on the slope of a calibration curve and the standard deviation of the response by utilising the  
87 formulas displayed in Table 1 for the LOD and LOQ, respectively [Swartz and Krull, 2012]. The  
88 sample was prepared by weighing  $\pm 5$  mg of each selected statin into a 100 ml volumetric flask, made  
89 up to volume with methanol, where after 1 ml of the stock solution was transferred to another 100 ml  
90 volumetric flask and made up to volume with methanol. A volume of the dilution was then placed in a  
91 HPLC vial and injected six times at injection volumes of 2.5  $\mu$ l, 5.0  $\mu$ l, 7.5  $\mu$ l and 10.0  $\mu$ l.

92 Precision for this analytical method was examined firstly, in terms of intra-day precision (repeatability)  
93 and secondly, according to inter-day precision (reproducibility). Triplicate samples were prepared of  
94  $\pm 160$   $\mu$ g/ml, 200  $\mu$ g/ml and 240  $\mu$ g/ml for each of the statins. From intra-day precision (repeatability),  
95 these samples were analysed in duplicate. The acceptance criteria for intra-day precision are  
96 considered to be a %RSD equal or less than 2.00% [APVMA 2004; Snyder et al. 1997b], and  
97 therefore, it could be concluded that each of the selected statins displayed satisfactory results (%RSD  
98  $\leq 2\%$ ) as displayed in Table 1.

99 The samples used to determine inter-day precision (reproducibility) were prepared similarly to those  
100 used for intra-day precision, except for this assay only the intermediate concentration (200  $\mu$ g/ml) was  
101 utilised. These three intermediate concentration samples were analysed in duplicate. By observing  
102 Table 1, it can be stated that the statins present with acceptable %RSD values within the acceptance  
103 criteria of equal or less than 3.00% [Rafael et al. 2007]. The sample stability (over 24 h) and system  
104 repeatability was also determined. Sample stability was performed by injecting a standard ( $\pm 20$  mg in  
105 100 ml methanol) hourly for 24 h. This analysis is performed so that the sample solution will not be  
106 used for a period longer than it takes to degrade by 2%. System repeatability was done by injecting  
107 the standard sample (as prepared for 24 h) seven consecutive times to evaluate the repeatability of  
108 peak areas, as well as retention times of samples under same day conditions.

109 As the acceptance criteria for sample stability is considered a %RSD of equal or less than 2% [Shabir,  
110 2003; Suresh et al. 2010] and that of system repeatability less than 1% [Shabir 2004], it can be  
111 concluded from Table 1 that the statins met the criteria from both the percentage recovery and peak  
112 area, as seen for sample stability, and retention time and peak area as displayed by system  
113 repeatability (Table 1). After completion of sample stability and system repeatability, specificity of the  
114 analytical method was determined.

115 Specificity of an analytical method can be defined as the ability of the method to accurately detect an  
116 analyte in the presence of degradants, the matrix or impurities [Chandran and Singh, 2007].  
117 Acceptable specificity can be seen as the pure identification of the target analyte in the presence of  
118 similarly structured compounds [ICH 2005], therefore, there should be no peak interference with the  
119 retention time of the API [Snyders et al. 1997].

120 Figures 1A-D indicates that additional peaks formed as a result of degradation caused by stressing  
121 the sample, however the respective statins peak retention times were not affected:

- 122 • Lovastatin:  $\pm 5.17$
- 123 • Mevastatin:  $\pm 4.75$
- 124 • Simvastatin:  $\pm 4.87$
- 125 • Rosuvastatin:  $\pm 3.09$

126 After completing the validation of the analytical method, the method was utilised during skin diffusion  
127 studies for analysis of diffusion (buffer phase), tape stripping and skin samples.

128 These chromatograms were obtained during skin diffusion studies performed on the respective  
129 statins. From Figures 2.A-D it is clear that the peak of the tape stripping, skin and buffer (receptor  
130 phase) samples, displayed the same retention time as the standard solutions. The peak visible to the  
131 right in (c) of Figures 2.A-C and to the left of Figure 2.D is due to the 3M Scotch® Magic™ Tape used  
132 during tape stripping.

### 133 **3. Experimental**

134 A Dionex UltiMate 3000 dual system was used during this study, which consisted of ternary gradient  
135 pumps, column ovens, auto sampler and diode array detectors operated on Chromeleon 7.2 data  
136 acquisition and analysis software (Thermo Fisher Scientific Inc., Waltham, MA). A Venusil XBP C<sub>18</sub>  
137 (2), (150 x 4.6 mm, 5  $\mu$ m) (Agela Technologies, Newark, DE) column was used for this method.  
138 Gradient elution was used with mobile phase A **(A)** consisting of Milli-Q water and 0.1%  
139 orthophosphoric acid and mobile phase B **(B)** consisting of HPLC grade acetonitrile. The flow rate  
140 was set to 1 ml/min at 45% acetonitrile, to increase linearly to 90% after 1 min, where it was held at  
141 90% for 6 min and then re-equilibrated at start conditions (45%). The default injection volume was set  
142 at 10  $\mu$ l and the UV detector was set at 240 nm. The retention times were  $\pm 3.090$ , 4.673, 4.867, and  
143 5.140 min for rosuvastatin, mevastatin, simvastatin and lovastatin, respectively, with a run time of 10  
144 min. Methanol was utilised as a solvent during the course of the method validation.

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150

151 **Disclaimer**

152 Any opinion, findings and conclusions, or recommendations expressed in this material are those of  
153 the authors and therefore the NRF does not accept any liability in regard thereto. The authors declare  
154 no conflict of interest.

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178 [/Guidelines/Quality/Q2\\_R1/Step4/Q2\\_R1\\_\\_Guideline.pdf](http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf) Date of access: 21 Nov. 2017.

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206 [Validation%20and%20Protocol%20in%20the%20Quality%20System%20Compliance%20Industry.pdf](http://www.ivtnetwork.com/sites/default/files/Step-byStep%20Analytical%20Methods%20Validation%20and%20Protocol%20in%20the%20Quality%20System%20Compliance%20Industry.pdf)  
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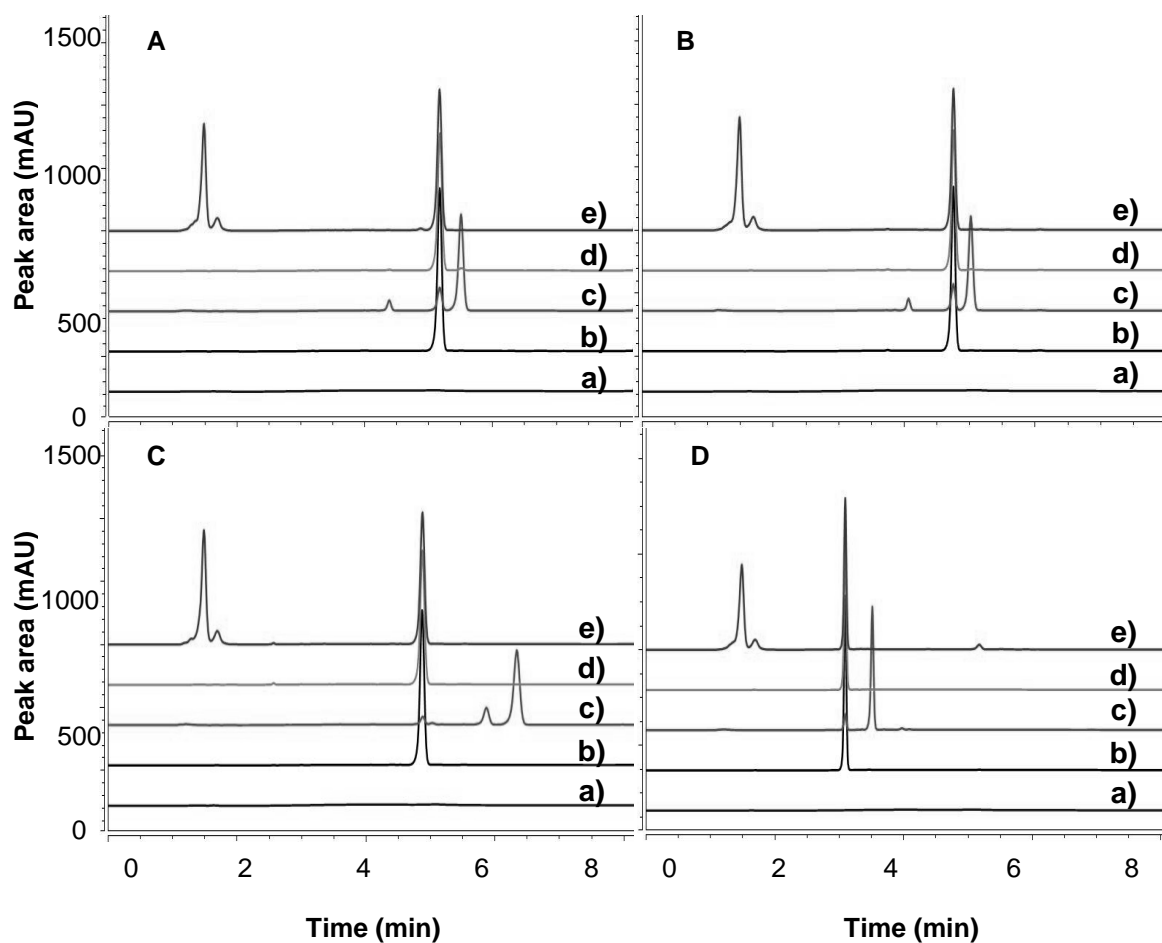
**Tables:**

**Table 1:** Validation parameters obtained for the four statins

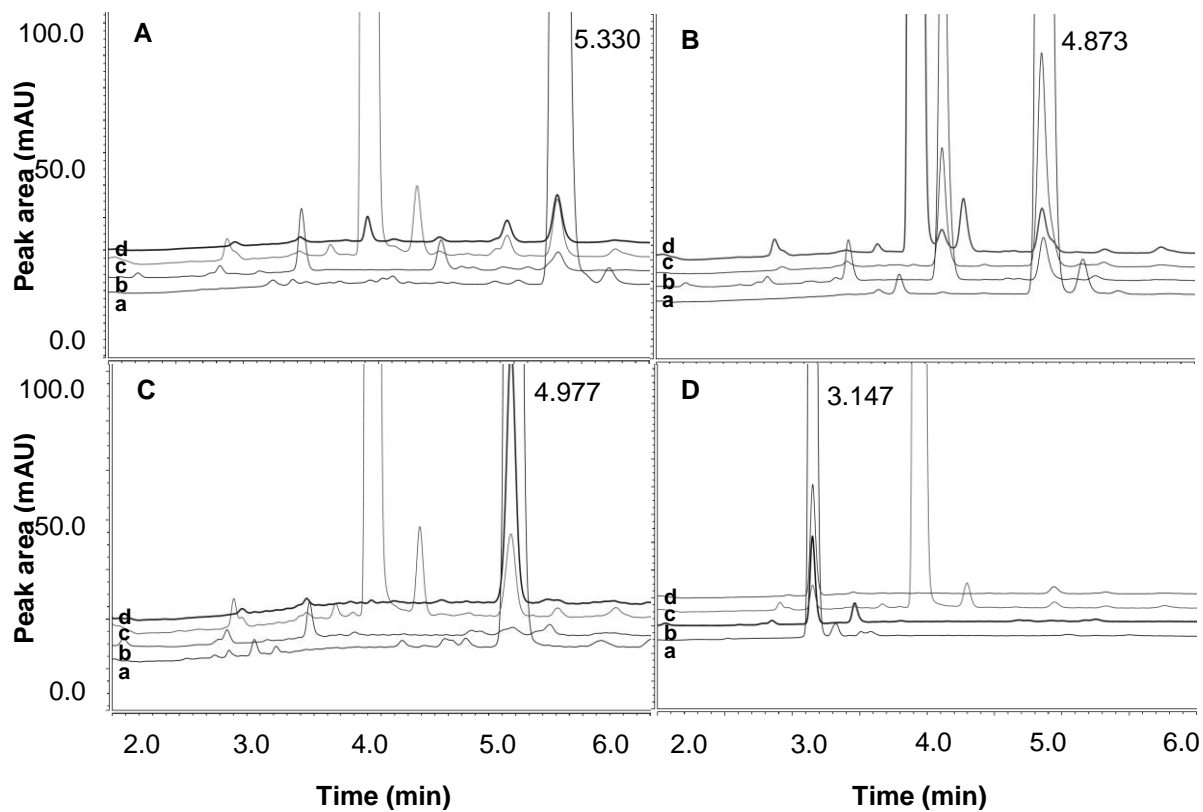
<b>Linearity</b>				
	<b>Lovastatin</b>	<b>Mevastatin</b>	<b>Rosuvastatin</b>	<b>Simvastatin</b>
<b>Concentration range (µg/ml)</b>	0.480-191.900	0.624-249.500	0.488-195.300	0.547-218.900
<b>Regression equation</b>	$y = 0.4916x + 0.0927$	$y = 0.3772x + 0.1555$	$y = 0.4159x - 0.0234$	$y = 0.4304x + 0.1386$
<b>Correlation coefficient (R<sup>2</sup>)</b>	1	1	1	0.9998
<b>LOD and LOQ</b>				
<b>LOD (µg/ml)</b> DL (detection limit) = 3.3 X $\sigma$ /S	0.0806	0.0148	0.0863	0.0138
<b>LOQ (µg/ml)</b> QL (quantification limit) = 10 X $\sigma$ /S	0.2441	0.0447	0.2615	0.0419
<b>Accuracy</b>				
<b>Mean recovery (%)</b>	99.7	98.6	95.9	100.6
<b>% RSD</b>	0.3	0.2	0.3	0.4
<b>Precision</b>				
<b>Intra-day precision (%RSD)</b>	0.73	0.70	0.79	0.58
<b>Inter-day precision (%RSD)</b>	2.72	2.48	2.65	1.12
<b>Sample stability over 24 h</b>				
<b>Percentage recovery (%RSD)</b>	0.99	1.23	0.61	0.74
<b>Peak area (%RSD)</b>	0.99	1.24	0.61	0.74
<b>System repeatability</b>				
<b>Retention time (%RSD)</b>	0.047	0.035	0.00	0.00
<b>Peak area (%RSD)</b>	0.92	0.10	0.12	0.08

( $\sigma$  = Standard deviation of y-intercepts, S = Slope, RSD = relative standard deviation)

Figures:



**Fig. 1:** HPLC chromatogram showing specificity data obtained: A) lovastatin, B) mevastatin, C) simvastatin and D) rosuvastatin. In addition for a) placebo solution, b) statin standard solution, following the sample solution of respective statin stressed with 200  $\mu$ l of c) HCl, d) H<sub>2</sub>O and e) H<sub>2</sub>O<sub>2</sub>



**Fig. 2:** Chromatographic representation of A) lovastatin, B) mevastatin, C) simvastatin and D) rosuvastatin. Chromatograms represents a) standard solution sample of respective statins, b) buffer (receptor phase) extraction sample, c) tape stripping sample of the statin and d) skin sample of the statin.

## *CHAPTER 4:*

### *Article for the publication in the International Journal of Pharmaceutics*

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This Chapter is written in article form for publication in The International Journal of Pharmaceutics. For the ease of reading the paragraphs of this chapter have been justified. The author's guide states that the article can be written in either UK or US English, and the authors decided to write in UK English. The complete author's guide is listed in Appendix G.

# **Nano-emulsions and nano-emulgels for transdermal delivery of statins**

Suzanne Marais, Jeanetta du Plessis, Lissinda du Plessis and Minja Gerber\*

Centre of Excellence for Pharmaceutical Sciences, North-West University, Private Bag  
X6001, Potchefstroom 2520, South Africa

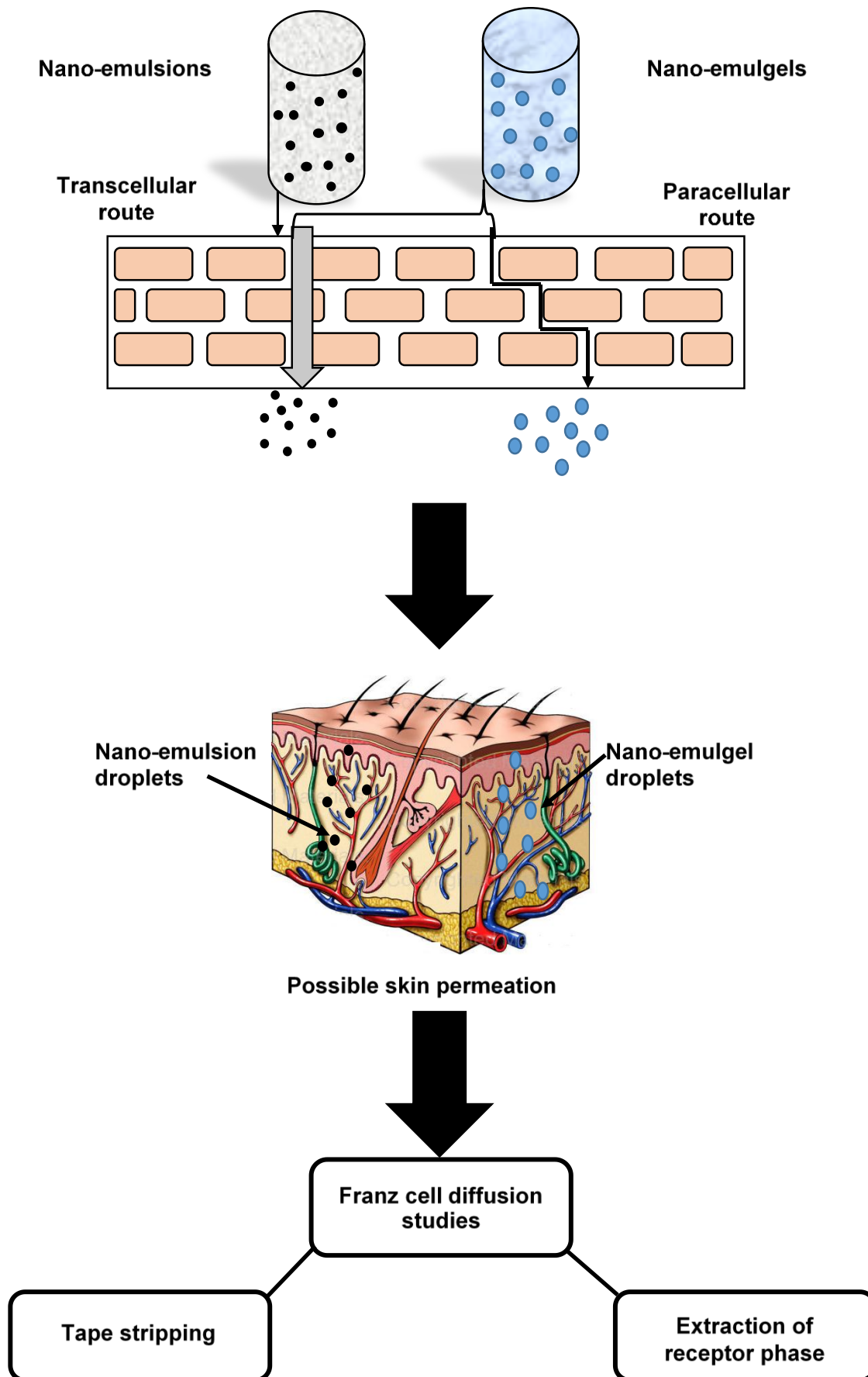
\* Corresponding author. Tel.: +2718 299 2328; Fax: +2787 231 5432. E-mail address:  
[Minja.Gerber@nwu.ac.za](mailto:Minja.Gerber@nwu.ac.za) (M Gerber).

## **Abstract**

Nano-emulsions and nano-emulgels were used as delivery systems to optimise the transdermal delivery of lovastatin, mevastatin, rosuvastatin and simvastatin. These delivery systems were tested, as it is suggested that the transdermal delivery of both lipophilic and hydrophilic active pharmaceutical ingredients (APIs) can be improved by utilising nano-emulsions and nano-emulgels as delivery vehicles. The aim of this study was therefore to formulate a nano-emulsion and nano-emulgel each containing 2% of the selected statins respectively, and determining which of the statins proved more favourable for transdermal delivery, and additionally, which vehicle proved most advantageous. During the membrane release studies, the highest flux was obtained by the nano-emulsion and nano-emulgel containing rosuvastatin. From the 12 h Franz cell diffusion studies, it was concluded that nano-emulgel containing simvastatin ( $3.244 \mu\text{g}/\text{cm}^2$ ) obtained the highest median amount per area diffused. Tape stripping followed, where the nano-emulgel containing simvastatin displayed the highest median concentration ( $7.517 \mu\text{g}/\text{ml}$ ) within the stratum corneum-epidermis, while the nano-emulsion containing simvastatin reached the highest median concentration within the epidermis-dermis. Although transdermal delivery was achieved with all of the respective formulas, it can be proposed that during this study, transdermal delivery of the statins was enhanced by the nano-emulgels with the exception of rosuvastatin.

*Keywords:* Statins, Transdermal delivery, Nano-emulsion, Nano-emulgel, Franz cell

# Graphical Abstract



## 1 **1 Introduction**

2 Familial hypercholesterolemia, a widespread genetic disorder, is associated with high blood  
3 cholesterol levels, which is most frequently caused by mutations in the low density  
4 lipoprotein receptor gene (LDLR) (Benito-Vicente *et al.*, 2018; Migliara *et al.*, 2017); other  
5 mutation in genes related to the low density lipoprotein (LDL) clearance, such as  
6 apolipoprotein B-100 (Apo B) and proprotein convertase subtilisin/kexin type 9 (PCSK9), can  
7 result in familial hypercholesterolemia (Alonso *et al.*, 2018). Consequently, high levels of  
8 LDL is associated with this receptor mutation (LDLR), which causes a disruption in normal  
9 cholesterol metabolism, and could result in early occurrence of cardiovascular disease  
10 (CVD) (Ravnskov *et al.*, 2018). Although the use of inhibitors of 3-hydroxy-3-methylglutaryl  
11 coenzyme A (HMG-CoA) reductase (statins) is considered the most applicable therapy to  
12 reduce serum cholesterol levels (Barale *et al.*, 2018), some adverse effects associated with  
13 the use of these drugs, resulted in patients discontinuing therapy, without proper medical  
14 consultation, and consequently increased the risk of CVD (Toth *et al.*, 2018). An increased  
15 risk of statins side-effects can be associated with first-pass metabolism by cytochrome P450  
16 (CYP450) enzymes, which is responsible for the metabolism of all statins (with the exception  
17 of pravastatin), consequently increased levels of statins can occur with an inhibition of these  
18 enzymes (Ramkumar *et al.*, 2016). Other side-effects, such as nausea, can present due to  
19 patients' inability to tolerate the dose required to reduce risk of CVD (Banach *et al.*, 2015).  
20 Therefore, considering an alternative route of administration, such as transdermal delivery,  
21 can prove beneficial in reducing side-effects, as this route avoids hepatic first-pass  
22 metabolism (thus unaffected by CYP450), and additionally requires a lower dose to be  
23 administered (N'Da, 2014).

24 The skin, covering an area of about  $2\text{m}^2$  in an average human adult, is considered the  
25 largest organ of the body (Raza *et al.*, 2015), acting as an effective barrier (Jassim *et al.*,  
26 2018). Although the skin is comprised of various layers, the most important barrier is posed  
27 by the outermost stratum corneum against exogenous chemical and physical effects, as well  
28 as dehydration. Subsequently, it can be proposed that the stratum corneum is the rate-

29 limiting step in percutaneous absorption, thus determining the systemic bioavailability of  
30 topically applied active pharmaceutical ingredients (APIs) (Jacques-Jamin *et al.*, 2017). To  
31 overcome this relatively impermeable barrier, the physiochemical properties of the API  
32 intended for transdermal delivery, should be evaluated, as such candidates are required to  
33 meet certain criteria (Szunerits & Boukherroub, 2018). These criteria entail a molecular  
34 mass of <500 Da, aqueous solubility of >1 mg/ml, log P of 1 to 3, and melting point of  
35 <200°C (Naik *et al.*, 2000). APIs meeting all these requirements can be considered ideal for  
36 transdermal delivery; however, very few APIs possess these ideal properties, and so several  
37 methods have been proposed to enhance transdermal delivery of non-ideal APIs (Dodou,  
38 2012). Formulation of a nano-emulsion can be considered such a method, as research  
39 suggests that nano-emulsions will enhance delivery of an API across the skin, compared to  
40 conventional formulation (Tsai *et al.*, 2014). The more efficient delivery of nano-emulsions  
41 can be ascribed to the excipients used in the formulation, such as surfactant, co-surfactant  
42 and oils, which can alter the lipid structure of and fluidity of the stratum corneum,  
43 subsequently acting as penetration enhancers (Su *et al.*, 2017). In addition, the nano-sized  
44 droplets of these systems can further contribute to the effective systemic delivery of the API,  
45 due to easier penetration of the skin (Chaudhary *et al.*, 2018). Nano-emulsions are  
46 considered to have a droplet size ranging from 20 to 200 nm (Ee *et al.*, 2008). Although  
47 nano-emulsions pose numerous other advantages (e.g. abstaining from flocculation and  
48 coalescence, and kinetic stability) (Roberts *et al.*, 2017), the major disadvantage is the low  
49 viscosity of these systems (Ali *et al.*, 2014), which can cause difficulty with application. This  
50 limitation can however be overcome, by incorporating a nano-emulsion with a gel, forming a  
51 nano-emulgel, which will improve the already advantageous nature of nano-emulsion (e.g.  
52 high solubilisation capacity), and additionally, enhance the stability of the formulation by  
53 increasing viscosity of the aqueous phase and reducing interfacial tension (Eid *et al.*, 2014).  
54 The aim of this study was thus to facilitate transdermal delivery by formulating a nano-  
55 emulsion and nano-emulgel containing lovastatin, mevastatin, rosuvastatin and simvastatin.  
56 Furthermore, it is important to determine whether a sufficient amount of the respective statin

57 was delivered to exhibit a therapeutic effect. Moderate-intensity statin therapy (lowering LDL  
58 by 30 to <50%) is considered an oral daily dose of 20 to 40 mg simvastatin, 5 to 10 mg  
59 rosuvastatin, and 80 mg of lovastatin (Karlson *et al.*, 2016). By administering these doses, a  
60 mean plasma concentration of 0.001 to 0.002 µg/ml will be reach for simvastatin, 0.003  
61 µg/ml for lovastatin (Björkhem-Bergman *et al.*, 2011), while rosuvastatin will attain a mean  
62 plasma concentration of approximately 0.001 µg/ml (DeGorter *et al.*, 2013). The mean  
63 plasma concentration was used rather than peak concentration ( $C_{max}$ ) in the aforementioned  
64 studies, since the peak concentration is significantly high compared to the mean plasma  
65 concentration, and will only be present for a short time (Björkhem-Bergman *et al.*, 2011),  
66 while *in vitro* skin diffusion studies will be conducted over 12 h. No data could be obtained  
67 on mevastatin.

## 68 **2 Materials and Methods**

### 69 **2.1 Materials**

70 Lovastatin, mevastatin, rosuvastatin calcium and simvastatin ammonium were obtained from  
71 DB Fine (Johannesburg, RSA). Apricot kernel oil was obtained from CJP Chemicals  
72 (Johannesburg, RSA), while Tween<sup>®</sup> 80, Span<sup>®</sup> 60, Carbopol<sup>®</sup> Ultrez 20 and analytical grade  
73 methanol were obtained from Sigma Aldrich (Johannesburg, RSA). Dihydrogen  
74 orthophosphate (KH<sub>2</sub>PO<sub>4</sub>) and sodium hydroxide (NaOH) used in the preparation of the  
75 phosphate buffered solution (PBS) was obtained from Sigma-Aldrich (Johannesburg, RSA).  
76 During this study, deionised high performance liquid chromatography (HPLC) grade water  
77 (Millipore, Milford, USA) was used.

### 78 **2.2 Methods**

#### 79 **2.2.1 Formulation of nano-emulsions and nano-emulgels**

80 During this study, eight formulas were developed containing the respective statins (2%). For  
81 each of the selected statins, an o/w nano-emulsion and nano-emulgel (semi-solid) was  
82 formulated. The nano-emulsions were formulated by firstly dissolving the excipients in the  
83 respective phase on a hot plate with a magnetic stirrer ( $\pm 40^{\circ}\text{C}$ ), Tween<sup>®</sup> 80 within the water  
84 phase and Span<sup>®</sup> 60 along with the respective statins within the oil phase (apricot kernel oil).

85 After the excipient within the respective phases was properly dissolved, the oil phase was  
86 added to the water phase in a drop wise manner, resulting in a coarse emulsion that was left  
87 to stir on the hotplate for another 5 min. To obtain a nano-emulsion, ultrasonication as a  
88 high-energy method was applied, by sonicating the dispersion for 3 min with 1 min intervals  
89 with an ultrasonic probe. The formulation of the nano-emulgels were performed slightly  
90 different, with the addition of Carbopol® Ultrez 20 to the water phase and the time used to  
91 dissolve the excipients within the respective phases was adjusted to 10 min, which ensured  
92 proper dissolution of the gelling agent in the water phase. Thereafter, before adding the oil  
93 phase to the water phase, the ultrasonicator (for  $\pm 2$  min) was used to free the water phase of  
94 any entrapped air due to the addition of the gelling agent (Carbopol® Ultrez 20). The beaker  
95 containing the water phase was then placed under an overhead mechanical stirrer at  $\pm 850$   
96 rpm, while the oil phase was added in a drop-wise manner. After addition of the oil phase,  
97 the formulation was left to stir for another 15 min to ensure proper mixing of the two phases.  
98 This results in a formulation with a higher viscosity than a nano-emulsion, but in such a  
99 manner that ultrasonication ( $\pm 3$  min) could still be applied successfully to obtain droplets  
100 within the nano-metric range. The final step to obtain a nano-emulgel was to adjust the pH  
101 of the formulations to that of their respective nano-emulsions. Adjustment of the pH is an  
102 essential step during the formulation of nano-emulgels; firstly, due to the fact that Carbopol®  
103 gels will only achieve optimum viscosity after neutralisation (Shin *et al.*, 2000) and secondly,  
104 due to the low pH of these formulations (2.5 to 3.5) before neutralisation of Carbopol® Ultrez  
105 20 (Lubrizol, 2009), consequently adjustment of pH ensures safe application to the skin (Nair  
106 *et al.*, 2013).

107 Table 1:

108 Ingredients used during the formulation of the nano-emulsions and the nano-emulgels

109

110 After formulation the nano-emulsions containing the respective statins were referred to as  
111 **(NEL1)** (lovastatin nano-emulsion), **(NEM1)** (mevastatin nano-emulsion), **(NER1)**  
112 (rosuvastatin nano-emulsion) and **(NES1)** (simvastatin nano-emulsion). The nano-emulgels

113 will be referred to as **(NEGL)** (lovastatin nano-emulgel), **(NEGM)** (mevastatin nano-emulgel),  
114 **(NEGR)** (rosuvastatin nano-emulgel) and **(NEGS)** (simvastatin nano-emulgel).

### 115 **2.2.2 Analysis of mevastatin, lovastatin, rosuvastatin and simvastatin**

116 An HPLC method was developed and validated for the determination of the selected statins.  
117 The Dionex UltiMate 3000 dual system with ternary gradient pumps, column ovens, auto  
118 sampler and diode array detectors operated on Chromeleon 7.2 data acquisition and  
119 analysis software (Thermo Fisher Scientific Inc., Waltham, MA) were used as HPLC. A  
120 Venusil XBP C18(2), 150 x 4.6 mm, 5 µm (Agela Technologies, Newark, DE) column was  
121 utilised and the UV detector was set at a wavelength of 240 nm, the flow rate at 1.0 ml/ml  
122 and default injection volume at 10 µl. The mobile phase comprised of acetonitrile and water  
123 with 0.1% orthophosphoric acid. Gradient elution was utilised by starting at 45% acetonitrile,  
124 while increasing linearly to 90% after 1 min, then holding it at 90% until 6 min and thereafter  
125 re-equilibration took place at start conditions (total runtime of 10 min). The retention time  
126 was ±3.090, 4.673, 4.867 and 5.140 min for rosuvastatin, mevastatin, simvastatin and  
127 lovastatin, respectively; methanol was used as a solvent. Table 2 illustrates the LOD (limit of  
128 detection) and LOQ (limit of quantification) of the statins.

129 Table 2

130 Lowest limit of detection (LOD) and lowest limit of quantification (LOQ) of statins

131

132 All analytical tests performed during this study were in a controlled temperature of 25°C.

### 133 **2.2.3 Standard preparation**

134 The standard preparation was done by firstly weighing approximately 20 mg of each statin  
135 into a 100 ml volumetric flask respectively, where after it was made up to volume with  
136 methanol. This solution was then used to prepare an additional two dilutions in order to  
137 obtain a standard range of 0.5-200.0 µg/ml. The standard solution, along with the two  
138 dilutions, were each injected at different volumes (2.5, 5.0, 7.5, 10.0 µl) to obtain a standard  
139 curve.

140

## 141 **2.2.4 Physicochemical properties**

### 142 **2.2.4.1 Aqueous solubility**

143 The experiment was conducted by firstly pre-heating a water bath equipped with a rotating  
144 device (to ensure proper mixing) to 32°C, which resembles *in vivo* human conditions.  
145 Thereafter, three clean glass tubes (triplicate) were filled with 5 ml PBS (pH 7.4), and an  
146 additional three tubes (triplicate) were filled with PBS:ethanol (9:1). An excess amount of  
147 the selected statin was placed in each of the six tubes in order to obtain a saturated solution,  
148 where after the samples were placed in the preheated water bath and left to rotate for 24 h.  
149 The samples were examined regularly during the course of the test, to ensure the sample  
150 remained saturated. After 24 h, the samples were removed and centrifuged (4500 rpm for  
151 15 min). After centrifugation was completed, each respective sample was extracted from the  
152 tube by means of a syringe and filtered through a 0.45 µm polytetrafluoroethylene (PTFE)  
153 filter into an HPLC vial to ensure that no undissolved particles reached the HPLC. Each of  
154 the respective samples were then analysed in duplicate. It is important to note that this  
155 process was performed for each of the statins (lovastatin, mevastatin, rosuvastatin and  
156 simvastatin).

### 157 **2.2.4.2 Octanol-buffer distribution coefficient (log D)**

158 Before the log D values of the respective statins could be determined by means of the shake  
159 flask method, the solubility of the statins within *n*-octanol was determined. Performing a  
160 solubility test provided information with regard to the amount of statin that should be used  
161 during the determination of log D. This test was performed similar to the method utilised in  
162 Section 2.2.4.1 (aqueous solubility). A dilution was prepared after removal of the test tubes  
163 for the centrifuge, by placing 1 ml (of test tube sample) in a 25 ml volumetric flask, where  
164 after it was made up to volume with methanol to ensure the safety of the vials for HPLC  
165 injection. After solubility results were obtained, equal volumes (100 µl) of PBS (pH 7.4) and  
166 *n*-octanol were placed in a separating funnel to equilibrate for 24 h, ensuring co-saturating of  
167 the two phases. It was determined (by means of the *n*-octanol solubility tests) that 40 mg of  
168 mevastatin and lovastatin, respectively, would be placed in beakers containing 20 ml of the

169 pre-saturated *n*-octanol, whereas due to weaker solubility only 4 mg of simvastatin and  
170 rosuvastatin would be added to their respective beakers. After proper dissolution, 3 ml of  
171 respective pre-saturated *n*-octanol/statin solutions were placed in three different test tubes,  
172 to which 3 ml of the pre-saturated PBS (pH 7.4) was added respectively. The samples (test  
173 tubes) were then placed in the preheated water bath (32°C) and left to rotate overnight  
174 (approximately 8 h). After removal, 1 ml of the octanol phase from each of the test tubes  
175 (triplicates) was transferred to a 10 ml volumetric flask and made up to volume with  
176 methanol to dilute the *n*-octanol and ensure safety of injections on the HPLC. An amount of  
177 each volumetric flask was then placed in a HPLC vial. The PBS (pH 7.4) phase was  
178 extracted without dilution and placed in a HPLC vial. All the respective vials were then  
179 analysed by means of HPLC, to determine the concentration of the respective statins. After  
180 HPLC analysis, the log D values could be determined by utilising the logarithmic ratio of the  
181 concentration statin in *n*-octanol phase and concentration in PBS phase. Note that this  
182 process was performed for each of the respective statins.

### 183 **2.3 Characterisation of pravastatin formulations**

184 The eight formulas were characterised in terms of pH, droplet size and zeta-potential, and  
185 viscosity. Additionally, transmission electron microscopy (TEM) was performed on the  
186 respective nano-emulsions.

#### 187 **2.3.1 TEM**

188 A FEI Tecnai G2 20S-Twin 200 kV high-resolution transmission electron microscope  
189 (HRTEM) (Czech Republic, EU) with an Oxford INCA X-Sight EDS System was used to  
190 obtain micrographs of the respective nano-emulsions. A dilution (of the nano-emulsions)  
191 was made with Milli-Q® water, where after a small volume of each of the respective dilutions  
192 was placed on a microscopic carbon-coated 300 mesh copper grid, by means of a  
193 micropipette and left to dry for 10 min (excess removed with filter paper). Osmium tetroxide  
194 was used to stain samples.

195

196 **2.3.2 pH**

197 A Mettler Toledo® pH meter (Mettler Toledo, CU) equipped with a Mettler Toledo® InLab®  
198 410 electrode (Mettler Toledo, CU) was utilised to acquire these values. Calibration of the  
199 apparatus was performed before triplicate measurements took place at 32°C.

200 **2.3.3 Viscosity**

201 A Brookfield Viscometer DV2T LV Ultra (Middleboro, Massachusetts, USA), connected to a  
202 thermostatic water bath was used to acclimatise the formulas to  $\pm 25^{\circ}\text{C}$ , by placing the  
203 formulation in the bath prior to the test. Thereafter, the viscosity was determined by  
204 immersing the specific spindle into the formulas (or sample chamber in the case of nano-  
205 emulsions) and measuring the resistance to the rotating spindle, which turns at a specific  
206 rate set prior to the test (measured in rpm). Additionally, it was specified that measurements  
207 should be taken every 10 sec for 3 min; consequently, 18 readings were obtained and an  
208 average viscosity could be determined.

209 **2.3.4 Droplet size**

210 A Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) was utilised to  
211 establish the droplet size of the eight formulas. Three readings were taken from the eight  
212 freshly prepared samples, which were diluted (1 ml with 20 ml of Milli-Q® water). These  
213 dilutions were analysed by placing 2 ml in a clear disposable zeta-cell (DTS1070 folded  
214 capillary cell), where after an average droplet size could be determined.

215 **2.3.5 Zeta-potential**

216 A Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) was utilised to  
217 determine the zeta-potential of the eight formulas. Triplicate measurements were taken  
218 between 3.5 to 65.5 rpm over a period of 300 sec at room temperature. Samples were  
219 prepared utilising the method as described in Section 2.3.4 and placed in a clear disposable  
220 zeta-cell (DTS1070 folded capillary cell). Approximately 30 readings were taken of each  
221 formula and an average zeta-potential calculated.

222

## 223 **2.4 Diffusion experiments**

### 224 **2.4.1 Membrane release studies**

225 The eight formulas were formulated and pre-heated (to 32°C in a water bath), while the  
226 receptor phase (PBS 7.4 pH and 10% denatured ethanol) was pre-heated in a second water  
227 bath to 37°C prior to commencing with the membrane release study. A vertical Franz cell  
228 was used during this study, consisting of two compartments, namely a donor and receptor  
229 compartment. Dow Corning® high vacuum grease was applied to the each of the  
230 compartments on the connecting sides, and a magnetic stirrer was placed in the receptor  
231 compartment before placing the synthetic membrane (polyvinylidene fluoride (PVDF), Pall®  
232 Life Sciences, Michigan, USA synthetic membranes, with a pore size of 0.45 µm and 25 mm  
233 diameter) on the greased side of the receptor compartment. Following the placement of the  
234 membrane, the donor compartment was placed on top of the receptor compartment and  
235 vacuum grease was utilised to thoroughly seal the sides of the two connected compartments  
236 (to void leakage) before the addition of a horseshoe clamp (donor on top of receptor  
237 compartment) to tightly fasten the two. Before filling of the receptor compartment (filling  
238 capacity of ±2 ml), 2 ml PBS/10% ethanol was accurately measured by means of a pipet into  
239 12 polytops, where after extraction took place to fill the individual receptor compartments.  
240 Prior to filling the donor compartment with the respective formula (±1 ml), the receptor  
241 compartment was firstly inspected to ensure no air bubbles were trapped within. A piece of  
242 Parafilm® and a plastic cap were used to cover each of the donor compartments of the  
243 assembled and prepared Franz cells to avoid loss of constituents. The Franz cells were  
244 fitted onto a Franz cell stand and placed into the pre-heated water bath (Grant Instruments,  
245 UK) at 37°C on a magnetic stirring plate (Variomag, USA). For the following 6 h, the entire  
246 receptor phase was extracted hourly and refilled with the PBS/10% ethanol that was kept at  
247 a constant temperature of 37°C. The hourly extracted samples were placed in individual  
248 HPLC vials and analysed by means of HPLC to determine the concentration of the API that  
249 permeated through the synthetic membrane into the receptor fluid along with API release  
250 rate.

#### 251 **2.4.2 Skin preparation**

252 The skin obtained from abdominoplasty, after informed consent (Caucasian females: 21-80  
253 years), was utilised for the purpose of this study (ethical approval reference number: NWU-  
254 00111-17-A1-03). The utilisation of only female skin minimises the variation in the results  
255 obtained and in addition, is easier to obtain, as abdominoplasty in South Africa is generally  
256 only performed on females. A Dermatome™ (Zimmer TDS, United Kingdom) was used to  
257 obtain dermatomed skin samples of  $\pm 400 \mu\text{m}$  by pressing the Dermatome™ onto the skin at  
258 an angle of approximately  $45^\circ$  prior to conducting skin diffusion studies. The dermatomed  
259 skin samples were then placed on Whatman® filter paper and enfolded within aluminium foil  
260 to be further stored at  $-20^\circ\text{C}$  until needed for skin diffusion studies. The amount of samples  
261 needed were thawed and cut into circles before each respective study, in such a manner  
262 that each circle could be fitted between the receptor and donor compartment of a vertical  
263 Franz cell. Before fitting the samples between the donor and receptor, visual examination  
264 was performed to exclude samples with visible defects.

#### 265 **2.4.3 Skin diffusion**

266 The same technique, as explained in Section 2.4.1, used during the membrane release  
267 studies was implemented for the *in vitro* skin diffusion studies. The only variance between  
268 the two methods was that the PVDF synthetic membranes were substituted with  
269 dermatomed skin samples, and placed between the receptor and donor compartments with  
270 the stratum corneum facing upwards. The receptor compartment was filled with PBS/10%  
271 ethanol (9:1), and extraction took place 12 h after initiation of the study. These extracted  
272 samples were placed in HPLC vials and analysed by means of HPLC to establish the  
273 amount of API that diffused through the skin after 12 h (Baert *et al.*, 2011).

#### 274 **2.4.4 Tape stripping**

275 For topical and transdermal research, it is essential to quantify the API in the skin (Escobar-  
276 Chávez *et al.*, 2008). Tape stripping (after 12 h diffusion studies) will provide more specific  
277 data on whether topical delivery of the API within either the stratum corneum-epidermis  
278 (SCE), or epidermis-dermis (ED) occurred (OECD, 2004).

279 Subsequent to the skin diffusion study, the Franz cell compartments were separated and the  
280 skin was visually inspected before tape stripping commenced. The skin samples were  
281 removed and pinned to a piece of Parafilm® on a solid surface, and any remaining  
282 formulation or dispersion was gently dabbed off using a piece of paper towel. Sixteen pieces  
283 were acquired by cutting 3M Scotch® Magic™ into strips that could sufficiently cover the  
284 diffusion area. The first strip was discarded to avoid possible contamination and the  
285 remaining 15 strips, containing the API and SCE, were placed in a polytop containing 5 ml of  
286 methanol, serving as the extraction solution. Another polytop with 5 ml extraction solution  
287 was used for the remaining ED, which was cut into pieces (to enhance the surface area)  
288 before being placed in the polytop. These polytops were then placed in the fridge ( $\pm 4^{\circ}\text{C}$ )  
289 and left for approximately 8 h, thereafter, a volume ( $\pm 1.5$  ml) was extracted by means of a  
290 syringe from each of the polytops and filtered through a  $0.45\ \mu\text{m}$  PTFE filter into an HPLC  
291 vial for analysis (Pellett *et al.*, 1997).

## 292 **2.5 Data analysis**

293 The samples of each membrane release study were analysed by means of HPLC to obtain a  
294 raw data, which was analysed by means of a linear line obtained from injecting a standard  
295 solution, as described in Section 2.2.3, at different injection volumes. Consequently, the  
296 concentration (i.e. drug flux) of each of the selected statins in the respective receptor phase  
297 of the Franz cells could be established using the slope of the linear line (Ng *et al.*, 2010).  
298 The results obtained from analysis could subsequently be examined with regards to the  
299 average cumulative amount of selected statin per area ( $\mu\text{g}/\text{cm}^2$ ), which had diffused through  
300 the membrane, plotted against time (h) (Shakeel *et al.*, 2007). During skin diffusion studies,  
301 results were determined in terms of the average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of the  
302 respective statin that diffused through the skin, in conjunction with average concentration  
303 ( $\mu\text{g}/\text{ml}$ ) of the respective statin that diffused through the skin after 12 h.

## 304 **2.6 Statistical analysis**

305 Descriptive analysis entails the calculation of mean (which includes standard deviation) and  
306 median (middle score of distribution) values relating to the flux value obtained during

307 membrane studies, and concentration values where skin diffusion is concerned (Sheskin,  
308 2000). Box-plots were used for the purpose of this study to illustrate summarised data in  
309 terms of the lower quartile, media, upper quartile, extreme value (Krzywinski & Altman,  
310 2014) and mean. Log transformation was performed on data before utilising analysis of  
311 variance (ANOVA) to determine whether statistically significant effects occurred between the  
312 different factors, consequently p-values were obtained. A p-value of equal or less than 0.05  
313 is considered a statistical significant effect (Concato & Hartigan, 2016). A statistically  
314 significant ( $p < 0.05$ ) interaction was found between the formula type (nano-emulsions and  
315 nano-emulgels) and the statins during membrane studies, hence one-way ANOVAs (two)  
316 were performed to establish the effect of statins for each nano-emulsions and nano-emulgel,  
317 which also presented statin effects ( $p < 0.05$ ). Thereafter, Tukey's honest significance  
318 difference (HSD) tests were used to determine pairwise differences between the four statin  
319 means. The same method was implemented during skin diffusion studies. The interaction  
320 between the type formula, statins and skin layer showed statistical significance ( $p < 0.05$ )  
321 during analysis of tape stripping result, therefore one-way ANOVAs (four) were performed, to  
322 determine the effect of statins within the respective formula on the respective skin layer.

### 323 **3 Results and Discussion**

#### 324 **3.1 Formulation of nano-emulsions and nano-emulgels**

325 The eight formulas contained an amount of 2% active ingredient, which consisted of four  
326 nano-emulsions and four nano-emulgels (two formulas per statin). The nano-emulsions  
327 were translucent, homogenous and displayed low viscosity, while the nano-emulgels  
328 exhibited a higher viscosity, which could provide a more effortless application. The nano-  
329 emulgels were formulated in an attempt to overcome the main limitation of the nano-  
330 emulsions, which is considered the rheological properties of these dispersions (Chellapa *et*  
331 *al.*, 2015).

332

## 333 **3.2 Physicochemical properties**

### 334 **3.2.1 Aqueous solubility**

335 The solubility of the selected statins in PBS (pH 7.4) at 32°C was determined to be  
336  $0.001\pm 0.008$  mg/ml,  $0.002\pm 0.001$  mg/ml,  $0.310\pm 0.066$  mg/ml and  $0.243\pm 0.058$  mg/ml for  
337 mevastatin, lovastatin, rosuvastatin and simvastatin, respectively. As Naik *et al.* (2000)  
338 proposed that ideal permeation of molecules through the skin would occur if the API  
339 possessed a solubility of equal or more than 1 mg/ml, the aforementioned solubility of the  
340 statins can be seen as not being ideal.

### 341 **3.2.2 Log D**

342 Log D can be described as the distribution ratio of all species (both ionised and unionised) at  
343 pH 7.4 (or determined pH) between two immiscible phases, such as non-polar or oil (e.g. *n*-  
344 octanol) and aqueous buffer, consequently log D is the logarithmic of the determined ratio.  
345 At pH 7.4 (physiological pH), the log P and log D can be seen as corresponding (Andrés *et*  
346 *al.*, 2015; Vraka *et al.*, 2017).

347 The value (log P at a specific pH) can be indicative of a drug or molecule ability to serve as a  
348 candidate for transdermal permeation, as a hydrophilic compound will possess a lower log P  
349 value. Subsequently, it can be proposed that transdermal delivery of a compound with a log  
350 P value of under -1 will prove difficult, while a compound with a log P higher than -1 to a  
351 maximum of 3 can express sufficient lipophilic and hydrophilic characteristics, and could be  
352 considered a transdermal candidate (N'Da, 2014). The log D values obtained for the  
353 respective statins were 4.49, 3.80, -0.20 and 1.80 for lovastatin, mevastatin, rosuvastatin  
354 calcium and simvastatin ammonium, respectively. Consequently, it can be stated that  
355 rosuvastatin calcium and simvastatin ammonium are more suitable candidates for  
356 transdermal delivery when compared to the more lipophilic lovastatin and mevastatin.

### 357 **3.2.3 Characterisation of semi-solid formulations**

358 The data obtained from the pH, viscosity, droplet size and zeta-potential analysis are  
359 summarised in Table 3.

360

361 Table 3:  
362 Summary of the characteristics of the nano-emulsions and the nano-emulgels  
363  
364 Additionally, the result obtained from TEM performed on the nano-emulsions is displayed in  
365 Table 4 and Fig. 1.

366 Table 4  
367 Results obtained from TEM performed on the nano-emulsions  
368

369 Fig. 1: Micrographs of oil droplets captured with the TEM: a) **(NEL1)**, b) **(NEM1)**, c) **(NER1)**  
370 and d) **(NES1)** and the size thereof. Scale bars for magnification are indicated for each  
371 micrograph.

372 Where the pH of a formulation intended for transdermal delivery is concerned, two aspects  
373 have to be considered. Firstly, the pH of the skin is suggested to range between 4 and 7  
374 (Ng & Lau, 2015); consequently, it can be proposed that formulations intended for topical  
375 application should retain pH value within this range, as a pH of above 9 and under 3 can  
376 compromise the integrity of skin (Naik *et al.*, 2000). Secondly, the dissociation of a molecule  
377 (weak acid or base) is determined by pH, consequently the degree of ionisation. The  
378 ionisation of a compound is of particular relevance when attempting transdermal delivery, as  
379 this aim can be achieved more effectively when the API is unionised (Li *et al.*, 2012), due to  
380 the lipophilic barrier posed by the skin (Williams, 2003).

381 Effective permeation and diffusion in transdermal and topical delivery can be achieved when  
382 the API is unionised (Li *et al.*, 2012; Williams, 2003). Therefore, it can be stated, that at the  
383 pH displayed in Table 3, only a small percentage of rosuvastatin and simvastatin will be  
384 unionised (0.30% and 2.14%, respectively), whereas lovastatin and mevastatin will retain a  
385 high percentage unionised species of 99.99%. The zeta-potential of both the dispersions  
386 and formulation is considered favourable, as formulas with a zeta-potential of more or equal  
387 to  $\pm 30$  mV can remain stable over time (Eid *et al.*, 2014; Silva *et al.*, 2012), although it  
388 should be stated that the zeta-potential of the nano-emulgels presented as more negative,

389 which could indicate that these semi-solids are more stable than the dispersions. In addition  
390 to the zeta-potential, droplet sizes of the formulas were determined. The dispersions and  
391 formulation displayed average droplet sizes considered well within the accepted range  
392 proposed for nano-emulsions, of 50-200 nm (Tadros *et al.*, 2004) and nano-emulgels of 100-  
393 500 nm (Drais & Hussein, 2017). These readings correlated with the results obtained from  
394 TEM performed on the dispersions (Table 4 and Fig. 1), although TEM could not be  
395 performed on the nano-emulgels. The viscosity measurement of the dispersions correlated  
396 with what is suggested in literature, as very low viscosity is predicted for o/w nano-emulsions  
397 due to the large water component in the dispersions (Chime *et al.*, 2014). The high viscosity  
398 readings of nano-emulgels, after neutralisation of the gelling agent, further correlated with  
399 what is proposed in literature (Lubrizol, 2009).

### 400 **3.3 Membrane diffusion experiments**

401

402 Fig. 2: Box-plot indicating the flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) of: a) the nano-emulsions ((**NEL1**), (**NEM1**),  
403 (**NES1**) and (**NER1**)), as well as b) the nano-emulgels ((**NEGL**), (**NEGM**), (**NEGS**) and  
404 (**NEGR**)) after 6 h

405 During the membrane diffusion experiment, flux data was obtained for all the respective  
406 formulas. Subsequently, it can be proposed that release of the API from the vehicle  
407 occurred, followed by diffusion through the PVDF synthetic membranes. However, it can be  
408 stated that nano-emulsions exhibited a higher average %released, when compared to the  
409 respective nano-emulgels, which could be explained by the lower viscosity of nano-  
410 emulsions (Arora *et al.*, 2014; Begur *et al.*, 2015) and the fact that nano-emulgels will  
411 release the API in a more controlled manner (Chellapa *et al.*, 2015). The median flux  
412 indicated that (**NER1**) obtained the highest median flux ( $413.650 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ), followed by  
413 (**NEGR**) ( $281.937 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ), (**NES1**) ( $272.851 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ), (**NEGS**) ( $98.442 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ),  
414 (**NEM1**) ( $18.970 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ), (**NEL1**) ( $8.647 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ), (**NEMG**) ( $4.867 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ) and lastly,  
415 (**NELG**) with the lowest median flux ( $4.651 \mu\text{g}/\text{cm}^2\cdot\text{h}$ ).

416

## 417 **3.4 Diffusion experiment**

### 418 **3.4.1 Diffusion study**

419 As it was predetermined, by a pilot study conducted by Burger *et al.* (2015), that only one  
420 extraction after 12 h should be performed; therefore, no flux values could be obtained during  
421 the skin diffusion studies, and it could only be determined whether any of the statins within  
422 the respective formulas diffused through the skin.

423

424 Fig. 3: Box-plot indicating the amount per area diffused ( $\mu\text{g}/\text{cm}^2$ ) present in the receptor  
425 phase of: a) the nano-emulsions ((**NEL1**), (**NEM1**), (**NES1**) and (**NER1**)), as well as b) the  
426 nano-emulgels ((**NEGL**), (**NEGM**), (**NEGS**) and (**NEGR**)) after 12 h

427 Comparing the mean and median amounts per area, a significant difference can be  
428 observed, more specifically when observing (**NEGS**), (**NEGR**) and (**NEGM**). Therefore, it is  
429 essential to report the median values as the true concentration, which is unaffected by  
430 outliers, as the mean value can be influenced by skewed distribution and consequently lead  
431 to inaccurate estimation of true concentration values (Dawson & Trapp, 2004). When  
432 comparing all the formulas in terms of the median amount diffused per area ( $\mu\text{g}/\text{cm}^2$ ), it can  
433 be stated that (**NEGS**) ( $3.244 \mu\text{g}/\text{cm}^2$ ) obtained the highest median amount diffused per  
434 area, followed by (**NER1**) ( $2.146 \mu\text{g}/\text{cm}^2$ ), (**NEGM**) ( $0.846 \mu\text{g}/\text{cm}^2$ ), (**NES1**) ( $0.654 \mu\text{g}/\text{cm}^2$ ),  
435 (**NEM1**) ( $0.4783.244 \mu\text{g}/\text{cm}^2$ ), (**NEGL**) ( $0.440 \mu\text{g}/\text{cm}^2$ ), (**NEGR**) ( $0.319 \mu\text{g}/\text{cm}^2$ ) and lastly,  
436 (**NEL1**) ( $0.297 \mu\text{g}/\text{cm}^2$ ) with the lowest amount per area diffused. Comparing the nano-  
437 emulsions to the respective nano-emulgels, with the exception of (**NEGR**), the nano-  
438 emulgels displayed the highest median amount diffused per area, which coincides with the  
439 suggestions in literature, as high solubilisation capacity (characteristic of nano-emulsion) in  
440 conjunction with the increased skin adhesion of these semi-solids can result in enhanced  
441 skin permeation (Eid *et al.*, 2014). The deviation of (**NEGR**) from the trend, observed with  
442 the other nano-emulgels, can possibly be the result of physiological differences between the  
443 donor skin (e.g. skin thickness) used during the respective studies (Dev *et al.*, 2015).

444 When comparing the statins, it can be proposed overall simvastatin attained the highest

445 median amount diffused, which can possibly be attributed to the ideal log D value of this API.  
446 The fact that all the respective statins diffused through the skin, despite the limitations  
447 proposed by the physiochemical properties of these compounds, could be attributed to the  
448 utilisation of nano-emulsions as vehicles, as it has the capacity to dissolve lipophilic drugs  
449 and enhance skin permeation (Elmataeeshy *et al.*, 2018). Additionally, the excipients used  
450 in a nano-emulsion, such as the surfactant and apricot kernel oil, can increase membrane  
451 permeability, consequently assisting transdermal delivery (Aparna *et al.*, 2015). The  
452 formulation of nano-emulgels from these dispersions will add to the advantages of nano-  
453 emulsions (Basera *et al.*, 2015; Eid *et al.*, 2014; Khullar *et al.*, 2012). Utilising these nano-  
454 systems can facilitate transdermal delivery of molecules that would normally not cross the  
455 skin barrier (Gadkari *et al.*, 2018). The lower diffusion observed with the **(NEL1)** and  
456 **(NELG)**, could be attributed to the more lipophilic nature of lovastatin compared to other  
457 statins, and possibly to the high affinity of the epidermis for lipophilic drugs, consequently  
458 limiting deeper penetration (Schulz *et al.*, 2017).

459 Table 5:

460 The concentration ( $\mu\text{g/ml}$ ) of the selected statins within the respective formulas that diffused  
461 through the skin after 12 h

462

463 From Table 5, it can be concluded that the respective statins attained higher concentrations  
464 within the receptor phase compared to the mean plasma concentrations after oral  
465 administration. This could not be established for the mevastatin-containing formulas, as not  
466 all mean plasma concentrations could be obtained from literature. Consequently, it can be  
467 proposed that the lovastatin, rosuvastatin and simvastatin formulas may obtain therapeutic  
468 concentrations after transdermal delivery.

### 469 **3.5 Tape stripping**

470

471 Fig. 4: Box-plot indicating the concentration ( $\mu\text{g/ml}$ ) present in: a) the SCE with the nano-  
472 emulsions (**(NEL1)**, **(NEM1)**, **(NES1)** and **(NER1)**); b) the SCE with the nano-emulgels

473 ((**NEGL**), (**NEGM**), (**NEGS**) and (**NEGR**)); c) the ED with the nano-emulsions ((**NEL1**),  
474 (**NEM1**), (**NES1**) and (**NER1**)), and d) the ED with the nano-emulgels ((**NEGL**), (**NEGM**),  
475 (**NEGS**) and (**NEGR**)) after tape stripping was performed

### 476 **3.5.1 Stratum corneum-epidermis**

477 The tape stripping results for the SCE are depicted in Fig. 4.a and b, and indicate that all the  
478 respective formulas accumulated in the SCE.

479 From the result obtained for median statin concentration within the SCE, it can be stated that  
480 (**NEGS**) (7.517 µg/ml) obtained the highest median concentration, followed by (**NEGL**)  
481 (3.475 µg/ml), (**NES1**) (2.121 µg/ml), (**NEM1**) (1.871 µg/ml), (**NER1**) (1.620 µg/ml), (**NEGR**)  
482 (1.434 µg/ml), (**NEGM**) (1.416 µg/ml) and lastly, (**NEL1**) (0.783 µg/ml), which obtained the  
483 lowest median concentration within the SCE. The simvastatin formulas ((**NES1**) and  
484 (**NEGS**)) obtained the highest median concentration when compared to the other nano-  
485 emulsion and nano-emulgel formulas. When comparing the dispersions and semi-solid  
486 formulations, it can be stated that (**NEM1**) and (**NER1**) penetrated the SCE better than the  
487 semi-solid formulation, and the (**NEGS**) and (**NEGL**) penetrated the SCE better than the  
488 dispersion. No definite consensus could therefore be drawn to indicate whether the nano-  
489 emulgels or nano-emulsions attained higher concentrations within the SCE, although  
490 literature suggests that in theory, nano-emulgels should attain higher concentrations within  
491 SCE, as the addition of the gelling agent can increase the affinity for the formulation for the  
492 stratum corneum (Zheng *et al.*, 2016).

493 When comparing the respective statins, irrespective of the formulas, simvastatin reached the  
494 highest concentration within the SCE, which can be attributed to the ideal physiochemical  
495 properties of this statin. These properties include a log D between 1 and 3 (1.80 (Section  
496 3.2.2)), a melting point under 200°C (135-138°C (Oh *et al.*, 2006)), molecular weight (418.6  
497 Da (Bhagat & Sakhare, 2014)) under 500 Da, aqueous solubility higher than 1 mg/ml (as  
498 discussed in Section 3.2.1). Additionally, physiochemical properties of lovastatin and  
499 mevastatin particularly log D (Section 3.2.2), indicating higher lipophilicity of the  
500 aforementioned statins compared to rosuvastatin, can explain the higher concentration of

501 lovastatin and mevastatin attained in the SCE, when compared to rosuvastatin. As  
502 rosuvastatin, is moderately hydrophilic, it can be proposed that limited partitioning occurred  
503 from the vehicle to the stratum corneum, since highly hydrophilic molecules will exhibit a  
504 complete inability to partition into stratum corneum (Vitorino *et al.*, 2015).

### 505 **3.5.2 Epidermis-dermis**

506 In Fig. 4.c and d, it is clear that the mean concentration obtained in the ED from highest to  
507 lower were as follows: **(NES1)** (10.680 µg/ml), **(NEGS)** (9.518 µg/ml), **(NER1)** (3.901 µg/ml),  
508 **(NEGM)** (3.429 µg/ml), **(NEGL)** (2.413 µg/ml), **(NEGR)** (1.932 µg/ml), **(NEL1)** (1.622 µg/ml),  
509 and lastly, **(NEM1)** (1.338 µg/ml). Although literature suggests that nano-emulgels will  
510 penetrate the ED better due to the possible transformation (depending on the concentration  
511 gelling agent) of the transdermal vehicle to a more topical vehicle gelling agent (Zheng *et al.*,  
512 2016), a consensus could not be made regarding which vehicle attained the highest median  
513 concentration within ED; this as a result of **(NEGL)** and **(NEGM)** reaching a higher  
514 concentration within the ED than their dispersion-counterparts, whereas **(NER1)** and **(NES1)**  
515 attained the highest concentration, compared to their semi-solid formulation-counterparts.

516 The fact that the simvastatin formulas (**(NES1)** and **(NEGS)**) attained the highest  
517 concentration within the ED overall, could be due to the ideal log D value of the API. The  
518 ideal log D value is indicative of a API that retains both hydrophilic and lipophilic  
519 characteristics, consequently the lipophilic characteristic will assist in the penetration of the  
520 stratum corneum, while the hydrophilic characteristic will facilitate permeation to the other  
521 layers of the skin (Perrie *et al.*, 2012).

## 522 **3.6 Statistical analysis**

### 523 **3.6.1 Membrane release studies**

524 During the ANOVA performed on formula type and statins, the interaction was of the flux  
525 value where the statistical significance was  $p < 0.05$ . Thereafter, one-way ANOVAs were  
526 performed to establish the effect of statin on each of the nano-emulsions and nano-  
527 emulgels. As there were statistical significant ( $p < 0.05$ ) effects during both ANOVAs,  
528 pairwise differences were determined between the four statin means by utilising Tukey's

529 HSD tests. The means of the nano-emulsions ((**NEL1**), (**NEM1**), (**NER1**), and (**NES1**)) were  
530 mutually significantly different on a 0.05 level of significance. The Tukey's HSD tests  
531 performed on the nano-emulgels indicated no significant difference between (**NEGL**) and  
532 (**NEGM**), while a significant difference, at 0.05 level of significance, could be noted between  
533 (**NEGS**) and (**NEGR**), and between these two dispersions and the aforementioned (**NEGL**)  
534 and (**NEGM**).

### 535 **3.6.2 Skin diffusion studies**

536 Statins effects ( $p < 0.05$ ) existed during both one-way ANOVAs, consequently Tukey's HSD  
537 tests followed to determine pairwise differences between the four statin means. During the  
538 analysis of the nano-emulsions, it was evident there were no significant differences between  
539 the mean values of (**NEL1**) and (**NEM1**), and between (**NES1**) and (**NEM1**). However, the  
540 mean value of (**NEL1**) were significantly different (on a 0.05 level of significance) from  
541 (**NES1**) and (**NER1**). The mean value of (**NER1**) were also significantly different from  
542 (**NEM1**) and (**NES1**).

### 543 **3.6.3 Tape stripping**

544 Since the three-way ANOVA applied on the formula type, statin and SCE and ED indicated a  
545 statistical significant difference ( $p < 0.05$ ), one-way ANOVAs were performed to determine the  
546 effect of the statin for each of the four combinations of formula type and skin layer during  
547 tape stripping studies. No statistical significance was observed between the nano-emulsions  
548 and SCE, while statistical significant effects were observed between the nano-emulsions and  
549 ED ( $p = 0.0142$ ); the nano-emulgels and SCE ( $p < 0.01$ ), and the nano-emulgels and ED  
550 ( $p < 0.01$ ). Tukey's HSD tests were performed to determine pairwise differences between  
551 the four statins means due to the significant difference of the three aforementioned  
552 ANOVAs, the results were as follows: the mean values of (**NEL1**) and (**NES1**) in SCE were  
553 significantly different on a 0.05 level of significance. Nano-emulgels and SCE, showed  
554 (**NEGM**) and (**NEGR**). However, significant differences (on a 0.05 level of significance) were  
555 observed when comparing the aforementioned to (**NEGS**) and (**NEGL**), and (**NEGS**) and  
556 (**NEGL**). Lastly, when comparing the nano-emulgels and ED, no significant difference was

557 observed between the mean values of **(NEGL)**, **(NEGM)** and **(NEGR)**, however **(NEGS)**  
558 differed significantly from the aforementioned formulas.

559 Furthermore, t-tests were performed, to compare all the formulas in terms of SCE (group 1)  
560 and ED (group 2) respectively. Significant differences were observed for **(NEGM)**  
561 ( $p=0.0003$ ), **(NES1)** ( $p=0.0057$ ) and **(NER1)** ( $p=0.0362$ ) when compared to SCE/ED. T-tests  
562 were also performed to compare SCE and ED respectively, in terms of formula (nano-  
563 emulsions=group 1, and nano-emulgels=group 2). Statistical significant differences were  
564 noted between SCE and lovastatin formulas (**(NEL1)** and **(NEGL)**) ( $p=0.0003$ ), and SCE and  
565 simvastatin formulas (**(NES1)** and **(NEGS)**) ( $p=0.0001$ ). In ED, a significant difference  
566 showed only between the ED and rosuvastatin formulas (**(NER1)** and **(NEGR)**) ( $p=0.0314$ ).

#### 567 **4 Conclusion**

568 By means of characterisation, an optimised formula for the nano-emulsions was selected,  
569 where after nano-emulgels were formulated and characterised by utilising the optimised  
570 formula of the nano-emulsions. During the membrane release studies, it was evident that  
571 better release of the respective statin occurred from the nano-emulsions compared to the  
572 nano-emulgel formulas. This correlated with the predictions of literature, as a nano-emulgel  
573 will release an API in a more controlled manner (Chellapa *et al.*, 2015). Although release  
574 occurred from all the respective formulas, the significantly lower flux values displayed by the  
575 mevastatin and lovastatin formulas, compared to the flux observed with rosuvastatin and  
576 simvastatin formulas, can possibly be ascribed to the low solubility of lovastatin and  
577 mevastatin in the receptor phase (see Section 3.2.1). The highest flux values were obtained  
578 by **(NER1)** and **(NEGR)**. It can further be proposed that nano-emulsions attained higher flux  
579 value than the respective nano-emulgels.

580 It can be concluded from the skin diffusion studies that amounts ( $\mu\text{g/ml}$ ) were quantified of  
581 each statin formula within the receptor phase, above the LOD and LOQ, although **(NEL1)**  
582 attained an amount under the LOQ of lovastatin and above the LOD. Subsequently, it can  
583 be suggested that the aim of transdermal delivery was achieved with both the nano-  
584 emulsions and the nano-emulgels. Significantly higher median amounts per area diffused

585 were obtained by **(NEGS)** (3.244  $\mu\text{g}/\text{cm}^2$ ) and **(NER1)** (2.146  $\mu\text{g}/\text{cm}^2$ ), when comparing all  
586 the formulas, while **(NEL1)** (0.297  $\mu\text{g}/\text{cm}^2$ ), displayed the lowest amount. It can therefore be  
587 proposed that higher median amounts diffused per area were observed with nano-emulgels,  
588 although an exception was seen with **(NER1)**. This deviation can possibly be attributed to  
589 higher concentrations attained by **(NER1)** within the SCE and ED, since diffusions are  
590 concentration dependant (Barry, 2002). The low viscosity and zeta-potential of **(NEGR)**,  
591 compared to the other nano-emulgels, could influence the adhesion and stability of this  
592 formulation. In addition, low concentration of **(NEL1)** can be related to poor physiochemical  
593 properties (e.g. lipophilicity and poor aqueous solubility) (Naik *et al.*, 2000), while the  
594 possible affinity of lovastatin for the oil phase or penetration enhancer, could also have  
595 resulted in poor skin diffusion (Haque & Talukder, 2018).

596 Following skin diffusion, tape stripping was performed to determine the amounts of statin  
597 retained within the SCE and ED. The highest median concentration obtained by **(NEGS)** in  
598 the SCE, can be ascribed to the ideal properties of simvastatin, such as log D, molecular  
599 eight, aqueous solubility and melting point (Naik *et al.*, 2000). When comparing **(NEGR)** and  
600 **(NEGM)** to **(NEGL)**, it can be proposed that lovastatin, due to lipophilicity, expressed a  
601 higher affinity for the stratum corneum (Förster *et al.*, 2009), while enabling more effortless  
602 penetration of this layer (Sugibayashi, 2017). The log D value of mevastatin contradicts the  
603 concentrations of **(NEGM)** found within the SCE; however, due to the higher concentration of  
604 **(NEGM)** within the ED and receptor phase compared to the respective nano-emulsion, it can  
605 be suggested that the semi-solid improved the delivery of mevastatin through the skin layers  
606 to the systemic circulation in a more significant manner than the counter-part **(NEM1)**. The  
607 highest median concentrations obtained within the ED were displayed by the simvastatin  
608 formulas (**(NES1)** and **(NEGS)**), which might be related to the ideal physiochemical  
609 properties of this API (Naik *et al.*, 2000). It can further be observed that **(NER1)** attained a  
610 higher concentration in the ED and additionally the receptor phase, compared to nano-  
611 emulgel-counterpart; hence, it can be suggested that the more hydrophilic rosuvastatin in  
612 **(NER1)** expressed a higher affinity for the hydrophilic layers and systemic circulation

613 (Jankowski *et al.*, 2017), and that the delivery of rosuvastatin might not be improved by the  
614 semi-solid formulation. The higher concentration of simvastatin and rosuvastatin within the  
615 hydrophilic ED and receptor phase can further be attributed to the salt forms of these APIs  
616 (simvastatin ammonium salt and rosuvastatin calcium salt), presenting with higher solubility  
617 than the respective acid or base forms (Serajuddin, 2007). Therefore, it can be proposed  
618 that transdermal delivery results of lovastatin and mevastatin might be altered when utilising  
619 the respective salt forms of these APIs, as proper solubility of these APIs within the receptor  
620 phase can provide sink conditions required to consider the permeation methodology as  
621 suitable (Khandavilli & Panchagnula, 2006).

622 The exception of lovastatin, when observing the overall higher concentrations of the  
623 remaining statins within the ED compared to the SCE, can be attributed to the more lipophilic  
624 nature of this API, as partitioning into the ED could have become the rate-limiting step  
625 (Tashiro *et al.*, 2001). The overall higher concentrations in the ED of the remaining statins  
626 however can be ascribed to the presence of apricot kernel oil in the formulation comprised of  
627 fatty acids, causing a disruption in the intercellular lipid matrix of the stratum corneum,  
628 consequently acting as penetration enhancers (Choi *et al.*, 2012; Van Zyl *et al.*, 2016).  
629 Additional penetration enhancing effect can be caused by surfactants such as Tween<sup>®</sup> 80  
630 (Van Zyl *et al.*, 2016), which was used in the formulation of both the nano-emulsions and  
631 nano-emulgels. Finally, it should be stated that the ideal physiochemical properties of  
632 simvastatin, could be proposed as the main factor resulting in the generally higher amounts  
633 of this API within the SCE, ED and receptor phase.

