UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DE RIBEIRÃO PRETO

LÍVIA SALOMÃO CALIXTO

Desenvolvimento de formulações cosméticas contendo ativos de origem natural: avaliação das propriedades físico-mecânicas, sensoriais e eficácia clínica

Lívia Salomão Calixto

Ribeirão Preto
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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Ciências Farmacêuticas de Ribeirão Preto/USP em 29/07/2019 para obtenção do Título de Doutor em Ciências

Área de Concentração: Medicamentos e Cosméticos.
Orientada: Lívia Salomão Calixto

Orientadora: Profª. Drª. Patrícia Maria Berardo Gonçalves Maia Campos

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FOLHA DE APROVAÇÃO

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Aprovado em:

Banca Examinadora

Prof. Dr. _____

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Dedicatória

Dedico esse trabalho à minha mãe Rachel.

Desde que nasci me pareço fisicamente com você.

Com o passar do tempo fui espelhando minha força na sua

e de repente me percebi parecida com você como mulher.

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“A alegria não chega apenas no encontro do achado, mas faz parte do processo da busca. E ensinar e aprender não pode dar-se fora da procura, fora da boniteza e da alegria.”

Paulo Freire

Resumo

CALIXTO, L. S. **Desenvolvimento de formulações cosméticas contendo ativos de origem natural: avaliação das propriedades físico-mecânicas, sensoriais e eficácia clínica.** 2019. 164 f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2019.

A preocupação com a hidratação da pele e retardar o envelhecimento cutâneo é cada vez maior entre os consumidores e, com isso, existe a busca por produtos que aliem várias vantagens em uma única formulação, os chamados produtos multifuncionais. Existem vários desafios no desenvolvimento desses produtos, como por exemplo, contemplar os diferentes tipos de pele, estabilizar diversos ingredientes e ainda possuir um sensorial agradável. O apelo da textura mostrou ter relação com uma aceitação maior do produto pelo consumidor. Dessa forma, a análise do perfil de textura é um importante passo tanto no desenvolvimento de produtos, quanto na predição da percepção sensorial e até mesmo na otimização dos processos de produção. Nesse contexto, o objetivo do presente trabalho foi desenvolver formulações cosméticas multifuncionais, buscando atender os diferentes tipos de pele e avaliar as propriedades físico-mecânicas e a eficácia clínica dessas formulações. Para tal, as formulações foram elaboradas com matérias primas biocompatíveis e substâncias ativas inovadoras e a escolha dos principais ingredientes da formulação foi realizada com ajuda de um planejamento completo de experimentos. Após a realização de testes de segurança *in vitro* e estabilidade física por determinação da reologia, as formulações foram consideradas seguras e estáveis. O sistema de análise das propriedades físico-mecânicas - texturômetro foi essencial para esse projeto, uma vez que, por meio de inúmeros testes, possibilitou obter informações importantes sobre o comportamento das formulações. Estudos de eficácia clínica imediata e percepção sensorial foram realizados com participantes franceses e brasileiros e as formulações tiveram performances parecidas. Por fim, eficácia clínica em longo prazo foi avaliada por técnicas de biofísica e analise de imagem da pele e os tratamentos propostos se mostraram eficazes na melhora da hidratação, do brilho e do controle de manchas na pele. Em síntese, o presente projeto contemplou diversas etapas do processo de Pesquisa & Desenvolvimento de cosméticos e, como resultado, foram desenvolvidas formulações estáveis, seguras, eficazes e com sensorial agradável.

Palavras-chave: Análise de textura; Formulações cosméticas; Eficácia clínica; Análise sensorial; Estabilidade; Reologia

Abstract

CALIXTO, L. S. **Development of cosmetic formulations containing natural active ingredients: evaluation of the physical and mechanical properties, sensory and clinical efficacy.** 2019. 164 f. Thesis (Doctoral). School of pharmaceutical sciences of Ribeirão Preto – University of São Paulo, Ribeirão Preto, 2019.

