Formulation, characterisation and topical delivery of salicylic acid containing whey-protein stabilised emulsions

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B.Pharm

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It all starts here ™

PREFACE

This dissertation was written in article format. The candidate, Johann Combrinck, was the primary author of the article (chapter 3) and all other chapters included in this thesis and performed all the experimental work under supervision and assistance of all promoters.

- Chapter 2 represents a literature overview of emulsions,
- Chapter 3 includes methods, results and discussion of the investigation of different biopolymer emulsifiers on release and topical delivery, written in article format and submitted to AAPS PharmSciTech (Status: Published), and
- Chapter 4 presents methods, results and discussion of the investigation of the effect of pH of unsaturated aqueous solutions of salicylic acid on release of the active. It is also written in an article format; however additional studies, including computational modelling, will be conducted before submission. These additional studies were not part of the current project to obtain the Master degree and were rather suggestions for further investigations as a result of this study.

The article manuscript was formatted according to a standard format chosen for this dissertation. However, the reference style was maintained according to the guideline for authors of AAPS PharmSciTech. The guideline for authors was added in the Appendices.

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TABLE OF CONTENT

	Preface	**I**
	Acknowledgements	**II**
	Table of content	**III**
	Abstract	**VII**
	Uittreksel	**IX**
1	**Aims and objectives**	**1**
2	**Emulsions as topical delivery systems: Effects of emulsifier on release and topical performance**	**3**
2.1	Emulsions	3
2.1.1	Introduction	3
2.1.2	Emulsion instabilities	5
2.1.2.1	Creaming/Sedimentation	6
2.1.2.2	Coalescence	6
2.1.2.3	Flocculation	7
2.1.2.4	Phase inversion	7
2.1.3	Emulsifiers	7
2.2	The effect of emulsifier on release	12
2.3	The effect of emulsifier on transdermal delivery	14
2.3.1	Modelling	14
2.3.2	Solid particles	15
2.3.3	Emulsifier mixture HLB value	15
2.3.4	Droplet charge	16
2.3.5	Surfactant association structure	16
2.3.6	Hydrophilic chain length	17
2.4	Polymers for transdermal delivery systems with emulsifying properties	17
2.4.1	Introduction	17

2.4.2	Layer-by-layer technique	18
2.4.3	Whey proteins	19
2.4.4	Chitosan	20
2.4.5	Carrageenan	20
2.5	Conclusion	21
	References	21
3	**Whey protein / polysaccharide-stabilized emulsions: Effect of polymer type and pH on release and topical delivery of salicylic acid**	**27**
3.1	Introduction	28
3.2	Materials and Methods	32
3.2.1	Materials	32
3.2.2	Aqueous and Oil Phase Preparation	32
3.2.3	Emulsion Preparation	33
3.2.4	Particle Size Analysis	34
3.2.5	Zeta Potential Measurements	34
3.2.6	Viscosity Measurements	34
3.2.7	Creaming Stability	34
3.2.8	Turbidity	35
3.2.9	Release of Active from the Formulations	35
3.2.10	Skin Preparation	35
3.2.11	*In Vitro* Skin Absorption Study	36
3.2.12	Skin Sample Preparation	37
3.2.13	Statistical Analysis	37
3.2.14	HPLC-UV Method	37
3.3	Results	38
3.3.1	Characterization of Emulsions	38
3.3.2	Turbidity	40
3.3.3	Release of Active from the Formulations	41

3.3.4	*In Vitro* Skin Absorption	46
3.4	Discussion	48
3.4.1	Characterization of Emulsions	48
3.4.2	Turbidity	50
3.4.3	Release of Active from the Formulations	51
3.4.4	*In Vitro* Skin Absorption	53
3.5	Conclusion	54
3.6	Acknowledgements	54
	References	55
4	**The effect of pH on release of salicylic acid from unsaturated, aqueous donor solutions**	**58**
4.1	Introduction	58
4.2	Materials and Methods	59
4.2.1	Materials	59
4.2.2	Aqueous Phase Preparation	59
4.2.3	Solubility Determination of Salicylic Acid	59
4.2.4	Oil-Water Phase Partition Coefficient Determination of Salicylic Acid	60
4.2.5	Determination of percentage ionized salicylic acid	60
4.2.6	*In Vitro* Release Study	60
4.2.7	Monitoring of pH of Donor Solution	61
4.2.8	Swelling Degree (*SD*)	61
4.2.9	Scanning Electron Microscope (SEM)	61
4.2.10	Statistical Analysis	61
4.2.11	HPLC-UV Method	61
4.3	Results	62
4.4	Discussion	66
4.5	Conclusion	68
	References	68

5	**Conclusion and Future perspectives**	**69**
	Appendix A: Instructions for Authors - American Association of Pharmaceutical Scientists	**71**
	Appendix B: HPLC Validation	**84**
	Appendix C: Droplet Size	**90**
	Appendix D: Zeta Potential	**92**
	Appendix E: Microscopy Images of Emulsions – Day 1	**93**
	Appendix F: Photos indicating Creaming – Day 7	**94**
	Appendix G: Turbidity	**95**
	Appendix H: Cumulative release data of salicylic acid from emulsions	**96**
	Appendix I: Skin absorption data of salicylic acid from emulsions	**98**
	Appendix J: Cumulative release data of aqueous solutions of salicylic acid	**100**
	Appendix K: pH values of donor phase during release study from aqueous solutions	**101**

ABSTRACT

Emulsions are widely used as topical formulations in the pharmaceutical and cosmetic industry. They are thermodynamically unstable and require emulsifiers to stabilize them physically. A literature survey has revealed that emulsifiers could have an effect on topical delivery. Therefore, the overall aim of this research project was to investigate and to understand the various effects of biopolymers, chosen for this study as emulsifiers, on the release and the topical delivery of an active ingredient from emulsion-based delivery systems. Emulsions were stabilized by either whey protein alone or in combination with chitosan or carrageenan. Salicylic acid was chosen as a model drug. Furthermore, the emulsions were prepared at three different pH values (pH 4, 5 and 6) in order to introduce different charges to the polymeric emulsifiers and subsequently determine the effect of pH on release as well as on dermal and transdermal delivery. Emulsion characteristics, such as droplet size, zeta potential, viscosity and stability against creaming and coalescence were ascertained. In addition, turbidity was determined to evaluate the degree of insoluble complex formation in the aqueous phase of the emulsions. A high pressure liquid chromatographic (HPLC) method was validated for the quantitative determination of salicylic acid in the release, skin and transdermal perfusate samples. Nine emulsions were formulated, utilizing the layer-by-layer (LbL) self-assembly technique, from which the release of salicylic acid was determined. These release studies were conducted, utilizing nitrocellulose membranes (0.2 µm pore size) with the use of Franz-type diffusion cells in four replicates per formulation over a time period of 8 hours. Based on the emulsion characterization and release data, six formulations, including the oil solution, were chosen to determine dermal and transdermal delivery of salicylic acid. During the diffusion studies, the effect of different pH (whey protein pH 4.00, 5.00 and 6.00), different polymers and different polymer combinations were investigated. These diffusion studies were conducted with the use of dermatomed (thickness ~400 µm), human abdominal skin and Franz-type diffusion cells over a period of 24 hours. The characterization of the emulsions revealed no significant differences in the droplet size and viscosity between the various formulations. All emulsions showed stability towards coalescence over a time period of 7 days; however, not all the emulsions showed stability towards creaming and flocculation. The results of the release studies indicated that an increase in emulsion droplet charge could have a negative effect on the release of salicylic acid from these formulations. In contrast, positively charged emulsion droplets could enhance the dermal and transdermal delivery of salicylic acid from emulsions. It was hypothesized that electrostatic complex formation between the emulsifier and salicylic acid could affect the release, whereas electrostatic interaction between emulsion droplets and skin could influence dermal/transdermal delivery of the active. Furthermore, the degree of ionization of salicylic acid played an important role in the dermal and transdermal delivery of salicylic acid from the various emulsions.

KEYWORDS

Emulsion, release, topical, transdermal, salicylic acid, LbL self-assembly

UITTREKSEL

Emulsies word algemeen gebruik as topikale formulerings in die farmaseutiese en kosmetiese industrie. Emulsies word as termodinamies onstabiel geklassifiseer en benodig emulsifiseerders om hulle te stabiliseer. 'n Literatuurstudie het getoon dat emulsifiseerder 'n effek om die dermale aflewering van geneesmiddels het. Die doelwit van hierdie navorsingsprojek is om die verskillende parameters wat topikale aflewering van emulsiesisteme beïnvloed, wat gestabiliseer is deur verskillende polimere, te ondersoek en te verstaan. Emulsies was gestabiliseer deur slegs van wei-proteïene gebruik te maak, of in kombinasie met kitosaan of karrageenaan. Salisielsuur is gekies as 'n model geneesmiddel. Die emulsies is berei by drie verskillende pH-waardes (pH 4.00, 5.00 en 6.00), sodat die polimere verskillende ladings kan besit en dus verskillende effekte op die dermale en transdermale aflewering bepaal kon word. Emulsie-eienskappe, byvoorbeeld die druppelgrootte, zetapotensiaal, viskositeit en die stabiliteit teen oproming en koagulering is bepaal. Die troebelheid is bepaal as maatstaf van onoplosbare kompleksvorming in die waterfase. 'n Gevalideerde Hoë-verrigting-vloeistofkromatografiese (HDVK) metode is ontwikkel vir kwantitatiewe bepaling van salisielsuur in die dermale, transdermale en diffusiemonsters. Nege emulsies is geformuleer deur die laag-op-laag-bedekkingsmetode. Membraandiffusiestudies is uitgevoer op al die formulerings, insluitend 'n olie-oplossing met die ooreenstemmende konsentrasie salisielsuur as wat by die emulsies aangewend is. Nitrosellulosemembrane (0.2 µm poriegrootte) is aangewend met behulp van Franz-tipe diffusieselle om die membraandiffusiestudies oor 'n periode 8 ure te ondersoek. Vier herhalings per formulering is ondersoek. As voortvloeisel uit die emulsie-eienskappe sowel as die membraandiffusiestudies, is ses formulerings, insluitend die olie-oplossing, gekies om die dermale en die transdermale aflewering van die salisielsuur te bepaal. Tydens die diffusiestudies is die effek van pH (wei-proteïene pH 4.00, 5.00 en 6.00) asook die effek van die polimeer (pH 6.00 wei-proteïene, wei-proteïene in kombinasie met kitosaan of karrageenaan) bepaal. Gedermatoomde (~400 µm) abdominale, menslike huid is met behulp van Franz-tipe diffusieselle aangewend vir die diffusiestudies oor 'n periode van 24 uur. Die karakterisering van die emulsies het geen beduidende verskille in die druppelgrootte en die viskositeit getoon nie. Al die emulsies het stabiliteit teenoor koagulering oor 'n periode van 7 dae getoon, maar nie al die formulerings was stabiel teenoor oproming en flokkulasie nie. Die membraandiffusiestudies het getoon dat 'n emulsiedruppellading 'n negatiewe effek kan veroorsaak op die vrystelling van salisielsuur vanuit die formulering. Daar was verder waargeneem dat positief-gelaaide emulsiedruppels die dermale en transdermale aflewering van die geneesmiddel kan bevoordeel. Dit is vermoed dat 'n elektrostatiese kompleksvorming tussen die emusifiseerder en die salisielsuur die vrystelling van geneesmiddel vanuit die formulering kan beïnvloed. Die elektrostatiese interaksie tussen die emulsiedruppels en die huid, asook die graad van ionisasie

van die geneesmiddel, kan die dermale en transdermale aflewering van 'n geneesmiddel beïnvloed vanuit verskillende formulerings.

Soekwoorde

Emulsie, vrystelling, topikaal, transdermaal, salisielsuur, laag-op-laag bedekking

CHAPTER 1

AIMS AND OBJECTIVES

Aims

Emulsion-based delivery systems employing biopolymers have mainly been investigated for oral delivery; however, data for topical delivery systems are limited. Therefore, it would be of a valuable contribution to investigate various biopolymers, employed as emulsifiers, for the formulation of topical delivery systems and to test their effect on release as well as on dermal and transdermal delivery. Since the overall characteristics of the multilayer assembly of biopolymers at the interface (e.g. charge, thickness and permeability) could have an influence on the properties of the resulting formulation and its performance, it is also of fundamental interest to investigate the influence of formulation parameters (e.g. pH) on emulsion properties, release and topical delivery.

Therefore, the overall aim of the research project was to investigate and to understand the various effects that influence the release and topical performance of emulsion-based delivery systems employing biopolymers as emulsifiers. The knowledge could contribute to appropriate modification of the topical delivery systems in order to achieve efficient, controlled and targeted delivery to the designated site.

Objectives

- Survey literature to gain knowledge about topical emulsion formulation and their effect on release and topical delivery. The focus of the literature study should be on the effect of emulsifiers on release and topical delivery as well as on biopolymers as possible emulsifiers.

- Formulate stable, salicylic acid-containing emulsions using whey proteins alone and in combination with either chitosan or carrageenan as emulsifiers. The following formulation and preparation parameters should be investigated in this study:

    - Different emulsifiers (whey proteins, whey proteins in combination with chitosan, whey proteins in combination with carrageenan)

    - pH of aqueous phase

- Develop and validate a suitable analytical method for the quantitative determination of salicylic acid in the emulsions, in release samples as well as human skin and transdermal perfusate samples.

- Determine emulsion properties (e.g. droplet size, zeta potential, viscosity, stability against coalescence and creaming), insoluble complex formation between salicylic acid and the different polymers as well as release of salicylic acid from the resultant emulsions.
- Assess the delivery of salicylic acid from selected emulsions into and through skin. The selection of the formulations to be tested shall depend on the outcome of the emulsion characterization as well as stability and release results.
- Interpret the data and investigate the effect of the different formulation and preparation parameters (emulsifier, pH) on emulsion properties, release and topical delivery.

CHAPTER 2

Emulsions as topical delivery systems: Effects of emulsifier on release and topical performance

2.1 Emulsions

2.1.1 Introduction

Emulsions can be defined as immiscible droplets dispersed in a continuous phase which usually consists of an oil phase and an aqueous phase. Emulsions are used in a variety of fields including cosmetic, pharmaceutical and food industry. Research has been conducted on transdermal emulsions and recently, focus has shifted to micro- and nanoemulsions for transdermal delivery (Ru *et al.*,2009:399; He *et al.*, 2011:521).

Emulsions can be classified according to the composition of phases and droplet size. Emulsions containing of aqueous droplets dispersed in an oil phase are known as water-in-oil (w/o) emulsions, whereas oil-in-water (o/w) emulsions consist of oil droplets dispersed in an aqueous phase. The formation of multiple emulsions can be achieved by aqueous droplets dispersed in an oil phase which is again dispersed in an aqueous phase, resulting in a water-in-oil-in-water (w/o/w) emulsion. Conversely, oil droplets dispersed in water droplets which in turn are again dispersed in an oily continuous phase yielding oil-in-water-in-oil (o/w/o) emulsions. It should also be noted that emulsions can be formed from two immiscible oil phases resulting in oil-in-oil (o/o) emulsions (Tadros, 2009:1).

Emulsions can also be classified according to droplet size, such as macro-, micro- or nanoemulsions. The terminology of these emulsions may be confusing as the prefix indicates size. However, in practise droplets in microemulsions could be as small as or even smaller than those in nanoemulsions. McClements (2012:1725) stated the reason for this confusion as being a result of the history of emulsion preparation. The term nanoemulsion is only recently being used. Even though researchers manufactured nanoemulsions, they were still termed microemulsions. Thus, this term has become generally used. No exact values for critical droplet size could be agreed upon to distinguish between micro- and nanoemulsions. McClements (2012:1726) defines the upper limit of the droplet size of microemulsions similar to the one of nanoemulsions (radius <100 nm). The determining difference between micro- and nanoemulsion is the thermodynamic stability. Microemulsions are thermodynamically stable and are formed spontaneously by bringing two immiscible phases together with a surfactant at a specified temperature. On the contrary, nanoemulsions are not thermodynamically stable and always require external energy for the

formation. In general, at a high surfactant-to-oil ratio, preferentially microemulsions are formed, whereas at low surfactant-to-oil ratio, nanoemulsions might be formed (McClements, 2012:1726). Consequently, the long-term stability of micro- and nanoemulsions differs. The spherical or non-spherical particles of microemulsions do not change during prolonged storage. In contrast, due to Ostwald ripening, flocculation, creaming/sedimentation and gravitational separation, the morphology and size distribution of the spherical particles of nanoemulsions change during prolonged storage. Owing to the difference in thermodynamic stability of the two emulsions, the properties of the microemulsions should remain the same in contrast to nanoemulsions, when exposed to external stress factors, such as mechanical agitation, heating or cooling (McClements, 2012:1728). Macroemulsions are also known as conventional emulsions and their droplet size varies between 0.15 µm and 100 µm. These emulsions are, like nanoemulsions, thermodynamically unstable and necessitate external energy for their formation. However, nanoemulsions have better stability against droplet aggregation and gravitational separation because of a high kinetic stability (McClements, 2012:1727).

Various methods can be used for the preparation of emulsions. With these methods, external energy is applied to the system in order to break-up the dispersed phase into droplets. Basic methods include (Gopal, 1968:5; Billany, 2007:395):

- Dispersion method:
  This is regarded as the conventional method of emulsion preparation by using brute force to break-up the interface to form emulsion droplets (Gopal, 1968:6).
- The intermitted shaking method:
  For the preparation of small amounts of emulsions this method can be very successful. Shaking the two phases in a tube for short intervals (20-30 seconds) over a period of about 2 minutes can yield stable emulsions (Gopal, 1968:6).
- Mixers:
  Turbulence in the container is applied in order to force the formation of emulsion droplets (Gopal, 1968:8).
- Colloidal mills:
  The formation of emulsion droplets is achieved by a strong shearing flow between a rotar and stator surface at high speeds (Gopal, 1968:9).
- Homogenizers:
  With the forcing of two immiscible phases through a small orifice at high pressure, emulsion droplets can be formed (Gopal, 1968:11).

- Condensation method:
  By injection of a vapour into another liquid, the vapour becomes supersaturated and forms micron-sized emulsion droplets (Gopal, 1968:5).
- Ultrasonification:
  The agitation of the two phases by a sonicator containing a piezoelectrical quartz crystal, which responses to alternating electrical voltage, can contract and expand which lead to mechanical vibrations when the tip of the sonicator comes into contact with the liquid (Maa and Hsu, 1999:234).
- Phase Inversion Temperature (PIT) Method:
  An o/w emulsion, stabilized by non-ionic emulsifiers, can be converted to a w/o emulsion by heating of the emulsion above the phase inversion temperature. Subsequently rapid cooling will form finely dispersed o/w emulsions. The hydrophilic-lipophilic balance (HLB) value of non-ionic surfactants will decrease with temperature and become hydrophilic, resulting in the phase inversion (Billany, 2007:395).

As the work in this dissertation included macroemulsions, the introduction chapter continues with the focus on macroemulsions, which are referred to as emulsions.

2.1.2 Emulsion instabilities

Emulsions are thermodynamically instable which is caused by huge differences in surface tension and interfacial tension. Physical instabilities that may occur are creaming/sedimentation, coalescence, flocculation and phase inversion (Fig. 1). In addition, chemical instabilities could occur, which include oxidation of the oil phase, microbiological contamination and adverse storage conditions (Billany, 2007:401).

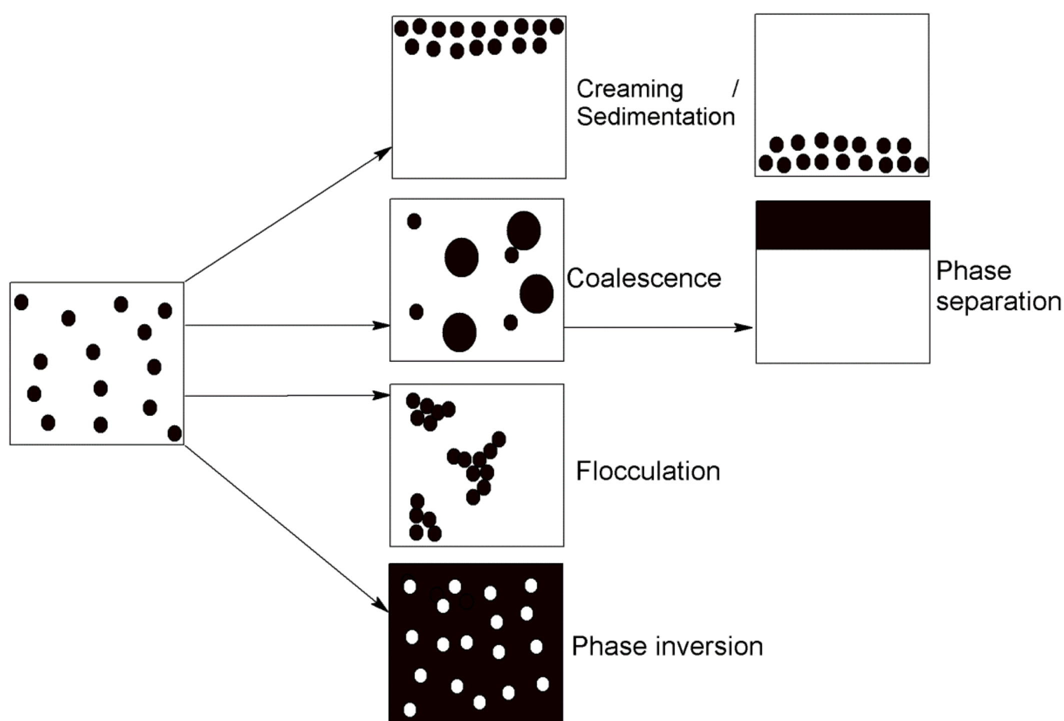

Fig. 1. Instabilities of emulsions.

2.1.2.1 Creaming/Sedimentation

Creaming or sedimentation occurs when dispersed droplets rise to the top of the continuous phase in the case of o/w emulsions (creaming) or sink to the bottom of the continuous phase in the case of w/o emulsions (sedimentation), where in general the oil phase has a lower density than the water phase. Though creaming/sedimentation of emulsions is not a serious instability, it may lead to coalescence of the dispersed phase. Creaming/sedimentation can be reversed by gently mixing or shaking of the formulation. Creaming/sedimentation can be reduced by (Billany, 2007:400; Tadros, 2009:35):

- Reducing droplet size;
- Increasing viscosity of the continuous phase;
- Reducing density differences between the two phases and;
- Increasing the volume fraction of the dispersed phase.

2.1.2.2 Coalescence

Coalescence is also known as breaking of emulsions. Increased droplet size is caused by fusion of droplets, due to thinning and disruption of the emulsifier layer. It may lead to total separation of the dispersed phase and the continuous phase which is irreversible. Coalescence can be resisted by adsorption of a mechanically strong layer of emulsifier. It has been reported that large zeta potentials of positive or negative polarity (e.g. above +30 mV and below -30 mV, respectively) can

cause electrostatic repulsion between emulsion droplets (Guzey & McClements, 2007:482). Steric hindrance is another option to overcome the Van der Waals attraction to minimise the fusion of emulsion droplets. Extreme fluctuations in temperature and chemical changes of the emulsifier can lead to coalescence and phase inversions (Billany, 2007:395; Sherman,1968:136). In a study performed by Shinoda and Arai (1964:3487-3490), to determine the PIT of emulsions comprising non-ionic surfactants, it was observed that different o/w emulsions at different temperatures. Benzene had the lowest inversion temperature at ~ 20°C, whereas hexadecane had the highest at ~110 °C. This indicated that the oil phase that is being used will also affect the stability of emulsions.

2.1.2.3 Flocculation

Flocculation is the process where droplets aggregate without disruption of the emulsifier layer. It is caused by Van der Waals attractions between the emulsion droplets (Tadros, 2009:7). Flocculation of droplets can be reversed by gentle mixing or shaking of the emulsion as with creaming. Flocculation of droplets is not a serious stability problem; however, it can result in coalescence of the droplets if the emulsifier showed inadequate mechanical resistance. A zeta potential value close to zero could increase the risk of flocculation.

2.1.2.4 Phase inversion

Phase inversion occurs when an o/w emulsion changes to a w/o emulsion and *vice versa*. It can be caused by a high concentration of dispersed phase (≥70%) or by changing the hydrophilic/lipophilic properties of the emulsifier (e.g. HLB value of surfactants or contact angle of solid particles) (Billany, 2007:401).

2.1.3 Emulsifiers

Emulsifiers are required for the formation of emulsions as well as for the stabilization of thermodynamically unstable emulsions. Emulsifiers reduce interfacial tension and form a layer around the droplets, facilitating separation of the dispersed and continuous phases. Emulsifiers could have additional properties, such as gelling, thickening or penetration enhancing, making them multifunctional (Rodríguez *et al.*, 2002:271; De Ruiter & Rudolph, 1997:389). Emulsifier concentration must be sufficient to form a protective layer around the dispersed phase. McClements (2009:13) indicated that the employment of a single emulsifier may not yield adequate stabilization of emulsions and suggested the use of combinations of emulsifiers.

The hydrophilic-lipophilic balance (HLB) scale was developed for the selection of appropriate surfactants on a semi-empirical basis, where the relative percentage of hydrophilic to lipophilic groups in the surfactant molecule is represented by the HLB value (Tadros, 2009:25). The HLB

value is based on the concept that surfactants contain both, hydrophilic and lipophilic groups (Sherman, 1968:140). An emulsifier with an HLB value of 3-6 will result in w/o emulsions, whereas emulsifiers with an HLB value of 8-13 will result in o/w emulsions (Gopal, 1968:15). There also exists the possibility for the combination of two surfactants from opposite ends of the HLB scale, e.g. Tween 80 and Span 80, at various ratios in order to cover a wide range of HLB values (Tadros, 2009:25).

There are different mechanisms for emulsifiers to stabilize emulsions. Emulsion droplets may attract each other due to Van der Waals attraction (Tadros, 2009:7). To prevent flocculation and eventually coalescence, the Van der Waals attractions should be overcome by keeping a minimum distance between emulsion droplets, for instance by either electrostatic repulsion or steric hindrance (Tadros, 2009:9-12). Droplets with low to zero net electrostatic charge may have an increased tendency for flocculation, which in turn may lead to coalescence. By the addition of either an ionic surfactant or a charged polymer, an increased net droplet charge can be achieved (Tadros, 2009:9). The increased droplet charge forces the emulsion droplets away from one another (steric repulsion), decreasing the probability of flocculation of the droplets. Steric repulsion can be realised by either unfavourable mixing of the surfactant/polymer chains or by entropic volume restriction (Tadros, 2009:11). Furthermore, some emulsifying agents contain thickening properties to increase the viscosity of the emulsions, which will aid to the emulsion stabilization (Perez *et al.*, 2011:306 & Rodríguez *et al.*, 2002:271). For example, particles or polymers could develop a three-dimensional structure in the continuous phase (Fig. 2) thereby increasing the viscosity (Aveyard *et al.*, 2003:510-511). Increased viscosity decreases the chance for emulsion droplets to collide because the velocity of emulsion droplets is reduced.

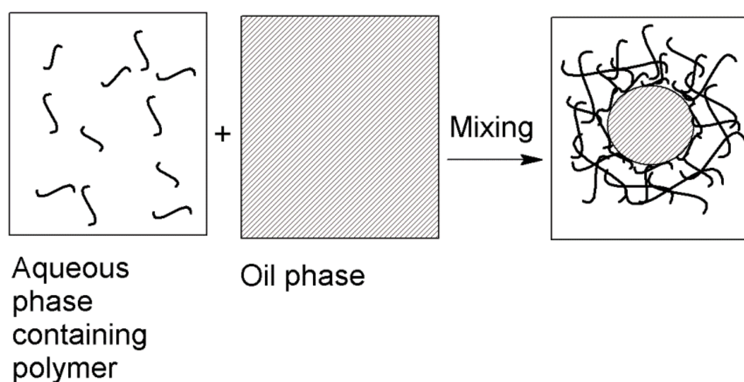

Fig. 2. Schematic presentation of polymer-stabilized emulsions forming a three-dimensional network in the continuous phase.

Emulsifiers also form a protective layer around the emulsion droplets resulting in a mechanical protective layer, thereby increasing the stability of emulsions (Kitchener & Musselwhite, 1968:79).

This protective layer can be formed by surfactants, polymers or solid particles (Billany, 2007:395-398). The emulsifying agents can either arrange in monolayers or multilayers.