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644 **Conflict of Interest**

645 The authors declare no conflict of interest.

646

647 **References**

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## Tables

Table 1:

Ingredients used during the formulation of the nano-emulsions and the nano-emulgels

	<b>Nano-emulsion</b>	<b>%m/m</b>	<b>Nano-emulgel</b>	<b>%m/m</b>
	Statin	2.00%	Statin	2.00%
<b>Phase A</b>	Apricot kernel oil	8.00%	Apricot kernel oil	8.00%
	Span® 60	6.00%	Span® 60	1.00%
	Milli-Q® water	78.00%	Milli-Q® water	77.54%
<b>Phase B</b>	Tween® 80	6.00%	Tween® 80	6.00%
			Carbopol® Ultrez 20 polymer	0.46%

Table 2:

Lowest limit of detection (LOD) and lowest limit of quantification (LOQ) of statins

<b>Statins</b>	<b>Lovastatin</b>	<b>Mevastatin</b>	<b>Simvastatin</b>	<b>Rosuvastatin</b>
<b>LOD (<math>\mu\text{g/ml}</math>)</b>	0.0806	0.0148	0.0863	0.0138
<b>LOQ (<math>\mu\text{g/ml}</math>)</b>	0.2441	0.0447	0.2615	0.0419

Table 3:

Summary of the characteristics of the nano-emulsions and the nano-emulgels

	<b>pH</b>	<b>Zeta-potential (mV)</b>	<b>Droplet size (nm)</b>	<b>Viscosity (cP)</b>
<b>(NEL1)</b>	5.83	-43.77	162.60	4.64
<b>(NEGL)</b>	5.82	-52.67	172.10	46090.00
<b>(NEM1)</b>	6.13	-49.57	147.17	4.05
<b>(NEGM)</b>	6.05	-58.07	213.93	27985.71
<b>(NES1)</b>	5.87	-43.53	153.10	8.38
<b>(NEGS)</b>	5.82	-54.87	201.40	19151.43
<b>(NER1)</b>	6.52	-34.35	169.60	7.87
<b>(NEGR)</b>	6.56	-39.57	149.83	8358.43

Table 4:

Results obtained from TEM performed on the nano-emulsions

<b>Dispersions</b>	<b>TEM (nm)</b>
<b>(NEL1)</b>	51.40-207.10
<b>(NEM1)</b>	60.61-139.94
<b>(NES1)</b>	25.24-203.85
<b>(NER1)</b>	44.28-79.12

Table 5:

The concentration ( $\mu\text{g/ml}$ ) of the selected statins within the respective formulas that diffused through the skin after 12 h

	<b>Concentration (<math>\mu\text{g/ml}</math>)</b>
<b>Mean plasma concentration lovastatin</b>	0.003 <sup>a</sup>
<b>Mean plasma concentration simvastatin</b>	0.001-0.002 <sup>a</sup>
<b>Mean plasma concentration rosuvastatin</b>	0.001 <sup>b</sup>
<b>(NEGS)</b>	4.260
<b>(NER1)</b>	1.327
<b>(NEGR)</b>	0.858
<b>(NEGM)</b>	0.800
<b>(NES1)</b>	0.379
<b>(NEGL)</b>	0.308
<b>(NEM1)</b>	0.266
<b>(NEL1)</b>	0.147

<sup>a</sup>Björkhem-Bergman *et al.*, 2011:165; <sup>b</sup>DeGorter *et al.*, 2013:402

**Figures:**

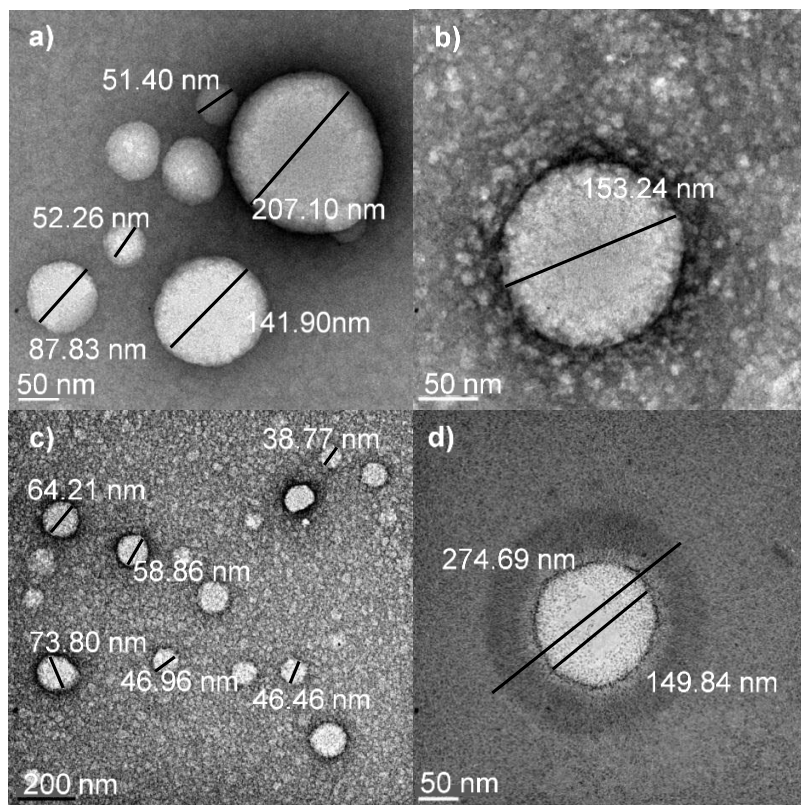


Fig. 1: Micrographs of oil droplets captured with the TEM: a) **(NEL1)**, b) **(NEM1)**, c) **(NER1)** and d) **(NES1)** and the size thereof. Scale bars for magnification are indicated for each micrograph.

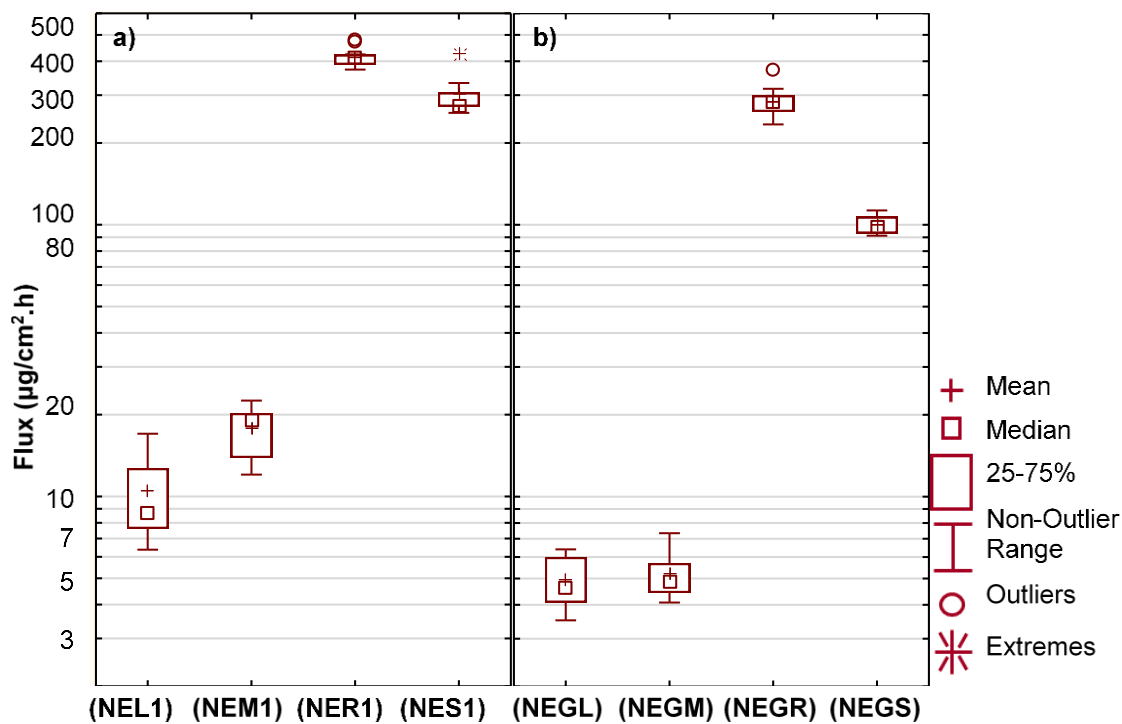


Fig. 2: Box-plot indicating the flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) of: a) the nano-emulsions ((NEL1), (NEM1), (NES1) and (NER1)), as well as b) the nano-emulgels ((NEGL), (NEGM), (NEGS) and (NEGR)) after 6 h

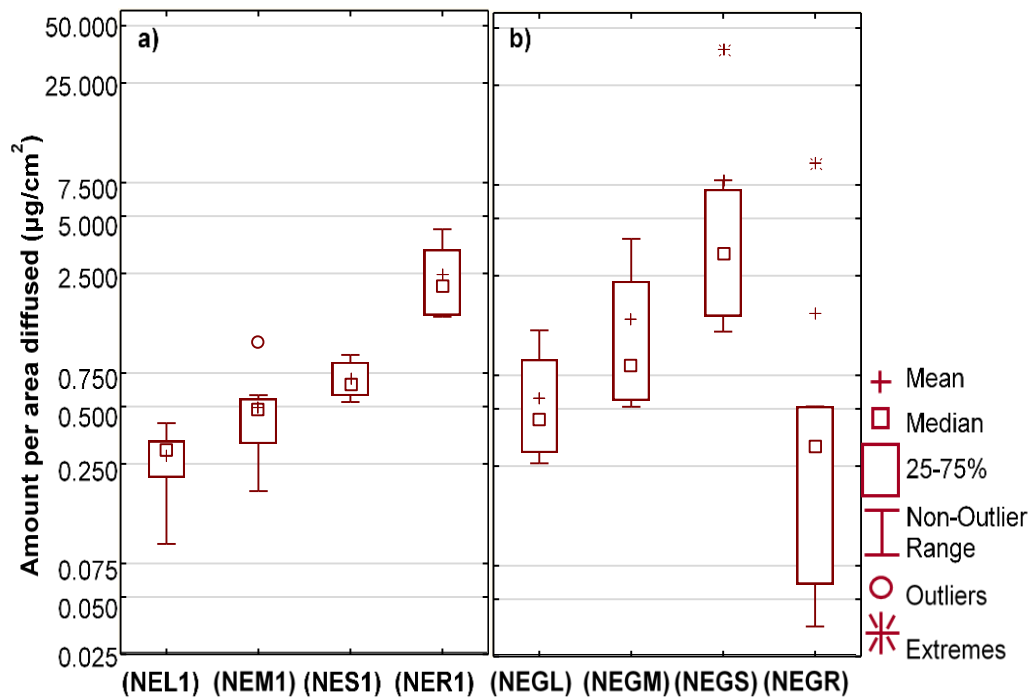


Fig. 3: Box-plot indicating the amount per area diffused ( $\mu\text{g}/\text{cm}^2$ ) present in the receptor phase of: a) the nano-emulsions ((NEL1), (NEM1), (NES1) and (NER1)), as well as b) the nano-emulgels ((NEGL), (NEGM), (NEGS) and (NEGR)) after 12 h

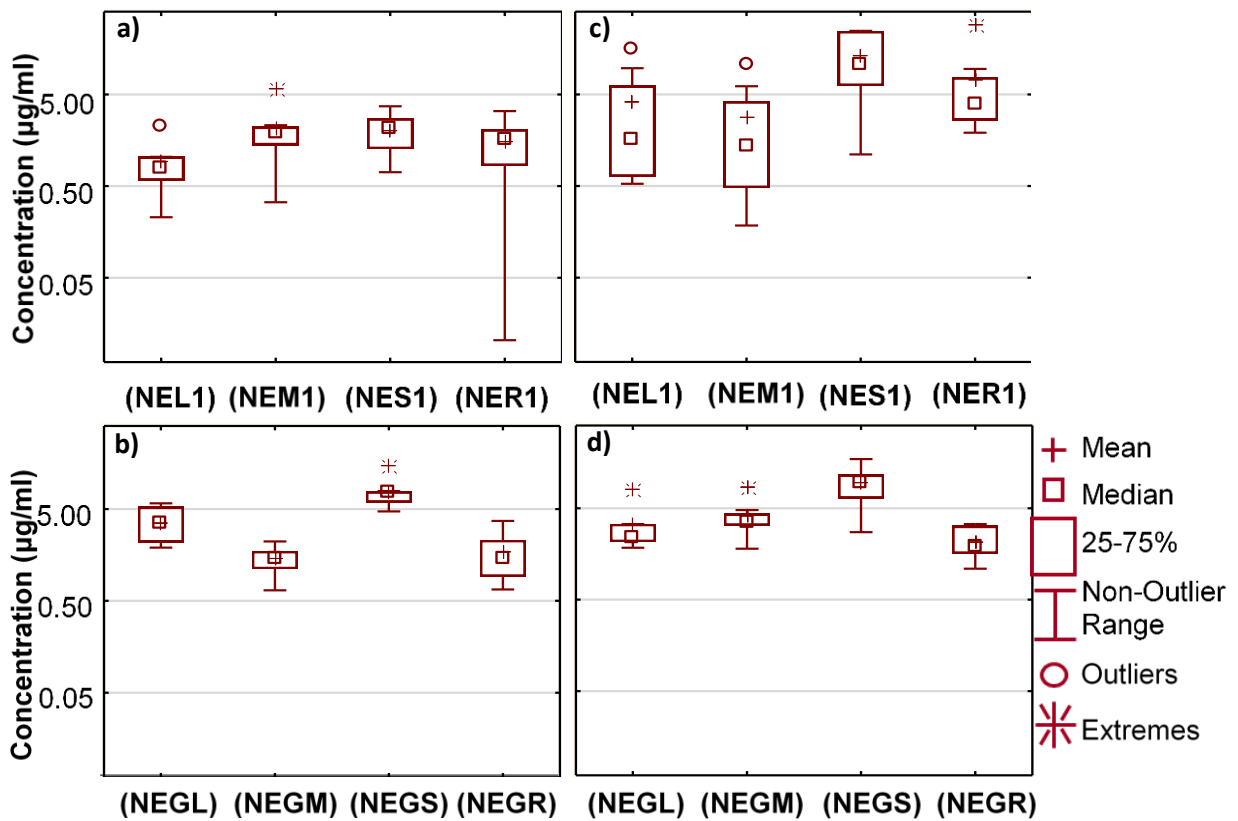


Fig. 4: Box-plot indicating the concentration (µg/ml) present in: a) the SCE with the nano-emulsions ((NEL1), (NEM1), (NES1) and (NER1)); b) the SCE with the nano-emulgels ((NEGL), (NEGM), (NEGS) and (NEGR)); c) the ED with the nano-emulsions ((NEL1), (NEM1), (NES1) and (NER1)), and d) the ED with the nano-emulgels ((NEGL), (NEGM), (NEGS) and (NEGR)) after tape stripping was performed

## *Chapter 5:*

# *Conclusion and future prospects*

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Familial hypercholesterolemia is considered one of the most common inherited metabolic disorders, and is associated with defective lipoprotein metabolism (Mytilinaiou *et al.*, 2018:1). The defect in lipoprotein metabolism is caused by mutation in genes of proteins that regulate LDL metabolism PCSK9 and Apo B-100, and additionally, LDL receptor (LDLR) genes (Harada-Shiba *et al.*, 2018:751). Consequently, FH is associated with very high levels of LDL (Kim *et al.*, 2018), due to gene mutations causing abnormally low uptake of LDL within the liver (Mytilinaiou *et al.*, 2018:1). Hence, accumulation of LDL in the plasma occurs, increasing the risk of premature CVD (Krogh *et al.*, 2018:1398). Currently HMG-CoA reductase inhibitors statins are considered as the first line treatment of FH (Zodda *et al.*, 2018:1), which are administered orally. Although the lipid lowering efficacy of statin has been reported on several occasions, a significant amount of patients is unable to tolerate statins due to side-effects, particularly at doses required to reach the satisfactory lipid levels (Stoekenbroek *et al.*, 2018:205).

It is suggested that the transdermal route is the favoured alternative to oral administration, as it can avoid systemic side-effects associated with oral administration (Sharma & Khandelwal, 2018:405), such as gastrointestinal effects (Buchanan *et al.*, 2018:2). For transdermal delivery, the API, should pass through the stratum corneum to reach the systemic circulation (Sharma & Khandelwal, 2018:405). Consequently, selection of an API retaining the ideal properties and vehicle selection for delivery (dosage form) is essential (Mbah *et al.*, 2011:684). Conventional dosage forms, such as ointments and cream, have been used and investigated extensively as transdermal delivery systems; however, numerous limitations are associated with these current dosage forms (Kathe & Kathpalia, 2017:488). It is proposed that these limitations can be overcome by using nanoparticles systems (Szunerits & Boukherroub, 2018:3); nano-emulsions are considered one such nanoparticle system (Rizvi & Saleh, 2018:68). The nanometric droplets associated with these systems, resulting in a larger surface area and promoting contact of the dispersion with the skin, can enhance percutaneous delivery of APIs (Da Silva Marques *et al.*, 2018:2828).

The aim of this study was to investigate the transdermal delivery of the selected statins (lovastatin, mevastatin, rosuvastatin, and simvastatin) by means of nano-emulsions containing apricot kernel oil as a penetration enhancer. Consequently, o/w nano-emulsions of different formulas were formulated and characterised, resulting in the selection of an optimised nano-emulsion. This formula was then utilised in the formulation of nano-emulgels, which was characterised and compared to the optimised nano-emulsion. These formulations were investigated by *in vitro* skin diffusion studies and cytotoxicity studies were conducted to

determine if these formulations and the excipients used in the formulation could be considered safe for application to the skin.

Subsequently, certain objectives were set and met, to achieve the aforementioned aim.

- Development and validation of an HPLC analytic method to determine and quantify the concentrations of the selected statins within the formulations (nano-emulsions and nano-emulgels), and the samples obtained from membrane release and *in vitro* skin diffusion studies.
- Ascertaining the aqueous solubility and log D of the selected statins.
- Formulation of both the nano-emulsions and the semisolid nano-emulgel containing the selected statins respectively, incorporated into the oil phase (apricot kernel oil) additionally acting as a penetration enhancer.
- Characterisation of both the nano-emulsion formulas (pre-formulated and optimised) with regard to TEM, pH, droplet size, zeta-potential, viscosity, entrapment efficacy and visual examination.
- Characterisation of nano-emulgels with regard to light microscopy, pH, droplet size, zeta-potential, viscosity and visual examination.
- Performing membrane release studies to determine the release of the respective statins from the nano-emulsions and nano-emulgels.
- Conducting skin diffusion studies by means of vertical Franz cells combined with tape stripping, to establish transdermal and topical delivery of the respective statins.
- Determining the cytotoxic effects of the nano-emulsion, statins (separately) and the surfactant(s) or excipients used in the nano-emulsion, by using *in vitro* cell cultures, specifically pre-malignant HaCaT cell lines, and conducting MTT.

An HPLC method was developed and validated for the analyses of the selected statins. During the course of validation, this method was found to be sensitive, precise and reliable for the accurate quantification of the statins.

When considering transdermal delivery of an API, certain characteristics (physiochemical properties) of the API have to be evaluated, which can effect successful delivery if not ideal such as log D and aqueous solubility. The log D value of the statins were determined to be  $4.49 \pm 0.039$ ,  $3.80 \pm 0.128$ ,  $1.80 \pm 0.001$ , and  $-0.20 \pm 0.001$  for lovastatin, mevastatin, simvastatin and rosuvastatin respectively. Lovastatin, mevastatin, simvastatin and rosuvastatin depicted a solubility in PBS (pH 7.4) of  $0.001 \pm 0.008$  mg/ml,  $0.002 \pm 0.001$  mg/ml,  $0.243 \pm 0.058$  mg/ml and  $0.310 \pm 0.066$  mg/ml, respectively. When considering both the log D

and aqueous solubility of the statins, it can be stated that none of these molecules exhibited an ideal aqueous solubility of greater than 1 mg/ml. While the log D of simvastatin could be considered ideal for skin permeation (between 1 and 3) (Naik *et al.*, 2000:319), mevastatin and lovastatin could present as too lipophilic and rosuvastatin as too hydrophilic.

For the purpose of this study, optimised o/w nano-emulsions were formulated containing 2% (w/w) of the respective statins and equal ratios of Span<sup>®</sup> 60 and Tween<sup>®</sup> 80 (1:1) and 8% (w/w) apricot kernel oil. This formula was then utilised to formulate semi-solid formulations, i.e. nano-emulgels. These nano-emulgels (**NEG1s**) comprised the same amount of statin, apricot kernel oil and surfactant, although Carbopol<sup>®</sup> Ultrez 20 was added to the water phase of the dispersions. Characterisation of these nano-emulgels was indicative of advantageous properties. Consequently, *in vitro* Franz cell diffusion studies were conducted and analysed by means of the validated HPLC method.

The pre-formulation stage resulted in the formulation of the optimised dispersion (**NEF1**), which exhibited ideal characteristics, such as a droplet size range within the acceptance criteria of 50 – 200 nm (Tadros *et al.*, 2004:303), and highly negative zeta-potential of below - 30 mV (Eid *et al.*, 2014:2; Silva *et al.*, 2012:860). Consequently, successful skin permeation can be proposed when taking into account both of these characteristics (Abolmaali *et al.*, 2011:140; Duangjit *et al.*, 2011:6). Additionally, high entrapment of the statins within the (**NEF1s**), were established, while pH of the dispersions ranging between 5.83 and 6.52 was considered safe for application to the skin (Barry, 2002:512). The viscosity of (**NEF1s**) was characteristically low (4.05 – 8.38 cP) (Shakeel *et al.*, 2007:E6); therefore, the aim was to formulate (**NEG1s**) and increase viscosity, which was achieved (8358.43 – 46090.00 cP), along with highly negative zeta-potential (-39.57 to -58.07 mV) and droplets within the acceptance range of 100 – 500 nm (Drais & Hussein, 2017:10).

Subsequent to the characterisation of the respective formulas, membrane release studies were conducted to determine the release of the API from the nano-emulsions containing lovastatin, mevastatin, rosuvastatin and simvastatin ((**NEL1**), (**NEM1**), (**NER1**) and (**NES1**), respectively) and nano-emulgels containing lovastatin, mevastatin, rosuvastatin and simvastatin ((**NEGL**), (**NEGM**), (**NEGR**) and (**NEGS**), respectively). Overall, the (**NER1**) (409.880 µg/cm<sup>2</sup>.h) and (**NEGR**) (306.560 µg/cm<sup>2</sup>.h) formulas attained the highest average flux values over a period of 6 h, followed by (**NES1**) (297.010 µg/cm<sup>2</sup>.h), (**NEGS**) (107.280 µg/cm<sup>2</sup>.h), (**NEM1**) (17.892 µg/cm<sup>2</sup>.h), (**NEL1**) (10.899 µg/cm<sup>2</sup>.h), (**NEGM**) (5.458 µg/cm<sup>2</sup>.h) and lastly, (**NEGL**) (5.306 µg/cm<sup>2</sup>.h). Additionally, it can be stated that the (**NEF1s**) depicted higher flux than the respective (**NEG1s**).

Following the membrane release studies of the eight formulas, *in vitro* skin diffusion studies and tape stripping were conducted to assess transdermal and topical delivery respectively. Concentration of the respective statins within all eight formulas was quantified within the receptor phase, stratum corneum epidermis (SCE) and epidermis dermis (ED). Within the receptor phase, the highest median amount was quantified for **(NEGS)** (3.244  $\mu\text{g}/\text{cm}^2$ ), followed by **(NER1)** (2.146  $\mu\text{g}/\text{cm}^2$ ), **(NEGM)** (0.846  $\mu\text{g}/\text{cm}^2$ ), **(NES1)** (0.654  $\mu\text{g}/\text{cm}^2$ ), **(NEM1)** (0.478  $\mu\text{g}/\text{cm}^2$ ), **(NEGL)** (0.440  $\mu\text{g}/\text{cm}^2$ ), **(NEGR)** (0.319  $\mu\text{g}/\text{cm}^2$ ) and lastly, **(NEL1)** (0.297  $\mu\text{g}/\text{cm}^2$ ).

From the data obtained from tape-stripping, it was evident that **(NEGS)** (7.517  $\mu\text{g}/\text{ml}$ ) yielded the highest median concentration within the SCE, while **(NES1)** (10.680  $\mu\text{g}/\text{ml}$ ) yielded the highest median concentration within the ED. Additionally, it can be stated that **(NEGS)** and **(NES1)** yielded the highest concentration in both the SCE and ED compared to the other **(NEG1s)** and **(NEF1s)**, respectively. A consensus could however not be drawn about which vehicle (**(NEF1s)** or **(NEG1s)**) displayed higher concentrations within the respective layers. Overall, irrespective of the formulas, it should be stated that simvastatin attained the highest amount within the SCE, ED, and receptor phase, which can be attributed to the ideal physiochemical properties of this API, compared to the other statins.

Lastly, *in vitro* cytotoxicity studies were conducted on the **(NEF1s)**, a placebo dispersion **(PNEF1)**, excipients used in the formulation (Tween<sup>®</sup> 80, Span<sup>®</sup> 60, and Span<sup>®</sup> 60:apricot kernel oil combination) and the statins alone. These cytotoxicity studies were performed on HaCaT cells as a representative of the *in vitro* keratinocyte model. The methods used included the methylthiazol tetrazolium (MTT) assay, assessing mitochondrial activity of cells, and the neutral red (NR)-assay, assessing the lysosomal activity of cells. The %cell viability over the respective concentration range of the treatments was calculated from the respective absorbance of the dyes. The concentrations exposed exceeded the concentrations that diffused through the skin. The MTT colorimetric assay established **(NEL1)**, **(NEM1)** and **(NES1)** dispersions as cytostatic at 0.0100% and cytotoxic between 0.0200 – 0.0400%. The remaining **(NER1)** was non-cytotoxic, between the entire concentration range (0.0025 – 0.0400%). The NR-assay however indicated **(NEL1)**, **(NEM1)** and **(NER1)** as cytostatic between 0.0100 – 0.0200%, while cytotoxicity was observed at the highest concentration (0.0400%). The result obtained for **(NES1)** correlated with MTT. For the statins alone, the MTT assay established that lovastatin, mevastatin and simvastatin displayed cytotoxicity at highest concentration exposed (0.01200%), while rosuvastatin displayed no cytotoxicity between the concentration range (0.00075 – 0.01200%), while NR-assay found the four statins as cytostatic at highest concentration. The MTT-assay found Tween<sup>®</sup> 80 and Span<sup>®</sup> 60 as cytotoxic at highest concentration exposed of 0.1200%, while NR-assay established the aforementioned excipients and Span<sup>®</sup> 60:apricot kernel oil as only cytostatic or weak cytotoxic (60 – 80%) at highest concentration.

The cell viability data from both colorimetric assays was additionally used to determine the half maximal inhibitory concentration ( $IC_{50}$ ) for each of the **(NEF1s)**, excipients, and statins (lovastatin, mevastatin, rosuvastatin and simvastatin). When considering  $IC_{50}$  value obtained from both colorimetric assays, it can be concluded that **(PNEF1)** required the highest concentration to inhibit half of the test cells, while rosuvastatin, as an API, and Span<sup>®</sup>60:apricot kernel oil as an excipient, required the highest concentration to inhibit half of the test cells.

The  $IC_{50}$  of lovastatin during MTT (78.6  $\mu\text{g/ml}$ ) was lower, while during the NR-assay, a higher value (236.9  $\mu\text{g/ml}$ ) was obtained than was proposed in literature on HeLa cells (160  $\mu\text{g/ml}$ ) (Bhargavi *et al.*, 2016:3800). Mevastatin obtained higher  $IC_{50}$  during both MTT- (0.0777 mg/ml) and NR- (0.2369 mg/ml) assays compared to the  $IC_{50}$  obtained from literature of 4.335  $\mu\text{g/ml}$  determined on BT474A breast cancer cells (Glynn *et al.*, 2008:4). Rosuvastatin displayed higher  $IC_{50}$  values than those stated in literature of > 20  $\mu\text{g/ml}$  in HEp-2, KB and HeLa cells (Campos-Lara & Mendoza-Espinoza, 2011:191) during MTT- (305.9  $\mu\text{g/ml}$ ) and NR- (245.3  $\mu\text{g/ml}$ ) assays. The  $IC_{50}$  of simvastatin obtained for both MTT- (7.48  $\mu\text{g/ml}$ ) and NR- (168.2  $\mu\text{g/ml}$ ) assays however, was significantly lower than that stated in literature on MCF-7 breast cancer cells of 43.2 mg/ml (Safwat *et al.*, 2017:1122). Consequently, it can be proposed that lovastatin, mevastatin, and rosuvastatin might be less cytotoxic on HaCaT cells, while simvastatin might be more cytotoxic.

It should be stated that all the formulas **(NEF1s)** and **(NEG1s)** successfully achieved the aim of transdermal delivery of the selected statins, as treatment of hypercholesterolemia requires the delivery of statins to the systemic circulation.

Future prospects might include:

- Performing compatibility studies on the excipients (Thermal activity monitor (TAM)).
- Performing long-term stability tests on formulas, to investigate possible instabilities (e.g. flocculation, phase inversion, or creaming).
- Investigation of other natural oils, acting as penetration enhancers to possibly improve the transdermal delivery compared to apricot kernel oil.
- Utilising the salt forms of lovastatin and mevastatin, to enhance solubility of these statins.
- Investigate other methods of formulating a nano-emulgel.
- Performing additional cytotoxicity assays on nano-emulsions and individual excipients, such as lactate dehydrogenase (LDH).

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# *Appendix A:*

## *The validation of a high performance liquid chromatographic assay for the selected statins*

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### **A.1 Purpose of validation**

The purpose of the validation of an HPLC assay was to verify if the intended analytical method was sufficiently sensitive, reliable and suitable for quantification of the intended statins in the different semi-solid formulations (ICH, 2005:1; ICH, 2005:6). Where validation of an analytical method is concerned, certain criteria has to be examined, which according to the ICH (2005:2), includes linearity, accuracy, precision, ruggedness, detection limit and quantification limit. The samples collected after *in vitro* diffusion studies were also analysed by means of the HPLC to establish and quantify the respective statin concentration in the receptor phase.

### **A.2 Chromatographic conditions**

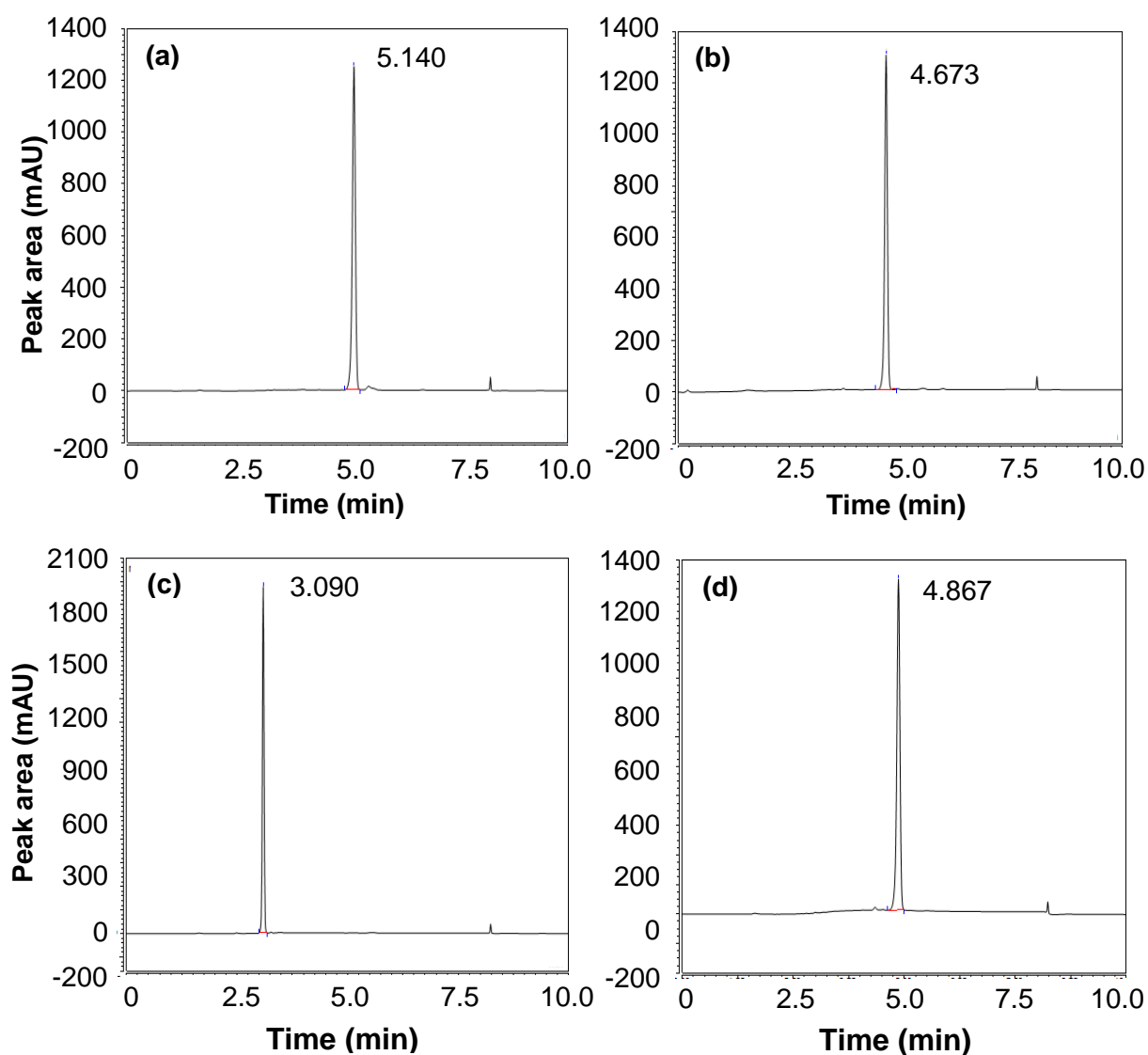
Professor Jan du Preez of the North-West University (NWU), Potchefstroom, designed and developed the analytical HPLC method; this process was carried out in the Analytical Technology Laboratory (ATL). Chromatographic conditions that were considered and controlled during the development and validation of this analytical method for the selected statins was the analytical instrument, the column, mobile phase, flow rate, injection volume and detection wavelength,. Specific factors of the chromatographic conditions had been taken into account when this method of analysis was developed and these were:

<b>Analytical instrument:</b>	Dionex UltiMate 3000 dual system with ternary gradient pumps, column ovens autosampler and diode array detectors operated on Chromeleon 7.2 data acquisition and analysis software (Thermo Fisher Scientific Inc., Waltham, MA).
<b>Column:</b>	Venusil XBP C18(2), 150 x 4.6 mm, 5 µm (Agela Technologies, Newark, DE)
<b>Mobile phase:</b>	acetonitrile/water with 0.1% orthophosphoric acid
<b>Gradient:</b>	Start at 45% acetonitrile and increase linearly to 90% after 1 min. Hold at 90% until 6 min and then re-equilibrate at start conditions.
<b>Flow rate:</b>	1.0 ml/min
<b>Injection volume:</b>	10 µl
<b>Detection:</b>	UV at 240 nm

**Retention time:**  $\pm$  3.090, 4.673, 4.867 and 5.140 min for rosuvastatin, mevastatin, simvastatin and lovastatin, respectively.

**Stop time:** 10 min

**Solvent:** Methanol



**Figure A.1:** HPLC chromatograms representing a) lovastatin, b) mevastatin, c) rosuvastatin and d) simvastatin standard solution peaks and retention times

### A.3 Preparation of standard and samples

#### A.3.1 Standard preparation

The standard preparation of the selected statins (lovastatin, mevastatin, rosuvastatin and simvastatin) is as follows:

1. Weigh approximately 20 mg of each of the selected statins accurately in a 100 ml volumetric flask.
2. Dissolve and fill to volume with methanol (Standard 1).
3. Dilute 5 ml of the solution to 50 ml with methanol (Standard 2).
4. Dilute 5 ml of the solution (Standard 2) to 50 ml with methanol (Standard 3).
5. Transfer the standards into auto sampler vials and analyse.

### A.3.2 Preparation of samples for the analysis of formulations

The ingredients and quantities as shown in Table A.1 were weighed off. Phases A and B were both heated separately while stirring. When all of the ingredients in the respected phases were dissolved properly, the oil phase was added slowly to the water phase. An oil-in-water (o/w) nano-emulsion was obtained thereafter; 1 g was weighed into a 100 ml volumetric flask and diluted with methanol.

**Table A.1:** Nano-emulsion (o/w) standard formula

Ingredients		%m/m
A	Apricot kernel oil	7.961%
	Span <sup>®</sup> 60	6.000%
	Statin	2.000%
B	Tween <sup>®</sup> 80	6.000%
	Milli-Q water	78.039%

Table A.2 displays the different ingredients used to obtain a nano-emulgel. Firstly, phases A and B were heated and stirred separately, thus the same method was used as with the nano-emulsion, but phase A was added to phase B using an homogeniser and left to stir for 15 min to obtain a nano-emulgel. After the emulgel was obtained, 1 g weighed into a 100 ml volumetric flask and diluted with methanol.

**Table A.2:** Nano-emulgel standard formula

Ingredient		%m/m
A	Apricot kernel oil	7.961%
	Span <sup>®</sup> 60	6.000%
	Statin	2.000%
B	Tween <sup>®</sup> 80	6.000%
	Milli-Q water	77.579%
	Carbopol 20 polymer	0.460%

### **A.3.3 Placebo preparation**

The aforementioned method was used to prepare the placebo, with the exception of adding the API.

### **A.3.4 Sample preparation for diffusion studies**

Samples were collected from Franz diffusion cells, which were transferred into auto sampler vials and analysed without any further processing.

## **A.4 Validation parameters**

### **A.4.1 Linearity**

The ability of an analytical procedure to obtain a test result that is directly equivalent to the concentration analyte in a sample is considered as the linearity of the method. In order to establish linearity, the data plots formed by the peak areas against the analyte concentration ( $\mu\text{g/ml}$ ) on the linear regression curve should be analysed; a straight line should be formed by the plot with an acceptable coefficient of determination ( $R^2 \geq 0.99$ ) (Johnson & Buskirk, 1998:90). If the coefficient of determination is within the acceptance criteria, it suggests there is a direct relationship between response and concentration of the analyte, thus a high degree of linearity.

The linearity of the selected statins was determined by making a combined stock solution. This stock solution was prepared by weighing and dissolving approximately 20 mg of each statin in a 100 ml volumetric flask, after which it is made up to volume with methanol. This stock solution was used thereafter to prepare two separate dilutions:

1. Dilution 1 was prepared by transferring 5 ml of the stock solution into a 50 ml volumetric flask, and made up to volume with methanol.
2. Dilution 2 was prepared by transferring 5 ml of Dilution 1 into a 50 ml volumetric flask and made up to volume with methanol.

These two dilutions, together with the stock solution were injected into the HPLC eight times. With each sample, the method was set to use four different injection volumes in duplicate:

- 2.5  $\mu\text{l}$
- 5.0  $\mu\text{l}$
- 7.5  $\mu\text{l}$
- 10.0  $\mu\text{l}$

By using this method, different concentration ranges was obtained for each of the selected statins:

- Rosuvastatin: 0.488 – 195.300 µg/ml
- Mevastatin: 0.624 – 249.500 µg/ml
- Lovastatin: 0.480 – 191.900 µg/ml
- Simvastatin: 0.547 – 218.900 µg/ml

Figures A.2 – A.5 along with Tables A.3 – A.6 demonstrate the linearity of the individual statins over the aforementioned concentration ranges. These figures and tables demonstrate that rosuvastatin, mevastatin and lovastatin obtained an  $R^2$  value of 1, while simvastatin obtained a value of 0.9998. These values are indicative of a flawless positive linear relationship.

#### **A.4.1.1 Linear regression analysis**

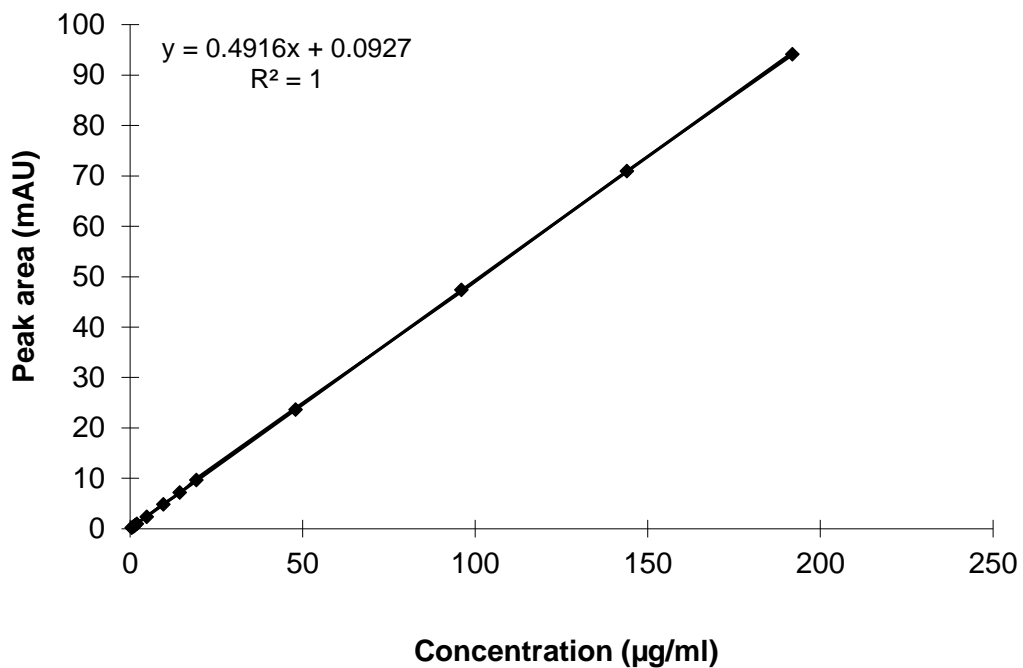
The peak area versus the analyte concentration (µg/ml) was utilised for interpretation of the regression curve, with peak areas representing the y-axis and API concentration plotted on the x-axis. The data can best be described by the linear regression equation:

$$y = mx + c$$

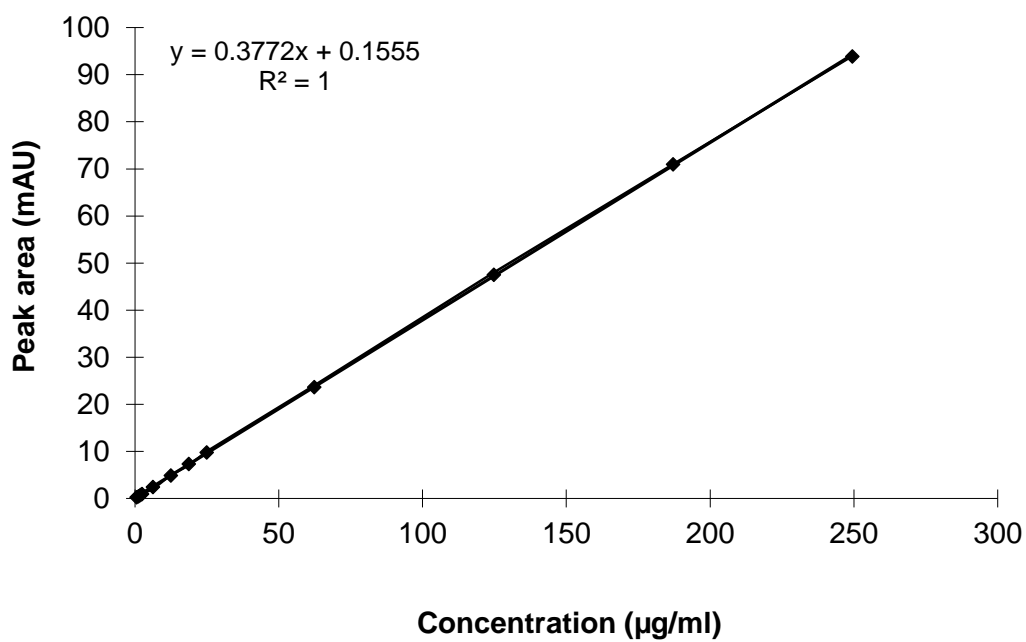
**Equation A.1**

Where:

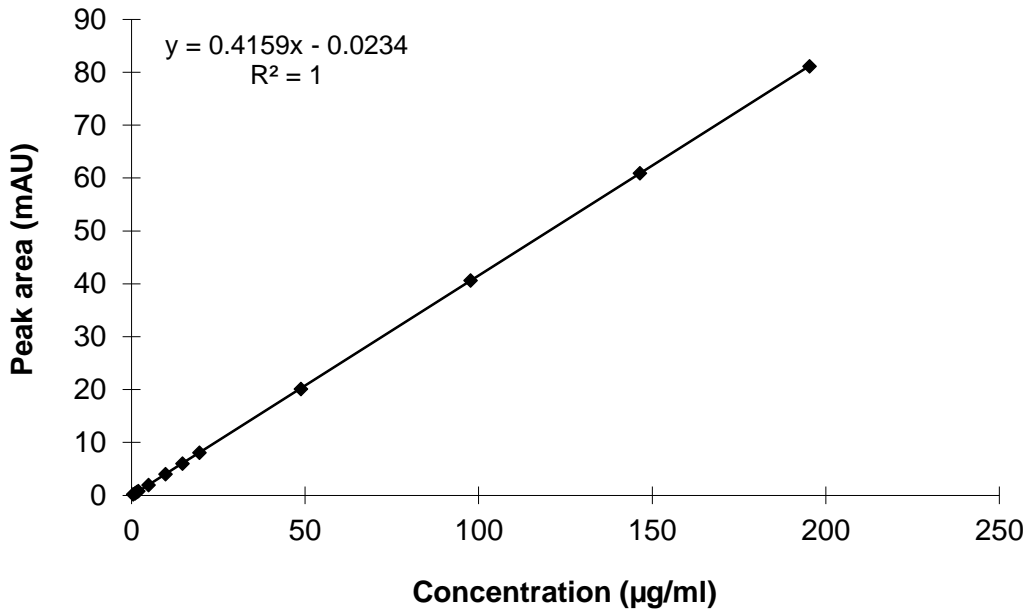
- y = Peak area ratios of the different API
- m = Slope
- x = Concentration of the different API in µg/ml
- c = y-intercept



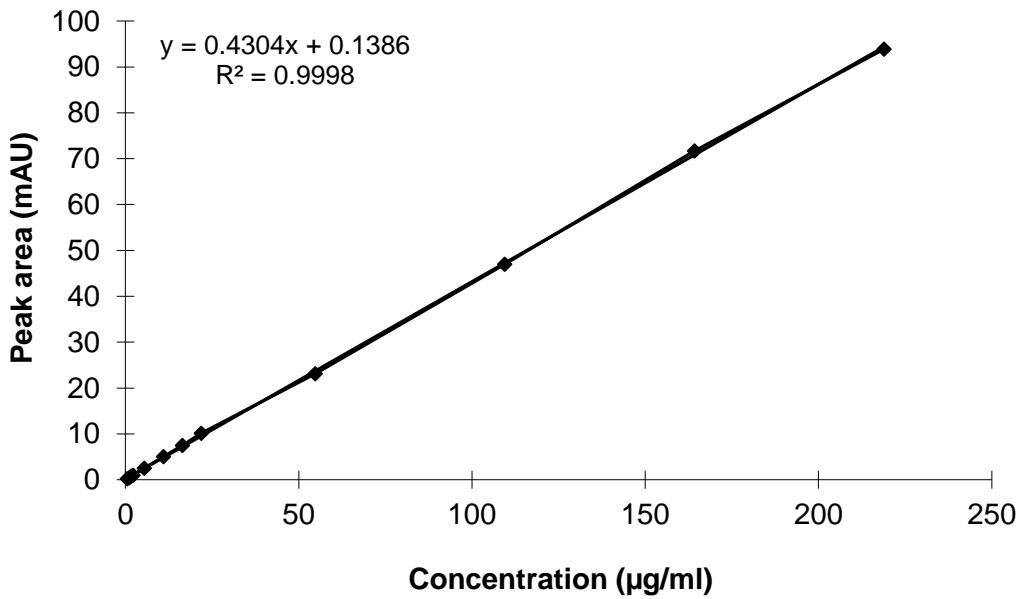
**Figure A.2:** Linear regression curve of lovastatin standards



**Figure A.3:** Linear regression curve of mevastatin standards



**Figure A.4:** Linear regression curve of rosuvastatin standards



**Figure A.5:** Linear regression curve of simvastatin standards

The coefficient of determination or regression value ( $R^2$ ) from the aforementioned statins was well within the acceptance criteria as demonstrated by Figures A.2 – A.5. These values indicate a high degree of linearity and a stable analysis system.

**Table A.3:** Linearity results of lovastatin

Standard ( $\mu\text{g/ml}$ )	Peak area
0.480	0.2
0.960	0.5
1.439	0.7
1.919	1.0
4.798	2.4
9.595	4.8
14.393	7.2
19.190	9.7
47.975	23.7
95.950	47.4
143.925	71.0
191.900	94.2
Slope	0.4916
y-intercept	0.0927
R <sup>2</sup>	1

**Table A.4:** Linearity results of mevastatin

Standard ( $\mu\text{g/ml}$ )	Peak area
0.624	0.2
1.248	0.5
1.871	0.7
2.495	1.0
6.238	2.4
12.475	4.9
18.713	7.4
24.950	9.8
62.375	23.7
124.750	47.5
187.125	71.0
249.500	93.9
Slope	0.3772
y-intercept	0.1555
R <sup>2</sup>	1

**Table A.5:** Linearity results of rosuvastatin

<b>Standard (<math>\mu\text{g/ml}</math>)</b>	<b>Peak area</b>
0.488	0.2
0.977	0.4
1.465	0.6
1.953	0.8
4.883	2.0
9.765	4.0
14.648	6.0
19.530	8.1
48.825	20.1
97.650	40.7
146.475	61.0
195.300	81.2
Slope	0.4159
y-intercept	0.0234
R <sup>2</sup>	1

**Table A.6:** Linearity results of simvastatin

<b>Standard (<math>\mu\text{g/ml}</math>)</b>	<b>Peak area</b>
0.547	0.2
1.095	0.4
1.642	0.7
2.189	1.0
5.473	2.5
10.945	5.0
16.418	7.4
21.890	10.1
54.725	23.1
109.450	47.0
164.175	71.7
218.900	93.9
Slope	0.4304
y-intercept	0.1386
R <sup>2</sup>	0.9998

#### A.4.1.2 Lower limit of detection and quantification

The lowest amount of an analyte in a sample that can be quantitatively determined with suitable accuracy and precision is seen as the lowest limit of quantification (LLOQ) (Rozet *et al.*, 2011:854; Wolfinger *et al.*, 2018:2). When referring to the lowest limit of detection (LLOD), it can be seen as the lowest amount of an analyte in a sample that cannot necessarily be quantified, although it can be detected (ICH, 2005:5). The method used to determine LLOQ was by preparing a solution containing 5 mg of the intended statin in a 100 ml volumetric flask made up to volume with methanol. Thereafter 1 ml of this stock solution was placed into another 100 ml volumetric flask by means of a micropipette and made up to volume with methanol. A volume of the diluted solution was then placed in a HPLC vial for analysis. The solution was injected six times at different injection volumes:

- 2.5  $\mu$ l
- 5.0  $\mu$ l
- 7.5  $\mu$ l
- 10.0  $\mu$ l

A %RSD of 20% or less is seen as acceptable (González *et al.*, 2014:16). The limit of detection (LOD) can be described, as the lowest concentration of an analyte in a sample that can be detected, but not quantified (APVMA, 2004:4; Snyder *et al.*, 1997b:659). Calculations of LOD and LOQ were based on the slope of a calibration curve and the standard deviation of the response by utilising these formulas for LOD (Equation A.2) and LOQ (Equation A.3), respectively (Swartz & Krull, 2012:70-71):

$$\text{DL (detection limit)} = 3.3 \times \sigma/S \quad \text{Equation A.2}$$

$$\text{QL (quantification limit)} = 10 \times \sigma/S \quad \text{Equation A.3}$$

Where:

$\sigma$  = Standard deviation of y-intercepts

S = Slope

**Table A.7:** Results obtained from injecting diluted sample of lovastatin at different injection volumes

Injection volume ( $\mu\text{l}$ )	2.5	5.0	7.5	10.0
Concentration ( $\mu\text{g/ml}$ )	0.144	0.288	0.431	0.575
Peak area	0.129	0.168	0.276	0.355
	0.107	0.217	0.255	0.351
	0.120	0.188	0.280	0.358
	0.119	0.182	0.240	0.334
	0.127	0.212	0.222	0.364
	0.118	0.189	0.231	0.326
Mean	0.120	0.190	0.250	0.348
SD	0.010	0.020	0.020	0.013
RSD%	6.050	8.760	8.700	3.817

**Table A.8:** Statistical analysis of lovastatin

Summary output		
Regression statistics		
Multiple R	0.999865	
R square	0.999729	
Adjusted R square	0.999594	
Standard error	0.001785	
Observations	4	
ANOVA	df	SS
Regression	1	0.023542
Residual	2	6.37E-06
Total	3	0.023548
	Coefficients	Standard error
Intercept	-0.0077	0.002186
X variable 1	0.521805	0.006071

Table A.8 displays the regression analysis data, which was obtained by utilising the data displayed in Table A.7. The standard error or deviation of the intercept ( $\sigma$ ) and slope (S) (highlighted in Table A.8 as X variable 1) was therefore obtained and could be utilised to determine the LOD and LOQ of lovastatin.

**Table A.9:** Results obtained from injecting diluted sample of mevastatin at different injection volumes

<b>Injection volume (µl)</b>	2.5	5.0	7.5	10.0
<b>Concentration (µg/ml)</b>	0.143	0.286	0.428	0.571
<b>Peak area</b>	0.053	0.128	0.191	0.266
	0.059	0.137	0.192	0.253
	0.053	0.128	0.182	0.252
	0.064	0.128	0.196	0.262
	0.055	0.129	0.209	0.249
	0.057	0.125	0.199	0.251
<b>Mean</b>	0.060	0.130	0.190	0.256
<b>SD</b>	0.000	0.000	0.010	0.006
<b>RSD%</b>	7.160	3.060	4.200	2.387

**Table A.10:** Statistical analysis of mevastatin

<b>Summary output</b>		
<b>Regression statistics</b>		
<b>Multiple R</b>	0.999755	
<b>R square</b>	0.99951	
<b>Adjusted R square</b>	0.999346	
<b>Standard error</b>	0.002593	
<b>Observations</b>	5	
<b>ANOVA</b>	<b>df</b>	<b>SS</b>
<b>Regression</b>	1	0.041129
<b>Residual</b>	3	2.02E-05
<b>Total</b>	4	0.041149
	<b>Coefficients</b>	<b>Standard error</b>
<b>Intercept</b>	-0.00113	0.002009
<b>X variable 1</b>	0.449262	0.005745

The data displayed in Table A.9 was analysed by means of regression, hence data in Table A.10 was obtained, where after the slope (X variable 1) and standard deviation of the intercept (highlighted in Table A.10) could be utilised to determine the LOD and LOQ of mevastatin.

**Table A.11:** Results obtained from injecting diluted sample of simvastatin at different injection volumes

Injection volume (µl)	2.5	5.0	7.5	10.0
Concentration (µg/ml)	0.135	0.271	0.406	0.541
Peak area	0.081	0.126	0.179	0.240
	0.053	0.075	0.190	0.222
	0.095	0.122	0.177	0.222
	0.055	0.117	0.187	0.227
	0.104	0.115	0.203	0.256
	0.114	0.111	0.186	0.229
Mean	0.080	0.110	0.190	0.233
SD	0.020	0.020	0.010	0.012
RSD%	27.670	15.110	4.590	5.183

**Table A.12:** Statistical analysis of simvastatin

Summary output		
Regression statistics		
Multiple R	0.99078604	
R square	0.98165697	
Adjusted R square	0.97554263	
Standard error	0.01420527	
Observations	5	
ANOVA	df	SS
Regression	1	0.032397
Residual	3	0.000605
Total	4	0.033003
	Coefficients	Standard error
Intercept	0.00905061	0.011003
X variable 1	0.42084017	0.033213

To establish the slope (X variable 1) and standard deviation of the y-intercept required to determine the LOD and LOQ of simvastatin, Table A.11 was analysed by means of regression, to obtain the data displayed in Table A.12.

**Table A.13:** Results obtained from injecting diluted sample of rosuvastatin at different injection volumes

Injection volume (µl)	2.5	5.0	7.5	10.0
Concentration (µg/ml)	0.132	0.263	0.395	0.526
Peak area	0.056	0.125	0.195	0.265
	0.059	0.127	0.195	0.265
	0.061	0.128	0.196	0.266
	0.055	0.127	0.196	0.266
	0.058	0.127	0.195	0.265
	0.055	0.126	0.196	0.265
Mean	0.060	0.130	0.200	0.265
SD	0.000	0.000	0.000	0.001
RSD%	4.040	0.710	0.300	0.209

**Table A.14:** Statistical analysis of rosuvastatin

Summary output		
Regression statistics		
Multiple R	0.999865	
R square	0.999729	
Adjusted R square	0.999594	
Standard error	0.001785	
Observations	4	
ANOVA	df	SS
Regression	1	0.023541669
Residual	2	6.37286E-06
Total	3	0.023548042
	Coefficients	Standard error
Intercept	-0.0077	0.002186241
X variable 1	0.521805	0.006070739

The regression analysis of rosuvastatin data, as displayed in Table A.13, was performed in the same way as mentioned before. Therefore, by means of regression analysis of the data displayed in Table A.13, the slope (X variable 1) and standard deviation (highlighted in Table A.14) could be obtained and utilised to determine the LOD and LOQ of rosuvastatin.

**Table A.15:** The lower limit of detection and quantification (LLOD and LLOQ) of the selected statins as determined by the linear curves procedure

Statin	Lovastatin	Mevastatin	Simvastatin	Rosuvastatin
<b>LOD</b> DL (detection limit) = $3.3 \times \sigma/S$	0.0806	0.0148	0.0863	0.0138
<b>LOQ</b> QL (quantification limit) = $10 \times \sigma/S$	0.2441	0.0447	0.2615	0.0419

Table A.15 displays the LOD and LOQ determined for the selected statin by means of the Equation A.2 (LOD) and Equation A.3 (LOQ), as described by Swartz & Krull (2012:70-71).

#### A.4.2 Accuracy

The accuracy of analytical methods can be described as the closeness in proximity of the test results obtained from the procedure, compared to that of the true value (APVMA, 2004:4; ICH, 2005:4). It is recommended that nine samples of three different concentrations should be analysed to cover the full calibration curve (Bansal & DeStefano, 2007:E111; Snyder *et al.*, 1997b:691). The percentage recovery after injection of the sample is required to be within 98 to 102%, as this percentage is seen as a ratio between the estimated and the obtained results, thus serving as an indicator of the methods accuracy (APVMA, 2004:5).

##### A.4.2.1 Accuracy analysis

For this a placebo nano-emulsion was prepared as follows:

1. Weigh approximately 400 mg of each statin into a 100 ml volumetric flask and dissolve with methanol. This will serve as the spike solution.
2. Transfer 0.8 ml, 1.0 ml and 1.2 ml of the placebo into 100 ml volumetric flasks in triplicate.
3. Transfer 4 ml of the spike solution with a glass pipette into each 100 ml volumetric flask containing 0.8 ml of the placebo and fill up to volume with methanol.
4. Transfer 5 ml of the spike solution with a glass pipette into each 100 ml volumetric flask containing 1.0 ml of the placebo and fill up to volume with methanol.
5. Transfer 6 ml of the spike solution with a glass pipette into each volumetric flask containing 1.2 ml of the placebo and fill up to volume with methanol.
6. Repeat this process for each of the selected statins, thus acquiring nine samples per statin.

This sample preparation will yield concentrations of approximately 160, 200 and 240 µg/ml of each statin. These samples will then be placed in auto sampler vials and analysed against a standard solution of approximately 20 mg/100 ml. Each sample will be injected twice at an injection volume of 10 µl.

**Table A.16:** Accuracy results of lovastatin

Concentration spiked µg/ml	Peak area			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
160.5	82.500	82.400	82	160.4	100.0
160.5	82.333	82.389	82	160.3	99.9
160.5	82.735	82.798	83	161.1	100.4
200.6	102.676	102.955	103	200.1	99.7
200.6	102.634	102.806	103	199.9	99.7
200.6	102.657	102.738	103	199.9	99.6
240.8	122.911	122.876	123	239.2	99.4
240.8	123.256	123.430	123	240.1	99.7
240.8	122.917	122.856	123	239.2	99.3

**Table A.17:** Statistical analysis results of lovastatin

Statistical analysis	
Mean	99.7
SD	0.3
%RSD	0.3
95% confidence intervals	
Lower limit	99.3
Upper limit	100.4
Estimated median	99.7
Confidence level (95.0%)	0.2

Lovastatin as demonstrated in Table A.17 yielded a mean percentage of 99.7%, which falls within the acceptance criteria of 98 to 102% (APVMA, 2004:5).

**Table A.18:** Accuracy results of mevastatin

Concentration spiked (µg/ml)	Peak area			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
160.7	73.427	73.549	73	158.6	98.7
160.7	73.335	73.520	73	158.5	98.7
160.7	73.455	73.611	74	158.7	98.8
200.8	91.674	91.770	92	198.0	98.6
200.8	91.906	91.814	92	198.3	98.7
200.8	91.922	91.899	92	198.4	98.8
241.0	109.833	110.002	110	237.3	98.5
241.0	110.268	110.328	110	238.1	98.8
241.0	109.354	109.659	110	236.4	98.1

**Table A.19:** Statistical analysis results of mevastatin

Statistical analysis	
Mean	98.6
SD	0.2
%RSD	0.2
95% confidence intervals	
Lower limit	98.1
Upper limit	98.8
Estimated median	98.7
Confidence level (95.0%)	0.2

The percentage recovery is required to be within 98 to 102%, as this percentage is seen as a ratio between the estimated results and the obtained results, thus serving as an indicator of the methods accuracy (APVMA, 2004:5). Shabir (2005:9) also stated that 90 – 100% could serve as an acceptable recovery range for APIs over a target concentration range of 80 – 100%. Table A19 indicates that a mean percentage of 98.6% was obtained over the chosen concentration ranges, thus falling into the acceptance criteria.

**Table A.20:** Accuracy results of simvastatin

Concentration spiked µg/ml	Peak area			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
160.6	74.500	74.200	74	162.0	100.9
160.6	74.372	74.558	74	162.3	101.1
160.6	74.581	74.690	75	162.6	101.3
200.7	92.584	92.623	93	201.8	100.5
200.7	92.153	92.265	92	200.9	100.1
200.7	92.247	92.141	92	200.9	100.1
240.9	110.626	110.773	111	241.2	100.1
240.9	111.259	111.242	111	242.4	100.6
240.9	111.354	111.056	111	242.3	100.6

**Table A.21:** Statistical analysis results of simvastatin

Statistical analysis	
Mean	100.6
SD	0.4
%RSD	0.4
95% confidence intervals	
Lower limit	100.1
Upper limit	101.3
Estimated median	100.6
Confidence level (95.0%)	0.3

Table A.21 indicated that simvastatin yielded a mean percentage (100.6%) within the acceptance criteria of 98 to 102%, as suggested by APVMA (2004:5).

**Table A.22:** Accuracy results of rosuvastatin

Concentration spiked (µg/ml)	Peak area			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
159.8	61.242	61.318	61	153.3	95.9
159.8	61.450	61.524	61	153.8	96.3
159.8	61.413	61.479	61	153.7	96.2
199.8	76.755	76.635	77	191.9	96.0
199.8	76.503	76.506	77	191.4	95.8
199.8	76.315	76.255	76	190.8	95.5
239.7	91.906	91.644	92	229.6	95.8
239.7	91.827	91.808	92	229.7	95.8
239.7	91.601	91.292	91	228.8	95.4

**Table A.23:** Statistical analysis results of rosuvastatin

Statistical analysis	
Mean	95.9
SD	0.3
%RSD	0.3
95% confidence intervals	
Lower limit	95.4
Upper limit	96.3
Estimated median	95.8
Confidence level (95.0%)	0.2

Although the percentage recovery (as seen in Table A.23) of rosuvastatin (95.9%) was lower compared to results obtained from the other statins, this is still seen as acceptable according to Shabir (2005:322), as 90 – 100% could serve as acceptable recovery range for APIs over a target concentration range of 80 – 100%.

#### A.4.3 Precision

The precision of an analytical procedure can be expressed as the closeness of agreement obtained between a series of measurements from multiple sampling of the same standardised sample, under the stipulated conditions (ICH, 2005:4; Snyder *et al.*, 1997b:690; Viganò *et al.*, 2018:3). Precision for this analytical method was examined firstly, in terms of intra-day precision (repeatability) and secondly, according to inter-day precision (reproducibility).

#### A.4.3.1 Intra-day precision (repeatability)

The repeatability of an analytical method describes precision (intra-day) of the method while under similar operating conditions during a short period. This assay can be performed by preparing a minimum of nine samples consisting of three different concentrations. Samples were prepared as described in Section A.4.2.1 and analysed in duplicate by means of HPLC on the same day. The acceptance criteria for intra-day precision are considered as an RSD equal or less than 2.00% (APVMA, 2004:5; Snyder *et al.*, 1997b:691).

**Table A.24:** Repeatability results of mevastatin

Concentration spiked µg/ml	Peak area			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
162.268	73.5	73.2	73.3	163.4	100.7
162.268	73.6	73.7	73.7	164.1	101.1
162.268	73.9	74.4	74.1	165.2	101.8
202.835	92.5	92.7	92.6	206.3	101.7
202.835	92.7	92.6	92.7	206.5	101.8
202.835	91.8	91.9	91.8	204.6	100.9
243.402	109.1	109.2	109.1	243.2	99.9
243.402	109.1	109.2	109.1	243.2	99.9
243.402	109.8	109.5	109.6	244.3	100.4
				<b>Mean</b>	100.92
				<b>SD</b>	0.71
				<b>%RSD</b>	0.70

**Table A.25:** Repeatability results of lovastatin

Concentration spiked µg/ml	Peak area			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
160.140	81.1	81.3	81.2	166.9	104.2
160.140	81.5	81.3	81.4	167.3	104.5
160.140	81.8	81.8	81.8	168.1	105.0
200.175	100.7	100.7	100.7	206.9	103.4
200.175	101.1	101.2	101.1	207.9	103.9
200.175	101.5	101.6	101.6	208.7	104.3
240.210	119.9	120.0	119.9	246.5	102.6
240.210	120.8	120.7	120.8	248.2	103.3
240.210	120.1	120.1	120.1	246.9	102.8
				<b>Mean</b>	103.77
				<b>SD</b>	0.75
				<b>%RSD</b>	0.73

**Table A.26:** Repeatability results of simvastatin

Concentration spiked µg/ml	Peak area			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
162.228	73.3	73.5	73.4	166.5	102.7
162.228	74.1	74.0	74.0	167.9	103.5
162.228	74.2	74.3	74.3	168.4	103.8
202.785	93.0	92.9	93.0	210.8	104.0
202.785	92.5	92.6	92.6	209.9	103.5
202.785	93.1	92.9	93.0	210.9	104.0
243.342	109.6	109.7	109.7	248.7	102.2
243.342	108.9	108.9	108.9	247.0	101.5
243.342	110.8	111.1	110.9	251.6	103.4
				<b>Mean</b>	103.18
				<b>SD</b>	0.82
				<b>%RSD</b>	0.79

**Table A.27:** Repeatability results of rosuvastatin

Concentration spiked µg/ml	Peak area			Recovery	
	Area 1	Area 2	Mean	µg/ml	%
159.780	62.353	62.269	62.3	155.8	97.5
159.780	62.481	62.425	62.5	156.2	97.7
159.780	62.119	62.046	62.1	155.2	97.2
199.725	76.901	76.951	76.9	192.4	96.3
199.725	77.073	77.226	77.1	192.9	96.6
199.725	76.997	77.090	77.0	192.7	96.5
239.670	92.673	92.715	92.7	231.8	96.7
239.670	92.334	91.786	92.1	230.2	96.1
239.670	92.184	92.142	92.2	230.5	96.2
				<b>Mean</b>	96.74
				<b>SD</b>	0.57
				<b>%RSD</b>	0.58

From Tables A.24 – A.27, it can be observed that the results obtained from this procedure were satisfactory for each of the intended statins (RSD ≤ 2%):

- Mevastatin: 0.70%
- Lovastatin: 0.73%
- Simvastatin: 0.79%
- Rosuvastatin: 0.58%

#### A.4.3.2 Inter-day precision (reproducibility)

Reproducibility of the analytical methods was performed by analysis of three homogenous samples with approximately equivalent known concentrations on three consecutive days. This data expressed the analytical method precision between different laboratories.

Samples were prepared as stated in Section A.4.2.1, but only the intermediate concentration was prepared for the purpose of this assay. These samples were analysed in duplicate on the HPLC. According to Rafael *et al.* (2007:100), acceptable criteria for inter-day precision should be equal to or less than 3.00%.

**Table A.28:** Reproducibility results of mevastatin

	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	
	101.7	96.7	95.6	
	101.8	97.0	95.9	
	100.9	97.4	96.0	
				<b>Between days</b>
<b>Mean</b>	101.46	97.04	95.85	98.12
<b>SD</b>	0.41	0.26	0.17	2.43
<b>%RSD</b>	0.41	0.27	0.17	2.48

**Table A.29:** Reproducibility results of lovastatin

	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	
	103.4	99.5	97.1	
	103.9	98.7	97.9	
	104.3	98.6	97.7	
				<b>Between days</b>
<b>Mean</b>	103.84	98.92	97.57	100.11
<b>SD</b>	0.36	0.38	0.37	2.72
<b>%RSD</b>	0.35	0.38	0.38	2.72

**Table A.30:** Reproducibility results of simvastatin

	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	
	104.0	98.5	98.1	
	103.5	98.4	97.8	
	104.0	98.7	97.9	
				<b>Between days</b>
<b>Mean</b>	103.84	98.55	97.96	100.11
<b>SD</b>	0.22	0.13	0.14	2.65
<b>%RSD</b>	0.21	0.14	0.14	2.65

**Table A.31:** Reproducibility results of rosuvastatin

	Day 1	Day 2	Day 3	
	96.3	95.4	94.3	
	96.6	94.4	94.1	
	96.5	94.1	93.9	
				<b>Between days</b>
<b>Mean</b>	96.46	94.66	94.09	95.07
<b>SD</b>	0.11	0.56	0.18	1.07
<b>%RSD</b>	0.12	0.59	0.19	1.12

The results as observed from Tables A.28 – A.31 exhibit that each of the selected statins inter-day precision fell within the acceptance criteria ( $RSD \leq 3.00\%$ ):

- Mevastatin: 2.48%
- Lovastatin: 2.72%
- Simvastatin: 2.65%
- Rosuvastatin: 1.12%

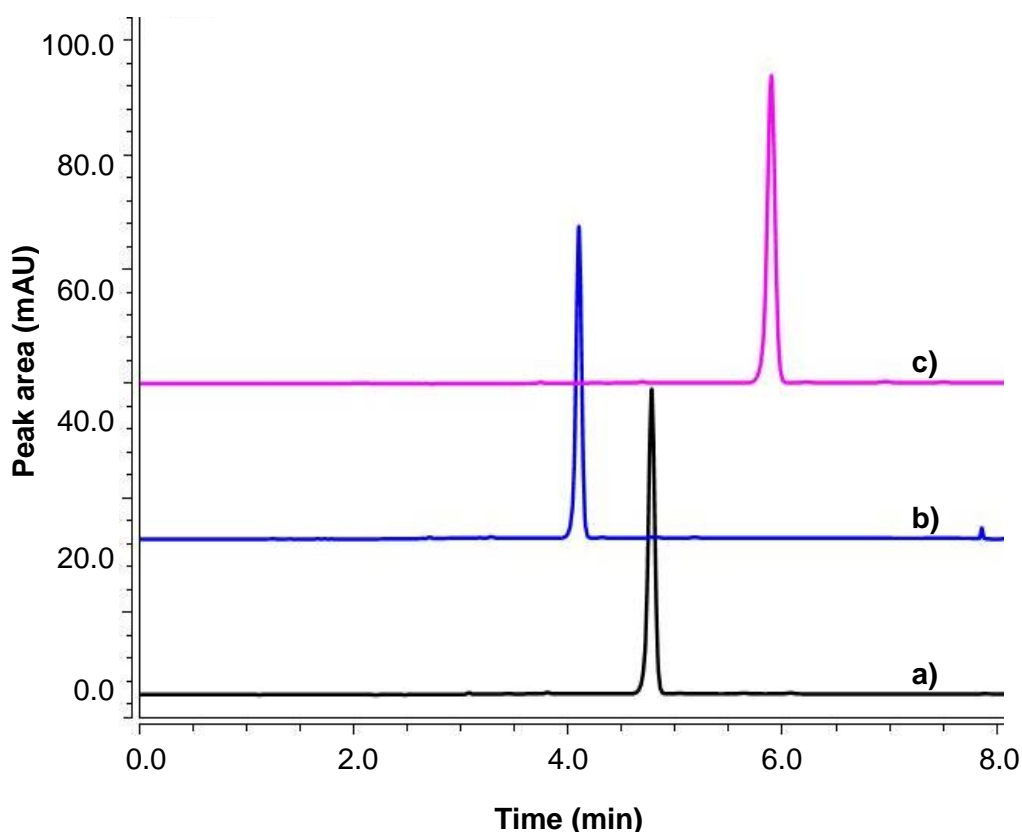
#### A.4.4 Robustness

The capacity of an analytical method to be impervious to small though deliberate changes in the parameters of the method is seen as robustness of the analytical method (Naseef *et al.*, 2018:3). This is indicative of the reliability during normal usage (ICH, 2005:5). For this validation test, a standard solution was prepared by placing 20 mg of each of the selected statins into 100 ml volumetric flask respectively. The 100 ml volumetric flasks were then made up to volume with methanol and placed in an ultrasonic bath to ensure proper dissolution. A volume of each standard solution was then placed in HPLC flasks for analysis.