The concern with skin hydration and delay skin aging is increasing among the consumers. This way, there is a search for products that combine several advantages in one formulation, the called multifunctional products. There are several challenges in the development of these products, for example, contemplate the different types of skin, stabilize the active ingredients and have an agreeable sensory. The texture profile showed to be related to a greater acceptance of the product by the consumer. So, the analysis of the texture profile is an important step in both the development of new products and the prediction of sensory perception and even in the optimization of production processes. In this context, the objective of the present study was to develop multifunctional cosmetic formulations, seeking to attend to the different types of skin and to evaluate the physical-mechanical properties and clinical efficacy of these formulations. For this purpose, the formulations were prepared with biocompatible raw materials and innovative active substances and the choice of the main ingredients was carried out by full design of experiments. After *in vitro* safety tests and physical stability studies by rheology determination, the formulations were considered safe and stable. The system of analysis of physical-mechanical properties - texturometer was essential for this project, since through numerous tests, it was possible to obtain important information on the behavior of the formulations. Immediate clinical efficacy and sensory perception studies were performed with French and Brazilian participants and the formulations had similar performances. Finally, the clinical efficacy was evaluated by biophysical techniques and skin image analysis and the treatments showed to be effective in improving hydration, brightness and skin spots control. In summary, this project contemplated several stages of the Research & Development of cosmetics and as a result, stable, safe, effective and pleasant formulations were developed.

Keywords: Texture analysis; Cosmetic Formulations; Clinical efficacy; Sensory analysis; Stability; Rheology

Lista de figuras

Lista de tabelas

Lista de abreviaturas e siglas

FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
ABNT	Associação Brasileira de Normas Técnicas
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
TEWL	Perda Transepidérmica de Água
MCR	Microscopia Confocal de Reflectância a laser
mg	miligrama
g	grama
UV	Ultravioleta

Lista de símbolos

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SUMÁRIO

1. INTRODUÇÃO

A crescente mercado cosmético está relacionada às mudanças no perfil de consumo e aumento do interesse dos consumidores pelos tipos de produtos disponíveis. Isso fez com que o rigor do processo de pesquisa & desenvolvimento de cosméticos aumentasse, pois consumidores mais exigentes demandam por produtos mais adaptados às suas necessidades (INFANTE; CALIXTO; MAIA CAMPOS, 2016).

Ao idealizar um cosmético, é necessário considerar os diferentes fatores responsáveis pela eficácia e aceitação de um produto pelo seu público alvo. Gênero, idade, cultura e sensibilidade da pele são exemplos de questões que exercem um papel importante nesse processo (DRAELOS; THAMAN, 2005). Por isso, o surgimento dos produtos multifuncionais veio como solução para atender as necessidades desse público que almeja por um produto cosmético, que proteja sua pele e ainda atenda suas necessidades específicas (DRAELOS, 2011).

Um produto multifuncional pode ser composto de substâncias ativas (como anti-aging e clareadores), filtros solares (químicos e físicos), pigmentos, maquiagens, entre outros, que combinados à modernos processos de fabricação, resultam em produtos que simultaneamente cuidam da pele, tratam demandas dermatológicas e a embelezam esteticamente (TAMILVANAN, 2009; SCHUELLER; ROMANOWSKI, 2016). Porém, para garantir a qualidade dos produtos desenvolvidos, é necessária a preocupação com as suas características dentro e fora do laboratório, sua segurança e principalmente, sua eficácia.

Dentre as principais dificuldades encontradas no desenvolvimento tecnológico de formulações cosméticas podemos destacar a seleção e o balanço adequado de matérias-primas do veículo e o processo de preparo da formulação. Embora a escolha das substâncias ativas do produto seja muito importante para garantir a eficácia desejada, se as mesmas forem acrescidas a um veículo instável e com um sensorial desagradável, o produto não terá aceitação por parte do consumidor e, portanto, a eficácia proposta não será alcançada (BRUMMER; GODERSKY, 1999; SAVARY; GRISEL; PICARD, 2013; CALIXTO; MAIA CAMPOS, 2017).

Nesse contexto, a análise do perfil de textura é de grande valia na etapa de bancada do desenvolvimento de novos produtos e também na otimização dos processos industriais. Além disso, a correlação existente entre os parâmetros da análise do perfil de textura, com a avaliação sensorial subjetiva por voluntários treinados já foi

comprovada por estudos científicos (CALIXTO; INFANTE; MAIA CAMPOS, 2018; DUBUISSON et al., 2018; EUDIER et al., 2018). Ainda, essa análise representa um excelente indicador para a avaliação da estabilidade física de formulações cosméticas especialmente quando aliada à análises como a reologia. Com a avaliação rápida e precisa dessas técnicas, é possível avaliar sinais de instabilidade relacionados à alteração do pH, variação de temperatura e alteração das características organolépticas. Tal análise também pode ser de grande utilidade para estudar os efeitos da adição de princípios ativos na estabilidade das formulações, uma vez que, ativos como extratos vegetais, sais e outros, podem alterar significativamente a consistência do produto e, consequentemente, a estabilidade, textura e sensorial do mesmo, comprometendo a qualidade e reprodutibilidade das formulações (GIANETI et al., 2012; GILBERT et al., 2013a; CALIXTO et al., 2018).