Surfactants contain both hydrophilic and lipophilic groups. With the adsorption of a surfactant to the interface, the surfactant molecules rearrange themselves such that the hydrophilic groups orientates towards the aqueous phase and the lipophilic groups towards the oil phase (Fig. 3) (Tadros, 2009:25). Surfactants decrease the interfacial tension between two separate phases (Attwood, 2007:85) and therefore aid the emulsification and prevention of coalescence of the emulsion droplets. When surfactants are in excess in the aqueous phase, they may arrange in liquid crystalline structures thereby increasing the viscosity and the stability of emulsions (Friberg and Solans, 1986:121).

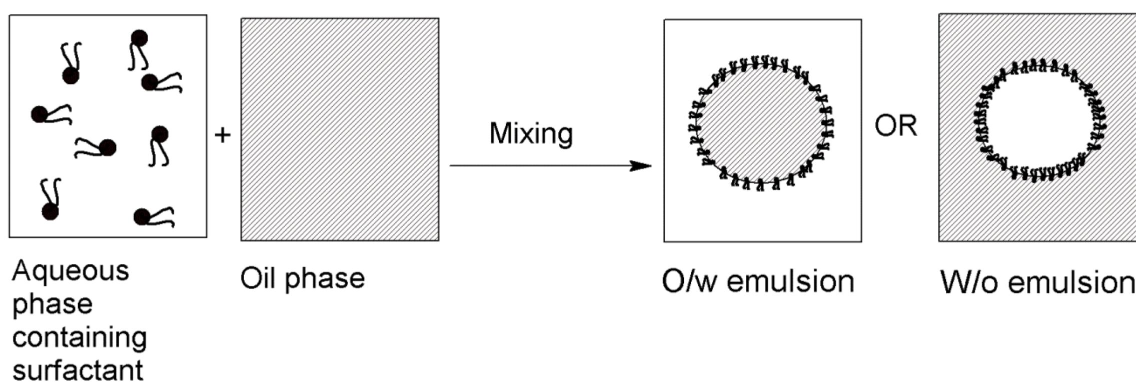

Fig. 3. Schematic presentation of surfactant-stabilized emulsions.

Solid particles can also be used to stabilize emulsions (Fig. 4). These emulsions that are solely stabilized by solid particles are called Pickering emulsions. Solid particles such as silica (Binks & Lumsdon, 2000:2539), chitosan (Wei *et al.*, 2012:1229), starch (Marku *et al.*, 2012:1) and microcrystalline cellulose (Oza & Frank, 1989:163) have been used to stabilize emulsions.

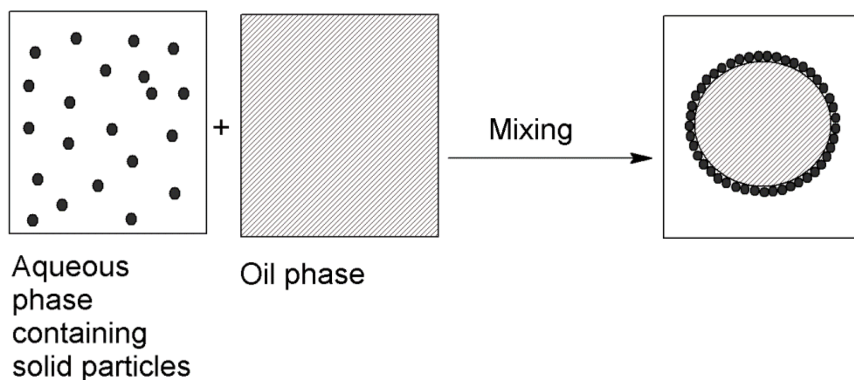

Fig. 4. Schematic presentation of Pickering emulsions.

An important parameter to take into consideration when formulating Pickering emulsions is the contact angle, θ of the solid particles at the oil-water interface (Binks & Clint, 2002:1270; Melle *et al*. 2005:2158). According to Melle *et al*. (2005:2158), θ should be approximately 90° for the formation of stable emulsions. In general, hydrophilic particles that show a θ lower than 90° preferably form o/w emulsions, whereas w/o emulsion can be formed with hydrophobic particles that show a θ higher than 90° (Fig. 5) (Binks & Clint, 2002:1270).

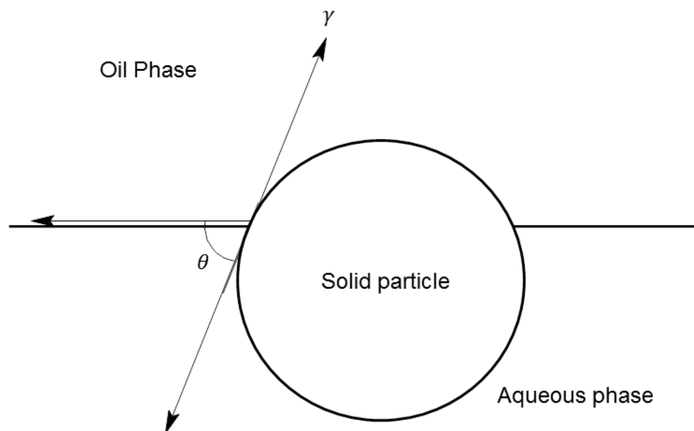

Fig. 5. Schematic presentation of emulsion droplets stabilized by solid particles (Redrawn from Binks & Clint, 2002:1270).

Some examples of emulsifiers and their possible mechanisms of emulsion stabilization are listed in Table 1.

Table 1. Comparison of different emulsifying agents and their proposed mechanisms of stabilization.

Emulsifier	Examples	Reference	Mechanism of stabilization
Cationic surfactant	Alkali metals Ammonium soaps	Attwood, 2007:86	Reduction of interfacial tension
Anionic surfactant	Alkylpyridinium chloride Alkylmethylammonium bromide	Attwood, 2007:86	Reduction of interfacial tension and tendency for liquid crystal formation.
Non-ionic surfactant	Propylene glycol Long chain alcohols	Attwood, 2007:86	Reduction of interfacial tension and tendency for liquid crystal formation
Natural and semi-synthetic polymers	Tragacanth Acacia Methylcellulose Carmellose sodium	Billany, 2007:398	Formation of a multimolecular, protective film around o/w emulsion droplets.
Proteins	Whey Soybean	He *et al.,* 2011:522	Formation of a mechanical, protective layer and viscosity increase
Cationic polysaccharides	Chitosan	Rodríguez *et al.*, 2002:271	Electrostatic repulsion, formation of a mechanical layer and viscosity increase.
Anionic polysaccharides	Carrageenan Pectin Alginate	De Ruiter & Rudolph, 1997:392 Perez *et al.*, 2011:306-307	Electrostatic repulsion, formation of a mechanical layer and viscosity increase.
Non-ionic polysaccharides	Starch Dextran Cellulose	Chanamai & McClements, 2002:120	Reduction of interfacial tension, steric hindrance and viscosity increase.
Sterol containing substances	Beeswax Wool fat	Billany, 2007:398 Chao *et al.*, 2010:493	Reduction of interfacial tension.
Solid particles	Silica Chitosan	Binks & Lumsdon, 2000:2539-2540 Wei *et al.*, 2012:1229-1230	Formation of a protective layer around emulsion droplets and possible viscosity increase.

2.2 The effect of emulsifier on release

Mathematical models were designed to describe release rates from formulations. In an article by Dash *et al.* (2010: 223), two models for the release of drugs from emulsions were described. The most well-known release model for emulsions is the Higuchi-model (1961:875), which is based on the following assumptions:

- The initial concentration of drug in the matrix is much higher than the solubility;
- The diffusion of drug occurs only in one dimension;
- The particle size is much smaller than the system thickness;
- The matrix swelling and dissolution are negligible;
- The drug diffusivity is constant and
- The perfect sink conditions are always attained in the release environment.

The Higuchi model is described by Eq. 1:

$$f_t = Q = A\sqrt{D\,(2C - C_s)C_s\,t} \qquad \text{Eq. 1}$$

where f_t is the cumulative amount of drug released in time t per surface area A. C is the drug initial concentration, C_s is the drug solubility in the matrix media and D is the diffusion coefficient of the drug molecule in the solvent. This model can be simplified to Eq. 2 which is known as the simplified Higuchi model:

$$f_t = Q = K_H\sqrt{t} \qquad \text{Eq. 2}$$

where K_H is the Higuchi dissolution constant. Data obtained can be plotted as cumulative amount of drug released per surface area against square root of time and should yield a linear correlation. The Higuchi model can be used to describe diffusion-controlled release from emulsions under sink conditions.

The release of actives from emulsion-based formulations is dependent on the initial concentration and solubility of the active (Martin, 1993:504-505). A variation of the Higuchi model, given by Eq. 3, can be utilized for calculating the effective diffusion constant, D_e.

$$Q = 2\,C_0\sqrt{\frac{D_e t}{\pi}} \qquad \text{Eq. 3}$$

where Q is the amount of active released per unit area, C_0 is the initial concentration and t is the time after application of formulation. If the internal phase consists of a small volume, D_e can be calculated from Eq. 4, or if $D_2 >> D_1$, from Eq. 5.

$$D_e = \frac{D_1}{\emptyset_1 + K\emptyset_2}\left[1 + 3\emptyset_2\left(\frac{KD_2 - D_1}{KD_2 + 2D_1}\right)\right] \quad \text{Eq. 4}$$

$$D_e = \frac{D_1 + 3\emptyset_2}{\emptyset_1 + K\emptyset_2} \quad \text{Eq. 5}$$

where $\emptyset$ is the volume fractions of the internal and external phases. The subscripts 1 and 2, respectively, represent the external and internal phases and K is the partition coefficient between the two phases.

According to Martin (1993:504), release takes place at two different rates. Initially (approximately within the first 30 minutes), actives are released at a nonlinear rate which provides immediate availability of the active for absorption from the external phase and is then followed by a linear, diffusion-controlled rate. Eq.3 is only applicable to the linear, diffusion-controlled part of the graph.

The release kinetics of drugs from transdermal formulations, e.g. emulsions, can also be predicted by the octanol-water partition coefficient (log K_{ow}) (Simovic & Prestidge 2007:39). Drugs with a log K_{ow} < 9 were generally found to be rapidly released from conventional emulsions and lacked sustained release (Nishikawa *et al.*, 1998:99-118), whereas drugs with a high log K_{ow} > 9 showed a decreased release rate due to the retention of the drug in the emulsion droplets (Simovic & Prestidge, 2007:39).

With the stabilization of emulsions by solid particles, it is assumed that the solid particles have an effect on the release of drugs from Pickering emulsions. Some researchers investigated these effects of the solid particles on the release and compared it to conventional, surfactant-stabilized emulsions. Simovic and Prestidge (2007:39-47) investigated the effect of interfacial layers of silica nanoparticles on release of the lipophilic active ingredient, dibutyl phthalate (DBP), from oil-in-water-emulsions. At lower DBP loading levels, resulting in sink conditions in the release medium, sustained release was obtained, due to rigid interfacial multilayers of hydrophobic silica nanoparticles, when compared to non-coated droplets and droplets coated with permeable layers of hydrophobic or hydrophilic silica particles. However, at higher DBP loading levels, yielding no sink conditions, hydrophilic and hydrophobic permeable silica layers could significantly enhance release. Simovic and Prestidge (2007:44) indicated that in the presence of silica solid particles, the dissolution velocity and soluble drug fraction increased, yielding in the higher release. In another study, Frelichowska *et al.* (2009:7-15) investigated the differences in the release and permeation of caffeine from conventional w/o emulsions compared to Pickering emulsions, stabilized by silica particles. The Pickering emulsions showed a slower release of caffeine compared to conventional emulsions hypothesizing that the solid particles obstructed interfacial diffusion. However, the coverage of aqueous droplets with these silica particles resulted in sustained release of caffeine.

This study also indicated that even though the Pickering emulsions showed slower release than the conventional emulsions, they revealed a three-fold higher flux of caffeine through skin. (Frelichowska *et al.* (2009:7-15).

2.3 The effect of emulsifier on transdermal delivery

2.3.1 Modelling

Transdermal drug transfer is the process of which the drug is released from formulations, transported into the skin and eventually through the skin. This process is in some extent dependent on the vehicle properties (Bernardo & Saraiva; 2008:3781). The Higuchi model for the release of a drug from matrix system can be used or manipulated for modelling of transdermal delivery of a drug (Dash *et al.*, 2010:219-220).

Recently, Bernardo and Saraiva (2008:3781-3806) formulated a theoretical model for transdermal drug delivery from emulsions. Formulation heterogeneity and the prediction of transdermal delivery of drugs as function of emulsion composition are included in this model. It should, however, be noted that no provision is made for penetration enhancers. This model may be used to evaluate the differences in drug release because of resistance of interfacial layers on drug transfer (Bernardo and Saraiva, 2008:3805).

Grégoire *et al.* (2009:80-91) also proposed a model for the transport of drugs into and through the skin from cosmetic and dermatological formulations. The degree of ionization and the properties of the vehicle were taken into consideration for the development of this model. The following assumptions were made for this model:

- steady-state diffusion was applicable even with finite dosage forms;
- the penetration enhancing properties of the formulation excipients were insignificant;
- all vehicles could be approached as o/w emulsions and
- only the fraction of the drug dispersed in the continuous phase could be available for penetration into the skin

It should be noted that even though this model assumed that the penetrating enhancing effects of excipients are insignificant, some emulsifiers contain penetration enhancing effects. Results from this model were related to experimental data and showed a correlation of above 90% (Grégoire *et al.* 2009:89). However, it should be noted that some drugs may be available from both, the continuous phase as well as the dispersed phase.

Studies have shown that emulsifiers could affect dermal and transdermal delivery and the next chapter will illustrate some examples from literature. Since emulsions are multicomponent systems

and various emulsion excipients could contribute to the topical delivery, the main focus was set on studies with a more systematic approach i.e., studies investigating emulsions with the same oil and aqueous phase and only differing in the emulsifier component.

2.3.2 Solid particles

In a study reported by Frelichowska *et al.* (2009:7-15), a threefold higher flux of caffeine was observed form a Pickering emulsion when compared to a conventional emulsion. The transdermal delivery of caffeine through full-thickness porcine skin from o/w emulsions stabilized with silica particles were compared to conventional emulsions stabilized by Abil® EM 97 and Abil® Wax 9810. The higher flux was attributed to a higher adhesion of silica particle-stabilized water droplets onto the surface of the skin. In addition, it was hypothesized that caffeine could have been transported into the skin by means of adsorption onto the silica particles, which were found to penetrate into the upper layers of the *stratum corneum*. Ghouchi Eskandar *et al.* (2007:1764-1775) compared the dermal delivery of all-*trans*-retinol containing emulsions stabilized by solid particles (silica) as well as surfactants (lecithin or oleylamine) to non-silica coated emulsions using excised porcine skin. Targeted and enhanced transport of all-*trans*-retinol into the skin from silica coated o/w emulsions was found. It was found that the silica particles improved physical stability of the emulsion carrier and influenced the hydration of the skin barrier.

Another study, using Pickering emulsions with methyl salycilate as active ingredient and starch particles as emulsifier, evaluated the effect of three different oils, Miglyol, paraffin and shea nut oil, on topical delivery of the drug (Marku *et al.;* 2012:1). The study was conducted by use of porcine skin (500 µm) in flow-through cells. O/w emulsions with a percentage of oil phase as high as 56% v/v could be formulated. These emulsions showed high stability towards creaming or sedimentation, changes in droplet size and alteration in rheology properties for over 8 weeks. Marku *et al.* (2012:6) also indicated a doubling in flux of methyl salicylate when compared to previous studies. It was assumed that the reason for this was the adsorption of solid particles to the skin surface.

2.3.3 Emulsifier mixture HLB value

Various studies have examined the effect of the HLB value on dermal and transdermal delivery (Nam *et al.*, 2012:51; Cho *et al.*, 2012:1). Nam *et al.* (2012:55-56) evaluated the dermal delivery of tocopheryl acetate from, o/w emulsion, stabilized by a mixture of unsaturated phospholipids and polyethylene oxide-block-poly(ε-caprolactone) (PEO-*b*-PCL). Enhanced dermal delivery of tocopheryl acetate was found with the combination of unsaturated phospholipids and PEO-*b*-PCL. It was further suggested that the emulsifier contain penetration enhancing effects contributing to enhanced drug delivery.

Cho *et al.* (2012:1) described the dermal delivery of retinol from o/w emulsions which were stabilized by Tween 20 and biodegradable poly(ethylene oxide)-block-poly(ε-caprolactone)-blockpoly(ethylene oxide) (PEO-PCL-PEO) triblock copolymers having different lengths of the hydrophobic PCL block. By addition of the triblock copolymer and by increasing the PCL block length, the retinol transport into artificial skin could be enhanced (Cho *et al.*, 2012:6).

In a study conducted by Wu *et al.* (2001:63) it was indicated that surfactant mixtures with a low HLB value showed significantly higher permeation of inulin from w/o emulsions in comparison to surfactant mixtures with a high HLB value. The transport of inulin from w/o emulsions via the hair follicles (transfollicular route) was assumed to be increased, when the HLB value of the oil phase was compatible with the sebum environment.

2.3.4 Droplet charge

The charge of emulsion droplets should be considered when formulating topical emulsions as studies indicated that the charge could influence dermal and transdermal delivery of active ingredients. In literature, reports from Youenang Piemi *et al.* (1999:177-187) and Ghouchi Eskandar *et al.* (2009:1764-1775) indicated that positively charged emulsion droplets could enhance dermal and transdermal delivery. Ghouchi Eskandar *et al.* (2009:1771-1773) contributed the higher skin retention of actives to the positively charged oleylamine, interacting with the negatively charged skin, as well as to the penetration enhancing properties of oleylamine. It was assumed that electrostatic interactions between the negatively charged skin (Abdel-Mottaleb *et al.* 2012:4231) and positively charged emulsion droplets could increase dermal and transdermal delivery. Youenang Piemi *et al*, (1999:183-186) found that positively charged emulsion droplets enhanced the delivery of econazole and miconazole nitrate into and through the skin. It was suggested that positively charged emulsions droplets could promote skin absorption due to a superior binding of the positively charged droplets to the negatively charged skin surface.

2.3.5 Surfactant association structure

Skin permeation could be affected by the surfactant association structures formed in emulsions. Some surfactants may arrange in liquid crystalline structures in the aqueous phase when in excess which may contribute to the stabilisation of emulsions (Friberg & Solans, 1986:121). Otto *et al.* (2010:273-282) investigated the effect of liquid crystalline structures of five o/w emulsions on dermal and transdermal delivery. They found an enhanced dermal and transdermal delivery for octadecenedioic acid and hydroquinone from liquid crystalline emulsions compared to conventional o/w emulsion without liquid crystalline structures in the water phase. However, the emulsions containing salicylic acid showed no differences in the dermal and transdermal delivery with or without liquid crystalline structures. The variance in interactions between actives and surfactants

was assumed to be the reason for the differences in the dermal and transdermal delivery. In another study done by Brinon *et al.* (1998:1-11), it was also indicated that liquid crystalline structures increased the flux of actives. This was explained by the altering of the partitioning of the active between the skin and the formulations, as well as by the modified interactions between the surfactant and the *stratum corneum*. From these two studies it can be seen that differences in topical delivery may occur when interactions between actives and surfactants are involved; however, it also indicates that liquid crystalline structures could affect dermal and transdermal delivery.

2.3.6 Hydrophilic chain length of non-ionic surfactants

Studies by Förster *et al.* (2011: 858-872) and Oborska *et al.* (2004:35:42) indicated that the permeation was inversely related to the hydrophilic chain length of the non-ionic surfactants. The evaluation of three different polyoxyethylene cetostearyl ethers containing different oxyethylene chain lengths in o/w emulsions revealed a decrease in release of rutin and quercetin through liposome model membranes with increasing oxyethylene chain lengths (Oborska *et al.* 2004:35). These results could be explained by an increasing solubilization effect of the non-ionic surfactant micelles with increasing length of the oxyethylene chain, without an apparent interaction between the surfactants and the *stratum corneum* lipids (Dalvi & Zatz, 1981:89-93). Similar results were obtained by Förster *et al.* (2011:858-872), who found a decrease in penetration into the skin with a decrease in polyethyleneglycol chain length of the polar head groups when o/w emulsions were stabilized with various polyethyleneglycol ester type surfactants. It was explained by the change in partitioning behaviour between the skin and formulation as no indication of disruption of the stratum corneum lipid structure by the emulsions was observed.

2.4 Polymers for transdermal delivery systems with emulsifying properties

2.4.1 Introduction

According to Billany (2007:393), a list of approved emulsifying agents does not exist for the use in pharmaceutical industry. Anionic and cationic surfactants possess the potential for low chronic skin irritancy, whereas non-ionic surfactants are regarded as more safe for the formulation of dermal and transdermal formulations (Williams & Barry, 2012:133). Because of the skin irritancy of surfactants, research has also focussed on the use of polymers as emulsifying agents in topical emulsions. It is suggested that polymers, including synthetic polymers, such as hydroxypropyl methylcellulose, polyester and acrylic fibres, are non-irritant to the skin because of the low tendency for polymers to penetrate the skin (Valenta & Auner, 2004:286).

In recent years research has also been focused on biopolymers as suitable emulsifiers for pharmaceutical delivery systems. For example, it was shown that proteins could be used to

stabilize emulsions (Li *et al.*, 2010:38). However, because biopolymers are derived from natural sources; they may contain impurities which could increase the risk of contamination and bacterial growth. Nevertheless, the advantages of biopolymers are non-irritability to skin, lower toxicity, biocompatibility and biodegradability. The stability of protein-stabilized emulsions could be improved by the electrostatic deposition of oppositely charged polysaccharides (e.g. pectin, alginate, and carrageenan) onto the proteins (multilayer assembly) (Guzey and McClements, 2006:30; Ru *et al.*, 2009:399). Moreover, it has been reported that substances, encapsulated by biopolymers, could be protected from oxidation, chemical or enzymatic degradation (He *et al.*, 2011:521). In overall, this could result in new emulsion-based delivery systems, especially for active ingredients that possess adverse physicochemical properties like chemical or enzymatic instability.

A wide variety of biopolymers, e.g. proteins and polysaccharides, exists and the three biopolymers, included in this study, are discussed in more detail below. Furthermore, the layer-by-layer technique was applied in this study to formulate multi-layer emulsions and therefore, the technique is also described below.

2.4.2 Layer-by-layer self-assembly technique

Various techniques exist for the preparation of emulsions resulting in different emulsion characteristics, including droplet size of the emulsions. The layer-by-layer (LbL) self-assembly technique, for instance, can be used in the formulation of biosensors (Ram *et al.*, 2001:849), hollow polyelectrolyte capsules (Gao *et al.*, 2001:21) and emulsions (McClements, 2009:13-15). McClements (2009:10) indicated the possibility to produce multi-layered emulsions consisting of oppositely charged biopolymers adsorbed to each other at the oil-water interface, with improved stability against environmental stresses or controlled release properties. These multi-layered emulsions are prepared by a multiple-step process (Fig. 6). The first step includes the preparation of primary oil-in-water emulsions, containing a water-soluble ionic emulsifier. This is followed by the addition of another biopolymer, preferably a biopolymer with opposite charge than that of the primary emulsions, to yield multi-layered emulsions known as secondary emulsions. This process can be repeated to yield more layers around the emulsion droplets. Gu *et al.* (2005:5752) prepared emulsions containing β-lactoglobulin as primary layer, ι-carrageenan as second layer and gelatin as tertiary layer with better stability towards aggregation than single-layer emulsions. Ionic strength, pH and temperature may alter the characteristics and functions of biopolymers and hence the stability of resulting emulsions (McClements, 2009:6). Furthermore, coatings formed by different biopolymers will have altered physicochemical properties, such as droplet charge, thickness of the biopolymer layer at the oil-water interface, its permeability and environmental responsiveness, which concomitantly will lead to changes in emulsion characteristics, such as stability and delivery (Cho *et al*: 2008:2655).

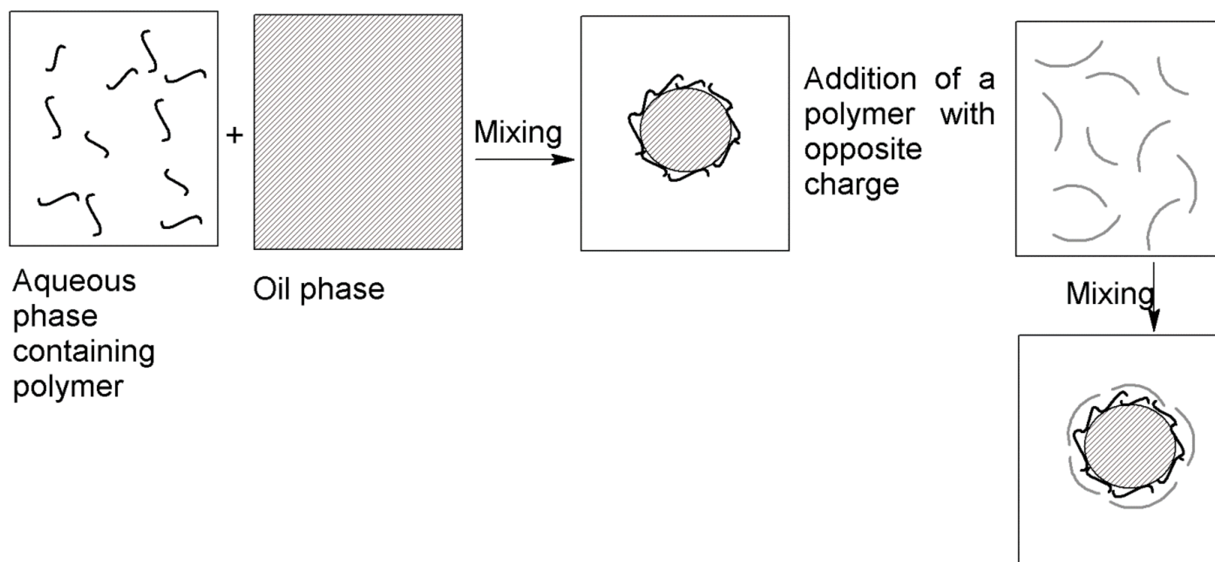

Fig. 6. Schematic presentation of the layer-by-layer self-assembly technique for the formation of multi-layered emulsions.

2.4.3 Whey proteins

Research on the stabilization of emulsions with whey proteins has been done, but mostly on oral emulsions in the pharmaceutical as well as the food industry (Bouyer *et al.*, 2012:359). Whey proteins are derived from milk, with two major proteins: *β*-lactoglobulin and *α*-lactalbumin (He *et al.*, 2011:522; Livney, 2010:74).

β-lactoglobulin is a small globular protein (M_w = 18.3 kDa), consisting of 162 amino acids with two disulphide bonds and one free cysteine group. At physiological pH, it mainly exists in the dimer form (M_w = 36.4 kDa) (Livney, 2010:74; Sawyer *et al.*, 1997:65). With an increase in temperature (above 80 °C), whey proteins tend to denature and increase the medium viscosity which may lead to increased stability (Ru *et al.*, 2009:402;He *et al.,* 2011:522).

The isoelectric point (p*I*) of whey proteins was reported to be p*I* ~5.2 (Ru *et al*, 2009:400; Bouyer *et al.*, 2012:365). He *et al.* (2011:525-532) found that whey proteins have better emulsifying capacities than traditional emulsifiers and whey protein-stabilized emulsions showed better resistance to gravitational separation. In a study done by Li *et al.* (2010:46), it was seen that with an increase in pH from 3.00 to 7.00, the droplet charge changed from positive to negative. At pH values close to the p*I* value of *β*-lactoglobulin, where the net charge of the droplets will approach zero, the emulsions showed instabilities towards droplet aggregation (Li *et al.*, 2010:46). It can be seen from these studies that even though whey proteins possess better emulsifying effects than conventional emulsifiers, whey proteins should rather be used in combination with polysaccharides to improve the emulsion stability. Polysaccharides may increase droplet surface charges, which

may lead to increased electrostatic repulsion between the droplets (Guzey & McClements, 2007:482)

2.4.4 Chitosan

Chitosan (Fig. 7), a cationic polysaccharide, is produced by deacetylation of chitin, which is found in the exoskeletal material of invertebrates (Kumar *et al*, 2004:6019; Lima *et al.*, 2012:322). It is used in a variety of fields, including food industry, cosmetics, agrochemicals and cell culture. Chitosan could be used as a gelling agent, emulsifying agent (Rodríguez *et al.*, 2002:271) and it also has penetration enhancing effects (Lima *et al.*, 2012:327). The pK_a value is ~6.5 resulting in a positive charge at pH values lower than pH 6.50 (Li *et al.*, 2010:39). Complexation of chitosan and *ß*-lactoglobulin in the aqueous phase at various pH values, as reported by Guzey and McClements (2006:130), could affect the stability of emulsions. For example, in a study by Li *et al.* (2010:39), it was found that *β*-lactoglobulin-chitosan-stabilized emulsions were more stable to droplet aggregation at pH values between 3.00 to 6.00, but less stable at pH values higher than 6, due to the loss of the positive charge of chitosan at pH > 6.0.