The standard solution for each of the selected statins was injected three times in total. The first injection was done under the normal chromatographic parameters (1.0 ml flow rate, 240 nm wavelength, and 45% acetonitrile) and utilised as a reference. The second injection (1.2 ml/min flow rate, 235 nm wavelength, and 40% acetonitrile) and third injection (0.8 ml/min flow rate, 230nm wavelength, and 37% acetonitrile) were performed using varied parameters. Tables A.32 – A.35 represent the robustness data obtained for each of the respective statins and Figures A.6 – A.9 the compared chromatograms obtained from the second and third injection to that of the standard injected under normal chromatogram parameters.

**Table A.32:** Robustness data for lovastatin

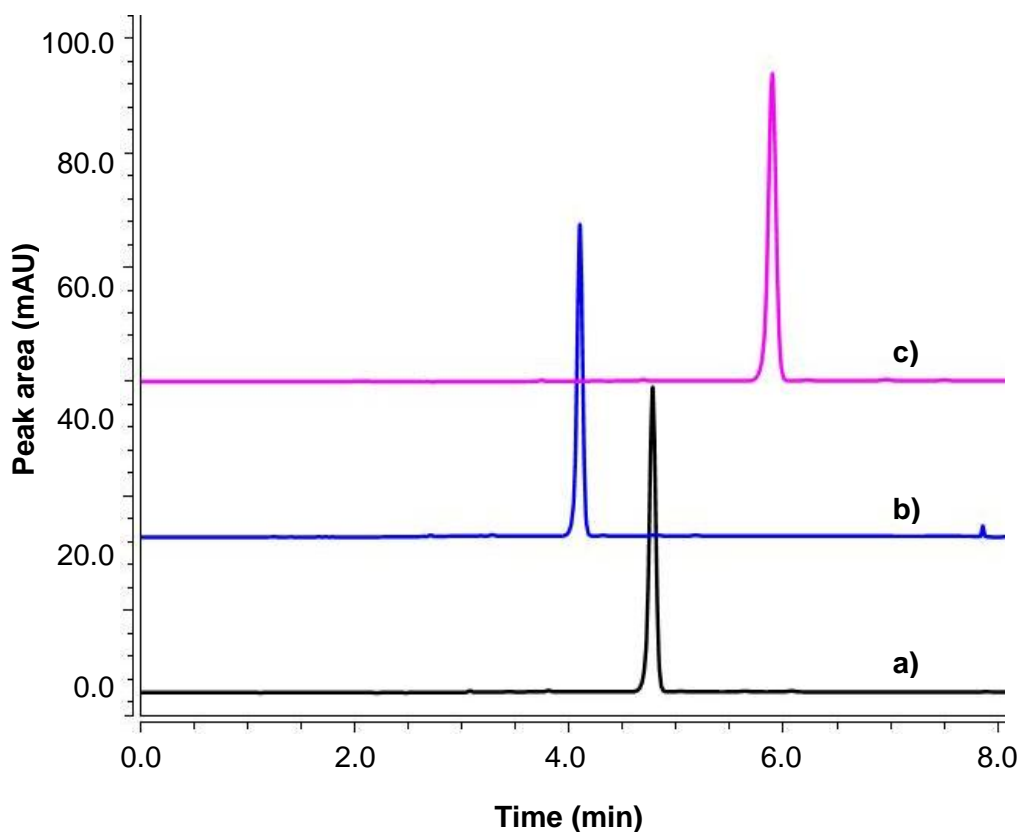
Sample method	HPLC parameters				
	%ACN mobile phase	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)	Peak area (mAU)
Standard method	45	1.0	240	5.150	99.003
Method 2	40	1.2	235	4.447	79.168
Method 3	37	0.8	230	6.407	104.917



**Figure A.6:** Lovastatin HPLC chromatogram representing the robustness data of a standard solution injected at different test parameters: a) normal conditions of 1.0 ml/min flow rate, 240 nm wavelength and 45% acetonitrile, b) 1.2 ml/min flow rate, 235 nm wavelength and 40% acetonitrile and c) 0.8 ml/min flow rate, 230 nm wavelength and 37% acetonitrile

**Table A.33:** Robustness data for mevastatin

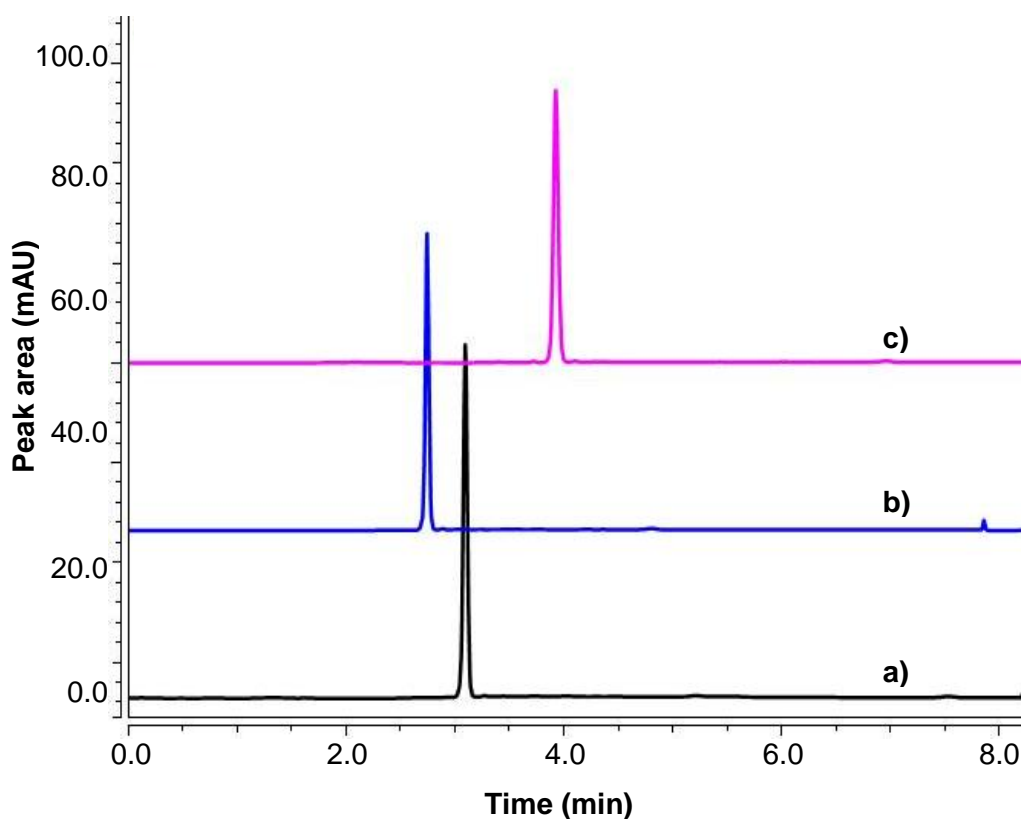
Sample method	HPLC parameters				
	%ACN mobile phase	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)	Peak area (mAU)
Standard method	45	1.0	240	4.730	94.272
Method 2	40	1.2	235	4.100	83.820
Method 3	37	0.8	230	5.897	112.216



**Figure A.7:** Mevastatin HPLC chromatogram representing the robustness data of a standard solution injected at different test parameters: a) normal conditions of 1.0 ml/min flow rate, 240 nm wavelength and 45% acetonitrile, b) 1.2 ml/min flow rate, 235 nm wavelength and 40% acetonitrile and c) 0.8 ml/min flow rate, 230 nm wavelength and 37% acetonitrile

**Table A.34:** Robustness data for rosuvastatin

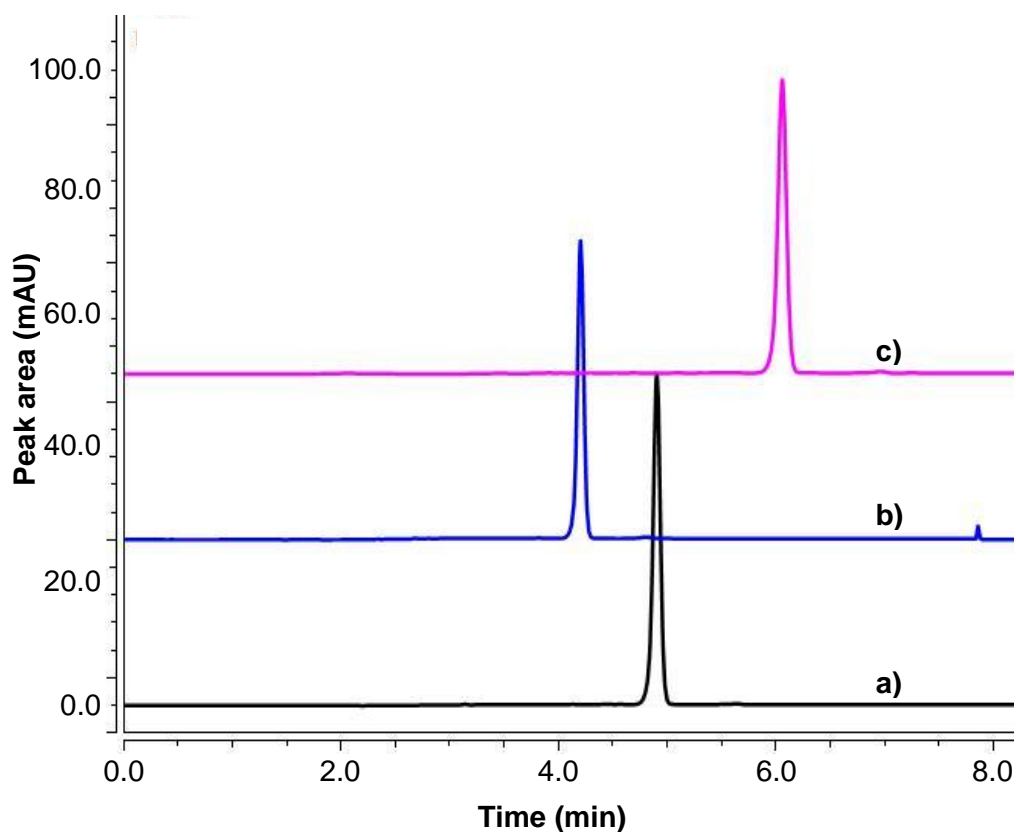
Sample method	HPLC parameters				
	%ACN mobile phase	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)	Peak area (mAU)
Standard method	45	1.0	240	3.093	75.941
Method 2	40	1.2	235	2.743	56.178
Method 3	37	0.8	230	3.927	70.729



**Figure A.8:** Rosuvastatin HPLC chromatogram representing the robustness data of a standard solution injected at different test parameters: a) normal conditions of 1.0 ml/min flow rate, 240 nm wavelength and 45% acetonitrile, b) 1.2 ml/min flow rate, 235 nm wavelength and 40% acetonitrile and c) 0.8 ml/min flow rate, 230 nm wavelength and 37% acetonitrile

**Table A.35:** Robustness data for simvastatin

Sample method	HPLC parameters				
	%ACN mobile phase	Flow rate (ml/min)	Wavelength (nm)	Retention time (min)	Peak area (mAU)
Standard method	45	1.0	240	4.853	88.397
Method 2	40	1.2	235	4.203	71.882
Method 3	37	0.8	230	6.057	98.685



**Figure A.9:** Simvastatin HPLC chromatogram representing the robustness data of a standard solution injected at different test parameters: a) normal conditions of 1.0 ml/min flow rate, 240 nm wavelength and 45% acetonitrile, b) 1.2 ml/min flow rate, 235 nm wavelength and 40% acetonitrile and c) 0.8 ml/min flow rate, 230 nm wavelength and 37% acetonitrile

From Tables A.32 – A.35 and Figures A.6 – A.9, it can be observed that the variation in peak areas and retention times for each of the selected statins during the second and third injection was minimal compared to that of the first injection utilising the normal chromatographic parameters. The shifts in peak retention times for each of the selected statins were as follows:

- Lovastatin: 4 – 7 min
- Mevastatin: 4 – 6 min
- Rosuvastatin: 2 – 4 min
- Simvastatin: 4 – 6 min

#### A.4.5 Ruggedness

The ruggedness of an analytical method can be defined as the ability of the method to reproduce results when used under actual conditions, hence determining the variability of the method (Snyder *et al.*, 1997b:701). Sample stability and system repeatability can be utilised for this test.

#### A.4.5.1 Sample stability

The ability of a sample to resist decomposition and physical disintegration or chemical changes is referred to as sample stability (UNODC, 2009:63). The sample stability is measured by determining the %RSD, which is indicative of the amount of degradation that occurred; this analysis is done under predetermined conditions and in hourly intervals. The acceptance criteria is considered to be a %RSD equal or less than 2% (Shabir, 2003:59; Suresh *et al.*, 2010:126). This analysis is performed so that the sample solution will not be used for a period longer than it takes to degrade by 2%.

**Table A.36:** Results of sample stability of lovastatin

Time (h)	Peak area	%Remaining
0	107.900	100.0
1	105.265	97.6
2	105.295	97.6
3	105.300	97.6
4	105.200	97.5
5	105.148	97.4
6	105.583	97.9
7	105.055	97.4
8	104.667	97.0
9	104.400	96.8
10	104.365	96.7
11	104.187	96.6
12	103.895	96.3
13	103.930	96.3
14	103.968	96.4
15	103.981	96.4
16	103.811	96.2
17	103.705	96.1
18	103.567	96.0
19	103.377	96.2
20	103.346	95.8
21	103.286	95.7
22	103.472	95.9
23	103.499	95.9
24	103.273	95.5
<b>Mean</b>	104.4	96.7
<b>SD</b>	1.03	0.96
<b>%RSD</b>	0.99	0.99

**Table A.37:** Results of sample stability of mevastatin

<b>Time (h)</b>	<b>Peak area</b>	<b>%Remaining</b>
0	100.5	100.0
1	101.7	101.2
2	100.4	99.9
3	100.0	99.5
4	100.0	99.5
5	99.8	99.3
6	99.5	99.0
7	99.4	98.9
8	99.2	98.7
9	99.2	98.7
10	98.9	98.4
11	99.0	98.5
12	98.8	98.3
13	98.6	98.1
14	98.7	98.2
15	98.4	97.9
16	98.1	97.6
17	98.3	97.8
18	99.9	99.4
19	97.6	97.1
20	97.3	96.8
21	97.4	96.9
22	97.2	96.7
23	96.9	96.4
24	96.7	96.2
<b>Mean</b>	98.9	98.4
<b>SD</b>	1.23	1.21
<b>%RSD</b>	1.24	1.23

**Table A.38:** Results of sample stability of simvastatin

<b>Time (h)</b>	<b>Peak area</b>	<b>%Remaining</b>
0	101.354	100.0
1	100.766	99.4
2	100.374	99.0
3	100.318	99.0
4	100.347	99.0
5	100.034	98.7
6	99.926	98.5
7	99.963	98.6
8	99.844	98.5
9	99.825	98.5
10	99.780	98.5
11	99.639	98.3
12	99.549	98.2
13	99.511	98.2
14	99.388	98.1
15	99.254	97.9
16	99.164	97.8
17	99.232	97.9
18	99.083	97.8
19	99.080	97.8
20	99.125	97.8
21	99.068	97.7
22	98.731	97.4
23	99.012	97.7
24	99.288	98.0
<b>Mean</b>	99.7	98.3
<b>SD</b>	0.61	0.60
<b>%RSD</b>	0.61	0.61

**Table A.39:** Results of sample stability of rosuvastatin

<b>Time (h)</b>	<b>Peak area</b>	<b>%Remaining</b>
0	80.764	100.0
1	80.440	99.6
2	80.301	99.4
3	80.003	99.1
4	79.856	98.9
5	79.978	99.0
6	79.830	98.8
7	79.686	98.7
8	79.747	98.7
9	79.599	98.6
10	79.405	98.3
11	79.171	98.0
12	79.171	98.0
13	79.157	98.0
14	79.155	98.0
15	78.993	97.8
16	78.886	97.7
17	78.737	97.5
18	78.728	97.5
19	78.878	97.7
20	78.848	97.6
21	78.956	97.8
22	78.703	97.5
23	78.645	97.4
24	78.899	97.7
<b>Mean</b>	79.4	98.3
<b>SD</b>	0.59	0.73
<b>%RSD</b>	0.74	0.74

Tables A.36 – A.39 display the results obtained after injecting each of the selected statins every hour for 24 h. These Tables illustrate that all the selected statins obtained results within the acceptance criteria of < 2% RSD.

#### A.4.5.2 System repeatability

With the purpose of determining the repeatability of the system, samples were prepared of each of the selected statins by weighing approximately 20 mg into a 100 ml volumetric flask, and made up to volume with methanol. These samples were injected into the HPLC seven consecutive times. This was done to evaluate the repeatability of peak areas as well as retention times of samples under same day conditions. Cowley (2012:114) stated a %RSD of 2.00% or less is acceptable.

**Table A.40:** Results of system repeatability of mevastatin

	Peak area	Retention time (min)
	96	4.767
	96	4.767
	96	4.767
	96	4.767
	96	4.763
	96	4.763
	96	4.763
<b>Mean</b>	96	4.765
<b>SD</b>	0.10	0.002
<b>%RSD</b>	0.10	0.035

**Table A.41:** Results of system repeatability of lovastatin

	Peak area	Retention time (min)
	113	5.197
	110	5.193
	110	5.190
	110	5.193
	110	5.190
	110	5.190
	110	5.190
<b>Mean</b>	110	5.192
<b>SD</b>	1.01	0.002
<b>%RSD</b>	0.92	0.047

**Table A.42:** Results of system repeatability of simvastatin

	<b>Peak area</b>	<b>Retention time (min)</b>
	95	4.883
	95	4.883
	95	4.883
	95	4.883
	95	4.883
	95	4.883
	95	4.883
<b>Mean</b>	95	4.883
<b>SD</b>	0.12	0.000
<b>%RSD</b>	0.12	0.000

**Table A.43:** Results of system repeatability of rosuvastatin

	<b>Peak area</b>	<b>Retention time (min)</b>
	5720.4	4.009
	5725.3	4.003
	5722.1	4.012
	5716.8	4.013
	5710.7	4.003
	5741.8	4.012
<b>Mean</b>	5722.9	4.009
<b>SD</b>	9.63	0.004
<b>%RSD</b>	0.17	0.105

By observing Tables A.40 – A.43, it is clear the system performed well within the acceptance criteria, with %RSD for peak area as well as retention times well below 2 for all the selected statins.

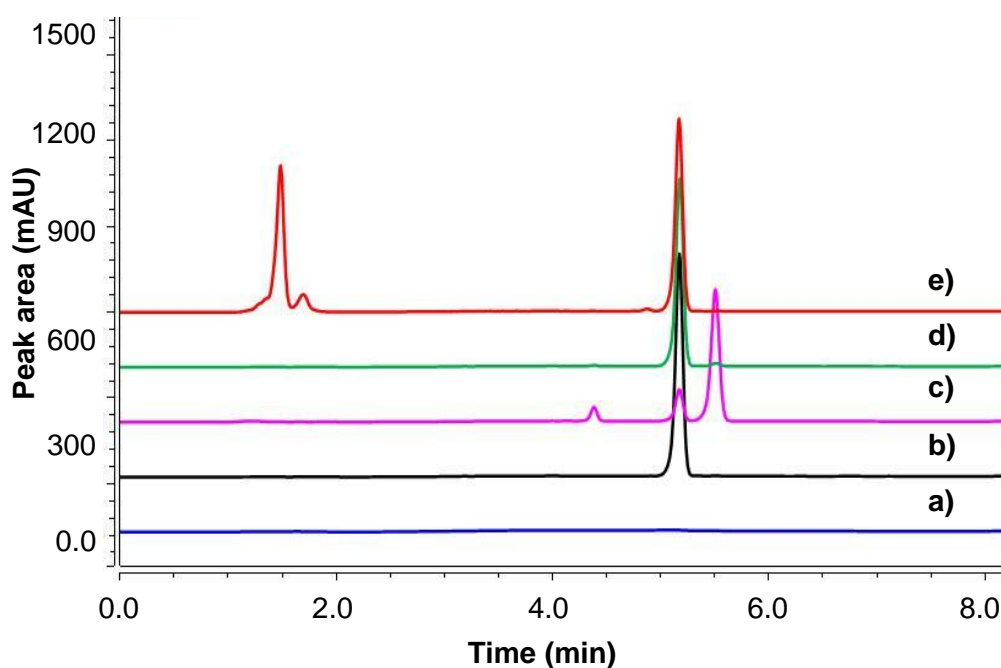
#### **A.4.6 Specificity**

Specificity of an analytical method can be defined as the ability of the method to detect an analyte accurately in the presence of degradants, the matrix or impurities. Acceptable specificity can be seen as the pure identification of the target analyte in the presence of similarly

structured compounds (ICH 2005:4), therefore there should be no peak interference with the retention time of the API (Snyders *et al.*, 1997b:700).

The six specificity samples were prepared as follows:

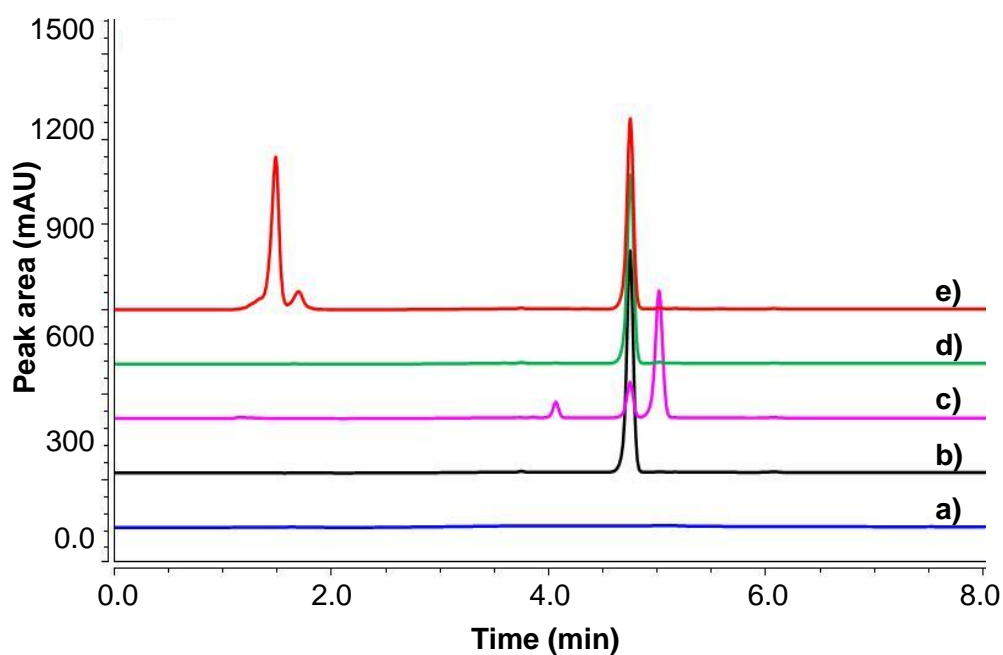
1. A placebo sample was prepared by filling a vial with methanol.
2. A standard solution was prepared by weighing approximately 20 mg of each of the selected statins into a 100 ml volumetric flask. A volume was placed in a HPLC vial.
3. The standard solution was used to prepare four samples by placing 1 ml into four individual test tubes. The standard solution was diluted by adding 200  $\mu$ l of water, 2 M hydrochloric acid (HCl), 2 M sodium hydroxide (NaOH) and 2 M hydrogen peroxide ( $H_2O_2$ ) into the respective test tubes and mixed by vortexing.
4. The test tubes were left to stand for a few minutes and thereafter a volume of each test tube was placed into a HPLC vial.
5. The samples were injected in duplicate into the HPLC with a run time of 10 min.



**Figure A.10:** HPLC chromatogram showing specificity data obtained for a) a placebo solution, b) lovastatin standard solution, following the sample solution of lovastatin stressed with 200  $\mu$ l of c) HCl, d)  $H_2O$  and e)  $H_2O_2$

**Table A.44:** Specificity data for lovastatin

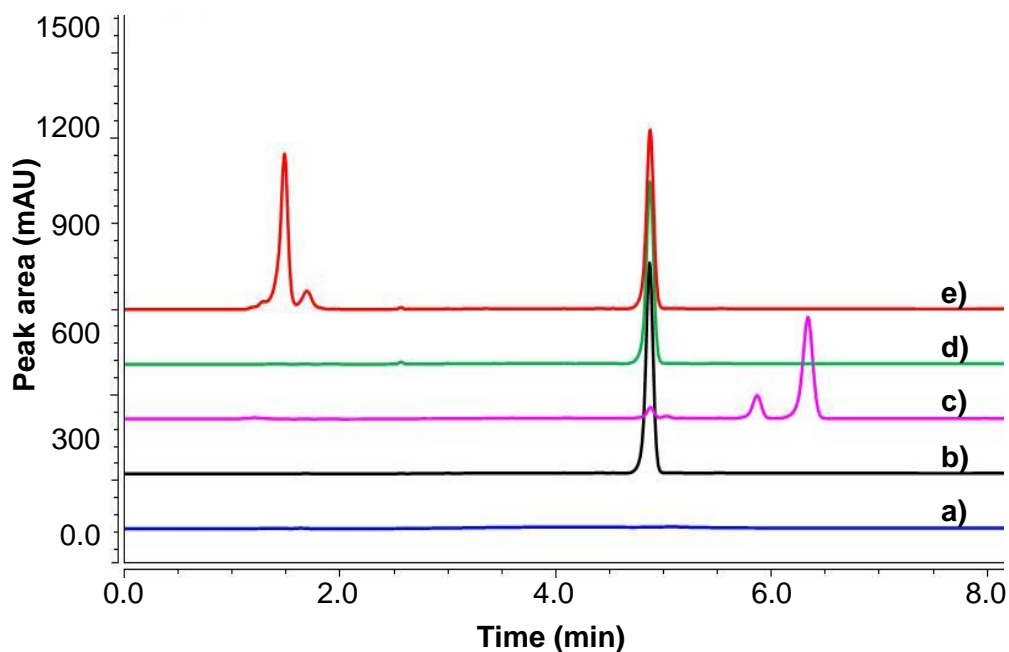
Sample	Peak area (mAU)			Retention time (min)
	1	2	Mean	
Placebo (a)	0.000	0.000	0.000	0.000
Standard solution (b)	111.019	111.024	111.021	5.175
HCl (c)	17.571	17.567	17.567	5.173
H <sub>2</sub> O (d)	94.406	94.334	94.370	5.170
H <sub>2</sub> O <sub>2</sub> (e)	96.299	96.435	96.367	5.173



**Figure A.11:** HPLC chromatogram showing specificity data obtained for a) a placebo solution, b) mevastatin standard solution, following the sample solution of mevastatin stressed with 200 µl of c) HCl, d) H<sub>2</sub>O and e) H<sub>2</sub>O<sub>2</sub>

**Table A.45:** Specificity data for mevastatin

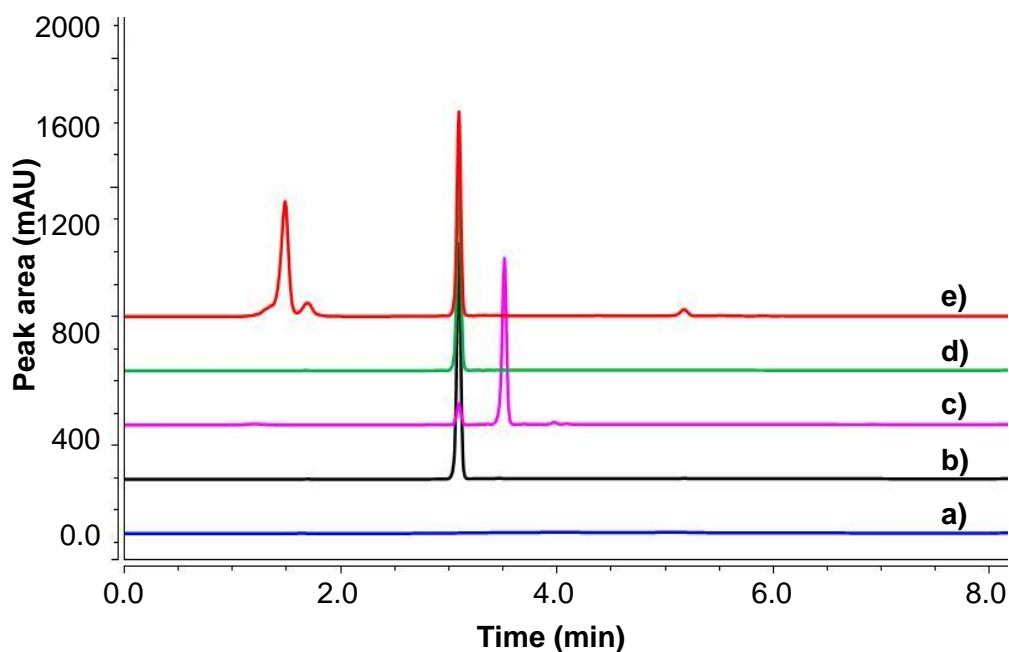
Sample	Peak area (mAU)			Retention time (min)
	1	2	Mean	
Placebo (a)	0.000	0.000	0.000	0.000
Standard solution (b)	98.835	98.395	98.615	4.750
HCl (c)	16.839	16.384	16.612	4.750
H <sub>2</sub> O (d)	84.204	84.075	84.140	4.753
H <sub>2</sub> O <sub>2</sub> (e)	85.237	85.446	85.342	4.753



**Figure A.12:** HPLC chromatogram showing specificity data obtained for, a) a placebo solution, b) simvastatin standard solution, following the sample solution of simvastatin stressed with 200  $\mu$ l of c) HCl, d) H<sub>2</sub>O and e) H<sub>2</sub>O<sub>2</sub>

**Table A.46:** Specificity data for simvastatin

Sample	Peak area (mAU)			Retention time (min)
	1	2	Mean	
Placebo (a)	0.00	0.00	0.00	0.00
Standard solution (b)	103.037	103.103	103.070	4.874
HCl (c)	5.877	6.174	6.026	4.877
H <sub>2</sub> O (d)	89.886	89.757	89.822	4.873
H <sub>2</sub> O <sub>2</sub> (e)	88.530	88.630	88.580	4.877



**Figure A.13:** HPLC chromatogram showing specificity data obtained for a) a placebo solution, b) rosuvastatin standard solution, following the sample solution of rosuvastatin stressed with 200  $\mu$ l of c) HCl, d) H<sub>2</sub>O and e) H<sub>2</sub>O<sub>2</sub>

**Table A.47:** Specificity data for rosuvastatin

Sample	Peak area (mAU)			Retention time (min)
	1	2	Mean	
Placebo (a)	0.00	0.00	0.00	0.00
Standard solution (b)	83.386	83.455	83.421	3.090
HCl (c)	8.346	8.053	8.199	3.093
H <sub>2</sub> O (d)	71.088	70.903	70.996	3.093
H <sub>2</sub> O <sub>2</sub> (e)	71.713	71.779	71.746	3.093

From Tables A.44 – A.47 and Figures A.10 – A.13, it can be observed that the placebo affected none of the selected statins. Although Figures A.10 – A.13 indicate additional peaks formed due to degradation caused by stressing the sample, the retention times of the respective statins were not affected:

- Lovastatin:  $\pm$  5.17 min
- Mevastatin:  $\pm$  4.75 min
- Simvastatin:  $\pm$  4.87 min
- Rosuvastatin:  $\pm$  3.09 min

The samples stressed with NaOH however displayed peak interference with that of the selected statin. From Tables A.44 – A.47 (peak areas), it is clear that in the samples containing HCl, more degradation occurred, but there was no interference with the retention times of the respective statins. This could be expected as simvastatin can degrade by 50% in the presence of 0.01 M HCl after 30 min, whereas 2.00 M was utilised in this test (Malenović *et al.*, 2018:542). Kaila *et al.* (2010:596) also established degradation of rosuvastatin in acidic (HCl) medium, in conjunction with a second peak, as seen in Figure A.13.c. Lovastatin formed two acid degradation (at  $\pm 4.5$  min and  $\pm 4.8$  min) products, which correlated with what is described in literature (Álvarez-Lueje *et al.*, 2005:641). No data could be found for mevastatin.

Snyders *et al.* (1997b:700) stated that if there is no peak interference with the retention time of the respective API, the analytical method can be considered specific. Hence, from Tables A44 – A.47 it can be concluded the method was specific.

## **A.5 Conclusion**

The HPLC method for the selected statins (mevastatin, lovastatin, rosuvastatin and simvastatin) was validated successfully. Hence, the method will be able to quantify the selected statins in both the nano-emulsions and semisolid dosage forms (nano-emulgels). The acceptance criteria were thus met by the method and this enables the usage of the method during the *in vitro* skin diffusion studies to quantify the selected statins.

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## *APPENDIX B:*

# *The formulation of o/w nano-emulsions separately containing the selected statins and apricot kernel oil*

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### **B.1 Introduction**

Transdermal drug delivery before 1980 was limited to a small number of compounds (e.g. oestradiol) that was formulated in basic gels and ointments. These conventional vehicles presented challenges, regarding inadequate control over the area of skin exposed, quantity of API applied, ineffectiveness and elegance. Hence, substantial inconsistencies occurred with respects to the extent and duration of the APIs effect (Wiedersberg & Guy, 2014:150).

Since transdermal drug delivery poses many advantages over other routes of administration (i.e. parenteral and oral), which includes avoidance of first-past metabolism, enhanced patient compliance, steady-state drug levels, and reduced gastrointestinal effects (Walter & Xu, 2015:1293), it can possibly be utilised to overcome adverse effects associated with the oral administration of statins, e.g. liver disease (Law & Rudnicka, 2006:58C) and gastrointestinal effects (Mancini *et al.*, 2013:1557). However, the one inevitable disadvantage is the barrier the skin poses (Lovelyn & Attama, 2011:630; Naik *et al.*, 2000:319).

This barrier, which is essential to overcome in transdermal delivery, is primarily caused by the outermost layer of the skin, namely the stratum corneum (Mueller *et al.*, 2016:2006). The stratum corneum causes this barrier (for an API to cross the skin layers) due to regulating of API flux and acting as a rate-limiting step (El Maghraby *et al.*, 2008:204; Williams, 2003:28).

Despite the considerable increase in the selection of APIs for transdermal use since 1979 (Walter & Xu, 2015:1293), the required characteristics for an API to be suitable for transdermal delivery (i.e. molecular mass of < 500 Da and aqueous solubility of > 1 mg/ml (Naik *et al.*, 2000:319)) still poses extreme challenges (Münch *et al.*, 2017:236). Ultimately, a meticulous investigation of the intended API's physiochemical properties is essential in the formulation of a product to obtain successful topical or transdermal delivery (Williams, 2013:685). Furthermore, the selection of an acceptable non-irritating delivery system, compatible with the intended API, is imperative (Weiss, 2011:471). For the purpose of this study, a nano-emulsion has been chosen as a possible transdermal delivery system. Nano-emulsions have shown to enhance the permeation of APIs, when compared to other conventional formulations (i.e. emulsions and gels). Currently, nano-emulsions are considered to act as penetration enhancers themselves due to the excipients used (Reza, 2011:1942). Lipophilic drugs, such as statins, can possibly

be delivered transdermally by utilising o/w nano-emulsions as delivery system (Chime *et al.*, 2014:98).

## **B.2 The purpose and selection of a novel delivery system**

The intended purpose of a new formulation should be well considered during the formulation process. The aim in this study was to investigate the transdermal delivery of the selected statins. Although the selected statins exhibit favourable physiochemical properties in some areas (i.e. molecular mass), others such as solubility, can impede transdermal delivery.

When attempting topical or transdermal delivery, the challenge lies not only with the intended API, but also with the selection of an appropriate vehicle with which the drug is formulated. This vehicle should not only accommodate the intended API, but should be cosmetically refined and non-irritating. The effectiveness of transdermal delivery can be defined as the efficient delivery of an API through the skin (Weiss, 2011:471). Effective release of the API, as well as numerous other aspects can influence drug flux and transdermal or topical delivery (Barry, 2002:508). Consequently, selection of the correct vehicle for delivery is essential to bring the API in contact with the skin effectively (Weiss, 2014:472). After effective release of the API from the vehicle or delivery system, it will commence to move through the skin layers, creating more difficulties in the delivery process (Weiss, 2014:471; Wiechers, 2008:7).

In view of this, nano-emulsions were selected as the drug delivery system in an attempt to penetrate the barrier of the skin successfully. An o/w nano-emulsion was formulated containing apricot kernel oil and each of the selected statins separately.

## **B.3 Excipients used to formulate a nano-emulsion**

The formulation of nano-emulsions necessitates the use of four main excipients or components, namely aqueous material or water, oil, surfactant(s) and co-surfactant(s) (Basera *et al.*, 2015:1878; Chime *et al.*, 2014:90; Reddy *et al.*, 2013:87; Setya *et al.*, 2014:2218; Thakur *et al.*, 2012:222). It is of utmost importance that the excipients chosen to formulate the nano-emulsion are compatible with one another, especially where the oil is concerned (Reddy *et al.*, 2013:87), as the chosen oil component can influence the topical or transdermal delivery in both w/o and o/w nano-emulsions (Zhang *et al.*, 2011:702). The use of surfactants in nano-emulsions contributes greatly to the successful formulation of these systems, since they cause a decrease in interfacial tensions between two immiscible liquids to make them miscible. The coalescence of newly formed drops is also prevented by these surface-active agents (Chime *et al.*, 2014:92; Reddy *et al.*, 2013:87; Setya *et al.*, 2014:2218; Tadros *et al.*, 2004:305). The choice between formulating an o/w or w/o nano-emulsions is largely determined by the API intended for use in the formulation, as the API will be incorporated into the core component, thus either being oil or

water (Chime *et al.*, 2014:77; Kela & Kaur, 2013:9203). In this study, the aim was to formulate an o/w nano-emulsion containing 2% (w/w) of each selected statin with both 8.0% (w/w) and 10.5% (w/w) apricot kernel oil. Different surfactant ratios were also applied between the two formulations and the optimal formulation was chosen accordingly. Table B.1 list the excipients used to formulate the o/w nano-emulsions.

**Table B.1:** The excipient used in the formulation of o/w nano-emulsions with their function, supplier and batch number

Excipient/Component	Function	Supplier	Batch number
Statin (lovastatin, mevastatin, rosuvastatin, and simvastatin)	API	DB Fine chemicals	20170423 (lovastatin) USMV-1703006 (mevastatin) ROC/1512B/0008J1 (rosuvastatin) IF-SI-170913 (simvastatin)
Apricot Kernel oil	Oil phase (natural oil) and penetration enhancer	CJP Chemicals	1512E036197
Tween <sup>®</sup> 80	Hydrophilic surfactant	Sigma-Aldrich	BCBT9142
Span <sup>®</sup> 60	Lipophilic surfactant	Sigma-Aldrich	SLBN0127V
Milli-Q <sup>®</sup> water	Water phase and solvent	In lab	Direct Pure UP

#### B.4.1 Statins

The chemical structure of the statins (HMG-CoA reductase inhibitors) will preside over the water solubility of the compound (Schachter, 2004:117). All the selected statins are lipophilic (lovastatin, mevastatin, rosuvastatin, and simvastatin) in nature, although rosuvastatin has a relatively hydrophilic nature (Schachter, 2004:117), as described in Section 2.6.4. Lipophilic APIs are largely formulated as o/w nano-emulsions (Kela & Kaur, 2013:9203), and therefore each of the selected statins were formulated into the oil phase of the nano-emulsion. The percentage of each respective API was kept at 2% for all the formulated nano-emulsions.

#### B.4.2 Apricot kernel oil

The oil or lipids used in the preparation of nano-emulsions can be characterised as fatty acids, fatty alcohols, vegetable oils, fatty acid esters, and medium chain glycerides (Pawar & Babu, 2014:429). To obtain appropriate characteristics when formulating nano-emulsions, the selection of the oil is essential as it should possess the ability to solubilise the selected API adequately (Sarker *et al.*, 2015:968). If the API has adequate solubility within the oil phase, it enables maximum drug loading (Debnath *et al.*, 2011:74; Reddy *et al.*, 2013:87). Apricot kernel

oil was selected to act as the oil phase of the o/w nano-emulsions, as the major fatty acids present are linoleic and oleic acid, which are classified as C<sub>18</sub>-unsaturated fatty acids that can act as penetration enhancers for transdermal delivery (Santoyo & Ygartua, 2000:245). By acting as penetration enhancers, it will cause a disruption in the barrier posed by the stratum corneum, and consequently lead to increased delivery of the API (Baibhav *et al.*, 2011:68).

#### **B.4.2.1 Solubility of the selected statins in apricot kernel oil**

When formulating a nano-emulsion, the compatibility as well as the solubility of the API in the particular oil is crucial factor (Chime *et al.*, 2014:91), as this information will enable the formulator to ascertain the maximum amount of the API that can be incorporated into the nano-emulsion. To determine the solubility of the selected statins in apricot oil, a solubility test was carried out. The test was done by preheating a water-shaker bath to 32 °C, while 5 ml of apricot oil was placed into clean test tubes. An excess amount of each of the selected statins was added, each to their own tube, and clearly marked to distinguish between them (triplicate tubes for each statin). These tubes were placed into the water-shaker bath for a period of 24 h, after which they were removed. These tubes were then placed into a centrifuge (4500 rpm for 15 min) and 1 ml of the supernatant was extracted and placed into a 25 ml volumetric flask using a micropipette. The weight of the extracted amount was noted for each sample. Each of the volumetric flasks was made up to volume using tetrahydrofuran (THF). A volume of this dilution was then transferred into HPLC vials for analysis to determine the concentration of each statin in the apricot oil. After HPLC analysis was completed, it was determined that the concentration of each statin in the oil was as follows:

- Mevastatin: 0.9807 ± 0.043 mg/ml
- Lovastatin: 0.9836 ± 0.030 mg/ml
- Rosuvastatin: 0.1515 ± 0.016 mg/ml
- Simvastatin: 0.3789 ± 0.179 mg/ml

The results obtained during this test can be related to what was suggested in literature, as the higher solubility of lovastatin, mevastatin and simvastatin compared to rosuvastatin in apricot kernel oil can be due to the higher lipophilicity of the three compounds (Kim *et al.*, 2011:295).

#### **B.4.3 Emulsifiers**

Surfactants or surface acting agents are names more commonly used to describe emulsifiers (Setya *et al.*, 2014:2218). The characteristics of the specific application area, will determine the requirements with regard to the emulsifier. Emulsifiers or surfactant molecules contain properties that are soluble in water or oil. It is advantageous to determine these properties to be able to utilise the emulsifiers in the suitable area (Nagy *et al.*, 2015:580). Where nano-

emulsions are concerned, both a water and an oil phase are present, thus both a lipophilic and hydrophilic surfactant will be required for formulation (Magdassi & Garti, 1999:156; Reddy *et al.*, 2013:87) and stability of nano-emulsions will be improved by using a combination of surfactants (Setya *et al.*, 2014:2219). Eid *et al.* (2014:4) suggested that the total hydrophilic-lipophilic balance (HLB) of both surfactants should fall within 9 – 18, to enable the formulation of an o/w nano-emulsion. It is also important to consider the HLB of the oil used in the formulation, as Fernandes *et al.* (2013:108) stated that the combination HLB value of the emulsifiers should be close to that of the intended oil. The primary function of emulsifiers, when incorporated into a formulation, is to facilitate a decrease in interfacial tension, which is present between the two phases of the nano-emulsion (Setya *et al.*, 2014:2218). These agents will also endorse the emulsification process during the manufacturing of formulations (Baibhav *et al.*, 2011:68; Hyma *et al.*, 2014:4). During this study, the sorbitan esters, Span<sup>®</sup> 60 and Tween<sup>®</sup> 80, were selected as surfactants.

#### **B.4.3.1 Span<sup>®</sup> 60 (sorbitol monostearate)**

Span<sup>®</sup> 60, a non-ionic surfactant, possesses the ability to serve as an emulsifying, dispersing, suspending and wetting agent (Zhang, 2009b:675) and attributes to the stability of the formulation over time (Baibhav *et al.*, 2011:68). Literature suggests an emulsifying agent with an HLB value under 10 can be characterised as lipophilic (Reddy *et al.*, 2013:87; Zhang, 2009b:6780). Consequently, Span<sup>®</sup> 60, with a HLB of 4.7, will be incorporated into the oil phase of the o/w nano-emulsions, since it is more lipophilic in nature and will present favourable solubility in oil (Reddy *et al.*, 2013:87; Zhang, 2009b:6780).

#### **B.4.3.2 Tween<sup>®</sup> 80**

Tween<sup>®</sup> 80, also known as polysorbate 80, is commonly used as an emulsifier, stabiliser and to assist with solubility in the preparation of cosmetics, drugs and skin care products (Zhang; 2009a:550). Tween<sup>®</sup> 80 is classified as a polyoxyethylene 20 sorbitan and is considered a non-ionic surfactant. The HLB value of 15 is indicative of the hydrophilic nature of Tween<sup>®</sup> 80, and will consequently be incorporated into the water phase (Reddy *et al.*, 2013:87). As mentioned before, surfactants will enhance the stability of the nano-emulsion, therefore incorporating a hydrophilic surfactant in conjunction with lipophilic surfactant, will decrease the amount of energy needed to formulate a nano-emulsion (Chime *et al.*, 2014:91).

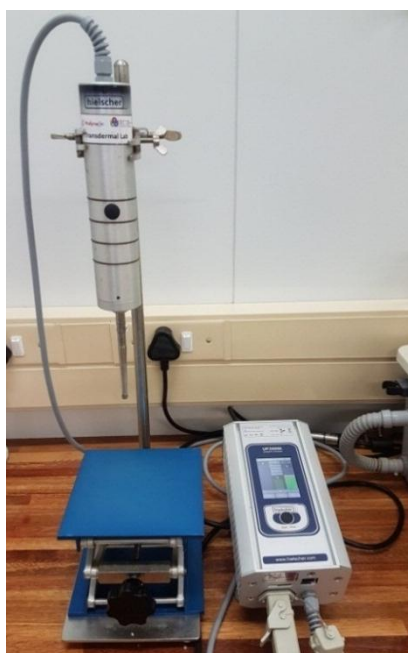
#### **B.4.4 Water**

Aqueous materials, such as water or alcohols, are used to obtain the continuous, water or hydrophilic phase of a nano-emulsion (Hyma *et al.*, 2014:4). This phase is of great importance during the formulation of an o/w nano-emulsion (Silva *et al.*, 2012:857), as this phase will serve as the largest part of the nano-emulsions. The higher the water concentration in the

formulation, the lower the viscosity of the final formulation will be (Lovelyn & Attama, 2011:626). During this study, Milli-Q® water was utilised to serve as the continuous phase of the nano-emulsions.

## B.5 Formulation of nano-emulsions

In the process to formulate a successful nano-emulsion, a trial-and-error approach was utilised to select a suitable formula by which the nano-emulsion displayed proper characteristics. While nano-emulsions are kinetically stable, they are not thermodynamically stable, as seen in the case of micro-emulsions (Kela & Kaur, 2013:9203), and therefore energy is needed for the formation of droplets in the nanometric range (Abolmaali *et al.*, 2011:141; Tadros *et al.*, 2004:303). The required energy can be applied through either low-energy emulsification or high-energy emulsification. The use of high-energy methods includes sonication, high-energy stirring and high-pressure homogenisation (Bhatt & Madhav, 2011:2294; Jaiswal *et al.*, 2015:124; Lovelyn & Attama, 2011:627; Tadros *et al.*, 2004:308), while application of low-energy emulsification includes phase inversion temperature and spontaneous emulsification. In some cases, a combination of the aforementioned is applied (Jaiswal *et al.*, 2015:124). High-energy emulsification methods are more advantageous to use as stated before, because of droplet control (small droplet size can be achieved) (Jafari *et al.*, 2007:734). Therefore, it was predetermined that high energy (by means of sonication) would be implemented as the emulsification method, because although it tends to be expensive, it is advantageous with regards to droplet control and provides a larger selection of integral components (Jafari *et al.*, 2007:734). Stable dispersions were obtained by sonicating the formulation for 3 min, with 1 min intervals, with an ultrasonic probe.



**Figure B.1:** High-energy emulsification method ultrasonicator (Model UP200St)

Hence, nanometric droplets were obtained by means of an ultrasonicator with a probe (Model UP200St, Hielscher Ultrasonics, Teltow, DE) (see Figure B.1.). The mechanism, by which these devices facilitate the creation of nano-sized droplets, is by means of high-energy forces (Bhatt & Madhav, 2011:2294; Debnath *et al.*, 2011:77), therefore an o/w nano-emulsion was formulated containing 2% (w/w) API by means of ultrasonication (high-energy emulsification). Two different surfactants, as well as oil ratios were investigated; the first formula contained 7.85% (w/w) oil and Tween<sup>®</sup> 80:Span<sup>®</sup> 60 in a ratio of 1:1, the second contained 10.78% (w/w) oil and Tween<sup>®</sup> 80:Span<sup>®</sup> 60 ratio of 1:2. The optimised formula between the two formulas was selected to formulate the semi-solid formulation and will be discussed later.

### **B.5.1 Formulation of pre-formulated o/w nano-emulsions**

The first o/w nano-emulsion formula containing 7.85% (w/w) oil and Tween<sup>®</sup> 80:Span<sup>®</sup> 60 in a ratio of 1:1 will be referred to as **(NEF1)**, which includes:

- **(NEL1)** (2% lovastatin in nano-emulsion formula 1);
- **(NEM1)** (2% mevastatin in nano-emulsion formula 1);
- **(NER1)** (2% rosuvastatin in nano-emulsion formula 1), and
- **(NES1)** (2% simvastatin in nano-emulsion formula 1).

The second formula containing 10.78% (w/w) oil and a Tween<sup>®</sup> 80:Span<sup>®</sup> 60 ratio of 1:2 will be referred to as **(NEF2)**, which includes:

- **(NEL2)** (2% lovastatin in nano-emulsion formula 2);
- **(NEM2)** (2% mevastatin in nano-emulsion formula 2), and
- **(NES2)** (2% simvastatin in nano-emulsion formula 2).

No characterisation of **(NER2)** could be performed, as no dispersion could be successfully formulated.

#### **B.5.1.1 Formulation of o/w nano-emulsions**

When preparing a nano-emulsion, four methods can be utilised. Although these methods vary from each other considerably, the main objective remains the same, which is to formulate a stable nano-emulsion with particles within the acceptable nanometre range. The selection of the appropriate method will depend greatly on the dosage form and the API intended for formulation (Sutradhar & Amin, 2013:99). Nano-emulsions is a two-phased heterogeneous system, formed by mixing main components (water and oil), and stabilised by the added surfactants (Abolmaali *et al.*, 2011:140; Chime *et al.*, 2014:77). As stated in Section B.5 it was

predetermined that sonication would be utilised to obtain nanometric droplets. The formulas based on the predetermined surfactant ratios are listed below in Tables B.2 and B.3.

**Table B.2:** Formula for **(NEF1)** dispersions (50 ml)

Phase	Excipient	Function	%(w/w)
<b>Oil phase (A)</b>	Statin	API	2.0% (1.00 g)
	Apricot kernel oil	Natural oil and penetration enhancer	8.0% (4.35 ml)
	Span <sup>®</sup> 60	Surfactant (lipophilic)	6.0% (3.00 g)
<b>Water phase(B)</b>	Milli-Q <sup>®</sup> water	Water phase and solvent	78.0% (39.00 ml)
	Tween <sup>®</sup> 80	Surfactant (hydrophilic)	6.0% (2.80 ml)

**Table B.3:** Formula for **(NEF2)** dispersions (50 ml)

Phase	Excipient	Function	%(w/w)
<b>Oil phase (A)</b>	Statin	API	2.0% (1.00 g)
	Apricot kernel oil	Natural oil and penetration enhancer	10.5% (5.98 ml)
	Span <sup>®</sup> 60	Surfactant (lipophilic)	7.0% (3.67 g)
<b>Water phase (B)</b>	Milli-Q <sup>®</sup> water	Water phase and solvent	77.0% (38.50 ml)
	Tween <sup>®</sup> 80	Surfactant (hydrophilic)	3.5% (1.71 ml)

#### B.5.1.2 Formulation method of a nano-emulsion

During the formulation of the o/w nano-emulsions (Tables B.2 and B.3) a number of methods were applied. Initially self-emulsification was used in the preparation of a coarse emulsion, followed by ultrasonication (high-energy emulsification) to obtain droplets within the required nanometric range. The stepwise method applied for both dispersions is listed below, followed by a diagrammatic representation of the entire process displayed in Figure B.2.

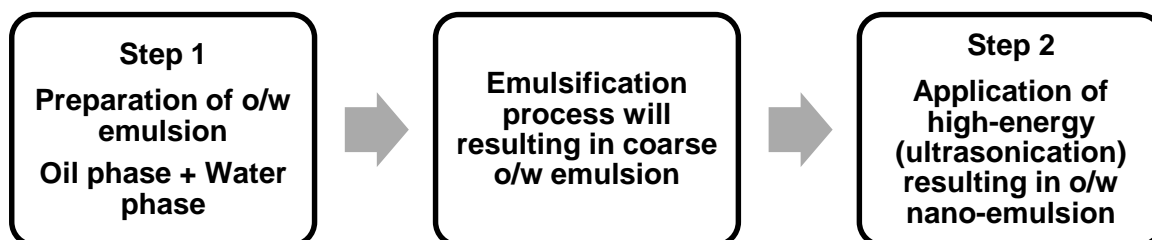
##### Step 1:

- A measuring cylinder was used to measure Milli-Q<sup>®</sup> water, which was transferred into a 100 ml beaker.
- A micropipette was then used to measure Tween<sup>®</sup> 80 (lipophilic surfactant), where after it was placed in the beaker with Milli-Q<sup>®</sup> water.
- This beaker was then placed on a hot plate with a magnetic stirrer to ensure continuous stirring of the water phase (B) until the Tween<sup>®</sup> 80 was dissolved entirely (Figure B.3.a)

- Preparation of the oil phase (A) was done by measuring apricot kernel oil by means of a micropipette and transferring it into a beaker, after which the beaker was placed on a hotplate (Figure B.3.b).
- Span<sup>®</sup> 60 was then weighed and added to the beaker containing apricot kernel oil.
- The beaker was then placed on a hot plate equipped with a magnetic stirrer until the Span<sup>®</sup> 60 was adequately dissolved (Figure B.3.c).
- The intended statin was weighed and added to the Span<sup>®</sup> 60-apricot kernel oil (phase A) and stirred on the hot plate until completely dissolved (Figure B.3.d).
- Phase A was then added drop wise to phase B with continuous stirring (Figure B.3.e).
- This final dispersion, known as the coarse emulsion, formed after phase A was completely added to phase B and left to stir on the hotplate for another 5 min (Figure B.3.f)

**Step 2:**

- The beaker containing the coarse emulsion was then removed and placed under the ultrasonic probe for a total of 3 min with 1 min rest intervals (Figure B.3.g).
- As a result, droplets within the nanometric range were obtained due to the application of high energy.



**Figure B.2:** Formulation of nano-emulsion as a diagrammatic representation

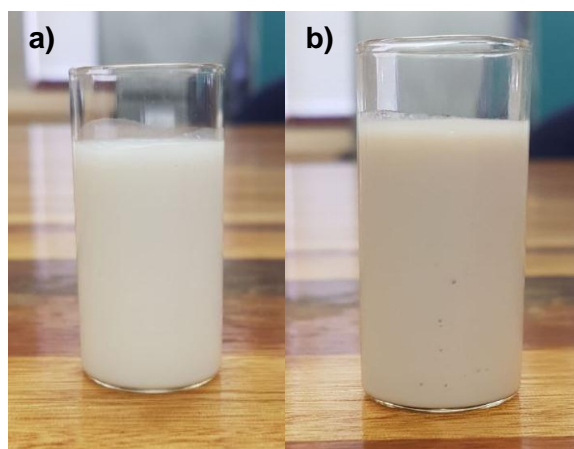


**Figure B.3:** Formulation method of the **(NEF1)**: a) Tween<sup>®</sup> 80 and water pre-heated (phase B); b) apricot kernel oil preheated; c) addition of Span<sup>®</sup> 60 to pre-heated apricot kernel oil (phase A); d) addition of the API to phase A; e) phase A added to phase B (drop wise); f) mixing of phases A and B together; g) sonication 3 min with 1 min intervals

### B.5.1.3 Outcome

During formulation, it became clear that rosuvastatin could not be formulated by utilising the **(NEF2)** formula, but although it proved challenging, the **(NEF1)** dispersion was prepared successfully as no aggregation, sedimentation or visible oil droplets were present. The remaining statins (lovastatin, mevastatin, and simvastatin) were formulated successfully utilising both **(NEF1)** and **(NEF2)** dispersions. Post formulation, during visual examinations, it was clear that the oil droplets properly dispersed into the water phase, as no oil droplets were visible. These formulations also showed no indication of the presence of sedimentation or aggregation.

The dispersions looked similar to one another, with the exception of **(NES1)** and **(NES2)**, as these dispersions displayed a beige colour, while the other dispersions were white. Examples of the dispersions are displayed in Figure B.4.



**Figure B.4:** The formulated dispersions: a) all **(NEF1)** and **(NEF2)** dispersions, except b) **(NES1)** and **(NES2)**

## B.6 Characterisation of the pre-formulated nano-emulsions

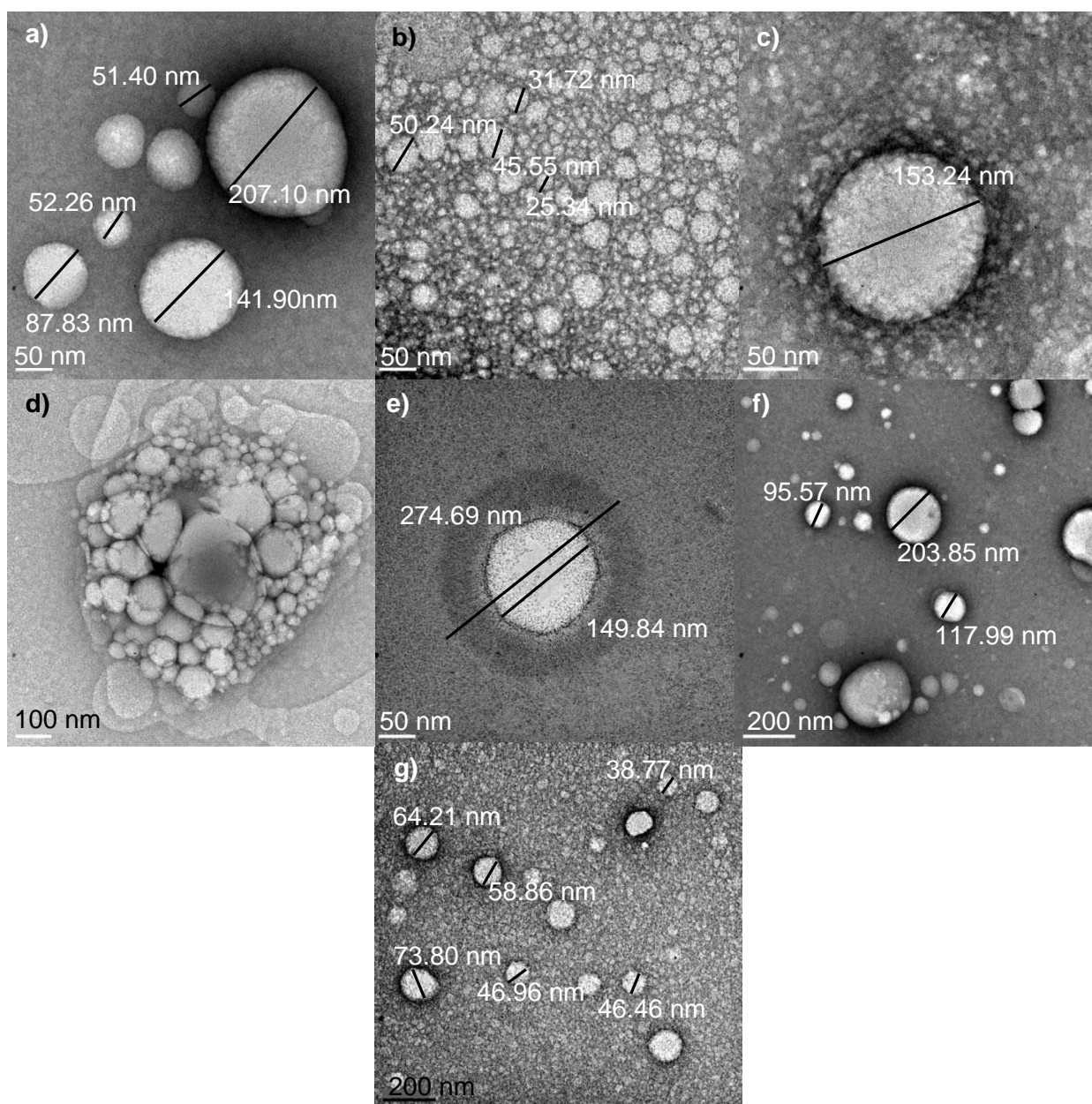
The characterisation of nano-emulsions will enable the formulator to determine which of the two formulas possess the properties to serve as the optimised formulation. The results of the different characterisation tests were compared, where after the optimised formula was selected and used in further investigations. The characterisation tests that were utilised during this study were:

- morphology;
- pH;
- droplet size and distribution;
- zeta-potential;
- viscosity, and
- drug entrapment efficiency.

### B.6.1 Morphology

Transmission electron microscopy (TEM) was utilised to further investigate the morphological characteristics of the **(NEF1)** and **(NEF2)** dispersions, such as the presence of droplets and if present, the size and structure thereof (Chime *et al.*, 2014:96; Gaur *et al.*, 2014:47). A FEI Tecnai G2 20S-Twin 200 kV high-resolution transmission electron microscope (HRTEM) (Czech Republic, EU) with an Oxford INCA X-Sight EDS System was used. Samples were prepared with API in both **(NEF1)** and **(NEF2)** containing each of the selected statins separately. A

dilution was made with Milli-Q® water for each of the samples, where after a small volume of each of the respective dilutions was placed on a microscopic carbon-coated 300 mesh copper grid, by means of a micropipette and left to dry for 10 min (excess removed with filter paper). Thereafter, staining was done with osmium tetroxide (Chime *et al.*, 2014:96), which provided the images with high-contrast quality. The osmium tetroxide also preserved any unsaturated fatty acids present, making viewing of oil droplets effortless (Nomaki *et al.*, 2015:33). This osmium stained carbon-coated grid was examined at sufficient voltage ( $\pm 200$  kV) under the TEM, after allowing it to dry adequately (20 – 30 min). A Gatan bottom mount camera and digital micrograph software was utilised to capture and process micrographs.



**Figure B.5:** Micrographs of oil droplets captured with the TEM: a) (NEL1), b) (NEL2), c) (NEM1), d) (NEM2), e) (NES1), f) (NES2) and g) (NER1) and size thereof. Scale bars for magnification are indicated for each micrograph.

Nano-emulsions are systems with a droplet size in the range of 50 – 200 nm (Tadros *et al*, 2004:303). Figures B.5.a to B.5.g exhibit the HRTEM results for the **(NEF1)** dispersions (mevastatin, lovastatin, rosuvastatin, and simvastatin) and the **(NEF2)** dispersions (mevastatin, lovastatin and simvastatin, as rosuvastatin could not be formulated using this formula). These micrographs display the morphology of the oil droplets dispersed within the water phase, and could therefore be utilised to determine both the size and shape of the droplets.

From Figures B.5.a – b it is clear that the droplet size of **(NEL1)** and **(NEL2)** are well within the intended range. Figure B.5.a displays a range of 51.40 – 207.10 nm and Figure B.5.b a range of 8.88 – 50.24 nm. In both Figures B.5.a and B.5.b, it appears that the droplets are spherically shaped. **(NEM1)** (Figure B.5.c) has spherical droplets in the size range of 60.61 – 139.94 nm. **(NEM2)** (Figure B.5.d) clearly show that coalescence of the droplets occurred, which can be indicative that **(NEM1)** is a more stable dispersion. **(NES1)** displayed perfectly spherical droplets with size of  $\pm 149.84$  nm (Figure B.5.e). These droplets appeared to have a dark shadow surrounding them, which might be the result of osmium tetroxide having a greater staining effect on the droplets. This dark shadow effect was also observed with **(NES2)** (Figure B.5.f), which exhibited droplets in the size range of 25.24 – 203.85 nm and coalescence of the droplets seems to have occurred already. Therefore, it can be proposed that **(NES1)** is the more stable nano-emulsion. Lastly, **(NER1)** (Figure B.5.g) displayed no coalescence and spherical droplets that ranged between 44.28 – 79.12 nm in size were obtained.

### B.6.2 pH

Measuring the pH value for topical formulations is essential to ensure the dispersions are safe to use on the skin and are non-irritating in nature (Basera *et al*, 2015:1881), therefore a Mettler Toledo® pH meter (Mettler Toledo, CU) equipped with a Mettler Toledo® InLab® 410 electrode (Mettler Toledo, CU) was utilised to acquire these values. According to Hach Company (2010:6), determining these values offers the necessary information as to whether the dispersion is acidic or basic of nature. The amount of hydrogen ions [H+] to that of hydroxide ions [OH-] will express the pH value, and a dispersion is seen as stable when these amounts remain constant (Hach Company, 2010:6).

The pH value was measured for the following dispersions:

- **(NEL1)** and **(NEL2)**
- **(NEM1)** and **(NEM2)**
- **(NES1)** and **(NES1)**
- **(NER1)**

Berg *et al.* (2009:276) stated that pH can have an impact on the zeta-potential of a formulation due to the influence of pH on the surface charge of a dispersion; as a result, it is important to measure pH before determining the zeta-potential.



**Figure B.6:** A Mettler Toledo<sup>®</sup> pH meter with a Mettler Toledo<sup>®</sup> InLab<sup>®</sup> 410 electrode

**Table B.4:** The average pH values of the respective nano-emulsions

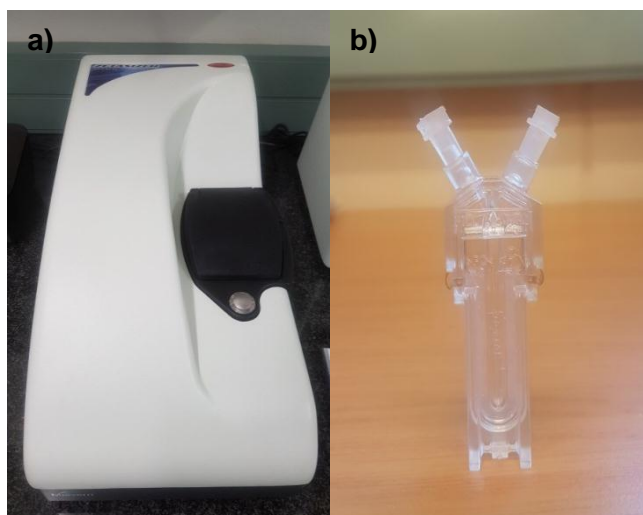
Nano-emulsion	Average pH value
(NEL1)	5.83 ± 0.033
(NEL2)	6.52 ± 0.008
(NEM1)	6.13 ± 0.017
(NEM2)	6.71 ± 0.005
(NES1)	5.87 ± 0.005
(NES2)	7.04 ± 0.008
(NER1)	6.52 ± 0.005

It can be observed from Table B.4 that all the respective dispersions presented with a pH that is within the acceptance range. Barry (2002:512) stated that a pH outside of the range of 3 – 9 could affect both the integrity and permeability of the skin. Hence, application of any of the respective dispersions should not bring about any skin sensitivity or irritation. Furthermore, it can be observed that the respective **(NEF2)** dispersions displayed higher pH values than the **(NEF1)** dispersions, which could be attributed to the higher Span<sup>®</sup> 60 content present in the **(NEF2)** dispersions. Danimayotsu *et al.* (2017:16) stated that the formulation containing the highest percentage of Span<sup>®</sup> 60 yielded the highest pH. The **(NEF1)** dispersions could accordingly be characterised as more acidic than the **(NEF2)** dispersions.

### B.6.3 Droplet size and distribution

The droplet size and distribution of a dispersion is mostly established by utilising instruments based on photon correlation spectroscopy (PCS), i.e. a Zetasizer. The basis on which PCS

functions, is the measurement of light fluctuations between dispersed droplets triggered by Brownian motion (Gaur *et al.*, 2014:40; Malvern Instruments Limited, 2015:15), enabling the determination of droplet size, by measuring the z-average, which indicates the average diameter (d.nm) of the droplets. PCS measures and expresses the droplets distribution as the polydispersity index (PDI). Establishing these characteristics is essential to acquire an ideal formulation (Gaumet *et al.*, 2008:2).

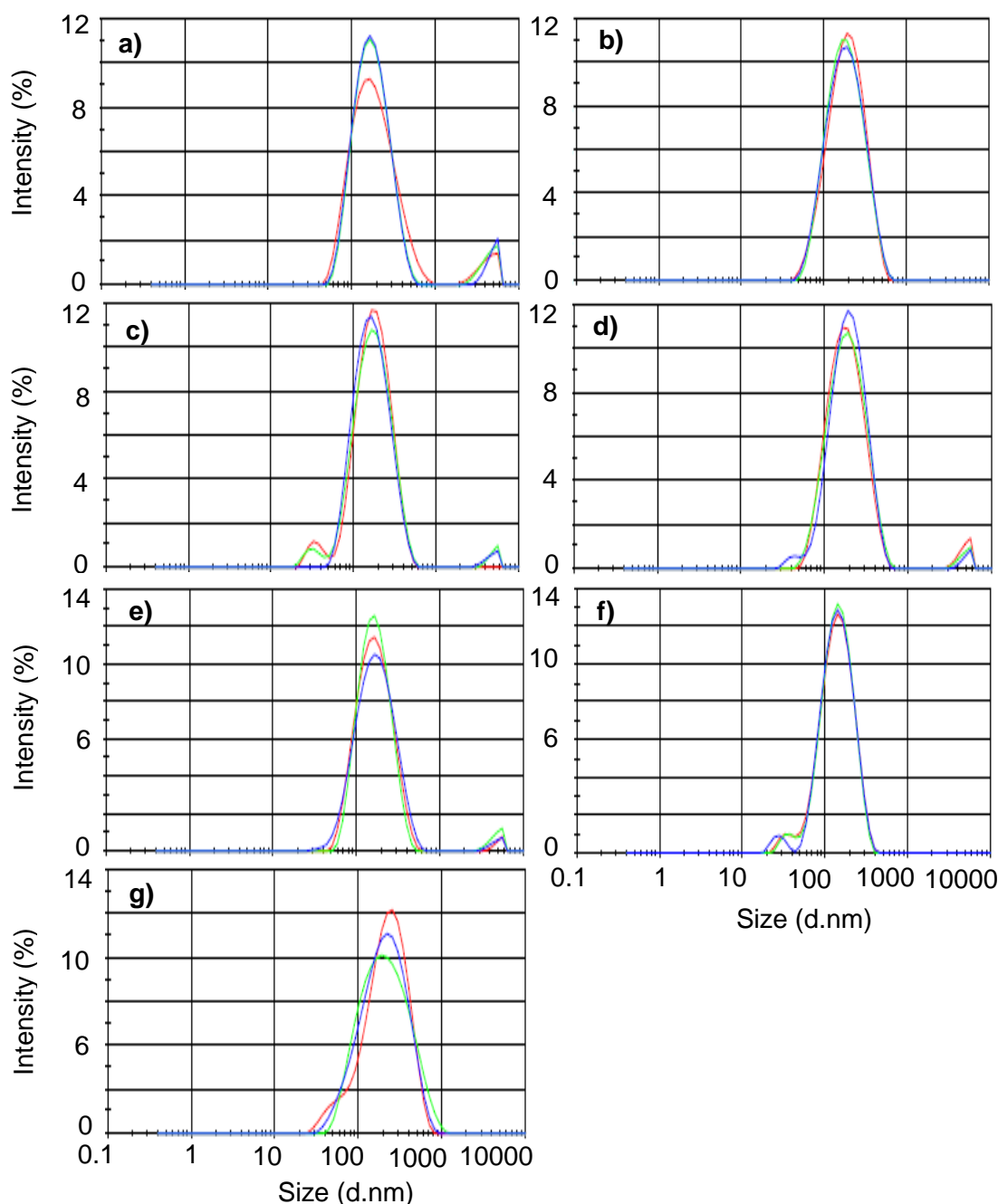


**Figure B.7:** a) Malvern Zetasizer Nano ZS and b) a clear disposable DTS1070 folded capillary zeta-cell

For the purpose of this study, the Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) (Figure B.7.a) was utilised to establish the droplet size and distribution of the **(NEF1)** and **(NEF2)** dispersions. Samples of **(NEF1)** and **(NEF2)** were prepared by placing 10 ml Milli-Q® water into polytops with one drop of each dispersion (**(NEF1)** or **(NEF2)**) respectively; followed by proper mixing. These dilutions were then analysed by placing 2 ml in a clear disposable zeta-cell (DTS1070 folded capillary cell) (Figure B.7.b) by means of a syringe. Measurement was performed in triplicate.