A determinação do comportamento reológico da formulação auxilia na avaliação do desempenho do produto final durante a aplicação, bem como a natureza física do veículo. Assim, ela nos possibilita detectar sinais precoces de instabilidade física, facilitando o controle de qualidade dos constituintes da formulação e dos produtos finais. Entre os parâmetros avaliados por meio do estudo reológico podemos mencionar a viscosidade aparente mínima, os índices de fluxo e consistência, tixotropia (JIAO; BURGESS, 2003; CORRÊA; JÚNIOR, 2005; GUARATINI; GIANETI; CAMPOS, 2006; GASPAR; CAMPOS, 2007; SOUZA; CAMPOS, 2017). Dessa forma, é possível predizer valores práticos relacionados ao consumidor a partir de dados instrumentais, o que é importante para direcionar as etapas de pesquisa & desenvolvimento, justificar a escolha de matérias primas e melhorar o sensorial de formulações. Correlacionando esses valores também é possível obter um padrão de textura para formulações cosméticas, bem como um modelo de interação produto-pele (GILBERT et al., 2012; GORE; PICARD; SAVARY, 2018; SAVARY et al., 2019).

No desenvolvimento de formulações cosméticas, além dos pontos tecnológicos anteriormente mencionados, é de fundamental importância considerar o impacto do conhecimento da biologia da pele para a obtenção de formulações mais eficazes e inovadoras.

Entre todos os órgãos, a pele ocupa uma posição única e extensa, e está em contato ao mesmo tempo com o meio externo e o meio interno. Devido a essa

particularidade, exerce várias funções como: proteção contra microrganismos, substâncias e radiações lesivas e prevenção da perda excessiva de água. A aparência da pele humana sofre modificações ao decorrer da vida, devido a fatores extrínsecos e intrínsecos, como por exemplo, o aparecimento de rugas, perda da elasticidade e redução na hidratação, o que leva à uma busca constante por produtos e substâncias ativas que atendam às necessidades da pele, para a melhoria de alterações de caráter inestético e manutenção da eudermia (MAIA CAMPOS; MERCURIO, 2009; GIANETI; MERCURIO; CAMPOS, 2013; MAIA CAMPOS; MELO; MERCURIO, 2016).

Dessa maneira, o desenvolvimento de formulações biocompatíveis acrescidas da associação de substâncias ativas de origem natural tais como extratos e vitaminas visando a obtenção de produtos multifuncionais eficazes para a manutenção e restauração da integridade da pele é imprescindível na área cosmético-dermatológica (GIANETI et al., 2012; MAIA CAMPOS et al., 2015). Dentre as substâncias utilizadas para tal finalidade, destacam-se o extrato seco de *Spirulina maxima* obtida por processo biotecnológico, o extrato da raiz de chicória, o extrato de alfafa e o extrato de alga vermelha que apresentam potencial de atuação na renovação celular, na hidratação, na proteção da função barreira, bem como no controle da hiperpigmentação e da oleosidade da pele.

A Spirulina (blue green algae), apresenta uma rica composição, em vitaminas, minerais, além de um alto conteúdo proteico, podendo proporcionar benefícios para os cuidados gerais da pele. Além da grande concentração proteica, o extrato é rico em polissacarídeos e pigmentos, entre eles β-caroteno, isto é, provitamina A, além de vitaminas do complexo B (MIRANDA et al., 1998; BECKER, 2007; BELO; GASPAR; CAMPOS, 2011; DELSIN et al., 2015). O extrato de *Spirulina* pode trazer benefícios para pele quando adicionado em formulações cosméticas possibilitando o desenvolvimento de um produto com características multifuncionais. Essas formulações são mais estáveis, seguras, com menor custo e menor número de substâncias ativas, o que torna de grande importância o desenvolvimento de formulações cosméticas para os cuidados da pele contendo o referido extrato (NETO; DE CAMARGO; MAIA CAMPOS, 2015; SOUZA; CAMPOS, 2017).