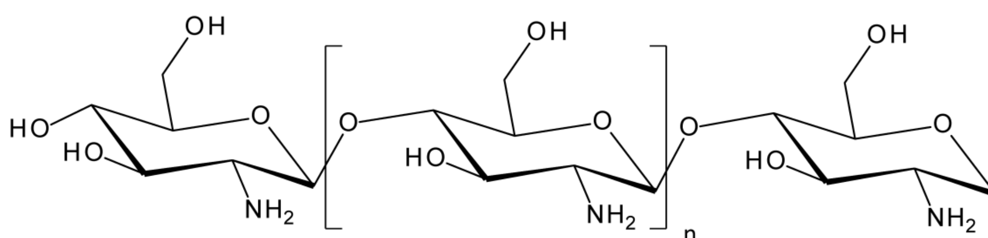

Fig. 7. Molecular structure of chitosan.

2.4.5 Carrageenan

Carrageenan is an anionic polysaccharide (Fig. 8) obtained from red algae (De Ruiter & Rudolph, 1997:391). Commercial carrageenan can be divided into three major groups (*κ*-, *ɩ*-, and *λ*-carrageenan) mainly differing in the amount of sulphate groups present and hence in the water solubility and gelling properties. As in the case of chitosan, carrageenan can also be used for increasing emulsion stability, thickening and gelling of food, as well as for pharmaceutical and cosmetic products (De Ruiter & Rudolph, 1997:389). Due to the sulphate groups present on the carrageenan and the pK_a value of ≈ 2 (Cho *et al.,* 2008:2655), carrageenan will be negatively charged at pH values higher than 2. Thus, all carrageenan that will be employed in dermal and transdermal delivery systems will always have a negative charge. Gu *et al.* (2004:3628-3631) conducted a study on the stabilization of emulsions containing *β*-lactoglobulin and *ɩ*-carrageenan. They observed the highest stability towards creaming at pH 6, most likely due to improved electrostatic repulsion between the emulsion droplets owing to highly charged droplet surfaces. Ru

et al. (2009:405) observed an increase in emulsions stability of emulsions containing *β*-lactoglobulin and *ι*-carrageenan at concentrations of carrageenan between 0.3-0.6 wt% at pH 4.00 whereas at pH 6.00, extensive flocculation occurred. The extensive flocculation at pH 6.00 was explained by the bigger droplet size possessing lower kinetic stability (Ru *et al.*, 2009:404)

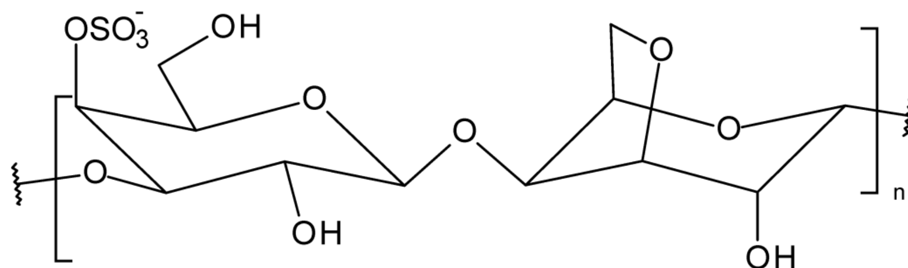

Fig. 8. Molecular structure of *κ*-carrageenan.

2.5 Conclusion

Many possibilities exist for the stabilization of emulsions (e.g. by surfactants, polymers or solid particles); however, these emulsifiers truly affect the release of actives from emulsions as well as the dermal and transdermal delivery of these actives. For example, the charge of emulsion droplets or the hydrophilic chain length of non-ionic surfactants could affect the topical delivery of actives. Solid particles that are used to stabilize emulsions, could also enhance dermal delivery. These factors should be kept in mind for optimal formulation of emulsion for topical delivery.

The use of polymers can be utilized to stabilize emulsions, however, not much is known on the effect of these polymers on release as well as on the dermal and transdermal delivery of actives.

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CHAPTER 3

Whey protein / polysaccharide-stabilized emulsions: Effect of polymer type and pH on release and topical delivery of salicylic acid

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ABSTRACT

Emulsions are widely used as topical formulations in the pharmaceutical and cosmetic industries. They are thermodynamically unstable and require emulsifiers for stabilization. Studies have indicated that emulsifiers could affect the topical delivery of active ingredients and this study was therefore designed to investigate the effects of different polymers, applied as emulsifiers, as well as the effects of pH on the release and topical delivery of the active ingredient. Oil-in-water emulsions were prepared by the layer-by-layer technique, with whey protein (whey) forming the first layer around the oil droplets, while either chitosan (Chi) or carrageenan (Car) were subsequently adsorbed to the protein at the interface. Additionally, the emulsions were prepared at three different pH values to introduce different charges to the polymers. The active ingredient,

salicylic acid, was incorporated into the oil phase of the emulsions. Physical characterization of the resulting formulations, i.e. droplet size, zeta potential, stability towards creaming and insoluble complex formation in the water phase, was performed. Release studies were conducted on the nine prepared emulsions, after which dermal and transdermal delivery studies were performed on the five most stable emulsions, by using Franz type diffusion cells and utilizing human, abdominal skin membranes. It was found that an increase in emulsion droplet charge could negatively affect the release of salicylic acid from these formulations. Contrary, positively charged emulsion droplets were found to enhance the dermal and transdermal delivery of salicylic acid from emulsions. It was hypothesized that electrostatic complex formation between the emulsifier and salicylic acid could affect its release, whereas electrostatic interaction between the emulsion droplets and skin could influence dermal/transdermal delivery of the active.

KEYWORDS

Emulsion, release, topical, transdermal, salicylic acid

3.1 INTRODUCTION

Penetration of active ingredients into and through the skin, respectively, is essential in the treatment of certain skin conditions, or to obtain systemic therapeutic effects. It is known that skin penetration not only depends upon the physicochemical properties of the active ingredient and the condition of the skin, but also on the design of the formulation in which the active ingredient is applied to the skin (1). Emulsions are widely used as topical formulations, due to their excellent solubilizing capabilities for lipophilic and hydrophilic active ingredients (2). However, because emulsions are thermodynamically unstable, they require emulsifiers to be stabilized. Various studies have shown that emulsifiers could affect dermal and transdermal delivery of actives from emulsions. Studies, in which the investigated emulsions had exactly the same composition, with variation only in the emulsifier component, showed that structure and physicochemical properties of emulsifiers, such as the hydrophilic-lipophilic balance (HLB) value, emulsifier charge, or solid particles *versus* surfactant, could affect skin absorption of the active. Youenang Piemi *et al.* (3), for

example, had found that positively charged emulsion droplets enhanced the delivery of econazole- and miconazole nitrates through and into the skin. It was hypothesized that positively charged emulsion droplets could promote skin absorption, due to a superior binding of the positively charged droplets to the negatively charged skin surface. Another study indicated that Pickering emulsions, which are solely stabilized by solid particles, could enhance percutaneous penetration. Frelichowska *et al.* (4) investigated the skin absorption of caffeine from silica particle-stabilized water-in-oil emulsions and compared it to the absorption from surfactant-stabilized emulsions. They observed a three-fold higher flux of caffeine from the Pickering emulsion, which was explained by a higher adhesion of the silica particle-stabilized water droplets onto the skin surface. In addition, it was hypothesized that caffeine may have been transported into the skin by means of adsorption onto the silica particles, which were found to penetrate into the upper layers of the stratum corneum. Studies by Förster *et al.* (5) and Oborska *et al.* (6) indicated that permeation was inversely related to the hydrophilic chain length of the non-ionic surfactants. These results could be explained by an increasing solubilization effect of the non-ionic surfactant micelles with increasing length of the oxyethylene chain, without an apparent interaction between the surfactants and the stratum corneum lipids (7).

In recent years, research has also focussed on biopolymers, derived from natural, renewable sources, as suitable emulsifiers for pharmaceutical delivery systems. Proteins and polysaccharides could, for example, be used to stabilize emulsions (8,9). According to He *et al.* (10), the improved stability of protein-stabilized emulsions relative to surfactant-stabilized emulsions could be a result of the higher surface potential of proteins. Furthermore, the layer-by-layer technique could be employed to produce multi-layered emulsions, consisting of oppositely charged biopolymers, adsorbed to each other at the oil-water interface, with improved stability against environmental stresses, or controlled release properties (11). Such multi-layered emulsions are prepared by a multiple-step process. First, primary oil-in-water emulsions are prepared with a water soluble ionic emulsifier, e.g. whey proteins. Secondary emulsions are subsequently formulated by adding another biopolymer (e.g. carrageenan or chitosan), preferably with an opposite charge than the droplets in the primary emulsions, to form multi-layered,

interfacial coatings. Ionic strength, pH and temperature may alter the characteristics and functions of biopolymers and hence the stability of resulting emulsions (11). In addition, coatings formed by different biopolymers would have altered physicochemical properties, such as droplet charge, thickness of the biopolymer layer at the oil-water interface, its permeability and environmental responsiveness, which concomitantly would lead to changes in emulsion characteristics, such as stability and delivery (12).

In this study, three different biopolymers were employed as potential emulsifiers, i.e. solely whey proteins (Whey emulsion), whey proteins layered with chitosan (Chi emulsion), and whey proteins layered with carrageenan (Car emulsion). Furthermore, three different pH values (pH 4, 5, and 6) were chosen for the emulsions in order to introduce different charges to the polymers. Whey proteins are derived from milk, comprising two major proteins, *β*-lactoglobulin and *α*-lactalbumin (13). Research on the stabilization of emulsions with whey proteins has been reported, but mostly in food science (8). It was found that whey proteins have a better emulsifying capacity than traditional emulsifiers and that *β*-lactoglobulin-stabilized emulsions showed higher resistance to gravitational separation (10). The isoelectric point (p*I*) of whey proteins was reported as p*I* ≈ 5.2 (8,14). At pH values higher than the p*I* (e.g. pH 6), the whey proteins have a negative charge, whereas at pH values below the p*I* (e.g. pH 4), they are positively charged. At pH 5, being close to the p*I* value, the whey proteins would have a net charge of around zero. Chitosan (Fig. 1) is a cationic biopolymer, produced by deacetylation of chitin, the skeletal material of invertebrates (15). The pK_a value of chitosan is ≈ 6.5 and therefore chitosan would be positively charged at all pH values chosen for this study (pH < 6.5). Chitosan has further shown to have emulsifying properties (16) and penetration enhancing effects (17). Different states of complexation between chitosan and *ß*-lactoglobulin in the aqueous phase at various pH values were reported (18) that could affect emulsion stability. In a study by Li *et al.* (9), for example, it was found that *β*-lactoglobulin-chitosan-stabilized emulsions were more stable to droplet aggregation at pH values between 3 – 6, but less stable at pH values higher than 6, due to the loss of the positive charge of chitosan at pH > 6. In contrast, carrageenan (Fig. 1) is an anionic polysaccharide obtained from red algae (19). The three main commercial carrageenans are *κ*-, *ι*-, and *λ*-carrageenan, which mainly differ in the

amount of sulfate groups present and hence in their water solubility and gelling properties. Carrageenan is also utilized for increasing the stability, thickening and gelling of food, pharmaceutical and cosmetic products (19). The negative charge of the anionic carrageenan is due to the sulfate groups present and with a pK_a value of ≈ 2 (12), carrageenan would be negatively charged at all pH values used throughout this study (pH > 2). A study by Ru *et al.* (14) indicated that stable emulsions could be obtained with a combination of *β*-lactoglobulin and *ι*-carrageenan at pH values of 3.4 and 4, depending on the protein/polysaccharide ratio used. Contrary, it was found that extensive flocculation occurred at pH 6. A different trend in *β*-lactoglobulin-*ι*-carrageenan-stabilized emulsions was observed during a study by Gu *et al.* (20), where the most stable emulsions towards creaming were found at pH 6, most likely due to improved electrostatic repulsion between the emulsion droplets, owing to highly charged droplet surfaces.

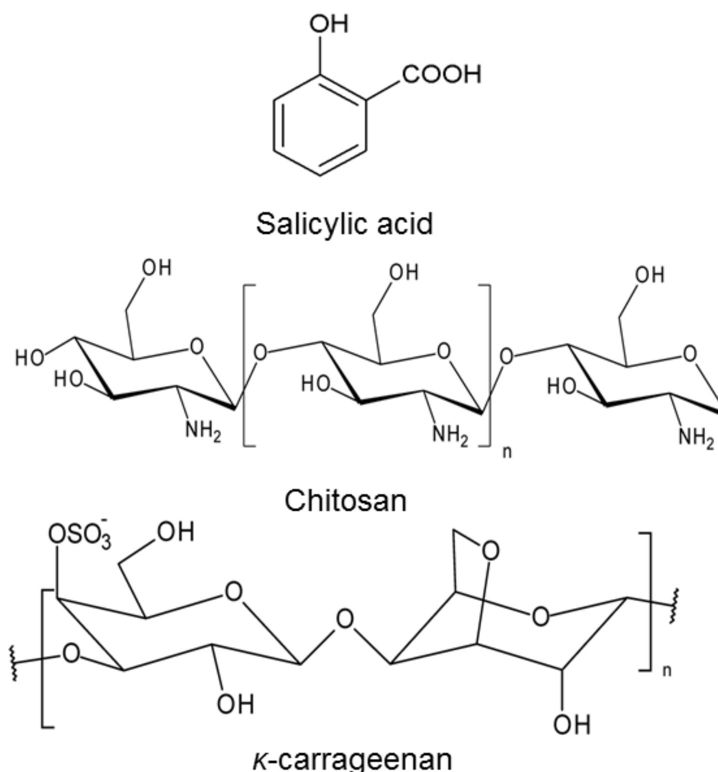

Fig 1. Structures of salicylic acid, chitosan and *κ*-carrageenan.

This study was designed to evaluate the release and the topical delivery of salicylic acid-containing emulsions, by utilizing whey proteins as emulsifier, solely and in conjunction with chitosan or carrageenan at different pH values. Characterization of the various emulsions was performed, i.e. droplet size, zeta potential and viscosity, to further investigate the effects of the various polymers and pH on the release of the active and also on the dermal and transdermal delivery thereof.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Whey protein isolate (BiPro®) was kindly donated by Davisco Foods International (Le Sueur, MN, USA). As per the manufacturer, the whey protein isolate powder comprised at least 97% of dry basis protein, with the main proteins being *β*-lactoglobulin and *α*-lactalbumin. Miglyol 812 N® was kindly donated by Cremer (Hamburg, Germany). Carrageenan (commercial grade, type I, predominantly *κ*-carrageenan, lesser amounts of *λ*-carrageenan) and chitosan glutamate were purchased from Sigma Aldrich (Johannesburg, RSA) and CarboMer (San Diego, CA, USA), respectively. Salicylic acid (99+%) was acquired from SAFC (St. Louis, MO, USA). Potassium chloride and citric acid anhydrous were also purchased from Sigma Aldrich (Johannesburg, RSA). Sodium dihydrogen orthophosphate, di-sodium hydrogen orthophosphate anhydrous, propylene glycol, 1 N hydrochloric acid (HCl), 1 N sodium hydroxide (NaOH) and methanol were purchased from ACE Chemicals (Johannesburg, RSA). **Acetonitrile** (LiChrosolv®, gradient grade for liquid chromatography) and acetic acid (glacial) were acquired from Merck Chemicals (Johannesburg, RSA).

3.2.2 Aqueous and Oil Phase Preparation

The buffer solution used for the preparation of the aqueous phase of emulsions was prepared by dissolving 0.01 mol of citric acid and 0.02 mol of dibasic sodium phosphate in deionized water. The solutions were mixed in order to attain citrate-phosphate buffer solutions at pH 4, 5 and 6. The whey protein solution was then prepared by dispersing 3.5% *wt.* whey protein powder into citrate-phosphate buffer on a mixing plate at room temperature for approximately two hours to

ensure complete hydration. 1% *wt.* chitosan glutamate and 0.5% *wt.* carrageenan were separately dissolved in citrate-phosphate buffer at 60°C and the resultant solutions were allowed to cool to room temperature.

The oil phase consisted of 2% *wt.* salicylic acid in Miglyol 812 N®. Salicylic acid was left to dissolve in Miglyol 812 N® overnight at 37°C.

3.2.3 Emulsion Preparation

The emulsions were prepared in two phases. First, primary emulsions were prepared by ultrasonication for 30 seconds (Model UP200St, Hielscher Ultrasonics, Teltow, Germany) at room temperature to yield 30% *wt.* oil-in-water emulsions. The aqueous phase used for the primary emulsion was the 3.5% *wt.* whey protein solution. The secondary emulsion was then prepared by diluting the primary emulsion 1:1 (*v/v*), either with pure citrate-phosphate buffer, or 1% chitosan solution, or 0.5% carrageenan solution byultrasonication for 30 seconds at room temperature. After the secondary emulsion was prepared, the pH of the emulsion was adjusted to pH 4, 5 and 6 by adding 1 N HCl or NaOH. These emulsions were then again sonicated for 30 seconds to yield the final formulations. The compositions of the final emulsions are listed in Table I. An oil solution, containing the same concentration of salicylic acid as the final emulsions, was prepared as a control formulation.

Table I. Composition of secondary emulsions and the oil solution

Ingredient % *wt.*	**Whey protein (Whey)**	**Whey protein / chitosan (Chi)**	**Whey protein / carrageenan (Car)**	**Oil solution (Oil)**
Oil phase				
Salicylic acid	0.3	0.3	0.3	0.3
Miglyol 812 N®	14.7	14.7	14.7	99.7
Water phase				
Whey protein	1.225	1.225	1.225	–
Chitosan glutamate	–	0.5	–	–
Carrageenan	–	–	0.25	–
Water	83.775	83.275	83.525	–

3.2.4 Particle Size Analysis

Droplet size was determined by using the Malvern Mastersizer 2000, equipped with a wet cell Hydro 2000 SM dispersion unit (Malvern Instruments, Worcestershire, UK). The emulsions were diluted with deionized water to yield obscuration values between 10 - 20%. Particle size measurements were performed on days 0 (day of preparation), 1 and 7 to determine the changes in particle size over time. Measurements were taken from two freshly prepared samples per emulsion, with two readings made per sample.

The particle size was confirmed on day 0 by means of microscopy. A Motic microscope (Motic, Hong Kong) was used, equipped with a Moticam 3 camera and Motic Images Plus 2.0 software.

3.2.5 Zeta Potential Measurements

The zeta potential was determined, using a Malvern Zetasizer 2000 (Malvern Instruments, Worcestershire, UK). Prior to analysis, emulsions were diluted 1:3,000 (*v/v*) with citrate-phosphate buffer at the appropriate pH. Zeta potential measurements were taken on day 0 from one prepared sample per emulsion, with three readings per sample.

3.2.6 Viscosity Measurements

The apparent viscosity was determined with a Brookfield DV-II+ Viscometer + (Middleboro, MA, USA), equipped with a LV1 spindle. Measurements were taken at 100 rpm every 10 seconds over a time period of 100 seconds at room temperature. The average of 10 readings taken for each emulsion was calculated. The percentage torque was recorded between 7.8% - 28.9%.

3.2.7 Creaming Stability

Three ml of each emulsion was transferred into a separate test tube and sealed with plastic wrap. These samples were kept at room temperature for one week and the height of the emulsion and of the creaming layer were measured after 7 days.. The extent of creaming was assessed by using the creaming index (*CI*), which was calculated as per Equation 1:

$$CI = \frac{Height\ of\ creaming\ layer}{Total\ heigth\ of\ emulsion} \times 100\%$$ Eq. 1

3.2.8 Turbidity

The aqueous phases of the different emulsions, as well as aqueous solutions of pure salicylic acid and of the different pure biopolymers at pH 4, 5 and 6 were subjected to turbidity measurements. The samples were analyzed using a UV-VIS spectrophotometer (Specord 200 Plus, Analytic Jena, Germany) at 600 nm. Two readings were performed per sample.

3.2.9 Release of Active from the Formulations

The release of the active ingredient from all nine emulsions, as well as from the oil solution were determined, using Franz type diffusion cells having a diffusion area of 1.13 cm^2 and cellulose nitrate membranes (0.2 µm pore size, Whatman, Dassel, Germany). The release experiments were conducted at 37 ± 1°C in a heated water bath for 8 hours. The membranes were soaked overnight in the receptor phase, consisting of phosphate buffer pH 7.4 and propylene glycol (PG) (1:1, *v/v*). 50% of PG was included into the receptor phase to increase the solubility of salicylic acid therein to ensure sink conditions throughout the duration of the study. The receptor compartment was filled with 2 ml of preheated and degassed receptor phase and left to equilibrate before adding the donor phase. The donor compartment was filled with 1 ml of emulsion. The receptor fluid was stirred with a magnetic stirrer at 750 rpm. Extractions were taken at 0.5, 1, 1.5, 2, 3, 4, 6 and 8 hours. The samples were analyzed by HPLC.

3.2.10 Skin Preparation

White female abdominal skin was obtained from cosmetic surgeries. The utilization of human skin was approved by the Ethics Committee of the North-West University, Potchefstroom, South Africa (Ethics number: NWU-00114-11-A5) and skin was obtained with the informed consent of the donors. The skin was thawed at room temperature. Split thickness skin (400 µm), containing stratum corneum, viable epidermis and upper dermis, was removed with an electric dermatome (Zimmer Inc. Warsaw, IN, USA). The skin membrane was then placed with stratum corneum

facing upwards on filter paper, wrapped in aluminium foil and frozen at -20°C until use. Prior to the diffusion experiments, the skin membrane was thawed at room temperature and cut into the required size (circular pieces of approximately 1.5 cm in diameter). The time from skin collection to use of the skin did not exceed six months.

3.2.11 In Vitro Skin Absorption Study

A randomized, complete block design was used to assess the effect of skin donor variability, as well as the effect of formulation on dermal and transdermal delivery. Three different skin donors were used per formulation and each formulation was applied in duplicate on each donor skin, giving a total of six replicates per formulation.

Based on the results of the release and emulsion characterization data, five formulations, plus the oil solution were selected for the skin diffusion study. Whey emulsions at pH 4, 5, and 6, the Chi emulsion at pH 6 and the Car emulsion at pH 6 were chosen. Skin diffusion studies were performed using Franz type diffusion cells with a diffusion area of 1.13 cm^2. The study was conducted at 37 ± 1°C in a heated water bath for 24 hours. Prior to the skin diffusion study, the skin integrity was assessed by measuring electric resistance across the skin. For skin resistance, the donor and the receptor compartments were both filled with a 0.9% potassium chloride solution and placed in the heated water bath for an equilibration period of 30 minutes. Electrical resistance was measured by using a Tinsley LCR Databridge Model 6401 (Tinsley Precision Instruments, Croydon, UK). The reading was determined at 1 kHz with a maximum voltage of 300 mV root-mean-square in the parallel equivalent circuit mode, using an alternating current (21). Skin samples with resistance values below 10 kΩ were rejected from the study. After completion of the resistance measurements, both the donor and the receptor compartments were emptied. The receptor compartment was filled with 2 ml of preheated and degassed receptor phase (phosphate buffer pH 7.4 and propylene glycol (PG) (1:1, *v/v*)) and left for 30 minutes to equilibrate before adding the donor phase. The donor compartment was filled with 1 ml of emulsion. The receptor fluid was stirred, using a magnetic stirrer at 750 rpm. Receptor samples were taken at times 3, 6, 9, 12, 18 and 24 hours. The samples were analyzed by HPLC.

3.2.12 Skin Sample Preparation

After completion of the 24 hour diffusion studies, the emulsions were removed from the donor compartment and the skin membranes removed from the cells and pinned onto filter paper with the stratum corneum facing upwards. The remainder of the emulsion was removed by gently dabbing the skin surface with paper towel. Sixteen strips of 3M Scotch® Magic™ tape, of which the first strip was discarded, were used to remove the stratum corneum. The remainder of the skin after the tape stripping was cut into pieces to increase the surface area for extraction. The tape strip samples as well as the cut skin pieces were separately placed into methanol in order to extract the salicylic acid over a period of at least 12 hours. The samples were then vortex mixed and filtered through hydrophilic PVDF pre-filters with a pore size of 0.45 µm (Agela Technologies Inc., Wilmington, DE, USA) and subsequently analyzed by HPLC.

3.2.13 Statistical Analysis

Data from the release study was analyzed by means of one-way analysis of variance (ANOVA) and the data from the skin absorption study was analyzed by two-way ANOVA, using STATISTICA® (StatSoft Inc., Tulsa, OK, USA). For the skin absorption results, the formulation was employed as fixed effect and the skin donor as random effect. Subsequently, Tukey's Honestly Significant Difference (HSD) test was performed on both data sets to compare the various formulations with each other and data with $p < 0.05$ were considered statistically significant.

3.2.14 HPLC-UV Method

HPLC analysis of salicylic acid was performed using an Agilent® 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a high performance, silica based, reversed phase C18-2 column (150 x 4.60 mm) with 5 µm particle size (Venusil XBP Agela Technologies, Wilmington, DE, USA), in a controlled laboratory environment at 25°C. This instrument was equipped with a G1311A quaternary pump, G1313A autosampler and a G1315A multi wavelength, diode array detector. The mobile phase consisted of 1% acetic acid, 45% acetonitrile and 54% Milli-Q® water and was degassed after preparation. Flow rate was set at 1 ml

min^{-1} with a run time of approximately 6 minutes. The UV-detector was set at 236 nm. All samples were injected in duplicate. Chromatograms were analyzed, using ChemStation Rev. A.10.03 software.

3.3 RESULTS

3.3.1 Characterization of Emulsions

The data obtained from the zeta potential analysis, droplet size measurement, viscosity and creaming index (*CI*) are summarized in Table II. The data illustrate that within each emulsion set (Whey, Chi and Car emulsions) the zeta potential decreased with increasing pH. Furthermore, the addition of chitosan to the whey protein emulsions resulted in increased zeta potentials at all pH values, whereas the addition of carrageenan decreased the zeta potential. The results of the droplet size analyses were presented as volume weighted means (D [4,3]) and surface weighted means (D [3,2]). Table II shows that the Chi emulsions initially yielded the largest droplets (with volume weighted means between 25 – 46 µm on day 0), followed by whey and Car emulsions (with volume weighted means between 2 – 3 µm). The droplet size of the whey emulsions at pH 4 and 6, the Chi emulsion at pH 6 and all Car emulsions did not change significantly over the time period of 7 days. All other emulsions exhibited an increase in droplet size over time. Furthermore, shortly after preparation of the emulsions, the droplet size data obtained by laser light scattering, were confirmed microscopically (Fig. 2). The microscopy images of the whey emulsions at pH 4, the Car emulsion at pH 4 and all of the Chi emulsions showed aggregation of emulsion droplets. From the creaming index (*CI*) it could be seen that the whey emulsions at pH 4 and 6 and the Chi emulsion at pH 6 revealed the best stability against creaming. The apparent viscosity indicated that all nine formulations had similar, very low viscosity values.

Table II. Physical characterization of emulsions containing salicylic acid presented as average ± S.D.