The determination of droplet size was concluded by means of the PCS (Zetasizer). For the dispersions to be categorised as nano-emulsions, the droplet size is required to fall within the range of 50 – 200 nm (Tadros *et al.*, 2004:303). For **(NER1)** no comparison could be drawn as formulation of **(NER2)** proved to be unsuccessful. Comparisons were drawn between the following:

- **(NEL1)** and **(NEL2)**
- **(NEM1)** and **(NEM2)**
- **(NES1)** and **(NES2)**



**Figure B.8:** Average droplet size measured per droplet radius of a) **(NEL1)**, b) **(NEL2)**, c) **(NEM1)**, d) **(NEM2)**, e) **(NES1)**, f) **(NES2)** and g) **(NER1)**

Figures B.8.a – B.8.g illustrate the triplicate curves obtained during the determination of droplet size of each of the respective **(NEF1)** and **(NEF2)** dispersions combined into one figure. For **(NEL1)** and **(NEL2)**, all three of the curves on the respective figures seem relatively symmetrical, although those of **(NEL2)** seem more symmetrical than **(NEL1)**. This could be the result of variation between droplets within the three readings of **(NEL1)**. The triplicate curves on the respective figures of **(NEM1)** and **(NEM2)** seem largely symmetrical, with little to no difference between the dispersions. **(NES1)** and **(NES2)** displayed triplicate curves that appear relatively symmetrical, although those of **(NES2)** appear more symmetrical than those of

**(NES1)**. The three curves of **(NER1)** seem relatively symmetrical, but with a noticeable difference that could indicate a mentionable variation between the droplets between the three readings. From these figures, it can be observed that all the formulated nano-emulsions displayed a radius of average droplet size within the nanometre range.

**Table B.5:** Average droplet size and Pdl of **(NEF1)** and **(NEF2)** dispersions.

Nano-emulsions	Average droplet size(nm)	Average Pdl
<b>(NEL1)</b>	162.60 ± 1.735	0.335 ± 0.003
<b>(NEL2)</b>	160.80 ± 2.571	0.198 ± 0.009
<b>(NEM1)</b>	147.17 ± 1.850	0.248 ± 0.001
<b>(NEM2)</b>	172.90 ± 1.345	0.237 ± 0.009
<b>(NES1)</b>	153.10 ± 1.249	0.238 ± 0.009
<b>(NES2)</b>	120.10 ± 0.819	0.195 ± 0.007
<b>(NER1)</b>	169.60 ± 2.615	0.279 ± 0.004

From Table B.5 it can be observed that all the formulated nano-emulsions had a droplet size within the required range. From the quality reports, the Malvern Zetasizer revealed all the samples were “good”, serving as confirmation that the results were acceptable.

The Pdl value of a dispersion is indicative of the dispersity of droplets in the water phase, thus how uniform droplets are in the entire dispersion (Bali *et al.*, 2011:52; Shakeel *et al.*, 2007:E6). Pdl is determined by means of a scale ranging from 0 – 1, where a low Pdl (closer to 0) is indicative of monodispersed or homogenous dispersions, and a Pdl closer to 1 suggesting a more polydispersed dispersion (Gaumet *et al.*, 2008:5). From Table B.5, it can be observed that all dispersions (**(NEL2)**, **(NEM1)**, **(NEM2)**, **(NES1)**, **(NES2)**, and **(NER1)**) displayed a Pdl value closer to 0 with the exception of **(NEL1)**. This indicates that the first mentioned dispersions are fairly monodispersed, and **(NEL1)** is more polydispersed. Dispersions can be expected to be stable against aggregation and sedimentation over time when the Pdl value is closer to 0. Although the majority of the nano-emulsions presented as monodispersed, it is important to take note that nano-emulsions are characteristically seen as heterogeneous dispersions and therefore will never be fully monodispersed (Abolmaali *et al.*, 2011:140; Chime *et al.*, 2014:77).

#### **B.6.4 Zeta-potential**

When the surface charge of a dispersion is determined, it is known as the zeta-potential of the dispersion (Eid *et al.*, 2014:2; Thakur *et al.*, 2012:223). The zeta-potential of a dispersion is indicative of the electrostatic charges present between droplets (Malvern Instruments Limited, 2015:10), and typically caused by Brownian motion and Van der Waals forces (Cao & Wang,

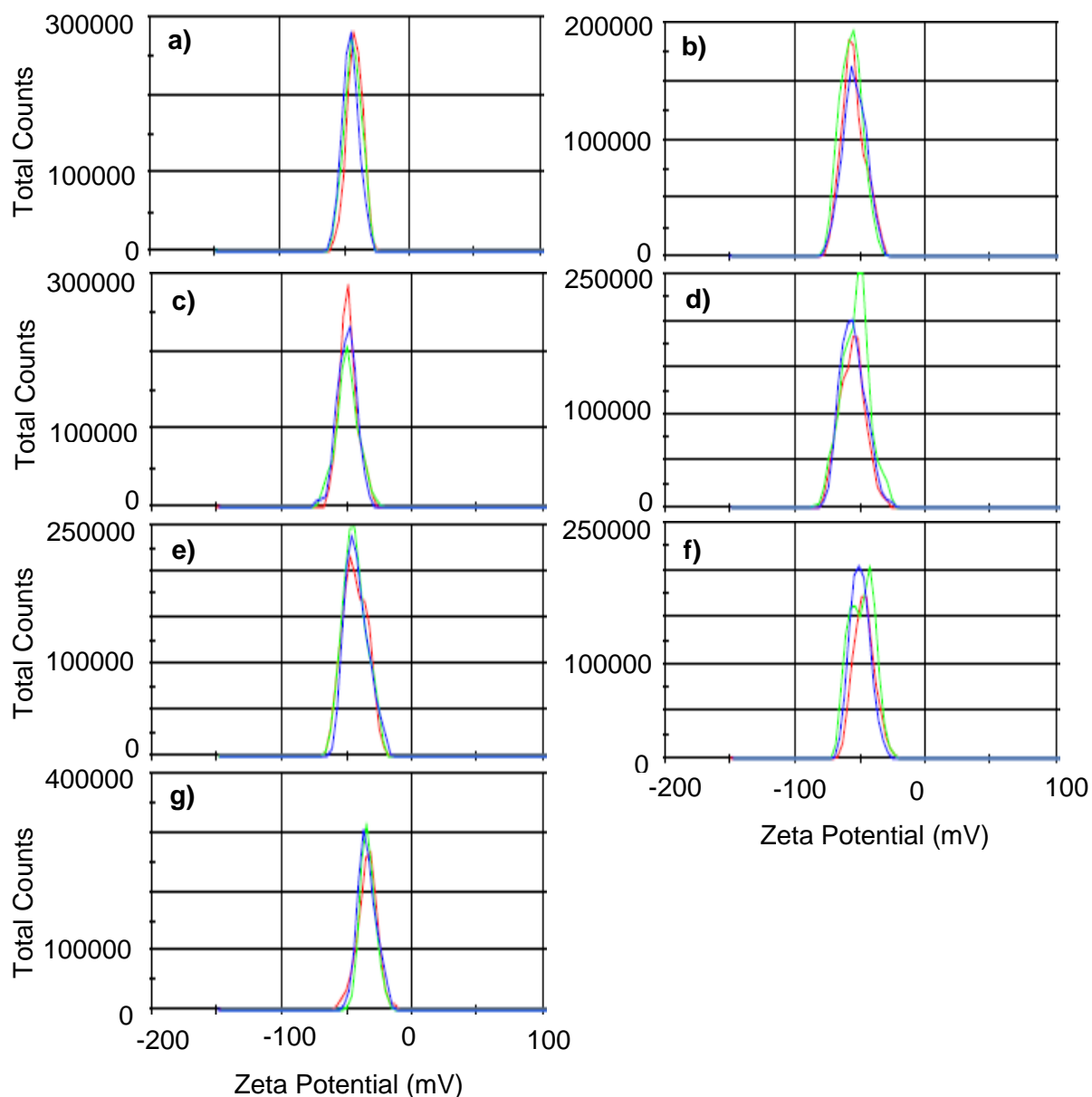
2011:43). Determining the zeta-potential is an essential part of any formulation, as it can influence both stability and drug entrapment of the formulation (Bhatt & Madhav, 2011:2296; Eid *et al.*, 2014:2; Klang *et al.*, 2015:262). It is proposed that dispersions with a zeta-potential of more than 30 mV or less than - 30 mV can remain stable over time (Eid *et al.*, 2014:2; Silva *et al.*, 2012:860). Furthermore, it is proposed that negatively charged molecules could lead to enhanced diffusion and subsequently improve API flux (Sinico *et al.*, 2005:129). The method as described in Section B.6.3 for the determination of droplet size was utilised to determine the zeta-potential on a Malvern Zetasizer Nano ZS and by means of a clear disposable DTS1070 folded capillary zeta-cell

**Table B.6:** The comparison of zeta-potential average between **(NEF1)** and **(NEF2)**

Nano-emulsion	Zeta-potential			
	Reading 1	Reading 2	Reading 3	Average
<b>(NEL1)</b>	-42.2	-43.7	-45.4	-43.77 ± 1.601
<b>(NEL2)</b>	-55.2	-57.1	-54.4	-55.57 ± 1.387
<b>(NEM1)</b>	-48.9	-49.7	-50.1	-49.57 ± 0.611
<b>(NEM2)</b>	-56.2	-53.8	-55.7	-55.23 ± 1.266
<b>(NES1)</b>	-43.9	-44.1	-42.6	-43.53 ± 0.815
<b>(NES2)</b>	-47.2	-48.9	-50.1	-48.73 ± 1.457
<b>(NER1)</b>	-34.5	-34.6	-33.96	-34.35 ± 0.344

From Table B.6 it is observed that both **(NEF1)** and **(NEF2)** displayed negative zeta-potential values well within the acceptance criteria; hence, it can be stated that a stable dispersion was formed with adequate repulsion between the droplets with a lesser probability of flocculation and sedimentation (Silva *et al.*, 2012:860).

Figures B.9.a – B.9.g illustrate the zeta-potential values of the respective nano-emulsions (**(NEL1)**, **(NEL2)**, **(NEM1)**, **(NEM2)**, **(NES1)**, **(NES2)** and **(NER1)**). Table B.6 displays the triplicate zeta-potential values obtained along with the average zeta-potential value for each of the respective nano-emulsions. As stated above, all dispersions possessed a zeta-potential within the required range (below - 30 mV), although, **(NEF2)** presented with lower zeta-potential values and could therefore be more stable dispersions. When observing Figures B.9.a – B.9.g, it can be stated that the curves appear symmetrical, which is indicative of a narrow data distribution. As all the respective **(NEF2)** dispersions presented with more negative zeta-potential values, it can be suggested that these dispersions are more stable and ideal compared to the **(NEF1)** dispersions.



**Figure B.9:** The average zeta-potential (mV): a) **(NEL1)**, b) **(NEL2)**, c) **(NEM1)**, d) **(NEM2)**, e) **(NES1)**, f) **(NES2)** and g) **(NER1)**.

### B.6.5 Viscosity

With visual examination, nano-emulsions appear watery, and as a result usually present with low viscosity values (Thakur *et al.*, 2012:223). The viscosity on a nano-emulsion can be indicative of the excipients utilised in the formulation of the dispersion, formulation stability and in what manner the API is released from the delivery system (Chime *et al.*, 2014:97). Very low viscosity is observed with o/w nano-emulsions, due to the large water phase that contains the dispersed oil droplets (Chime *et al.*, 2014:97). Subsequently, when referring to nano-emulsions, the viscosity can be used as a characteristic of these systems (Shakeel *et al.*, 2007:E6).



**Figure B.10:** A Brookfield Viscometer DV2T LV Ultra connected to a water bath

To establish the viscosity of the **(NEF1)** and **(NEF2)** dispersions, a Brookfield Viscometer DV2T LV Ultra (Middleboro, Massachusetts, USA), connected to a thermostatic water bath (Figure B.10) was used to acclimatise the **(NEF1)** and **(NEF2)** dispersions to  $\pm 25$  °C by placing the formulation in the bath 45 min prior to the test. A small volume of the nano-emulsions was placed in the sample chamber, where after it was fitted to the small sample adapter of the viscometer. The SC4-18 cylindrical spindle was inserted into the formulation and coupled to the viscometer. Viscosity was then measured by programming the spindle to rotate at a speed of 200 rpm. Centipoise (cP) at room temperature ( $\pm 25$  °C) is used to express the viscosity readings. Rheocalc T1.2.19 was preprogrammed to read and gather multipoint data at 10s intervals for 3 min. These readings were used to determine average viscosity values. The average viscosity measurements for the **(NEF1)** and **(NEF2)** formulas are presented in Table B.7.

**Table B.7:** Average viscosity (cP) and torque (%) measurements of the **(NEF1)** and **(NEF2)** dispersions

Nano-emulsion	Viscosity (cP)	Torque (%)
<b>(NEL1)</b>	4.64 ± 0.064	12.25 ± 0.418
<b>(NEL2)</b>	8.97 ± 0.030	59.83 ± 0.216
<b>(NEM1)</b>	4.05 ± 0.006	27.00 ± 0.055
<b>(NEM2)</b>	9.31 ± 0.026	62.03 ± 0.175
<b>(NES1)</b>	8.38 ± 0.027	55.83 ± 1.770
<b>(NES2)</b>	9.16 ± 0.017	61.10 ± 0.126
<b>(NER1)</b>	7.87 ± 0.084	52.45 ± 0.544

From Table B.7 it can be observed that the viscosity measurements differ between the respective nano-emulsions containing different statins, as well as different surfactant ratios. Table B.8 also indicates that for all the selected statins, the **(NEF2)** dispersions presented with a higher viscosity compared to the **(NEF1)** dispersions. This increased viscosity, as seen with the **(NEF2)** dispersions, could be due to more apricot kernel oil used during formulation of the **(NEF2)** (Ali *et al.*, 2014:1134; Dluzewska *et al.*, 2006:147). The torque between the dispersions ranged between 12.25 – 62.03%.

#### B.6.6 Drug entrapment efficiency

The difference between the total amount of drug entrapped within the nano-emulsion and that of the free drug within the sample is known as the entrapment efficacy (EE%), representing the yield obtained. The HPLC was utilised to determine the EE% of the **(NEF1)** and **(NEF2)** dispersions. The samples used for analysis by means of HPLC were prepared by placing 20 ml of each formulated nano-emulsion (**(NEL1)**, **(NEL2)**, **(NEM1)**, **(NEM2)**, **(NES1)**), **(NES2)** and **(NER1)**) into Eppendorf® tubes, where after they were centrifuged in an Optima L-100 XP ultracentrifuge (Beckman Coulter, South Africa) at a speed of 25 000 g for 45 min at room temperature. After centrifuging, it was possible to distinguish between the unentrapped API (supernatant) fluid and the fluid containing the entrapped API.

The samples intended for analysis by means of the HPLC were prepared by extracting 200 µl of the supernatant and diluting it with tetrahydrofuran (THF). Thereafter, a small volume was placed in a HPLC vial for analysis.

$$\%EE = [(C_t - C_f)/C_t] \times 100$$

**Equation B.1**

A standard linear curve obtained from a standard solution (as described in Appendix A) for each of the selected statins was used to analyse the data obtained in conjunction with Equation B.1

to calculate the %EE. Loureiro *et al.* (2015:96) stated that a %EE of 100%, or close to 100%, can be accomplished when a lipophilic API is formulated into a nano-emulsion. The results of the calculated entrapment efficacy are listed in Table B.8.

**Table B.8:** The entrapment efficacy (%EE) as calculated for **(NEF1)** and **(NEF2)** dispersions

Nano-emulsion	Entrapment efficacy (%EE)
<b>(NEL1)</b>	99.546
<b>(NEL2)</b>	99.551
<b>(NEM1)</b>	98.940
<b>(NEM2)</b>	99.270
<b>(NER1)</b>	99.034
<b>(NES1)</b>	95.289
<b>(NES2)</b>	94.216

It can be observed from Table B.8 that both the **(NEF1)** and **(NEF2)** dispersions yielded a high %EE. Kurakula *et al.* (2012:37) stated that if dispersion yields a high %EE, it ensures a larger amount of the respective API can be delivered to the intended target site. This result of nearly a 100% entrapment is also indicative that each of the selected statins were practically fully incorporated with the oil phase in both the **(NEF1)** and **(NEF2)** dispersions (Loureiro *et al.*, 2015:96).

### B.7 Decision on final formula to be used

The droplet sizes of both the **(NEF1)** and **(NEF2)** dispersions were determined by means of TEM and a Zetasizer. This test was performed to ensure that the droplet size of the various dispersions fell within the required criteria of 50 – 200 nm (Tadros *et al.*, 2004:303). These characterisation tests concluded that all the respective dispersions fell within the required range with the exception of **(NEM2)**, as TEM of this dispersions displayed coalescence of droplets. Pdl was also determined and the result was indicative of monodispersed dispersions (measurement closer to 0), although the **(NEF2)** dispersions displayed the lowest values, therefore it could be proposed that these dispersions could be more stable.

Establishing the pH of dispersions for transdermal use is essential, as pH not within the acceptable safe range of 3 – 9 (Barry, 2006:7; Barry, 2007:576; Malan *et al.*, 2002:388) could cause irritation or damage to the skin. Both the **(NEF1)** and **(NEF2)** dispersions displayed values safe for transdermal use.

The zeta-potential obtained met the required criteria of below -30 mV or above 30 mV. Consequently, these dispersions can be seen as stable over time and efficient in permeating the skin, although **(NEF2)** dispersions more than **(NEF1)** dispersions, as these dispersions displayed a more negative value. The more efficient permeation of skin can be attributed to the skin being mainly negatively charged. Subsequently, enhanced diffusion and therefore increased API flux can possibly be achieved with high zeta-potential values or highly negatively charged molecules (Duangjit *et al.*, 2011:6; Sinico *et al.*, 2005:129).

The viscosity of the respective dispersions displayed results as expected. The **(NEF2)** dispersions showed a higher viscosity than the **(NEF1)** dispersions, which is the result of the higher oil component in **(NEF2)**. Although **(NEF2)** appears to be optimal when comparing certain characteristics, the coalescence observed by means of TEM of **(NEM2)** and the fact that rosuvastatin could not be formulated with success into the **(NEF2)** formula, **(NEF1)** was selected as the optimal formula. This formula met all the required characteristics and no coalescence of droplets was visible for any of the dispersions (**(NEL1)**, **(NEM1)**, **(NER1)** and **(NES1)**) during TEM.

**Table B.9:** Summary of the characteristics of the **(NEF1)** and **(NEF2)** dispersions

Formula	TEM (nm)	pH	Droplet size (nm)	Zeta-potential (mV)	Viscosity (cP)	(%EE)
<b>(NEL1)</b>	51.40 - 207.10	5.83 (± 0.033)	162.6 (± 1.735)	-43.77 (± 1.601)	4.64 (± 0.064)	99.546
<b>(NEL2)</b>	8.88 - 50.24	6.52 (± 0.008)	160.8 (± 2.571)	-55.57 (± 1.387)	8.97 (± 0.030)	99.551
<b>(NEM1)</b>	60.61 - 139.94	6.13 (± 0.017)	147.17 (± 1.850)	-49.57 (± 0.611)	4.05 (± 0.006)	98.940
<b>(NEM2)</b>	coalescence	6.71 (± 0.005)	172.9 (± 1.345)	-55.23 (± 1.266)	9.31 (± 0.026)	99.270
<b>(NER1)</b>	44.28 – 79.12	6.52 (± 0.005)	169.6 (± 2.615)	-34.35 (± 0.344)	7.87 (± 0.084)	99.034
<b>(NES1)</b>	±149.84	5.87 (± 0.005)	153.1 (± 1.249)	-43.53 (± 0.815)	8.38 (± 0.027)	95.289
<b>(NES2)</b>	25.24 - 203.85	7.04 (± 0.008)	120.1 (± 0.819)	-48.73 (± 1.457)	9.16 (± 0.017)	94.216

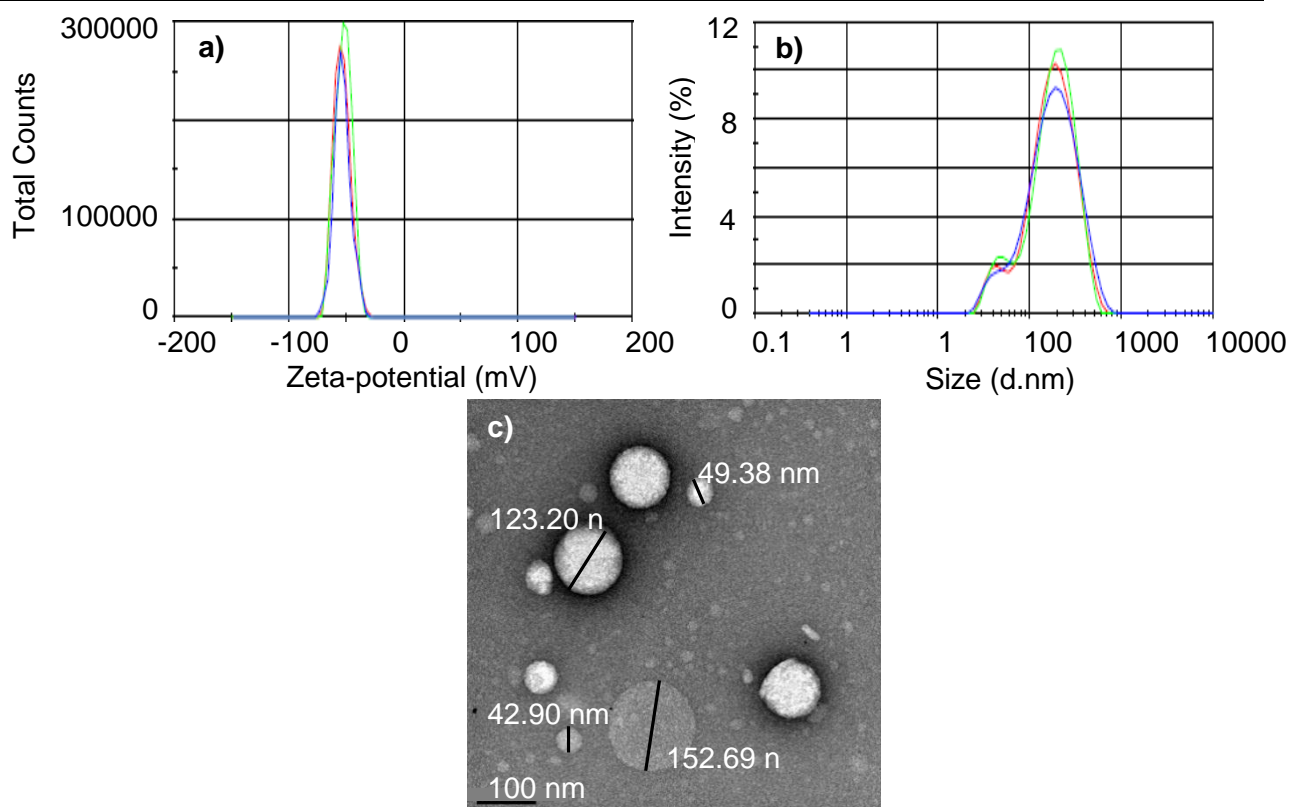
## B.8 Characterisation of chosen optimised nano-emulsion placebo

During membrane release and skin diffusions studies, the placebo of the optimised nano-emulsion serves as a control, necessitating its characterisation. The placebo will be referred to as **(PNEF1)** and formulated in a similar manner as **(NEF1)**, with the exception of including the

respective statins. Table B.10 and Figure B.11 contain a summary of the characterisation results obtained for **(PNEF1)**.

**Table B.10:** Characterisation summary of **(PNEF1)**

Formula	TEM (nm)	pH	Droplet size (nm)	Zeta-potential (mV)	Viscosity (cP)
<b>(PNEF1)</b>	18.70 – 152.69	$6.63 \pm 0.009$	$141.0 \pm 0.374$	$-53.3 \pm 0.509$	$4.15 \pm 0.015$



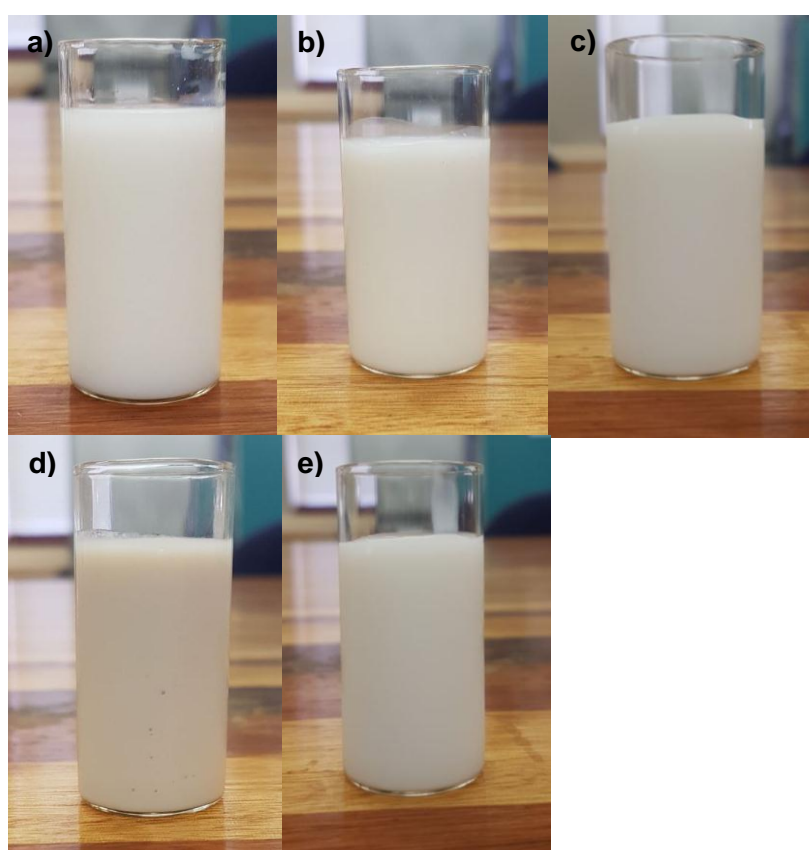
**Figure B.11:** a) Average zeta-potential; b) average droplet size measured per droplet radius and c) TEM micrograph of **(PNEF1)**

According to Tadros *et al.* (2004:303), the droplet range of nano-emulsions is normally between 50 – 200 nm. It can be stated that when observing the result obtained from both TEM and droplet size on the Zetasizer, **(PNEF1)** falls well within the range of a nano-emulsions. By measuring the droplet size on the Zetasizer, the Pdl of the dispersion was also determined to be  $0.272 \pm 0.007$ , hence this value is closer to 0 which may refer to a homogeneous or monodispersed dispersion (Gaumet *et al.*, 2008:5). **(PNEF1)** displayed a pH of  $6.63 \pm 0.009$ , which is within the desired pH range that is believed would not influence skin integrity (pH 3 – 9) (Barry, 2006:7; Barry, 2007:576; Malan *et al.*, 2002:388; Williams, 2003:38). Furthermore, a zeta-potential of  $-53.3 \pm 0.509$  was measured, therefore this dispersion can be considered to be stable over time, as the zeta-potential value was greater than - 30 mV (Da Costa *et al.*, 2014:5). Lastly, the viscosity of **(PNEF1)** was  $4.15 \pm 0.015$  cP, correlating well with what is suggested in

literature, which indicates the viscosity of nano-emulsions will increase with the increase of the oil content (Ali *et al.*, 2014:1134). As the oil component of the nano-emulsion is relatively low, low viscosity measurements are to be expected. Chime *et al.* (2014:97) also stated that the use of an adequate amount of surfactant in the formulation leads to lower viscosity due to interfacial tension.

## B.9 Conclusion

When comparing the **(NEF1)** dispersions and **(PNEF1)** firstly by means of visual examination, it can be observed that the placebo formulation **(PNEF1)** is more translucent than the dispersions containing API (see Figure B.12). The decrease in the translucent nature of the dispersions can possibly be attributed to the incorporation of 2% (m/m) API.



**Figure B.12:** The formulated dispersions a) **(PNEF1)**, b) **(NEL1)**, c) **(NEM1)**, d) **(NES1)** and e) **(NER1)**

By comparing TEM micrographs, and average droplet size obtained from measurements on the Zetasizer, it can be stated that all the respective **(NEF1)** and **(PNEF1)** dispersions displayed droplet sizes well within the range to be characterised as nano-emulsions by Tadros *et al.* (2004:303) of 50 – 200 nm. All the respective dispersions achieved a zeta-potential of greater than - 30 mV and, as a result, these nano-emulsions can be considered stable (Da Costa *et al.*, 2014:5). Finally, the viscosity measurements of all the respective dispersions (**(NEF1)** and

**(PNEF1)** displayed values (low viscosity) correlating with that suggested in literature, due to the large water component and small oil component in the dispersions. When comparing the **(NEF1)** dispersions to **(PNEF1)**, the addition of lovastatin, rosuvastatin and simvastatin increased the viscosity of the formulation, as **(NEL1)** had an average viscosity of  $4.64 \pm 0.064$ , **(NER1)** of  $7.87 \pm 0.084$  and **(NES1)** of  $8.38 \pm 0.027$ . Although the addition of mevastatin ( $4.05 \pm 0.006$ ) had a lower value than **(PNEF1)**, the difference in values is insignificantly small.

## References

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## Appendix C:

# *Formulation and characterisation of a semi-solid dosage form of an o/w nano-emulsion separately containing the selected statins and apricot kernel oil*

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### C.1 Introduction

During pre-formulation, an optimised nano-emulsion was selected and characterised. This optimised nano-emulsion (**NEF1**) contained 8.0% (w/w) apricot kernel oil and equal ratios of hydrophilic and lipophilic surfactants (Tween<sup>®</sup> 80 and Span<sup>®</sup> 60, respectively). Nano-emulsions present numerous advantages due to the small droplet size that produces a system that is kinetically stable and that abstains from, i.e. flocculation and coalescence (Roberts *et al.*, 2017:94). However, nano-emulsions have very low viscosity (Ali *et al.*, 2014:1128). Nevertheless, as Singh *et al.* (2017:29) stated, this obstacle can be overcome by transforming nano-emulsions into a variety of dosage forms (i.e. gels, creams and foams) that can be administered through several routes. It is proposed that incorporating a nano-emulsion with a gel, resulting in a nano-emulgel, can improve the formulation in terms of stability (by increasing viscosity of the aqueous phase and reducing interfacial tension), while better skin adhesion of nano-emulgel in combination with the high solubilisation capacity (as seen with nano-emulsions) can facilitate in better skin penetration (Eid *et al.*, 2014:1). Furthermore, the application of a semi-solid dosage form to the skin is much more effortless (Pund *et al.*, 2015:152).

As of yet there is no transdermal therapy available for the treatment of hypercholesterolemia and although statins (HMG-CoA reductase inhibitors) caused a revolution in the treatment of this condition, they are associated with systemic adverse effects (Thompson *et al.*, 2016:2395). The most common statin-associated side effects are liver and muscle toxicity, however gastrointestinal side effects (i.e. nausea, abdominal pain and flatulence) can also occur (Kiortsis *et al.*, 2007:7-8), hence, exploring other routes of administration could be advantageous with regard to the use of statins.

Transdermal delivery poses numerous advantages above other routes of administration (self- and pain-free administration). The most important advantage of this route, specifically with regard to statins, is the avoidance of the gastrointestinal tract and first-pass metabolism (Paudel *et al.*, 2010:109), which are the main causes of statin-associated adverse effects. As mentioned before, the formulation of a semi-solid dosage form will overcome the low viscosity associated with nano-emulsions, but in addition to this, a semi-solid dosage form will increase

the direct contact between the API and the skin, hence acting as a carrier (Gupta & Garg, 2002:144). The objective in this section was to incorporate the optimised nano-emulsion (**NEF1**) into a semi-solid dosage form in order to increase viscosity. In addition, formulation of an API into a semi-solid can facilitate penetration of the layers of the skin (Mahalingham *et al.*, 2008:268).

## **C.2 Intended purpose of the formulation**

The implementation of nano-emulsions as transdermal or topical delivery vehicles poses many challenges. With observation of a nano-emulsion, this delivery system presents all the ideal properties of a liquid vehicle, i.e. high solubilisation capacity for both hydrophilic and lipophilic ingredients, nanometric droplet size and effortless formation (Kong *et al.*, 2011:838). Although all these properties of nano-emulsions are favourable for efficient drug delivery, considering the rheology properties of a nano-emulsion is important. These rheology properties of nano-emulsions, i.e. low spreadability and low viscosity, pose the main limitation with the clinical application of nano-emulsions (Chellapa *et al.*, 2015:44). Consequently, the intended purpose during formulation will be to increase the viscosity of the formulation by incorporating a gelling agent into the nano-emulsion to overcome this limitation. Furthermore, integrating a nano-emulsion system into a hydrogel matrix may enhance skin penetration (Chellapa *et al.*, 2015:44).

### **C.2.1 Semi-solid dosage form selection**

Semi-solid dosage forms for topical or transdermal use are presented in various forms, i.e. ointments, creams, gels and pastes (Bora *et al.*, 2014:3594). Each of these dosage forms has unique characteristics (Gupta & Garg, 2002:144), which will assist in the selection of an appropriate dosage form. A semi-solid dosage form can be prepared using a variety of raw materials, apart from the usual pharmaceutical ingredients and are unique to its composition. Drug delivery requirements will serve as the basis on which raw materials will be selected for formulation development (Gupta & Garg, 2002:144). For the purpose of this study, a nano-emulgel will be investigated for the possible transdermal delivery of the selected statins.

### **C.2.2 Gels as a semi-solid dosage form**

Gels (semi-solid) can be defined as polymeric matrices (three-dimensional) that contain small amounts of a solid, which are dispersed in a fairly large amount of liquid (Das Neves & Bahia, 2006:1). Gels can be either hydrophilic or hydrophobic of nature (Rehman & Zulfakar, 2014:433). By utilising gels as a delivery system, direct contact can be established between the site of absorption (skin) and the API (Mahalingam *et al.*, 2008:288). Gels prove to be more advantageous when compared to ointments and creams. Mainly this comes as a result of the

higher aqueous component present in gels, as this permits enhanced dissolution of API and more effortless movement of the API through the vehicle (Khullar *et al.*, 2012:63). The main limitation with these systems is the delivery of hydrophobic drugs, hence the preparation of emulgels (Khullar *et al.*, 2012:63).

#### **C.2.2.1 Emulgel**

As mentioned before, the incorporation of the selected statins into a gel will not be possible due to the limitation of gels with regard to hydrophobic drugs, thus preparing emulgels was considered. Emulgels generally consist of an o/w or w/o emulsion combined with a gelling agent. Compared to other topical delivery systems, emulgels demonstrate improved drug release due to the absence of excess oily bases and insoluble excipients. Due to the presence of a gel phase, emulgels are non-greasy, which improves patient compliance (Ajazuddin *et al.*, 2013:122; Yadav *et al.*, 2017:15). Although there are many advantages in using emulgels, there are also certain disadvantages, such as the large particle size of emulgels that may influence permeation through the skin negatively (Yadav *et al.*, 2017:16).

#### **C.2.2.2 Nano-emulgel**

The purpose of nano-emulgels, as a delivery system, is to improve both the therapeutic profile and the systemic delivery of lipophilic drugs. As with emulgels, nano-emulgels consist of two phases, and although very similar, the difference lies with the finely dispersed nanometric droplets of the nano-emulsion. This allows enhanced skin permeability of the formulation (Sengupta & Chatterjee, 2017:353). Although nano-emulsions have various advantages, amalgamating a gelling agent with these systems will further improve these systems (i.e. better stability due to the decrease in surface and interfacial tension, easier administration due to increased viscosity). As stated in Section C.1, the high solubilisation capacity and enhanced skin adhesion will improve skin permeation (Basera *et al.*, 2015:1873; Eid *et al.*, 2014:1; Khullar *et al.*, 2012:63). In addition, a nano-emulgel will provide all the advantageous properties as that of a gel formulation (i.e. good spreadability and non-greasy), only these already favourable properties will be improved (Basera *et al.*, 2015:1873; Eid *et al.*, 2014:1). This delivery system is also advantageous with regard to APIs with a short half-life, as controlled release can be attained (Malay *et al.*, 2018:3; Panwar *et al.*, 2011:337).

#### **C.2.3 Suitable semi-solid dosage form**

Although nano-emulsions have presented several advantages, disadvantages (such as poor skin retention and spreadability problems as a result of low viscosity) limits the clinical use of nano-emulsions as topical delivery systems (Mou *et al.*, 2008:271). Baibhav *et al.* (2011:67) suggested that incorporating a nano-emulsion with a gelling agent may be utilised as a strategy

to overcome these problems. The use of hydrogels is limited due to their inability to incorporate lipophilic molecules (Begur *et al.*, 2015:70), therefore formulating a nano-emulgel will resolve limitations of both the hydrogel and that of the nano-emulsion, as statins are lipophilic of nature.

The **(NEF1)** optimised and formulated dispersion formula was consequently utilised to formulate a nano-emulgel for each of the selected statins and compared to the nano-emulsion of the respective statin. From this point onwards, the nano-emulgels (**(NEG1)**) containing the respective statins will be referred to as:

- Lovastatin nano-emulgel **(NEGL)**
- Mevastatin nano-emulgel **(NEGM)**
- Rosuvastatin nano-emulgel **(NEGR)**
- Simvastatin nano-emulgel **(NEGS)**

### **C.3 Excipients used to formulate the nano-emulgels**

#### **C.3.1 General excipients used for nano-emulgel**

The conversion of an emulsion to an emulgel occurs with the presence of a gelling agent in the water phase (Purushottam *et al.*, 2013:233). As emulgels and nano-emulgels both consist of two phases (water and oil) (Sengupta & Chatterjee, 2017:353), with the addition of a high energy or low energy methods to obtain nanometric droplets (Lovelyn & Attama, 2011:627), the general excipients used to formulate nano-emulgels can be categorised as aqueous material, oils, emulsifiers, gelling agents and penetration enhancers (Pant *et al.*, 2015:1734). In the following section, the ingredients used to formulate the nano-emulgel **(NEG1)** will be discussed more thoroughly.

#### **C.3.2 Excipients used to formulate a nano-emulgel**

The excipients used in the formulation of the nano-emulgels (**(NEGL)**, **(NEGM)**, **(NEGR)** and **(NEGS)**) are listed in Table C.1.

**Table C.1:** The excipients utilised for the formulation of **(NEG1)** in conjunction with batch numbers, suppliers and function

Excipients	Batch number	Supplier	Function
Apricot kernel oil	1512E036197	CJP Chemicals	Oil phase (natural oil) and penetration enhancer
Milli-Q® water	Direct Pure UP	In lab	Water phase/solvent
Tween® 80	BCBT9142	Sigma-Aldrich	Hydrophilic surfactant/emulsifier
Span® 60	SLBN0127V	Sigma-Aldrich	Lipophilic surfactant/emulsifier
Carbopol® Ultrez 20	0102052576	Sigma-Aldrich	Gelling agent/ thickening agent
Statins	Lovastatin (20170423) Mevastatin (USMV-1703006) Rosuvastatin (ROC/1512B/000 8J1) Simvastatin (IF – SI - 170913)	DB Fine chemicals	API

### C.3.2.1 Oils (apricot kernel oil)

This excipient will form the oily phase of the nano-emulgel. For semi-solid formulation (nano-emulgel) intended for external use, a variety of oils can be utilised (i.e. mineral oils, alone or in combination with hard paraffin) (Bhavesh & Shah, 2016:348). Van Zyl *et al.* (2016:192) however stated that formulations containing essential fatty acids (EPAs), specifically linoleic and oleic acid, showed enhanced transdermal delivery. Hence, as apricot kernel oil contains both oleic (51.0 – 83.3%) and linoleic acid (9.6 – 45.9%) (Gupta *et al.*, 2012:366-367), this oil will be utilised as the oil phase for the formulation of the **(NEGs)**. In addition, the use of natural oil (containing essential fatty acid) is also considered safe for transdermal use (Fox *et al.*, 2011:10528) and both delivery of lipophilic and hydrophilic compound will be enhanced.

### C.3.2.2 Emulsifiers

The purpose of emulsifiers (surface acting agents), as stated by Setya *et al.* (2014:2218), is to decrease the interfacial tension present between the two phases (o/w interface). In addition to this function, emulsifiers will also contribute or improve the long term stability of a product (Baibhav *et al.*, 2011:68; Sengupta & Chatterjee, 2017:355). For the purpose of this study Tween® 80 and Span® 60 will be utilised as the hydrophilic and lipophilic emulsifiers, respectively.

### C.3.2.3 Gelling agent

Gelling agents are commonly used in conventional and novel dosage forms and are otherwise known as stabilisers, solidifiers and thickening agents (Shah *et al.*, 2014:319). The last mentioned comes as a result of the swelling characteristics of these agents, leading to increased or enhanced viscosity (Mahalingam *et al.*, 2008:293; Mitsui, 1997:138). The term stabilisers stem from the ability of these agents to prevent the separation of emulsified particles (Mitsui, 1997:138). Commercially, numerous gelling agents have been used in topical gels (Jantrawut & Ruksiriwanich, 2014:231) and for the purpose of this study Carbopol® Ultrez 20 will be used.

Carbopol® Ultrez 20 is comprised of a polyacrylic acid crosslink and is seen as a synthetic polymer. Due to its synthetic nature, it provides advantages above that of natural gelling agents, i.e. highly controllable and reproducible physical properties during manufacturing and better stability when preparing gels (Jantrawut & Ruksiriwanich, 2014:231). As Carbopol® Ultrez 20 is an anionic polymer, neutralisation is required for the polymer to become gelified (Sail Aja & Supraja, 2016:18). These polymers, or carbomer polymers, have the potential to be employed in variety of areas in both the pharmaceutical and dermocosmetic fields. These polymers are compatible with many APIs and high viscosity can be achieved at low concentrations. Other advantages include good patient compliance to its exceptional organoleptic characteristics (Islam *et al.*, 2004:1192).

### C.3.2.4 Water

Milli-Q® water is used as a solvent in conjunction with the gelling agent (Carbopol® Ultrez 20) to form the hydrogel. The Milli-Q® water forms the major part of the nano-emulgel.

## C.4 Formulation of a nano-emulgel

Certain aspects have to be considered when selecting/designing a transdermal delivery system, i.e. the target-site, properties of the delivery system and the aim of the intended formulation (Khullar *et al.*, 2011:119). Due to the limitations of nano-emulsions (i.e. low viscosity) (Khurana *et al.*, 2013:383), it was proposed that the formulation of a nano-emulgel could overcome these limitations. Utilising the optimised nano-emulsion formula (**NEF1**), a nano-emulgel was formulated for each on the selected statins, which were compared to each other.

### C.4.1 Formulation method

The methods commonly used to prepare nano-emulgels can be seen as either a three steps/two steps process. These methods involve formulating a nano-emulsion and hydrogel base separately, where after the nano-emulgel will be formed by incorporating the nano-

emulsion into the hydrogel (Basera *et al.*, 2015:1877-1878; Choudhury *et al.*, 2017:1741). However, Purrushottam *et al.* (2013:233) proposed that a nano-emulgel could be formed by incorporating the gelling agent into the water phase of the nano-emulsion; this concept was therefore utilised to design a method for the formulation of the nano-emulgels.

#### C.4.2 Formula used for preparation of (NEG1)

By utilising the optimised nano-emulsion formula (NEF1), a formula was derived for the formulation of (NEG1); the major modification to the (NEF1) formula being the addition of a gelling agent to the water phase.

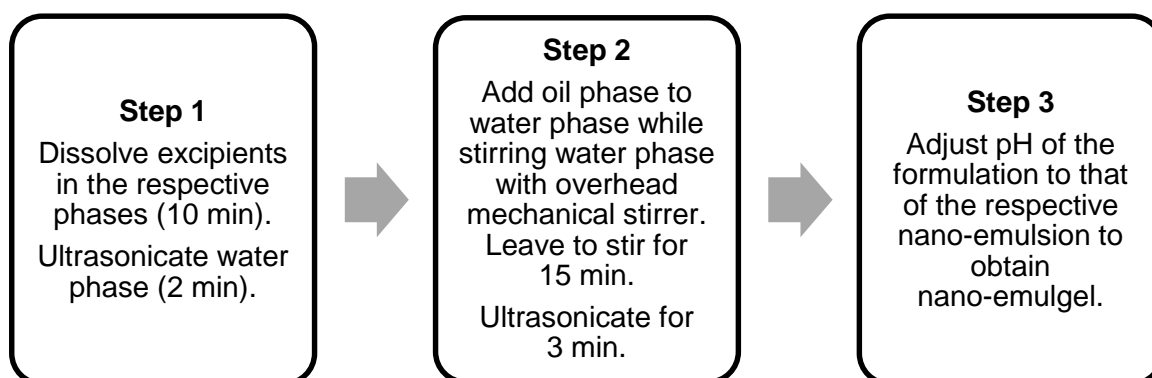
**Table C.2:** Formula used to formulate (NEG1) (100 ml)

Phase	Ingredient	Function	%m/m
A	Apricot kernel oil	Oil phase and penetration enhancer	8.000% (4.350 ml)
	Span® 60	Lipophilic surfactant/emulsifier	6.000% (3 g)
	Statin	API	2.000% (1 g)
B	Tween® 80	Hydrophilic surfactant/emulsifier	6.000% (2.804 ml)
	Milli-Q® water	Water phase/solvent	77.540%
	Carbopol® Ultrez 20 polymer	Gelling agent/thickening agent	0.460%

#### C.4.3 Formulation method used for (NEG1)

The formulation of (NEG1) was initiated by weighing and measuring all the excipients of both phases A and B. Firstly, the excipients were dissolved in the respective phases on a hotplate heated to  $\pm 40$  °C. This step was however adjusted to 10 min to ensure proper dissolution of the Carbopol® Ultrez 20 in the water phase. Before adding the oil phase to the water phase, the ultrasonicator was used to free the water phase of any entrapped air due to the addition of the gelling agent. Secondly, the oil phase was added to the water phase while stirring with an overhead mechanical stirrer at  $\pm 850$  rpm (Figure C.2). After the oil phase was added to the water phase, the formulation was left to stir for 15 min to ensure adequate mixing of the two phases. This results in a formulation with a higher viscosity than a nano-emulsion, but in such a manner that ultrasonication could still be applied successfully to obtain droplets within the nanometric range. The final and third step to obtaining a nano-emulgel was to adjust the pH of the formulations to that of their respective nano-emulsions. Shin *et al.* (2000:307) stated that neutralisation of pH of Carbopol® Ultrez 20 gels will result in an increase of viscosity. The technical data sheet obtained from Lubrizol (2009:1) stated that the average pH before neutralisation of formulations varies between 2.5 and 3.5. Hence, aside from the viscosity, the

pH has to be adjusted so that the formulations are safe for application to the skin (Nair *et al.*, 2013:425). Figure C.1 serves as a diagrammatic representation of the aforementioned method to obtain the **(NEG1)**.



**Figure C.1:** Diagrammatic representation of the formulation process used to obtain **(NEG1)**



**Figure C.2:** Mechanical Heidolph RZR 2041 overhead stirrer (Heidolph Instruments GmbH & Co. KG, Germany), used in the formulation of nano-emulgel **(NEG1)**.

### C.5 Outcome

The formulation of a semi-solid dosage form was to achieve the aim of developing a transdermal delivery system for the treatment of hypercholesterolemia. Consequently, a nano-

emulgel was formulated for each of the selected statins by utilising the respective optimised o/w nano-emulsion. The **(NEG1)** formulation for each of the respective statins resulted in a smooth homogeneous gel. **(NEGL)**, **(NEGM)** and **(NEGR)** were white in colour, while **(NEGS)** had a beige colour, similar to that of the APIs powder. These nano-emulgels were then characterised due to the importance to establish or predict whether the skin can be reached, if a therapeutic effect will be achieved and if the formulations are stable and safe (pH) for use on human skin. These characteristics of the nano-emulgels were then compared to those of the respective nano-emulsions in the following sections.

## **C.6 Characterisation of the nano-emulgels (semi-solids)**

As stated in Section C.4.2, the optimised nano-emulsions formula was used to formulate nano-emulgels for each of the selected statins. Consequently, characterisation tests had to be performed on these nano-emulgels (holds both gel and nano-emulsion characteristics). The nano-emulgels (**(NEGL)**, **(NEGM)**, **(NEGR)** and **(NEGS)**) were investigated in terms of:

- morphology (light microscopy);
- pH;
- droplet size and distribution;
- zeta-potential, and
- viscosity.

Valentine (2014:153) stated that these characteristics, along with others (i.e. the interfacial tension between the two phases), can have a significant impact on the release of the API.

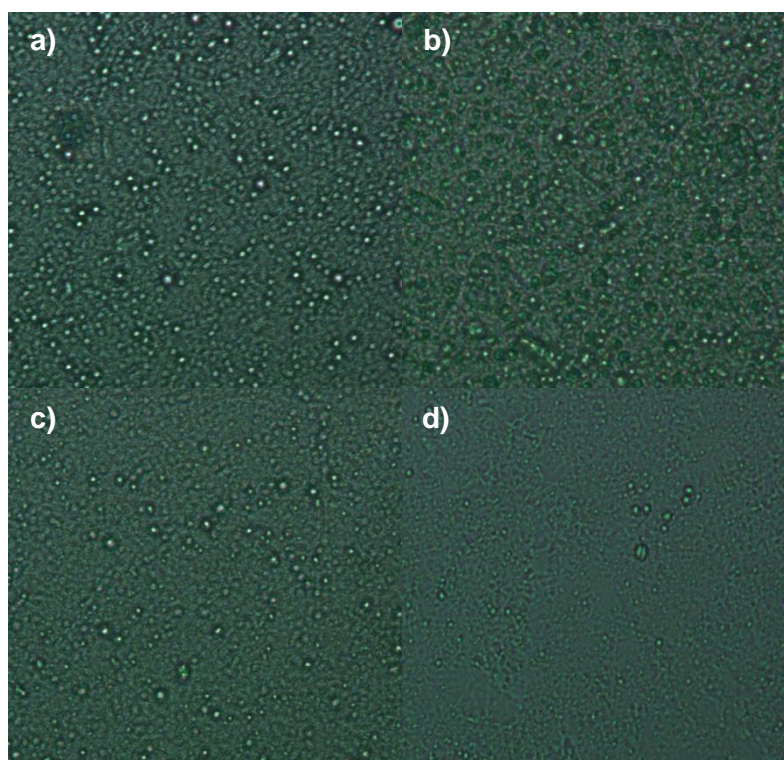
### **C.6.1 Light microscopy**

Light microscopy was utilised to observe the morphology of the nano-emulgels. This characterisation test was performed by utilising a Nikon Eclipse 50i microscope, fitted with a Nikon DSfi1 camera (Nikon, Japan Linkam THMS600), equipped with a T95 LinkPad temperature controller (Surrey, ENG), as displayed in Figure C.3. The micrographs were captured by utilising NIS-elements D (4.00.01, 64-bit) software. To prepare the samples intended for investigation, a small volume of the respective nano-emulgels (**(NEGL)**, **(NEGM)**, **(NEGS)** and **(NEGR)**) were placed on microscope slides, and covered with a 16 mm cover slip. The slides were then examined on the microscope individually, by implementing several magnifications, but it was concluded that a 50x magnification was ideal to investigate the morphology of the nano-emulgels. The respective nano-emulsions (**(NEL1)**, **(NEM1)**, **(NES1)** and **(NER1)**) were not investigated by means of light microscopy, as the morphology of these dispersions was investigated by means of TEM. TEM was not performed on the nano-emulgels

due to the viscosity of the formulations, which requires alteration in sample preparation. These alterations involve dissolving the viscous formulation in ethanol, to separate and destroy the nanoparticles from the emulsion matrix, consequently changes in the properties of the nanoparticle within the sample can occur (Klang *et al.*, 2013:117).



**Figure C.3:** Nikon Eclipse 50i microscope



**Figure C.4:** Light microscopy micrographs of: a) (NEGL), b) (NEGM), c) (NEGS) and d) (NEGR)

The micrographs (Figures C.4.a – C.4.d) were taken to illustrate the droplet size of the respective nano-emulgels. From Figure C.4, it can be stated that small droplets were visible. It should be noted that due to magnification differences between TEM (higher magnification capability) and light microscopy, these droplets may appear small, compared to the TEM micrographs.

### C.6.2 pH

The pH of formulations before neutralisation varies between 2.5 – 3.5, due to the incorporation of Carbopol® Ultrez 20 as a gelling agent (Lubrizol, 2009:1). Consequently, it is essential to determine and adjust the pH to ensure that the formulations are safe for transdermal application (Nair *et al.*, 2013:425). A Mettler Toledo® pH meter (Mettler Toledo, CU) fitted with a Mettler Toledo® InLab® 410 electrode (Mettler Toledo, CU) was utilised for this determination. The electrode was placed in the semi-solid, where after a reading was obtained. Sodium hydroxide was then utilised to adjust the pH to the desired values. Triplicate readings of the adjusted pH were taken.

**Table C.3:** The average pH values of the respective nano-emulsions and nano-emulgels

Formula	Average pH value
(NEL1)	5.83 ± 0.033
(NEGL)	5.82 ± 0.005
(NEM1)	6.13 ± 0.017
(NEGM)	6.05 ± 0.005
(NER1)	6.52 ± 0.005
(NEGR)	6.56 ± 0.005
(NES1)	5.87 ± 0.005
(NEGS)	5.82 ± 0.005

The pH of the nano-emulgels was adjusted to that of its respective nano-emulsion. This was firstly done to neutralise the Carbopol® Ultrez 20 and secondly to ensure the pH was within a safe range for application to the skin. Furthermore, adjusting the pH of the nano-emulgels to that of their respective nano-emulsion would ensure that a more accurate comparison could be drawn between the dispersions and semi-solid formulation during characterisation and *in vivo* skin diffusion studies.

### C.6.3 Droplet size and distribution

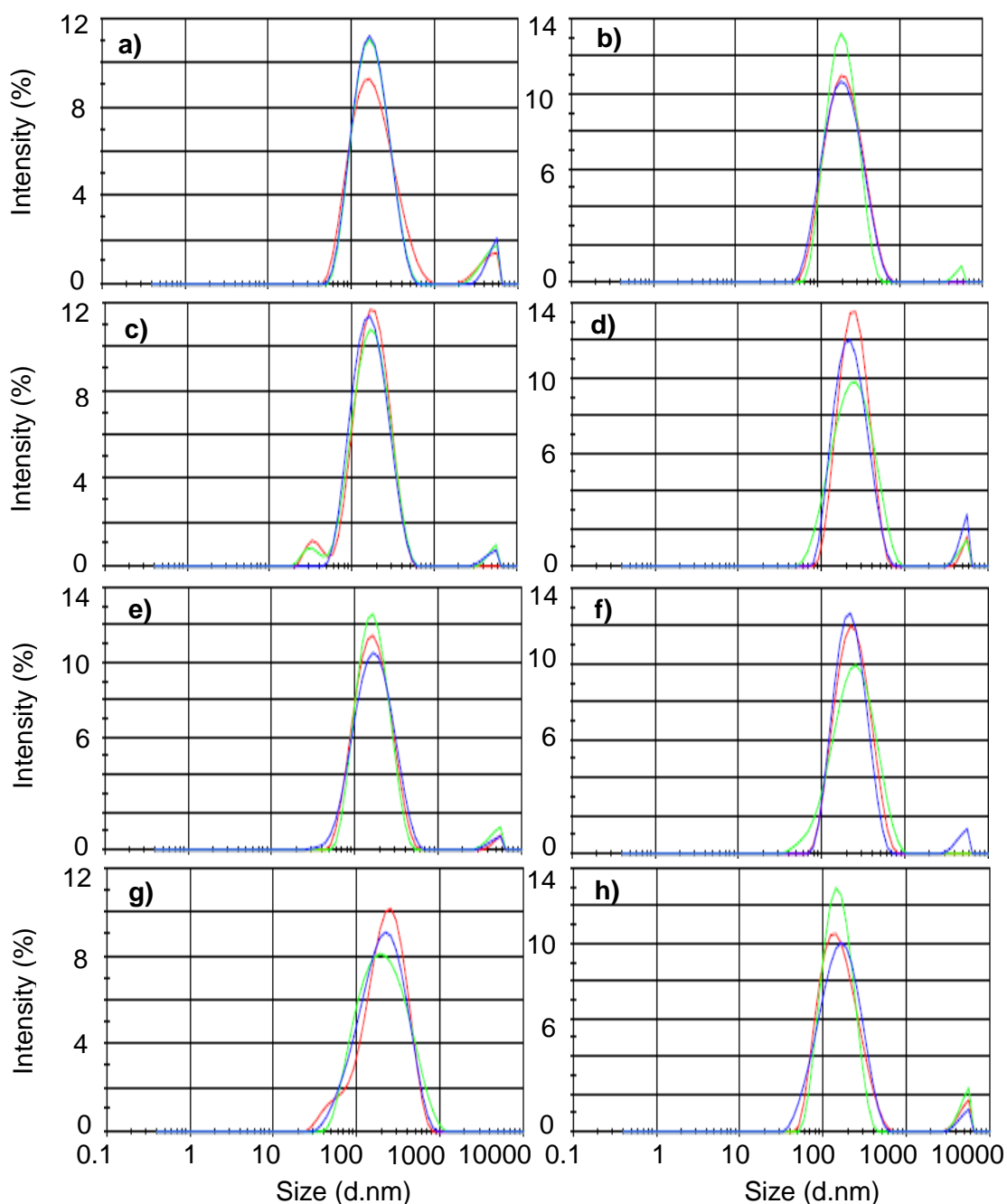
A technique called PCS was employed by utilising a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK), which determines droplet size and distribution by calculating the light scatter variation of droplets caused by Brownian motion (Chime *et al.*, 2014:96; Patravale *et al.*, 2004:833). Therefore, this characterisation test provided important information

on two essential aspects, droplet size and distribution of droplets. Droplet size will provide information on solubility and dissolution, but most importantly, the stability of the formulation (Patravale *et al.*, 2004:833; Reddy *et al.*, 2013:88). Where nano-emulgels are concerned, this will indicate whether the droplet size fell within the acceptance criteria, as droplet size determination by means of TEM, will prove to be difficult because of the viscosity of these formulations. Droplets size that is seen as acceptable, ranges between 100 – 500 nm (Drais & Hussein, 2017:10), but Baresa *et al.* (2015:1871) stated that droplet size can be seen acceptable from 10 – 100  $\mu\text{m}$ . For the purpose of this study, the droplet range of 100 – 500 nm will be used for the acceptance criteria. Droplets size distribution (Pdl) will be indicative of the uniformity in which droplet sizes are distributed (Chime *et al.*, 2014:97; Patravale *et al.*, 2004:833); hence, establishing whether the semi-solid is monodispersed or polydispersed (Gaumet *et al.*, 2008:3-4; Gaur *et al.*, 2014:36). A dilution (in a polytop) was made for each of the respective nano-emulgels ((**NEGL**), (**NEGM**), (**NEGR**) and (**NEGS**)) where after 2 ml was extracted and placed in clear disposable zeta-cells (DTS1070 folded capillary cell) for analysis in triplicate.

By formulating a conventional emulgel, effective skin permeation will prove to be difficult due to the large droplet size of this delivery system (Baibhav *et al.*, 2011:66). Consequently, this restriction can be overcome by formulating a nano-emulgel due to the small nanometric droplets size of this system; however, literature suggests that an increase in droplet size can occur in nano-emulgels compared to dispersions with the addition of a polymer (Eid *et al.*, 2014:5). Table C.4 along with Figure C.5 displays the average droplets size and Pdl obtained for both the (**NEF1**) dispersions and (**NEG1**) formulation. The Pdl is used to describe the dispersity of the droplets within the aqueous phase (Shakeel *et al.*, 2007:E6). Pdl is measured on a scale ranging from 0 – 1, where formulation measuring closer to 0 is considered to be monodispersed and those closer to 1 are seen as polydispersed (Gaumet *et al.*, 2008:5; Shaw, 2016). In conjunction with determining the droplet size, the Pdl was also established for each of the (**NEF1**) dispersions and (**NEG1**) formulations as tabulated in Table C.4.

**Table C.4:** Average droplet size and Pdl of (**NEF1**) and (**NEG1**) dispersions

Formula	Average droplet size(nm)	Average Pdl
( <b>NEL1</b> )	162.60 $\pm$ 1.735	0.335 $\pm$ 0.003
( <b>NEGL</b> )	172.10 $\pm$ 3.550	0.236 $\pm$ 0.005
( <b>NEM1</b> )	147.17 $\pm$ 1.850	0.248 $\pm$ 0.001
( <b>NEGM</b> )	213.93 $\pm$ 5.762	0.347 $\pm$ 0.003
( <b>NES1</b> )	153.10 $\pm$ 1.249	0.238 $\pm$ 0.009
( <b>NEGS</b> )	201.40 $\pm$ 1.805	0.267 $\pm$ 0.014
( <b>NER1</b> )	169.60 $\pm$ 2.615	0.279 $\pm$ 0.004
( <b>NEGR</b> )	151.05 $\pm$ 1.050	0.285 $\pm$ 0.017



**Figure C.5:** Average droplet size measured per droplet radius of: a) **(NEL1)**, b) **(NELG)**, c) **(NEM1)**, d) **(NEMG)**, e) **(NES1)**, f) **(NESG)**, g) **(NER1)** and h) **(NERG)**

From Table C.4, it can be observed that both the nano-emulsions and the nano-emulgels obtained droplet sizes well within the acceptance criteria, with little to no change between the nano-emulsion and nano-emulgel after the incorporation of Carbopol® Ultrez 20 (Drais & Hussein, 2017:10). The increase in droplet size as seen with **(NEGL)** correlated with literature, as the addition of a gelling agent increases the viscosity due to a higher degree of cross-linking (Eid *et al.*, 2014:5). The Pdl of both the dispersion and formulation is indicative of a relatively monodispersed formula, hence, it can be seen as ideal (Shakeel *et al.*, 2007:E6). An increase in droplet size is observed with the formulation of **(NEGM)** in Table C.4; this could be ascribed

to the possibility that this specific nano-emulgel requires increased stirring speed (Baudonnet *et al.*, 2002:499) to obtain droplets closer to the of the respective dispersion (**NEM1**). Even though an increase of droplet size is observed, the droplet size of the formulation still falls within the acceptance criteria of 100 – 500 nm (Drais & Hussein, 2017:10). It can further be noted that with the increase in droplet size with (**NEGM**), an increase in Pdl also occurred, resulting in a less monodispersed formulation, although the value is still closer to 0, as seen with (**NEM1**). If the stirring rate is increased (to decreasing droplets size), it could have a negative impact on the viscosity (lower viscosity), while lower stirring speeds will result in a decrease in Pdl and constant viscosity, even though the droplet size decreases (Baudonnet *et al.*, 2002:499).

From Table C.4, it can be stated that the incorporation of Carbopol® Ultrez 20, had no significant effect on the droplet size or Pdl of the (**NEGR**) formulation. However, the droplet sizes of both the (**NER1**) dispersion and (**NEGR**) formulation was well within range as both displayed a droplet size of < 200 nm, which is ideal. The Pdl of both formulas is indicative of a monodispersed dispersion (0.248) and formulation (0.285). The same conclusion can be drawn for (**NES1**) and (**NESG**) when observing the mevastatin formulas ((**NEGM**) and (**NEM1**)). The difference however with (**NESG**) is that a significant variation is observed between the droplet size of the dispersion (**NES1**) and formulation (**NEGS**). Consequently, the droplet size in the case of (**NEGS**) could be decreased by increasing the stirring speed rather than the stirring time (Baudonnet *et al.*, 2002:499).

#### C.6.4 Zeta-potential

The electrokinetic potential, i.e. the surface charge, that exists within a colloidal system can be described as the zeta-potential (Eid *et al.*, 2014:2; Silva *et al.*, 2012:860; Thakur *et al.*, 2012:223). The zeta-potential of both the dispersions and the semi-solids (nano-emulgels) was determined by utilising a Malvern Zetasizer ZS (Malvern Instruments, Worcestershire, UK). The Zetasizer can establish zeta-potential by means of a process called PCS, which measures the light fluctuations between dispersed droplets (Gaur *et al.*, 2014:40). The samples for analysis of the semi-solids were prepared by diluting one drop of each respective nano-emulgel in a polytop with 20 ml Milli-Q® water. A syringe was then used to extract and transfer 2 ml of each respective dilution into a zeta-cell (DTS1070 folded capillary cell). The measurement took place in triplicate, and one day after the initial preparation of the nano-emulgels.

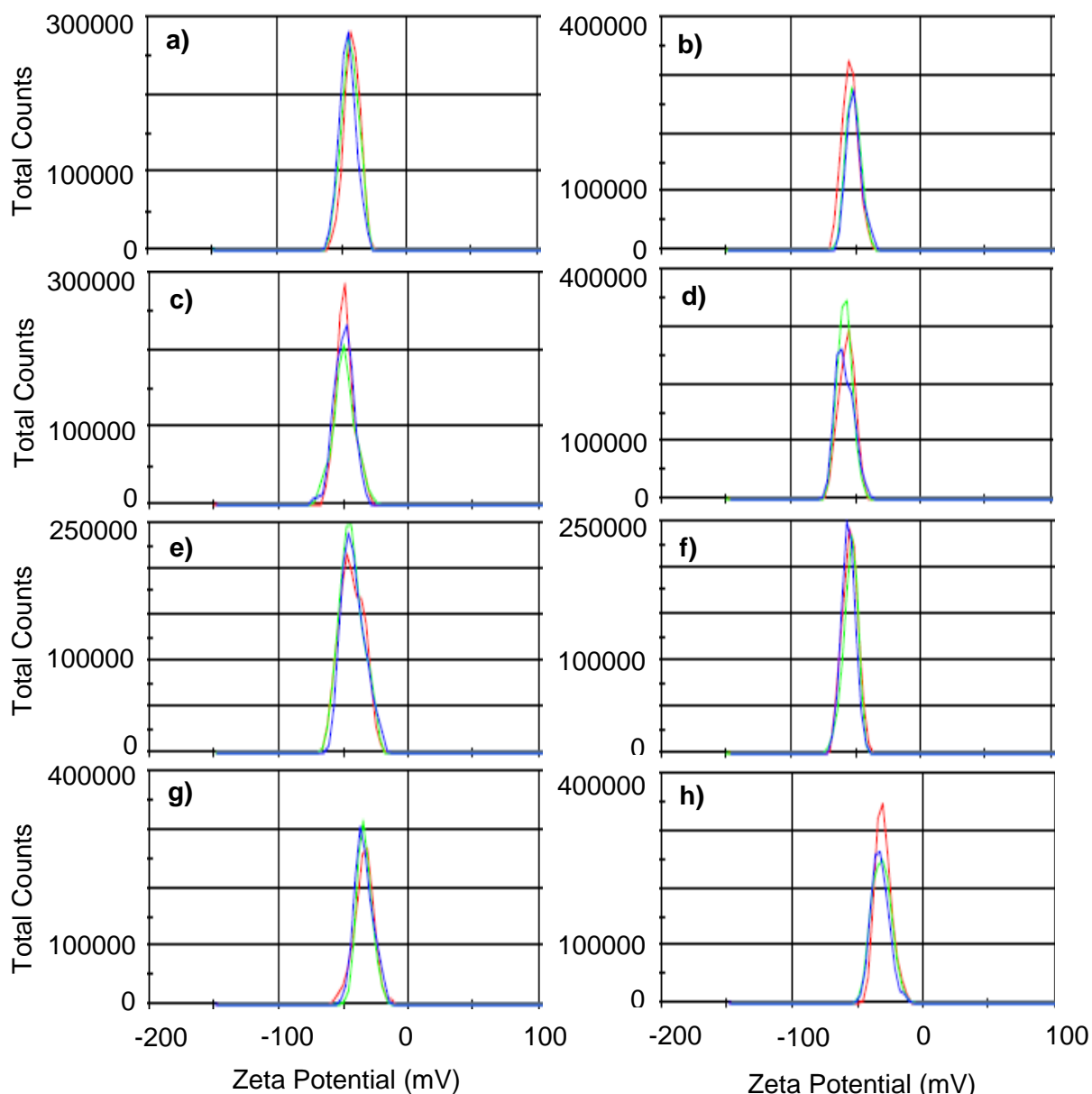
The addition of Carbopol® Ultrez 20 can cause an increase in the zeta-potential of the formulation compared to that of the dispersions, due to the influence of this gelling agent on the surface charger of the droplets (Eid *et al.*, 2014:5). This is however advantageous, as formulas with a zeta-potential of  $\leq \pm 30$  mV can remain stable over time (Eid *et al.*, 2014:2; Silva *et al.*,

2012:860), in addition, diffusion and API flux can be improved with negatively charged molecules (Sinico *et al.*, 2005:129).

**Table C.5:** The comparison of zeta-potential average between **(NEF1)** and **(NEG1)** dispersions

Formula	Zeta-potential			
	Reading 1	Reading 2	Reading 3	Average
<b>(NEL1)</b>	-42.2	-43.7	-45.4	-43.77 ± 1.601
<b>(NEGL)</b>	-54.6	-54.6	-54.6	-52.67 ± 1.389
<b>(NEM1)</b>	-48.9	-49.7	-50.1	-49.57 ± 0.611
<b>(NEGM)</b>	-57.1	-57.1	-57.1	-58.07 ± 0.694
<b>(NES1)</b>	-43.9	-44.1	-42.6	-43.53 ± 0.815
<b>(NEGS)</b>	-54.5	-54.5	-54.5	-54.87 ± 0.665
<b>(NER1)</b>	-34.5	-34.6	-33.9	-34.35 ± 0.344
<b>(NEGR)</b>	-39.6	-38.9	-40.2	-39.57 ± 0.531

From Table C.5, it can be observed that an increase in zeta-potential occurred with the incorporation of Carbopol® Ultrez 20 into the water phase of **(NEGL)** from - 43.77 mV to - 52.67 mV. Therefore, it can be proposed that this increase in zeta-potential led to an increase in the stability of the formulation (Eid *et al.*, 2014:5; Silva *et al.*, 2012:860). **(NEMG)** shows an increased zeta-potential when compared to **(NEM1)**, consequently correlating with the prediction stated in the literature; both **(NEM1)** and **(NEGM)** can be purported to be stable over time, as both obtained zeta-potential well below - 30 mV (Eid *et al.*, 2014:5; Silva *et al.*, 2012:860). The zeta-potential of **(NEGS)** also increased due to the addition of Carbopol® Ultrez 20 as observed in Table C.5, and it can be proposed that **(NEGS)** is a more stable formula than **(NES1)**, as stated in literature (Eid *et al.*, 2014:5); both **(NES1)** and **(NEGS)** can be viewed as stable formulas due to the fact that both the dispersion and formulation displayed highly negative zeta-potential values of - 43.53 mV and - 54.87 mV, respectively. From Table C.5 it can be observed that **(NEGR)** obtained a more negative zeta-potential when comparing the formulation to the respective nano-emulsion (**(NER1)**), hence proposing that **(NEGR)** is a more stable formula than **(NER1)**.



**Figure C.6:** The average zeta-potential (mV) of a) (NEL1), b) (NELG), c) (NEM1), d) (NEMG), e) (NES1), f) (NESG), g) (NER1) and h) (NERG)

### C.6.5 Viscosity

To achieve an increase in viscosity, the mixing speed and time are important considerations. It is proposed that 800 – 1200 rpm for  $\pm 20$  min is optimal. Although extremely high shear mixers will shorten the mixing time, it is not recommended, as this type of intense mixing can result in permanent loss of viscosity (Lubrizol, 2009:1). Furthermore, neutralisation of Carbopol® Ultrez 20 by means of a suitable alkali or amine base is essential to obtain an increased viscosity (Lubrizol, 2009:2); for example, unneutralised Carbopol® 934 NF with a viscosity of 390 cP can increase to 50 000 cP when neutralised to pH 7 (Lubrizol, 2011:1). When incorporating a gelling agent, the nano-emulgel should be highly viscous, uniform and consistent (Dhawan *et al.*, 2014:71). Consequently, in addition to the low pH of the semi-solids

after formulation, the adjustment of pH is essential to obtain the desired viscosity due to the occurrence of neutralisation of Carbopol® Ultrez 20 (Shin *et al.*, 2000:307). Therefore, viscosity could only be determined after the pH was adjusted and measured by means of a Brookfield Viscometer DV2T LV Ultra (Middleboro, Massachusetts, USA) fitted to a thermostatic bath at a controlled temperature of  $\pm 25$  °C. Before measurements could take place, the appropriate spindle for each of the respective nano-emulgels had to be selected and fitted to the viscometer. T-bar spindles in conjunction with the Helipath Stand accessory enables the measurement of viscosity of formulations that are non-flowing or slow flowing, i.e. creams, pastes, and gels. Due to the unique geometry of the T-bar spindles, which prevents the calculation of shear stress and shear rate, results are only considered “apparent” (Brookfield, 2014:5).