A vitamina D apresenta potencial e grande interesse para aplicação em cosméticos, uma vez que regula os processos de manutenção da função barreira da pele, por meio da inibição da expressão de genes responsáveis pela proliferação de queratinócitos e indução da expressão de genes responsáveis pela diferenciação destes (BIKLE, 2011). Durante o processo de envelhecimento cutâneo e com a exposição solar reduzida, em função de medidas de fotoproteção excessivas, ocorre uma diminuição da síntese de vitamina D e do seu receptor específico, o que pode representar um impacto negativo na função barreira da pele (GIANETI; MAIA CAMPOS, 2014).

Assim, o estímulo do receptor de vitamina D (VDR) é um alvo terapêutico de grande interesse para a clínica dermatológica atual. Essa vitamina é capaz de regular o crescimento e diferenciação de queratinócitos e demonstrou efeito benéfico sobre a permeabilidade na barreira epidérmica (SEARING; LEUNG, 2010; BIKLE, 2011). Porém, a instabilidade da vitamina D dificulta o seu emprego em formulações cosméticas, o que desencadeou recentemente a busca de substâncias que atendam ao conceito “Vitamina D-like”, como o extrato de raiz de chicória.

Devido às suas propriedades como ser fotoestável, fotoprotetor e prevenir a formação do eritema induzido por radiação UVB, possui grande capacidade de aplicação na área cosmética (ENK et al., 2004). O extrato de raiz de chicória *Cichorium intybus* L., é rico em oligofrutoses, possui atividade semelhante à vitamina D, uma vez que atua no aumento da síntese de VDR, estimula a expressão de genes envolvidos nos processos de cornificação e descamação (KLF4, Citoqueratina 1, Involucrina, Cistatina E/M, KLK), e possui atividade *in vitro* no aumento da espessura da epiderme e síntese de filagrina (ENK et al., 2004; BASSMANN, 2013; EL-SAYED et al., 2015; MAIA CAMPOS et al., 2017).

O extrato de Alfafa (*Medicago sativa*) foi proposto pelo efeito semelhante ao Retinol (“retinol-like”), apresentando potencial para estimular a atividade celular que diminui durante o processo de envelhecimento, favorecendo a renovação da epiderme e regulando a diferenciação dos queratinócitos. Além disso, possui ação antioxidante, podendo proteger e reparar a derme por meio da estimulação da síntese de Colágeno I e reduzir a atividade das metaloproteinases responsáveis pela destruição das fibras de elastina. Consequentemente, a pele é revitalizada, a função barreira da pele é restaurada

e as rugas são atenuadas (RANA et al., 2010; SILVA et al., 2013; PANCHENKO; MURATOVA; TURKOVSKAYA, 2017).

Além dos benefícios acima propostos, a ação clareadora é de extrema importância para a obtenção de um produto final multifuncional que atenda às finalidades propostas. Nesse contexto, a fração de oligossacarídeos ricas em xilose e galactose obtidas de *Palmaria palmata*, uma alga vermelha, vem sendo proposta com uma alternativa segura e inovadora com atividade clareadora. Estudos *in vitro* demonstram os seus efeitos na inibição da enzima tirosinase, e do complexo proteico responsável pela transferência de melanossomos e pela inibição do "Stem Cell factor" após indução por radiação ultravioleta (HARNEDY et al., 2014). Portanto, a fração de oligossarídeos atua em diferentes etapas envolvidas no processo de pigmentação cutânea: melanogênese, transporte de melanossomas e pigmentação induzida pela radiação ultravioleta.

Como parte do protocolo de Pesquisa & Desenvolvimento de Cosméticos, temos ainda a avaliação da eficácia e segurança dos produtos em desenvolvimento. As técnicas de biofísica e análise de imagem da pele são técnicas *in vivo* com grande aplicação no estudo da eficácia clínica de produtos cosméticos. Elas são técnicas não invasivas, que a partir de equipamentos com diferentes princípios físicos e/ou físico-mecânicos, permitem determinar como os produtos em teste podem atuar na pele em tempo real (GASPAR; CAMPOS, 2007; GASPAR et al., 2008; MAIA CAMPOS et al., 2012; WAGEMAKER et al., 2015). Desta forma, é possível avaliar e correlacionar parâmetros como conteúdo aquoso do estrato córneo, brilho da superfície da pele, perda de água transepidérmica, o microrrelevo cutâneo, dentre outros, utilizando equipamentos como Corneometer®, Glossymeter®, Tewameter® e Visioscan® (BERARDESCA et al., 1997; LEVEQUE, 1999; ROGIERS, 2001; CALIXTO et al., 2018).