	Whey			Chi			Car		
	pH 4	**pH 5**	**pH 6**	**pH 4**	**pH 5**	**pH 6**	**pH 4**	**pH 5**	**pH 6**
Zeta Potential[a] [mV]									
With Sal	23.9 ± 0.8	3.8 ± 1.8	-8.6 ± 1.4	32.4 ± 2.0	21.6 ± 0.8	14.9 ± 3.6	15.5 ± 0.8	-13.0 ± 1.2	-32.5 ± 12.2
Without Sal	23.7 ± 0.5	6.4 ± 2.2	-9.7 ± 0.6	26.7 ± 1.3	20.7 ± 1.5	9.5 ± 3.1	22.5 ± 0.3	-18.7 ± 5.5	-21.0 ± 2.1
Particle Size[b] [µm]									
Day 0									
D [4,3]	2.61 ± 0.09	2.85 ± 0.06	2.47 ± 0.01	25.77 ± 3.21	46.07 ± 1.28	38.34 ± 3.01	3.09 ± 0.03	2.54 ± 0.02	2.39 ± 0.06
D [3,2]	1.85 ± 0.02	2.08 ± 0.02	1.80 ± 0.01	5.19 ± 0.41	5.84 ± 0.08	14.37 ± 0.83	2.31 ± 0.03	1.99 ± 0.02	1.90 ± 0.06
Day 1									
D [4,3]	3.63 ± 1.12	94.13 ± 2.87	2.50 ± 0.03	46.77 ± 4.47	70.69 ± 7.32	36.36 ± 0.68	9.09 ± 4.92	16.22 ± 12.1	7.71 ± 6.18
D [3,2]	1.82 ± 0.02	21.68 ± 1.01	1.82 ± 0.02	7.66 ± 0.13	11.45 ± 0.08	13.76 ± 0.20	2.50 ± 0.09	2.12 ± 0.12	2.07 ± 0.13
Day 7									
D [4,3]	3.69 ± 1.21	93.64 ± 6.71	2.38 ± 0.03	47.23 ± 4.85	58.02 ± 3.00	37.25 ± 1.57	7.64 ± 4.95	2.69 ± 0.04	2.26 ± 0.01
D [3,2]	1.83 ± 0.04	18.02 ± 2.92	1.79 ± 0.02	7.61 ± 0.19	10.60 ± 0.36	13.69 ± 0.30	2.46 ± 0.10	1.99 ± 0.01	1.74 ± 0.01
CI[c] [%]	0	47.3	0	57.1	Flocs	0	73.2	83.9	91.1
Viscosity[d] [mPa s]	7.88 ± 0.03	19.24 ± 0.71	4.69 ± 0.04	17.16 ± 0.09	5.99 ± 0.02	13.92 ± 0.08	13.65 ± 0.03	13.31 ± 0.09	10.18 ± 0.06

[a] n = 3; [b] n = 4; [c] determined after 7 days; [d] n = 10

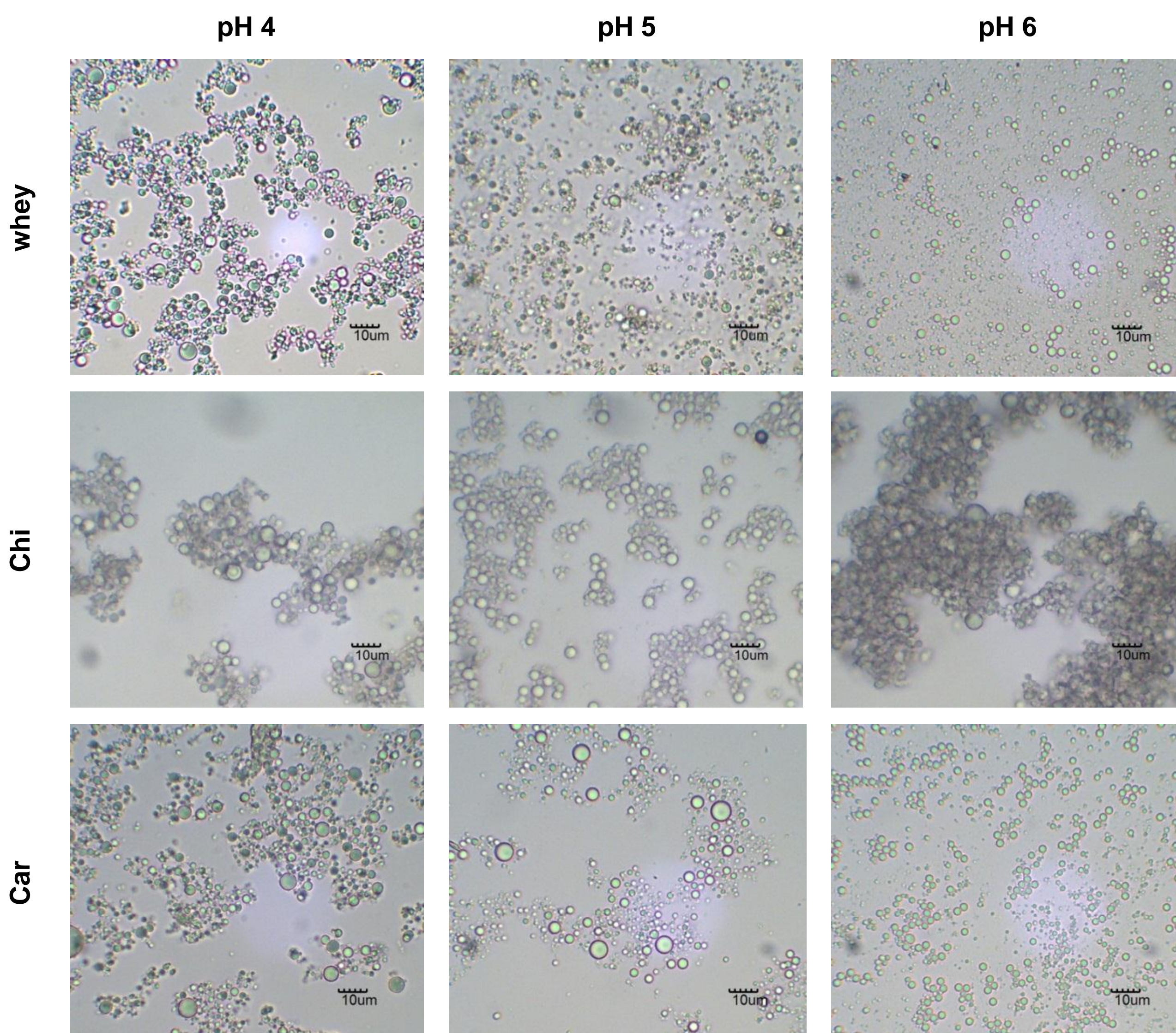

Fig. 2. Light microscopy images of whey, Chi and Car emulsions at pH 4, 5 and 6. The scale bar in each photograph represents 10 μm.

3.3.2 Turbidity

Data from the turbidity measurements are presented in Table III. They illustrate that the highest turbidity for the aqueous solution of whey proteins was obtained at pH 5, whereas the turbidity of the chitosan and the carrageenan solutions increased with pH. In addition, with an increase in pH, the insoluble complex formation between whey protein and chitosan increased. Contrary, the insoluble complex formation between whey protein and carrageenan decreased with increasing pH. The absorbance value for the whey-carrageenan solution at pH 4 was very high (> 9). However, the samples were not diluted and the original polymer concentrations in the various aqueous solutions were maintained in order to enable a comparison between them. It was further

noted that the addition of salicylic acid to the aqueous solutions of the various emulsifiers (either solely whey, whey + Chi, or whey + Car) did not result in a significant change in turbidity, except for whey-Chi at pH 4 and whey-Car at pH 6.

Table III. Turbidity (absorbance values at 600 nm) of aqueous solutions presented as average ± S.D. (n = 2).

Turbidity [cm^{-1}]	pH 4	pH 5	pH 6
Sal	0.008 ± 0.001	0.300 ± 0.002	0.339 ± 0.007
Whey	0.745 ± 0.007	2.430 ± 0.003	0.437 ± 0.001
Whey + Sal	0.749 ± 0.0435	2.464 ± 0.011	0.469 ± 0.017
Chi	0.067 ± 0.013	0.945 ± 0.002	1.331 ± 0.022
Whey + Chi	0.302 ± 0.014	1.674 ± 0.039	2.990 ± 0.003
Whey + Chi + Sal	1.878 ± 0.017	2.720 ± 0.023	2.825 ± 0.001
Car	0.026 ± 0.001	0.319 ± 0.002	0.396 ± 0.004
Whey + Car	> 9	4.335 ± 0.487	0.450 ± 0.002
Whey + Car + Sal	> 9	3.789 ± 0.141	3.310 ± 0.163

3.3.3 Release of Active from the Formulations

All release samples taken at the different time points had salicylic concentrations below 10% of the solubility of salicylic acid in the receptor phase (c_s = 31 mg ml^{-1}), which indicated that sink conditions in the receptor phase were maintained throughout the release study.

The results of the release study were divided into two groups, i.e. the effect of pH of each emulsion group (Fig. 3) and the effect of polymer at each pH value (Fig. 4). The results of the emulsions were also compared to the oil solution containing the same concentration of salicylic acid as the emulsions. The cumulative amount of salicylic acid released per surface area was plotted against the square root of time, according to Equation 2, which represents the simplified Higuchi model.

$$f_t = Q = K_H t^{1/2} \qquad \textit{Eq. 2}$$

where f_t is the cumulative amount of salicylic acid released per surface area, K_H is the Higuchi dissolution constant and $t^{1/2}$ represents the square root of time. The release rate K_H was calculated from the linear portion ($t_{0.5h} - t_{8h}$) of the correlation between the cumulative amount of released salicylic acid per surface area and square root of time. Calculated release rates and regression coefficients are presented in Table IV. As illustrated by Fig. 3 and 4, as well as by the regression coefficients in Table IV, the amount of salicylic acid released per surface area showed a linear relationship with the square root of time ($R^2 \geq 0.99$) for all formulations tested according to the Higuchi model.

During the first set of experiments, in which the effect of pH (Fig. 3) on the release of salicylic acid from the emulsions was investigated, it was found that for all three emulsion types, the release of salicylic acid was similar at pH 5 and 6, whilst the emulsions at pH 4 consistently obtained a statistically significantly lower release than those at pH 5 and 6 ($p < 0.05$). The second set of results, representing the effect of polymer (Fig. 4) on the release of the active, showed no differences between the different emulsion types at pH 4 and 5. However, at pH 6 the polymer affected the release of salicylic acid, i.e. the addition of carrageenan resulted in an increase, whereas the addition of chitosan reduced the release of salicylic acid from the emulsions. The differences between the Chi and Car emulsions at pH 6 were statistically significant ($p = 0.019$). It was also noted that the cumulative release of salicylic acid from the oil solution (ca. 24% salicylic acid in 8 hours) was similar to those from the various emulsions at pH 4 (ca. 23%), but lower in comparison with the emulsions at pH 5 (32 - 35%) and pH 6 (25 - 31%).

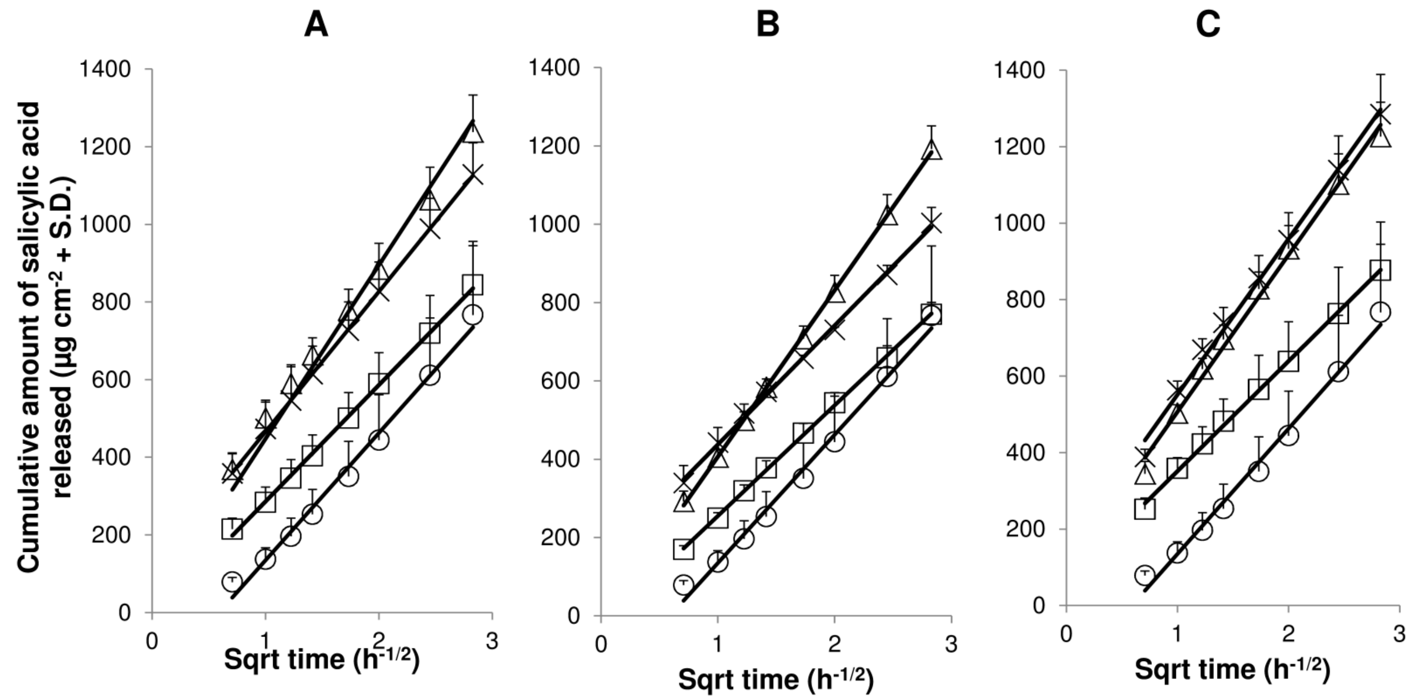

Fig. 3. *In vitro* release of salicylic acid through cellulose nitrate membranes for (A) whey emulsions, (B) Chi emulsions and (C) Car emulsions. Effect of pH: (□) pH 4, (∆) pH 5 and (X) pH 6. The oil solution (○) served as control. The release data are presented as linear fit against square root of time (according to simplified Higuchi equation). Average ± S.D. (n = 4).

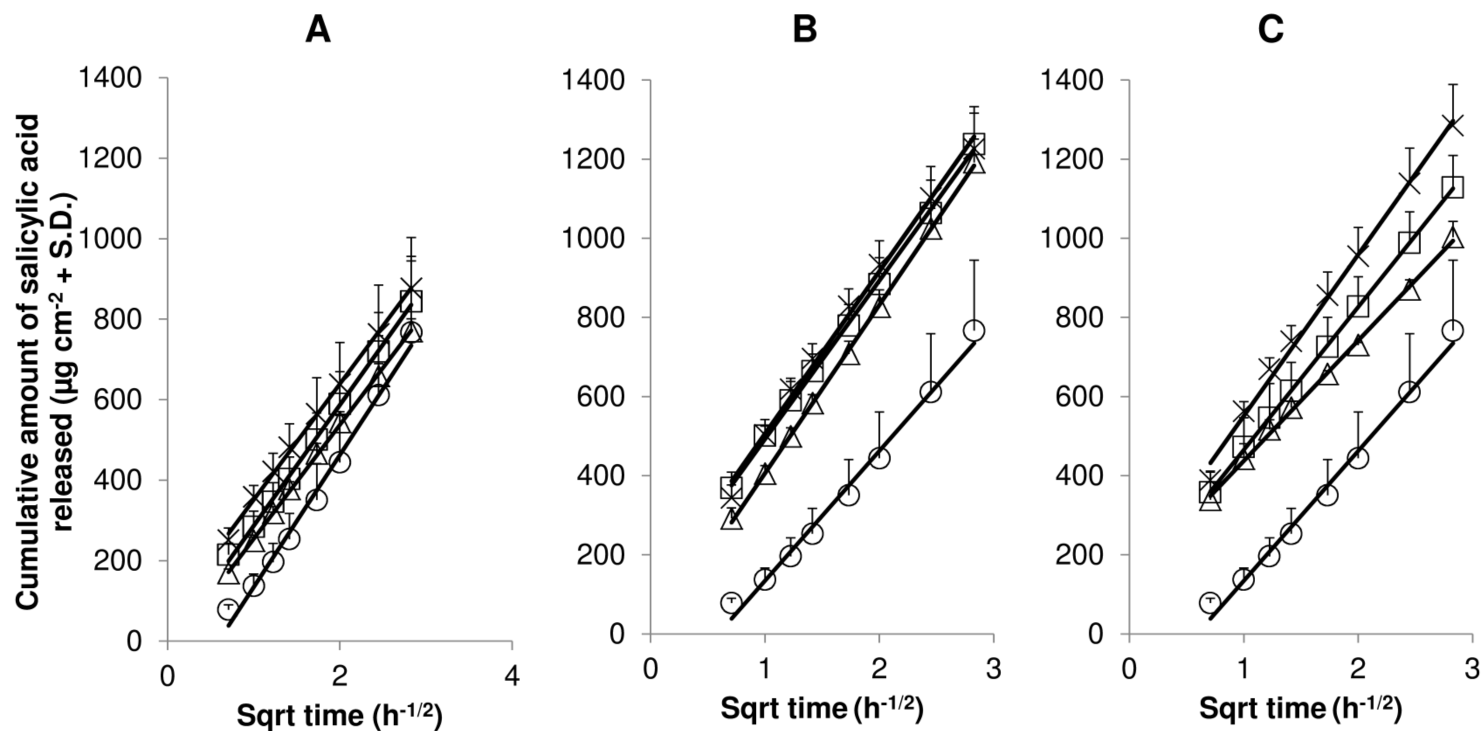

Fig. 4. *In vitro* release of salicylic acid through cellulose nitrate membranes for emulsions at (A) pH 4, (B) pH 5 and (C) pH 6. Effect of polymer: (□) whey, (∆) Chi and (X) Car. The oil solution (○) served as control. The release data are presented as linear fit against square root of time (according to simplified Higuchi equation). Average ± S.D. (n = 4).

Table IV. Release rate and cumulative release in 8 hours through cellulose nitrate membranes presented as average ± S.D. (n = 4) and steady state flux through human skin presented as least square means (LSM) (formulation effect) ± 95% confidence interval (C.I.) (n = 6).

		***In vitro* release through cellulose nitrate membranes**			***In vitro* steady state flux trough human skin**		
		k_H[a] [µg cm^{-2} h$^{-1/2}$]	**R^2**	**Cumulative release in 8 h[a] [%]**	**J_{SS}[b] [µg cm^{-2} h^{-1}]**		**R^2**
					LSM	**95% C.I.**	
whey	**pH 4**	300.35 ± 42.78	0.99	22.6 ± 2.6[3]	10.87[7]	7.72 – 15.29	0.99
	pH 5	400.48 ± 46.94	0.99	34.6 ± 2.3	0.75[8]	0.54 – 1.06	0.98
	pH 6	361.15 ± 12.75	0.99	30.4 ± 1.9	0.26	0.18 – 0.37	0.95
Chi	**pH 4**	282.80 ± 10.59	1.00	22.9 ± 0.8[4]		n.d.	
	pH 5	425.40 ± 33.90[1]	1.00	33.8 ± 1.5		n.d.	
	pH 6	303.98 ± 33.93	0.99	25.7 ± 0.9	0.75[9]	0.54 – 1.06	0.98
Car	**pH 4**	287.73 ± 48.97[2]	0.99	22.7 ± 2.8[5]		n.d.	
	pH 5	410.58 ± 35.29	0.99	32.0 ± 2.0		n.d.	
	pH 6	407.73 ± 44.63	0.99	31.2 ± 2.2[6]	0.17	0.12 – 0.24	0.97
Oil	**-**	328.13 ± 70.30	0.99	23.9 ± 4.8	1.16	0.83 – 1.64	0.98

[a] n = 4; [b] n = 6; n.d. not determined

[1] $p < 0.05$ against Chi pH 4 & 6; [2] $p < 0.05$ against Car pH 5 & 6

[3] $p < 0.05$ against whey pH 5 & 6; [4] $p < 0.05$ against Chi pH 5 & 6; [5] $p < 0.05$ against Car pH 5 & 6; [6] $p < 0.05$ against Chi pH 6

[7] $p < 0.05$ against whey pH 5 & 6; [8] $p < 0.05$ against whey pH 4 & 6; [9] $p < 0.05$ against whey & Car pH 6

3.3.4 In Vitro Skin Absorption

The results of the skin absorption experiments are depicted in Fig. 5 and 6 and are expressed as the total amount of active delivered over 24 hours of permeation as least square means (formulation effect) ± 95% confidence interval (C.I.) obtained after two-way ANOVA. As with the release data, the results of the skin absorption study were also divided into two groups, i.e. to investigate the effect of pH of whey emulsions (Fig. 5) and to study the effect of polymers at pH 6 (Fig. 6). Furthermore, the steady state flux was calculated from the linear portion of the permeation plots (cumulative amount of salicylic acid permeated per surface area plotted against time) between t_{3h} – t_{24h} and their calculated values, together with the regression coefficients, are summarised in Table IV.

The first set of results, investigating the effect of pH on topical delivery (Fig. 5), showed that the pH significantly influenced skin absorption of salicylic acid, showing a decline in skin absorption with increasing pH. Statistical analysis revealed that dermal and transdermal delivery were statistically significantly different among all pH values ($p < 0.05$). When comparing the oil solution with the whey emulsions at various pH, it was ascertained that the dermal and transdermal delivery of the active from the oil solution were similar to those of the emulsion at pH 5, which were much lower than at pH 4, but higher than at pH 6. It was further found that the trend for dermal and transdermal delivery was similar for the various whey emulsions at different pH.

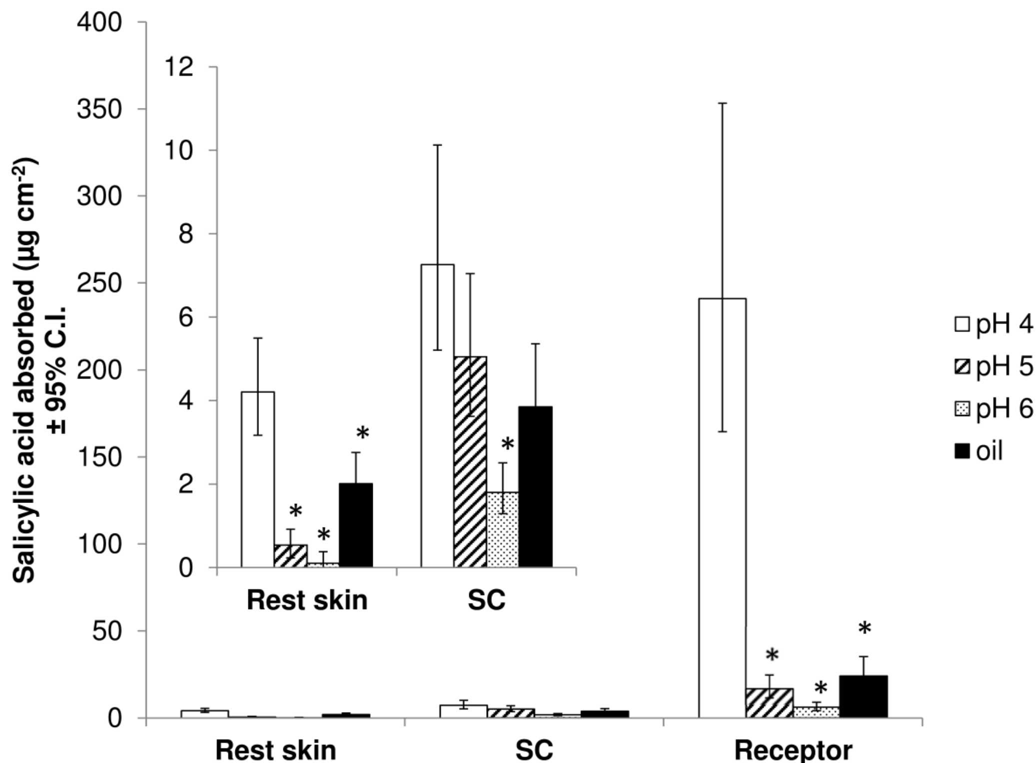

Fig. 5. *In vitro* skin absorption data of salicylic acid from whey emulsions through human abdominal skin, expressed as total amount delivered over 24 hours as least square means (formulation effect) ± 95% confidence interval (C.I.), obtained after two-way ANOVA (n = 6). The graph represents the effect of pH on skin absorption. The oil solution served as control.

However, in the second set of results, in which the effect of different polymers on topical delivery of salicylic acid was compared, the trend for dermal and transdermal delivery was different. The Chi emulsion statistically significantly enhanced permeation of the active through the skin (p = 0.009 against whey and p = 0.0002 against Car), whereas the Car emulsion statistically significantly increased the delivery of salicylic acid into the viable epidermis (p = 0.0009 against whey and p = 0.003 against Car). In addition, the oil solution resulted in the highest dermal and transdermal delivery, compared to the various emulsions. However, there was no statistically significant difference in transdermal delivery between the oil solution and the Chi emulsion, nor in dermal delivery between the oil solution and the Car emulsion.

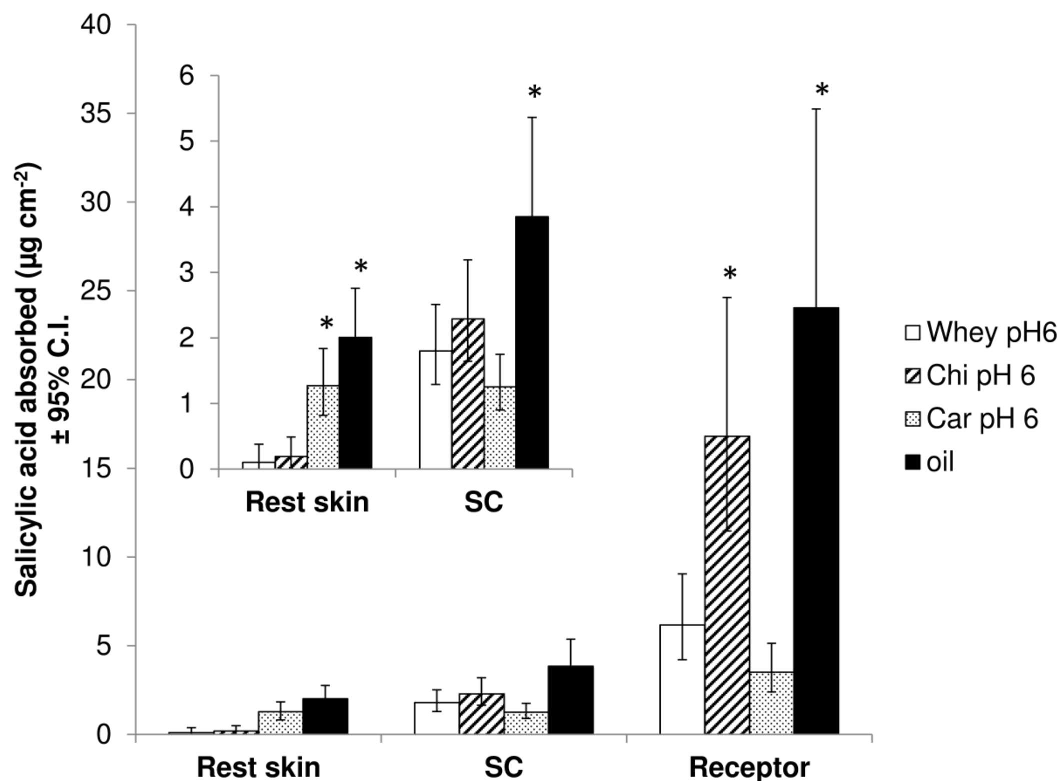

Fig. 6. *In vitro* skin absorption data of salicylic acid from different emulsions at pH 6 through human abdominal skin, expressed as total amount delivered over 24 hours as least square means (formulation effect) ± 95% confidence interval (C.I.), obtained after two-way ANOVA (n = 6). The graph represents the effect of polymer on skin absorption. The oil solution served as control.

3.4 DISCUSSION

3.4.1 Characterization of Emulsions

Emulsion preparation was based on the principles of the layer-by-layer technique (22), in which the oil droplets were coated with a layer of whey proteins and then with a layer of polysaccharides, either chitosan or carrageenan. The increase in zeta potential after addition of positively charged chitosan to whey-stabilized emulsions and similarly, the decrease of zeta potential after addition of negatively charged carrageenan to the primary emulsions at the three pH values of 4, 5 and 6 (Table II), was indicative of the successful layering of the polysaccharides onto the whey proteins at the oil-water interface, by means of electrostatic

complexation. An increase in zeta potential of a mixed aqueous solution of chitosan and *β*-lactoglobulin between pH 4 - 7, compared to a pure aqueous *β*-lactoglobulin solution, was also reported by Guzey and McClements (18) and was related to electrostatic complexation between the two polymers. Furthermore, Cho *et al.* (12) reported similar results for the adsorption of carrageenan onto *β*-lactoglobulin coated-lipid droplets, as the addition of carrageenan to the primary emulsion yielded more negative zeta potential values. Although the zeta potential of the whey emulsion was already negative at pH 6, it was evident from the results that anionic carrageenan was additionally adsorbed onto the whey proteins. It was very likely that positively charged groups were still available at the whey protein surface to interact with the anionic carrageenan (20). The same assumption could be made regarding the adsorption of cationic chitosan onto positively charged whey proteins at pH 4.