The sample chamber was not utilised during the viscosity measurement of the semi-solids due to the high viscosity of these formulations, hence it was kept in the initial container. The viscosity was then calculated for **(NELG)**, **(NEMG)**, **(NESG)** and **(NERG)** by adjusting the Rheocalc-T 1.2.19 software to multipoint readings every 20 s for 3 min with the following settings:

**Table C.6:** Settings used on Rheocalc-T 1.2.19 to measure the viscosity of the respective nano-emulgels

Nano-emulgel	Spindle	Speed (RPM)
<b>(NEGL)</b>	T-E	10
<b>(NEGM)</b>	T-F	30
<b>(NEGR)</b>	T-E	20
<b>(NEGS)</b>	T-C	10

According to Brookfield (2018:3), the full scale viscosity range (in cP) of these spindles can be determined by the following equation:

$$\text{Full scale range (cP)} = \text{Spindle coefficient} / \text{Spindle speed} \quad \text{Equation C.1}$$

**Table C.7:** Average viscosity (cP) and torque (%) measurements of **(NEF1)** dispersions and **(NEG1)** formulations

Formula	Viscosity (cP)	Torque (%)
<b>NEL1</b>	4.64 ± 0.06	12.25 ± 0.42
<b>NEGL</b>	46090.00 ± 164.40	98.44 ± 0.44
<b>NEM1</b>	4.05 ± 0.01	27.00 ± 0.06
<b>NEGM</b>	27985.71 ± 262.72	89.61 ± 0.80
<b>NER1</b>	7.87 ± 0.08	52.45 ± 0.54
<b>NEGR</b>	8358.43 ± 280.27	89.58 ± 3.01
<b>NES1</b>	8.38 ± 0.03	55.83 ± 1.77
<b>NEGS</b>	19151.43 ± 53.57	81.53 ± 0.51

By utilising Equation C.1, it was determined that the respective spindle used to determine viscosity of the nano-emulgels obtained values within the intended range. From Table C.7, it is clear that the incorporation of a gelling agent into the water phase of the nano-emulsion resulted in a notable increase in viscosity reading. It can also be noted that although the same amount of Carbopol® Ultrez 20 was incorporated into each of the respective nano-emulgels (**(NEGL)**, **(NEGM)**, **(NEGS)** and **(NEGR)**), the viscosity reading varied considerably between the nano-emulgels. The aim to increase the viscosity of the respective nano-emulsion by incorporating a gelling agent into the water phase (as stated in Section C.1) was however achieved when observing Table C.7.

### C.7 Discussion and conclusion

The use of a nano-emulgel increased the viscosity (Table C.8) of the aqueous phase due to the decrease in surface and interfacial tension, hence, serving as an improvement on a stable nano-emulsion. Therefore, this semi-solid formulation will improve the nano-emulsions in terms of stability, as the oil droplets serving as a carrier for lipophilic drugs will be distributed into a gel network. Nano-emulgels enhance drug delivery, for example through better skin adhesion, and this will improve patient compliance, as this semi-solid formulation is non-sticky and easy to spread compared to other similar dosage forms (Chellapa *et al.*, 2015:44). When comparing the zeta-potential between the respective dispersions and formulations, it can be observed that an increase in zeta-potential occurred in the case of the formulations. This correlated with what is suggested in literature, as the addition of Carbopol® Ultrez 20 can cause an increase in zeta-potential, as a result of the influence of this gelling agent on the surface charge of the droplets (Eid *et al.*, 2014:5). It can be proposed that the nano-emulgel formulation is more stable than the respective nano-emulsion, due to the more negative zeta-potential values displayed by these formulations (Eid *et al.*, 2014:2; Silva *et al.*, 2012:860), although, **(NEGR)** displayed a less

significant difference. The droplet size measurements of the nano-emulgels **(NEGL)**, **(NEGM)** and **(NEGS)** showed an increase in the average droplet size when compared to the respective dispersions, which can be explained, as Eid *et al.* (2014:5) stated that an increase in droplet size can occur due to the incorporation of a polymer. Although, a similar conclusion could not be made in the case of **(NEGR)**, the smaller droplet size of this nano-emulgel could possibly be attributed to the lower viscosity of **(NEGR)** when compared to the other nano-emulgels, as Eid *et al.* (2014:5) stated that an increase in viscosity could increase droplet size due to a higher degree of crosslinking. All the respective nano-emulgels, however, obtained an average droplet size within the acceptance range of 100 – 500 nm (Drais & Hussein, 2017:10). Furthermore, it can be concluded that the aim (to increase the viscosity), as stated in Section C.1, was achieved when observing Table C.8.

Finally, it can be concluded that the formulation of a nano-emulgel from a stable nano-emulsion enhanced the stability of the nano-emulsion (in terms of zeta-potential). It can also be suggested that this semi-solid formulation will increase patient compliance, due to ease of application and the non-sticky nature of these formulations (Chellapa *et al.*, 2015:45).

**Table C.8:** Summary of the characteristics of the **(NEF1)** dispersions and **(NEG1)** formulations

Formula	pH	Droplet size (nm)	Zeta-potential (mV)	Viscosity (cP)
<b>(NEL1)</b>	5.83 ± 0.03	162.60 ± 1.74	-43.77 ± 1.60	4.64 ± 0.06
<b>(NEGL)</b>	5.82 ± 0.01	172.10 ± 3.55	-52.67 ± 1.39	46090.00 ± 164.40
<b>(NEM1)</b>	6.13 ± 0.02	147.17 ± 1.85	-49.57 ± 0.61	4.05 ± 0.01
<b>(NEGM)</b>	6.05 ± 0.01	213.93 ± 5.76	-58.07 ± 0.69	27985.71 ± 262.73
<b>(NES1)</b>	5.87 ± 0.01	153.10 ± 1.25	-43.53 ± 0.82	8.38 ± 0.03
<b>(NEGS)</b>	5.82 ± 0.01	201.40 ± 1.81	-54.87 ± 0.67	19151.43 ± 53.57
<b>(NER1)</b>	6.52 ± 0.01	169.60 ± 2.62	-34.35 ± 0.34	7.87 ± 0.08
<b>(NEGR)</b>	6.56 ± 0.01	149.83 ± 1.92	-39.57 ± 0.53	8358.43 ± 280.27

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## *APPENDIX D:*

# *Franz cell diffusion studies of an o/w nano-emulsion and nano-emulgels dosage forms containing the selected statins and apricot kernel oil*

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### **D.1 Introduction**

The skin as a site of administration is both convenient and accessible. Due to this, much time and investments in both the past and the present have been made, with constant improvements of new and ground-breaking approaches in the transdermal field. The aim of this field is to develop safe and efficient methods from drug delivery across the skin (Alkilani *et al.*, 2015:438) at a predetermined and controlled rate (Rastogi & Yadav, 2012:161). As the system enables the transport of an API through the skin into the systemic circulation at a fixed or controlled rate, it can possibly be utilised to improve the characteristics of the approximately 74% of orally taken drugs not exhibiting the desired efficacy (Marwah *et al.*, 2016:564).

Although statins (HMG-CoA inhibitors) are considered the primary treatment for hypercholesterolemia (Mach *et al.*, 2018:2526), the safety of treatment with statins has become an important issue (Jacobson, 2006:44C). The concern regarding statins safety was brought about by reports of statin associated side effects (also known as statin intolerance), resulting in discontinuation without proper consultation (Raju *et al.*, 2013:977). The most common side effects include gastrointestinal (such as nausea and abdominal pain) (Kiortsis *et al.*, 2007:8), myositis and myalgias (Russo *et al.*, 2013:680). In addition, statin provoked hepatotoxicity has been documented, although the incidence is rare (Cueto *et al.*, 2008:20). Due to the clearly documented correlation between dose administered and statins associated side effects (Maji *et al.*, 2013:642), attempting the transdermal delivery of the selected statins could prove to be advantageous, as this will limit the hepatic first-pass metabolism, therefore lower doses than with oral formulations could be utilised to attain the desired plasma level (Isaac & Holvey, 2012:256).

While there are many advantages in utilising the transdermal route of administration, it is of great importance that the API selected for transdermal delivery should possess the desired physiochemical properties (Tanwar & Sachdeva, 2016:2283) to facilitate permeation and absorption of an API through the skin, which regulates or limits the extent of API permeation (Williams, 2013:680). In addition, selection of an appropriate vehicle for transdermal delivery is essential, as manipulation of the API or vehicle becomes essential when the API does not

exhibit the ideal physiochemical properties. One approach of vehicle manipulation is utilising nano-emulsions (Mbah *et al.*, 2011:684-686). Furthermore, a two-phase nano-emulsion possesses both hydrophilic and lipophilic characteristics, which can aid in the delivery of the API through the stratum corneum and to the underlying layers (Gaur *et al.*, 2014:37), which specifically proves advantageous in the formulation of lipophilic molecules (such as the statins), by enhancing their solubility and subsequently, the bioavailability (Nastiti *et al.*, 2017:2). Consequently, nano-emulsions were formulated containing the respective statins, and nano-emulgels were formulated to improve the application of nano-emulsions to the skin. The delivery of transdermal systems is evaluated by means of *in vitro* diffusion studies, hence, *in vitro* membrane release and skin diffusion studies by means of the vertical Franz cell method (Ng *et al.*, 2010b:1432; Salamanca *et al.*, 2018:3; Williams, 2013:683), were performed during this study. To determine whether release from the vehicle (nano-emulsion and nano-emulgel) occurred, membrane release studies were performed prior to the skin diffusion studies. The last-mentioned, in conjunction with tape stripping, was performed to establish whether transdermal delivery and/or topical delivery was achieved with the respective nano-emulsions and nano-emulgels.

## D.2 Methods

### D.2.1 HPLC analysis of the selected statin samples

An HPLC method was developed and validated, which was utilised to determine and quantify the amount of each respective statin (lovastatin, mevastatin, rosuvastatin and simvastatin) present in the samples collected during the *in vitro* diffusion studies. As described in Appendix A, these methods were successfully validated and could therefore be used to quantify the concentration of each selected statin present in the **(NEF1)** dispersions and **(NEG1)** semi-solid formulations, due to its reliable, responsive and sensitive nature.

The samples intended for analysis by means of HPLC were collected from the receptor compartment of the Franz cells and analysed with a Dionex UltiMate 3000 dual system. This system was fitted with ternary gradient pumps, column ovens, autosampler and diode array detectors. The system was operated on Chromeleon 7.2 data acquisition and analysis software (Thermo Fisher Scientific Inc., Waltham, MA). A Venusil XBP C<sub>18</sub>(2), 150 x 4.6 mm, 5 µm (Agela Technologies, Newark, DE) was fitted to the system along with acetonitrile (HPLC grade) and Milli-Q<sup>®</sup> water (in-lab) with 0.1% orthophosphoric acid, which served as the mobile phase. Controlled temperature (25°C) in the laboratory, along with specific chromatographic conditions (listed in Table D.1) were maintained throughout the duration of analysis.

During each analysis, a standard dilution of the respective statin was prepared ( $\pm$  20 mg statin in 100 ml methanol) followed by two dilutions. Dilution 1 was prepared by placing 5 ml of the

standard solution in a 50 ml volumetric flask, where after it was made up to volume with methanol. The second dilution (Dilution 2) was prepared by placing 5 ml of Dilution 1 into a 50 ml volumetric flask, where after it was made up to volume with methanol. The standard dilution along with dilutions 1 and 2 were injected as follows to obtain a standard curve for analysis:

- 2.5  $\mu$ l
- 5.0  $\mu$ l
- 7.5  $\mu$ l
- 10.0  $\mu$ l

**Table D.1:** The chromatographic conditions used during the analysis of samples obtained from the receptor phase to determine the concentration of the selected statin

<b>Run time</b>	10.00 min
<b>Injection volume</b>	10 $\mu$ l
<b>Flow rate</b>	1.00 ml/min
<b>Detection wavelength</b>	240 nm
<b>Retention times</b>	Lovastatin: $\pm$ 5.290 min Mevastatin: $\pm$ 4.840 min Rosuvastatin: $\pm$ 3.110 min Simvastatin: $\pm$ 4.867 min

## D.2.2 Physicochemical properties of the selected statins

### D.2.2.1 Solubility in various solvents

During this study, the solubility was calculated in PBS (pH 7.4), PBS:ethanol (9:1 at pH 7.4) and *n*-octanol. To obtain a standard curve for analysis, a standard solution was prepared by weighing  $\pm$  5 mg of the respective statin in a 100 ml volumetric flask, which was made up to volume with methanol and injected at different injection volumes (2.5, 5.0, 7.5 and 10.0  $\mu$ l).

#### D.2.2.1.1 Aqueous solubility

The aqueous solubility was examined to establish the solubility of each of the selected statins in PBS (phosphate buffer solution) at pH 7.4. It has been proposed that a direct correlation exists between the aqueous solubility and permeation of a compound (Encyclopaedia Britannica, 2016); consequently, it is a vital physicochemical property to consider when formulating a product intended for transdermal or topical use.

The experiment was conducted by pre-heating a water bath equipped with a rotating device (to ensure proper mixing) to 32 °C (temperature on top of the skin) (Williams, 2013:685). Three clean glass tubes (for each respective statin) were used and filled with 5 ml PBS (pH 7.4). An excess amount of the selected statin was placed in each of the three tubes to obtain a saturated solution, and placed in the preheated water bath and left to rotate for 24 h. The samples were examined regularly during the experiment to ensure they were saturated. The samples were removed (after 24 h) and centrifuged (4 500 rpm for 15 min). After centrifugation was completed, each respective sample was extracted from the tube by means of a syringe and filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter into an HPLC vial to ensure no undissolved particles reached the HPLC. Each of the respective samples were then analysed in duplicate. Note that this process was performed for each of the selected statins (lovastatin, mevastatin, rosuvastatin and simvastatin) respectively.

#### **D.2.2.1.2 Solubility in PBS:ethanol (9:1 at pH 7.4)**

Since lovastatin, mevastatin and simvastatin are soluble in organic solvents, such as methanol and ethanol (Cayman chemicals, 2008; O'Neil, 2006:968,1472), and rosuvastatin is only sparingly soluble in it (O'Neil, 2006:1428), a solubility test was also performed to determine if the addition of 10% ethanol to the PBS at pH 7.4 (10 ml of ethanol in 90 ml of PBS) would increase the solubility of the respective statins, which would then be utilised in the receptor phase. Hence, the same method as previously described in Section D.2.2.1.1, where 100% PBS (pH 7.4) was used.

#### **D.2.2.1.3 Solubility in *n*-octanol**

To establish whether lovastatin, mevastatin, simvastatin and rosuvastatin would be better soluble within a lipophilic phase, the same method as explained in Section D.2.2.1.1 was applied, except 100% *n*-octanol instead of 100% PBS (pH 7.4) was used. After the removal of the test tubes from the centrifuge, 1 ml of the *n*-octanol-statin mixture was dissolved with methanol in a 25 ml volumetric flask to ensure that the HPLC was not damaged. The solubility in *n*-octanol was then utilised to calculate the appropriate amount of statin to be used during the log D experiments.

#### **D.2.2.2 Octanol-buffer distribution coefficient**

The distribution coefficient (D) or log D, the logarithm thereof, can be described as the ratio of the total concentration of a molecule that is distributed between the aqueous buffer phase and the hydrophobic organic phase, which is dependent on the log P (hydrophilic or lipophilic) of the compound and pH of the buffer solution (Krishnamoorthy *et al.*, 2018:1). The golden standard method used to determine the log D is considered to be the traditional shake flask method (Liu

*et al.*, 2011:967; Wenlock *et al.*, 2011:348). When a negative value is obtained for log D, it can possibly be indicative that the compound or drug will be more susceptible to a higher aqueous solubility (Bhal, 2007:3), whereas a log D value above 3 is indicative of low solubility (Mondal *et al.*, 2008:180).

Equal volumes of PBS (pH 7.4) and *n*-octanol were placed in a separating funnel to equilibrate for 24 h to ensure co-saturation of the two phases. Thereafter, the two phases (top layer consisted of *n*-octanol and the bottom layer of PBS at pH 7.4) were separated by means of the separating funnel. It was determined (by means of solubility test performed in Section D.2.2.1.3) that 40 mg of lovastatin and mevastatin would be placed in respective beakers containing 20 ml of the pre-saturated *n*-octanol, whereas only 4 mg of simvastatin and rosuvastatin would be added to their respective beakers (due to poor solubility) containing 20 ml of the pre-saturated *n*-octanol. The pre-saturated *n*-octanol/statin mixtures (3 ml) were placed in three different test tubes (for each statin), to which 3 ml of the pre-saturated PBS (pH 7.4) was added respectively. The samples were placed in the preheated water bath (32 °C) and left to rotate overnight ( $\pm$  8 h). After removal from the water bath, 1 ml of the octanol phase from each of the test tubes (three replicates per statin) was placed in a 10 ml volumetric flask and made up to volume with methanol to dilute the *n*-octanol and ensure the safety of the HPLC. An amount of each volumetric flask was then placed in a HPLC vial (three vials per statin). The PBS (pH 7.4) phase from each test tube was extracted and placed in a HPLC vial without dilution. All the respective vials were then analysed by means of HPLC (Andrés *et al.*, 2015:181). The log D was established by means of the ratio of the respective statin concentration present in the *n*-octanol phase to the concentration of the statin present in the PBS (pH 7.4) phase. The application of Equation D.1 can be utilised to determine the log D.

$$\text{Log D} = \frac{\text{Concentration in } n\text{-octanol}}{\text{Concentration in PBS (pH 7.4)}} \quad \text{Equation D.1}$$

A standard curved was obtained by preparing standard samples as discussed in Section D.2.1.

### **D.2.3 *In vitro* diffusion studies: vertical Franz cell method**

Although *in vivo* studies prove to be the most advantageous for drug development and risk assessment by measuring the rate and extent of drug absorption through the skin, *in vitro* studies are frequently preferred due to the economic and ethical aspects involved in *in vivo* studies (Karadzovska & Riviere, 2013:568). The accurate prediction of the drug outcome *in vivo* is essential in the development of suitable transdermal or topical formulations, necessitating robust and validated *in vitro* techniques and models (Bartosova & Baljar, 2012:4671). The most commonly used *in vitro* model or method, utilised to assess the formulation intended for skin application, is the diffusion cell (Karadzovska & Riviere, 2013:568;

Simon *et al.*, 2016:235). It is suggested that Franz cells are the most frequently utilised as a diffusion cell, consisting of a donor and receptor compartment, which are separated by either a synthetic membrane (as used in release studies) or human skin samples (used for permeation studies) (Simon *et al.*, 2016:235).

It is essential that when conducting an *in vitro* diffusion study, that it should resemble *in vivo* conditions as closely as possible (Modi & Shah, 2015:1). Consequently, one vital factor that should be monitored is temperature, as fluctuation in temperature could significantly influence the rate of diffusions and therefore the results obtained from the respective *in vitro* study (Shahzad *et al.*, 2015:2). During a diffusion study, a water bath pre-heated to 37 °C (normal *in vivo* conditions) to submerge the receptor phase of the Franz cell, and a second water bath pre-heated to 32 °C (normal external skin temperature) (Williams, 2013:685) to pre-heat the formulas, are used.

### **D.2.3.1 Vertical Franz cell components**

Klein (2013:565) proposed that skin penetration by means of an *in vitro* method can be determined successfully when utilising a vertical Franz cell diffusion system. Consequently, this system was suitable to determine the release of the selected statin from both the **(NEF1)** dispersions and **(NEG1)** formulations respectively. In addition to the release of the statins, it could also be used to establish whether the API was delivered topically and/or transdermally.

#### **D.2.3.1.1 Preparation of receptor phase**

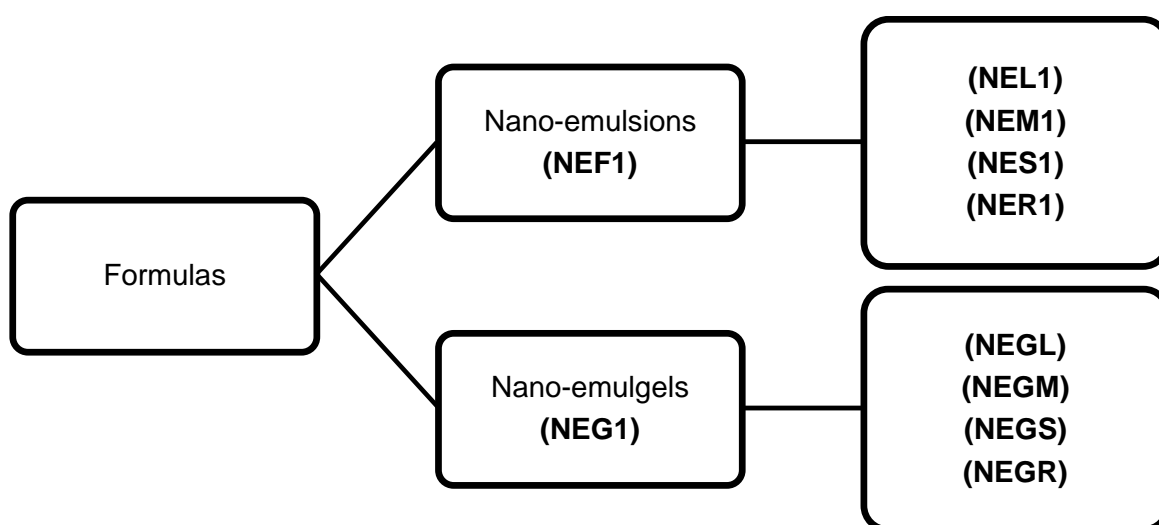
The solubility of an API is a very important physiochemical characteristic to consider when formulating a transdermal or topical product. With regards to diffusion studies, the importance of solubility in the receptor phase is essential, as it can affect the dissolution process and therefore the result obtained from the experimental diffusion study.

The PBS at pH 7.4 (1 000 ml) was prepared by dissolving 1.5 g NaOH in 400 ml Milli-Q<sup>®</sup> water with a magnetic stirrer. Thereafter, 6.5 g potassium dihydrogen orthophosphate (KH<sub>2</sub>PO<sub>4</sub>) was dissolved in 250 ml Milli-Q<sup>®</sup> water using a magnetic stirrer. The NaOH solution was then added to the KH<sub>2</sub>PO<sub>4</sub> solution while continuously stirring the solution. To this solution, a final volume of 350 ml Milli-Q<sup>®</sup> water was added, whereafter the pH was measured and adjusted to 7.4 by adding either orthophosphoric acid or NaOH using a Mettler Toledo<sup>®</sup> pH meter (Mettler Toledo, CU), equipped with a Mettler Toledo<sup>®</sup> InLab<sup>®</sup> 410 electrode (Mettler Toledo, CU). Thereafter, 100 ml of ethanol was added to 900 ml of PBS (pH 7.4) to attain the PBS:ethanol (9:1 at pH 7.4), as selection of a receptor phase into which a compound can freely partition is recommended for relatively lipophilic compounds, such as the statins (Collier & Bronaugh,

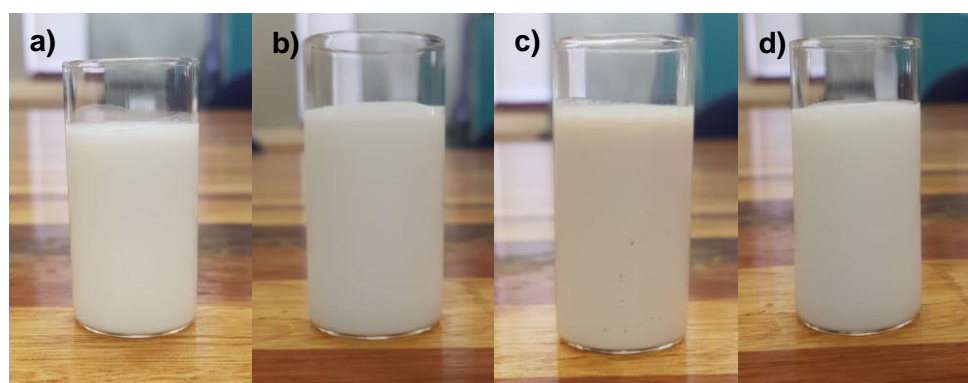
1991:47). Furthermore, it should be stated that Thomas *et al.* (2014:2533) found the addition of 10% ethanol to PBS acceptable, as it did not compromise the skin.

#### D.2.3.1.2 Test formulations and the preparation of the donor phase

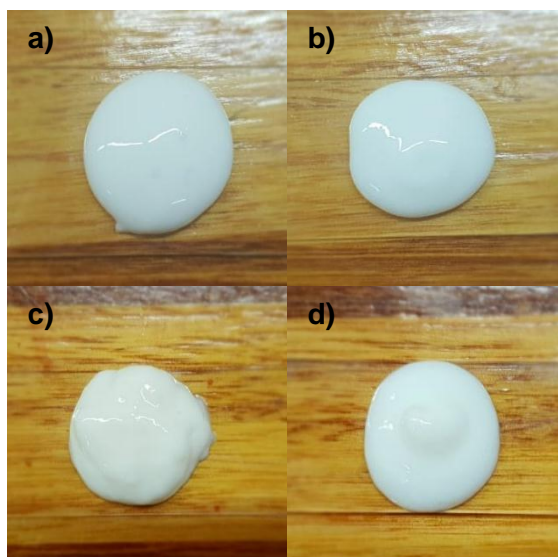
The nano-emulgels (**NEG1**) were formulated by utilising the (**NEF1**) dispersions (as discussed in Appendix C). The nano-emulsion (**NEF1**) formulas and nano-emulgel (**NEG1**) formulas containing the selected statins were separately tested during the *in vitro* diffusion studies. Hence, eight formulas containing the different statins, together with two placebos (containing no API), i.e. (**PNEF1**) and (**PNEG**) serving as a control, were tested and placed in the donor phase during each study. Figures D.2 and D.3 represent the dispersions and formulations, respectively, that were tested in both the membrane release and skin diffusions studies.



**Figure D.1:** Diagrammatic representation of the formulas tested during membrane release studies and skin diffusion studies



**Figure D.2:** Nano-emulsion formula (**NEF1**) tested: a) (**NEL1**), b) (**NEM1**), c) (**NES1**) and d) (**NER1**)



**Figure D.3:** Nano-emulgel formula (**NEG1**) tested: a) (**NEGL**), b) (**NEGM**), c) (**NEGS**) and d) (**NEGR**)

#### **D.2.3.2 Membrane release studies**

During the membrane release studies, conducted for each of the respective statin (**NEF1**) dispersions and (**NEG1**) semi-solid formulations, 12 vertical Franz cells were used. Two Franz cells contained only a placebo dispersion (**PNEF1**), or placebo semi-solid formulation (**PNEG1**), hence serving as a control and the other 10 Franz cells contained either the respective nano-emulsions (**NEF1**) or nano-emulgels (**NEG1**). All formulas were pre-heated to 32 °C in a water bath prior to commencing with the membrane release study. The receptor phase (PBS:ethanol (9:1 at pH 7.4)) was pre-heated in a second water bath to 37 °C. A vertical Franz cell consists of two compartments, namely a donor and receptor compartment. Dow Corning® high vacuum grease was applied to the each of the compartments on the connecting sides. A magnetic stirrer was then placed into the receptor compartment prior to placing the synthetic membrane (polyvinylidene fluoride (PVDF), Pall® Life Sciences, Michigan, USA synthetic membranes, with a pore size of 0.45 µm and 25 mm diameter) on the greased side of the receptor compartment. Thereafter, the donor compartment was placed on top of the receptor compartment and vacuum grease was used to thoroughly seal the sides of the two connected compartments to avoid leakage. Lastly, a horseshoe clamp was placed over the connecting compartments (donor on top of receptor compartment) to tightly fasten the two compartments (this was performed on all 12 Franz cells). The receptor compartment (1.075 cm<sup>2</sup> diffusion surface) has a filling capacity of ± 2ml. Therefore, 2 ml PBS:ethanol (9:1 at pH 7.4) was accurately measured by means of a pipette and used to fill the individual receptor compartments.



**Figure D.4:** Apparatus and materials utilised during membrane release studies in order of use  
 a) Franz cell with donor (top) and receptor compartment (bottom), b) PVDF synthetic membrane, c) Dow Corning® high vacuum grease, d) horseshoe clamp to fasten Franz cell compartments, e) Franz cell after filling the compartments, f) Grant® water bath, g) assembled Franz cells in Franz cell stand, placed on a magnetic stirrer plate within the water bath and h) syringes used for 1 h extractions for 6 h

After filling each receptor compartment, they were inspected to ensure that no air bubbles were trapped within the compartment. Each of the respective donor compartments was then filled with 1 ml of the formulation. Thereafter a piece of Parafilm® and plastic cap was used to cover each of the donor compartments of the assembled and prepared Franz cells. The Franz cells were placed in a Franz cell stand within the pre-heated water bath (Grant Instruments, UK) at 37 °C on a magnetic stirring plate (Variomag, USA). Every hour for the following 6 h, the entire receptor phase was extracted and refilled with the PBS:ethanol (9:1 at pH 7.4), was kept at a constant temperature of 37 °C. The extracted hourly samples were placed in individual HPLC vials and analysed by means of HPLC, as discussed in Section D.2.1.

### **D.2.3.3 *In vitro* skin diffusion**

By performing *in vitro* Franz cell skin diffusion studies and tape stripping, it can be established whether the API reached the receptor phase, hence if transdermal delivery was achieved, or if the API was retained within the stratum corneum epidermis (SCE) or epidermis dermis (ED), which represents topical delivery. Data was obtained after the analysis of the respective samples by means of HPLC.

#### **D.2.3.3.1 Skin ethics and collection**

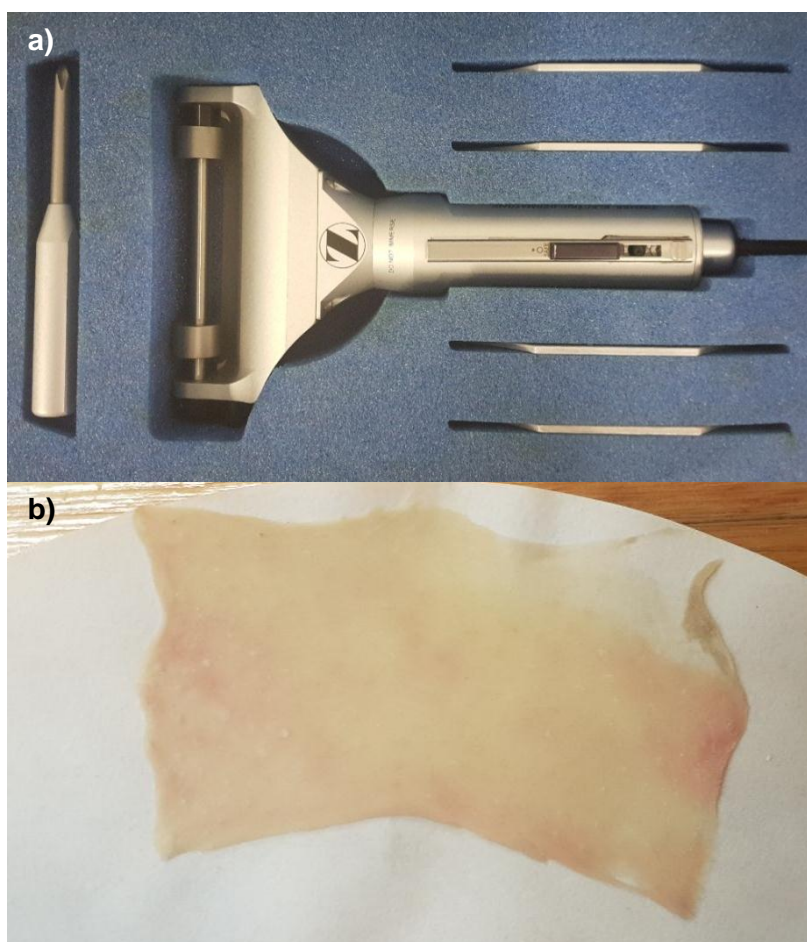
Informed consent was obtained from the participant, which was also signed by the practitioner performing the surgery in conjunction with a witness. The practitioner explained the informed consent to the patient and that participating in this study was in no way mandatory. In addition, the participant was allowed to withdraw from the study at any time. It is of great importance to protect the confidentiality of the participant and therefore all patient information was removed from the donated skin and replaced with a reference number. The patient's name along with the respective reference number was documented in a Donor File (log book) to ensure that if the participant wanted to withdraw, the correct skin could be identified. This information, however, was only accessible to authorised personnel and students. Although the participants were not formally recruited to partake in the study, they had to willingly donate skin (with informed consent) and comply with the predetermined criteria.

The skin obtained from abdominoplasty (Caucasian females: 21 – 80 years) was utilised for this study. As variations in skin can occur from region to region on the same individual, and more so between individuals, the utilisation of only female skin should minimise the variation in the results obtained and in addition, be easier to obtain since abdominoplasty is generally performed on females. Lastly, performing these studies only on skin obtained from abdominoplasty will allow the results to be compared to those published internationally. Ethics approval has been granted by the Health Research Ethics Committee (HREC) under the larger study titled "The use of human skin (biological waste) to study transdermal and topical delivery"

(Ethics no: NWU-00111-17-A1). Individual ethics approval was also obtained from HREC (Ethics no: NWU-00111-17-A1-03) for this study. When collecting the skin, it was transported in an appropriate container clearly marked as bio-waste, where after the skin was stored at - 20 °C until utilised.

#### D.2.3.3.2 Preparation of dermatomed skin

Before skin diffusion studies could be initiated, dermatomed skin samples were prepared. A Dermatome™ (Zimmer TDS, United Kingdom), as displayed in Figure D.5, was used to obtain dermatomed skin samples of  $\pm 400 \mu\text{m}$  by pressing the Dermatome™ on the skin at an angle of approximately 45 degrees. The samples of dermatomed skin was then placed on Whatman® filter paper and enfolded with aluminium foil to store the samples further at - 20 °C up until needed for skin diffusion studies. The skin samples needed were cut into circles before each respective study, in such a manner that each circle could be fitted between the receptor and donor compartment of a vertical Franz cell.



**Figure D.5:** a) Dermatome™ (Zimmer TDS, United Kingdom) and b) dermatomed skin samples of  $\pm 400 \mu\text{m}$  on Whatman® filter paper

#### **D.2.3.3.3 Skin diffusion studies**

Assessing skin permeability by means of *in vitro* diffusion cells can provide fundamental information on the relationship between the intended API, the delivery system and the skin (Ng *et al.*, 2010b:1432). By utilising these *in vitro* methods, percutaneous absorption can be established by means of passive diffusion through the non-viable stratum corneum (Venter *et al.*, 2001:169).

The same technique as explained in Section D.2.3.2 used during the membrane release studies, was implemented for the *in vitro* skin diffusion studies. The only alteration between the two methods were that the PVDF synthetic membranes were substituted with dermatomed skin samples. These dermatomed skin samples were placed between the receptor and donor compartments with the stratum corneum facing upwards. It was predetermined that one extraction of the receptor phase would take place 12 h after the study was initiated, based on a study performed by Burger (2014:108). Hence, after 12 h the entire volume of the receptor compartment was extracted and HPLC was utilised to analyse the samples collected by means of skin diffusion studies (Baert *et al.*, 2011:472, 473).

#### **D.2.3.3.4 Tape stripping**

For topical and transdermal research, it is essential to quantify the API in the skin (Escobar-Chávez *et al.*, 2008:104). Tape stripping more specifically will provide data on whether topical delivery of the API within either the SCE or the ED occurred (OECD, 2004:24). The purpose of this study, however, was to facilitate the transdermal delivery of the selected statins. Nevertheless, due to the physiochemical properties (lipophilicity) of the selected statins, the possibility existed that the statins could be retained by the lipophilic layer of the skin.

After completion of the skin diffusion study, the Franz cell compartments were separated and the skin was visually inspected before tape stripping commenced. The skin samples were removed and pinned to a piece of Parafilm® on a solid surface and by using a piece of paper towel, any remaining formulation or dispersion was gently dabbed off. 3M Scotch® Magic™ tape was cut into 16 strips that could sufficiently cover the diffusion area. To avoid possible contamination, the first strip was discarded and the remaining 15 strips, containing the API, stratum corneum and fractions of the epidermis were placed in a polytop containing 5 ml of methanol, serving as the extraction solution. The remaining skin was cut into pieces and placed into another polytop containing 5 ml of the extract solution. These polytops were then placed in the in the fridge ( $\pm 4$  °C) and left for approximately 8 h. Thereafter, a volume was extracted by means of a syringe from each of the polytops, respectively, and filtered through a 0.45 µm PTFE filter into an HPLC vial for analysis (Pellett *et al.*, 1997:91). This process was performed on each of the Franz cell samples of the respective study. By analysing the samples obtained

from the polytops, the tape strips and that of the polytops containing the remaining skin, it was possible to determine the concentration of the respective statins in the SCE and the ED.

#### **D.2.3.4 Data analysis**

The samples of each study (membrane release, and skin diffusion) were analysed by means of HPLC to obtain a linear line. This linear line could then be utilised to establish the concentration (i.e. drug flux) of each of the selected statins in the respective Franz cells (Ng *et al.*, 2010a:213). Consequently, the results obtained could be examined with regard to the average cumulative amount of the selected statin per area ( $\mu\text{g}/\text{cm}^2$ ), which had diffused through the membrane or skin, plotted against time (h) (Shakeel *et al.*, 2007:E3). Therefore, the average drug flux could be established by utilising the slope of the linear line (Ng *et al.*, 2010a:213). For the membrane release studies, the average flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) through the PVDF synthetic membrane of each of the selected statins was determined by means of linear regression attained at hourly intervals for 6 h.

During the skin diffusion studies, results were determined for each of the respective statins in terms of the amount per area diffused ( $\mu\text{g}/\text{cm}^2$ ) and the concentration ( $\mu\text{g}/\text{ml}$ ) for each Franz cell in conjunction with the average amount per area diffused ( $\mu\text{g}/\text{cm}^2$ ) and the average concentration ( $\mu\text{g}/\text{ml}$ ) of all the Franz cells together that diffused through the skin after 12 h.

By performing membrane release and skin diffusion studies, the release of the API from the dosage forms could be determined and proved especially beneficial during the testing of nano-particle (Jug *et al.*, 2017:177), while the use of human skin samples is considered the most advantageous *in vitro* method, to predict *in vivo* permeation of topically applied APIs (Simon, 2016:235). The aforementioned is also important in determining whether the target-site was reached, as for topical delivery, systemic absorption was not intended, while transdermal delivery requires a topically applied formulation to penetrate beyond the stratum corneum and epidermis layers (Kanfer, 2017:53).

#### **D.2.3.5 Statistical analysis**

Procedures ascribed to descriptive analysis include the calculation of the median (middle score in distribution) and mean concentrations, which includes the standard deviation (SD) (Sheskin, 2000:1, 4). The describing data only in terms of mean could be problematic, as data distribution and outliers can have a significant effect on the mean, while the median is less affected (Driscoll *et al.*, 2000:275). Box-plots will be used to display a summary of the data, by utilising the lower quartile (Q1 or 25<sup>th</sup> percentile), median (Q2 or 50<sup>th</sup> percentile), upper quartile (Q3 or 75<sup>th</sup> percentile) and extreme values (Krzywinski & Altman, 2014:119) and additionally the mean. The box extends over the middle 50% of the data, with the outer edges indicating the 25<sup>th</sup>

percentile and 75<sup>th</sup> percentile, while the middle line of the box is indicative of the median or 50<sup>th</sup> percentile (Nuzzo, 2016:269). In addition, to the box, whiskers are added to the edges of the 25<sup>th</sup> and 75<sup>th</sup> percentile and extend to what is considered extreme (usually with a distance of 1.5 interquartile range (IQR)), to display the extent of the data range for the remaining 50% of the data. Data is marked as individual points, when beyond the extremes, and considered as outliers (Dawson & Trapp, 2004:39; Nuzzo, 2016:269).

In addition, statistically significant effects of different factors were determined by utilising the analysis of variance (ANOVA), from which p-values were obtained for each effect. A p-value of equal or less to 0.05 is considered a statistical significant effect (Concato & Hartigan, 2016:1166). The eight formulas were compared during membrane diffusion studies in terms of the respective formula (nano-emulsion or nano-emulgel) and the respective statins. During the skin diffusion studies, diffusion data was also compared in terms of the formulas and the respective statins, although tape stripping data was compared in terms of the SCE and ED, in combination with the respective formulas and statins. This was performed using a three-way ANOVA by means of Statistica (Statsoft, 2008), where after a one-way or two-way was performed depending on the requirements. Although the median is considered a more accurate method to determine flux and concentration in cases where there is a substantial difference between the mean and median values (Dawson & Trapp, 2004:30), both the median and mean will be used throughout this study to describe statistical data.

### **D.3 Results and discussion**

#### **D.3.1 Solubility in various solvents**

Naik *et al.* (2000:319) suggested that amongst other characteristics, an API is considered ideal for transdermal delivery if the aqueous solubility of the compound is above 1 mg/ml. The aqueous solubility of the statins obtained from literature however suggests that only rosuvastatin has an ideal solubility (7.8 mg/ml) (Crestor, 2009). Lovastatin (0.004 mg/ml), mevastatin (0.032 mg/ml) and simvastatin (0.03 mg/ml), conversely do not possess ideal aqueous solubility (Drugbank, 2005; O'Neil, 2006:968; O'Neil, 2006:1472). During this study, the experimental aqueous solubility of the statins was determined and displayed in Table D.2.

**Table D.2:** Solubility (mg/ml) of the selected statins in PBS (pH 7.4), PBS:ethanol (9:1 at pH 7.4) and *n*-octanol

Statin	Solubility in PBS at pH 7.4 (mg/ml)	Solubility in PBS:ethanol (9:1 at pH 7.4) (mg/ml)	Solubility in <i>n</i> -octanol (mg/ml)
Lovastatin	0.001 ± 0.008	0.001 ± 0.001	8.901 ± 0.408
Mevastatin	0.002 ± 0.001	0.003 ± 0.001	7.674 ± 0.705
Simvastatin	0.243 ± 0.058	2.444 ± 0.020	0.641 ± 0.020
Rosuvastatin	0.310 ± 0.066	1.682 ± 0.040	0.276 ± 0.049

Lynch *et al.* (2001:1549) stated that the solubility of an API in an aqueous medium could be influenced significantly by pH and temperature. Consequently, the differences in the aqueous solubility presented in literature and those determined experimentally could be ascribed to pH differences between the tests performed in literature and those performed experimentally during this study. By observing the results displayed in Table D.2 for the solubility in PBS (pH 7.4), it can be stated that the solubility of the statins is not ideal (1 mg/ml) for permeation through the skin.

From Table D.2, it can be concluded that the solubility of mevastatin, simvastatin and rosuvastatin increased in the PBS:ethanol (9:1 at pH 7.4), however, no difference was observed for lovastatin. It can further be suggested that with the addition of 10% ethanol to the PBS, rosuvastatin and simvastatin displayed solubility, which is considered as ideal according Naik *et al.* (2000:319). For relatively lipophilic compounds, such as the statins, selection of a receptor phase into which a compound can freely partition is recommended (Collier & Bronaugh, 1991:47)

The solubility of all the statins were higher in *n*-octanol than in PBS (pH 7.4), while the solubility of lovastatin and mevastatin was higher in *n*-octanol than in PBS:ethanol (9:1 at pH 7.4) and simvastatin and rosuvastatin was higher in PBS:ethanol (9:1 at pH 7.4) than in *n*-octanol. The higher solubility of rosuvastatin and simvastatin within the aqueous buffer can be attributed to the salt forms of the APIs (rosuvastatin calcium salt and simvastatin ammonium salt), as salt formations of ionisable APIs will increase solubility of the compounds (Elder *et al.*, 2013:88).

### D.3.2 Octanol-buffer distribution coefficient

Naik *et al.* (2000:319) stated that an API with a log P value of between 1 and 3 is considered ideal for transdermal delivery, however, literature also suggests that log P between 1 and 4 could also be considered acceptable (Williams, 2013:680).

**Table D.3:** Experimentally determined log D value of statins

Statin	Log D
Lovastatin	4.49 ± 0.039
Mevastatin	3.80 ± 0.128
Simvastatin	1.80 ± 0.001
Rosuvastatin	- 0.20 ± 0.001

From Table D.3, it can be stated that lovastatin and mevastatin are more lipophilic when compared to simvastatin and rosuvastatin; however, rosuvastatin can be considered the most hydrophilic statin, which correlates with the value suggested in literature of - 0.3 (Wong *et al.*, 2008:245). The log D obtained for simvastatin also correlated with the value (1.6) that was suggested in literature (Wong *et al.*, 2008:245), with a minor difference. Conversely, lovastatin displayed a value significantly higher than the log D suggested in literature of 1.8 (Joshi *et al.*, 1999:270) and no data was available in literature for mevastatin. Table D.3 suggests that lovastatin and mevastatin are more soluble in the *n*-octanol phase than in the PBS phase (buffered at pH 7.4). The opposite is observed with simvastatin and rosuvastatin. It can also be stated that only the log D values of mevastatin and simvastatin are considered ideal for transdermal delivery when compared to the aforementioned values, as rosuvastatin is too hydrophilic and lovastatin too lipophilic. The significant differences found in the literature between the log D values for lovastatin and the value determined experimentally can be ascribed to the different buffers utilised during the determinations.

### D.3.3 Membrane release studies

The results on **(NEF1)** and **(NEG1)** obtained from the membrane release studies are summarised in Table D.4, which indicates the average %formulation/dispersion released over a period of 6 h, in conjunction with the average and median flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) of the respective formulas.

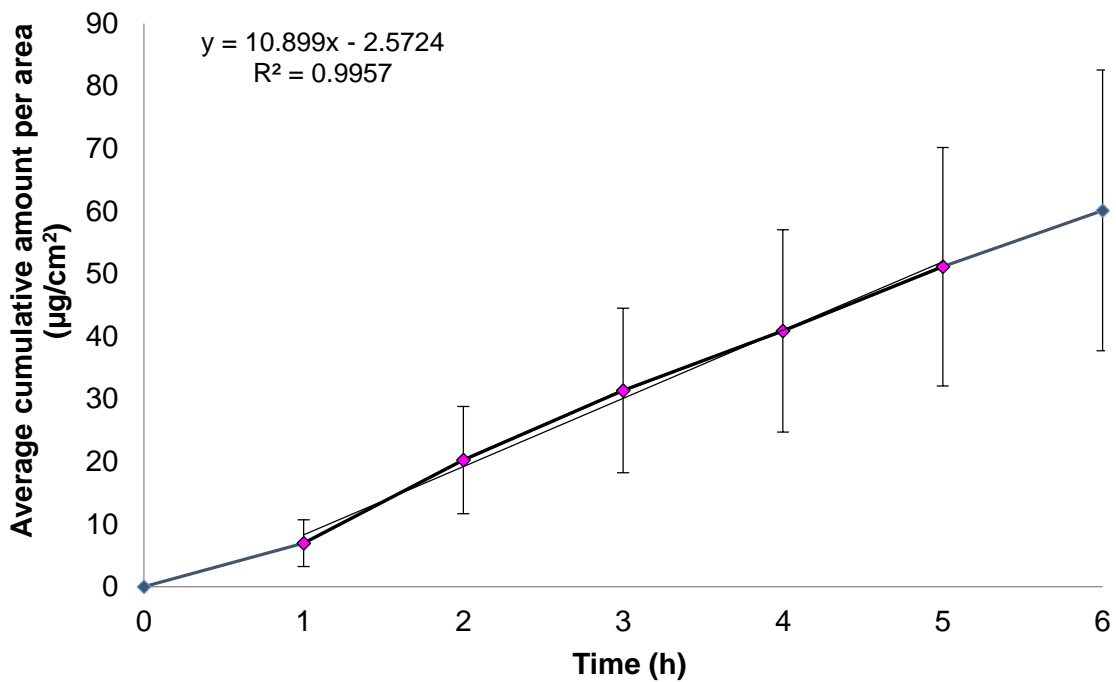
In Table D.4, it can be observed that concentrations were detected of each of the respective statins during the eight release studies that were performed on the **(NEF1)** dispersions and **(NEG1)** formulations. Consequently, it can be stated that the release of the API from the vehicle occurred, followed by diffusion through the PVDF synthetic membranes. It can be stated that the **(NEF1)** dispersions displayed a higher average %released and a higher average flux when compared to the respective **(NEG1)** formulations. This could be explained by the fact that nano-emulgels will release the API in a more controlled manner (Chellapa *et al.*, 2015:45). Arora *et al.* (2014:9) and Begur *et al.* (2015:78) found similar results and stated that the higher flux observed with nano-emulsions compared to the respective nano-emulgel could possibly be attributed to the higher viscosity of the nano-emulgel formulas. From the median flux values, it

can be concluded that **(NER1)** obtained the highest median flux (413.650  $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) followed by **(NEGR)**, **(NES1)**, **(NEGS)**, **(NEM1)**, **(NEL1)**, **(NEMG)** and lastly, **(NELG)** with the lowest median flux (4.651  $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ).

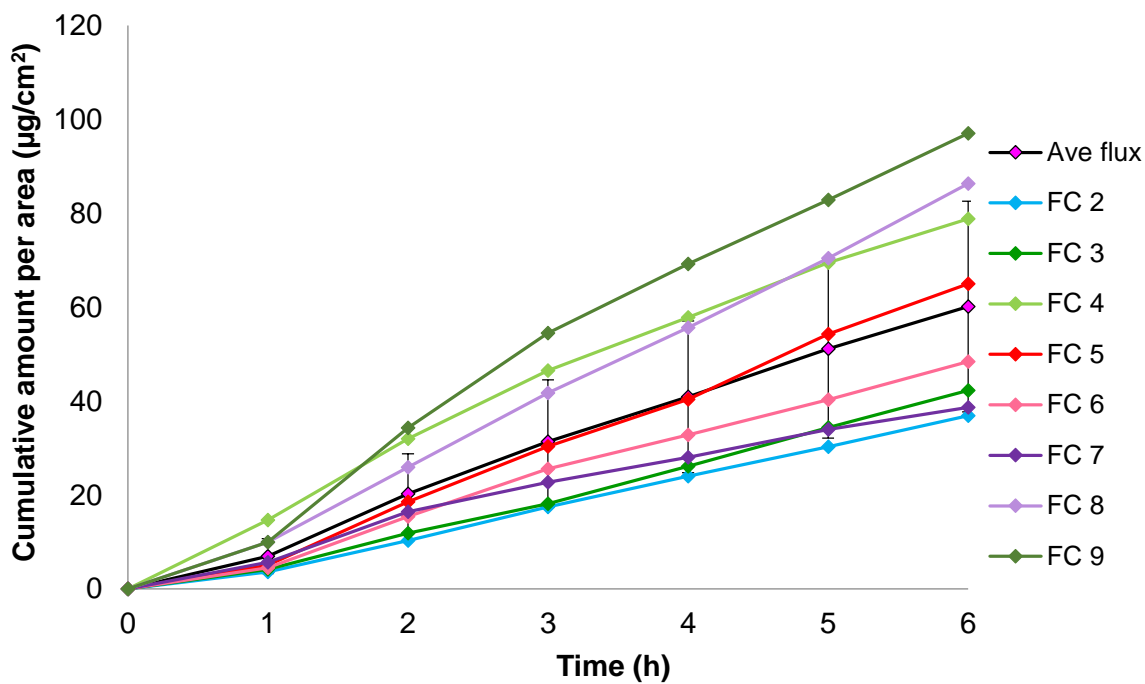
**Table D.4:** The average %released, the average and median flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) for each of the formulas after a 6 h membrane release study

<b>Formulas</b>	<b>Average %released</b>	<b>Average flux (<math>\mu\text{g}/\text{cm}^2\cdot\text{h}</math>)</b>	<b>Median flux (<math>\mu\text{g}/\text{cm}^2\cdot\text{h}</math>)</b>	<b>n</b>
<b>(NER1)</b>	6.696 $\pm$ 0.667	409.880 $\pm$ 27.906	413.650	12
<b>(NEGR)</b>	4.988 $\pm$ 0.674	306.560 $\pm$ 34.822	281.937	12
<b>(NES1)</b>	4.786 $\pm$ 0.827	297.010 $\pm$ 51.167	272.851	9
<b>(NEGS)</b>	1.765 $\pm$ 0.228	107.280 $\pm$ 9.066	98.442	8
<b>(NEM1)</b>	0.310 $\pm$ 0.085	17.892 $\pm$ 3.901	18.970	9
<b>(NEMG)</b>	0.090 $\pm$ 0.023	5.458 $\pm$ 1.303	4.867	10
<b>(NEL1)</b>	0.162 $\pm$ 0.060	10.899 $\pm$ 4.018	8.647	9
<b>(NELG)</b>	0.082 $\pm$ 0.028	5.306 $\pm$ 1.255	4.651	11

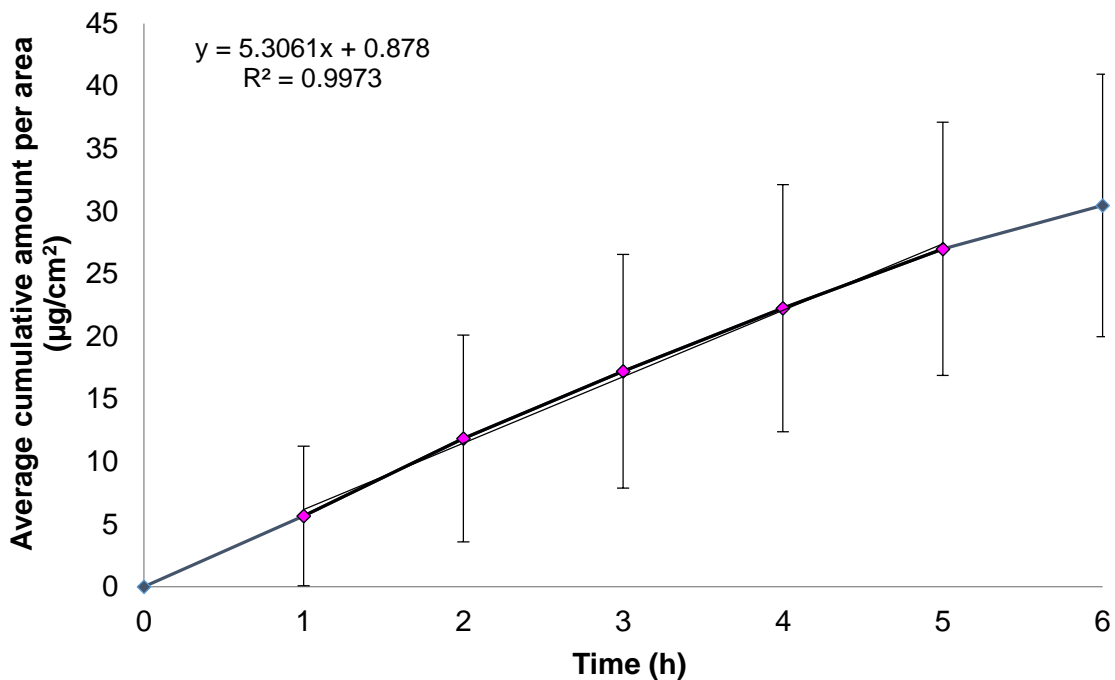
(n = number of cells used with success during the study, without any leakage)



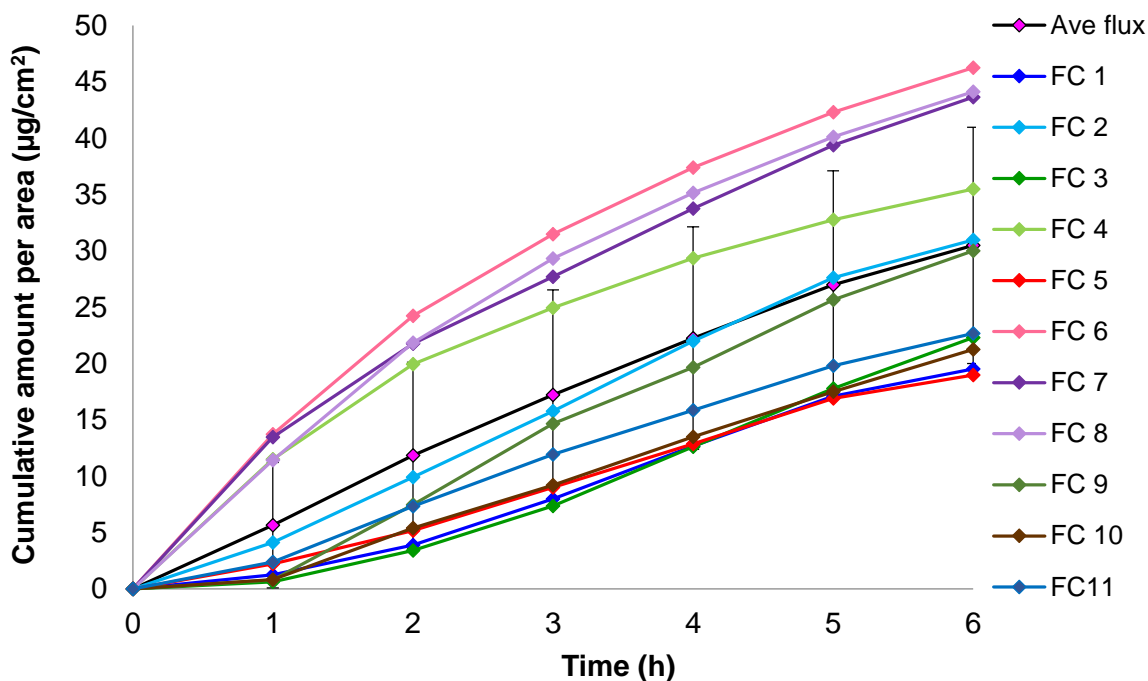
**Figure D.6:** Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of lovastatin permeated from the **(NEL1)** through the membrane as a function of time to illustrate the average flux from 1 – 5 h ( $n = 9$ )



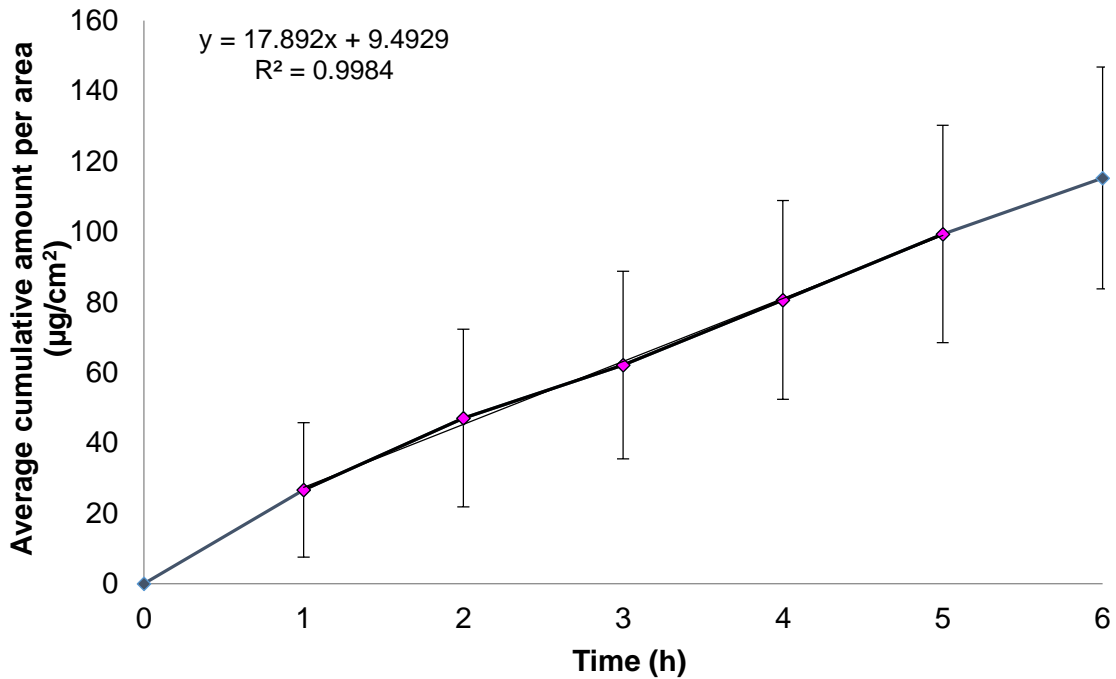
**Figure D.7:** Cumulative amount of lovastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the **(NEL1)** ( $n = 9$ )



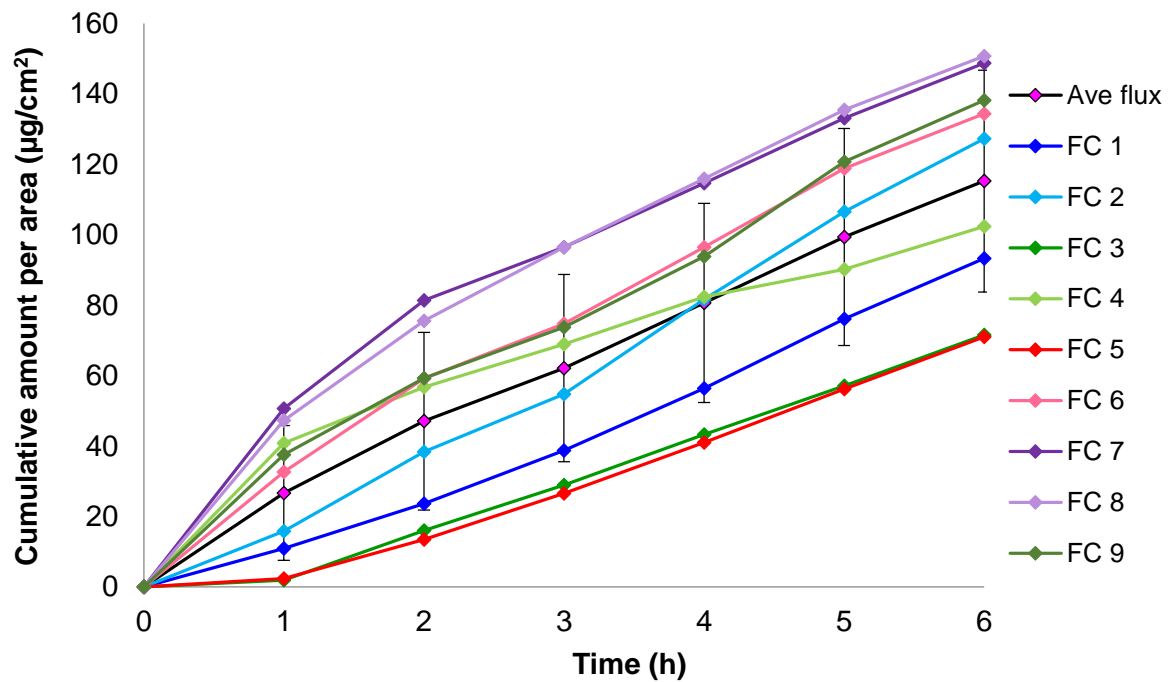
**Figure D.8:** Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of lovastatin permeated from the **(NEGL)** through the membrane as a function of time to illustrate the average flux from 1 – 5 h ( $n = 11$ )



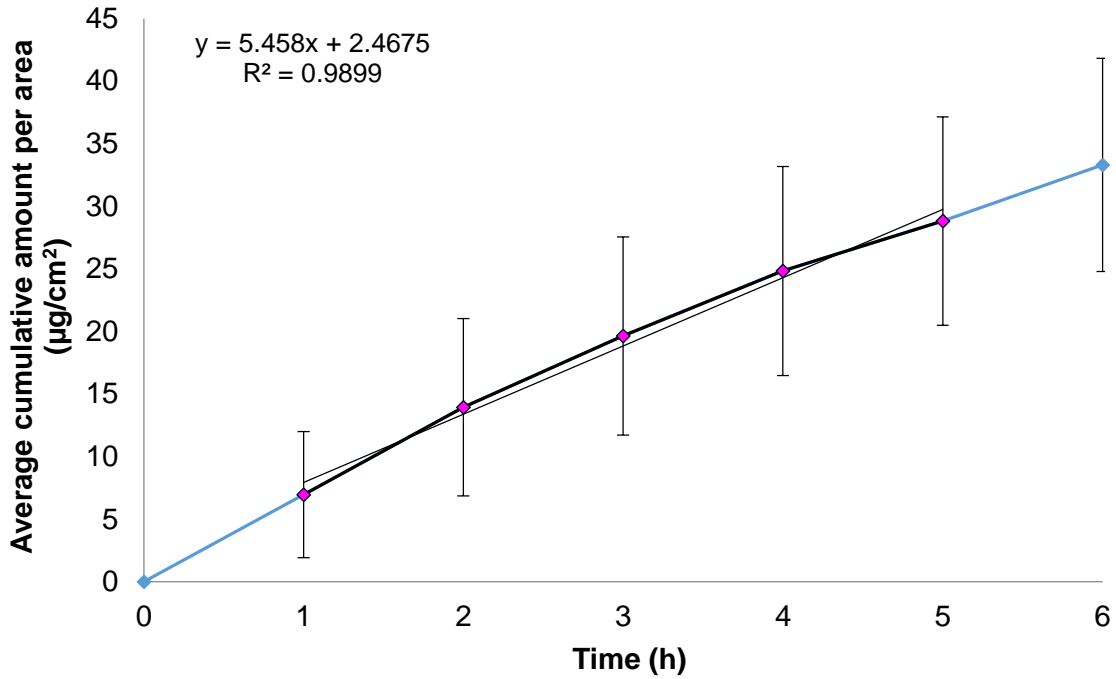
**Figure D.9:** Cumulative amount of lovastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the **(NEGL)** ( $n = 11$ )



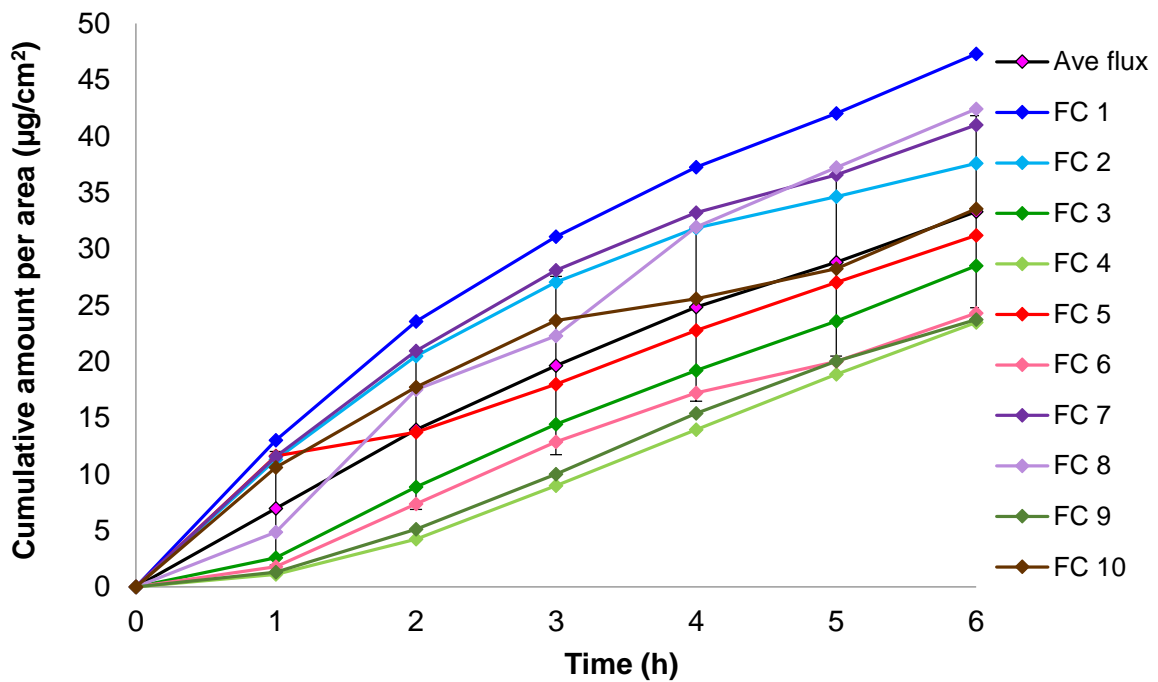
**Figure D.10:** Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of mevastatin permeated from the **(NEM1)** through the membrane as a function of time to illustrate the average flux from 1 – 5 h ( $n = 9$ )



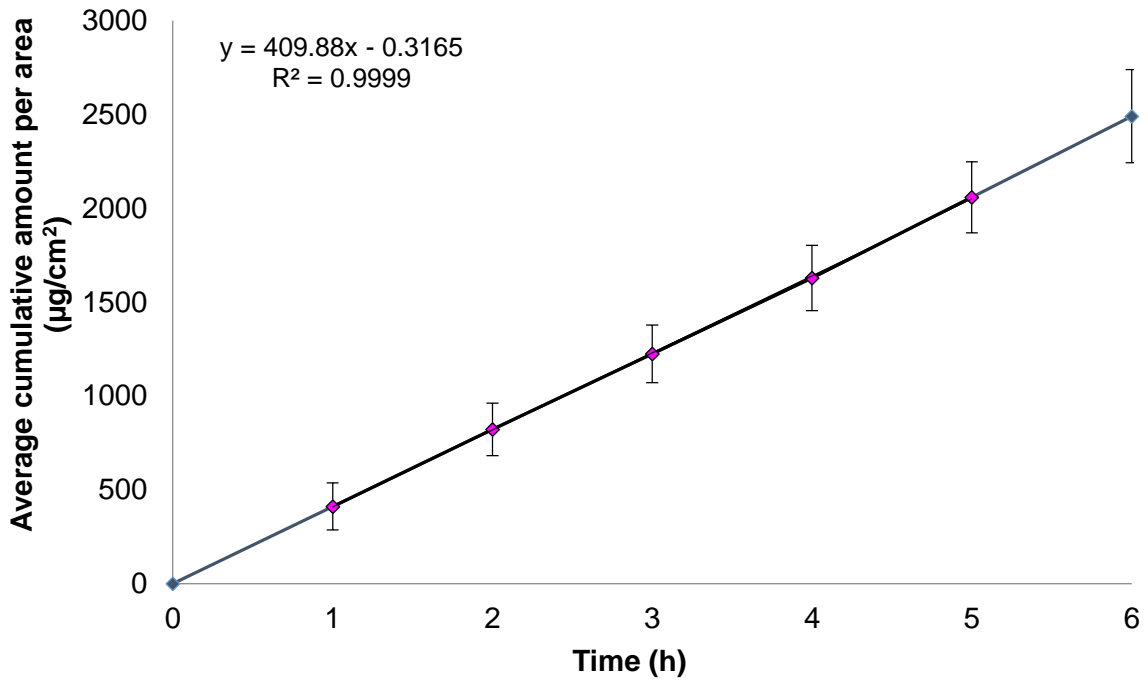
**Figure D.11:** Cumulative amount of mevastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the **(NEM1)** ( $n = 9$ )



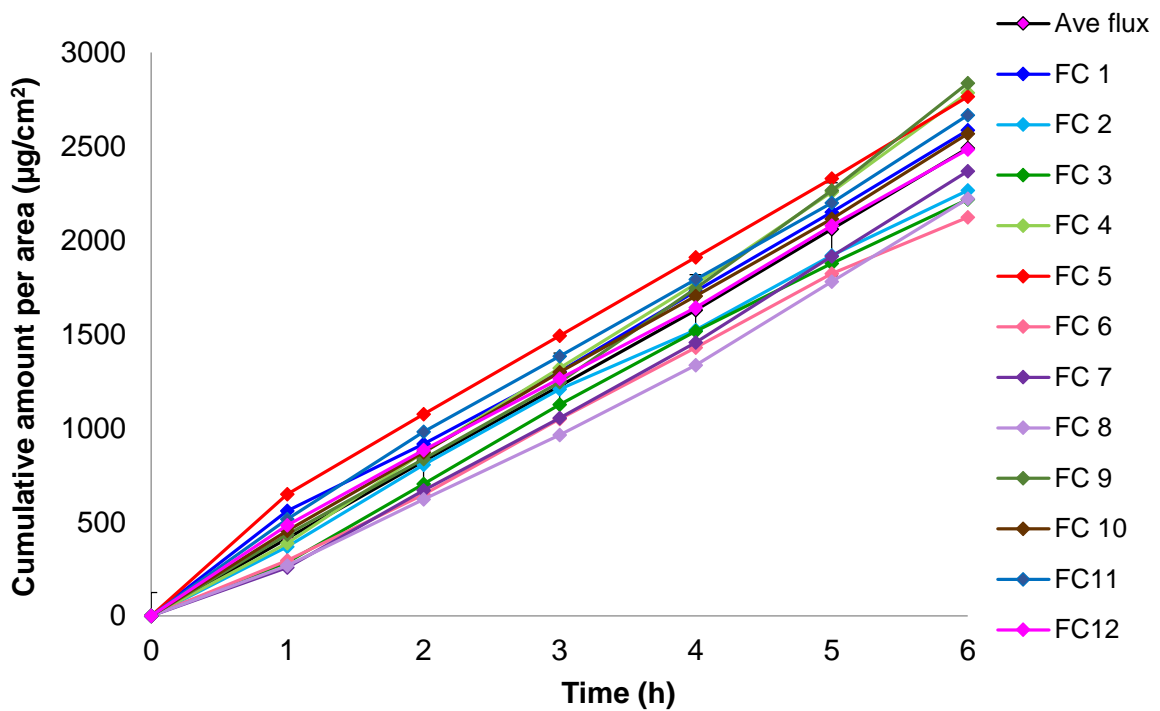
**Figure D.12:** Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of mevastatin permeated from the **(NEGM)** through the membrane as a function of time to illustrate the average flux from 1 – 5 h (n = 10)