Além disso, com as técnicas avançadas de análise de imagem, como o ultrassom de alta frequência e o microscópio confocal de reflectância, é possível avaliar as características morfológicas e estruturais da derme e epiderme, com a obtenção de dados conclusivos na avaliação das características da pele e eficácia clínica de produtos (WAGEMAKER et al., 2017; MARTINI; MAIA CAMPOS, 2018).

A microscopia confocal de reflectância a laser (MCR) é uma técnica inicialmente aplicada em diagnósticos dermatológicos e representa um avanço em

estudos de avaliação cutânea e testes de eficácia clínica de cosméticos. O Vivascope®1500 é um exemplo de aparelho utilizado para esta análise. Trata-se de uma análise sem a necessidade de tratamento tecidual, que é capaz de identificar, entre outras estruturas, as diferentes camadas da pele, áreas hiperqueratóticas, glândulas sebáceas, poros e microcomedões. Sendo assim, é uma técnica de grande interesse a ser utilizada na avaliação dos feitos de produtos cosméticos na pele (MAIA CAMPOS; MELO; MERCURIO, 2016).

Em paralelo aos estudos de eficácia clínica, a análise sensorial é uma importante ferramenta que permite estudar a percepção dos produtos por pessoas que tomam a decisão final sobre a compra de cosméticos. Mesmo que um produto tenha claims que prometam inúmeros benefícios, que pareça promissor e tenha um preço acessível, se ele não possuir um sensorial agradável, o consumidor não vai aderir ao tratamento, interromper o uso e vai trocar de produto (PARENTE; GÁMBARO; ARES, 2008; WANG; ADHIKARI, 2008; PENSÉ-LHÉRITIER, 2015; INFANTE; CALIXTO; MAIA CAMPOS, 2016).

Dependendo do objetivo, a análise sensorial pode ser realizada com participantes treinados ou não treinados. No primeiro caso, participantes são formados em determinadas propriedades que interessam o pesquisador. Eles devem estudar diferentes padrões para cada característica e aprender à classificá-la dentro de uma escala. Após o treinamento eles são chamados de “experts” e compõem um painel treinado. Esse tipo de análise é interessante quando se pesquisa uma propriedade sensorial específica ou quando se deseja validar uma medida instrumental a correlacionando com dados sensoriais (RISVIK; MCEWAN; RØDBOTTEN, 1997; ALBERT et al., 2011; PARENTE; MANZONI; ARES, 2011; SAVARY; GRISEL; PICARD, 2013; CALIXTO; INFANTE; MAIA CAMPOS, 2018).

O outro tipo de análise é feito com um painel não treinado, ou seja, com consumidores de certo tipo de produto que não receberam treinamento à respeito daquilo que se está pesquisando, por isso, eles formam o chamado painel consumidor (CHAO; SCHOR, 1998; ALMEIDA; GAIO; BAHIA, 2008; CALIXTO; MAIA CAMPOS, 2017; KWAK et al., 2017). As vantagens de trabalhar com esse tipo de painel são principalmente a facilidade em encontrar participantes, rapidez e baixo custo da análise e liberdade na coleta de respostas. Todavia, existem autores que questionam a

diferença entre esses dois tipos de painel (WORCH; LÊ; PUNTER, 2010; VARELA; ARES, 2012).

Para se assegurar da aplicabilidade dos dados, pesquisadores realizam estudos multicêntricos para assim verificar se um produto produzido para determinada população consegue agradar pessoas de outras regiões que possuem culturas, línguas e padrões de consumo diferentes (SOUIDEN; DIAGNE, 2009; HERSLETH et al., 2013; KIM et al., 2013; MONTEIRO et al., 2017).

Por fim, antes dos estudos clínicos e análise sensorial, é necessário realizar testes de segurança *in vitro* que garantam que nenhum efeito nocivo será causado aos participantes devido à aplicação do produto sobre a pele. A toxicidade induzida pela luz pode promover a geração de espécies reativas de oxigênio (ERO) e causar alta penetração na pele ou nos olhos. Como método alternativo ao uso de animais, a avaliação toxicológica de produtos com baixo potencial de irritação ocular é realizada com auxílio das técnicas de ensaio da membrana cório-alantoide (HET-CAM) e ensaio de fototoxicidade *in vitro* 3T3 NRU (BENEVENUTO; GASPAR, 2017; GASPAR; KAWAKAMI; BENEVENUTO, 2017; CAMPOS et al., 2019).