The increase in droplet size over time for the whey emulsion at pH 5 and for the Chi emulsions at pH 4 and 5 could either be attributed to coalescence, or flocculation of the emulsion droplets. The light microscopy images in Fig. 2, however, revealed no apparent increase in droplet size due to droplet coalescence, but rather due to flocculation. The whey emulsion at pH 5, which showed an increase in droplet size over time, did not show any flocculation in the microscopy images taken on day 0, indicating that flocculation only occurred after 1 day, as was confirmed by the Mastersizer results. The results for the whey emulsions were congruent with findings in previous studies, during which whey protein-stabilized emulsions had been unstable to droplet aggregation at pH values close to the isoelectric point (e.g. pH 5), owing to a low net charge of the droplets and therefore less repulsive interactions between emulsion droplets (12, 20). In contrast, the microscopy images of all Chi emulsions, the Car emulsion at pH 4 and the whey emulsion at pH 4 indicated immediate flocculation, which was not always congruent with the droplet size results obtained by laser light scattering, e.g. for whey emulsion at pH 4 and Car emulsion at pH 4. This was most likely due to a break-up of the emulsion droplet aggregates, as a result of dilution of the formulations prior to particle analysis. Li *et al.* (9) investigated *β*-lactoglobulin-chitosan-stabilized emulsions and found that the adsorption of chitosan onto *β*-lactoglobulin increased the stability of the emulsions towards droplet aggregation between pH 3

– 6, which was a function of the chitosan concentration in the emulsions. These findings were in contrast to the results obtained during this study. Different results were also obtained for β-lactoglobulin-carrageenan-stabilized emulsions. In a study by Cho *et al.* (12), the addition of carrageenan to β-lactoglobulin resulted in extensive droplet aggregation at pH values below 6. Gu *et al.* (20) also found extensive droplet flocculation between pH values 3 – 5, but not between pH 6 – 8, and it was further demonstrated that the droplet aggregation was dependent on the carrageenan concentration. In this study, however, no extensive droplet aggregation was observed for the Car emulsions between pH 4 – 6. These different outcomes could have been due to differences in polymer concentration, the emulsion preparation technique and ionic strength of the aqueous phase.

3.4.2 Turbidity

Turbidity can be used as a measurement for insoluble complex formation in the aqueous phase. However, this provides no information regarding complex formation that is soluble in the aqueous phase (18). It also has to be kept in mind that complex formation between the whey proteins and polysaccharides could be different in the emulsion formulations, compared to the aqueous solutions, because the structure of the whey proteins may differ between the two systems, e.g. native *versus* denatured form (20). The trend in turbidity of the whey and chitosan solutions across the analyzed pH range was similar to data presented by Guzey and McClements (18). The highest turbidity of the aqueous whey solution was found at pH 5, close to the isoelectric point of whey proteins. The reduced net charge of the proteins at this pH minimized electrostatic repulsion between the protein molecules and therefore enhanced aggregation. Similarly, the aqueous chitosan solutions became more turbid with increasing pH, due to the loss of positive charges of amino groups of chitosan when approaching the pK_a value ($pk_a \approx 6.5$) (18).

The increase in insoluble complex formation between whey proteins and positively charged chitosan with an increase in pH of the aqueous phase could be explained by an increased electrostatic interaction between whey and chitosan, due to an increase in negative charge of

the whey proteins with increasing pH. In contrast, the insoluble complex formation in the aqueous phase between whey proteins and negatively charged carrageenan decreased with an increase in pH, because of a decrease in the positive charge of the whey proteins. This alteration in turbidity with a change in pH could be indicative of a successful layering of the polysaccharides onto the whey proteins.

3.4.3 Release of Active from the Formulations

In a preliminary study (data not presented here – See Chapter 3), during which the release of salicylic acid from aqueous solutions at pH 4, 5 and 6 had been investigated, the release rate was determined to be in the order: pH 4 > pH 5 and pH 6, hence opposite to the trend observed for the release of salicylic acid from emulsions. The preliminary release study had been performed similarly to this emulsion study, with the only difference being the donor phase that had consisted of the aqueous phase of the emulsion, containing 1 mg ml^{-1} salicylic acid, but no emulsifiers. The concentration of 1 mg ml^{-1} of salicylic acid, which was below the saturation concentration, was assumed as the concentration present in the aqueous phase of the emulsions in equilibration with the oil phase (according to the oil/water phase partition coefficient). The decrease in release of the active with an increase in pH could be explained by a decrease in thermodynamic activity with increasing pH values of the aqueous donor phase, due to the increasing solubility of salicylic acid with increasing pH (23).

The opposite trend in release, being observed for the various emulsions at different pH, must therefore have been related to some effect within the emulsion. No correlation could be found between the release and droplet size, apparent viscosity and turbidity data. However, the active's release from the emulsion could be related to the zeta potential of the emulsions. According to the Henderson-Hasselbalch equation, more than 90% of the salicylic acid would be ionized in the aqueous phase of the emulsions between pH 4 - 6. Since the pK_a of salicylic acid is ≈ 3, the salicylic acid was negatively charged at all three pH values of 4, 5 and 6. It is assumed that the negatively charged salicylic acid was electrostatically interacting with the positively charged emulsion droplets at pH 4 for all three emulsions, thus resulting in a lower

release. In contrast, the more negatively charged emulsion droplets at pH 5 and 6 could have yielded a reduced electrostatic interaction between emulsion droplets and salicylic acid, which therefore hindered the release to a lesser extent. However, it was noted that the zeta potential of the emulsions containing salicylic acid and of those emulsions without salicylic acid were similar (Table II). It should, however, also be kept in mind that the emulsions were highly diluted prior to zeta potential analysis and therefore the effect of salicylic acid could have been weakened. Furthermore, the proposed electrostatic interaction between the emulsifier of the emulsion droplets and salicylic acid did not always result in insoluble complex formation, as indicated by the consistent turbidity results. No insoluble complex formation was, for example, observed between whey proteins and salicylic acid at all three pH values, since the addition of salicylic acid to the aqueous phase of whey proteins did not result in an increase in turbidity (Table III).

A comparison of the different emulsions, containing different polymers at the same pH, showed no correlation between release and droplet size, apparent viscosity and turbidity data. Again, the electrostatic interaction between the emulsifiers and active ingredient could have influenced the release. At pH 4, for example, the release of salicylic acid was similar for the different emulsions (Fig. 4A). Similarly, the zeta potential among the various emulsions did not differ significantly (Table II). In contrast, at pH 6 the release of salicylic acid increased (Chi < Whey < Car) (Fig. 4C), with a decline in zeta potential (Chi > Whey > Car) (Table II), indicating that a lower zeta potential yielded a reduced electrostatic interaction between emulsion droplets and salicylic acid, which therefore improved release.

All of the emulsions during this study performed equally or better in terms of release, than the corresponding oil phase. The increase in release of salicylic acid from emulsions could have been due to an increased surface area, owing to the presence of oil droplets. Figures 3 and 4 also showed a longer lag time for release of salicylic acid from the oil solution, which could have been caused by a less favorable partitioning of salicylic acid between the oil and the aqueous receptor phase-saturated membrane, compared to the partitioning between the aqueous phase of the emulsion and the aqueous receptor phase-saturated membrane.

3.4.4 In Vitro Skin Absorption

The comparison of skin absorption data of the whey-stabilized emulsions at three different pH values revealed that dermal and transdermal delivery were significantly increased by the whey-stabilized emulsion at pH 4, although the release was the lowest for this formulation. This could be related to the fact that salicylic acid had the lowest degree of ionization at pH 4. According to the Henderson-Hasselbalch equation, approximately 91% of salicylic acid would be ionized at pH 4, whereas more than 99% would be ionized at pH 5 and 6. The skin absorption results were congruent with the pH partition hypothesis, which states that the unionized form of actives is the preferred form for permeation, as was also demonstrated by the transdermal permeation of salicylic acid during a study by Smith and Irwin (24). However, the increase in skin permeation of salicylic acid was higher than what was expected solely from the effect of ionization. Another formulation factor may therefore have influenced skin absorption. It was hypothesized that the emulsion droplet charge (as indicated by the zeta potential) could have contributed to the increased delivery of salicylic acid from emulsions with decreasing pH. As shown in Table II, the zeta potential for the whey emulsion was positive at pH 4, whilst showing a zero net charge at approximately pH 5, with negative values at pH 6. Since the skin surface is negatively charged (25), it is assumed that electrostatic interactions between the negatively charged skin and positively charged emulsion droplets at pH 4 favored the transport of salicylic acid into and through the skin. Similar results had been obtained during studies by Youenang Piemi *et al.* (3) and Ghouchi Eskandar *et al.* (26), during which positively charged emulsion droplets had yielded increased dermal and transdermal delivery in comparison with negatively charged emulsion droplets.

A comparison of the skin absorption data of the three emulsions at pH 6, containing different biopolymers, revealed an enhanced transdermal delivery for the Chi emulsions, whereas the Car emulsions improved dermal delivery. As with the whey emulsions at various pH values, the transdermal delivery data of the emulsions containing different polymers correlated well with the zeta potential data. It was found that with decreasing zeta potential (Table II) (Chi > Whey > Car), the transdermal delivery into the receptor phase was reduced (Fig. 6) (Chi > Whey > Car).

It was assumed that the positively charged amino groups of chitosan interacted with the negatively charged skin surface, which enhanced transdermal delivery. The enhanced skin permeation could also be related to the fact that chitosan is known to lower the diffusion resistance of the stratum corneum and therefore enhance penetration into the skin (17). The reduced permeation of salicylic acid from the Car emulsion at pH 6 could also have been a result of the insoluble complex formation between whey-carrageenan and salicylic acid, as demonstrated by the increased turbidity data (increase in absorbance from 0.45 cm^{-1} for whey-Car without salicylic acid to 3.31 cm^{-1} for whey-Car with salicylic acid) (Table III), resulting in a reduced amount of salicylic acid available for absorption.

3.5 CONCLUSION

This study showed that the type of polymer used as emulsifier in the preparation of emulsions, as well as the pH of the formulation, influenced the release, as well as the topical delivery of the active ingredient. More specifically, it was found that electrostatic interactions between the different emulsifiers of the emulsion droplets and salicylic acid influenced the release of the active from the emulsions. Depending on the charge of the emulsion droplets, introduced by alteration of the pH of the formulation, increased electrostatic interactions resulted in a reduced release of the active ingredient. Furthermore, the extent of ionization, together with the charge of the emulsion droplets, played an important role in the dermal and transdermal delivery of salicylic acid from the different emulsion formulations. It was indicated that an increase in emulsion droplet charge could result in an increase in electrostatic interaction between emulsion droplets, containing the active ingredient, and negatively charged skin, resulting in enhanced topical delivery.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4

The effect of pH on release of salicylic acid from unsaturated, aqueous donor solutions

4.1 INTRODUCTION

In the previous chapter, the release of salicylic acid from various emulsions through cellulose nitrate membranes was determined. The study was designed in order to investigate, among other things, the effect of pH on release of salicylic acid and it was found that the release decreased with decreasing pH values. Several explanations are possible. For example, the donor pH could have affected the release rate:

- by changing the solubility of salicylic acid in the aqueous phase of the emulsions and therefore altering the driving force (thermodynamic activity) for diffusion or
- by altering the physico-chemical properties of the cellulose nitrate membranes (e.g. pore size, swelling degree) which could affect the permeability of the membranes or
- by modifying emulsion characteristics, such as droplet charge, polymer structure at interface, interactions between excipients and active ingredient, and thereby influencing the release from these emulsions.

With the intention of distinguishing between the various, possible pH effects on release, the current study was designed to investigate the release of salicylic acid from aqueous solutions of different pH (resembling the aqueous phase of the emulsions without the presence of emulsifiers) through cellulose nitrate membranes. The aim was to ascertain what effect the pH has on solubility of salicylic acid in the donor phase and therefore on the release. Additionally, it was further intended to study the effect of pH of the donor solution on the properties of cellulose nitrate membranes.

Previous studies have shown that the pH could affect the release of salicylic acid from aqueous solutions through different types of membranes. For example, a study by Asman *et al.* (2008a:3295) showed that an increase in pH decreased the amount of salicylic acid released through poly(vinyl alcohol-*g*-itaconic acid) membranes. In another study by Asman *et al.* (2008b:3005), poly(vinyl alcohol-*g*-acrylamide) membranes were prepared and subjected to release of salicylic acid. In the latter study, they found the opposite trend in release of salicylic acid from unsaturated, aqueous solutions, resulting in enhanced release with increasing pH.

Bhunia *et al.* (2012:E188) investigated the effect of swelling of poly(vinyl alcohol) hydrogel membranes on the release of diltiazem hydrochloride through these membranes. It was found

that the degree of swelling and the molecular relaxation of the membrane macro-chains could influence the release of the drug through the membranes.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Miglyol 812 N® was kindly donated by Cremer (Hamburg, Germany). Salicylic acid (99+%) was obtained from SAFC (St. Louis, MO, USA). Potassium chloride and citric acid anhydrous were purchased from Sigma Aldrich (Johannesburg, RSA). Sodium dihydrogen orthophosphate, di-sodium hydrogen orthophosphate anhydrous, propylene glycol, 1 N hydrochloric acid, 1 N sodium hydroxide solution and methanol were purchased from ACE Chemicals (Johannesburg, RSA). **Acetonitrile** (LiChrosolv®, gradient grade for liquid chromatography) and acetic acid (glacial) were purchased from Merck Chemicals (Johannesburg, RSA).

4.2.2 Aqueous Phase Preparation

The buffer solution used for the preparation of the aqueous phase was prepared by dissolving 0.01 mol citric acid and 0.02 mol dibasic sodium phosphate in deionized water. The solutions were mixed in order to obtain citrate phosphate buffer solutions at pH 4, pH 5 and pH 6. A concentration of 1 mg ml^{-1} were prepared by adding salicylic acid into citrate buffer (pH 4, 5 and 6) and stirring the solution on a stirrer plate for 12 hours. The concentration of 1 mg ml^{-1} was calculated to be the theoretical concentration of salicylic acid in the aqueous phase of 15% oil-in-water emulsion after equilibration with the oil phase containing initially 2% salicylic acid. The theoretical value of the salicylic acid concentration in the aqueous phase was determined according to the oil/water partition coefficient.

4.2.3 Solubility Determination of Salicylic Acid

Saturated solutions of salicylic acid were prepared by continuous adding of salicylic acid separately to Miglyol 812 N®, citrate-phosphate buffer (pH 4, 5 and 6) and receptor phase containing of phosphate buffer and propylene glycol (1:1, v/v) at pH 7.4. The solutions were mixed on magnetic stirrer plates in a heated water bath at 37 ± 1 °C. With the dissolution of salicylic acid the pH of the citrate-phosphate buffer solutions decreased and therefore, the pH values of the aqueous solutions were continuously adjusted to required pH values. After 24 hours, the solutions were filtered through hydrophilic polyvinylidene fluoride (PVDF) prefilters with a pore size of 0.45 µm (Agela Technologies Inc., Newmark, USA) and diluted 1:100 with methanol. The samples were then analyzed by HPLC. The solubility experiments were performed in triplicates.

4.2.4 Oil-Water Phase Partition Coefficient Determination of Salicylic Acid

Equilibration between the aqueous phase and the oil phase was reached by mixing 5 ml citrate-phosphate buffer at the various pH levels (pH 4, 5 and 6) with 5 ml Miglyol 812 N® for 24 hours on magnetic stirrer plates at room temperature. The two phases were then separated and saturated solutions of salicylic acid were prepared by continuous adding of salicylic acid to the different solutions to ensure saturation of thereof. All experiments were carried out in triplicates. After 24 hours mixing on magnetic stirrer plates at room temperature, the solutions were filtered through hydrophilic PVDF prefilters with a pore size of 0.45 μm (Agela Technologies Inc., Newmark, USA). The samples were diluted with methanol and subsequently analyzed by HPLC.

4.2.5 Determination of percentage ionized salicylic acid

The percentage of ionized salicylic acid in the citrate buffer solutions at various pH values were determined using the Henderson-Hasselbalch equation (Equ. 2):

$$pH - pKa = log\frac{[A^-]}{[HA]} \qquad \text{Equ. 3.}$$

Where pKa is the logarithmic acid dissociation constant, $[A^-]$ is the molar concentration of the conjugate base and $[HA]$ is the molar concentration of the undissociated weak acid.

4.2.6 *In Vitro* Release Study

All three salicylic acid solutions were subjected to the release study using Franz type diffusion cells with a diffusion area of 1.13 cm^2 and cellulose nitrate membrane filters (0.2 μm pore size, Whatman, Dassel, Germany). Four cells per solution were used. The study was conducted at 37 ± 1 °C in a heated water bath for 12 hours. The membranes were soaked in phosphate buffer / propylene glycol (PG) (1:1, v/v) at pH 7.4 (receptor phase) overnight. The receptor compartment was filled with 2 ml of preheated receptor phase and left to equilibrate before adding the donor phase. The donor compartment was filled with 1 ml of solution. Prior to the release experiments, the receptor phase was degassed by placing it in an ultrasonic bath for 10 min. The receptor fluid was stirred with a magnetic stirrer at 750 rpm. Extractions were taken at 1, 2, 3, 4, 6, 8 and 12 hours. The samples were analyzed by HPLC. Furthermore, the cellulose nitrate membranes were removed, dried and subjected to SEM analysis.

In addition, two Franz cells per pH were also run with blank donor solutions containing citrate-phosphate buffer at pH 4, 5 and 6 but without salicylic acid. No receptor samples were withdrawn during the study. However, after 12 hours release study the cellulose nitrate membranes were removed, dried and subsequently analyzed by SEM.

4.2.7 Monitoring of pH of Donor Solution

The pH of the various donor solutions were measured prior to application into the donor compartment (time 0) and at each extraction time point in the donor compartment (time x)

4.2.8 Swelling Degree (*SD*)

After the release study was conducted, the membranes were removed from the cells and immediately weighed (wet mass). In addition, a non-treated, dry cellulose nitrate membrane was weighed (dry mass). The swelling degree (*SD*) was calculated according to Equation 3:

$$SD\% = \frac{W - W_0}{W_0} \times 100\% \qquad \text{Equ. 3}$$

where W is the wet mass and W_0 the dry mass of the membranes.

4.2.9 Scanning Electron Microscope (SEM)

The dried membranes sections were mounted on double sided carbon tape. Au/Pd (67/33) was sputtered on the membranes forming a 15 nm thick coating. The membranes were viewed using a FEI Quanta FEG 250 Scanning electron microscope at 5kV under high vacuum mode.

4.2.10 Statistical Analysis

Data from the release study were analyzed by one-way analysis of variance (ANOVA) using STATISTICA® (StatSoft Inc., Tulsa, OK, USA). Subsequently, Tukey's HSD (Honestly Significant Difference) test was performed to compare the various aqueous solutions with different pH values with each other. Data with $p < 0.05$ were considered statistically significant.

4.2.11 HPLC-UV Method

HPLC analysis of salicylic acid was performed using an Agilent® 1100 Series HPLC system (Agilent Technologies, Palo Alto, CA, USA) fitted with a high performance silica based, reversed phase C18-2 column (150 x 4.60 mm) with 5 µm particle size (Venusil XBP Agela Technologies, Wilmington, DE, USA) in a controlled laboratory environment at 25 °C. The instrument was equipped with a G1311A quaternary pump, G1313A autosampler and a G1315A multi wavelength diode array detector. The mobile phase consisted of 1% acetic acid, 45% acetonitrile and 54% Milli-Q® water and was degassed after preparation. Flow rate was set at 1 ml min^{-1} with a run time of approximately 6 min. The UV-detector was set at 236 nm. Chromatograms were processed using ChemStation Rev. A.10.03 software.

4.3 RESULTS

Table 1. Saturated solubility of salicylic acid in the aqueous phases at different pH values, receptor phase and oil phase, observed oil-water phase partition coefficients (Log *P*) of salicylic acid at different pH values presented as average ± S.D. (n = 3) as well as calculated degree of ionization of salicylic acid at different pH values.

	Solubility [mg ml^{-1}]	**Log *P***	**Ionized [%]**
pH 4	30.6 ± 1.7	1.03 ± 0.02	91.5
pH 5	> 34.9 ± 3.2	0.98 ± 0.01	99.1
pH 6	> 47.8 ± 1.1	0.99 ± 0.02	99.9
Receptor phase	31.0 ± 0.4	-	-
Miglyol 812 N®	38.8 ± 1.8	-	-

The solubility, oil-water phase partition coefficient and degree of ionization of salicylic acid at different pH values are summarized in Table 1. The saturated solubility of salicylic acid could not be determined accurately because of experimental difficulties. With an increase of added salicylic acid to the aqueous phases, the pH of the solutions dropped and necessitated a re-adjustment of the pH, which subsequently resulted in the solubilization of more salicylic acid and again a drop in pH. The continuous dissolution of salicylic acid in the aqueous phase at pH 5 and 6 yielded the formation of a paste-like mass which was difficult to separate from the supernatant. However, a trend could be observed, i.e. the solubility of salicylic acid increased with an increase in pH. Similar results were also observed by Serajuddin and Jarowski (1985:150), though their solubility values were higher at the indicated pH values. The increase in solubility with increasing pH could be explained by a rise in the degree of ionization of salicylic acid (as indicated in Table 1). The pK_a of salicylic acid is ≈ 3 resulting in high percentages (≥ 91.5%) of ionized drug at the used pH values (pH 4 - 6). Furthermore, for the determination of Log *P*, no adjustments were made to the pH of the aqueous solutions.

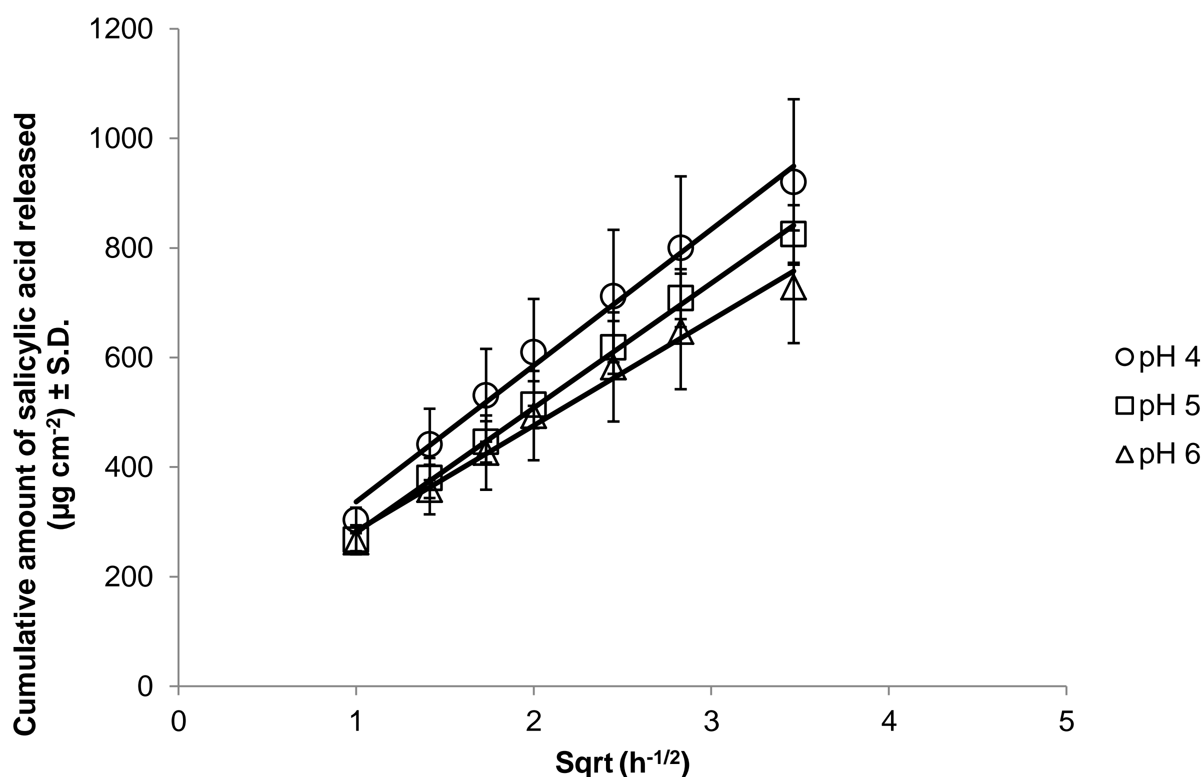


Figure 1. *In vitro* release of salicylic acid from unsaturated aqueous solutions (containing 1 mg ml^{-1} salicylic acid) at different pH values through cellulose nitrate membranes. The data are presented as linear fit against square root of time (according to Equ. 2) as average ± S.D. (n = 3).

The cumulative amount of salicylic acid released per surface area was plotted against the square root of time, see Equation 2, which represents the simplified Higuchi model.

$$f_t = Q = K_H t^{1/2} \qquad \textit{Equ. 2}$$

where f_t is the cumulative amount of salicylic acid released per surface area, K_H is the Higuchi dissolution constant and $t^{1/2}$ represents the square root of time. The release rate K_H was calculated from the linear portion (t_{1h} – t_{12h}) of the correlation between the cumulative amount of released salicylic acid per surface area and square root of time. Calculated K_H and corresponding regression coefficients as well as the cumulative amount of released salicylic acid in 12 hours are presented in Table 2. As seen from Fig. 1 and the regression coefficients in Table 2, the amount of salicylic acid released per surface area showed a linear relationship with the square root of time ($R^2 \geq 0.99$) for all three solutions tested according to the Higuchi model.

From Fig. 1 and Table 2 a trend can be seen in that the solution at pH 4 had the highest release, whereas the solution at pH 6 resulted in the lowest release of salicylic acid from unsaturated aqueous solutions. However, no statistically significant differences were found in

the release rate nor cumulative amount of salicylic acid released over 12 hours between the different pH values ($p > 0.05$).

Table 2. Release rate and cumulative release in 12 hours through cellulose nitrate membranes presented as average ± S.D. (n = 4), swelling degree presented as average ± S.D. (n = 4) and change of pH of donor solution over time presented as average ± S.D. (n = 4)

	k_H [µg cm^{-2} h$^{-1/2}$]	R^2	Cumulative release in 12 h [%]	SD [%]	pH of donor solution at time 0	pH of donor solution after 12 h release
pH 4	248.7 ± 51.0	0.99	81.3 ± 13.3	234.3 ± 37.9	4.0	4.3 ± 0.04
pH 5	227.6 ± 22.1	1.00	72.9 ± 4.6	195.7 ± 8.4	5.3	5.7 ± 0.00
pH 6	192.3 ± 36.1	0.99	64.4 ± 9.1	187.4 ± 10.6	6.0	6.2 ± 0.04

Although the SD values were high with values between 187 – 234% (Table 2), no significant differences in SD were be observed between the different pH values. Furthermore, the pH of the donor solutions at all pH values remained fairly stable over the 12 hours release study, only showing a slight increase.

Fig. 2 shows the SEM images of the cellulose nitrate membranes after completion of the release study. No differences in membrane structure were observed between the various pH values for the membranes that were subjected to donor solutions containing salicylic acid. However, the cellulose nitrate membranes that were subjected to blank donor solutions revealed a change in pore size. The blockage of pores was more pronounced at pH 6 than at pH 4 and 5.