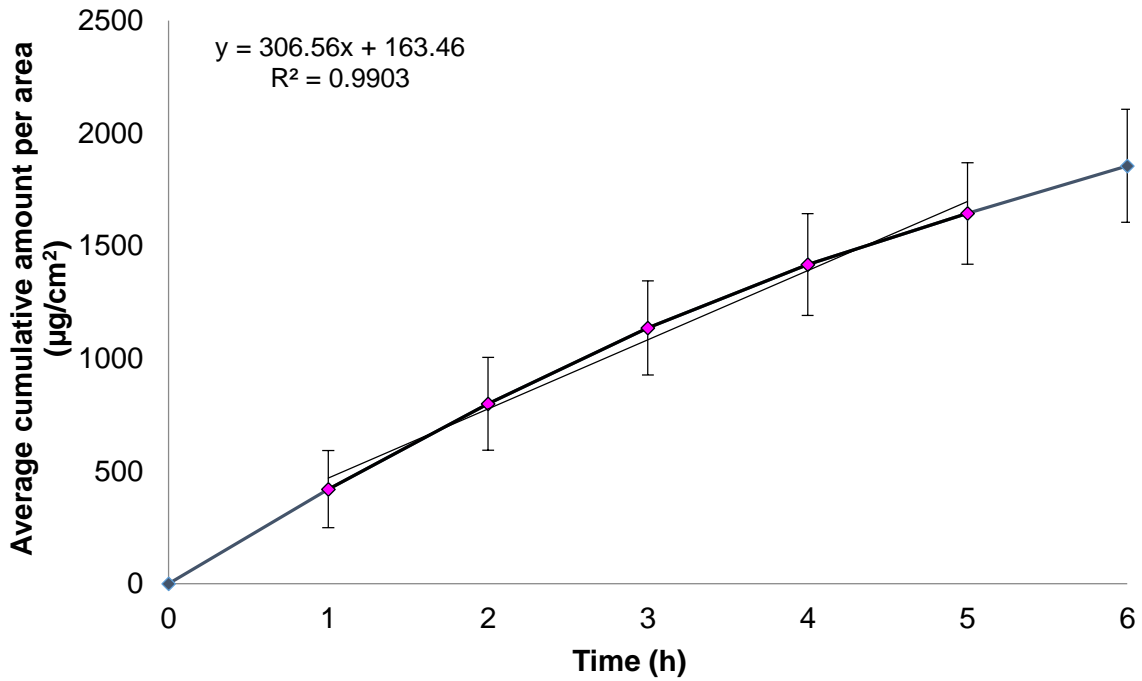
**Figure D.13:** Cumulative amount of mevastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the **(NEGM)** (n = 10)



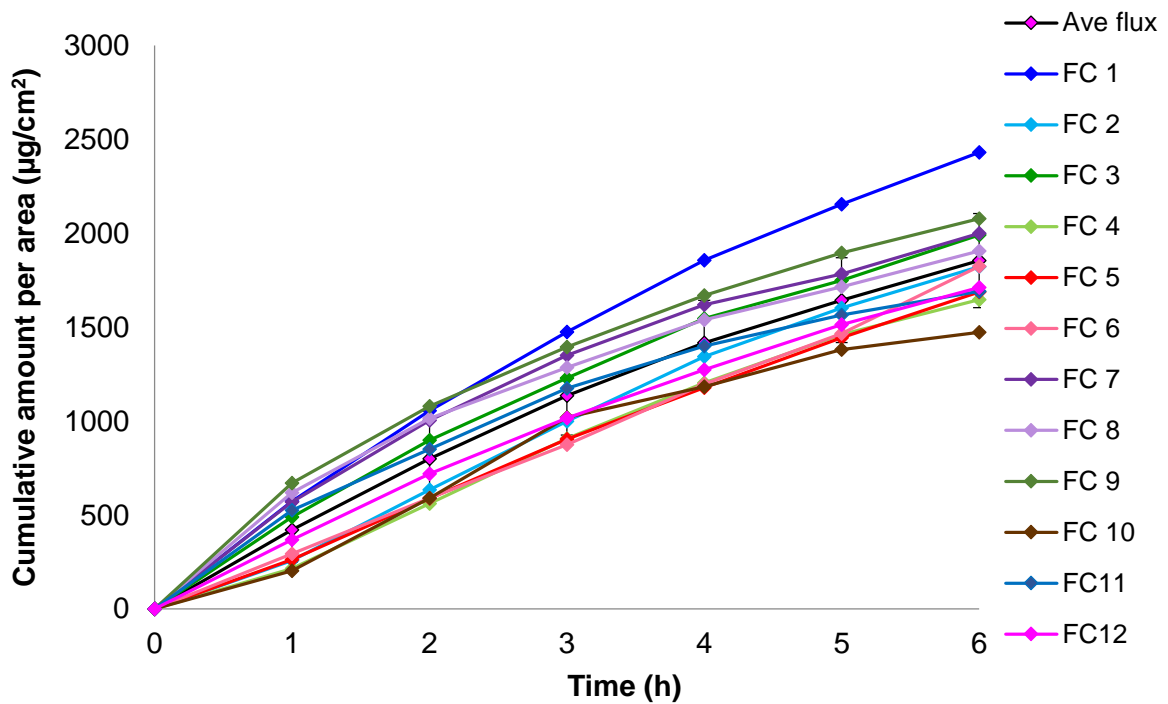
**Figure D.14:** Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of rosuvastatin permeated from the **(NER1)** through the membrane as a function of time to illustrate the average flux from 1 – 5 h ( $n = 12$ )



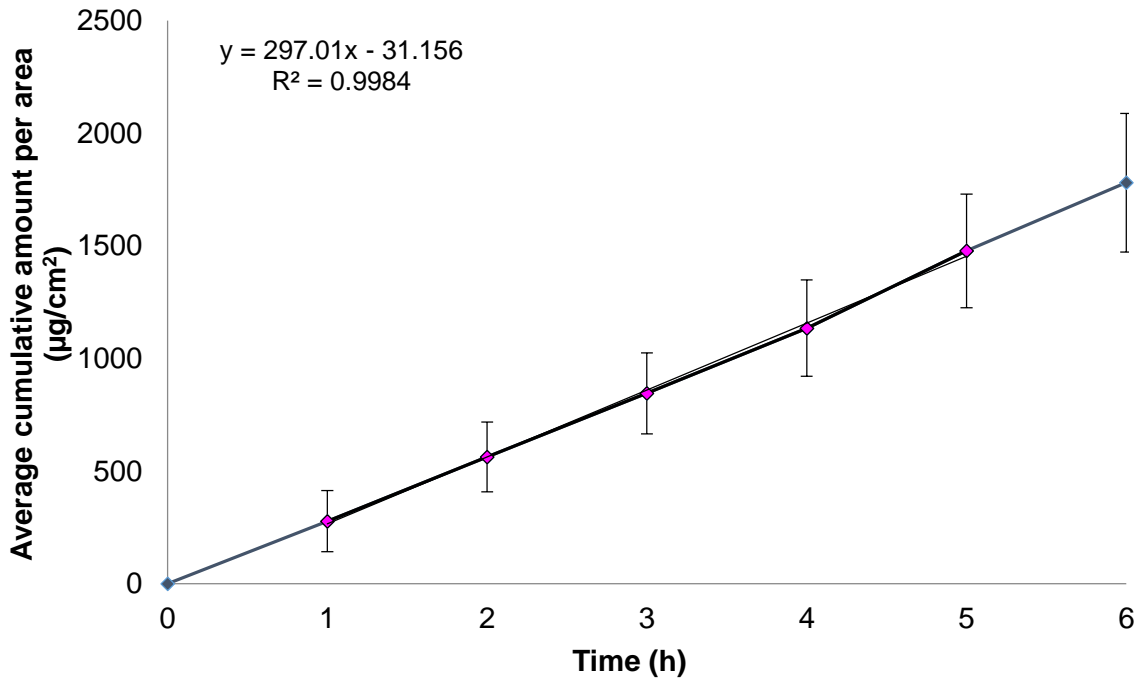
**Figure D.15:** Cumulative amount of rosuvastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the **(NER1)** ( $n = 12$ )



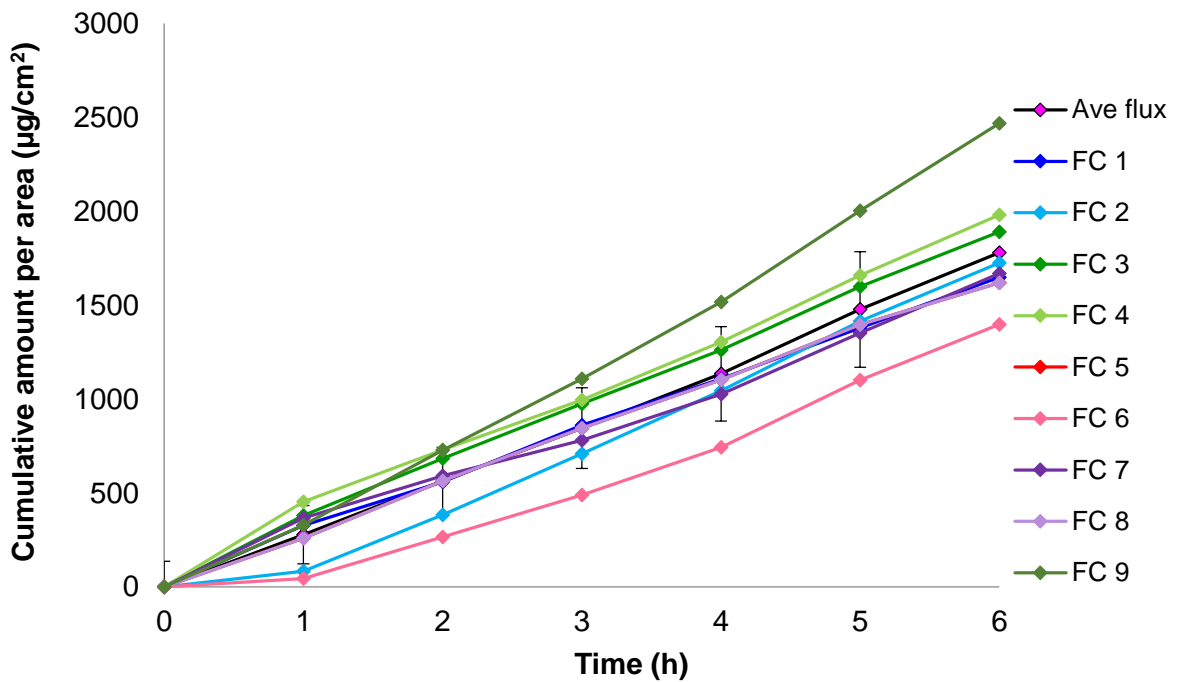
**Figure D.16:** Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of rosuvastatin permeated from the **(NEGR)** through the membrane as a function of time to illustrate the average flux from 1 – 5 h ( $n = 12$ )



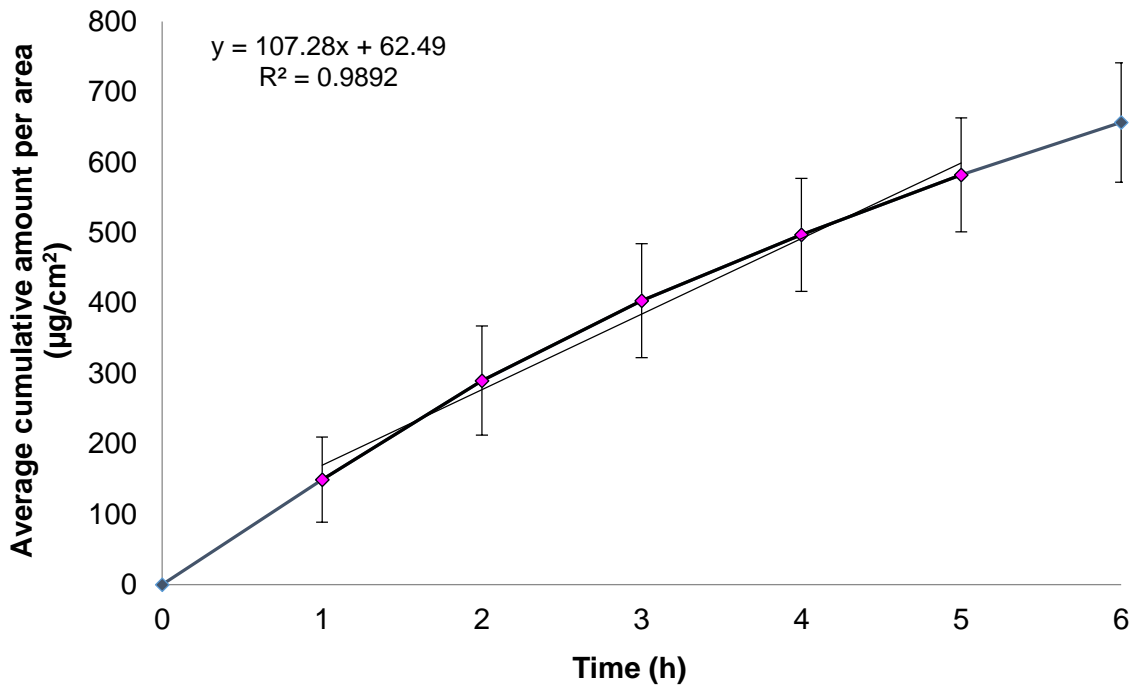
**Figure D.17:** Cumulative amount of rosuvastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the **(NEGR)** ( $n = 12$ )



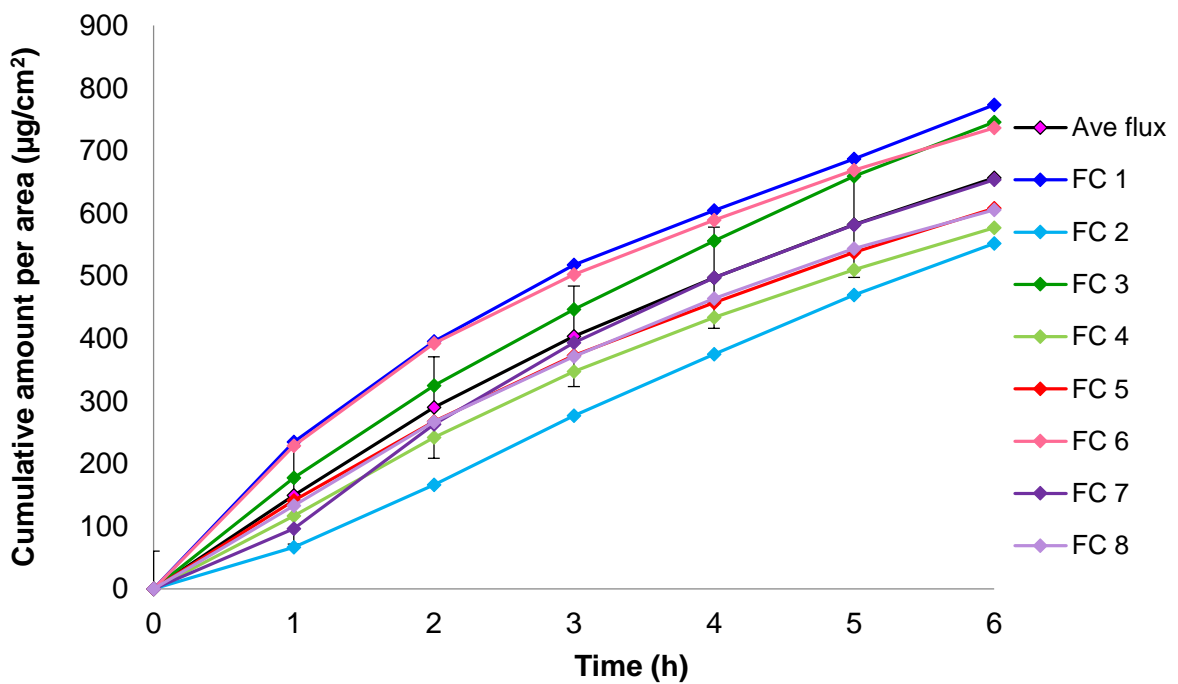
**Figure D.18:** Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of simvastatin permeated from the **(NES1)** through the membrane as a function of time to illustrate the average flux from 1 – 5 h ( $n = 9$ )



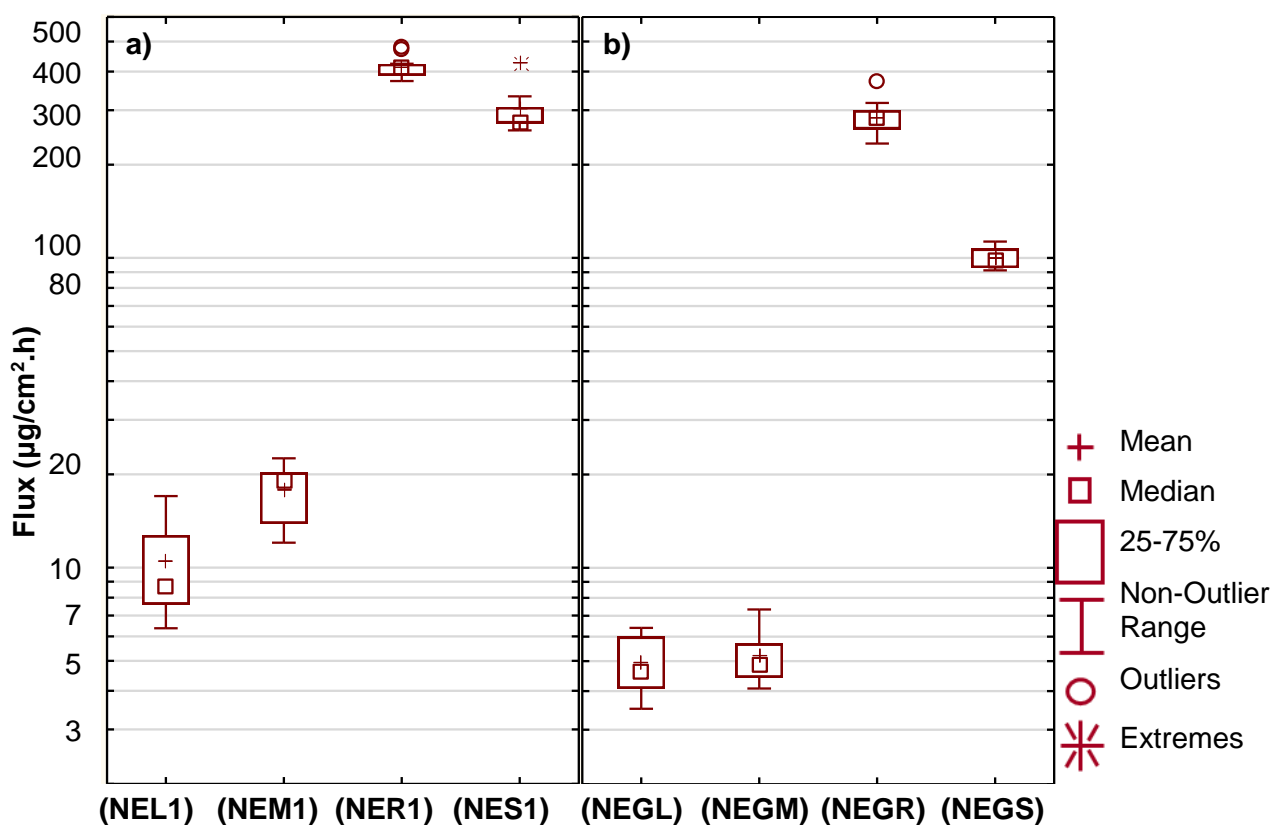
**Figure D.19:** Cumulative amount of simvastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the **(NES1)** ( $n = 9$ )



**Figure D.20:** Average cumulative amount per area ( $\mu\text{g}/\text{cm}^2$ ) of simvastatin permeated from the **(NEGS)** through the membrane as a function of time to illustrate the average flux from 1 – 5 h ( $n = 8$ )



**Figure D.21:** Cumulative amount of simvastatin per area ( $\mu\text{g}/\text{cm}^2$ ) for each individual Franz cell that permeated through the membrane over 6 h from the **(NEGS)** ( $n = 8$ )



**Figure D.22:** Box-plot indicating the flux ( $\mu\text{g}/\text{cm}^2\cdot\text{h}$ ) of: a) the nano-emulsions ((**NEL1**), (**NEM1**), (**NES1**) and (**NER1**)) and b) the nano-emulgels ((**NEGL**), (**NEGM**), (**NEGS**) and (**NEGR**)) after 6 h.

From Table D.4, it can be concluded that the statin that obtained the highest median flux was rosuvastatin, with (**NER1**) being the highest, followed by simvastatin, with (**NES1**) being higher than its respective nano-emulgel formula (**NEGS**). These results correlate with what is suggested in literature, as Chime *et al.* (2014:97) stated that lower viscosity results in quicker and improved release of the API; it can further be explained by the fact that droplet size increased with the addition of a polymer (Eid *et al.*, 2014:7). The fact that (**NEGR**) obtained a higher flux than (**NES1**) regardless of the higher viscosity of (**NEGR**) can possibly be attributed to the smaller droplet size of (**NEGR**) ( $149.83 \pm 1.922 \mu\text{g}/\text{cm}^2$ ) when compared to (**NES1**) ( $153.10 \pm 1.249 \mu\text{g}/\text{cm}^2$ ), hence a decrease in permeation occurred with the increase in droplet size (Baibhav *et al.*, 2011:66). Both (**NEM1**) and (**NEL1**) obtained higher flux than their respective nano-emulgels ((**NEGM**) and (**NEGL**)). The low diffusivity of (**NEM1**) and (**NEL1**) compared to that of (**NER1**) and (**NES1**) can possibly be attributed to the weak solubility of mevastatin and lovastatin in the buffer solution. According to FDA (2015:11), rosuvastatin calcium is an amorphous white powder, with sparing solubility in water and methanol, and slightly soluble in ethanol. Mevastatin compactin is a crystalline solid (Cayman Chemical, 2008) along with lovastatin (Yoshida *et al.*, 2011:657), and both are practically insoluble in water (Serajuddin *et al.*, 1991:830). The difference in solubility due to variations in the chemical

structure, can be attributed to the fact that the amorphous forms with non-crystalline structures and less stable crystal forms are supposed to have higher solubility (Taskinen & Norinder, 2007:628).

### D.3.4 Skin diffusion studies

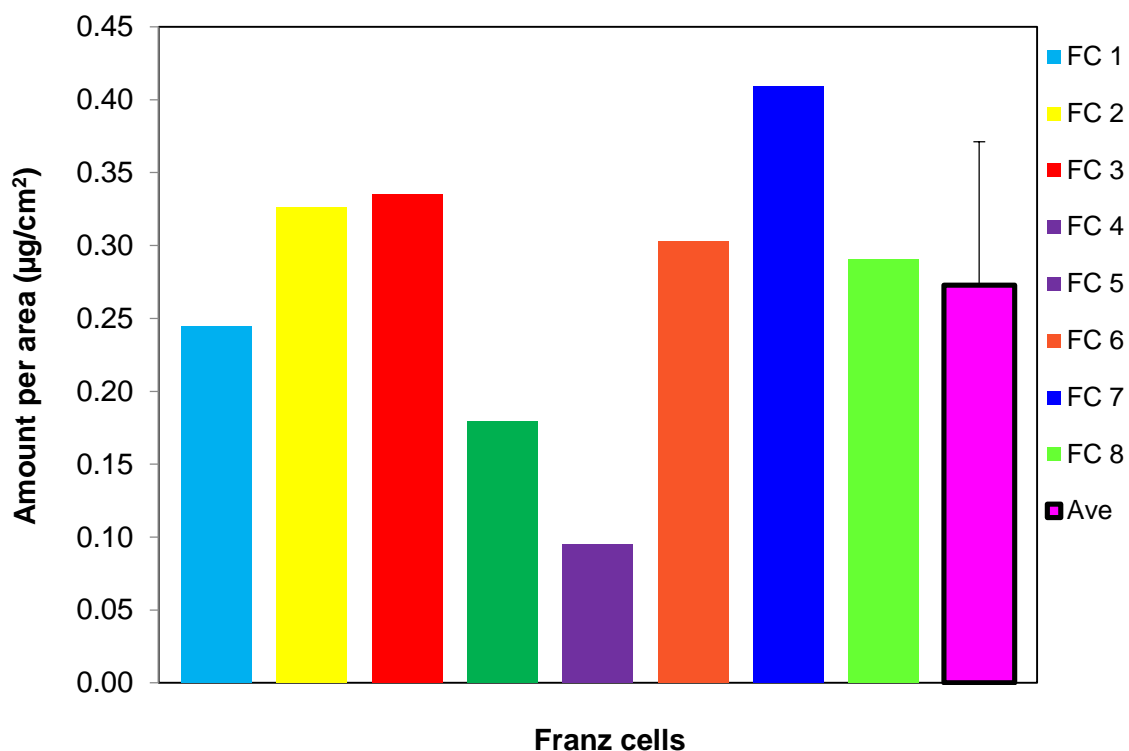
It is of importance to determine whether data will be described in terms of mean or median, which are both considered central tendencies. The decision lies on the appropriateness of utilising either mean or median to described data, although mean is commonly used and serves as a good representation of data (due to utilisation of all values), it is extremely sensitive to outliers. Therefore, it is not appropriate to use the mean value in the case of a skewed distribution (Manikandan, 2011:140), as seen with the skin diffusion data; for example, all data sets followed the same trend as the median values, except for **(NEGR)**. When the median and average values are compared, **(NEGR)** has the second lowest median value, but the third highest average concentration diffused and average amount per area diffused, this is due to outliers in the data and therefore, data will be described in terms of the median in this chapter. The mean value will however still be displayed in Table D.5.

The data collected by means of HPLC analysis was processed and presented in Table D.5 in terms of average %diffused, average concentration diffused ( $\mu\text{g/ml}$ ), average amount per area diffused ( $\mu\text{g/cm}^2$ ) and median amount per area diffused ( $\mu\text{g/cm}^2$ ) after 12 h for each of the respective formulas containing the different statins. Ten (eight containing API and two placebos) Franz cells were used in each diffusion studies, rather than the norm of 12 Franz cells due to the shortage of skin. Franz cells that leaked or had other complications before the completion of the study were excluded from the dataset. It can be observed that during the diffusion studies performed on **(NEGR)**, **(NES1)** and **(NEGL)**, one Franz cell was excluded from each study due to leakage.

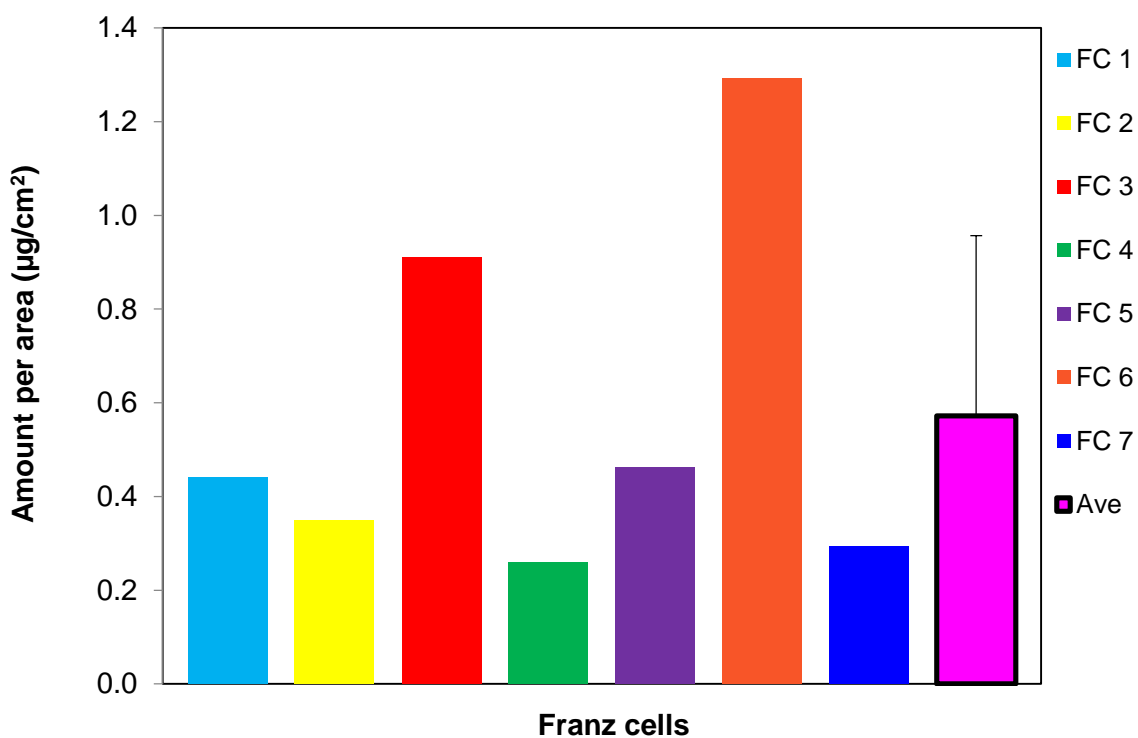
**Table D.5:** Transdermal data for all the formulas containing different statins after the 12 h skin diffusion study

Formulas	Average %diffused	Average concentration diffused ( $\mu\text{g/ml}$ )	Average amount per area diffused ( $\mu\text{g/cm}^2$ )	Median amount per area diffused ( $\mu\text{g/cm}^2$ )	n
<b>(NER1)</b>	0.007 $\pm$ 0.003	1.327 $\pm$ 0.567	2.467 $\pm$ 1.055	2.146	8
<b>(NEGR)</b>	0.004 $\pm$ 0.010	0.858 $\pm$ 1.935	1.596 $\pm$ 3.598	0.319	7
<b>(NES1)</b>	0.002 $\pm$ 0.000	0.379 $\pm$ 0.081	0.705 $\pm$ 0.151	0.654	7
<b>(NEGS)</b>	0.021 $\pm$ 0.034	4.260 $\pm$ 6.745	7.921 $\pm$ 12.542	3.244	8
<b>(NEM1)</b>	0.001 $\pm$ 0.001	0.266 $\pm$ 0.149	0.495 $\pm$ 0.278	0.478	8
<b>(NEGM)</b>	0.004 $\pm$ 0.004	0.800 $\pm$ 0.712	1.487 $\pm$ 1.324	0.846	8
<b>(NEL1)</b>	0.001 $\pm$ 0.000	0.147 $\pm$ 0.053	0.273 $\pm$ 0.098	0.297	8
<b>(NEGL)</b>	0.002 $\pm$ 0.001	0.308 $\pm$ 0.207	0.572 $\pm$ 0.385	0.440	7

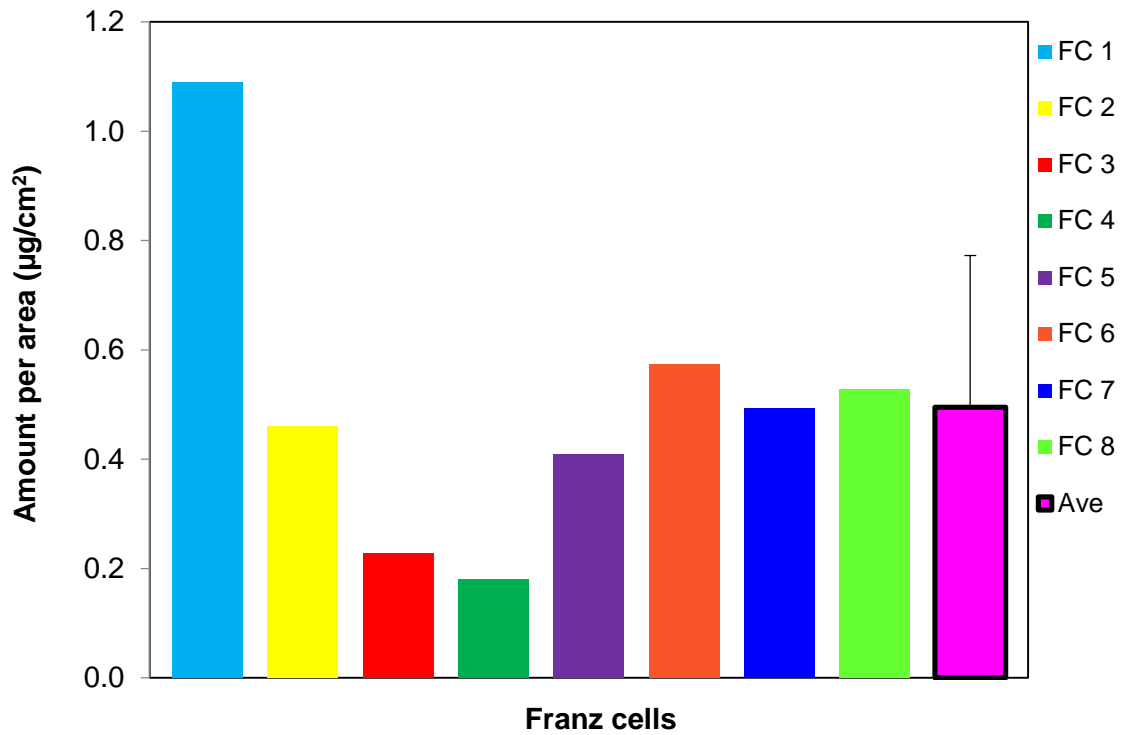
(n = number of cells used with success during the study, without any leakage)



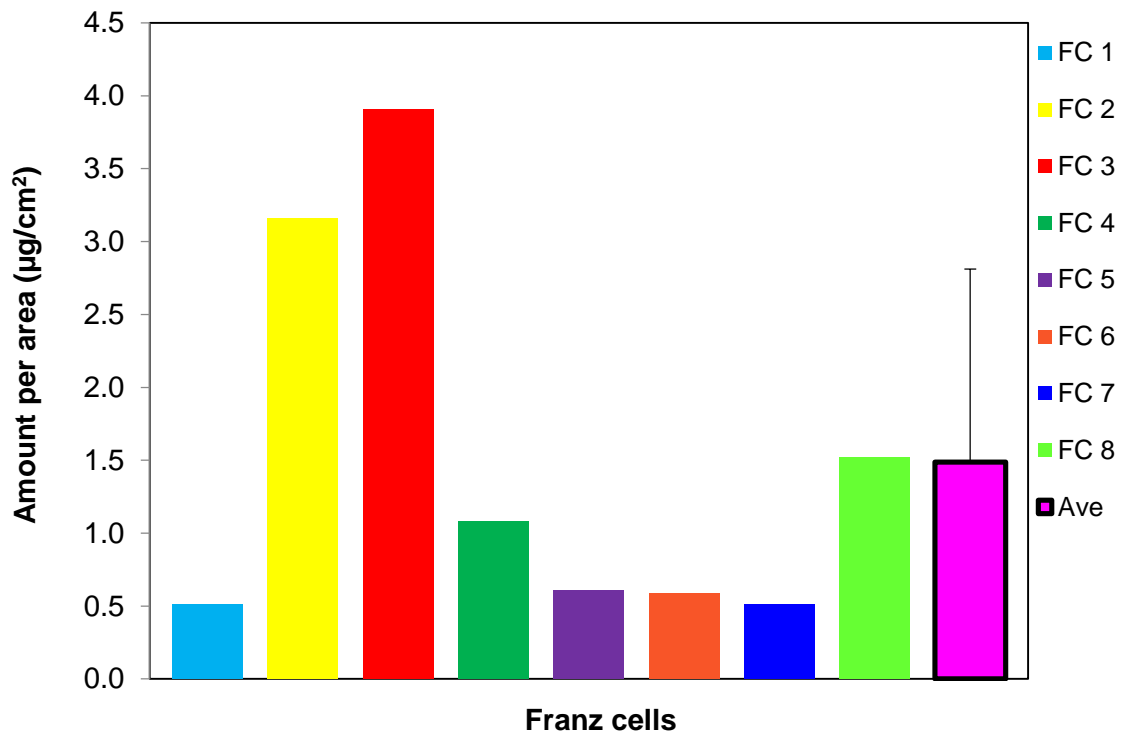
**Figure D.23:** Lovastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for **(NEL1)** (n = 8)



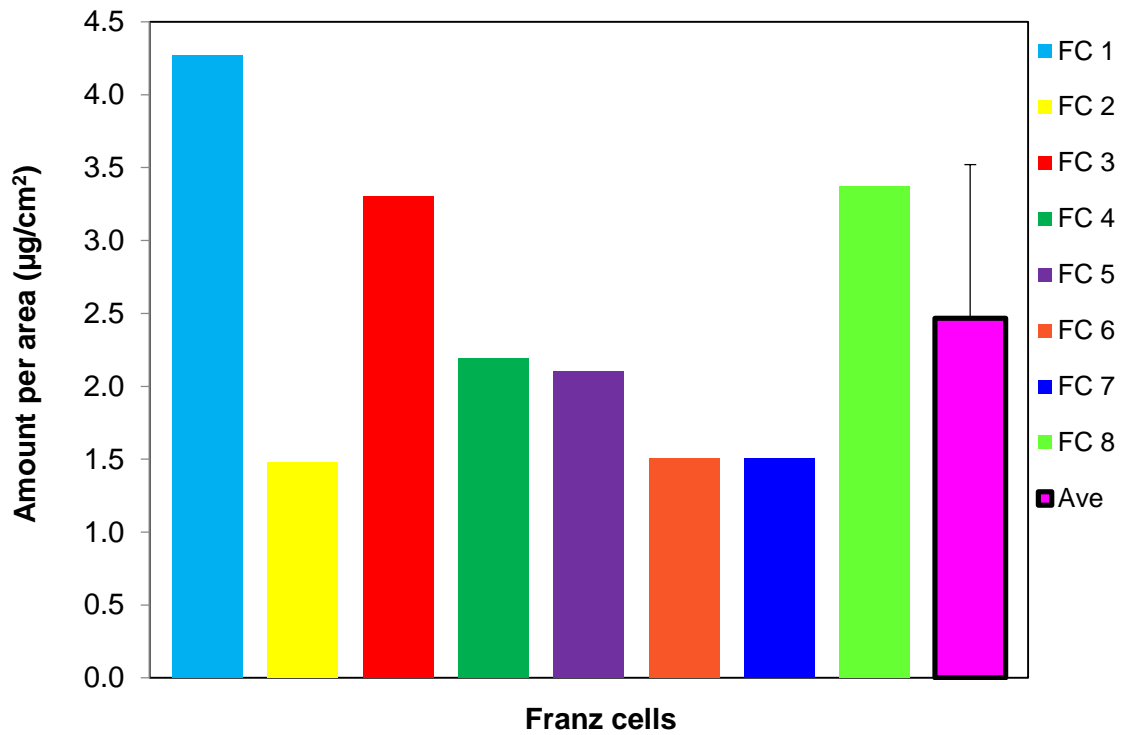
**Figure D.24:** Lovastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for **(NEGL)** (n = 7)



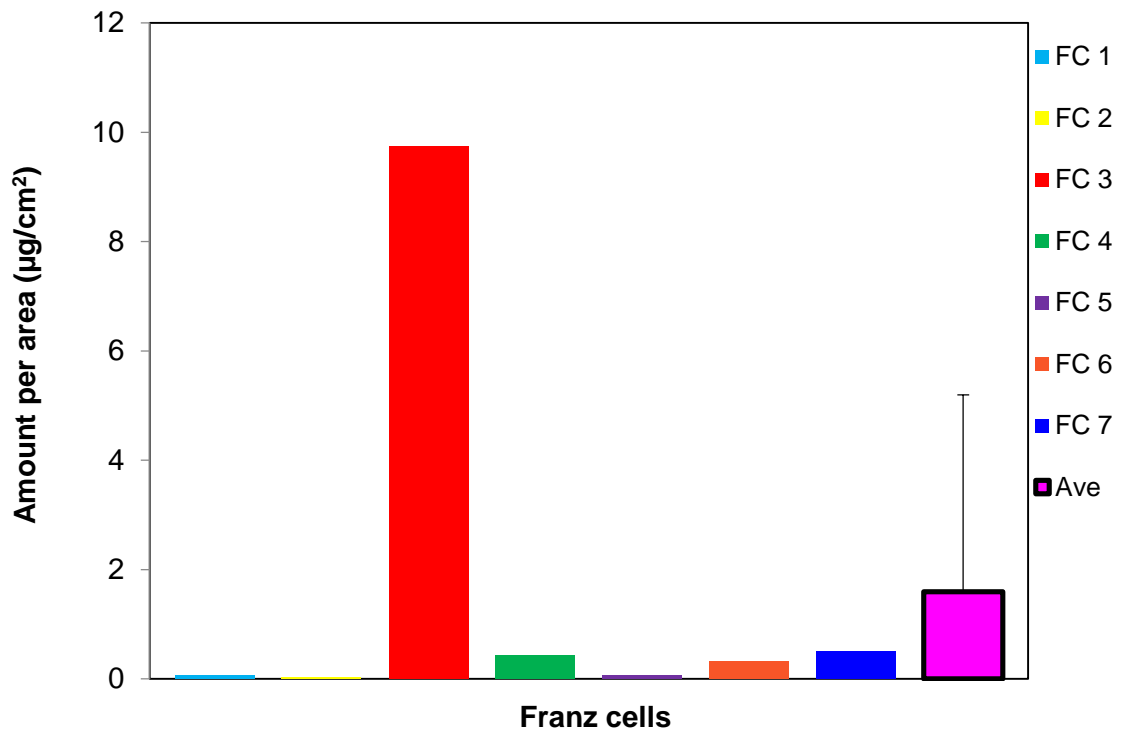
**Figure D.25:** Mevastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for **(NEM1)** (n = 8)



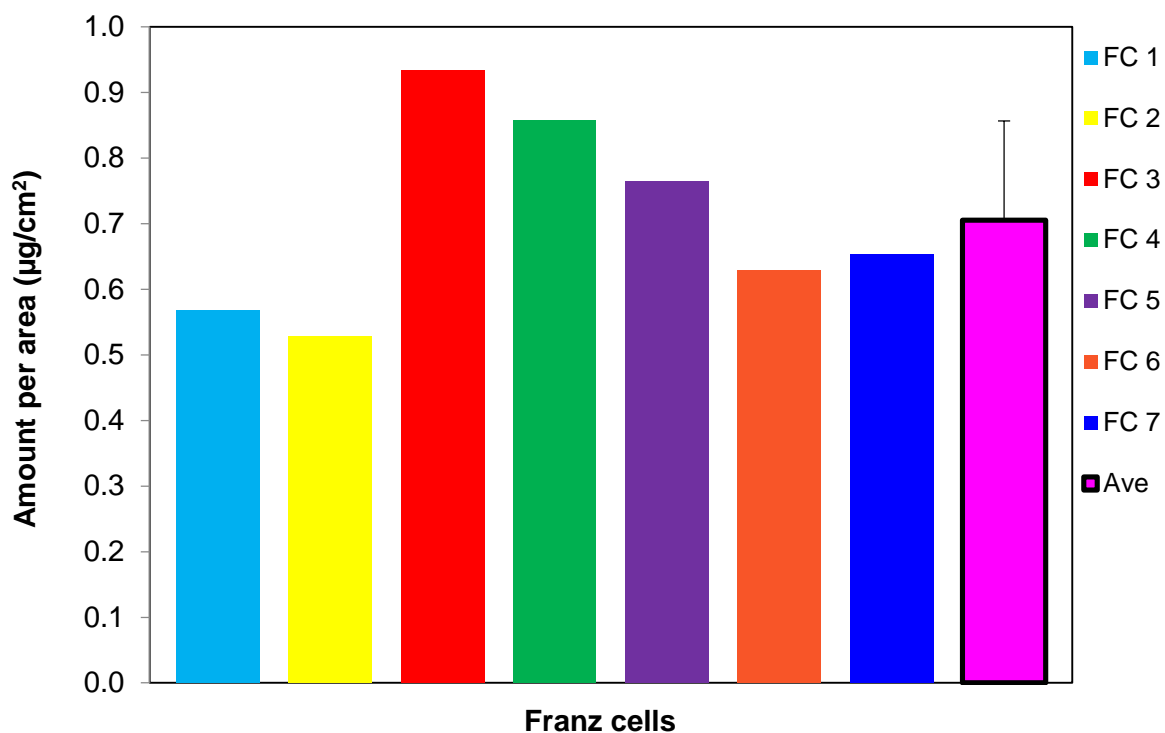
**Figure D.26:** Mevastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for **(NEGM)** (n = 8)



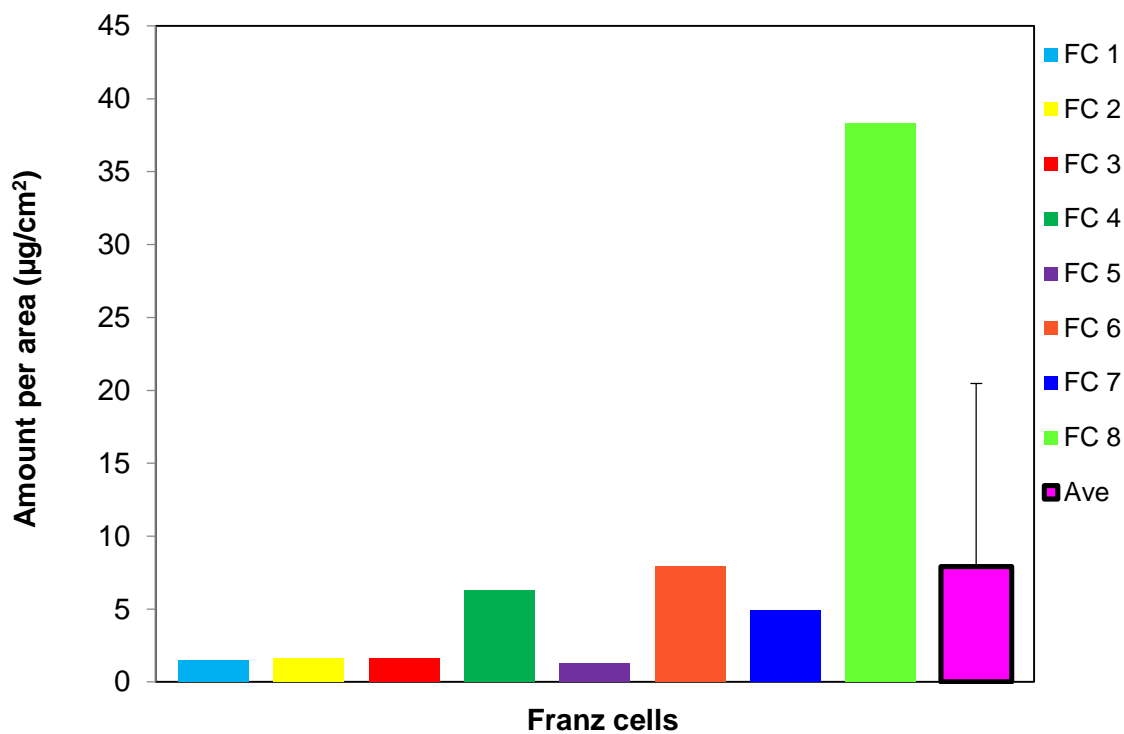
**Figure D.27:** Rosuvastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for **(NER1)** (n = 8)



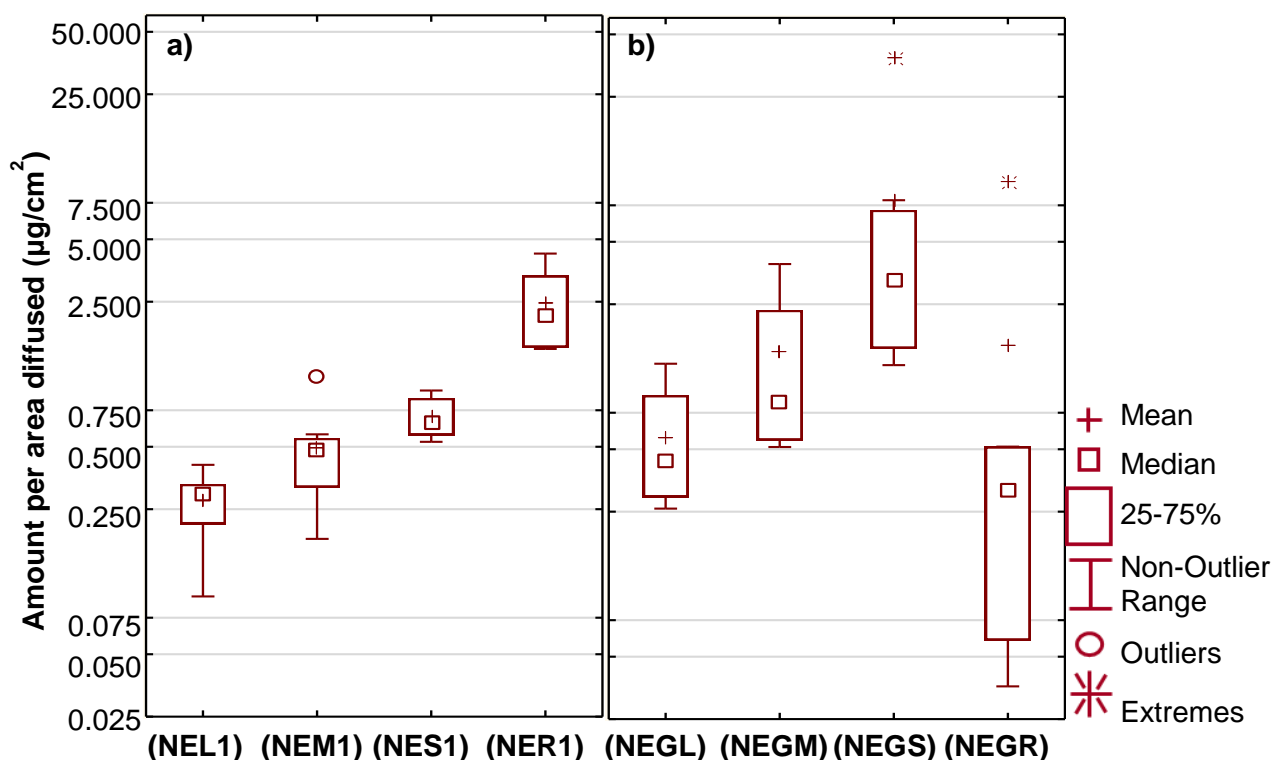
**Figure D.28:** Rosuvastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for **(NEGR)** (n = 7)



**Figure D.29:** Simvastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for **(NES1)** (n = 7)



**Figure D.30:** Simvastatin concentration after 12 h in the receptor phase of the Franz cells during the diffusion study performed for **(NEGS)** (n = 8)



**Figure D.31:** Box-plot indicating the amount per area diffused ( $\mu\text{g}/\text{cm}^2$ ) present in the receptor phase of: a) the nano-emulsions ((**NEL1**), (**NEM1**), (**NES1**) and (**NER1**)  $n = 8$ , except (**NES1**) was  $n = 7$ ) and b) the nano-emulgels ((**NEGL**), (**NEGM**), (**NEGS**) and (**NEGR**)) ( $n = 8$ , except (**NEGR**) and (**NEGL**) were  $n = 7$ ) after 12 h.

From Table D.5, it is clear that after HPLC analysis, the concentration ( $\mu\text{g}/\text{ml}$ ) of all the selected statins were quantified in the receptor phase, although it should be stated the (**NEL1**) obtained a value under the LOQ ( $0.2441 \mu\text{g}/\text{ml}$ ), but above the LOD ( $0.0806 \mu\text{g}/\text{ml}$ ) determined for lovastatin as stated in Section A.4.1.2. Although a percentage of each respective statin was quantified in the receptor phase, the average %diffused was very low, compared to the initial %API present in the dispersions and formulations; hence, only a small amount of the respective API reached the systemic circulation as observed from Table D.5.

It can be stated, that when comparing the eight formulas tested, (**NEGS**) obtained the highest median amount per area diffused ( $3.244 \mu\text{g}/\text{cm}^2$ ), followed by (**NER1**), (**NEGM**), (**NES1**), (**NEM1**), (**NEGL**), (**NEGR**) and lastly, (**NEL1**) with the lowest median amount per area diffused ( $0.297 \mu\text{g}/\text{cm}^2$ ). When comparing the (**NEF1**) dispersions, (**NER1**) displayed the highest amount per area diffused ( $2.146 \mu\text{g}/\text{cm}^2$ ), followed by (**NES1**) ( $0.654 \mu\text{g}/\text{cm}^2$ ), (**NEM1**) ( $0.478 \mu\text{g}/\text{cm}^2$ ) and lastly, (**NEL1**) ( $0.297 \mu\text{g}/\text{cm}^2$ ). This could possibly be attributed to the better solubility of rosuvastatin and simvastatin within the receptor phase (Section D.3.1), while the slight difference between (**NES1**) and (**NEM1**) can possibly be explained by the fact that both simvastatin and mevastatin retain more ideal log D value for skin diffusion (Naik *et al.*,

2000:319; Williams, 2013:680). Lastly, the significant higher amount per area diffused by **(NER1)**, can possibly be attributed firstly, to the nano-emulsion as a vehicle, as the excipient used in the formulation of this system will act as penetration enhancers; secondly, the more hydrophilic nature of rosuvastatin (as described in Section D.3.2) can facilitate better diffusion within the more aqueous layers (N'Da, 2014:20782), after passing the lipophilic stratum corneum. The low concentration quantified for lovastatin can be attributed to the weak aqueous solubility and lipophilic nature as described in Sections D.3.1 and D 3.2, respectively.

The **(NEG1)** semi-solid formulations displayed a different trend than the **(NEF1)** dispersions, as **(NEGS)** obtained the highest median amount per area diffused, followed by **(NEGM)** (0.846  $\mu\text{g}/\text{cm}^2$ ), **(NEGL)** (0.440  $\mu\text{g}/\text{cm}^2$ ), and lastly, **(NEGR)** (0.319  $\mu\text{g}/\text{cm}^2$ ). The difference in the trend observed for nano-emulgels with particularly **(NEGR)**, may be attributed to the different donor skin used between studies as physiological differences between the donors' skin (e.g. different skin-thickness) can influence permeability (Dev *et al.*, 2015:65), while the increase in the median amount per area diffused concerning the remaining three nano-emulgels can be related back to literature, as the enhancement of skin adhesion and high solubilisation capacity (characteristic of nano-emulsion) can facilitate better skin permeation (Eid *et al.*, 2014:1). The fact that **(NEGS)** attained the highest amount diffused when the nano-emulgels were compared, may be related to the ideal log D value of simvastatin (as described in Section D.3.2), as the more lipophilic lovastatin and mevastatin, can be retained within the more hydrophilic layer of the skin (Tampucci *et al.*, 2018:10).

Although small, concentrations within the receptor phase were quantified for all of the statins from their respective formulas; hence, target delivery of the highly lipophilic APIs were possible, due to these APIs being formulated into the oil phase (Hörmann & Zimmer, 2016:87). The small concentration quantified could be the result of non-ideal physiochemical properties (as discussed in Section 2.6). Consequently, it can be proposed that the formulation of the APIs in the nano-emulsions aided in the transdermal delivery of statins (or molecules that do not otherwise permeate the skin) (Nastiti *et al.*, 2017:1), due to small droplets size, prominent interaction with skin cells, efficient permeation ability and its fluidic nature (Rai *et al.*, 2018:204), but skin diffusion was limited due to obstacles posed by the properties of these API (especially lovastatin and mevastatin). This correlated with what is suggested in literature, as permeation of a drug is dependent on both the formulation composition and the physiochemical properties of the API (Aliyar & Schalau, 2015:827).

Overall, when comparing the statins within the respective formulations, it can be stated that simvastatin displayed better skin permeation, which can be related back to the more ideal physiochemical properties retained by this API in comparison to the other three statins.

### D.3.5 Tape stripping

As stated in Section D.3.4, describing data in terms of the mean value in the case of a skewed distribution (Manikandan, 2011:140) is not appropriate practice. Consequently, as in the case of skin diffusion, tape stripping data will be described in terms of the median as differences in the in the mean (average) concentration in the respective layer and the median concentration were significant, particularly where the ED is concerned. Although the median values will be discussed, the mean values will still be displayed in Table D.6.

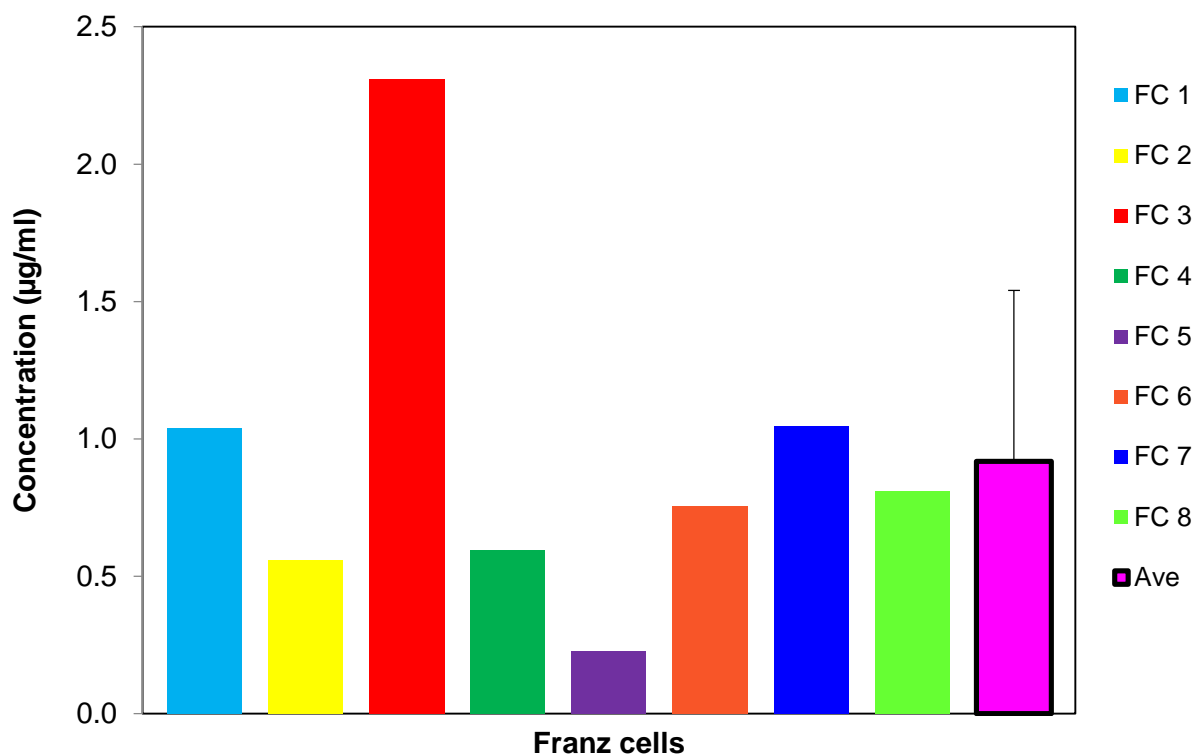
Table D.6 is representative of the results obtained by applying the tape stripping technique, to determine the average concentration ( $\mu\text{g/ml}$ ) and median concentration ( $\mu\text{g/ml}$ ) of the selected statins within the respective formulas in the SCE and ED.

**Table D.6:** The average concentration of the selected statins present in the SCE and the ED collected by means of tape stripping after the 12 h skin diffusion studies

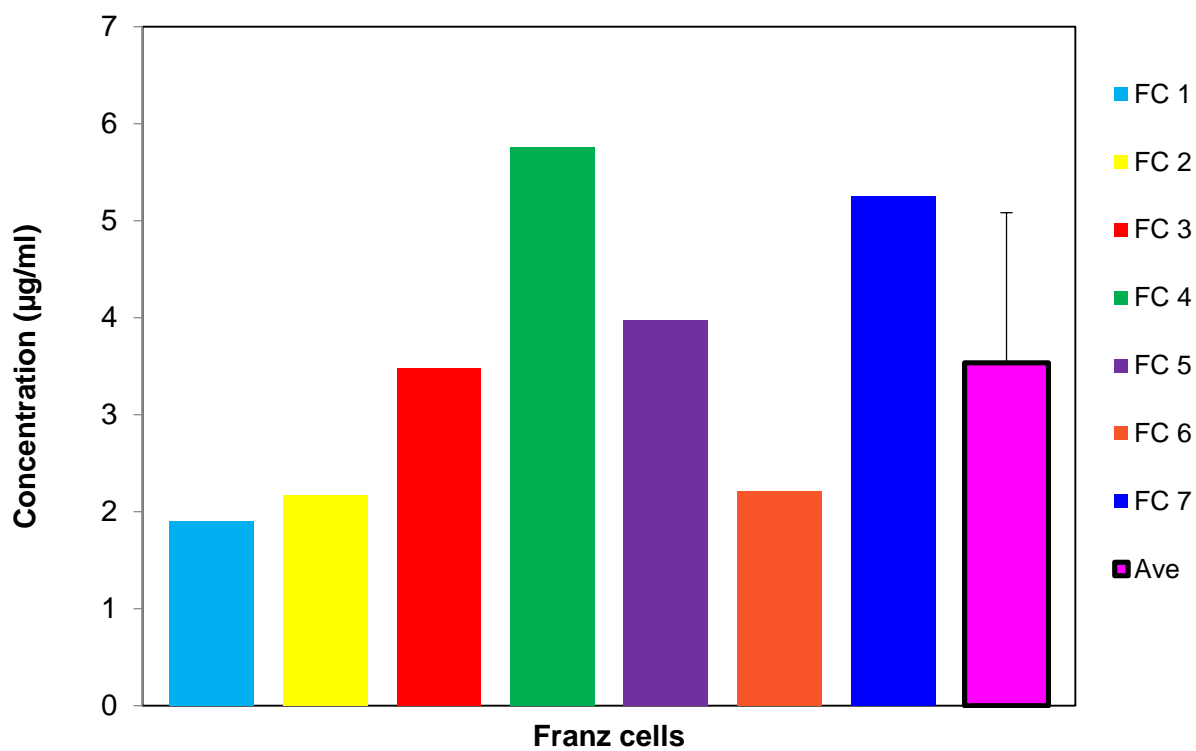
Formulas	Average concentration in SCE ( $\mu\text{g/ml}$ )	Median concentration in SCE ( $\mu\text{g/ml}$ )	Average concentration in ED ( $\mu\text{g/ml}$ )	Median concentration in ED ( $\mu\text{g/ml}$ )	n
(NER1)	$1.543 \pm 1.012$	1.620	$7.339 \pm 8.892$	3.901	8
(NEGR)	$1.668 \pm 1.032$	1.434	$2.139 \pm 0.831$	1.932	7
(NES1)	$2.053 \pm 1.036$	2.121	$13.290 \pm 9.073$	10.680	7
(NEGS)	$7.722 \pm 3.053$	7.517	$9.375 \pm 4.371$	9.518	8
(NEM1)	$2.144 \pm 1.616$	1.871	$2.867 \pm 3.715$	1.338	8
(NEGM)	$1.425 \pm 0.499$	1.416	$4.064 \pm 2.041$	3.429	8
(NEL1)	$0.918 \pm 0.623$	0.783	$4.188 \pm 5.661$	1.622	8
(NEGL)	$3.536 \pm 1.548$	3.475	$3.339 \pm 2.215$	2.413	7

#### D.3.5.1 Stratum corneum-epidermis concentration

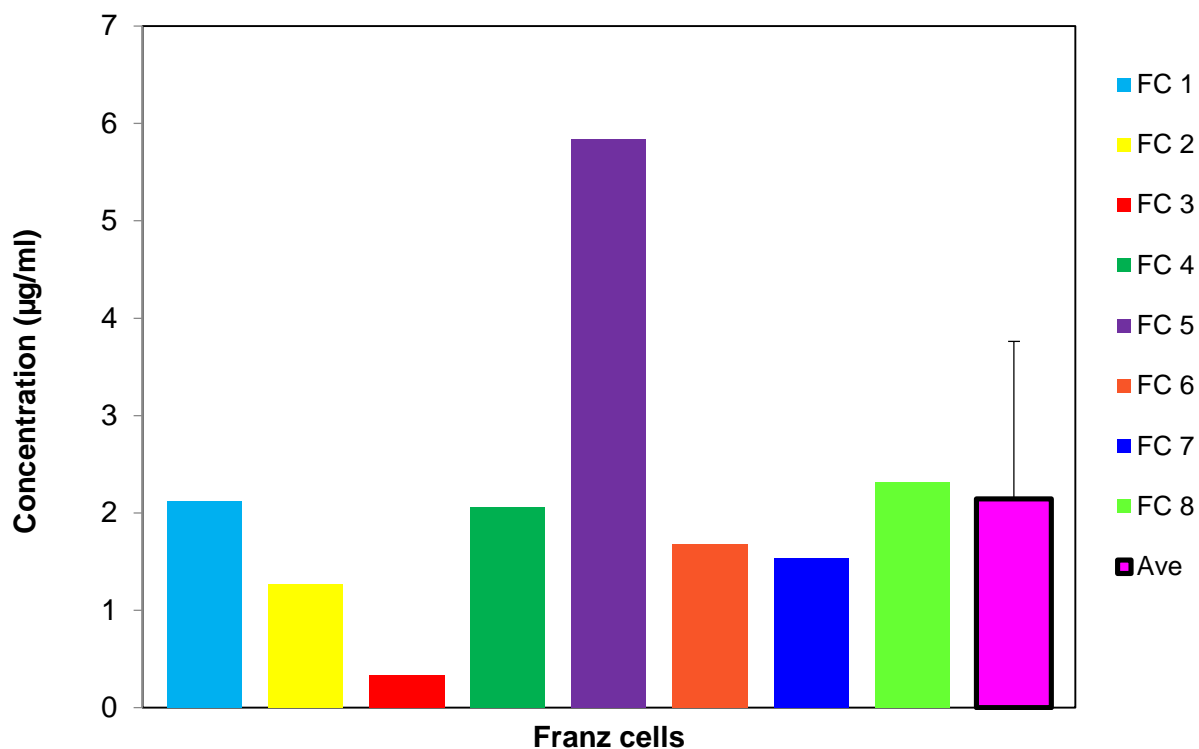
Figures D.32 – D.39 illustrate the results of the average statin concentration obtained in the SCE from the different formulas after tape stripping was performed.