Diante do exposto, o presente trabalho apresenta como contribuição o desenvolvimento de formulações cosméticas multifuncionais inovadoras, seguras e estáveis que tiveram sua eficácia comprovada em pele francesa e brasileira e sensorial aceito por consumidores dos dois países. Ainda, apresenta a aplicação da análise de textura como ferramenta na caracterização e na predição do aspecto sensorial de formulações cosméticas.

2. OBJETIVOS

2.1. Objetivo geral

Desenvolver formulações cosméticas multifuncionais estáveis com ativos de origem natural, caracterizar suas propriedades físico-mecânicas e sensoriais, bem como avaliar a eficácia clínica dessas formulações em curto prazo (efeitos imediatos) e em longo prazo.

2.2. Objetivos Específicos

- Desenvolver formulações cosméticas com ingredientes inovadores, seguros e estáveis;
- Racionalizar a escolha dos ingredientes por meio de design fatorial completo de experimentos;
- Realizar estudos completos de estabilidade e segurança;
- Caracterizar o comportamento das formulações por meio de testes micro e macroestruturais, com destaque para a análise de textura;
- Verificar a influência dos ingredientes adicionados à formulação veículo sobre o comportamento das formulações;
- Verificar, por meio de estudos clínicos e análises sensoriais, a eficácia das formulações e sua aceitabilidade em população brasileira e francesa;
- Correlacionar dados instrumentais e resultados *in vivo* à partir de ferramentas estatísticas.

3. TRABALHOS DESENVOLVIDOS

Essa tese foi dividida em sete capítulos que reúnem os sete artigos resultantes do presente projeto de doutorado (APÊNDICE 1). A divisão foi feita de acordo com as etapas de um processo de pesquisa e desenvolvimento de cosméticos e visa facilitar a leitura e compreensão da evolução do projeto.

Os capítulos 1, 2 e 3 contêm os três artigos que descrevem o processo de pesquisa e desenvolvimento das formulações estudadas. Nele, encontram-se os experimentos realizados nas etapas iniciais como o desenvolvimento da formulação veículo e os primeiros testes para a compreensão da análise de textura.

Após o desenvolvimento de um veículo estável, iniciou-se o processo de busca de ingredientes para comporem a formulação multifuncional. Por fim, as seguintes substâncias ativas foram escolhidas e acrescentadas à formulação veículo junto de filtros solares capazes de fornecer um FPS 30:

• Ingredientes ativos:

Extrato de Blue Green Algae - Spirulina dry extract - Ouro Fino Agronegócios

Extrato de Raiz de Chicória - *Cichorium intybus* (Chicory) root extract - Silab®

Extrato de Alfafa - *Medicago sativa* (Alfalfa) Extract - Silab®

Extrato de Alga Vermelha - *Palmaria palmata* extract - Silab®

• Filtros solares:

Ethylhexyl Salicylate - Neo Heliopan® OS - BASF

Bis-ethylhexyloxyphenol methoxyphenyl triazine - Tinosorb S® - BASF

Ethylhexyl Methoxycinnamate - Uvinul A Plus® - BASF

Ethylhexyl Triazone - Uvinul T150® - BASF

Para verificar o efeito de cada ativo sobre as propriedades em estudo, os quatro ingredientes ativos foram adicionados separadamente à formulação veículo e em combinação, totalizando 6 formulações (veículo; veículo + ativo 1; veículo + ativo 2; veículo + ativo 3; veículo + ativo 4; veículo + quatro ativos em combinação).

Da mesma maneira, estudou-se a influência dos filtros solares sobre todas as propriedades adicionando-os às seis formulações anteriores (veículo + filtros; veículo + ativo 1 + filtros; veículo + ativo 2 + filtros; veículo + ativo 3 + filtros; veículo + ativo 4 + filtros; veículo + quatro ativos em combinação + filtros), totalizando assim, 12 formulações em estudo.

Os ensaios de estabilidade em longo prazo e a caracterização micro e macroestrutural foram realizados para compreender a organização dos sistemas, bem como, a influência de cada ingrediente adicionado à formulação sobre propriedades importantes como reologia e textura. Como resultado, foram desenvolvidas formulações estáveis, com sensorial agradável e aptas à serem aplicadas in vivo.

Nos capítulos 4, 5, 6 e 7 serão abordados os testes de segurança in vitro realizados, bem como a avaliação da eficácia clínica imediata e em longo prazo e a análise sensorial com consumidores.

Após serem consideradas seguras, as formulações foram avaliadas por dois grupos de participantes: brasileiros e franceses. Eles formaram o painel consumidor que julgou a influência dos diferentes ingredientes nas formulações e determinou suas preferências em relação às opções disponíveis.