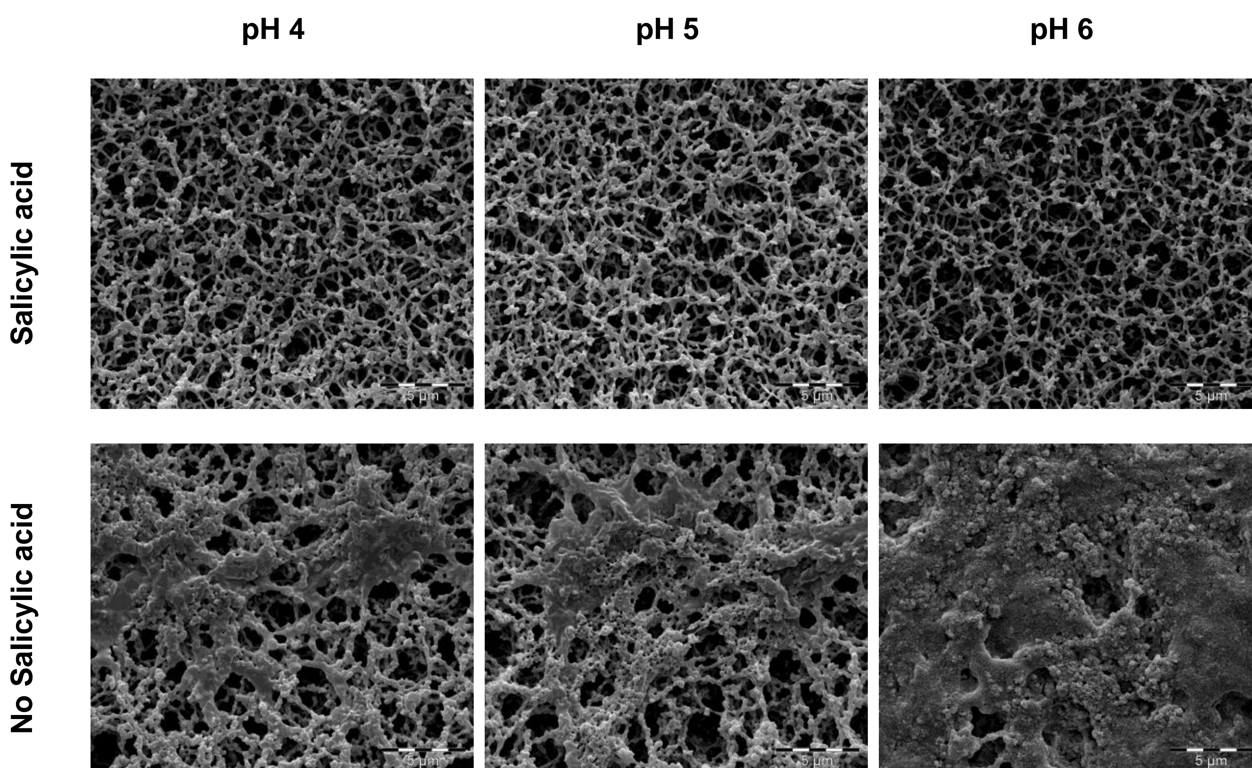
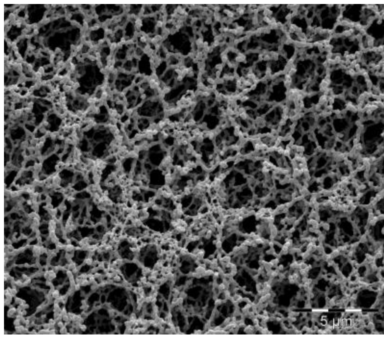


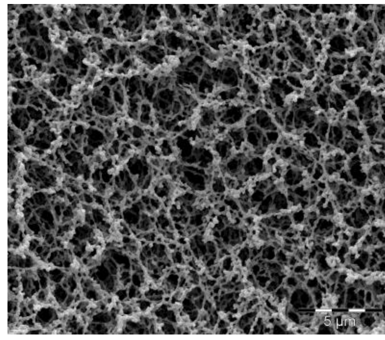
Figure 2. SEM images of cellulose nitrate membranes after completion of release study including donor solutions with 1 mg ml^{-1} salicylic acid and blank donor solutions without salicylic acid. The scale bar in each photograph represents 5 µm.

Cellulose nitrate membranes were subsequently subjected to further studies to determine which of the chemicals resulted in the blockage of the membranes. Therefore, the membranes were soaked separately in PBS pH 7.4, citrate phosphate buffer pH 4, 5 and 6 and in propylene glycol (PG) for 12 hours. The membranes were then dried, prepared in the same manner as the membranes from the release study and viewed with SEM (Fig. 3). The membranes that were soaked in PG could not be dried sufficiently and could hence not be viewed with SEM.

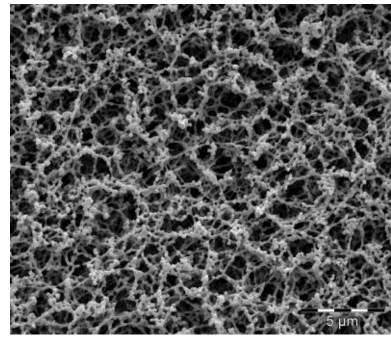
The images in Fig. 3 exhibited no differences in membrane structure between the cellulose nitrate membranes treated with either citrate phosphate buffer solutions at different pH or PBS at pH 7.4 and the untreated cellulose nitrate membrane. The membranes that were soaked in PG could not be viewed with SEM because of insufficient drying of the membranes. However, macroscopically shrinkage of the membranes during drying (although it was not complete) could be observed.



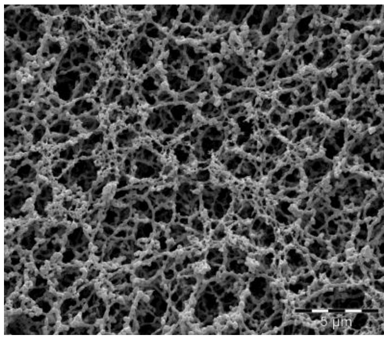
Citrate phosphate pH 4



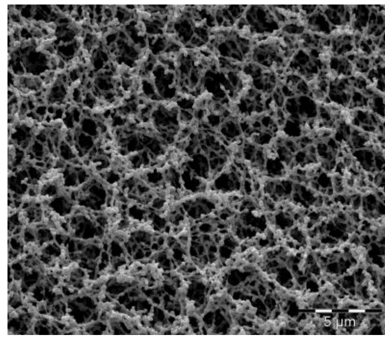
Citrate phosphate pH 5



Citrate phosphate pH 6



Untreated



PBS

Figure 3. SEM images of cellulose nitrate membranes after 12 h immersion in citrate phosphate buffer pH 4, 5 and 6 and PBS pH 7.4. An untreated membrane is also shown. The scale bar in each photograph represents 5 µm.

4.4 DISCUSSION

Although the differences in release of salicylic acid from the aqueous solutions at different pH values were not statistically significant, at least a trend of decreasing release with increasing pH could be observed. The decrease in release with an increase in pH could be explained by the difference in solubility of salicylic acid in the donor solutions at different pH values (Serajuddin & Jarowski, 1985:148). The significant increase in solubility of salicylic acid with increasing pH in the aqueous donor phase could have yielded a lower thermodynamic activity of salicylic acid in the donor solution and therefore a lower driving force for diffusion through cellulose nitrate membranes. No correlation was found between the release of salicylic acid and swelling degree. Furthermore, no differences in the cellulose nitrate membrane structure were observed at the different pH values, indicating that the pore size of the membrane was not affected by pH. However, it should be noted that the SEM images were taken in a dry state and could not give any information about the pore size of the membranes in wet state.

The outcome of the release study is congruent with results obtained by Asman *et al.* (2008a), where the release of salicylic acid from unsaturated aqueous donor phases through poly(vinyl

alcohol-*g*-itaconic acid) and poly(vinyl alcohol) membranes increased with decreasing pH. Different explanations were given for the two membranes. The decrease in release through poly(vinyl alcohol) membranes with increasing pH was related to a decline in the swelling degree which affected the free volume in the membrane that is available to the transfer of salicylic acid molecules. The release results, obtained from poly(vinyl alcohol-*g*-itaconic acid) membranes, however, were explained by electrostatic repulsion between the carboxylic groups of itaconic acid and salicylic acid. This lead to the conclusion that only the unionized molecules of salicylic acid determined the release of salicylic acid through the poly(vinyl alcohol-*g*-itaconic acid) membranes. However, it should be noted that in another study done by Asman *et al* (2008b:3008), contrasting results were found when poly(vinyl alcohol-*g*-acrylamide) membranes were used. In this study, an increase in pH resulted in an increase in release of salicylic acid which they contributed to the bonding of ionized salicylic acid to the membrane on the receptor side by hydrogen bonding. Furthermore, it was seen in this article that the percentage of salicylic acid released at pH 2.1 and 7.4 were similar, which was explained by the hydrolysis of the amide groups of the poly(vinyl alcohol-*g*-acrylamide) membranes at pH 2.1 resulting in higher swelling degrees. Both studies by Asman *et al* (2008a:3291; 2008b:3005) showed contrasting results, depending on the membranes used and it could be concluded that the membranes as well as the method affect the release.

Another finding of this study was the change in membrane structure of cellulose nitrate membranes when blank donor solutions were used in the release study. However, this altered membrane structure was not seen with the donor solutions containing salicylic acid. Neither, the cellulose nitrate membranes that were soaked in citrate phosphate buffer at different pH nor the membranes soaked in PBS at pH 7.4 showed blockages of the membranes, thus assuming that the change in membrane structure was not due to the buffer solutions and could have only resulted from the propylene glycol that was present in the receptor phase. Since no changes in the membrane structure were seen with the donor solution containing salicylic acid, it is hypothesized that interaction between salicylic acid, which has been released into the receptor phase, and propylene glycol occurred therefore preventing the interaction of propylene glycol with cellulose nitrate. According to the solubility of salicylic acid in water, 0.015 mol L^{-1} at room temperature (Pires & Franco, 2012:49), and propylene glycol, 1.5919 mol L^{-1} at room temperature (Jouyban *et al.*, 2011:15), salicylic acid would preferably interact with PG rather than with the aqueous buffer, which is present in the pores of the cellulose nitrate membranes, hindering the propylene glycol from interacting with the membrane. This theory still needs to be supported by data. Currently, computational modelling as well as interaction studies are ongoing to gain more knowledge. However, these studies were not part of this project to obtain a MSc degree. In a study done by Uragami *et al.* (1978:217), it was found that the interactions between cellulose nitrate membranes and the solvent or solute plays an important role in the

permeation of actives through the membranes. A much higher release was found through cellulose membranes compared to cellulose nitrate membranes, indicating different interactions between the groups on the different membranes with the solvent and solute.

4.5 CONCLUSION

The study revealed that an increase in pH reduced the amount of salicylic acid released from unsaturated, aqueous phases. No effect of the pH of the donor solution on cellulose nitrate membranes (swelling degree and pore size) could be observed. The decrease in release with increase in pH was, therefore, related to a lower driving force of salicylic acid, due to higher solubility of salicylic acid at higher pH values.

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CHAPTER 5

CONCLUSION

A comprehensive literature study on emulsions was conducted with the focus on emulsifiers and the effect of these emulsifiers on the release of actives from formulations as well as the effect on dermal delivery. It was found in numerous research articles that emulsifiers affect the stability of formulations, the release of actives, as well as the dermal and transdermal delivery of these actives from emulsions. For example, emulsion droplet charge, hydrophilic-lipophilic balance value (HLB) and the surfactant association structures are some of the emulsifier factors that may influence topical delivery.

In this study the effect of three different biopolymers (whey proteins, chitosan and carrageenan), that were employed as emulsifiers, on the physical properties of the resultant emulsions, release of the active ingredient from the emulsion as well as topical delivery of the active ingredient were investigated. Moreover, the emulsions were prepared at three different pH values to introduce different charges to the polymers in order to study the effect thereof on the emulsion properties as well as on delivery of the active ingredient. The active ingredient chosen for this study was salicylic acid.

Nine emulsions were formulated and characterized by droplet size, zeta potential, apparent viscosity and stability towards creaming and coalescence. All nine emulsions showed stability towards coalescence; however, not all of these emulsions showed stability towards creaming and flocculation over a period of 7 days. The pH value altered the charge of the emulsifiers at the oil-water interface which in turn affected the stability of the emulsions, due to modification of the repulsive forces between the emulsion droplets. Zeta potential measurement also indicated a successful adsorption of chitosan and carrageenan onto the whey proteins at the oil-water interface and in general, it was found that the three investigated biopolymers could be used for the formation and stabilization of emulsions. Turbidity, as a measurement for insoluble complex formation, was measured in the aqueous phase of the emulsions. The results showed no insoluble complex formation between salicylic acid and whey proteins at pH 4, 5 and 6. However, the addition of salicylic acid to whey-chitosan solutions at pH 4 resulted in insoluble complex formation, whereas insoluble complexes were formed by adding salicylic acid to whey-carrageenan solutions at pH 6.

An HPLC method for the quantitative determination of the concentration of salicylic acid in the emulsions, release samples, skin samples and transdermal perfusates samples was developed and validated. The method was found to be accurate, precise, reliable and repeatable and hence suitable for the proposed purpose.

The release of salicylic acid from all nine emulsions as well as from an oil solution, containing the same concentration of salicylic acid as the emulsions, was determined. No correlation could be found between the release and droplet size, apparent viscosity and turbidity data. However, it was found that the electrostatic interactions between the emulsifiers of the emulsion droplets and the salicylic acid influenced the release of salicylic acid from these emulsions, with increasing electrostatic interactions resulting in reduced release.

Six formulations, including the oil solution, were subjected to skin diffusion studies. It was found that the pH of the emulsions significantly influenced dermal and transdermal delivery of salicylic acid, with lower pH values of the emulsion yielding enhanced topical delivery. The enhanced delivery of salicylic acid at pH 4 could be explained by the lower degree of ionization of salicylic acid and the positively charged emulsion droplets which interacted with the negatively charged skin surface. Furthermore, biopolymers, such as chitosan which possess penetration enhancing effects, additionally enhanced the dermal and transdermal delivery of the salicylic acid.

FUTURE PERSPECTIVES

It is suggested to further investigate the effect of more biopolymers as well as the optimization of the formulation aspects of these emulsions. Different actives, e.g. with similar structures and only varying in specific functional groups, should also be included to determine the effect of the active on the interaction with emulsifiers and how these influence the release and topical delivery of actives from emulsions. Another suggestion for future projects could be the investigation of the interactions of the biopolymers with the skin, e.g. computational modelling.

APPENDIX A

INSTRUCTIONS FOR AUTHORS—EJOURNALS

Contents

Instructions for Authors—eJournals 72

Introduction 72

Type of Manuscripts 73

Manuscript Submission 75

Terms of Manuscript Consideration 76

Copyright Transfer 77

Ethics in Animal and Clinical Investigations 77

Originality of Manuscripts 78

Manuscript Organization 79

References 81

Tables 83

Figures 83

Footnotes 84

The following instructions pertain to submissions to the American Association of Pharmaceutical Scientists' (AAPS) electronic journals: *The AAPS Journal* and *AAPS PharmSciTech*.

Introduction

The AAPS Journal (ISSN 1550-7416) is a peer-reviewed online-only journal owned by the American Association of Pharmaceutical Scientists. The journal covers all areas of pharmaceutical research, except pharmaceutical technology and engineering, which are covered by its sister journal, *AAPS PharmSciTech*. The Journal is indexed by PubMed/Medline, Index Medicus, Institute of Scientific Information's Science Citation Index Expanded®, and Chem Abstracts.

Editor-in-Chief, Ho-Leung Fung, Ph.D., oversees an international editorial board of leading researchers in the pharmaceutical sciences. Fung is a professor of pharmaceutical sciences at the University at Buffalo, The State University of New York.

AAPS PharmSciTech (ISSN 1530-9932) is a peer-reviewed online-only journal owned by the American Association of Pharmaceutical Scientists. The journal's mission is to disseminate scientific and technical information on drug product design, development, evaluation, and processing to the global pharmaceutical research community. The Journal is indexed by PubMed/Medline, Index Medicus, Institute of Scientific Information's Science Citation Index Expanded®, and Chem Abstracts.

Editor-in-Chief, Lee E. Kirsch, Ph.D., oversees an international editorial board of leading researchers in the pharmaceutical sciences. Kirsch is professor of pharmacy at the University of Iowa, College of Pharmacy.

Type of Manuscripts

The AAPS Journal and *AAPS PharmSciTech* publish the following article types. For examples of published articles, please visit www.PharmaGateway.net.

Reviews

Reviews, usually by invitation and organized into themed issues, report on recent advances in pharmaceutical research. Unsolicited reviews are considered only if they are authored by investigators who have demonstrated expertise in the relevant areas.

Mini-Reviews

Mini-Reviews discuss a more narrowly focused topic of recent research. Unsolicited reviews are considered only if they are authored by investigators who have demonstrated expertise in the relevant areas.

Original Research Papers

Original Research Papers contain innovative, hypothesis-driven research that is supported by sound experimental design, methodology, and data interpretation.

Brief Technical Notes

Brief Technical Notes, normally more limited in scope than Original Research Papers, must be of high quality, general interest, and sufficient importance to warrant publication.

Rapid Communications

Rapid Communications provide a venue for fast-breaking research updates or other news items. The justification for rapid communication should be stated in the cover letter during submission.

Regulatory Notes

Regulatory Notes provide a summary of regulatory decisions made and rationale for the regulatory decision made on a product. These notes are typically submitted by invitation only, but Authors may propose notes to the Editors.

Editorials, Commentaries, or Summaries

Editorials, Commentaries, or Summaries are usually published by invitation only. These articles contain topical issues of public and scientific interest.

Meeting Reports

Meeting Reports on AAPS or AAPS-affiliated meetings provide readers with summaries of such meetings, including consensus views. When a meeting report purports to be a Consensus Report, authors should review and adhere to the Consensus Report Review and Publishing Guidelines available at http://www.aapspharmaceutica.com/inside/refguide/ConsensusReport-2007.pdf. The corresponding author must complete and submit the Corresponding Author's Consent to Publish Consensus Report (http://www.aapspharmaceutica.com/inside/refguide/Consensus_Rpt_-3) and all contributing authors must complete and submit the Contributing Author's Consent to Publish Consensus Report , available at

http://www.aapspharmaceutica.com/inside/refguide/Consensus_Rpt_-_Contributing_Author_Consent_Form.pdf.

Meeting Notices

A Meeting Notice provides readers with information on an upcoming AAPS or AAPS-affiliated meeting, including title of the meeting, date, time, location, an outline and description of meeting topics, and a list of invited speakers. If possible, Meeting Notices should include contact information for the organizers and a URL to the meeting's webpage. Meeting Notices should be submitted to the AAPS Editorial Office two to three months prior to the meeting.

Letters to the Editor

Letters to the Editor may be submitted by readers commenting on articles already published by the journal.

All articles published in the journal will follow the Springer Online First production workflow, enabling publication on the SpringerLink website after receipt of author corrections to page proofs.

Manuscript Submission

AAPS uses Editorial Manager™ as its peer review tracking system. Manuscripts must be submitted online by the Corresponding Author to the appropriate journal (see below).

The AAPS Journal: http://www.editorialmanager.com/aapsj/

AAPS PharmSciTech: http://www.editorialmanager.com/aapspt/

You may be required to register as a new user with Editorial Manager upon your first visit. Straightforward login and registration procedures can be found on the Web site. Editorial Manager allows authors to track the progress of manuscript review in real time. Detailed, step-by-step instructions for submitting manuscripts can be found on the Web site. Manuscripts must be created and saved in Microsoft® Office Word for Windows. All correspondence regarding your manuscript must go through Editorial Manager.

Special Features, Appendices, and Supplementary Material

Special features, appendices, and supplementary material can be accommodated and may contain highly interactive features or large databases. All authors are encouraged to take full advantage of the Web-only capabilities of online publishing, including 3-D, video, and interactive graphics. If a desired technical feature is not covered in the Author's Instructions, please contact the AAPS Editorial Office (see below).

The AAPS Journal: AAPSJsubmit@aaps.org

AAPS PharmSciTech: AAPSPTsubmit@aaps.org

All special features must be created by the Author.

Authors who wish to publish electronic supplementary material to their article (Excel files, images, audio/video files) must submit the supplementary files/materials with their manuscript submission via our online peer review tracking system, Editorial Manager. These files may be submitted as one file or multiple files as desired by the authors. Accepted formats for these files

include .doc, .docx, .xls, .xlsx, .jpg, .pdf, and for videos, .mpeg-3 format. Note that supplementary files are not automatically included in the reviewer's PDF. Therefore, please note in a cover letter if these materials should be evaluated by reviewers only and not be published should the manuscript be accepted, or if the supplemental files should be included for review and be published with the article should the manuscript be accepted for publication. If the supplemental files are intended for publication, callouts using, "supplemental 1," "supplemental 2," etc., should be placed in the appropriate location within the text of the manuscript body.

Hypertext Links

Authors may identify uniform resource locators (URLs) for websites that provide the reader with additional information on the topic addressed in the manuscript. Although URLs are an important feature of electronic publishing, authors are encouraged to be selective in their choice of sites to include. Do not include URLs for web pages with newspaper or journal articles that will be removed or archived to another Web page. Links to pharmaceutical manufacturers or other sources of product information are acceptable; however, providing a URL to the reader should not be substituted for adequate discussion within the manuscript itself. Do not include links to sites that are not accessible without a password.

Terms of Manuscript Consideration

AAPS Journals Ethics Policy

The Editors-in-Chief of the three Journals of AAPS, *Pharmaceutical Research*, *The AAPS Journal*, and *AAPS PharmSciTech*, along with the AAPS Publications Committee developed an integrated ethics policy to guide decision-making across the three journals. The document is based on the recommendations on publication ethics policies for medical journals published by the World Association of Medical Editors (WAME), posted at http://wame.org/resources/ethics-resources, and subscribed to by almost 1,000 journals.

Authors are required to review and adhere to the AAPS Journals Ethics Policy (http://www.aaps.org/journalsethics) in full prior to submitting manuscripts to *The AAPS Journal*, *AAPS PharmSciTech*, or *Pharmaceutical Research*. Excerpts from the Policy are outlined below.

Full Disclosure

During the manuscript submission process, all authors will be required to confirm that the manuscript has not been previously published in any language anywhere and that it is not under simultaneous consideration by another journal. Moreover, *The AAPS Journal* and *AAPS*

PharmSciTech policy regarding compound disclosure centers on the data presented. If specific data relating to the study compound are reported, then the Journal requires identification so that reviewers and readers can judge, based on general principles, whether the data are plausible and internally consistent, and to potentially allow future examination and/or validation of the results and conclusions reported. If, however, a library of 5 compounds is used to generate correlations, such as in QSAR when the scope and diversity of the compounds are described, then it is possible that the specific chemical compositions of the compounds need not be identified, subject to the discretion of the reviewers and the Editor.

Conflicts of Interest

Authors will be required to declare all conflicts of interest (or their absence) during the submission of a manuscript. This conflict declaration includes conflicts or potential conflicts of all listed authors. If any conflicts are declared, AAPS will publish them with the paper. In cases of doubt, the circumstance should be disclosed so that the editors may assess its significance.

Conflicts may be financial, academic, commercial, political or personal. Financial interests may include employment, research funding (received or pending), stock or share ownership, patents, payment for lectures or travel, consultancies, nonfinancial support, or any fiduciary interest in a company.

Copyright Transfer

The Copyright Revision Act of 1976 (Public Law 94-533) requires that Authors transfer their copyrights to the Publisher, American Association of Pharmaceutical Scientists, in order to provide for the widest possible dissemination of professional and scientific literature. A signed Transfer of Copyright form must be submitted online with the manuscript. The Transfer of Copyright form for an accepted manuscript must be on file with the AAPS Editorial Office prior to production for publication. Corresponding Authors may print and sign the form on behalf of all authors.

Ethics in Animal and Clinical Investigations

Human Subjects and Clinical Trials

AAPS Journals require author(s) at the time of manuscript submission to make a statement in the cover letter, indicating documented review and approval from a formally constituted review board (Institutional Review Board or Ethics committee) for all studies involving people, medical records, and human tissues, per the uniform guidelines from the World Medical Association (http://www.wma.net/en/30publications/10policies/HB-E-2011.pdf). Studies and research using

human subjects, medical records, and human tissues (including educational research) must also state this compliance within the Methods Section.

The AAPS Journals also require that controlled clinical trials must be registered in a publicly available database or the Journals will not publish the results of these trials. Manuscripts submitted to the Journals must include trial registration information in the cover letter. To register a clinical trial, authors should go to the NIH registry www.clinicaltrials.gov or the International Standard Randomized Controlled Trials database (http://isrctn.org). Further information can be obtained from the International Committee of Medical Journal Editors (ICMJE) at http://www.icmje.org/publishing_10register.html.

Animal Use and Assurances

AAPS Journals require author(s) at the time of manuscript submission to make a statement in the cover letter, indicating that animal experiments are conducted in full compliance with local, national, ethical, and regulatory principles and local licensing regulations, per the spirit of Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International's expectations for animal care and use/ethics (http://www.aaalac.org/education/module _1.cfm). Investigations using experimental animals (including educational research) must also state this compliance within the Methods Section

Originality of Manuscripts

Authors of manuscripts submitted to the American Association of Pharmaceutical Scientists are obliged to present accurate representation of the research performed along with an objective discussion of the significance of their findings. The author's submission should be original work that reflects research undertaken with integrity and honesty, and that conforms to ethical practices outlined in the AAPS Journals Ethics Policy. Authors should be willing to reply to any reasonable request from editors, referees, and scientist for materials, methods, or data necessary for verification of the conclusions reported in the paper.

Use of Copyrighted Tables and Figures

A copy of the granted permission to use copyrighted figures and tables must be included with the submitted manuscript.

Peer Review

All submissions will be reviewed anonymously by at least two independent Reviewers. Authors are encouraged to submit names and email addresses of expert reviewers, but selection remains a prerogative of the Editor(s). Authors may include supplementary notes to facilitate the

review process. If an accepted paper is cited that has not yet appeared in print and is required for evaluation of the submitted manuscript, authors should provide an electronic version for use by the Reviewers. Authors are responsible for all statements in their work, including changes made by the copyeditor after a manuscript is accepted.

Manuscript Organization

Several components of the manuscript must be submitted as individual files within Editorial Manager: cover letter, title page, manuscript body (including references list), individual figure files, and the Transfer of Copyright.

(*indicates item is required for all manuscript types, unless otherwise specified)

Cover Letter

A cover letter is recommended, but not required. Please note: a cover letter is required for a Rapid Communication submission.

Authors who wish to submit names and email addresses of recommended reviewers for the peer review process may also indicate those in the cover letter.

Title Page*

The title page must be submitted as a separate file, and should include the title of the article, author names with full first name (no degrees), each author's affiliation, and a suggested running head (of less than 50 characters, including spaces). The affiliation should comprise the department, institution (usually university or company), city, and state (or nation) and should be typed as a footnote to the author's name. For the corresponding author designated to correspond with the Editorial Office and review proofs, indicate his/her complete mailing address, office/cellular telephone number, fax number, and e-mail address.

Transfer of Copyright Form*

A signed copy of the Transfer of Copyright must be submitted online as part of the manuscript submission process. The Transfer of Copyright is available at http://www.aaps.org/journalsethics.

Abstract

Only Reviews, Mini Reviews, Original Research Articles, Rapid Communications and Meeting Reports require an abstract. The abstract is limited to 250 words or less. For Research Articles, the abstract should include a brief (2 to 3 sentences) statement for each of the following

sections: Introduction, Methods and Materials, Results and Discussion, and Conclusion written in paragraph form. All abstracts must be written in one paragraph, with no subheadings, equations, tables, reference citations or graphics.

*Keyword**

Provide a list of no more than 5 key words.

Introduction

Required for Reviews, Mini Reviews, Original Research Articles, and Meeting Reports only.

*Main Text Body**

Please include continuous line numbers in the manuscript body file.

For Original Research Articles, Brief Technical Notes, Rapid Communications, organize the main text as follows: Introduction, Materials and Methods, Results, Discussion, and Conclusion. Combining Results and Discussion is discouraged. The use of subheadings to divide the text is encouraged. Primary, secondary, and third level headings should be clearly defined, but do not use numbers or letters.

Recommended word counts are as follows:

Reviews, Original Research Articles and Meeting Reports	5000
Mini Reviews	2500
Brief Technical Notes, Meeting Reports, and Rapid Communications	1200

Use abbreviations sparingly, and define them at the first insertion in the text. Define all abbreviations used in tables within the table footnotes Use the metric system for all measurements. Express metric abbreviations in lowercase letters without periods (cm, mL, sec). Define all symbols used in equations and formulas. When symbols are used extensively, the authors may include a list of all symbols in a table.