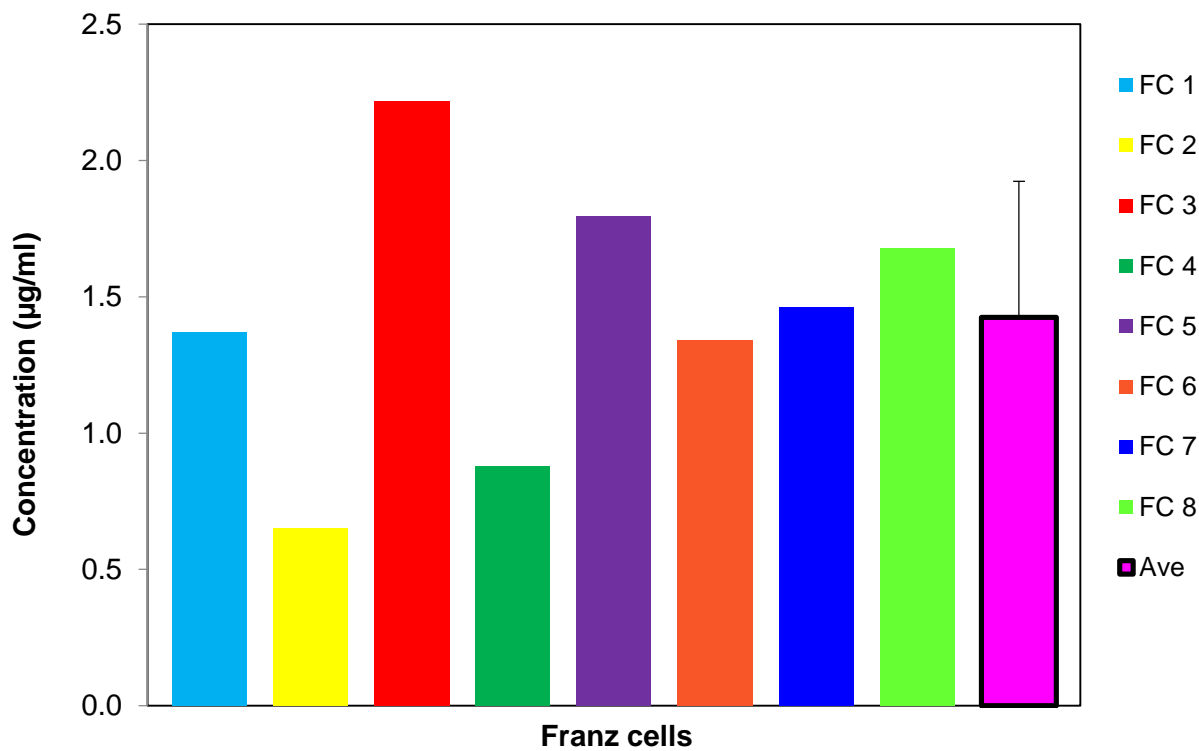
**Figure D.32:** Lovastatin concentration (µg/ml) from **(NEL1)** in the SCE after tape stripping (n = 8)



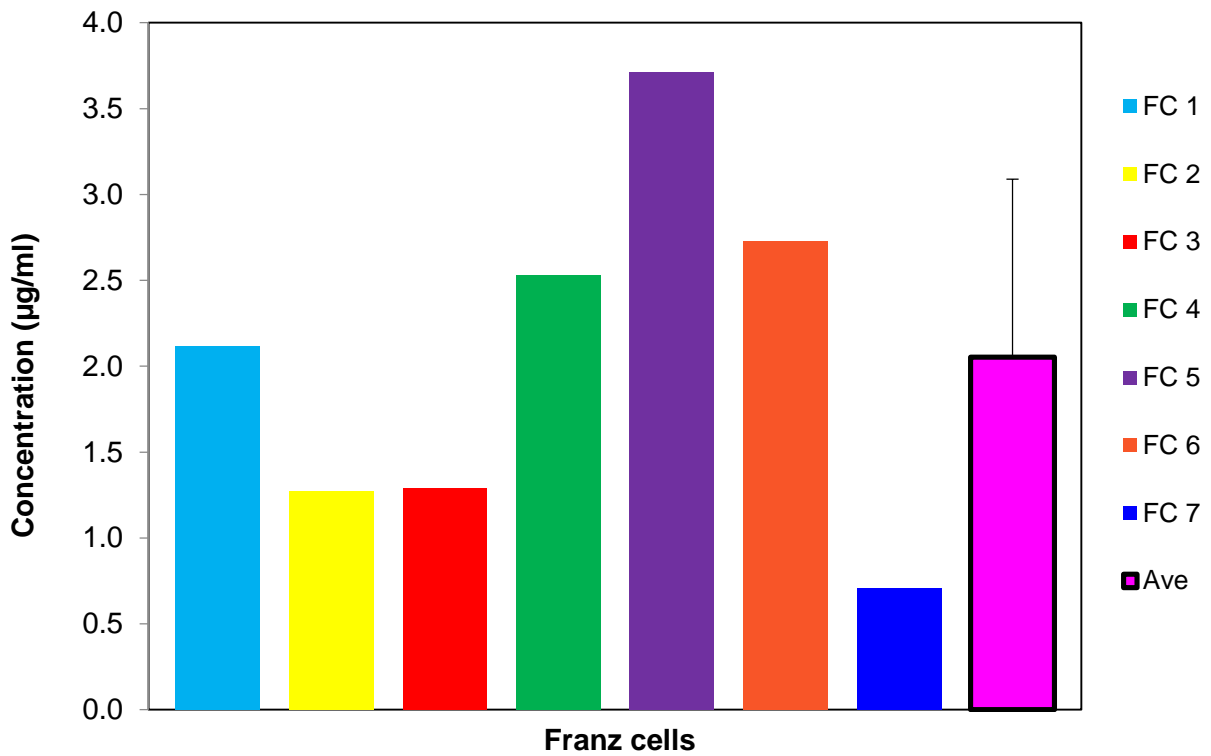
**Figure D.33:** Lovastatin concentration (µg/ml) from **(NEGL)** in the SCE after tape stripping (n = 7)



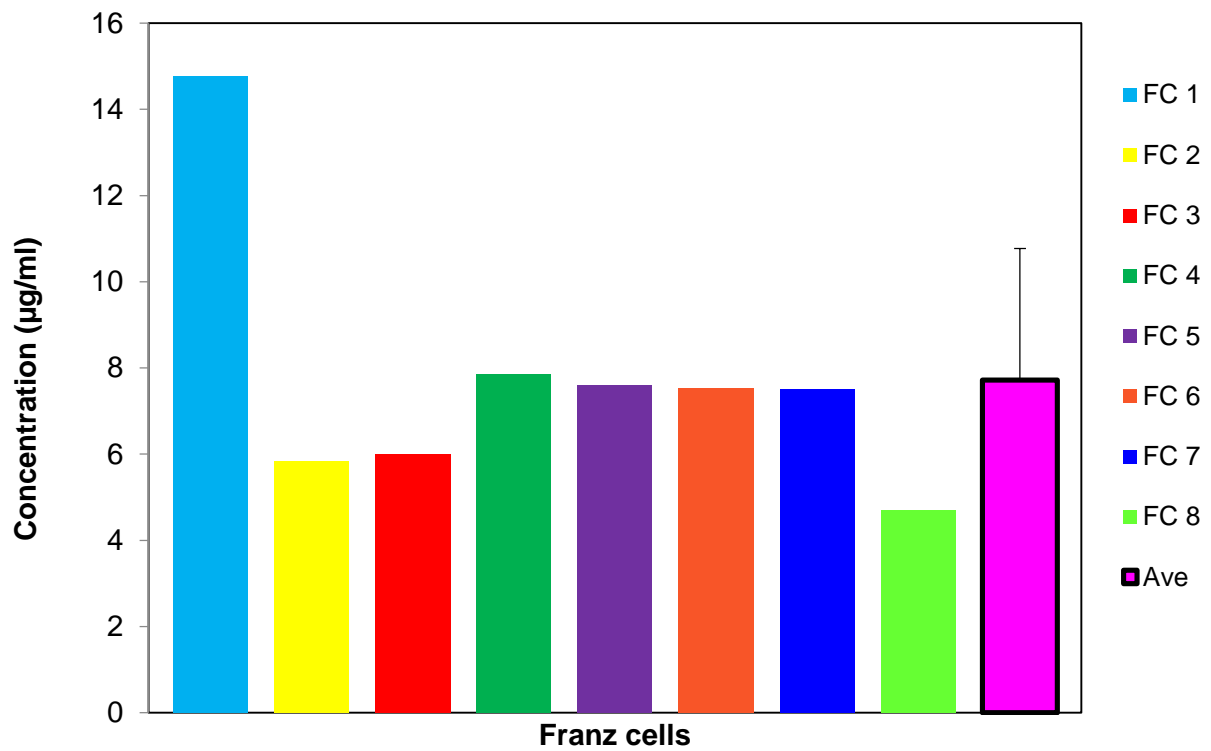
**Figure D.34:** Mevastatin concentration (µg/ml) from **(NEM1)** in the SCE after tape stripping (n = 8)



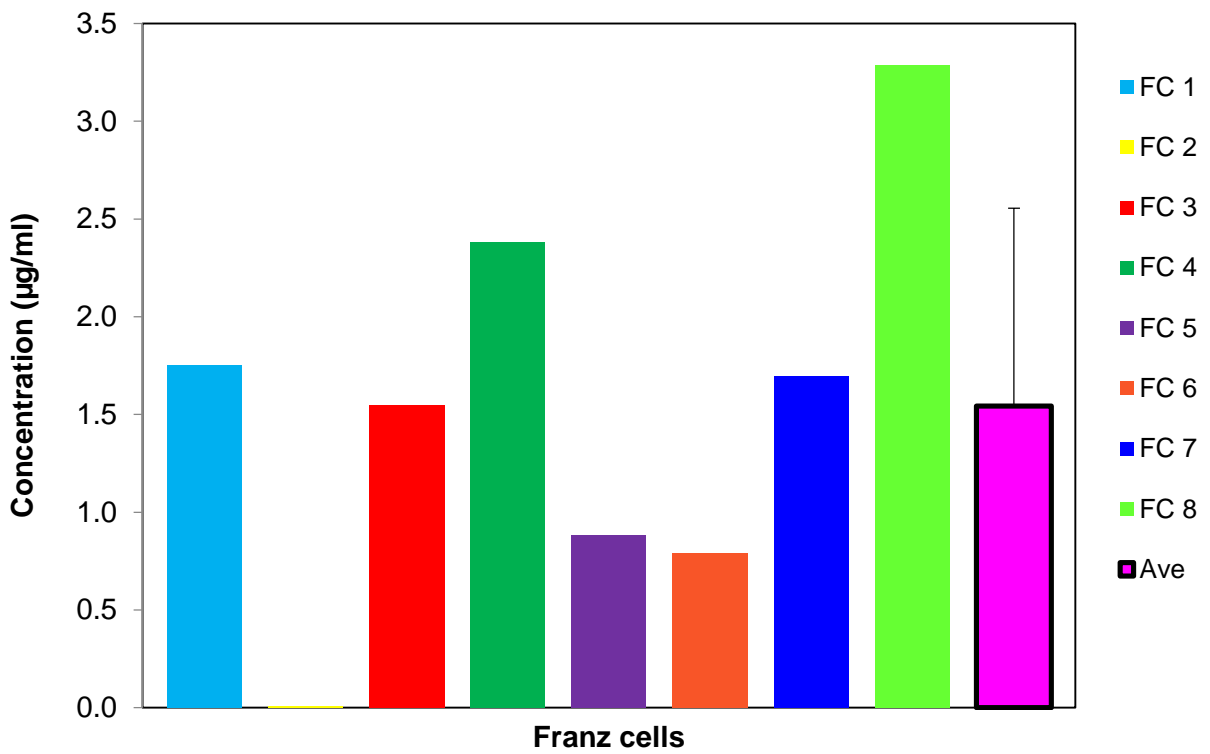
**Figure D.35:** Mevastatin concentration (µg/ml) from **(NEGM)** in the SCE after tape stripping (n = 8)



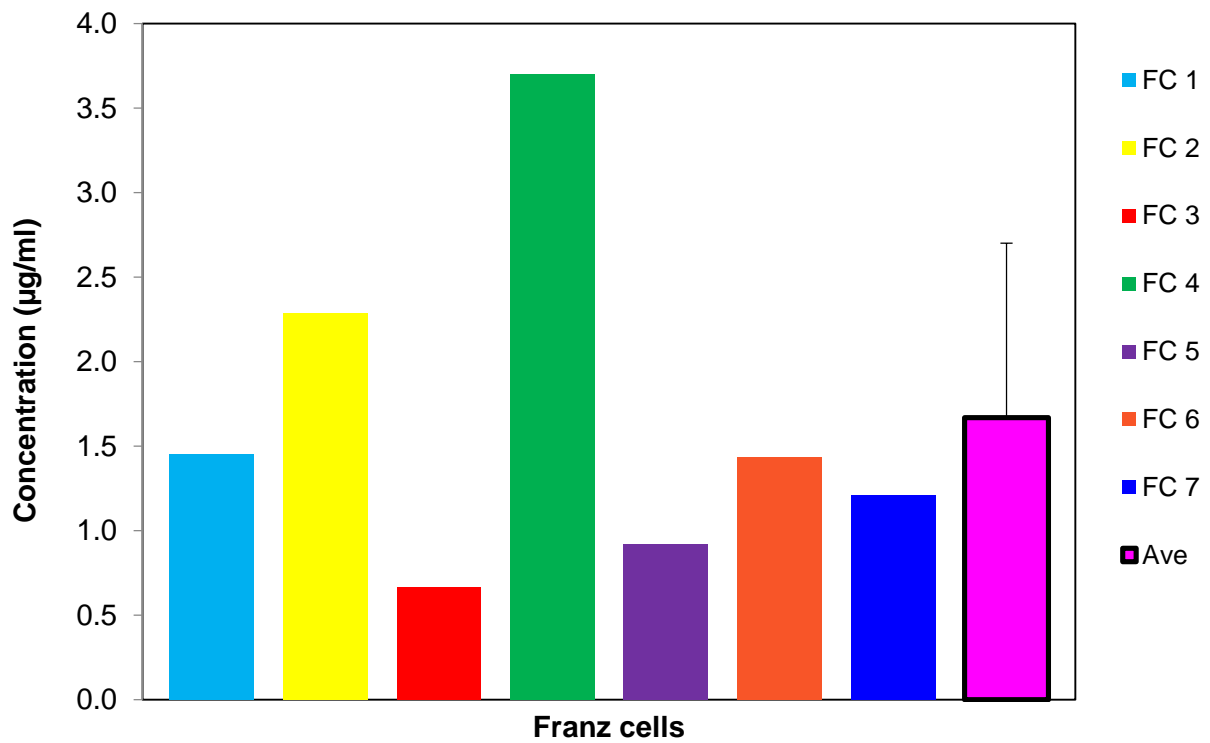
**Figure D.36:** Simvastatin concentration (µg/ml) from **(NES1)** in the SCE after tape stripping (n = 7)



**Figure D.37:** Simvastatin concentration (µg/ml) from **(NEGS)** in the SCE after tape stripping (n = 8)



**Figure D.38:** Simvastatin concentration (µg/ml) from **(NER1)** in the SCE after tape stripping (n = 8)

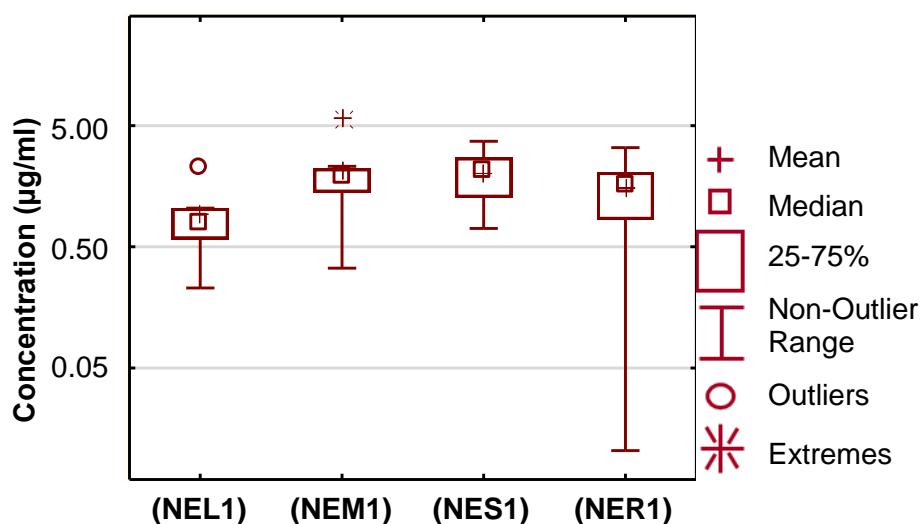


**Figure D.39:** Rosuvastatin concentration (µg/ml) from **(NEGR)** in the SCE after tape stripping (n = 7)

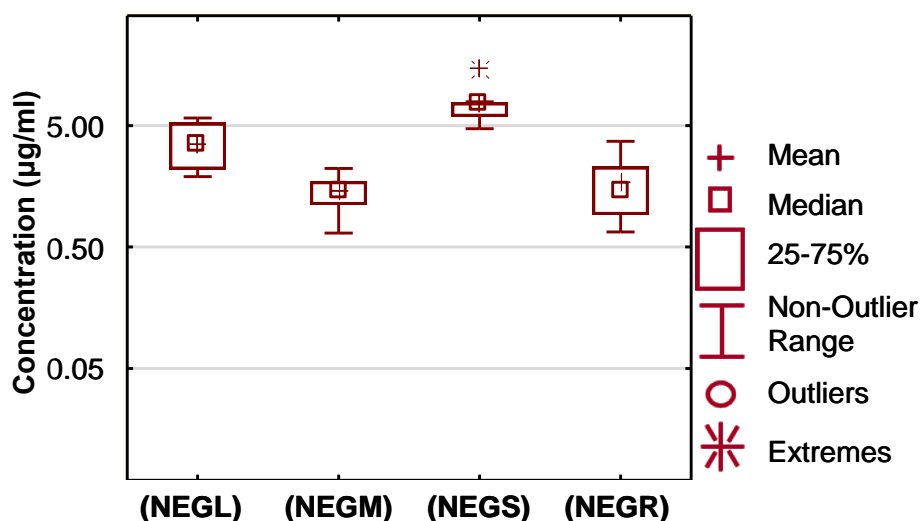
When comparing the median concentrations of all the respective formulas from the highest to the lowest median concentration obtained in the SCE, the **(NEGS)** obtained the highest median concentration, followed by **(NEGL) > (NES1) > (NEM1) > (NER1) > (NEGR) ≈ (NEGM)** and lastly, **(NEL1)**. When observing the **(NEF1)** dispersions, it can be stated that **(NES1)** obtained the highest median concentration, followed by **(NEM1) > (NER1) > (NEL1)**. When comparing the **(NEG1)** semi-solid formulations, **(NEGS)** yielded the highest median concentration, followed by **(NEGL) > (NEGR) > (NEGM)**, with **(NEGR)** and **(NEGM)** almost yielding the same (lowest) concentration within the SCE.

When comparing the **(NEG1)** semi-solid formulations and the **(NEF1)** dispersions, it is observed that the **(NEGS)** and **(NEGL)** penetrated the SCE better than their **(NEF1)** dispersion-counterparts, while **(NEM1)** and **(NER1)** penetrated the SCE better than their **(NEG1)** semi-solid formulation-counterparts. Hence, no definite consensus could be drawn to specify which vehicle (**(NEF1)** or **(NEG1)**) reached the highest concentration within the SCE, as theoretically the **(NEG1)** semi-solid formulations should exhibit a higher deposition of drug within the skin layers compared to the **(NEF1)** dispersions, as the addition of a gelling agent causes the formulation to exhibit a higher affinity for the stratum corneum (Zheng *et al.*, 2016:5982).

When the different statins are investigated, it can be stated that simvastatin displayed the highest concentration within the SCE, which can possibly be explained by the ideal physiochemical properties of the API (solubility in the receptor phase (2.444 mg/ml) being more than 1 mg/ml, the log D (1.80) was between 1 and 3, the molecular weight (418.6 Da) was under 500.0 Da and the melting point (135 – 138 °C) was lower than 200 °C (Bhagat & Sakhare, 2014:1051; Naik *et al.*, 2000:319; Oh *et al.*, 2006:1010). The higher lipophilicity of lovastatin (log D of 4.49) and mevastatin (log D of 3.80) with regard to rosuvastatin (log D of -0.20) may explain why these more lipophilic APIs attained a higher concentration within the lipophilic SCE (N'Da, 2014:20786), since highly hydrophilic molecules are unable to partition from the vehicle into the stratum corneum (Vitorino *et al.*, 2015:703); it can be suggested that this moderately hydrophilic API might have exhibited difficulty with partitioning.



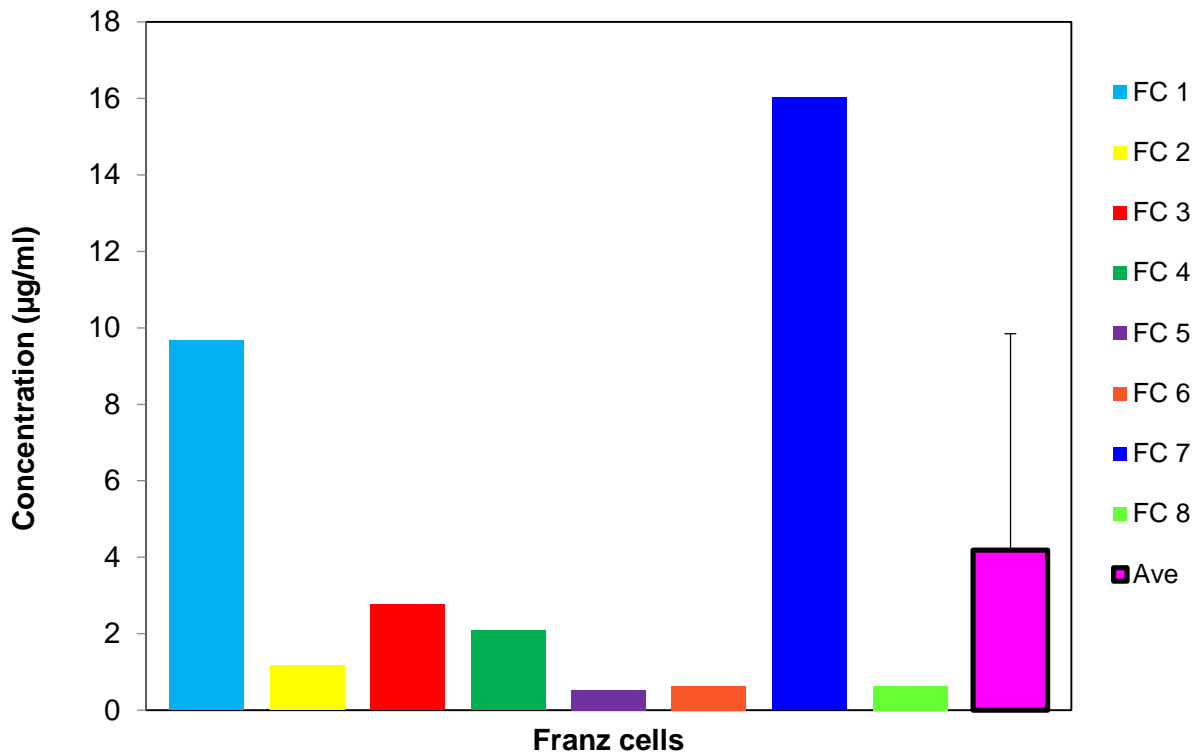
**Figure D.40:** Box-plot indicating the concentration (µg/ml) present in the SCE of the nano-emulsions ((NEL1), (NEM1), (NES1) and (NER1)) after tape stripping was performed (n = 8, except (NES1) was n = 7)



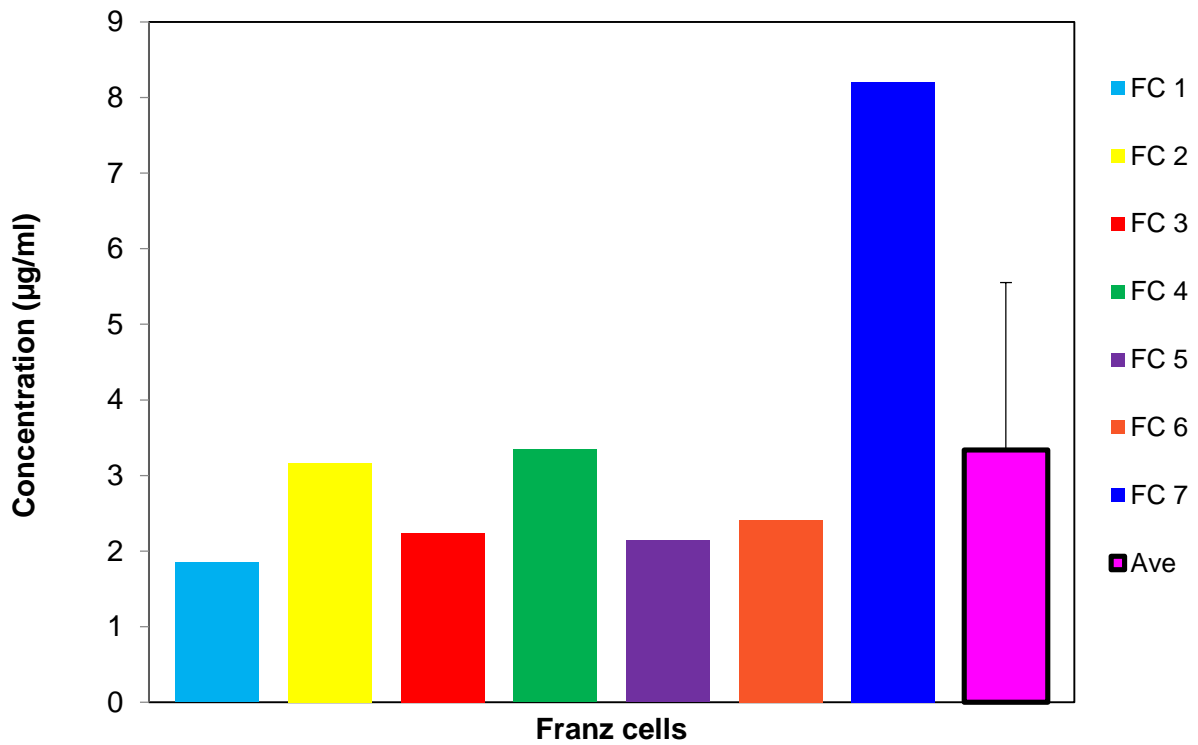
**Figure D.41:** Box-plot indicating the concentration (µg/ml) present in the SCE of the nano-emulgels ((NEGL), (NEGM), (NEGS) and (NEGR)) after tape stripping was performed (n = 8, except (NEGR) and (NEGL) were n = 7).

### D.3.5.2 Epidermis-dermis concentration

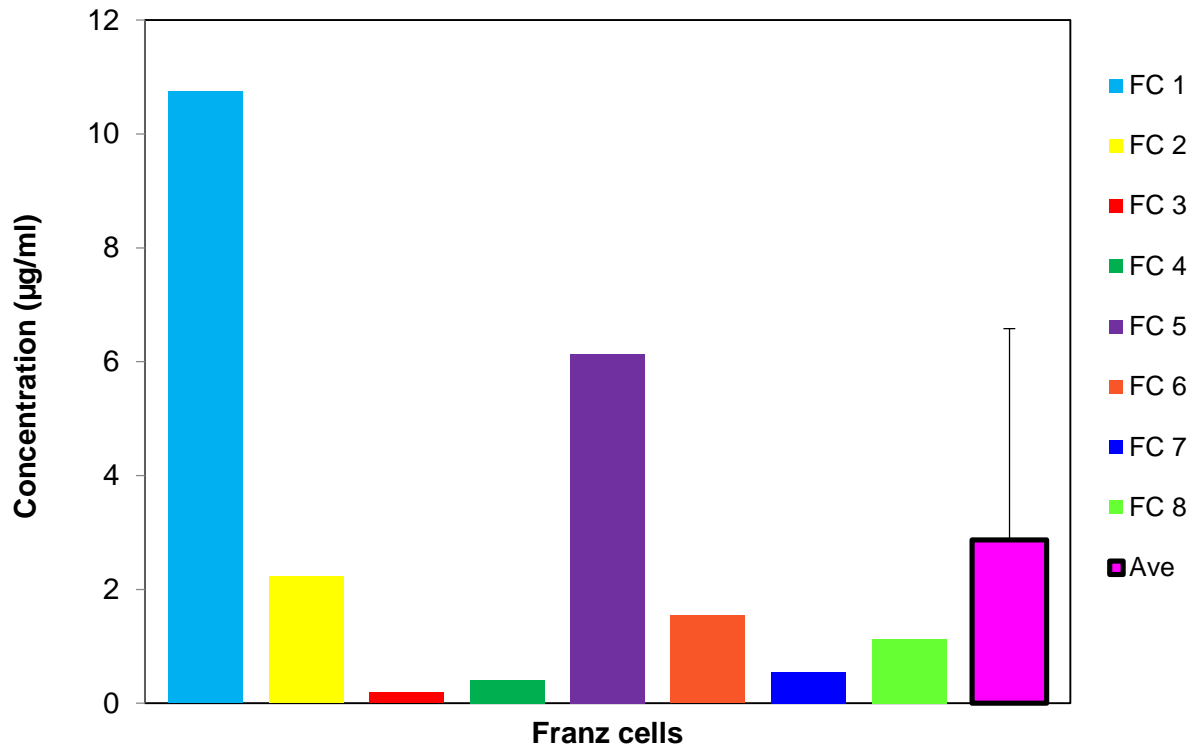
Figures D.42 – D.49 illustrate the results of the average statin concentration obtained in the ED from the different formulas after tape stripping was performed.



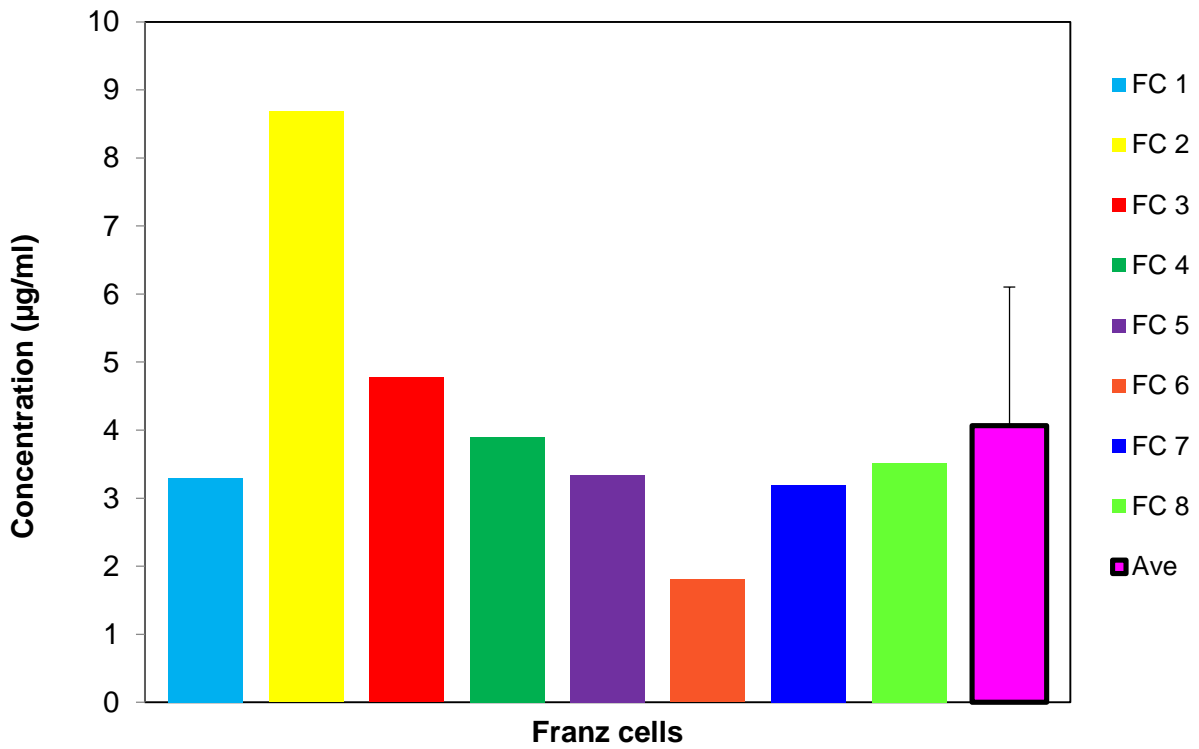
**Figure D.42:** Lovastatin concentration (µg/ml) from **(NEL1)** in the ED after tape stripping (n = 8)



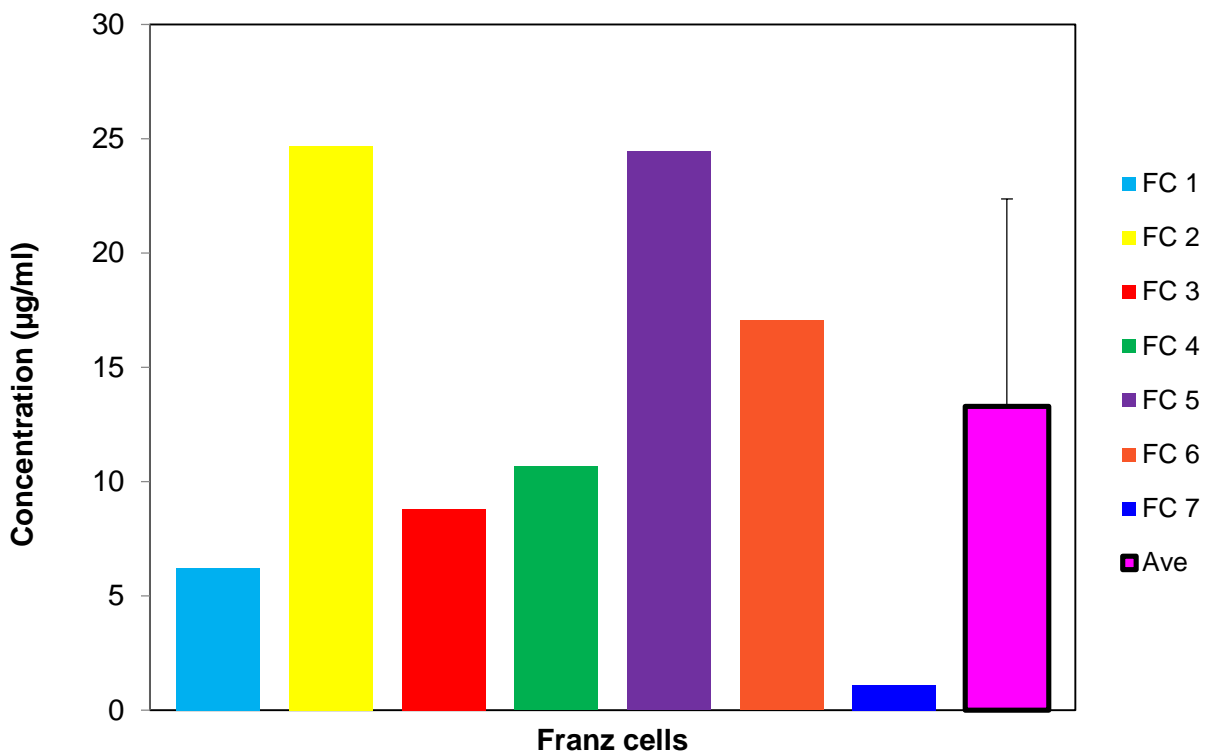
**Figure D.43:** Lovastatin concentration (µg/ml) from **(NEGL)** in the ED after tape stripping (n = 7)



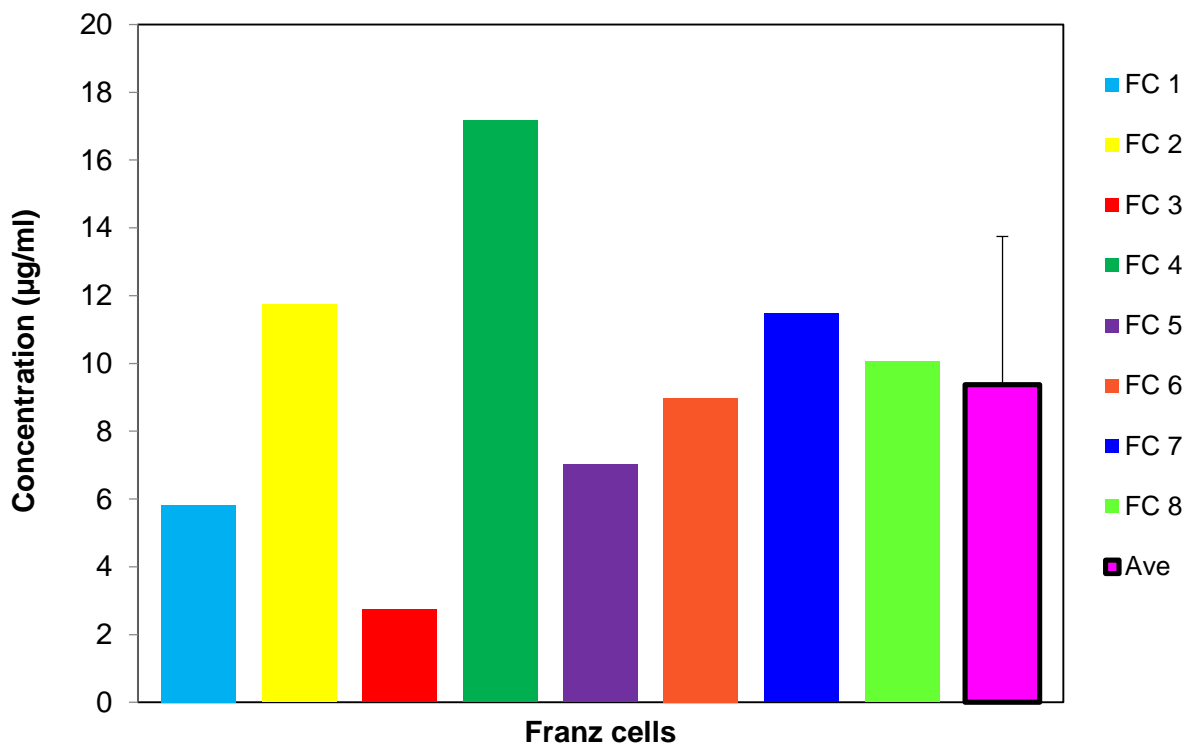
**Figure D.44:** Mevastatin concentration (µg/ml) from **(NEM1)** in the ED after tape stripping (n = 8)



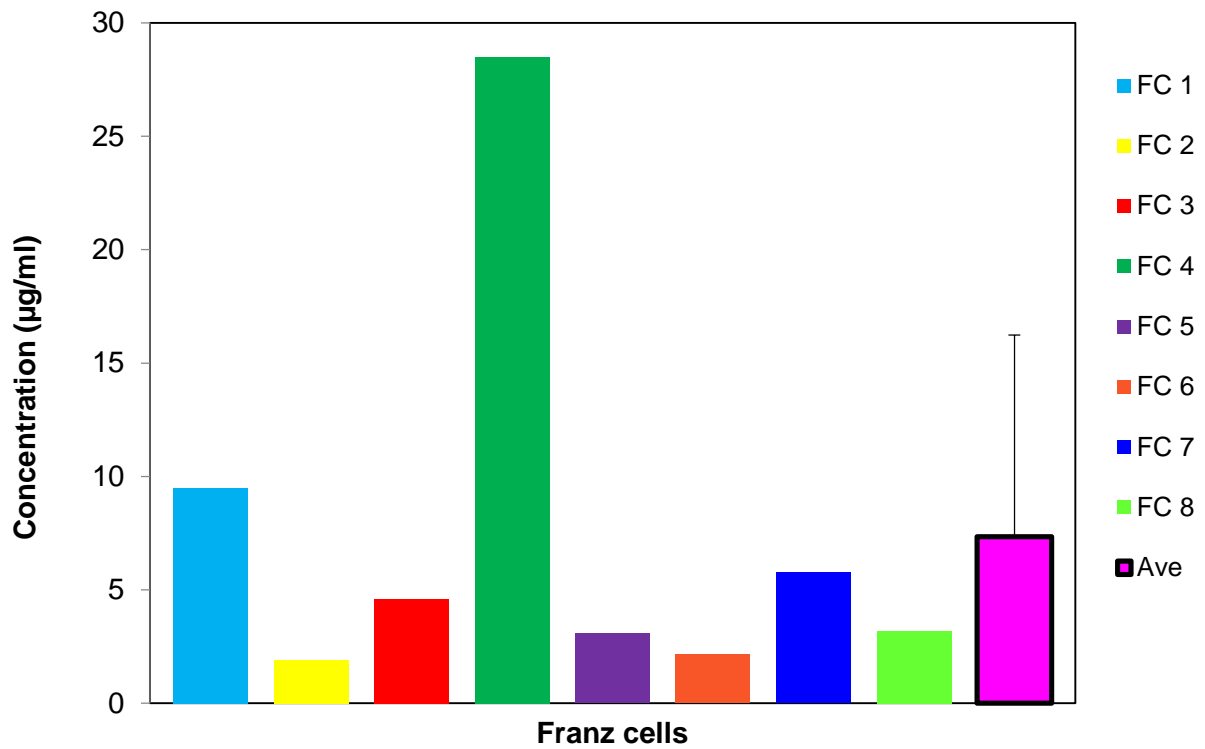
**Figure D.45:** Mevastatin concentration (µg/ml) from **(NEGM)** in the ED after tape stripping (n = 8)



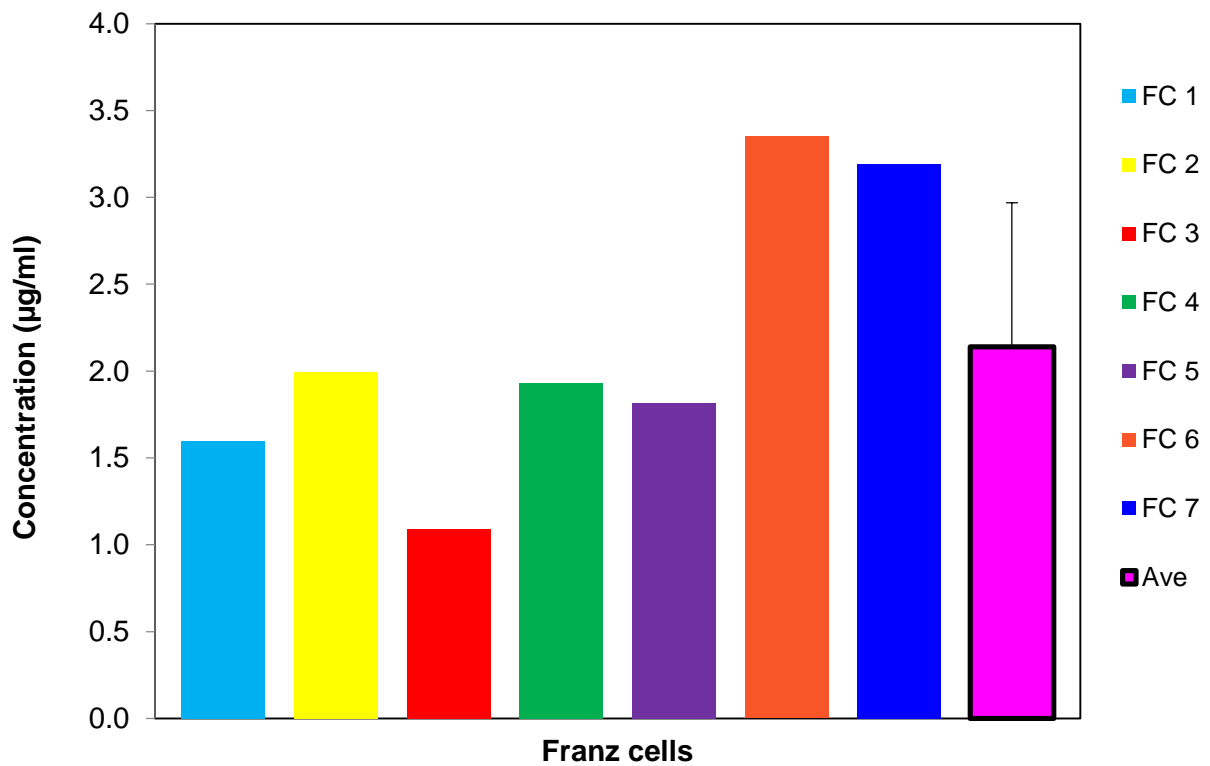
**Figure D.46:** Simvastatin concentration (µg/ml) from **(NES1)** in the ED after tape stripping (n = 7)



**Figure D.47:** Simvastatin concentration (µg/ml) from **(NEGS)** in the ED after tape stripping (n = 8)



**Figure D.48:** Rosuvastatin concentration ( $\mu\text{g/ml}$ ) from **(NER1)** in the ED after tape stripping ( $n = 8$ )

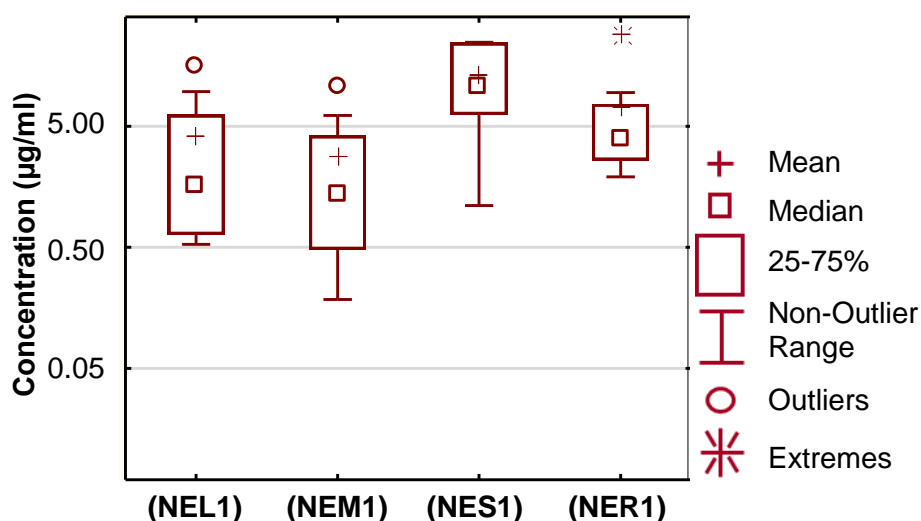


**Figure D.49:** Rosuvastatin concentration ( $\mu\text{g/ml}$ ) from **(NEGR)** in the ED after tape stripping ( $n = 7$ )

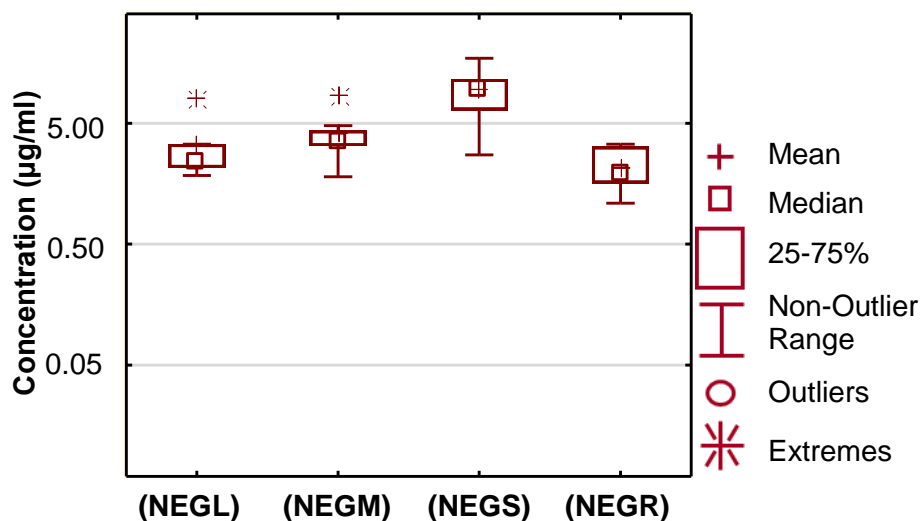
When observing Table D.6, it can be noted that all the average concentrations obtained for the respective dispersions and formulations were above the LOD and LOQ values of the selected statins. It can further be stated that when comparing all the respective formulas in terms of median concentration obtained in the ED, that **(NES1)** (10.680 µg/ml) obtained the highest median concentration, followed by **(NEGS)** > **(NER1)** > **(NEGM)** > **(NEGL)** > **(NEGR)** > **(NEL1)** and lastly, **(NEM1)**. Subsequently, it can be observed that when evaluating the **(NEF1)** dispersions that **(NES1)** yielded the highest median concentration, followed by **(NER1)** > **(NEL1)** and lastly, **(NEM1)**. When the **(NEG1)** semi-solid formulations were investigated, **(NEGS)** yielded the highest median concentration within the ED, followed by **(NEGM)** > **(NEGL)** and lastly, **(NEGR)**.

When comparing the **(NEF1)** dispersions and the **(NEG1)** semi-solid formulations, there was no exact indication to whether the semisolids or the dispersions reached higher concentrations within the ED, since the **(NES1)** and **(NER1)** penetrated the ED better than their **(NEG1)** semi-solid formulation-counterparts, while **(NEGM)** and **(NEGL)** penetrated the ED better than their **(NEF1)** dispersion-counterparts. Although literature predicts that a higher concentration could be attained within the ED for the nano-emulgels, as the addition of the gelling agent can transform the vehicle from a transdermal to a more topical vehicle depending on the concentration gelling agent used (Zheng *et al*, 2016:5983).

After comparing the different statins with each other, it can be stated that simvastatin obtained the highest concentrations within the ED. This result can be related to the better solubility of simvastatin within the more hydrophilic viable epidermis, which enables partitioning (Prakash *et al.*, 2016:357).



**Figure D.50:** Box-plot indicating the concentration (µg/ml) present in the ED of the nano-emulsions ((NEL1), (NEM1), (NES1) and (NER1)) after tape stripping was performed (n = 8, except (NES1) was n = 7).



**Figure D.51:** Box-plot indicating the concentration ( $\mu\text{g/ml}$ ) present in the ED of the nano-emulgels ((**NEGL**), (**NEGM**), (**NEGS**) and (**NEGR**)) after tape stripping was performed ( $n = 8$ , except (**NEGR**) and (**NEGL**) were  $n = 7$ ).

#### D.4 Statistical analysis

Log transformation can be useful when data is skewed or exponentially distributed, in particular for data with a small number of large inputs. In general, the differences between values are sustained, although the higher values become more compressed (or in bringing the outlier closer to the main group), while the lower values become more spread out (James, 2016:50-52). It was also established by Curran-Everett (2018:246) that log transformation will assist sample observation to fulfil the assumptions of certain statistical analyses. Additionally, it knows that logarithmic transformation of data, assists in improving the heterogeneity of variance (Hamada, 2018:22). Log transformation of data was used in this study to determine the statistically significance of effects during the membrane release studies, skin diffusion and tape stripping. Prior to performing any statistical analysis of data, a null hypothesis should be proposed (between the variables of interest; meaning there is no difference) (McCluskey & Lalkhen, 2007:209). Thereafter statistical analysis can be performed and results obtained interpreted by relying on the consideration and appreciation of the null hypothesis, p-value, pitfall of multiple comparisons, study power, concept of statistical significance and one as opposed to two-way tests, before performing the study (McCluskey & Lalkhen, 2007:208). The aforementioned

p-value can be defined as the probability that under the null hypothesis, a result can be obtained that is equivalent or more extreme than what was really observed. Additionally, it is proposed that the p-value evaluates the likeliness that the observed difference between groups is due to chance, hence p stands for probability, and can be seen as a value between 0 and 1. Consequently, values closer to 1 can suggest no difference (or significance), while those

close to 0 are indicative that the observed difference is not likely to be due to chance (Dahiru, 2008:22). Although a p-value smaller or equal to 0.05 is commonly considered statistically significant (Concato & Hartigan, 2016:1166), it is recommended that p-value be reported as very significant or highly significant, depending on the proximity to 0 rather than simple stating significant or not significant (Dahiru, 2008:22).

#### D.4.1 Membrane release studies

Since the interaction between formula type and statin was statistically significant ( $p < 0.05$ ), one-way ANOVAs were performed to determine the effect of the statin for each of the **(NEF1s)** and **(NEG1s)** during membrane release studies. As there were statin effects ( $p < 0.05$ ) in both ANOVAs, Tukey's honest significance difference (HSD) tests were performed to determine pairwise differences between the four statin means (averages).

**Table D.7:** Tukey's HSD-test performed on the **(NEF1s)**

Nano-emulsions	Mean log flux
<b>(NEL1)</b>	0.997 <sup>a</sup>
<b>(NEM1)</b>	1.238 <sup>b</sup>
<b>(NES1)</b>	2.474 <sup>c</sup>
<b>(NER1)</b>	2.616 <sup>d</sup>

a, b, c and d: denote statistically non-significant homogeneous groups on 0.05 level (i.e. all groups are significant)

It can therefore be stated that when observing Table D.7, that there were significant differences between all four the **(NEF1s)** during the membrane release studies.

**Table D.8:** Tukey's HSD-test performed on the **(NEG1s)**

Nano-emulgels	Mean log flux
<b>(NEGL)</b>	0.689 <sup>a</sup>
<b>(NEGM)</b>	0.707 <sup>a</sup>
<b>(NEGS)</b>	1.999 <sup>b</sup>
<b>(NEGR)</b>	2.453 <sup>c</sup>

a, b and c: denote statistically non-significant homogeneous groups on 0.05 level (i.e. first 2 groups are non-significant)

When observing Table D.8, it can be stated that when comparing **(NEGL)** and **(NEGM)**, there was no significant difference; although, **(NEGS)** differed from the aforementioned, as well as **(NEGR)**. It can further be observed that **(NEGR)** differed significantly from **(NEGL)**, **(NEGM)** and **(NEGS)** during the membrane release studies.

#### D.4.2 Skin diffusion

As there were statin effects ( $p < 0.05$ ) in both ANOVAs during the skin diffusion analysis, Tukey's HSD-tests were performed to determine pairwise differences between the four statin means.

**Table D.9:** Tukey's HSD-test performed on the **(NEF1s)**

Nano-emulsions	Mean log concentration
<b>(NEL1)</b>	- 0.598 <sup>a</sup>
<b>(NEM1)</b>	- 0.362 <sup>ab</sup>
<b>(NES1)</b>	- 0.160 <sup>b</sup>
<b>(NER1)</b>	0.359 <sup>c</sup>

a, b and c: denote statistically non-significant homogeneous groups on 0.05 level (i.e. first 2 groups are non-significant and groups 2 and 3 are non-significant)

From Table D.9, it can be proposed that between **(NEL1)** and **(NEM1)**, there were no significant differences, while **(NEL1)** displayed a significant difference when compared to **(NES1)** and **(NER1)**. It can be further stated that **(NES1)** did not differ significantly when compared to **(NEM1)** (indicated by ab), but a significant difference is noted when compared to **(NEL1)** and **(NER1)**. Lastly, a significance is observed between **(NER1)** and the other three dispersions.

**Table D.10:** Tukey's HSD-test performed on the **(NEG1s)**

Nano-emulsions	Mean log concentration
<b>(NEGL)</b>	- 0.314 <sup>a</sup>
<b>(NEGM)</b>	0.036 <sup>ab</sup>
<b>(NEGS)</b>	0.581 <sup>b</sup>
<b>(NEGR)</b>	- 0.570 <sup>a</sup>

a and b: denote statistically non-significant homogeneous groups on 0.05 level (i.e. groups 1, 2 and 4 are non-significant and groups 2 and 3 are non-significant)

The result as displayed in Table D.10, indicates that there is no significant difference between **(NEGL)**, **(NEGM)** and **(NEGR)**. In addition, when observing **(NEGS)** and **(NEGM)**, no significant difference can be noted; although **(NEGS)** differ significantly from both **(NEGL)** and **(NEGR)**.

#### D.4.3 Tape stripping

Since the interaction between formula type, statin and skin layer were statistically significant ( $p < 0.05$ ), one-way ANOVAs were performed to determine the effect of the statin for each of the four combinations of the formula type and the skin layer during tape stripping studies.

**Table D.11:** P-values obtained from the one-way ANOVA of all the combinations for the formula type and the skin layer during tape stripping

Combinations	p-value
(NEF1s) and SCE	0.3028
(NEF1s) and ED	0.0142
(NEG1s) and SCE	< 0.0100
(NEG1s) and ED	< 0.0100

When observing Table D.11, no statistical significance was observed within the SCE for (NEF1), although the remaining interactions was considered statically significant ( $p < 0.05$ ). Since there were statin effects ( $p < 0.05$ ) in three of the ANOVAs, Tukey's HSD-tests were performed to determine pairwise differences between the four statin means.

**Table D.12:** Tukey's HSD-test performed on the (NEF1s) and the SCE

Nano-emulsions	Mean log concentration
(NEL1)	- 0.116 <sup>a</sup>
(NEM1)	0.226 <sup>a</sup>
(NES1)	0.258 <sup>a</sup>
(NER1)	- 0.073 <sup>a</sup>

a: denote statistically non-significant homogeneous groups on 0.05 level (i.e. all 4 groups are non-significant)

Table D.12 serves as conformation of the results displayed in Table D.11 of the interaction between the (NEF1s) and SCE, that no statistical significance occurred.

**Table D.13:** Tukey's HSD-test performed on the (NEF1s) and the ED

Nano-emulsions	Mean log concentration
(NEL1)	0.129 <sup>a</sup>
(NEM1)	0.293 <sup>ab</sup>
(NES1)	0.975 <sup>b</sup>
(NER1)	0.683 <sup>ab</sup>

a and b: denote statistically non-significant homogeneous groups on 0.05 level (i.e. the first 2 groups and group 4 are non-significant and groups 2 and 3 are non-significant)

Table D.11 indicated that a very significant difference could be observed when comparing the (NEF1s) and ED ( $p = 0.0142$ ). However, when observing Table D.13, it can be stated that the main difference occurred between (NEL1) and (NES1).

**Table D.14:** Tukey's HSD-test performed on the **(NEG1s)** and the SCE

Nano-emulgel	Mean log concentration
<b>(NEGL)</b>	0.127 <sup>a</sup>
<b>(NEGM)</b>	0.512 <sup>b</sup>
<b>(NEGS)</b>	0.864 <sup>c</sup>
<b>(NEGR)</b>	0.160 <sup>b</sup>

a, b and c: denote statistically non-significant homogeneous groups on 0.05 level (i.e. groups 2 and 4 are non-significant)

From Table D.14, it can be observed that there is no significant difference between **(NEGM)** and **(NEGR)**. However, differences are observed when comparing the aforementioned to **(NEGS)** and **(NEGL)**, as these **(NEG1s)** differ from one another.

**Table D.15:** Tukey's HSD-test performed on the **(NEG1s)** and the ED

Nano-emulgels	Mean log concentration
<b>(NEGL)</b>	0.467 <sup>a</sup>
<b>(NEGM)</b>	0.570 <sup>a</sup>
<b>(NEGS)</b>	0.921 <sup>b</sup>
<b>(NEGR)</b>	0.302 <sup>a</sup>

a and b: denote statistically non-significant homogeneous groups on 0.05 level (i.e. the first 2 groups and group 4 are non-significant)

Although Table D.11 indicated a highly significant difference ( $p < 0.01$ ) when comparing **(NEG1s)** within the ED, it should be stated that when observing Table D.15, no significant difference is observed between **(NEGL)**, **(NEGM)** and **(NEGR)**; however, **(NEGS)** differed significantly from the aforementioned formulations. Consequently, it can be proposed that the highly significant p-value ( $< 0.01$ ), as displayed in Table D.11, could be caused by **(NEGS)**.

**Table D.16:** P-values obtained from t-tests performed to compare all the formulas in terms of SCE (group 1) and ED (group 2), respectively

Formulation	p-value
<b>(NEL1)</b>	0.0935
<b>(NEGL)</b>	0.6936
<b>(NEM1)</b>	0.6957
<b>(NEGM)</b>	0.0003
<b>(NES1)</b>	0.0057
<b>(NEGS)</b>	0.5800
<b>(NER1)</b>	0.0362
<b>(NEGR)</b>	0.2345

From Table D.16, it can be stated that for the nano-emulsions within the SCE and ED, **(NES1)** and **(NER1)** was found to be statically significant, while only **(NEGM)** was found significant when the **(NEG1s)** were compared. In addition, it can be proposed that **(NER1)** displayed a p-value that can be considered less significant due to the closer proximity to 0.05 rather than 0.

**Table D.17:** P-values obtained from t-tests performed to compare the specific skin layer (SCE or ED) in terms of the **(NEF1s)** (group 1) and the **(NEG1s)** (group 2)

Combinations	p-value
Lovastatin formulas ( <b>(NEL1)</b> and <b>(NEGL)</b> ) and SCE	0.0003
Lovastatin formulas ( <b>(NEL1)</b> and <b>(NEGL)</b> ) and ED	0.4546
Mevastatin formulas( <b>(NEM1)</b> and <b>(NEGM)</b> ) and SCE	0.4772
Mevastatin formulas( <b>(NEM1)</b> and <b>(NEGM)</b> )and ED	0.0653
Simvastatin formulas ( <b>(NES1)</b> and <b>(NEGS)</b> ) and SCE	0.0001
Simvastatin formulas ( <b>(NES1)</b> and <b>(NEGS)</b> ) and ED	0.7789
Rosuvastatin formulas ( <b>(NER1)</b> and <b>(NEGR)</b> ) and SCE	0.4736
Rosuvastatin formulas ( <b>(NER1)</b> and <b>(NEGR)</b> )and ED	0.0314

When comparing the respective **(NEF1s)** with the **(NEG1s)** containing the same statins within the SCE and ED, respectively (as displayed in Table D.17), only the lovastatin formulas (**(NEL1)** and **(NEGL)**) and simvastatin formulas (**(NES1)** and **(NEGS)**) within the SCE, along with the rosuvastatin formulas (**(NER1)** and **(NEGR)**) within the ED, displayed statistical significance. It should however be stated that the rosuvastatin formulas (**(NER1)** and **(NEGR)**) within the ED, displayed a less significant value compared to the aforementioned ( $p = 0.0314$ ).

## D.5 Conclusion

From the aqueous solubility data obtained for the statins, it can be stated that none of the selected statins possessed an ideal aqueous solubility ( $> 1\text{mg ml}$ ) as proposed by Naik *et al.* (2000:319), although rosuvastatin ( $0.310 \pm 0.066\text{ mg/ml}$ ) and simvastatin ( $0.243 \pm 0.058\text{ mg/ml}$ ) displayed a higher aqueous solubility compared to lovastatin ( $0.001 \pm 0.008\text{ mg/ml}$ ) and mevastatin ( $0.002 \pm 0.001\text{ mg/ml}$ ). Consequently, it can be stated that due to the low aqueous solubility, no permeation or poor permeation of skin is expected. From the experimental log D values obtained for the selected statins, it can be stated that only simvastatin ( $1.80 \pm 0.001$ ) possessed a log D value considered ideal for skin permeation (between 1 – 3), as suggested by Naik *et al.* (2000:319), as rosuvastatin ( $- 0.20 \pm 0.001$ ) can be considered too hydrophilic, while lovastatin ( $4.49 \pm 0.039$ ) and mevastatin ( $3.80 \pm 0.128$ ) are too lipophilic; although, according to Williams (2013:680), a log D between 1 and 4 can could still be seen as acceptable. Therefore, by determining the aqueous solubility and log D values, certain predictions could be made with regards to skin permeation of the APIs.

As stated in Section 1.3, the aim of this study was to facilitate the transdermal delivery of the selected statins, hence membrane release studies were performed firstly, followed by skin diffusion studies of the optimised **(NEF1s)** and **(NEG1s)**, as determined in Section C.7.

From the membrane release studies, it was evident that the release of all the APIs occurred from all the respective formulas that were tested, although, it can be stated that **(NER1)**, **(NEGR)**, **(NES1)** and **(NEGS)** presented with a significantly higher flux (**(NER1)** the highest), when compared to the lovastatin formulas (**(NEL1)** and **(NEGL)**) and mevastatin formulas (**(NEM1)** and **(NEGM)**). This difference could be attributed to the weaker solubility of mevastatin and lovastatin in the receptor buffer, due to the fact that these compounds are crystalline solids (Cayman Chemical, 2008; Yoshida *et al.*, 2011:657) compared to the more soluble salt forms of simvastatin and rosuvastatin, which could result in lower solubility of the crystalline solids (Taskinen & Norinder, 2007:628). It can further be stated that the nano-emulsions obtained higher flux values when compared to the respective nano-emulgels, which correlates with what is suggested in literature, as a nano-emulgel will release the API in a more controlled manner (Chellapa *et al.*, 2015:45). After completion of the membrane release studies, skin diffusion studies were performed.

During the skin diffusion studies, results indicated that an amount was quantified for each of the statins within each of the respective formulas. It can further be stated that the amount quantified ( $\mu\text{g}/\text{ml}$ ), however small, was higher than the respective LOD and LOQ values determined for the selective statins with the exception of **(NEL1)** that obtained an amount in the receptor phase, which was under the LOQ of lovastatin, but above the LOD. Therefore, it can be proposed that transdermal delivery was achieved with both the **(NEF1s)** and the **(NEG1s)**. When comparing all the respective formulas, it can be stated that **(NEGS)** ( $3.244 \mu\text{g}/\text{cm}^2$ ) and **(NER1)** ( $2.146 \mu\text{g}/\text{cm}^2$ ) attained significantly higher median amounts per area diffused compared to the other formulas tested, such as **(NEL1)**, which attained the lowest amount ( $0.297 \mu\text{g}/\text{cm}^2$ ) within the receptor phase. When comparing the median amount per area diffused of the **(NEF1s)** with their respective **(NEG1s)**, it can be concluded that a higher median value of the API diffused to the receptor phase during the skin diffusion studies from the **(NEG1s)** than from the **(NEF1s)**, with the exception of **(NER1)**, which attained a higher median value in the receptor phase than **(NEGR)**. This could possibly be ascribed to the higher concentration present in the SCE and ED for **(NER1)** compared to **(NEGR)**, as diffusion is dependent on the concentration gradient; hence, larger concentrations result in a larger diffusion gradient and consequently, better diffusion (Barry, 2002:512). In addition, this deviation from the trend by **(NEGR)**, can possibly be related to the lower viscosity and zeta-potential (thus lower stability) and viscosity (thus less adhesion) of this nano-emulgel compared to the other respective semi-solids. Furthermore, it can be suggested that the low concentration of **(NEL1)** obtained within the receptor phase can be attributed to the non-ideal physiochemical

properties (such as low aqueous solubility and high lipophilicity) (Naik *et al.*, 2000:319), while possible affinity of lovastatin for the oil phase (penetration enhancer) could also be a contributing factor, which could lead to poor skin permeation (Haque & Talukder, 2018:171). After extraction of the receptor phase for analysis by HPLC, tape stripping was conducted.

Although the aim of this study was to facilitate the transdermal delivery of the selected statins, concentrations of each of the statin within the respective formulas was quantified within the SCE and ED. **(NEGS)** yielding the highest concentration in the SCE can be attributed to the ideal physicochemical properties, i.e. log D, melting point, molecular weight and aqueous solubility of this API (Naik *et al.*, 2000:319). The fact that **(NEGL)** yielded a higher concentration compared to **(NEGR)** and **(NEGM)** could possibly be explained by the lipophilic nature of this API (**(NEGL)**), having a higher affinity for the lipophilic stratum corneum (Förster *et al.*, 2009:319) and will penetrate the stratum corneum more easily (Sugibayashi, 2017:246). Although the low concentration of **(NEGM)** within the SCE is contradictive when observing the log D value of this API (see Section D.3.2), it can be suggested that due to the increased droplet size of **(NEGM)** compared to the other **(NEG1s)**, this is a possible explanation as droplet size can influence penetration of skin layers (Sengupta & Chatterjee, 2017:361), and also the semi-solid formulation may have improved the delivery of mevastatin into the deeper layers and systemic circulation more than its **(NEF1)** dispersion-counterpart, which is observed with **(NEGM)** found in the ED and in the receptor phase.

It can further be stated that **(NES1)** and **(NER1)** attained higher median concentrations within the ED than their respective **(NEG1)** semi-solid formulation-counterparts. This might be due to the ideal physicochemical properties of simvastatin, according to Naik *et al.* (2000:319), since both **(NES1)** and **(NEGS)** delivered the highest median concentration of the statin into the ED. Furthermore, it is observed that the more hydrophilic rosuvastatin (from the **(NEF1)** dispersion rather than the **(NEG1)** semi-solid formulation) may have a higher affinity for hydrophilic ED (and hydrophilic receptor phase) (Jankowski *et al.*, 2017:495) and therefore improve diffusion into the ED (and systemic circulation). The higher concentrations of simvastatin and rosuvastatin within the ED and receptor phase (both hydrophilic in nature) can possibly be attributed to the salt forms of these APIs that were investigated in this study (rosuvastatin calcium salt and simvastatin ammonium salt), as salt forms will generally exhibit higher solubility, compared to the respective acid or base forms (Serajuddin, 2007:603). Consequently, it can be proposed that salt formation of lovastatin and mevastatin, could alter the transdermal diffusion results, as poor aqueous solubility within physiological buffers of the aforementioned compounds cannot provide the required sink conditions, and consequently can lead to unsuitability of the permeation methodology (Khandavilli & Panchagnula, 2006:471).

It can be concluded that overall (with the exception of lovastatin), higher concentrations of the respective statins were obtained within the ED compared to the SCE. The exception as seen with lovastatin, can however be explained by the higher lipophilicity of this statin compared to the others, subsequently it can be proposed that partition into the ED became the rate-limiting step (Tashiro *et al.*, 2001:278). This may be explained by the fact that each of the eight formulas contained apricot kernel oil, which comprised of fatty acids, hence, acting as a penetration enhancer. These fatty acids cause a disruption within the intercellular lipid matrix of the stratum corneum, consequently an increase in the API diffusion coefficient occurs. When reaching the lipid bilayer, fatty acids molecules form microcavities, resulting in an increased free volume for API diffusion (Choi *et al.*, 2012:88; Van Zyl *et al.*, 2016:192). Additionally, surfactants such as Tween<sup>®</sup> 80 used in the formulation of both nano-emulsions and nano-emulgels could act as penetration enhancers (Van Zyl *et al.*, 2016:192). It was also suggested that for lipophilic compounds, such as the statins, surfactants and fatty acids will primarily affect the lipids within the stratum corneum (Pham *et al.*, 2016:186). The overall higher amount of simvastatin present in the receptor phase and the respective skin layer can be attributed to the more ideal physiochemical properties of this API. In addition, the high amount in the ED can explain the higher amount in the receptor phase compared to the other statins, as Barry (2002:512) stated that larger concentrations result in larger diffusion gradients and consequently better diffusion; consequently diffusion of an API is dependent on the concentration gradient.

The following conclusions could be made with regards to this study:

- When the APIs are compared in terms of flux after a membrane release study, rosuvastatin ((**NER1**) and (**NEGR**)) obtained the highest flux, followed by simvastatin, mevastatin and lastly, lovastatin.
- When formulas are compared in terms of flux after a membrane release study, (**NEF1s**) obtained a higher flux than the respective (**NEG1s**).
- When the APIs are compared in terms of transdermal delivery (diffusion), simvastatin ((**NEGS**)) obtained the highest concentration within the receptor phase, while lovastatin obtained the lowest concentration in general.
- Comparing the formulas ((**NEF1s**) and (**NEG1s**)) in terms of transdermal delivery, it can be stated that in general, the (**NEG1s**) obtained higher concentrations within the receptor phase with the exception of (**NER1**) compared to their (**NEF1**) dispersion-counterparts.
- During tape stripping, simvastatin obtained the highest concentration within the SCE.
- The highest concentration quantified within the ED was attained by simvastatin.

- Within the SCE, the highest concentration was obtained by the nano-emulgels ((**NEGS**) and (**NEGL**)) compared to the respective nano-emulsions, while the nano-emulsions ((**NES1**) and (**NER1**)) attained the highest concentration within the ED compared to the respective nano-emulgels.
- Overall it can be stated that the aim of transdermal delivery was achieved for each of the selected statins within each of the respective formulas ((**NEF1s**) and (**NEG1s**)).

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## APPENDIX E:

# *Cytotoxicity studies performed on the optimised o/w nano-emulsions containing the selected statins*

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### E.1 Introduction

The effect of compounds on the *in vitro* growth, morphological effects and reproduction of tissue cells can be evaluated by means of cytotoxicity tests (Li *et al.*, 2015:617). Traditionally, toxicity assessments were performed relatively late in the discovery of new chemical entities or compounds. This approach caused drug attrition rate to increase, as toxicology assessments had an insignificant impact of drug design and therefore toxicology-related issues were exhibited (Peternel *et al.*, 2009:142). Consequently, both human suffering and the amount of resources and time can be positively influenced when cytotoxicity assessments are performed (Xu *et al.*, 2004:115). The positive impact regarding resources and time, is due to the fact that the stages between developing, initial testing and approval of a new therapeutic agent are lengthy and an expensive process. Therefore, it is essential to eliminate unsuitable compounds by means of preclinical testing before the operating expenses of clinical testing proceeds. As cultured cells can be selected to represent the area or disease of interest, it has become a popular tool in the initial preclinical screening of potential new therapeutic agents (Van Tonder *et al.*, 2015:2).

In addition, Astashkina *et al.* (2012:83) stated that when performing these assessments during the development of novel drug delivery systems, it could be determined whether any of the excipients of the formulation are toxic to humans or human tissue. The significance of the results obtained from these *in vitro* cell cultures can further be stressed, as it provides preliminary data for *in vivo* studies (Wang *et al.*, 2010:e10202). This data however is merely a prediction (Fotakis & Timbrell, 2005:171), due to the significant differences between *in vitro* and *in vivo* conditions (Yoon *et al.*, 2012:634).

The main focus of this study was to investigate the formulated o/w nano-emulsions containing 2% (w/w) of each selected statin, 6% (w/w) Tween<sup>®</sup> 80 and Span<sup>®</sup> 60 and 7.9% (w/w) apricot kernel oil. As these dispersions were intended for transdermal use, it was important to select an appropriate cell line. As the human skin consists of a variety of cell types, the most significant being keratinocytes (López-García *et al.*, 2014:44), HaCaT cells were utilised in this study due to its close similarities to that of normal keratinocytes (Van de Sandt *et al.*, 1999:727). For the purpose of this study, a MTT and NR-assay were performed to assess if the dispersions presented with cytotoxic effects. By utilising more than one assay during *in vitro* cell viability, the reliability of the results obtained will increase (i.e. utilising NR-assay in combination with

other assays is a useful tool to distinguish between organelle damage and cytotoxicity) (Fotakis & Timbrell, 2006:177). The respective semi-solid formulations were not investigated due to the viscous nature of the formulations, therefore resulting in its unsuitability to assess *in vitro*.

## **E.2 Cell culture toxicity studies**

### **E.2.1 The selection of an appropriate cell line**

The selection of a cell-line is commonly influenced by the aims of the specific project or study. However, for the results obtained to be significant, it is essential to select the correct representative biological cell line and assay conditions (Riss, 2005:16). Criteria is therefore utilised in the selection of an appropriate cell line, which includes functional characteristics, continuous or finite, type of cell specie and growth condition (Gibco®, 2016:18). For the purpose of this study, HaCaT cells (epidermal keratinocytes) were selected, as these cells are immortalised cells of a proliferative nature and primarily of human tissue and origin, and suitable for the *in vitro* cytotoxicity study of a transdermal delivery system.

### **E.2.2 Concentrations used for exposure**

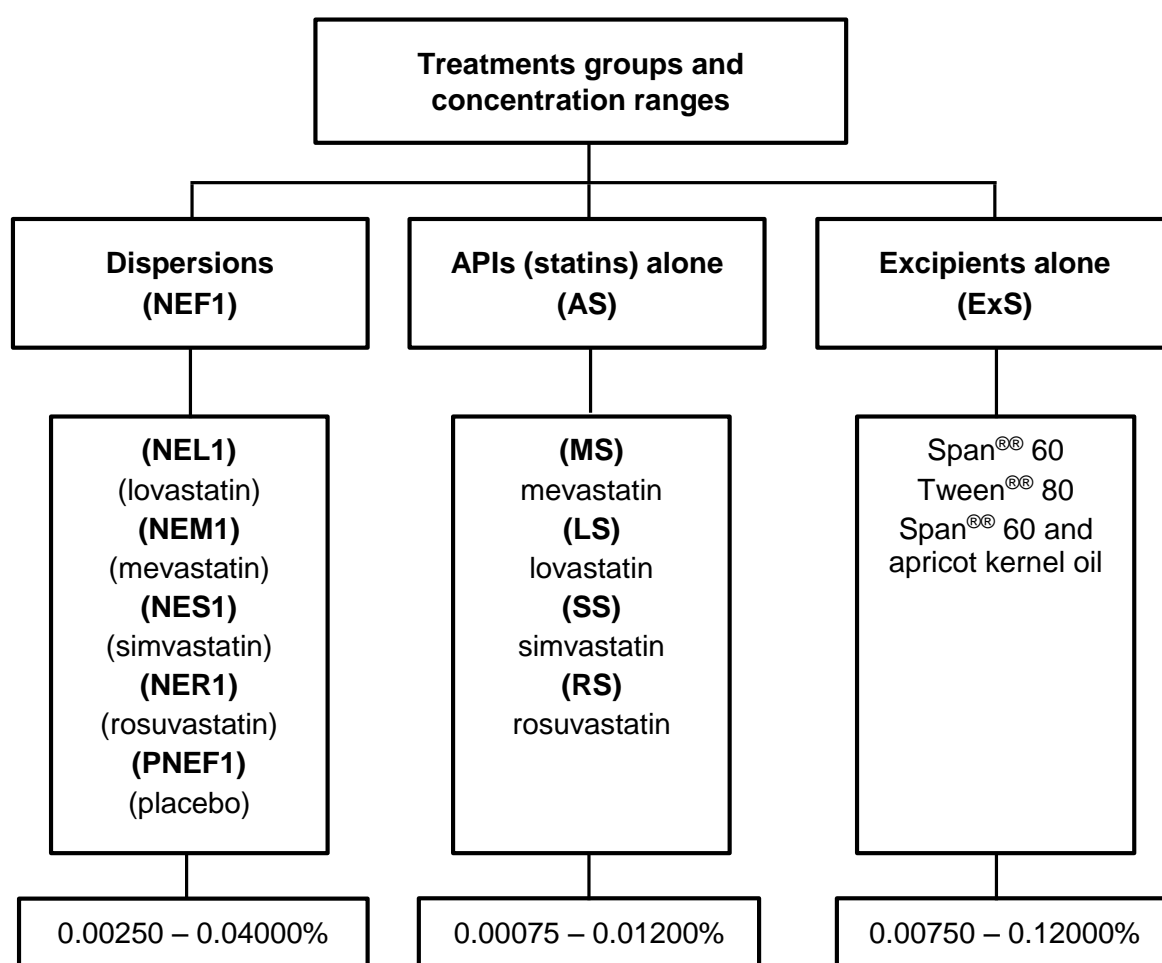
The API concentration used for the purpose of the cytotoxicity studies were less than the concentration API (2% w/w) present in the nano-emulsions. The reason for selecting these concentrations was based on data obtained from the *in vitro* skin diffusion studies (see Section D.3.4.1). The concentration for the nano-emulsions (**NEF1**) (dispersions containing statins) and (**PNEF1**) (placebo dispersion) ranged from 0.00250% (25.0 µg/ml) to 0.04000% (400.0 µg/ml) in a two-fold dilution. The APIs alone were also tested (mevastatin (**MS**), lovastatin (**LS**), rosuvastatin (**RS**) and simvastatin (**SS**)) in the range of 0.00075% (7.5 µg/ml) to 0.01200% (120.0 µg/ml) in two-fold dilution. Lastly, the excipients used to formulate the respective nano-emulsions were tested (Span® 60, Tween® 80, Span® 60:apricot kernel oil) in concentrations that ranged from 0.00750% (75.0 µg/ml) to 0.12000% (1 200.0 µg/ml) in two-fold dilution. Each treatment group consisted of three wells on a 96-well plate. Although no IC<sub>50</sub> value could be obtained for HaCaT cells, the IC<sub>50</sub> value of each of the respective statins were obtained from literature as follows:

- (**LS**) – 160.000 µg/ml on human cervix cancer cells (HeLa cells) (Bhargavi *et al.*, 2016:3800).
- (**MS**) – 11.1 ± 1.5 µM (4.335 ± 0.536 µg/ml) on human ductal carcinoma cells (BT474A) (Glynn *et al.*, 2008:4).
- (**SS**) – 43.000 ± 2.000 mg/ml on breast cancer cells (MCF-7) (Safwat *et al.*, 2017:1122).

- **(RS)** – > 20.000 µg/ml on HEp-2 (human epithelial type 2 carcinoma cells - HeLa contaminant), KB (sub line of the ubiquitous keratin-forming tumour cell line HeLa) and HeLa cells (Campos-Lara & Mendoza-Espinoza, 2011:191).

By taking the aforementioned into account, it can be proposed that treatment concentration when referring to the highest concentration used for the dispersions (400 µg/ml) of this study could be cytotoxic in the case of **(LS)**, **(MS)** and **(RS)**, with the exception of **(SS)**. Although Rinaldi *et al.* (2017:1265) found that cytotoxicity of an excipient could be reduced when utilising a nano-emulsion. Furthermore, a low probability of cytotoxicity can be prosed when testing the statins alone, as the IC<sub>50</sub> value of each respective are above the highest concentration tested.