A avaliação da eficácia clínica dos efeitos imediatos das formulações também foi feita em colaboração, tendo assim participantes dos dois países no estudo. Para os estudos de eficácia em longo prazo, foram testadas a formulação contendo o extrato de alga vermelha (Palmaria palmata) isolado e a formulação contendo todos os ativos em associação.

3.1. Capítulo 1 – Desenvolvimento da formulação veículo

3.1.1. Artigo 1:

CALIXTO, L. S. ; INFANTE, V. H. P. ; CAMPOS, P. M. B. G. M. ; Design and characterization of topical formulations: correlations between instrumental and sensorial measurements. AAPS Pharmscitech, v. 19, p. 1512-1519, 2018.

Design and characterization of topical formulations: correlations between instrumental and sensorial measurements

Abstract

The interaction between cosmetic emulsions and the skin's surface is an important factor to consider in the development of topical formulations. Two important ingredients in cosmetics formulations are waxes and polymers. The physical and mechanical properties of formulations directly impact the interface skin-formulation. To evaluate this interaction, it is important to study the rheology, texture and sensory properties. In this context, the aim of the study was to evaluate the influence of waxes and polymers on the rheological behavior, texture profile and sensorial properties of topical formulations and the correlation between these parameters. The best combination of a wax and a polymer was applied to develop eight formulations that were tested in relation to rheological, mechanical and sensorial properties. The polymer helps with the spreadability of the formulation, and the wax had a strong influence on the parameters related to the structure of emulsions. A correlation between these parameters was observed. This way, it was possible to compare theoretical and practical data, except between the flow index and the work of shear. Finally, it was possible to predict sensorial aspects from rheological and texture parameters, making the formulation process easier and more integrated with all stages of the development of new topical formulations. Thus, the present study introduces a new proposal in the development of cosmetics.

Keywords: polymers, waxes, topical formulations, rheological behavior, mechanical properties, sensory analysis

Introduction

Colloid science gives a basis for the development of numerous technologies. From the controlled release of a drug to the induction of plant growth, there are no limits for its application. There are several cosmetics based on colloidal dispersions, for example, gel, cream, hairspray and deodorant (1). Cosmetic creams are generally formed by an oil-in-water emulsion. Some are classified as a wax-in-water emulsion when a wax is utilized to stabilize them.

Ingredients as filters, stabilizers and surfactants can act as thixotropic modifiers by altering the physical structure of the complex (2,3,4). In emulsions, waxes can promote better adhesion of the particles and can interact with the emulsion interface, promoting a steric barrier to drop fusion (5). Furthermore, studies have shown that the addition of waxes to the emulsion promotes modification in rheological and physical properties, such as structural network strength (6,7,8). Polymers can also be used to stabilize the emulsion system because they promote steric stabilization through surface particles (1,9). In addition, studies have shown that a polymer can be a texture agent and influence texture and sensorial parameters, such as viscosity and consistency (10,11,12).

The adequate combination of polymers and waxes, as well as the balanced concentration of these components, is still a challenge in the development of stable and effective cosmetics products with an improved sensory. The oily content of the formulations appears to have a high influence on their physical properties. (13,14). Thus, the present study introduces a new proposal in the development of cosmetics by the prediction of formulations sensory through physical methods that impact the skin-formulation interface. (15,16,17). Polymers and waxes have aggregative action

on colloidal particles and can modify rheological, mechanical and sensory properties of formulations. However, it is not clear how much they affect these parameters and which of them has a stronger effect. Due to this, a combined rheology, texture and sensory analysis study is very important to elucidate these questions.

In the literature, there are several studies linking these techniques, but these studies are mostly in the food field (18,19,20). Lukic, Jaksic, Krstonosic, Cekic, and Savic (2012) demonstrated their utility in cosmetic studies as a sensitive tool, which allows one to optimize the structure of the formulation, to directly influence its behavior and stability and to provide adequate parameters for sensory use. They demonstrate the dependency of sensory properties in relation to physical and mechanical characteristics of emulsions (21).

Gilbert, Picard, Savary and Grisel (2013) verified the polymer's positive influence on viscosity and viscoelastic parameters and found a statistical correlation between rheology and texture analysis (22). Brenner et al (2014) showed correlations between similar empirical approaches that are useful to map the expected characteristics of a given formulation (23). Savary et al (2013) tested texture analysis to evaluate the spreading properties of cosmetic emulsions and found that the composition of the oily phase has a significant effect on spreadability, an important sensory attribute (24).