Numbers should be reported to reflect the precision of the instrumentation utilized. Calculated numbers, such as means and standard deviations, should be expressed to no more than 1 significant digit beyond the precision of the instrument. Normally, data reported to more than 3 significant figures should be justified. The precision of the variability (e.g., standard deviation) should not exceed that of the reported mean value.

Conclusion

The conclusion should be a brief paragraph, containing 3 to 4 sentences, that summarizes the findings presented.

Acknowledgments

Include funding source(s) and other contributions. If the work has been funded by NIH, please provide name(s) of funding institute(s) and grant number(s). This information is required for automatic deposit into PUBMED Central by the Publisher.

References

References should conform to Vancouver style and be numbered consecutively in the order in which they are cited in the text. Cite in the text by the appropriate Arabic numeral enclosed in parentheses, e.g., (1), (2-5), etc.

Maximum reference limits are as follows:

Reviews, Original Research Articles and Meeting Reports	100
Mini Reviews	40
Brief Technical Notes and Rapid Communications	20

References to unpublished peer-reviewed, personal communications, including conference abstracts, and papers in preparation or in review, cannot be listed, but they can be notated parenthetically in the text.

Abbreviations for journal names should conform to those of Vancouver style. The style and punctuation of the references should conform to the following examples:

Type Example

1. Journal article Smith JJ. The world of science. Am J Sci. 1999;36:234–5.

2. Journal article with DOI (and with page numbers) O'Mahony S, Rose SL, Chilvers AJ, Ballinger JR, Solanki CK, Barber RW, et al. Finding an optimal method for imaging lymphatic vessels of the upper limb. Eur J Nucl Med Mol Imaging. 2004;31:555–63. doi:10.1007/s00259-003-1399-3.

3. Journal article by DOI (before issue publication with page numbers) O'Mahony S, Rose SL, Chilvers AJ, Ballinger JR, Solanki CK, Barber RW, et al. Finding an optimal method for imaging

lymphatic vessels of the upper limb. Eur J Nucl Med Mol Imaging. 2004. doi:10.1007/s00259-003-1399-3.

4. Article in electronic journal by DOI (no paginated version) Slifka MK, Whitton JL. Clinical implications of dysregulated cytokine production. Dig J Mol Med. 2000. doi:10.1007/s801090000086.

5. Journal article in a supplement Frumin AM, Nussbaum J, Esposito M. Functional asplenia: demonstration of splenic activity by bone marrow scan. Blood 1979;59 Suppl 1:26–32.

6. Book chapter Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. In: Bourne GH, Danielli JF, Jeon KW, editors. International review of cytology. London: Academic; 1980. p. 251–306.

7. OnlineFirst chapter in a series (without a volume designation but with a DOI) Saito Y, Hyuga H. Rate equation approaches to amplification of enantiomeric excess and chiral symmetry breaking. Top Curr Chem. 2007. doi:10.1007/128_2006_108.

8. Book, authored Blenkinsopp A, Paxton P. Symptoms in the pharmacy: a guide to the management of common illness. 3rd ed. Oxford: Blackwell Science; 1998.

9. Online document Doe J. Title of subordinate document. In: The dictionary of substances and their effects. Royal Society of Chemistry. 1999. http://www.rsc.org/dose/title of subordinate document. Accessed 15 Jan 1999.

10. Online database Healthwise Knowledgebase. US Pharmacopeia, Rockville. 1998. http://www.healthwise.org. Accessed 21 Sept 1998.

11. Supplementary material/private homepage Doe J. Title of supplementary material. 2000. http://www.privatehomepage.com. Accessed 22 Feb 2000.

12. University site Doe, J.: Title of preprint. http://www.uni-heidelberg.de/mydata.html (1999). Accessed 25 Dec 1999.

13. FTP site Doe, J.: Trivial HTTP, RFC2169. ftp://ftp.isi.edu/in-notes/rfc2169.txt (1999). Accessed 12 Nov 1999.

14. Organization site ISSN International Centre: The ISSN register. http://www.issn.org (2006). Accessed 20 Feb 2007.

For a full description of the Vancouver reference style, including numerous examples, please access http://www.nlm.nih.gov/bsd/uniform_requirements.html.

Tables

Tables must be created in Microsoft Word table format. Tables should be numbered (with Roman numerals) and referred to by number in the text. Center the title above the table, and type explanatory footnotes (indicated by superscript lowercase letters) below the table. Data must be placed in separate cells of the table to prevent text and numbers from shifting when the table is converted for publication on the Internet. Empty cells may be inserted to create spacing. Tables should not duplicate information provided in the text. Instead, tables should be used to provide additional information that illustrates or expands on a specific point the author wishes to make. Each table should be self-explanatory.

Figures

The AAPS Journal and *AAPS PharmSciTech* offer authors the use of color figures in online published manuscripts, free of charge. Figures (as well as photographs, drawings, diagrams, and charts) are to be numbered in one consecutive series of Arabic numerals in the order in which they are cited in the text. The captions for illustrations and figures should be separated from the text and collated in a separate section called, ''Legend to Figures." All electronic artwork must be submitted online via our online peer review tracking system, Editorial Manager. Figure files should be submitted in .tiff or .eps format (1200 dpi for line and 300 dpi for half-tones and gray-scale art); however, .jpeg, .gif, and .bmp files may also be submitted as long as the dpi specifications above are met. Use of a professional graphics program such as Adobe® Photoshop to edit and/or save photographs and graphics is highly recommended. Because of difficulties with exporting graphics from Microsoft PowerPoint, original graphics (those imported into PowerPoint) must be saved in an acceptable file format (above). Microsoft PowerPoint and Microsoft Word figure files will not be accepted.

The maximum combined count for tables and figures are as follows:

Reviews, Original Research Articles and Meeting Reports	10 (suggested)
Mini Reviews	6
Brief Technical Notes, and Rapid Communications	6

Footnotes

Footnotes should be avoided. When their use is absolutely necessary, footnotes should be numbered consecutively using Arabic numerals and should be typed at the bottom of the page to which they refer. Place a line above the footnote, so that it is set off from the text. Use the appropriate superscript numeral for citation in the text.

APPENDIX B

Validation

I would like to thank and acknowledge Prof Jan du Preez from the ATL laboratory North West University Potchefstroom Campus for the assistance and method development with the HPLC method.

Linearity

For the calibration curve, approximately 100 mg of salicylic acid were dissolved in 100 ml methanol to yield a stock solution with a concentration of 1 mg ml^{-1}. Subsequently, the following standard solutions were prepared by appropriate dilution of the stock solution with methanol: 1.25 µg ml^{-1}, 2.5 µg ml^{-1}, 25 µg ml^{-1}, 62.5 µg ml^{-1}, 125 µg ml^{-1} and 250 µg ml^{-1}. Five calibration curves were determined on five different days. The linear regression between the concentration and the peak area was described by the following equation:

$$y = 73.384\,x - 6.839 \qquad \text{Equ. 1}$$

which where the average of the five calibration curves. The regression coefficients of all calibration curves were $R^2 \geq 0.999$ which indicated linearity between the concentration and the peak area for the concentration range 1.25 µg ml^{-1} – 250 µg ml^{-1}.
A calibration curve was also prepared in the receptor phase of the release and skin absorption studies containing of phosphate buffer pH 7.4/propylene glycol (1:1) and the following regression line was obtained:

$$y = 72.13\,x + 0.405 \qquad \text{Equ. 2}$$

with a regression coefficient exceeding 0.999. The regression lines for methanol and receptor phase were similar and therefore, further calibration curves were prepared in methanol.

Accuracy and precision

Accuracy describes the closeness of the mean concentration value obtained with the method to the true concentration, whereas precision is the relative standard deviation of repeatedly analysis of a sample. Accuracy and precision were calculated from five determinations per concentration. Three concentration levels (low, medium and high) of the calibration curve were used and the results are presented in Table 1:

Table 1. Determination of accuracy and precision.

Actual conc. [$\mu g\ ml^{-1}$]	Analyzed conc. [$\mu g\ ml^{-1}$]	Accuracy [%]	Precision [%]
1.25	1.16 ± 0.14	92.5	12.4
62.5	64.4 ± 2.2	103.1	3.4
250	249.8 ± 9.2	99.9	3.7

According to the FDA validation guideline for industry (United States, 2001), the mean value should be within 15% of the actual value (acceptance criteria for accuracy) and the precision should not exceed 15%. As seen from Table 1, the acceptance criteria were met at all three concentration levels and therefore the method was found to be accurate and precise for the analysis of salicylic acid within the concentration range of 1.25 $\mu g\ ml^{-1}$ – 250 $\mu g\ ml^{-1}$.

Lower Limit of Quantification (LLOQ)

LLOQ is the lowest concentration of the standard curve that can be analyzed with acceptable accuracy and precision (acceptance criteria for both parameters is set below 20% according to United States, 2001). As seen from Table 1, the analysis of the lowest concentration (1.25 $\mu g\ ml^{-1}$ resulted in an accuracy value within 20% of the actual concentration and in a precision value below 20% and was hence set as the lower limit of quantification.

Repeatability

The repeatability was determined by six consecutive injections of a sample with a concentration of 25 $\mu g\ ml^{-1}$. The analysis of six injections resulted in a measured concentration of 26.26 ± 0.03 $\mu g\ ml^{-1}$. As the resulting relative standard deviation (0.12%) was found to be below 2%, the repeatability was proven.

Selectivity

Blanco emulsions (containing no salicylic acid) as well as methanol solutions with skin from two different skin donors and tape strips were injected and compared to a standard sample of salicylic acid. No interferences were indicated at the retention time for salicylic acid (see Figure 1).

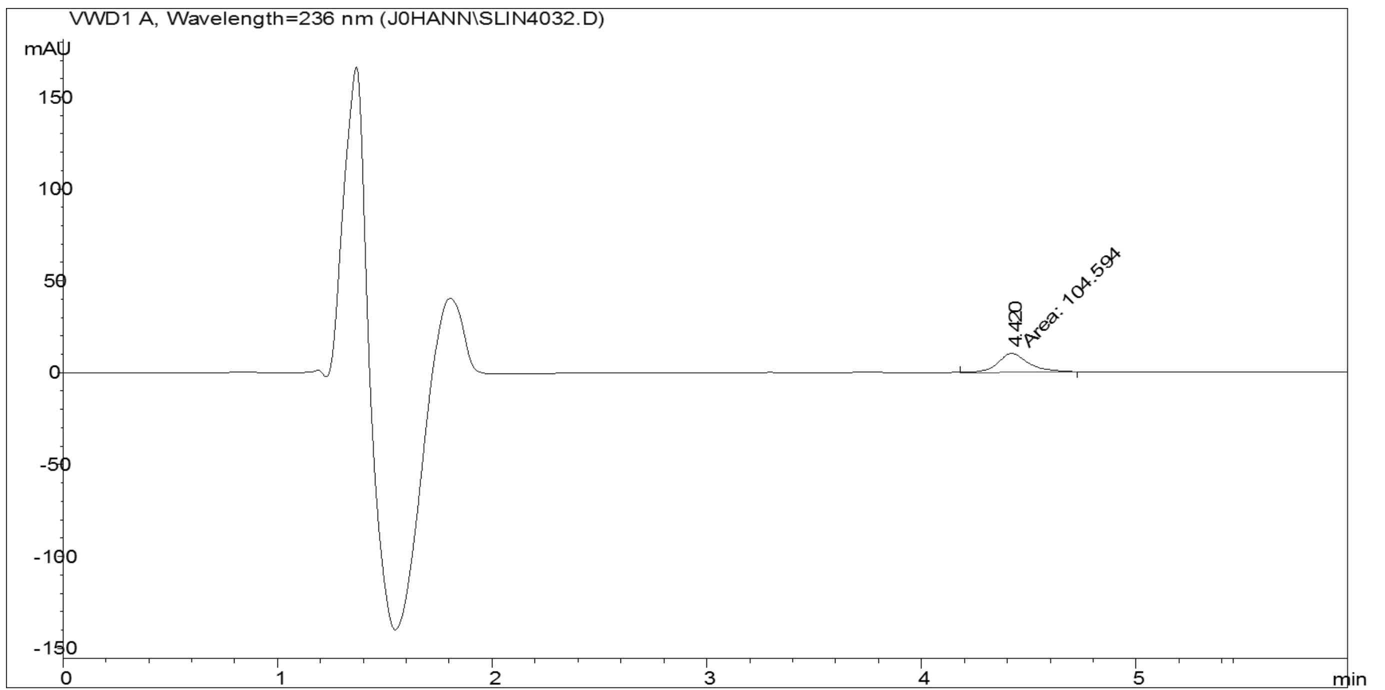


Figure 1. HPLC chromatograph of salicylic acid (1.25 µg ml^{-1}).

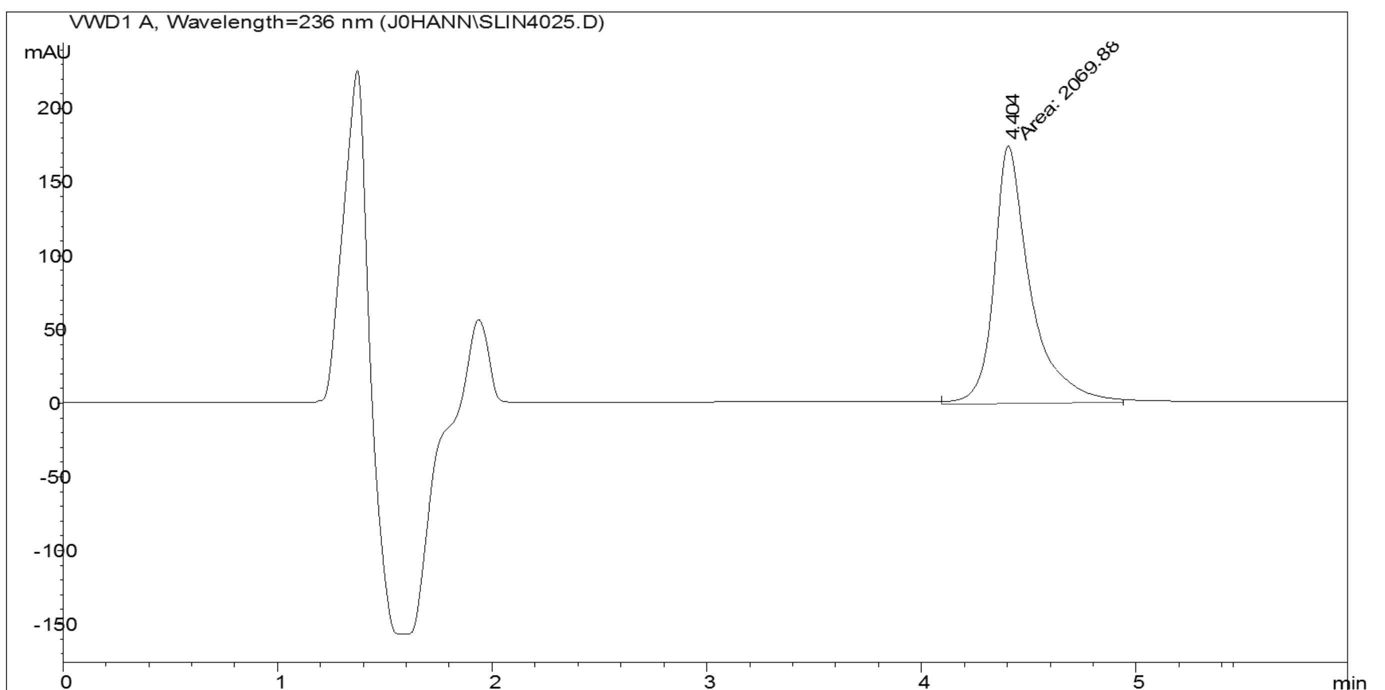


Figure 2. HPLC chromatograph of salicylic acid (25 µg ml^{-1}).

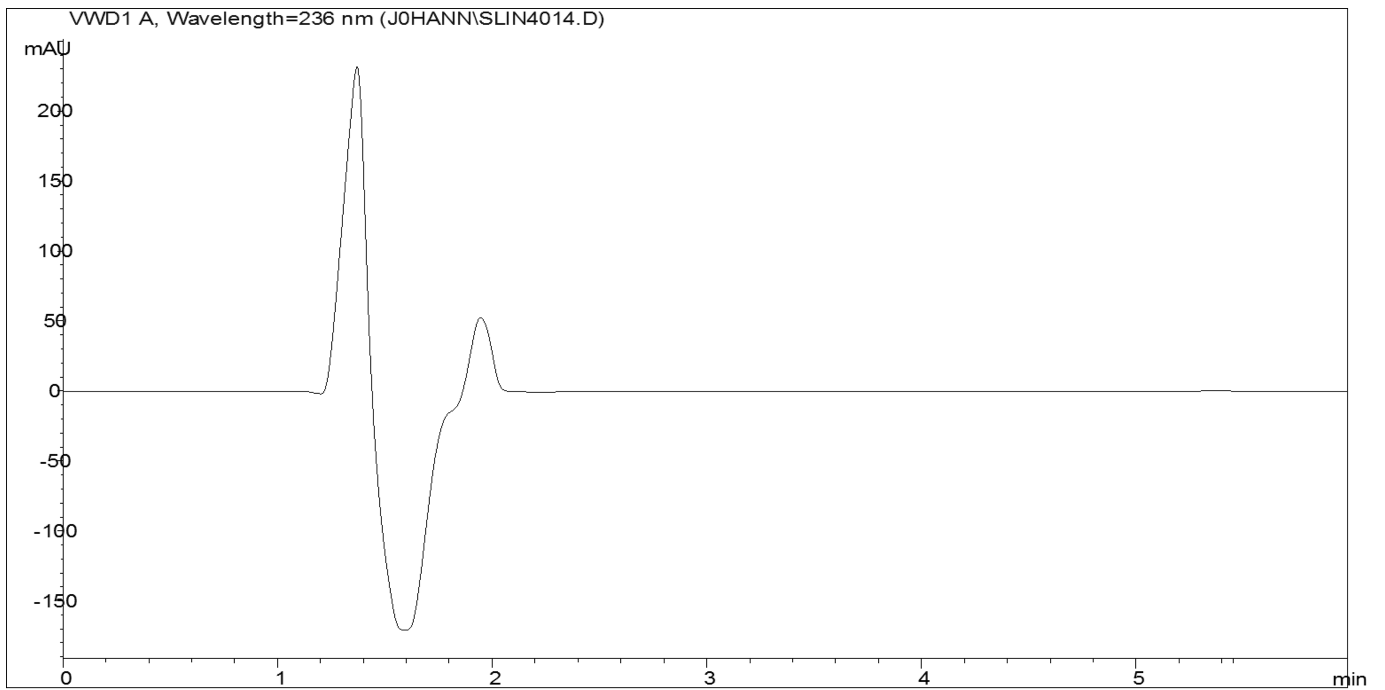


Figure 3. HPLC chromatograph of PBS:PG (1:1, v/v).

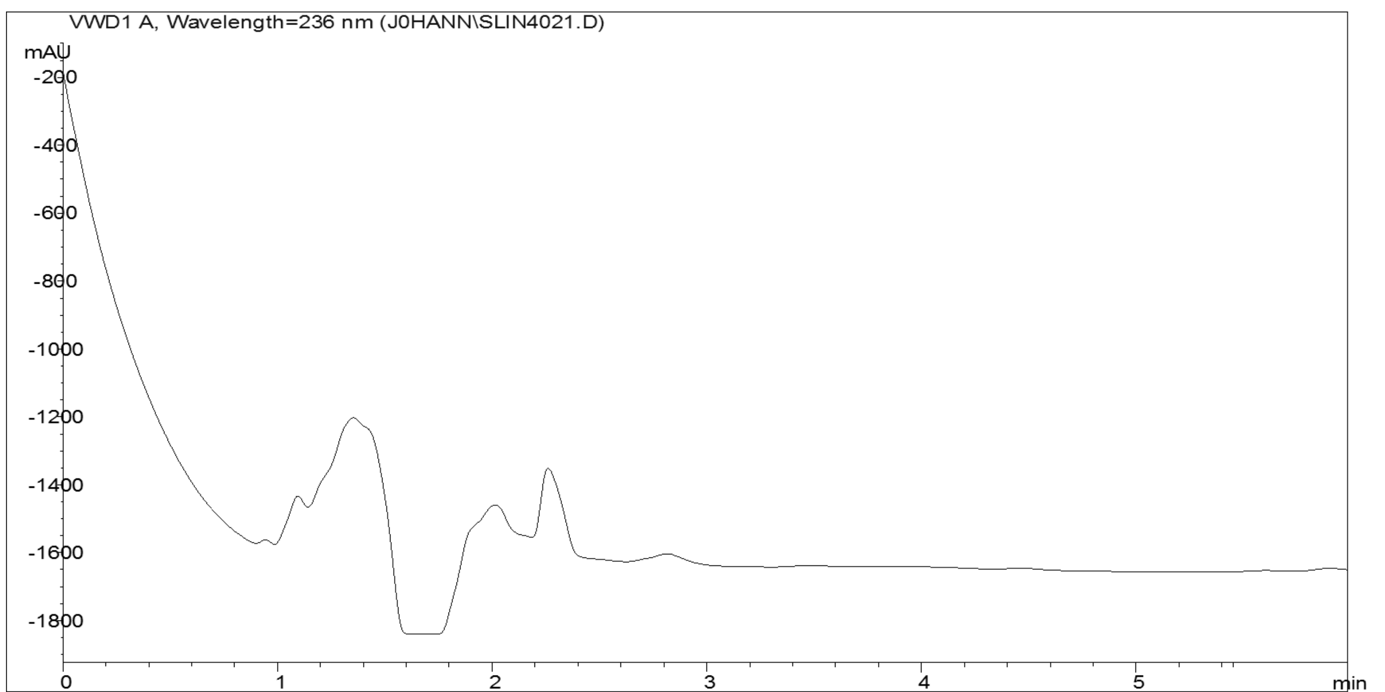


Figure 4. HPLC chromatograph of blank emulsion.

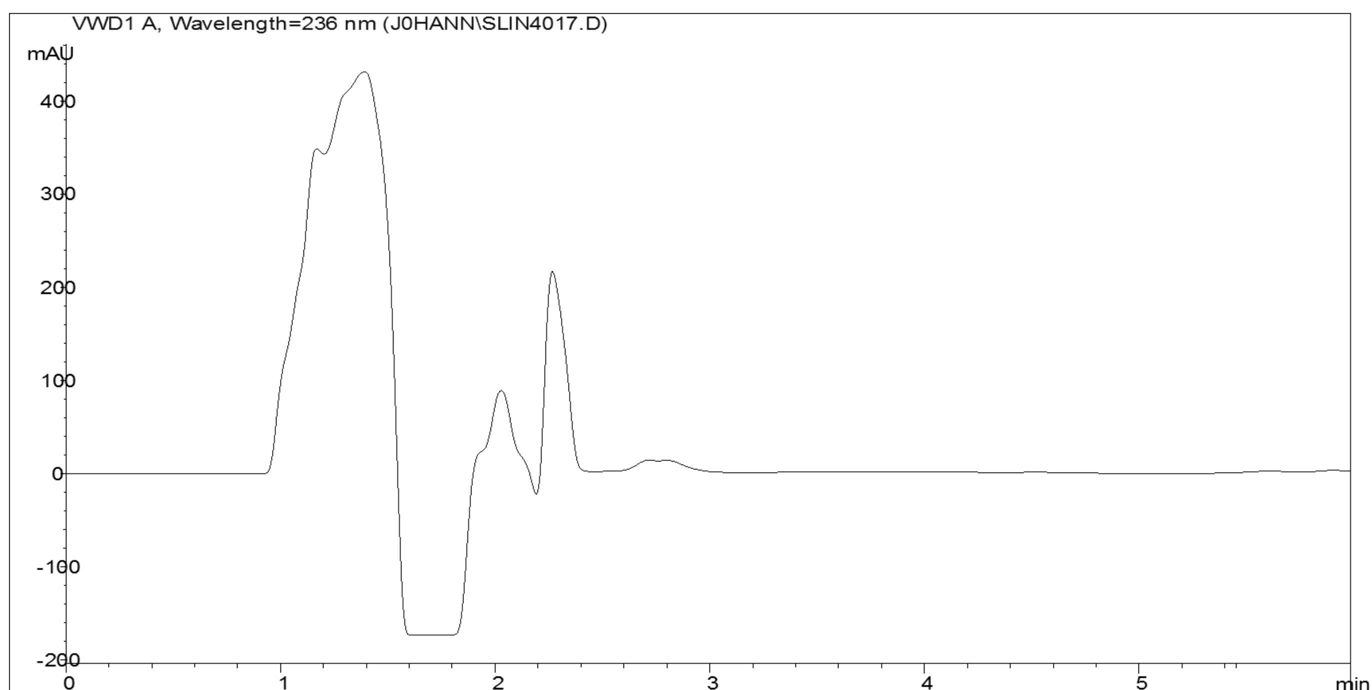


Figure 5. HPLC chromatograph of blank skin sample.

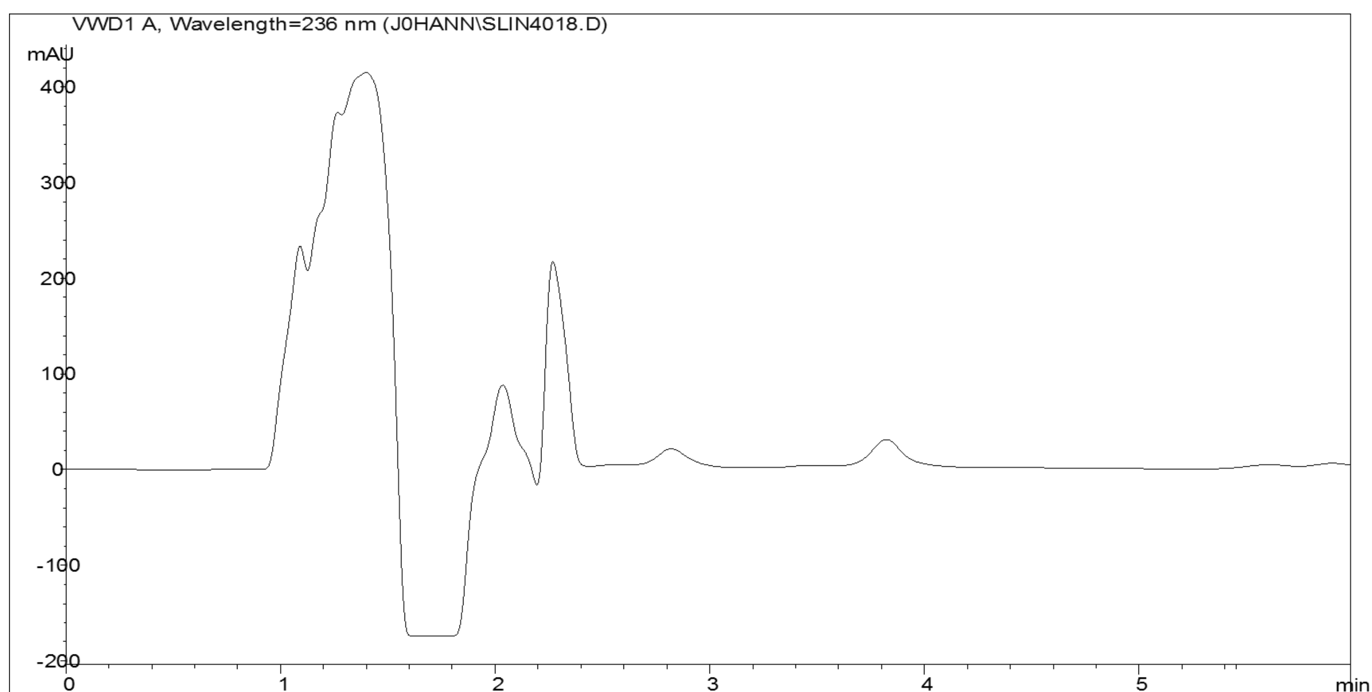


Figure 6. HPLC chromatograph of blank tape strip sample.

Stability

The stability of salicylic acid (250 µg ml^{-1}) in methanol and phosphate buffer pH 7.4:PG were determined every two hours for 24 hours and at 36 hours. The samples were kept at room temperature. The results are presented in Table 2:

Table 2. Determination of stability of salicylic acid in methanol and receptor phase.