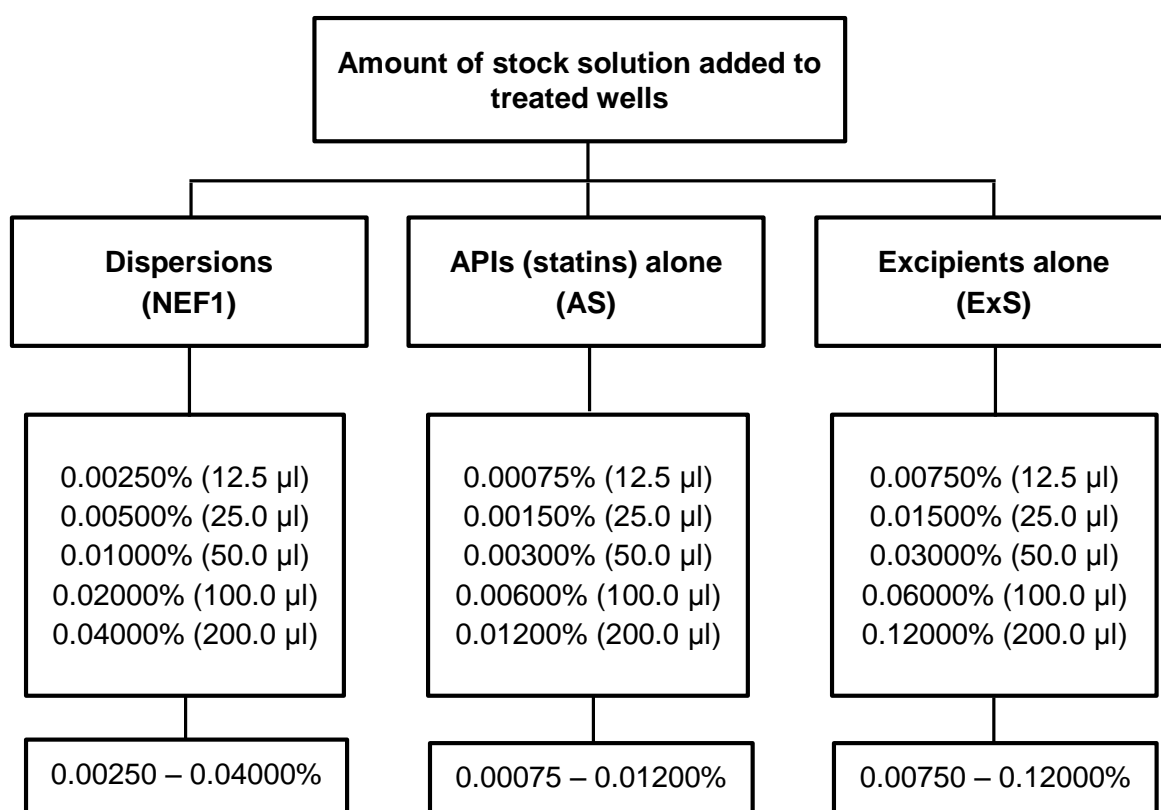
It should also be stated that due to the limited solubility of the respective statins (Petyaev, 2014:406) in aqueous solvents, testing of higher concentrations (e.g. 2% as used in dispersions) were not possible. Although statins pose an increased solubility, for example in dimethyl sulfoxide (DMSO) and ethanol, only small concentrations (DMSO 1% and ethanol 5% on e.g. HeLa cells) of these solvents can be used before compromising cell viability (Timm *et al.*, 2013:892).



**Figure E.1:** Diagrammatic representation of the treatment groups and concentrations utilised

### E.2.2.1 Treatment

The formulation of **(PNEF1)** and **(NEF1)** dispersions was discussed in Section B.5.1.2 (Appendix B). After the formulation, each dispersion was diluted (0.2000 ml nano-emulsions in 9.8000 ml Dulbecco's Modified Eagle Medium (DMEM)) to obtain the stock solution for each respective nano-emulsion. The stock solution of each selected statin **(AS)** was prepared by weighing 400 mg into a 100.0000 ml volumetric flask and made up to volume with methanol, where after 0.3000 ml was taken and diluted with 9.7000 ml DMEM to obtain the stock solution and to ensure the concentration methanol to which the cells are exposed is never greater than 5% (v/v). The stock solution for each of the excipients (Span<sup>®</sup> 60, Tween<sup>®</sup> 80, Span<sup>®</sup> 60:apricot kernel oil and apricot kernel oil) was prepared by firstly preparing an 8% solution of both Span<sup>®</sup> 60 and Tween<sup>®</sup> 80 in methanol. Thereafter each of the samples were diluted by adding 0.1200 ml concentrated stock to 7.8800 ml DMEM. The Span<sup>®</sup> 60:apricot kernel oil stock was prepared by dissolving 0.012 g Span<sup>®</sup> 60 in 17.48  $\mu$ l (0.16%) of apricot kernel oil and 9.9705 ml DMEM.



**Figure E.2:** Diagrammatic representation of the stock solution of each respective treatment group added to wells

The five different concentrations (as seen in Figure E.2) of the nano-emulsions (**(NEL1)**, **(NEM1)**, **(NER1)** and **(NES1)**), API alone (**(AS)**, stock solution) and the excipients alone (**(ExS)**; Span<sup>®</sup> 60, Tween<sup>®</sup> 80 and apricot kernel oil) were utilised to treat the HaCaT cells for 12 h at

37 °C (5% CO<sub>2</sub>, 95% humidity). Treatment was done over a period of 12 h, as *in vitro* skin diffusion studies are performed over the same time period (2.5.5.3). The cells were also treated with a placebo nano-emulsion (**PNEF1**), containing no API. The amount (µl) of the respective (**NEF1**), (**AS**) and (**ExS**) that had to be added to the well on the well plate was calculated in terms of the initial concentration present in the stock sample and the final required concentration needed; the amount added should be ≤ 200 µl after DMEM is added. The wells were treated for 12 h accordingly, whilst a group of cells were left untreated to serve as a control group.

### E.2.3 Non-assay experimental procedures

The experiments for the cytotoxicity study and procedures prior to the study (i.e. cell cultivation, treatment of cell lines, feeding and seeding of cell into 96-well plates) were performed and the reagents used are shown in Table E.1.

#### E.2.3.1 Materials

**Table E.1:** Reagents utilised during the *in vitro* cytotoxicity studies

Reagents	Supplier	Batch number
<b>DMEM with high glucose, 4.0 mM L-glutamine, sodium pyruvate</b>	HyClone™	AC11223315
<b>MEM Non-Essential Amino Acid (MEM NEAA) (100%)</b>	Lonza™	5MB124
<b>Penicillin/Streptomycin (Pen/Strep)</b>	Sigma-Aldrich®	SLBG0033v
<b>L-glutamine</b>	Lonza™	5MB180
<b>Foetal Bovine Serum (FBS)</b>	Gibco™	42Q9352K
<b>Trypan Blue solution (0.4%)</b>	Sigma-Aldrich®	RNBC9030
<b>Phosphate Buffered Saline (1x)</b>	HyClone™	AB212873
<b>MTT (methylthiazol tetrazolium)</b>	Sigma-Aldrich®	MKBX6716V
<b>Trypsin-Versene® (EDTA)</b>	Sigma-Aldrich®	Not available
<b>Dimethyl sulfoxide (DMSO)</b>	Sigma-Aldrich®	SHBH2447V
<b>Neutral Red Solution (NRS; 0.33%)</b>	Sigma-Aldrich®	RNBF9155
<b>Neutral Red Assay Fixative</b>	Sigma-Aldrich®	SLBT9587
<b>Neutral Red Assay Solubilisation Solution</b>	Sigma-Aldrich®	SLBT8171

The reagents displayed in Table E.1 were utilised to perform the cytotoxicity studies within a laminar airflow cabinet to avoid the contamination of the cell cultures and to ensure a sterile environment.

### E.3 *In vitro* toxicity testing

#### E.3.1 Determination of cell viability

The HaCaT cells were cultivated with DMEM or high glucose adequate growth medium, supplemented with 10% FBS, 1% MEMNEAA, 4 mM L-glutamine and 1% of Pen/Strep within a cell culture flask (75 cm<sup>2</sup>). The cell culture flask was incubated at 37°C, 95% humidity and 5% CO<sub>2</sub>.

Before seeding could be initiated, the amount of viable cells within the cell culture flask had to be established. The method as described by Louis and Siegel (2011:8) was utilised for this determination. This step is essential in determining the concentration of cell suspension needed, to enable the seeding of 15 000 cells per well. Cell counting was performed by means of the Trypan Blue exclusion test utilising a haemocytometer (Figure E.3). Firstly, 25 µl of Trypan Blue (0.4%) was mixed with 15 µl of Phosphate Buffered Saline and 10 µl of cell suspension. After proper mixing of the components, 10 µl was extracted and placed on the cover slip of the haemocytometer, expelled and consequently drawn into the chamber. The haemocytometer was then placed under a microscope and counted in terms of the nine large squares on both sides dividing the surface of the haemocytometer. Only the cells within the middle square and the corner squares were counted. The total cells after counting the five squares on both sides (ten squares) of the haemocytometer was then divided by two, which indicated the average viable cells on the two slides and thereafter divided by five to obtain the average per square. A dilution factor ( $5 \times 10^4$ ) multiplied with the final amount was used to determine the amount of cells present per millilitre of the cell suspension ( $C_1$ ). This cell concentration was then multiplied with the total volume of cell suspension to obtain the total number of cells present in the cell suspension. Lastly, the dilution required for seeding was determined by utilising the following equation:

$$C_1V_1 = C_2V_2$$

**Equation E.1**

Where:

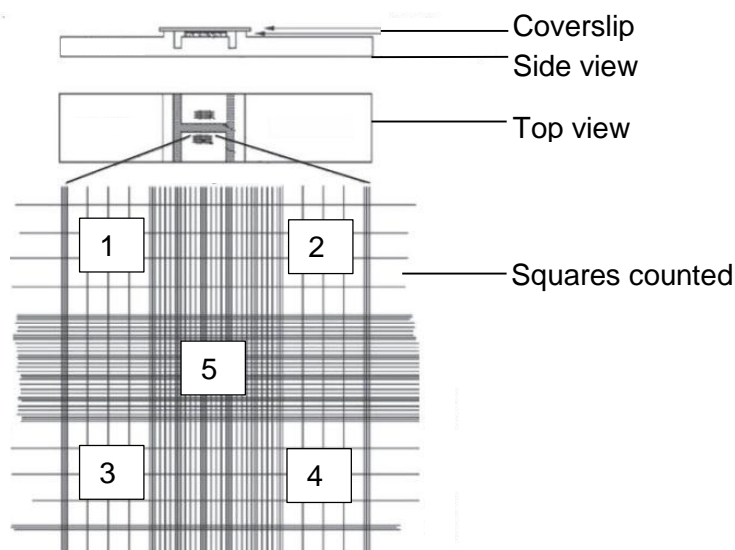
$C_1$  = Amount of cell present per millilitre of the cell suspension or cell concentration (cell/ml)

$C_2$  = Required cell density (15 000 cells per well)

$V_2$  = Total volume of cell suspension needed per well

The HaCaT cells were then utilised to perform *in vitro* cytotoxicity studies to determine whether any variation occurred in the quantity of viable cells. A MTT-assay in conjunction with a NR-assay was performed during the cytotoxicity studies. The cell was seeded at a density of 15 000 cell per well within the 96-well plate, where after the plates were placed in an incubator (37 °C, 5% CO<sub>2</sub>, 95% humidity) for 24 h to ensure the cells attached sufficiently to the surface

of the plates. After the plates were incubated for 24 h, exposure or treatment of cells could be initiated. This procedure was performed before both assays.



**Figure E.3:** Cell counting on one side of a haemocytometer (adapted from BioTek, 2014:1).

### E.3.2 MTT colorimetric assay

The water soluble tetrazolium, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is known to be converted by succinate dehydrogenase enzymes in the mitochondria of metabolically active cells to an insoluble purple formazan. Accumulation of the purple formazan occurs with the healthy cells due to the inability of formazan to permeate the cell membranes (Fotakis & Timbrell, 2006:172). Hence, cell viability measurements by means of a MTT-assay are determined in terms of the ability of metabolically active cells to transform yellow tetrazolium to purple formazan crystals. Thereafter, it can be solubilised with DMSO and quantified by means of spectrophotometry (Perez *et al.*, 2017:43).

The method as described by Mosman (1983:56) was utilised for the MTT-assay with minor modifications. For this assay, the amount of MTT solution needed had to be calculated and prepared on the day of the assay. Each inner well that was exposed or treated, along with the control group, received 100  $\mu$ l. As three plates were used with 72 wells in each, the amount of MTT solution needed could be calculated as seen in Table E.2.

**Table E.2:** Calculating the amount of MTT solution needed for the intended plates

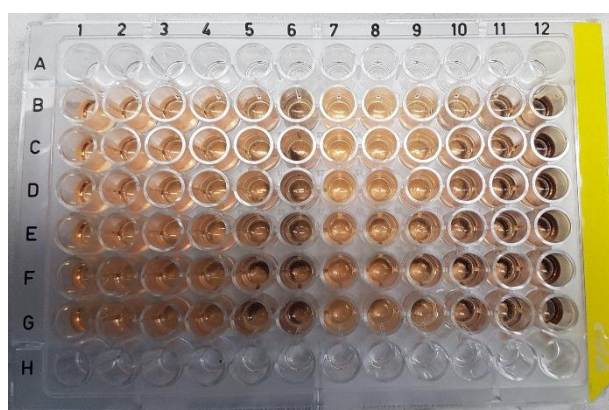
Number of plates	Wells used in plates	Volume needed for plates (ml)	Extra volume (ml)	Total volume MTT solution needed (ml)
3	216	21.6	3.4	25.0

The calculated volume assay media was consequently pipetted into a 50 ml centrifuge tube. The amount of MTT that had to be weighed and added to the assay media could then be calculated, as the MTT solution should have a concentration of 0.5 mg/ml.

$$\text{MTT (mg)} = \text{Total volume (ml)} \times 0.5 \text{ mg/ml} \quad \text{Equation E.2}$$

The total amount of MTT weighed was 12.5 mg. After adding the MTT to the assay media, the tube was covered (due to light sensitivity) and placed in the water bath (37°C) until utilised.

When the assay was started, the 96-well plates were removed from the incubator, 12 h after treatment. The plates were then inspected under a microscope to evaluate the viability of the cells after treatment. Media from the background wells was aspirated and the cells in these respective wells were killed with 200 µl of methanol (100%) and thereafter aspirated. The remaining cells were aspirated and washed with 150 µl of Phosphate Buffered Saline, followed by additional aspiration before MTT solution could be added. The MTT solution (100 µl per well) was then added to the treated wells and control wells, where after the plates were placed back in the incubator (37°C, 5% CO<sub>2</sub>, 95% humidity) and left for 2 h. After this time, the plates were inspected under a microscope for formazan crystals.



**Figure E.4:** Example of a 96-well plate, 2 h after adding the MTT solution and prior to aspiration and addition of DMSO

The yellow MTT solution (Figure E.4) was aspirated and gently tapped upside down on filter paper to ensure proper removal of the MTT solution. Lastly, the DMSO (200 µl) was added to the wells (including the control group) and placed on a shaker plate to guarantee adequate dissolution ( $\pm$  15 min). The plates were placed individually in a SpectraMax® Paradigm® Multi-Mode Microplate reader (Molecular Devices, California, USA) to measure absorbance at 560 nm and 630 nm respectively, hence, raw data was obtained.



**Figure E.5:** SpectraMax® Paradigm® Multi-Mode Microplate reader (Molecular Devices, California, USA) to measure absorbance

Equation E.3 was utilised and modified to determine the %viable cells (Karakas *et al.*, 2017:920) from the raw data.

$$\% \text{viable cells} = 100 \times (\text{sample abs}) / (\text{control abs}) \quad \text{Equation E.3}$$

Equation E.3 was modified to include the absorbance at 560 nm and 630 nm as well as the blank and negative control groups as follows:

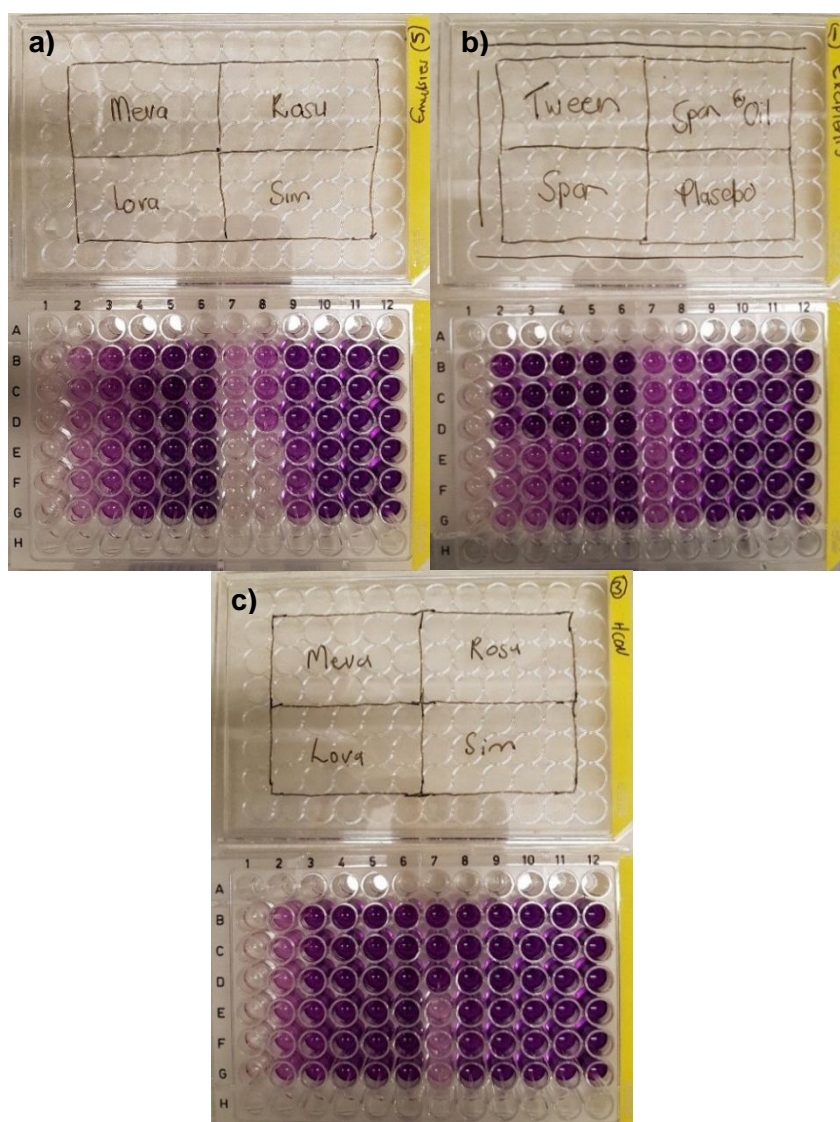
$$\% \text{viable cells} = \frac{((\text{absorbance } 560 \text{ nm} - 630 \text{ nm}) - \text{blank absorbance})}{(\text{Negative control absorbance} - \text{blank absorbance})} \times 100 \quad \text{Equation E.4}$$

### E.3.2.1 MTT colorimetric assay results and discussion

The cytotoxicity of a treatment (or exposure compound) can be determined in terms of the %cell viability of HaCaT cells after treatment with the **(NEF1)** dispersions (nano-emulsions) determined with the MTT method of cell viability, although they are generally cell line and assay specific (López-García *et al.*, 2014:44).

**Table E.3:** %Cell viability used to classify treatment cytotoxicity in this study

%Cell viability	Cytotoxicity
< 40%	Strong cytotoxic
40 – 60%	Moderate cytotoxic
60 – 80%	Weak cytotoxic
> 80%	Non-cytotoxic



**Figure E.6:** The three 96-well plates after the addition of DMSO: a) **(NEF1)** dispersions, b) excipients used in dispersions **(ExS)** and **(PNEF1)** and c) APIs alone **(AS)** (note that each sample was added from highest to lowest concentration)

Figure E.6.a displays the 96-well plate after exposure with **(NEM1)** **((MS))**, **(NEL1)** **((LS))**, **(NER1)** **((RS))** and **(NES1)** **((SS))**. When visually examining the plates, it can be stated that the intensity of the purple colour increased in the wells where the exposure concentration was lower. This correlates with what is suggested in literature, that in theory, it is indicative of cells that are more viable (Wang *et al.*, 2010:9). It can be proposed that an increased absorbance will be measured in the wells with more intensive purple colour, due to increased cell number, and consequently a higher amount of MTT formazan is formed (Baluchamy *et al.*, 2010:24770).

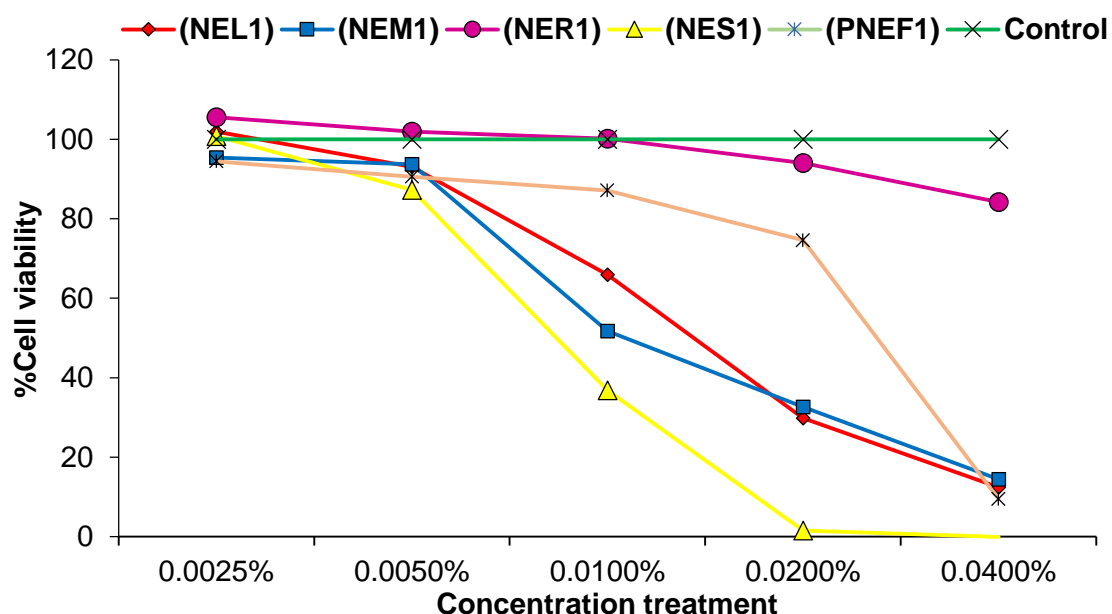
### E.3.2.2 MTT assay results on HaCaT cells

The %cell viability was assessed accordingly after 12 h exposure to the respective samples **((NEF1)**, **(AS)** and **(ExS))**. Tables E.4 – E.6, along with Figures E.7 – E.9, display the results obtained after exposure with the **(NEF1)** dispersions of the selected statins, **(AS)** of each statin

and the exposure with the excipients used in the formulation of the respective dispersions (**ExS**) with the five respective concentration ranges. Control wells were left untreated to serve as the control group.

**Table E.4:** The %cell viability of HaCaT cells after treatment with the (**NEF1**) dispersions (nano-emulsions) determined with the MTT method

%Cell viability	Exposure concentration	(NEL1)	(NEM1)	(NER1)	(NES1)	(PNEF1)	Control
	0.0025% (0.025 mg/ml)	101.887 (± 5.423)	95.398 (± 4.719)	105.532 (± 5.615)	100.784 (± 2.373)	94.467 (± 3.674)	100.000
0.0050% (0.050 mg/ml)	93.023 (±2.479)	93.716 (± 6.061)	101.956 (± 4.787)	87.2587 (± 0.789)	90.589 (± 5.743)		
0.0100% (0.100 mg/ml)	65.936 (± 11.165)	51.704 (± 18.305)	100.162 (± 0.256)	36.8322 (± 4.581)	87.105 (± 4.362)		
0.0200% (0.200 mg/ml)	29.792 (± 2.015)	32.612 (± 4.841)	94.025 (± 1.217)	1.552 (± 1.664)	74.607 (± 6.127)		
0.0400% (0.400 mg/ml)	12.453 (± 1.533)	14.429 (± 2.000)	84.199 (± 1.401)	-0.044 (± 0.214)	9.455 (± 1.633)		



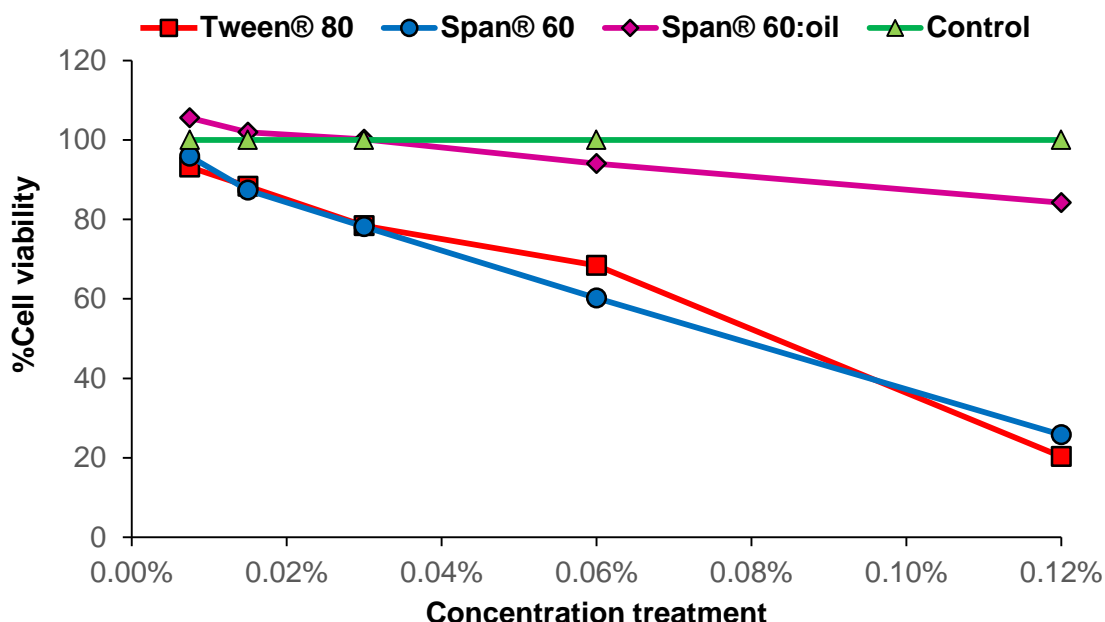
**Figure E.7:** The %cell viability after treatment with the five respective concentrations of (**NEL1**), (**NEM1**), (**NER1**), (**NES1**) and (**PNEF1**), determined with MTT

Table E.4 and Figure E.7 represent the %viable cells after exposure for 12 h (as determined by Equation E.4) with the respective (**NEF1**) dispersions and (**PNEF1**) at five different concentrations. From the data, it is clear that concentration depended on the decrease of %viable cell that occurred, although it was not particularly substantial for (**NER1**). When comparing the data to the guidelines suggested by López-García *et al.* (2014:44), (**NEL1**) and

(NEM1) became strong cytotoxic (< 40%) from 0.02% upward, whereas (NES1) from 0.01% upwards. When investigating the (PNEF1) dispersion, strong cytotoxicity is present from 0.04%, which could possibly be due to numerous interferences that can be experienced with MTT (Van Tonder *et al.*, 2015:4). It can be concluded that no noticeable difference in the %cell viability occurred for any of the dispersions when treating with concentrations 0.0025 – 0.0050%. Non-cytotoxicity (> 80%) was displayed for the entire concentration range (0.0025 – 0.0400%) with (NER1), resulting in the least cytotoxic dispersion.

**Table E.5:** The %cell viability of HaCaT cells after treatment with the excipients (ExS) used in the dispersions (nano-emulsions) determined with MTT

%Cell viability	Exposure concentration	Tween® 80	Span® 60	Span® 60:oil	Control
	0.0075% (0.075 mg/ml)	93.157 (± 4.768)	95.886 (± 4.886)	105.533 (± 1.019)	100.000
0.0150% (0.150 mg/ml)	88.290 (± 2.002)	87.338 (± 0.825)	101.956 (± 2.305)	100.000	
0.0300% (0.300 mg/ml)	78.378 (± 15.107)	78.184 (± 1.129)	100.160 (± 2.382)	100.000	
0.0600% (0.600 mg/ml)	68.431 (± 3.143)	60.184 (± 1.802)	94.025 (± 3.286)	100.000	
0.1200% (1.200 mg/ml)	20.283 (± 1.514)	25.792 (± 2.017)	84.199 (± 3.392)	100.000	

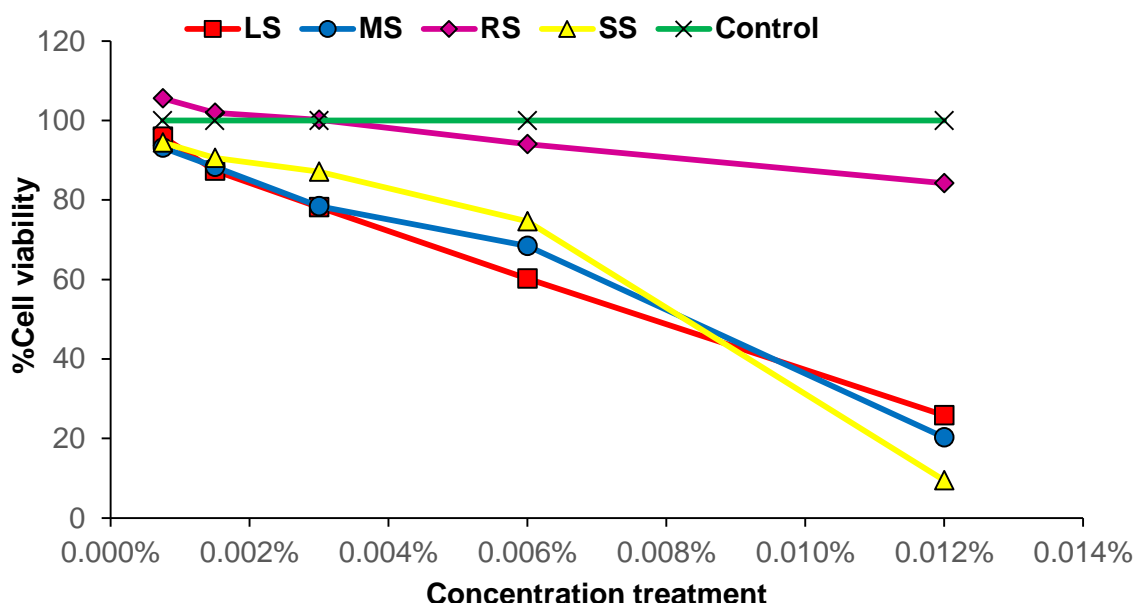


**Figure E.8:** The %cell viability after treatment with the five respective concentrations of excipients (ExS) determined with MTT

Table E.5 and Figure E.8 gives a representation of the %MTT cell viability obtained from testing the excipients used in the dispersions alone and in combination (Span® 60:apricot kernel oil). From the result it can be concluded that Tween® 80 and Span® 60 presented to be non-cytotoxic between 0.0075 and 0.0150%, weak cytotoxic between 0.0300 and 0.0600% and strong cytotoxic at 0.1200%. The Span® 60:apricot kernel oil sample presented as non-cytotoxic for the entire concentration range, which can possibly be attributed to this system interacting in a different way with the cell membranes (Warisnoicharoen *et al.*, 2003:866).

**Table E.6:** The %cell viability of HaCaT cells after treatment with the selected statins ((LS), (MS), (RS) and (SS)) alone (AS) determined with MTT

%Cell viability	Exposure concentration	(LS)	(MS)	(RS)	(SS)	Control
	0.00075% (0.0075 mg/ml)	95.8863 (± 4.886)	93.157 (± 4.768)	105.533 (± 1.019)	94.467 (± 3.674)	
0.00150% (0.0150 mg/ml)	87.3378 (± 0.825)	88.290 (± 2.002)	101.95 (± 2.305)	90.589 (± 5.743)		
0.00300% (0.0300 mg/ml)	78.1842 (± 1.129)	78.378 (± 15.107)	100.163 (± 2.382)	87.105 (± 4.362)		
0.00600% (0.0600 mg/ml)	60.1842 (± 1.802)	68.431 (± 3.143)	94.025 (± 3.286)	74.607 (± 6.127)		
0.01200% (0.1200 mg/ml)	25.7919 (± 2.017)	20.283 (± 1.514)	84.199 (± 3.392)	9.455 (± 1.633)		



**Figure E.9:** The %cell viability after treatment with the five respective concentrations of the APIs alone (AS) determined with MTT

Table E.6 and Figure E.9 indicate the %MTT cell viability obtained when exposing the HaCaT cells to the selected statins alone **(AS)**. From this data, it can be concluded that **(RS)** proved to be the least cytotoxic of all the respective APIs, as this yielded a percentage of > 80% for the entire concentration range, as seen with **(NER1)**. **(LS)** and **(MS)**, presented as non-cytotoxic between 0.00075 and 0.00150%, weak cytotoxic between 0.00300 and 0.00600% and strongly cytotoxic at 0.01200%. **(SS)** exhibited no cytotoxicity between 0.00075 and 0.00300%, weak cytotoxicity at 0.00600% and a large decrease in the %viable cell at 0.01200% was observed, which is indicative of strong cytotoxicity.

The result obtained from MTT-assay was further utilised to determine the IC<sub>50</sub> values for the respective samples that were tested. The IC<sub>50</sub> is used to describe the concentration of a drug or substance that leads to a 50% inhibition in cells (Koba *et al.*, 2009:31). Tables E.7 – E.9 represent the IC<sub>50</sub> values obtained after 12 h exposure to the **(NEF1)**, **(ExS)** and **(AS)** samples. The IC<sub>50</sub> values were determined by means of regression analysis (Shaikh *et al.*, 2012:141). Firstly, the %inhibited cells at each concentration were calculated by subtracting the %viable cells at the specific concentration for 100%. Regression analysis was then performed to determine the %inhibited cells (Y-value) and the concentration range (X-value) to obtain the slope and intercept, which was utilised to determine the IC<sub>50</sub> values.

**Table E.7:** IC<sub>50</sub> values obtained from MTT-assay of dispersions

<b>(NEF1) dispersions</b>	<b>IC<sub>50</sub> (mg/ml)</b>
<b>(NEL1)</b>	0.1994
<b>(NEM1)</b>	0.1901
<b>(NER1)</b>	0.1965
<b>(NES1)</b>	0.1369
<b>(PNEF1)</b>	0.7485

The difference in the concentration required to inhibit half of the test cells when observing **(NEL1)**, **(NEM1)** and **(NER1)** is slightly higher when compared to **(NES1)** (0.1369 mg/ml). It can be stated that **(NEL1)** required the highest concentration (0.1994 mg/ml) of the active containing dispersions in order to inhibit half of the test cells. **(PNEF1)** proved to be the least cytotoxic dispersion, which could be expected, as this dispersion was a placebo.

**Table E.8:** IC<sub>50</sub> values obtained from MTT-assay of excipients alone **(ExS)**

<b>Excipients</b>	<b>IC<sub>50</sub> (mg/ml)</b>
<b>Tween® 80</b>	0.7766
<b>Span® 60</b>	0.7864
<b>Span® 60:apricot kernel oil</b>	3.0591

Span® 60 required a slightly higher concentration to inhibit half of the test cells (0.7864 mg/ml) when compared to Tween® 80 (0.7766 mg/ml); Span® 60 can therefore be considered less cytotoxic, although the difference between the IC<sub>50</sub> obtained for Span® 60 and Tween® 80 is minor. The Span® 60:apricot kernel oil sample (3.0591 mg/ml) was established as the least cytotoxic sample, with a significant difference in the concentration required to inhibit half of the test cells when compared to Span® 60 and Tween® 80.

**Table E.9:** IC<sub>50</sub> values obtained from MTT-assay of the APIs alone **(AS)**

APIs alone	IC <sub>50</sub> (mg/ml)
<b>(LS)</b>	0.0786
<b>(MS)</b>	0.0777
<b>(RS)</b>	0.3059
<b>(SS)</b>	0.0748

**(RS)**, as seen in Table E.9, required the highest concentration (0.3059 mg/ml) in order to inhibit half of the test cells, although the difference in concentration of **(LS)**, **(MS)** and **(SS)** were minor; **(SS)** indicated the highest cytotoxicity.

The MTT cytotoxicity data, as discussed before, only provides relative identification of cytotoxicity. As the MTT-assay has been associated with certain limitations (Śliwka *et al.*, 2016:2) (e.g. certain APIs can cause a direct reduction in tetrazolium salts (Stockert *et al.*, 2012:793)), utilising a single assay can lead to incorrect interpretation of results. Therefore, an NR-assay was also conducted to support the results obtained from the MTT-assay.

### **E.3.3 Neutral red colorimetric assay**

The NR-assay, as described by the manufacturer (Sigma, 2018), was utilised. The NR-assay is frequently used and has a variety of applications, and functions on the ability of viable cells to accumulate and concentrate the dye within the lysosomes by means of non-ionic passive diffusion (Perez *et al.*, 2017:43; Repetto *et al.*, 2008:1125). As the cells uptake of NR depends on the cells viability and ability of maintain pH gradient through the production of ATP, pH gradient changes or cell death will cause an inability to retain the dye. In addition to viable cells, this assay also creates the possibility to distinguish between dead or damaged cells, as viable cells can undergo alteration to the cell membrane, which will influence the capacity for NR uptake (Repetto *et al.*, 2008:1125).

The NRS was prepared the day before the assay took place and left to incubate overnight (Repetto *et al.*, 2008:1129). The centrifuge tube containing the NRS was stored upright in the incubator. The NR dye is known to precipitate when stored, consequently, in preparing one day

before the assay, precipitation would have taken place, and the solution could be filtered (with a syringe filter) to ensure no crystals reached the cell cultures, as these crystals would interfere with the assay (Sigma, 2018). Before preparing the NRS, it had to be established what quantity of the solution would be needed to perform the assay on the respective plates (three). As 100 µl of the NRS will be added to each respective well, the quantity of solution needed for the assay can be determined as presented in Table E.10.

**Table E.10:** Calculating the amount of NR solution needed for the intended plates

Number of plates	Wells used in plates	Volume needed for plates (ml)	Extra volume (ml)	Total volume (NRS) needed (ml)
3	216	21.6	3.4	25.0

It is proposed by Sigma (2018) that the NRS (0.33%) should be added in an amount that is equivalent to 10% of the culture medium volume needed. Therefore, the amount of NRS required to obtain the final 25 ml assay solution to be used on the three plates could be determined as follows:

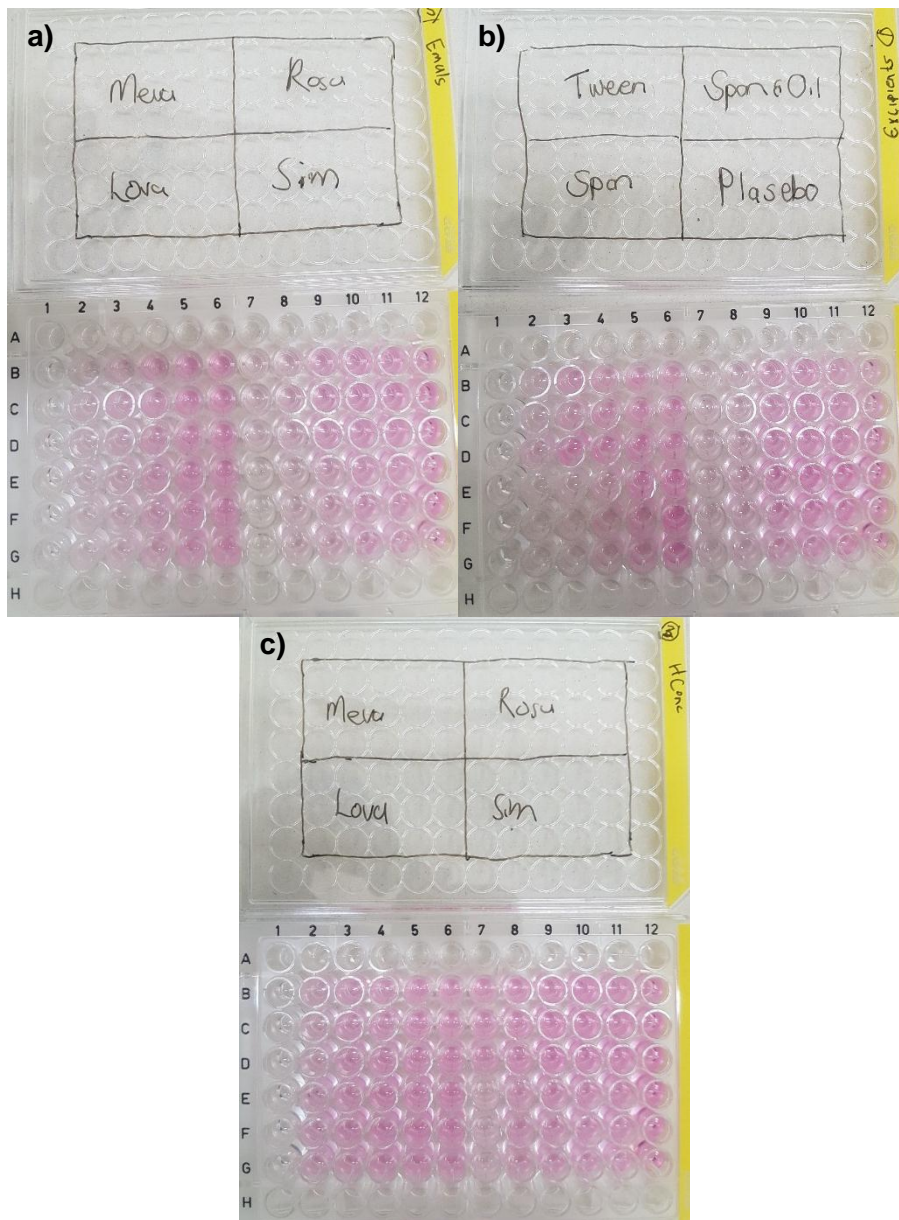
$$\text{Amount NR solution} = \text{Total amount} \times 10\% \quad \text{Equation E.5}$$

As a result, 2.5 ml of NRS will be added to 22.5 ml of DMEM.

The assay begun by removing the three respective plates from the incubator after 12 h of exposure or treatment and examining the plates to evaluate cell viability. The media from the background wells were aspirated and killed with 200 µl of 100% methanol. The exposed or remaining wells were also aspirated and washed with 150 µl Phosphate Buffered Saline and aspirated for a second time before adding 100 µl of the NRS. The plates were placed back into the incubator (37 °C, 5% CO<sub>2</sub>, 95% humidity) for 2 h. After this 2 h incubation period, the NRS was removed from the plates and the cells were washed immediately with the Neutral Red Assay Fixative. The Neutral Red Assay Fixative was aspirated and the Neutral Red Assay Solubilisation Solution was used to solubilise the incorporate dye. The plates were placed on a mechanical shaker plate for ± 15 min to ensure proper mixing, thereafter they were individually placed in a SpectraMax® Paradigm® Multi-Mode Microplate reader (Molecular Devices, California, USA) to measure absorbance spectrophotometrically at 560 nm and 630 nm respectively. The %cell viability was determined by means of Equation E.4.

### **E.3.3.1 Neutral red colorimetric assay results and discussion**

The guidelines as specified by López-García *et al.* (2014:44) will be used for the analysis of data obtained from the NR-assay, as discussed in Section E.3.2.1.



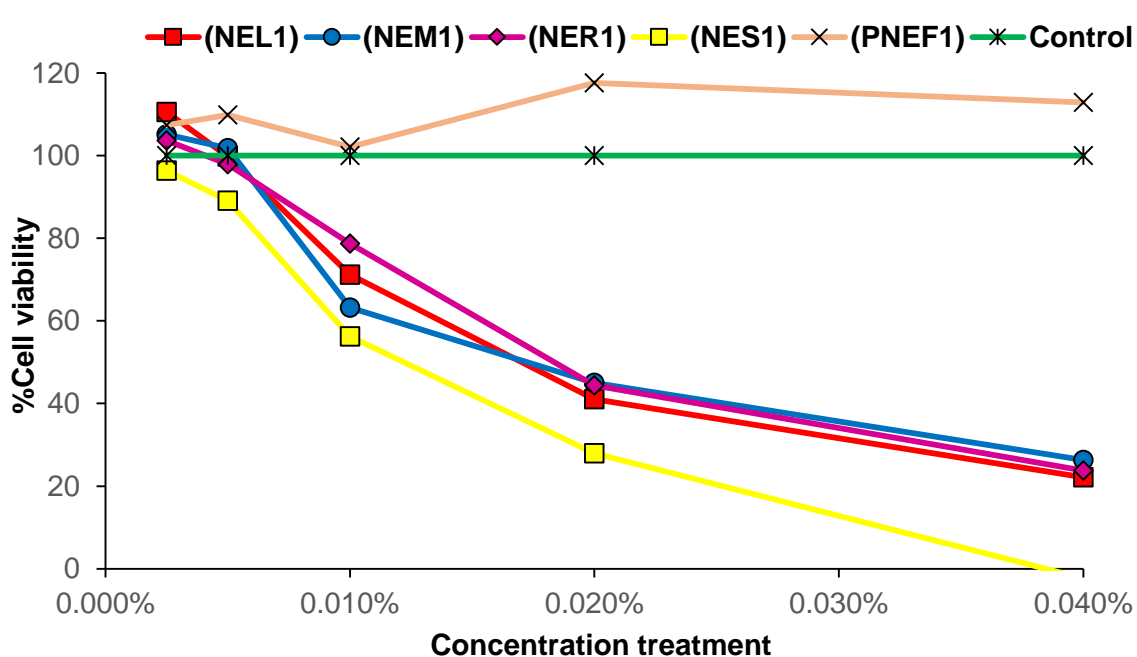
**Figure E.10:** The three 96-well plates after the addition of Neutral Red Assay Solubilisation Solution: a) **(NEF1)** dispersions, b) excipients used in dispersions **(ExS)** and **(PNEF1)**, and c) APIs alone **(AS)**

The same correlation as drawn with the colour of MTT plates can be made when conducting a NR-assay, as the intensity of the dye is directly proportional to the amount of living cells (Roesler *et al.*, 2010:817).

### E.3.3.2. Neutral red-assay results on HaCaT cells

**Table E.11:** The %cell viability of HaCaT cells after treatment with the **(NEF1)** dispersions (nano-emulsions) determined with NR

%Cell viability	Exposure concentration	(NEL1)	(NEM1)	(NER1)	(NES1)	(PNEF1)	Control
	0.0025% (0.025 mg/ml)	110.583 (± 4.058)	105.172 (± 2.201)	103.652 (± 4.769)	96.348 (± 3.407)	107.417 (± 11.217)	100.000
	0.0050% (0.050 mg/ml)	99.802 (± 5.198)	101.779 (± 5.045)	97.908 (± 4.943)	89.105 (± 2.089)	109.836 (± 8.570)	
	0.0100% (0.100 mg/ml)	71.228 (± 2.510)	63.236 (± 12.574)	78.699 (± 6.469)	56.223 (± 3.785)	102.152 (± 8.207)	
	0.0200% (0.200 mg/ml)	41.009 (± 5.079)	45.026 (± 7.264)	44.360 (± 2.938)	27.919 (± 0.216)	117.597 (± 17.395)	
	0.0400% (0.400 mg/ml)	22.112 (± 3.495)	26.296 (± 6.654)	23.757 (± 2.675)	-2.321 (± 2.829)	112.878 (± 9.683)	



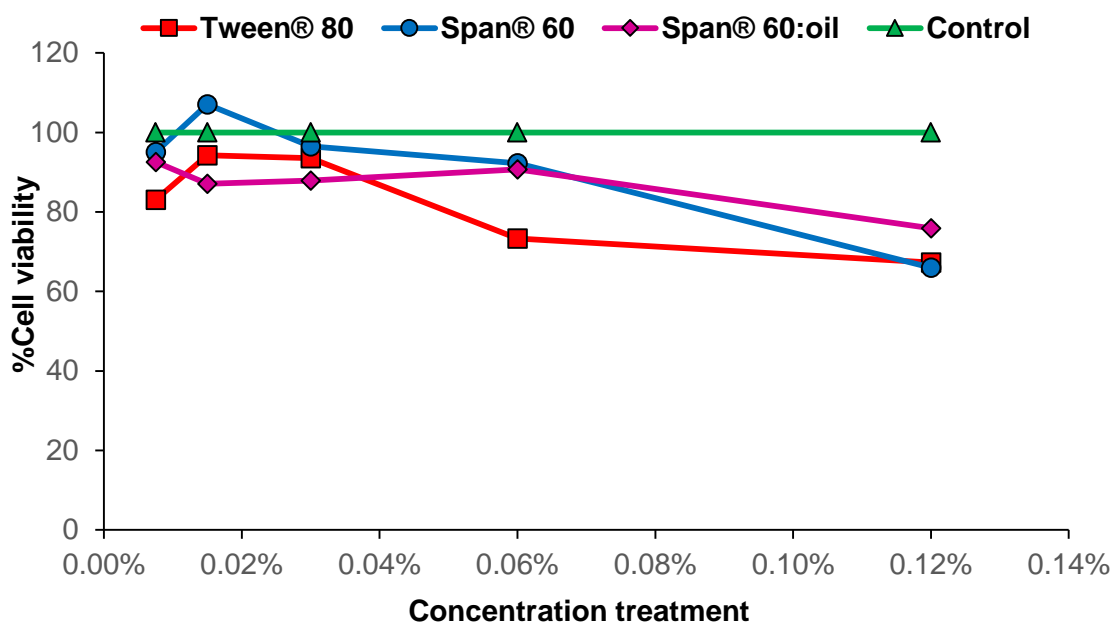
**Figure E.11:** The %cell viability after treatment with the five respective concentrations of **(NEL1)**, **(NEM1)**, **(NER1)**, **(NES1)** and **(PNEF1)** determined with NR

Table E.11 and Figure E.11 displays the NR cytotoxicity data obtained for the respective **(NEF1)** dispersions and the placebo dispersion **(PNEF1)**. From the data it can be observed that **(NEL1)**, **(NEM1)**, **(NER1)** and **(NES1)** exhibited no cytotoxicity between the concentration range 0.0025 and 0.0050%. For the concentration 0.0100%, **(NEL1)**, **(NEM1)** and **(NER1)** presented with weak cytotoxicity, whereas **(NES1)** exhibited moderate cytotoxicity. %NR cell viability decreased further at concentration of 0.0200%, where **(NEL1)**, **(NEM1)** and **(NER1)** displayed

moderate cytotoxicity and strong cytotoxicity at 0.0400%. It can be proposed that the difference in cytotoxicity between MTT and NR-assay of **(NER1)** is due to overestimation of cell viability by MTT (Stepanenko & Dmitrenko, 2015:193). **(NES1)** presented strong cytotoxicity from 0.0200% upward, **(PNEF1)** however, showed no cytotoxicity at any concentration.

**Table E.12:** The %cell viability of HaCaT cells after treatment with the excipients **(ExS)** used in the dispersions (nano-emulsions) determined with NR

%Cell viability	Exposure concentration	Tween® 80	Span® 60	Span® 60:oil	Control
	0.0075% (0.075 mg/ml)	83.039 (± 15.532)	95.105 (± 14.560)	92.583 (± 21.548)	100.000
0.0150% (0.150 mg/ml)	94.221 (± 4.700)	107.066 (± 5.717)	87.070 (± 22.694)		
0.0300% (0.30 mg/ml)	93.532 (± 14.218)	96.483 (± 7.873)	87.915 (± 12.652)		
0.0600% (0.600 mg/ml)	73.354 (± 16.709)	92.284 (± 12.686)	90.737 (± 10.734)		
0.1200% (1.200 mg/ml)	67.269 (± 15.267)	65.995 (± 5.557)	75.915 (± 6.109)		



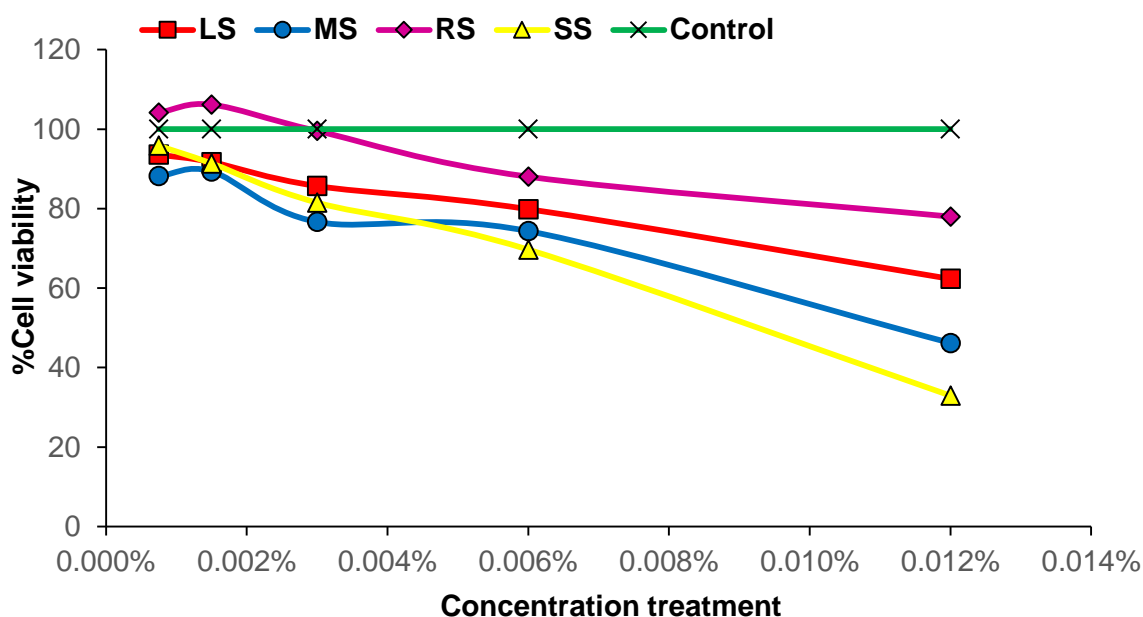
**Figure E.12:** The %cell viability after treatment with the five respective concentrations of excipients **(ExS)** determined with NR

The %NR cell viability performed on the excipients used in the formulation of the dispersions is represented by Table E.12 and Figure E.12. From this data it can be stated that Span® 60 and Span® 60:apricot kernel oil displayed no cytotoxicity between 0.0075 and 0.0600% and weak

cytotoxicity at the highest concentration of 0.1200%. Tween® 80 was non-cytotoxic between 0.0075 and 0.0030%, and weak cytotoxic at concentrations 0.0600 - 0.1200%.

**Table E.13:** The %cell viability of HaCaT cells after treatment with the selected statins ((**LS**), (**MS**), (**RS**) and (**SS**)) alone (**AS**) determined with NR

%Cell viability	Exposure concentration	(LS)	(MS)	(RS)	(SS)	Control
	0.00075% (0.0075 mg/ml)	93.573 (± 5.935)	88.195 (± 3.258)	104.184 (± 12.328)	95.816 (± 9.211)	100.000
0.00150% (0.0150 mg/ml)	91.696 (± 6.387)	89.362 (± 3.490)	106.153 (± 6.761)	91.367 (± 3.414)		
0.00300% (0.0300 mg/ml)	85.679 (± 10.277)	76.655 (± 5.204)	99.535 (± 8.821)	81.504 (± 4.988)		
0.00600% (0.0600 mg/ml)	79.863 (± 4.769)	74.339 (± 3.231)	88.031 (± 6.901)	69.690 (± 5.352)		
0.01200% (0.1200 mg/ml)	62.306 (± 3.475)	46.189 (± 4.714)	77.985 (± 4.721)	32.935 (± 1.086)		



**Figure E.13:** The %cell viability after treatment with the five respective concentrations of the APIs alone (**AS**) determined with NR

Table E.13 and Figure E.13 represent the %NR cell viability as obtained from the assay performed on the APIs alone (**AS**). (**LS**) and (**SS**) were found to be non-cytotoxic at concentrations 0.00075 - 0.00300% and (**MS**) between 0.00075 and 0.00150%. (**LS**), (**MS**) and (**SS**) showed weak cytotoxicity at 0.00600%. At a concentration of 0.01200%, the result differed significantly between the respective statins, as (**LS**) presented weak cytotoxicity, (**MS**) moderate

cytotoxicity and **(SS)** strong cytotoxicity. Conversely, **(RS)** exhibited no cytotoxicity between 0.00075 and 0.00600%, with only weak cytotoxicity at 0.01200%. Hence, **(RS)** can be concluded as the least cytotoxic API when compared to **(LS)**, **(MS)** and **(SS)**.

The result obtained from NR-assay was further utilised to determine the IC<sub>50</sub> values for the respective samples tested. As stated in Section E.3.2.2, the IC<sub>50</sub> is used to describe the concentration of a drug or substance that leads to a 50% inhibition in cells (Koba *et al.*, 2009:31). Tables E.14 – E.16 represent the IC<sub>50</sub> values obtained after 12 h exposure to the **(NEF1)**, **(ExS)** and **(AS)** samples. The IC<sub>50</sub> values were determined using the method described in Section E.3.2.2.

**Table E.14:** IC<sub>50</sub> values obtained from NR-assay of dispersions

<b>(NEF1) dispersions</b>	<b>IC<sub>50</sub> (mg/ml)</b>
<b>(NEL1)</b>	0.1655
<b>(NEM1)</b>	0.1134
<b>(NER1)</b>	0.2241
<b>(NES1)</b>	0.0907
<b>(PNEF1)</b>	-8.6707

From Table E.14 it can be concluded that **(NER1)** was the least cytotoxic active containing dispersion, as 0.2241 mg/ml was required to inhibit half of the test cells compared to **(NES1)**, where 0.0907 mg/ml inhibited half of the test cells. This correlates with the IC<sub>50</sub> results obtained from the MTT-assay, that **(NES1)** is the most cytotoxic nano-emulsion.

**Table E.15:** IC<sub>50</sub> values obtained from NR-assay of excipients alone **(ExS)**

<b>Excipients</b>	<b>IC<sub>50</sub> (mg/ml)</b>
<b>Tween® 80</b>	1.9536
<b>Span® 60</b>	1.8091
<b>Span® 60:apricot kernel oil</b>	3.5508

From Table E.15, it can be concluded that Tween® 80 was the least cytotoxic, as a higher concentration (1.9536 mg/ml) was needed to inhibit half of the test cells compared to Span® 60 (1.8091 mg/ml). The IC<sub>50</sub> results obtained from the MTT-assay differ from the results displayed in Table E.15, although, it can be stated that the difference in cytotoxicity between Tween® 80 and Span® 60 in both assays are minor. Lastly, it can be stated that Span® 60:apricot kernel oil is the least cytotoxic **(ExS)** sample tested, when compared to Tween® 80 and Span® 60, which corresponds with the IC<sub>50</sub> values determined during the MTT-assay.

**Table E.16:** IC<sub>50</sub> values obtained from NR-assay of the APIs alone (**AS**)

APIs alone	IC <sub>50</sub> (mg/ml)
<b>(LS)</b>	0.2369
<b>(MS)</b>	0.2429
<b>(RS)</b>	0.2453
<b>(SS)</b>	0.1682

In Table E.16, an insignificant difference can be observed between **(LS)**, **(MS)** and **(RS)** compared to **(SS)** which proved to be the most cytotoxic, although **(RS)** was the least cytotoxic API. This IC<sub>50</sub> results obtained from the NR-assay correlates with that of the MTT-assay, as both assays concluded that **(SS)** was the most cytotoxic API.

#### **E.4 Conclusion**

The MTT-assay, as well as the NR-assay, was performed to determine whether the **(NEF1)** dispersions, APIs alone (**AS**) or excipients used in the formulation of the dispersions (**ExS**) posed cytotoxic effects against HaCaT cells (epidermal keratinocytes). Although an MTT-assay is a comfortable and simple colorimetric method and the mitochondria is suggested to be the main site of MTT reduction, numerous other reductases (e.g. intercellular reductases) have been overlooked by MTT reduction. In addition, as literature suggests that intracellularly reduced nicotinamide adenine dinucleotide (NADH) is considered the major electron donor in MTT reduction, enhanced concentrations of NADH may lead to inaccurate results (Jo *et al.*, 2015:235). It is therefore proposed that to obtain more accurate and reliable cytotoxic results, other assays could be used to accompany the MTT-assay. Thus, MTT and NR-assay were performed, as the most common application of cytotoxicity is based on cell viability by measuring staining with these assays and the results of these are often comparable (Chiba *et al.*, 1998:257). In this study, most results obtained from the MTT and NR-assay were comparable. When the %cell viability of the **(NEF1)** dispersion between the MTT and NR was compared, it could be concluded that both the MTT and NR-assay found **(NES1)** to be the most cytotoxic. Although **(NER1)** and **(NEM1)** was found least cytotoxic by the MTT-assay and the NR-assay, respectively, it can be stated that the differences in %cell viability were minor. **(PNEF1)** was found to be weak cytotoxic at 0.02% (60 – 80% cell viability) and strong cytotoxic (<40% cell viability) at 0.04% during the MTT-assay; however, during the NR-assay the placebo dispersion was found non-cytotoxic (above 80% cell viability) throughout the entire concentration range. When comparing **(ExS)**, both the MTT and the NR-assay found Span® 60:apricot kernel oil non-cytotoxic (above 80% cell viability), to weak cytotoxic. The weak cytotoxicity of this sample was determined at the highest concentration (0.12%) of the NR-assay, yet there was only a slight difference in %cell viability at this concentration between the

MTT and NR-assay. The MTT found Span® 60 to be more cytotoxic than Tween® 80, while the opposite was found with the NR-assay. Nevertheless, it can be stated that the difference in %cell viability for these excipients at the various concentrations were minor. Lastly, both the MTT and NR-assay found the cytotoxicity of the APIs alone, in order of most cytotoxic, to be: **(SS)** > **(MS)** > **(LS)** > **(RS)**. Hence, **(RS)** was found to be the least cytotoxic API, while **(SS)** the most cytotoxic in terms of %cell viability at the highest concentration (0.012%).

When comparing the IC<sub>50</sub> value stated in literature for the respective statins with the IC<sub>50</sub> values obtained during this study, it can be stated that **(LS)** obtained a higher IC<sub>50</sub> (236.9 µg/ml) value when tested on HaCaT cells compared to the IC<sub>50</sub> value determined on HeLa cells (160 µg/ml) during the NR-assay. The opposite occurred during the MTT-assay, as **(LS)** displayed an IC<sub>50</sub> of 78.6 µg/ml compared to the IC<sub>50</sub> on HeLa cells of 160 µg/ml. **(SS)** displayed IC<sub>50</sub> values during MTT- (7.48 µg/ml) and NR- (168.2 µg/ml) assays, well below the IC<sub>50</sub> value determined for **(SS)** on MCF-7 breast cancer cells (43.2 mg/ml), as stated by Safwat *et al.* (2017:1122), indicating a higher cytotoxicity of **(SS)** on HaCaT cells compared to MCF-7 breast cancer cells. For **(RS)**, the IC<sub>50</sub> values obtained during MTT and NR-assay was 305.9 µg/ml and 245.3 µg/ml respectively on the HaCaT cells, and these values were significantly higher than those proposed in literature of > 20 µg/ml in HEp-2, KB and HeLa cells (Campos-Lara & Mendoza-Espinoza, 2011:191). During both the MTT- (0.0777 mg/ml) and NR- (0.2369 mg/ml) assays, **(MS)** obtained higher IC<sub>50</sub> values compared to the IC<sub>50</sub> value stated in the literature (4.335 µg/ml), which was determined on BT474A breast cancer cells (Glynn *et al.*, 2008:4), indicating that **(MS)** might be less cytotoxic on HaCaT cells.

Finally, it should be stated that when considering the amounts of the respective statins that diffused during the skin diffusions studies, the highest concentration tested still exceeded the amount of the statins that diffused as stated in Section D.3.4. Therefore, it can be suggested that little to no side effects or toxicity would occur at the target-site. It is however important to consider that *in vitro* determinations are not a direct indication of what would occur *in vivo*.

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# *Appendix F:*

## *Author guidelines: Die Pharmazie*

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### **F.1 Aim**

The journal DiePharmazie publishes reviews, experimental studies, letters to the editor, as well as book reviews.

The following fields of pharmacy are covered:

- Pharmaceutical and medicinal chemistry,
- pharmaceutical analysis and drug control,
- pharmaceutical technology, biopharmacy (biopharmaceutics, pharmacokinetics, biotransformation),
- experimental and clinical pharmacology,
- pharmaceutical biology (pharmacognosy), and
- history of pharmacy.

**Articles are published in English (preferred) or German and are classified as:**

#### **Reviews**

A summarizing presentation encompassing the current state of our knowledge and providing comprehensive interpretation with citation of the literature.

#### **Original articles**

Publications from all fields mentioned above.

#### **Short communications**

Brief publications about the fields mentioned above (see Preparation of manuscripts)

#### **Book reviews**

### **F.2 Conditions**

1. For submitted manuscripts, it is the responsibility of the author(s) to demonstrate novelty or a new approach taken in his research. The references should reflect the most recent relevant articles, and the discussion should compare the author's findings with the results of former investigations. For an experimental work, the data have to be

determined and classified in a suitable way, problems must be formulated in view of the data, hypotheses should be suggested an/or the author should give possible explanations for any inconsistencies.

If possible, the author(s) should perform mathematical or statistical calculations, fit the curves appropriate, and carry out the experiments under controlled conditions. Studies involving animals or human volunteers must include details of ethical approval.

2. Authors are requested to submit all manuscripts online.

Paper copy submissions are no longer acceptable.

Articles are considered for publication depending on their value and pharmaceutical relevance and with the understanding that they have not been published previously and are submitted exclusively to the journal Die Pharmazie.

3. All manuscripts are subject to experts review. Additional corrections may be done by the editors.
4. A PDF-file of the article is delivered free of charge after the paper has been published in the journal. Please note that, by copyright reasons, this is for personal use of the authors only and must not be made available, e.g. by posting on a freely accessible website.
5. Publication charges: Publication fees for publication in Die Pharmazie are 250 € per manuscript (excl.VAT), regardless of type and length. Authors will receive an invoice right after acceptance of their paper which will not be published before the fee has been paid.
6. Delivery of a PDF-file of the article is included in the publication fee. Please note that, by copyright reasons, this is for personal use of the authors only and must not be made available, e.g. by posting on a freely accessible website.
7. The quotation of registered names, trade names, trademarks, etc. in this journal does not imply, even in the absence of a specific statement, that such names are exempt from the relevant laws and regulations and therefore free for general use.

### **F.3 Preparation of manuscripts**

In order to achieve uniform presentation and to avoid unnecessary delays because of further inquiries, all authors are requested to observe the following guidelines:

1. Below the title, the surname(s) of the author(s) with initials should be given without academic and professional degrees. The full address of the author for correspondence should appear below author names. Details on the institution where the work was done are requested and should be given above the title.

2. Each manuscript should start with an abstract, containing the most essential results of the study. Extensive review papers and articles for continuous education should be preceded by an outline of topics.

Papers should be subdivided into chapters and subchapters according to the decimal system (e.g. 2.1.3.).

3. To achieve clarity and brevity of the presentation, original contributions should be subdivided after the abstract (see 2.) as follows:

- 3.1 Introduction: This should indicate the question under investigation which is generally based on a brief interpretation of the literature considering the current state of knowledge in the subfield and explaining the necessary theoretical foundations.

- 3.2 Investigations and results or synthesis of compounds: Methods should only be described generally (see "Experimental"), referring to previous or analogous studies. The presentation of results should be precise, with necessary formulas (numbered in sequence with Arabic numerals), diagrams, tables and figures added separately (together with the legend) to the manuscripts. Numerical values of results should generally be presented either in tables or curves (please mark statistical limits).

- 3.3 Discussion (unless covered by 2. as Investigations, results and discussion): It should not repeat results already given, but should state the conclusions drawn from the results or provide a theoretical debate and comparison with literature citations.

- 3.4 Experimental: This part describes briefly the detailed experimental conditions. Unless directions taken from literature have been modified, it suffices to refer to the original source. In the case of well known inorganic or organic compounds chemical formulae or common abbreviations may be used (e.g. NaCl, H<sub>2</sub>SO<sub>4</sub>, CH<sub>3</sub>OH, C<sub>6</sub>H<sub>6</sub>: Ac, Eth, Me, Phe, DMSO) under "Experimental". In other parts of the paper this is not desirable.

Results of elemental analyses can be omitted if it is stated that all the results were in an acceptable error range.

4. Short communications are published as rapidly as possible. The length of a manuscript is limited to 100 lines (including short summary; subdivisions are not required; the "Experimental" - if there is one - should be marked), up to 15 citations of literature and a maximum of 2 supplementary materials (schemes, figures, tables) are allowed.

5. Only the surnames of authors are given in the text. When there are more than two authors, only the name of the first one is used, followed by *et al.*
6. References in the text have to be cited by author and year, if there are three or more authors, use *et al.* (Miller 1997; Miller and Smith 2000; Miller *et al.* 2001). If the year is the same for several references identify these with a, b, c etc (Smith 1998a; Smith 1998b etc.) both in the text and in the reference list. At the end of the paper, references are listed in alphabetical order under the first authors' surname. If there are several references to items with the same first author, arrange these chronologically regardless the alphabetical order of the co-authors ("alphabetic-chronological" order).

Journal names should be abbreviated according to "Index Medicus" (Medline) or "Chemical Abstracts Service Source Index".

### **Quotations have to follow the following style:**

#### **Journal articles:**

Lee J (2002) Formulation development of epidermal growth factor. *Pharmazie* 57: 787-790.

Lee EB, Shin KH, Woo WS (1984) Pharmacological study on piperine. *Arch Pharm Res* 7: 127-132.

If each issue of a journal has its own pagination the issue number should be indicated in brackets after the volume number.

#### **Books/Book chapters**

Krishan K, Andersen ME (1994) Physiologically based pharmacokinetic modeling in toxicology. In: Hayes W (ed.) *Principles and methods of toxicology*, 3rd ed., New York, p. 149-187

Only if each issue of a journal has its own pagination the issue number should be indicated in brackets after the volume number.

7. For the identification of pharmaceutical substances, the International Nonproprietary Names (INN) proposed or recommended by the WHO should be used. Registered Trade Marks (usually indicated with R; in an article this sign should only be used when it is first mentioned or used in the summary), trivial names and chemical nomenclature can be added.
8. Nomenclature and spelling should conform to the directions given by IUPAC and IUB.
9. Units of measurement are determined by the directions of the International Units System SI as symbols; M instead of mol/l or mol \* l-1 is allowed

10. Botanical names (species, genus) should be marked in italics.
11. The following abbreviations should be used consequently (except in the title and all subtitles). All other abbreviations have to be explained in the manuscript at first usage, if aforementioned directions are not applicable. Abs. = absolute; anh. = anhydrous; b.p.; b.r. = boiling point, -range; calcd. = calculated; CC = column chromatography; conc. = concentrated; dec. = decomposition, eq. = equation; Fig. = figure; GC = gas chromatography, - chromatogram, HPLC = high performance liquid chromatography, - chromatogram; i.m. = intramuscular; i.p. = intraperitoneal; IR = infrared; i.v. = intravenous; m.p.; m.r. = melting point, -range; MS = mass spectrometry, mass spectrum; NMR = nuclear magnetic resonance spectrum; PC = paper chromatography, - chromatogram, % = per cent, percentage, p.o. = peroral; s.c. = subcutaneous; TLC = thin layer chromatography, - chromatogram; UV = ultraviolet.
12. Footnotes must be numbered consecutively and are to be added separately to the manuscript. They are printed following the "Experimental".
13. Dedications (e.g., on the occasion of the 60th or higher birthday) should be inserted between author(s) and summary.
14. Additions to legends of table should be marked by \*, \*\*, \*\*\* or a,b,c,d etc.
15. Figures have to be of sufficient quality for reproduction process. Even after size reduction the figures' key has to be easy to read. Manuscripts containing figures of insufficient quality cannot be accepted.

# *Appendix G:*

## *The International Journal of Pharmaceutics: Guide for authors*

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### **G.1 Introduction**

The International Journal of Pharmaceutics publishes innovative papers, reviews, mini-reviews, rapid communications and notes dealing with physical, chemical, biological, microbiological and engineering studies related to the conception, design, production, characterisation and evaluation of drug delivery systems in vitro and in vivo. "Drug" is defined as any therapeutic or diagnostic entity, including oligonucleotides, gene constructs and radiopharmaceuticals.

Areas of particular interest include: pharmaceutical nanotechnology; physical pharmacy; polymer chemistry and physical chemistry as applied to pharmaceutics; excipient function and characterisation; biopharmaceutics; absorption mechanisms; membrane function and transport; novel routes and modes of delivery; responsive delivery systems, feedback and control mechanisms including biosensors; applications of cell and molecular biology to drug delivery; prodrug design; bioadhesion (carrier-ligand interactions); and biotechnology (protein and peptide formulation and delivery).

Note: For details on pharmaceutical nanotechnology, see Editorials in 279/1-2 281/1, and 288/1.

### **G.2 Types of paper**

#### **(1) Full Length Manuscripts**

#### **(2) Reviews and Mini-Reviews**

Suggestions for review articles will be considered by the Review-Editor. "Mini-reviews" of a topic are especially welcome.

### **G.3 Ethics in publishing**

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

### **G.4 Studies in humans and animals**

If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The manuscript should

be in line with the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals and aim for the inclusion of representative human populations (sex, age and ethnicity) as per those recommendations. The terms sex and gender should be used correctly.

Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed. The sex of animals must be indicated, and where appropriate, the influence (or association) of sex on the results of the study.

Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

#### **G.5 Declaration of interest**

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Authors must disclose any interests in two places:

1. A summary declaration of interest statement in the title page file (if double-blind) or the manuscript file (if single-blind). If there are no interests to declare then please state this: 'Declarations of interest: none'. This summary statement will be ultimately published if the article is accepted.
2. Detailed disclosures as part of a separate Declaration of Interest form, which forms part of the journal's official records. It is important for potential interests to be declared in both places and that the information matches. More information.

#### **G.6 Submission declaration and verification**

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