	Area	Percentage of initial value [%]	Analyzed conc. [µg ml^{-1}]
Methanol	18703.8 ± 177.1	100.0 ± 1.0	254.97 ± 2.4
PBS:PG	18489.8 ± 73.0	100.6 ± 0.4	252.1 ± 1.0

From Table 2 it can be seen that no change in area occurred over time. Therefore, it could be concluded that salicylic acid was stable in methanol and receptor phase for at least 36 hours.

Reference

United States. Department of Health and Human Services. Food and Drug Administration. 2001. Guidance for industry-bioanalytical method validation. http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf.

APPENDIX C

Droplet size data of emulsions containing salicylic acid

Day 0

Emulsion		d (0.1) [µm]	d (0.5) [µm]	d (0.9) [µm]	Span	D [4,3] [µm]	D [3,2] [µm]	Obscuration [%]
Whey	**pH 4**	1.01 ± 0.03	2.21 ± 0.01	4.62 ± 0.17	1.64 ± 0.09	2.61 ± 0.09	1.85 ± 0.02	13.37 ± 0.22
	pH 5	1.16 ± 0.03	2.48 ± 0.03	5.03 ± 0.19	1.56 ± 0.07	2.85 ± 0.06	2.08 ± 0.02	18.49 ± 0.33
	pH 6	0.99 ± 0.004	2.15 ± 0.01	4.36 ± 0.02	1.57 ± 0.005	2.47 ± 0.01	1.80 ± 0.01	13.27 ± 0.06
Chi	**pH 4**	2.53 ± 0.20	9.38 ± 0.96	69.15 ± 17.10	7.03 ± 1.16	25.77 ± 3.21	5.19 ± 0.41	14.28 ± 1.22
	pH 5	2.26 ± 0.06	15.91 ± 0.30	133.40 ± 2.97	8.24 ± 0.10	46.07 ± 1.28	5.84 ± 0.08	12.11 ± 0.04
	pH 6	10.65 ± 0.63	31.57 ± 2.95	76.22 ± 5.75	2.08 ± 0.03	38.34 ± 3.01	14.37 ± 0.83	11.77 ± 0.29
Car	**pH 4**	1.31 ± 0.04	2.73 ± 0.01	5.35 ± 0.14	1.48 ± 0.06	3.09 ± 0.03	2.31 ± 0.03	13.43 ± 0.58
	pH 5	1.16 ± 0.03	2.30 ± 0.01	4.26 ± 0.10	1.35 ± 0.06	2.54 ± 0.02	1.99 ± 0.02	12.39 ± 0.80
	pH 6	1.13 ± 0.04	2.19 ± 0.06	3.94 ± 0.09	1.29 ± 0.01	2.39 ± 0.06	1.90 ± 0.06	12.38 ± 0.17

Day 1

Emulsion		d (0.1) [µm]	d (0.5) [µm]	d (0.9) [µm]	Span	D [4,3] [µm]	D [3,2] [µm]	Obscuration [%]
Whey	**pH 4**	0.96 ± 0.003	2.22 ± 0.01	4.95 ± 0.22	1.80 ± 0.09	3.63 ± 1.12	1.82 ± 0.02	12.92 ± 0.26
	pH 5	15.89 ± 1.03	65.11 ± 2.78	218.89 ± 14.62	3.12 ± 0.11	94.13 ± 2.87	21.68 ± 1.01	13.54 ± 1.07
	pH 6	1.00 ± 0.03	2.18 ± 0.004	4.43 ± 0.12	1.57 ± 0.07	2.50 ± 0.03	1.82 ± 0.02	13.42 ± 1.10
Chi	**pH 4**	3.92 ± 0.06	16.74 ± 0.46	126.73 ± 14.39	7.32 ± 0.67	46.77 ± 4.47	7.66 ± 0.13	12.23 ± 0.63
	pH 5	7.31 ± 0.06	33.67 ± 0.41	185.75 ± 26.45	5.31 ± 0.85	70.69 ± 7.32	11.45 ± 0.08	12.25 ± 0.59
	pH 6	10.37 ± 0.16	29.13 ± 0.61	72.85 ± 1.10	2.15 ± 0.02	36.36 ± 0.68	13.76 ± 0.20	11.63 ± 0.67
Car	**pH 4**	1.34 ± 0.03	2.95 ± 0.06	7.09 ± 1.16	1.94 ± 0.34	9.09 ± 4.92	2.50 ± 0.09	13.67 ± 0.13
	pH 5	1.16 ± 0.07	2.45 ± 0.07	5.48 ± 0.47	1.76 ± 0.12	16.22 ± 12.11	2.12 ± 0.12	15.47 ± 3.30
	pH 6	1.19 ± 0.07	2.35 ± 0.09	4.58 ± 0.42	1.44 ± 0.11	7.71 ± 6.18	2.07 ± 0.13	11.99 ± 0.25

Day 7

Emulsion		d (0.1) [µm]	d (0.5) [µm]	d (0.9) [µm]	Span	D [4,3] [µm]	D [3,2] [µm]	Obscuration [%]
Whey	pH 4	0.96 ± 0.04	2.20 ± 0.02	5.37 ± 0.96	2.00 ± 0.43	3.69 ± 1.21	1.83 ± 0.04	13.11 ± 0.12
	pH 5	12.54 ± 1.90	61.70 ± 6.27	216.27 ± 13.69	3.32 ± 0.31	93.64 ± 6.71	18.02 ± 2.92	14.82 ± 1.16
	pH 6	1.01 ± 0.03	2.10 ± 0.001	4.12 ± 0.12	1.48 ± 0.07	2.38 ± 0.03	1.79 ± 0.02	12.35 ± 0.32
Chi	pH 4	3.78 ± 0.11	17.19 ± 0.64	129.76 ± 15.71	7.31 ± 0.69	47.23 ± 4.85	7.61 ± 0.19	13.45 ± 0.70
	pH 5	6.65 ± 0.35	30.23 ± 1.36	146.19 ± 8.58	4.62 ± 0.22	58.02 ± 3.00	10.60 ± 0.36	11.83 ± 0.16
	pH 6	10.42 ± 0.18	28.71 ± 0.88	74.12 ± 3.43	2.22 ± 0.05	37.25 ± 1.57	13.69 ± 0.30	12.09 ± 0.06
Car	pH 4	1.32 ± 0.04	2.95 ± 0.06	6.96 ± 1.04	1.91 ± 0.30	7.64 ± 4.95	2.46 ± 0.10	13.58 ± 0.68
	pH 5	1.11 ± 0.03	2.36 ± 0.01	4.73 ± 0.17	1.53 ± 0.08	2.69 ± 0.04	1.99 ± 0.01	14.11 ± 0.89
	pH 6	1.00 ± 0.01	2.03 ± 0.01	3.85 ± 0.01	1.41 ± 0.01	2.26 ± 0.01	1.74 ± 0.01	13.09 ± 0.56

Droplet size data of emulsions without salicylic acid

Emulsion		d (0.1) [µm]	d (0.5) [µm]	d (0.9) [µm]	Span	D [4,3] [µm]	D [3,2] [µm]	Obscuration [%]
Whey	pH 4	1.04 ± 0.03	2.38 ± 0.004	5.29 ± 0.19	1.78 ± 0.09	2.87 ± 0.05	1.96 ± 0.02	13.32 ± 0.92
	pH 5	2.11 ± 0.09	6.53 ± 0.42	13.58 ± 1.00	1.76 ± 0.03	7.31 ± 0.50	3.95 ± 0.19	13.04 ± 0.21
	pH 6	1.06 ± 0.03	2.32 ± 0.004	4.67 ± 0.10	1.56 ± 0.06	2.64 ± 0.02	1.93 ± 0.03	12.67 ± 0.34
Chi	pH 4	1.17 ± 0.02	2.59 ± 0.07	5.45 ± 0.27	1.65 ± 0.05	3.15 ± 0.14	2.15 ± 0.05	13.52 ± 0.76
	pH 5	3.80 ± 0.05	14.02 ± 0.27	35.42 ± 1.23	2.26 ± 0.04	17.37 ± 0.47	6.92 ± 0.08	12.71 ± 0.59
	pH 6	4.95 ± 0.25	16.19 ± 0.51	42.61 ± 1.23	2.33 ± 0.07	22.52 ± 1.17	7.67 ± 0.21	12.52 ± 0.05
Car	pH 4	0.96 ± 0.04	1.93 ± 0.07	3.74 ± 0.40	1.44 ± 0.14	2.10 ± 0.01	1.68 ± 0.09	11.83 ± 0.14
	pH 5	0.92 ± 0.04	1.84 ± 0.06	3.44 ± 0.14	1.38 ± 0.08	2.04 ± 0.07	1.59 ± 0.05	12.60 ± 0.68
	pH 6	1.01 ± 0.04	2.07 ± 0.03	3.94 ± 0.09	1.42 ± 0.06	2.31 ± 0.03	1.76 ± 0.04	13.41 ± 1.28

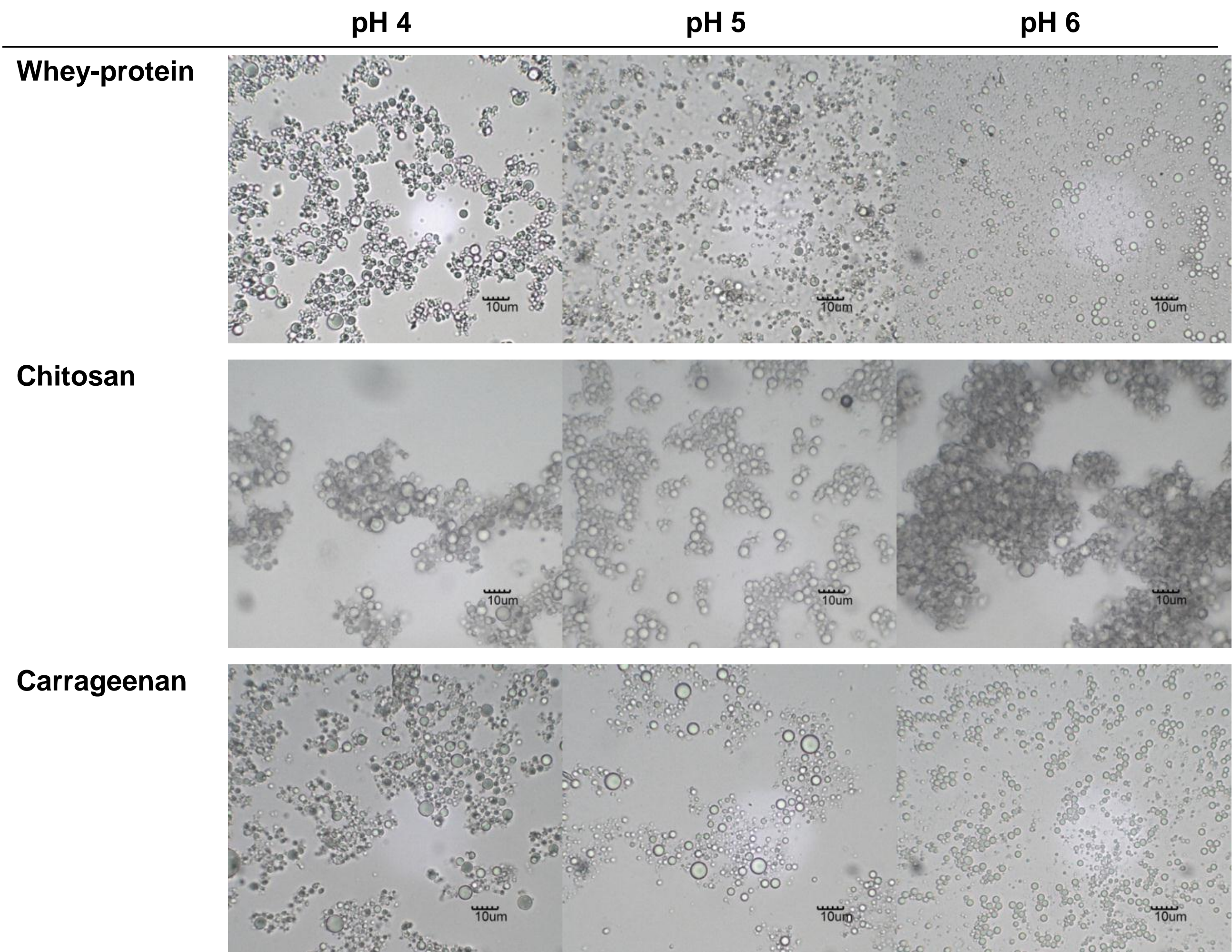
APPENDIX D

ζ-Potential data of emulsions

ζ-Potential [mV]		With salicylic acid	Without salicylic acid
		Day 0	Day 0
Whey	pH 4	23.9 ± 0.8	23.7 ± 0.5
	pH 5	3.8 ± 1.8	6.4 ± 2.2
	pH 6	-8.6 ± 1.4	-9.7 ± 0.6
Chi	pH 4	32.4 ± 2.0	26.7 ± 1.3
	pH 5	21.6 ± 0.8	20.7 ± 1.5
	pH 6	14.9 ± 3.6	9.5 ± 3.1
Car	pH 4	15.5 ± 0.8	22.5 ± 0.3
	pH 5	-13.0 ± 1.2	-18.7 ± 5.5
	pH 6	-32.5 ± 12.2	-21.0 ± 2.1

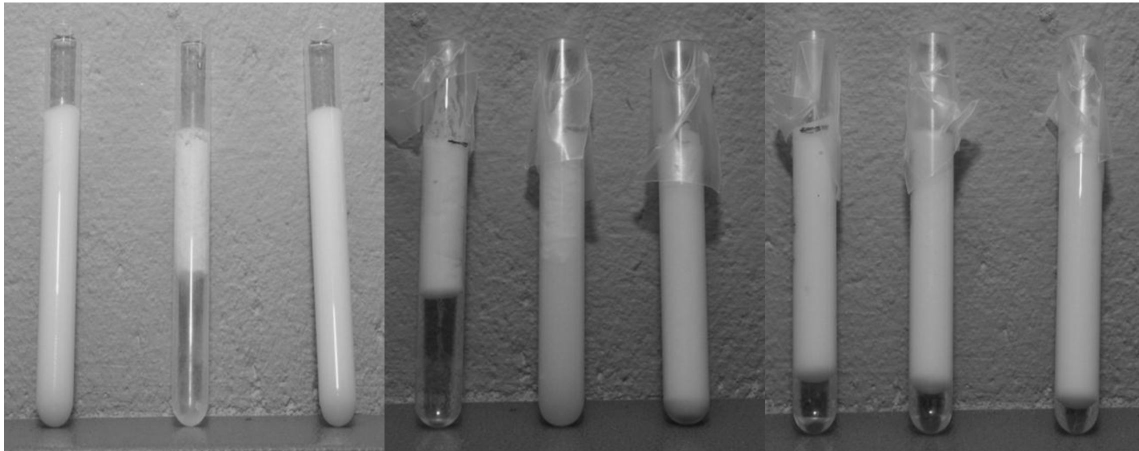
APPENDIX E

Microscopy images of emulsions – Day 1.



APPENDIX F

Photos indicating Creaming – Day 7.



Whey-protein Chitosan Carrageenan

APPENDIX G

Turbidity data (Absorbance value at 600 nm)

Turbidity [cm^{-1}]	pH 4		pH 5		pH 6	
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
Whey	0.737	0.752	2.433	2.428	0.437	0.437
Chi	0.081	0.054	0.943	0.947	1.309	1.353
Car	0.024	0.027	0.321	0.317	0.400	0.393
Sal	0.007	0.008	0.297	0.300	0.407	0.392
Whey + Chi	0.317	0.288	1.713	1.634	2.989	2.982
Whey + Car	> 9	> 9	4.822	3.848	0.448	0.452
Whey + Sal	0.705	0.792	2.454	2.475	0.451	0.486
Chi + Sal	0.022	0.027	0.587	0.573	1.255	1.214
Car + Sal	0.045	0.027	0.316	0.320	0.432	0.457
Whey + Chi + Sal	1.895	1.862	2.743	2.697	2.826	2.824
Whey + Car + Sal	> 9	> 9	3.930	3.649	3.469	3.143

APPENDIX H

Cumulative release data of salicylic acid from emulsions

		0.5h [μg cm^{-2}]	1h [μg cm^{-2}]	1.5h [μg cm^{-2}]	2h [μg cm^{-2}]	3h [μg cm^{-2}]	4h [μg cm^{-2}]	6h [μg cm^{-2}]	8h [μg cm^{-2}]	Rate [μg cm^{-2} h$^{-1/2}$]	R^2
Whey	pH 4	251.6	331.9	398.3	459.4	555.3	648.0	778.1	909.9	311.1	1.000
		185.7	235.4	281.8	328.3	409.7	480.6	593.9	710.1	249.9	0.997
		198.9	283.7	353.7	417.6	543.7	651.4	811.9	958.7	364.2	0.999
		222.0	284.3	349.4	405.6	493.6	576.7	693.1	796.8	276.2	1.000
	pH 5	369.8	493.4	580.6	648.3	726.2	816.0	979.8	1142.2	349.3	0.998
		420.5	553.6	654.9	717.2	825.6	910.1	1081.0	1235.1	372.1	0.999
		321.6	454.2	539.1	613.5	737.7	845.2	1019.4	1204.1	406.9	0.999
		362.4	502.5	584.7	675.5	825.4	965.3	1172.2	1368.6	473.6	0.999
	pH 6	410.4	558.1	658.3	711.2	825.3	925.6	1087.3	1225.3	373.5	0.998
		296.1	394.5	466.4	552.4	671.6	769.8	929.4	1074.5	369.8	1.000
		393.7	507.9	570.0	626.3	739.6	847.6	1015.2	1164.3	360.7	0.999
		332.1	429.2	489.1	565.8	666.7	767.0	920.5	1050.0	340.6	1.000
Chi	pH 4	175.0	258.6	322.8	386.0	478.2	554.0	661.9	779.6	283.4	0.999
		158.2	228.6	297.0	351.6	429.2	505.2	613.4	723.6	265.7	1.000
		162.9	247.9	320.4	379.6	474.0	553.7	676.3	786.1	294.2	1.000
		179.7	261.0	334.2	393.5	483.3	562.4	682.0	789.2	287.9	1.000
	pH 5	283.4	402.0	489.7	563.7	661.6	763.6	951.1	1115.7	384.9	0.999
		302.8	414.8	511.0	597.4	711.5	839.2	1040.3	1204.7	427.0	1.000
		258.8	376.6	473.8	564.6	722.0	860.6	1072.4	1259.4	478.0	1.000
		321.9	425.2	522.0	606.5	735.1	842.5	1032.9	1186.8	411.7	1.000

Cumulative release data (continued)

		0.5h [μg cm^{-2}]	1h [μg cm^{-2}]	1.5h [μg cm^{-2}]	2h [μg cm^{-2}]	3h [μg cm^{-2}]	4h [μg cm^{-2}]	6h [μg cm^{-2}]	8h [μg cm^{-2}]	Rate [μg cm^{-2} $h^{-1/2}$]	R^2
Chi	pH 6	325.9	432.0	512.0	573.9	657.2	725.5	855.0	982.3	299.6	0.998
		404.6	499.3	551.6	593.7	665.1	722.2	843.7	957.5	250.5	0.998
		320.2	418.7	494.5	561.5	659.5	733.3	886.5	1027.0	327.8	1.000
		304.2	419.8	508.1	557.8	644.1	737.9	896.1	1044.5	338.0	0.999
Car	pH 4	263.8	355.0	392.0	425.8	475.1	540.0	674.8	796.6	238.5	0.992
		239.6	342.1	414.5	474.2	553.6	633.3	760.2	859.5	288.5	0.999
		284.9	399.8	488.0	563.5	688.6	783.1	935.7	1060.1	366.7	0.999
		215.9	335.6	392.6	461.9	542.0	594.9	681.3	789.7	257.2	0.993
	pH 5	363.7	532.0	652.9	745.3	885.0	1005.7	1209.6	1354.8	463.1	0.998
		350.2	508.4	628.0	704.1	841.0	958.4	1106.6	1209.6	404.6	0.994
		296.1	483.8	589.9	666.0	794.6	892.6	1066.4	1195.5	410.8	0.995
		366.0	485.8	602.2	668.2	790.5	873.7	1023.4	1144.4	363.8	0.997
	pH 6	392.3	560.7	658.5	727.3	841.9	940.9	1131.6	1267.6	400.8	0.998
		410.3	595.2	712.0	798.8	939.5	1052.9	1242.7	1393.6	453.0	0.997
		389.5	558.2	650.6	711.3	800.2	877.4	1024.8	1148.3	337.8	0.994
		360.2	536.3	653.6	719.6	842.2	948.0	1154.7	1328.8	439.3	0.998
Oil		72.8	119.8	174.1	244.4	331.9	409.5	577.2	715.7	309.5	0.995
		74.9	144.2	214.8	271.0	377.1	483.2	660.2	843.9	362.8	0.995
		69.0	108.2	144.4	171.2	237.3	302.9	426.5	545.2	225.0	0.989
		95.9	175.4	251.3	325.4	454.7	579.8	779.4	961.1	415.2	0.997

APPENDIX I

Skin absorption data of salicylic acid from emulsions

		3h [µg/cm^2]	6h [µg/cm^2]	9h [µg/cm^2]	12h [µg/cm^2]	18h [µg/cm^2]	24h [µg/cm^2]	Flux [µg/cm^2/h^1]	Rest skin [µg/cm^2]	SC [µg/cm^2]	El. Res. [kΩ]
Whey	pH 4	19.16	51.63	85.25	123.89	198.20	276.41	12.31	3.64	6.37	18.1
		34.68	83.26	133.75	176.25	267.40	366.31	15.64	3.45	3.05	12.6
		4.08	20.19	41.98	65.62	115.89	171.00	8.05	4.51	8.49	25
		3.96	19.55	41.49	65.90	115.58	171.14	8.07	4.91	8.23	25.7
		17.81	52.42	91.17	125.99	200.95	277.00	12.36	5.34	11.42	29.3
		15.72	44.55	76.13	108.39	171.19	238.92	10.64	3.66	9.46	32.4
	pH 5	1.35	5.50	11.23	18.58	33.13	50.09	2.36	1.07	4.60	26.5
		4.63	8.21	10.28	10.90	22.44	23.82	0.98	1.27	12.17	20.5
		0.00	1.86	2.79	3.57	7.50	8.82	0.43	0.65	2.72	32.9
		0.00	1.51	2.55	2.86	6.66	8.05	0.39	0.68	4.04	35.1
		1.79	5.41	7.26	7.54	15.67	17.34	0.76	0.00	6.82	25.8
		2.47	5.82	6.48	7.15	14.08	15.32	0.63	0.00	3.96	28.3
	pH 6	15.11	22.90	24.96	26.35	28.61	31.10	0.64	0.80	0.62	11.8
		0.00	1.00	2.42	3.91	7.35	11.38	0.55	0.00	2.03	21.9
		0.00	0.00	0.00	0.27	1.38	2.79	0.14	0.00	1.72	30.6
		0.00	0.00	0.00	0.00	0.79	1.87	0.09	0.00	2.47	38.1
		0.00	0.00	0.51	1.25	2.83	4.73	0.24	0.00	2.38	26.9
		0.00	0.44	0.98	1.94	4.04	6.30	0.31	0.00	2.68	20.8

Skin absorption data (continued)

		3h [µg/cm²]	6h [µg/cm²]	9h [µg/cm²]	12h [µg/cm²]	18h [µg/cm²]	24h [µg/cm²]	Flux [µg/cm²/h¹]	Rest skin [µg/cm²]	SC [µg/cm²]	El. Res. [kΩ]
Chi	pH 6	1.35	5.50	11.23	18.58	33.13	50.09	2.36	0.72	2.73	15.2
		4.63	8.21	10.28	10.90	22.44	23.82	0.98	0.00	2.80	29.2
		0.00	1.86	2.79	3.57	7.50	8.82	0.43	0.00	1.76	27.8
		0.00	1.51	2.55	2.86	6.66	8.05	0.39	0.00	1.57	30.0
		1.79	5.41	7.26	7.54	15.67	17.34	0.76	0.67	2.90	21.8
		2.47	5.82	6.48	7.15	14.08	15.32	0.63	0.00	2.35	23.8
Car	pH 6	0.00	0.35	0.91	1.63	3.18	5.16	0.25	1.02	1.02	22.5
		0.00	0.00	0.00	0.32	1.05	2.14	0.10	1.05	1.04	36.4
		0.00	0.00	0.30	0.69	1.51	2.61	0.13	1.03	1.03	22.9
		0.00	0.00	0.34	0.79	1.88	3.31	0.16	1.25	1.25	18.8
		0.00	0.00	0.53	1.15	2.76	4.99	0.24	1.97	1.97	22.5
		0.00	0.00	0.45	0.94	2.16	3.91	0.19	1.45	1.45	27.9
Oil		2.17	14.60	25.85	38.40	63.79	90.52	4.20	2.91	2.03	14.1
		0.27	3.03	6.61	6.97	16.69	18.61	0.92	3.47	2.32	34.9
		0.60	4.40	6.41	7.64	16.30	17.34	0.83	1.41	3.69	28.1
		0.74	2.37	5.58	7.53	15.87	17.62	0.88	0.87	4.87	26.2
		2.51	5.98	9.54	11.15	22.44	23.82	1.09	3.35	6.93	32.9
		0.36	2.43	5.88	7.47	15.31	15.77	0.80	1.16	5.52	34.4

APPENDIX J

Cumulative release data of aqueous solutions of salicylic acid

	1h [µg/cm^2]	**2h [µg/cm^2]**	**3h [µg/cm^2]**	**4h [µg/cm^2]**	**6h [µg/cm^2]**	**8h [µg/cm^2]**	**12h [µg/cm^2]**	**Rate [µg/cm^2/h^1]**
pH 4	314.1	526.8	641.3	737.2	852.8	951.1	1099.1	308.2
	327.6	449.8	547.4	629.2	762.6	856.0	976.3	269.1
	297.6	416.8	490.6	557.7	656.2	740.9	857.8	226.5
	276.2	372.6	445.0	514.0	576.3	653.6	748.8	191.0
pH 5	245.4	348.2	395.8	453.3	551.3	634.0	749.2	204.4
	304.1	431.8	483.7	529.6	624.2	716.5	844.5	213.3
	269.4	378.6	464.1	552.8	665.6	757.9	869.1	249.6
	250.9	362.2	440.9	519.7	633.1	725.3	838.8	243.1
pH 6	238.8	293.1	329.5	378.2	441.7	498.1	582.3	141.8
	270.5	388.5	463.6	544.6	640.8	697.7	755.6	201.4
	268.9	364.9	432.8	495.4	585.1	655.7	755.3	198.9
	274.4	390.1	481.2	557.5	663.6	739.6	822.7	227.3

APPENDIX K

pH values of donor phase during release study from aqueous solutions

		pH Do 0h	pH Do 1h	pH Do 2h	pH Do 3h	pH Do 4h	pH Do 6h	pH Do 8h	pH Do 12h
Salicylic acid solution	pH 4	4.0	4.0	4.0	4.1	4.1	4.3	4.2	4.3
		4.0	3.9	4.0	3.9	3.9	4.3	4.1	4.3
		4.0	3.9	4.0	3.8	3.8	4.2	4.0	4.3
		4.0	3.9	4.0	4.1	4.1	4.2	4.0	4.2
	pH 5	5.3	5.3	5.8	5.4	5.3	5.4	5.6	5.7
		5.3	5.4	5.8	5.4	5.4	5.5	5.6	5.7
		5.3	5.4	5.5	5.4	5.4	5.6	5.6	5.7
		5.3	5.4	5.5	5.4	5.3	5.6	5.6	5.7
	pH 6	6.0	6.3	6.3	6.1	6.0	6.1	6.1	6.2
		6.0	6.2	6.2	6.1	6.2	6.1	6.3	6.2
		6.0	6.1	6.2	6.1	6.1	6.1	6.2	6.2
		6.0	6.1	6.2	6.1	6.0	6.1	6.2	6.3
Blank solution	pH 4	4.0	4.0	4.3	3.9	3.9	4.1	4.2	4.3
		4.0	4.1	4.2	4.0	3.9	4.0	4.0	4.2
	pH 5	5.0	5.1	5.3	5.0	5.0	5.0	5.2	5.1
		5.0	5.2	5.4	5.1	5.1	5.3	5.2	5.1
	pH 6	6.0	6.2	6.3	6.1	6.1	6.1	6.1	6.1
		6.0	6.3	6.4	6.2	6.1	6.2	6.1	6.1