The work of shear is a predictive parameter of spreadability (16,25) obtained from texture analysis. Due to the importance of the shear and spreadability characteristics of formulations, this parameter was chosen to compose a full factorial design of experiments to evaluate the influence of different waxes and

polymers in topical formulations. The statistical design allows for the study of the influence of different variables according to the desired responses, optimizing processes, reducing the number of experiments and saving time and money (26,27).

In this context, the aim of the present study was to evaluate the influence of wax and polymer in rheological behavior, texture profile and sensorial properties of topical formulations and the correlation between these parameters.

First, a full factorial experiment was designed with pre-formulations to evaluate the significance of waxes and polymers on the work of shear parameter. After that, formulations were developed with a wax and a polymer, which produced better results, to obtain texture patterns for a characterization and correlation study of rheological, textural and sensorial properties.

Materials and Methods

Development of pre-formulations

Before the development of the studied formulations, pre-formulations were developed to perform the full factorial design of experiments. It is extremely important to choose the correct wax and polymer to ensure a formulation is pleasant from a sensory perspective.

The raw materials currently available were studied, and two self-emulsifying waxes were chosen: cetearyl alcohol and dicetyl phosphate and ceteth-10 phosphate – Crodafos™CES/Croda inc (Wax 1) and mineral oil/paraffinum liquidum/cetearyl alcohol/ceteth-20/glyceryl stearate/PEG-40 hydrogenated castor oil/polyacrylic acid/sodium hydroxide/xylitol/caprylic acid – Emulfeel SSC/Chemyunion (Wax 2). They were provided by Croda do Brasil Ltda (Campinas, SP, Brazil) and

Chemyunion Ltda (Sorocaba, SP, Brazil) respectively. Two hydrophilic polymers were also selected: acrylates/C10-30 alkyl acrylate crosspolymer – Pemulen™ TR2/Lubrizol (Polymer 1) and sclerotium gum - Amigel®/Alban Muller (Polymer 2). They were provided by Lubrizol do Brasil Aditivos Ltda (Sao Paulo, SP, Brazil) and Pharmaspecial Especialidades Químicas e Farmacêuticas (Santana de Parnaiba, SP, Brazil) respectively.

Table I - Composition of formulations of design of experiments.

Ingredients	**Composition (w/w)**			
	F1	**F2**	**F3**	**F4**
Polymer 1	0.2%	-	0.2%	-
Polymer 2	-	1%	-	1%
Wax 1	-	5%	5%	-
Wax 2	6%	-	-	6%

The purpose was to combine the two selected polymers and the two selected waxes to obtain four emulsions stabilized with hydrophilic colloid. The pre-formulations were developed according to the specifications of the active substances studied, the sensory characteristics and the interaction of the raw materials used in formulations (Table I).

With the objective to develop a vehicle for the formulations, the following raw materials were used: Cyclopentasiloxane and Cyclomethicone (and) Dimethicone Crosspolymer that were provided by Dow Corning do Brasil Ltd. (Hortolandia, SP, Brazil). Butyl hydroxy toluene, glycerin, phenoxyethanol and parabens, ethyelenediamine tetraacetic acid, aminomethyl propanol, propyleneglycol, were provided from Mapric Produtos Farmacêuticos e Cosméticos (Sao Paulo, SP, Brazil). Ethylhexyl Salicylate was provided by Symrise (Galena, Campinas, SP, Brazil).

The aqueous phase was incorporated into the oil phase under heating at 70°C. The preparations were stirred for 20 minutes and then neutralized with AMP 95 to pH 5.5. The polymer, silicones and preservatives were then added. Subsequently, homogeneous and stable formulations were obtained. The formulations were tested in terms of preservation against bacterial development using standard tests performed by an external laboratory.

Full factorial design of experiments

To evaluate the effect of the addition of different waxes and polymers in topical formulations and the best combination of them, a full factorial design of experiments was drawn up using the Minitab software (Minitab 17, Minitab Inc., State College, PA, U.S.A.). The objective was to evaluate effects and interactions of the polymers and waxes. As spreadability is the ability to spread and deform the product with ease and uniformity, the variable "work of shear" was chosen as the sensory predictor. The lower work of shear, the better the spreadability of the formulation (16,28). The factorial design used was a "factor 2-level" full factorial, with four runs and four replicates. There was no central point, and the number of blocks was one.

Two categoric factors were evaluated, "polymer" and "wax," and also an answer, "work of shear", which is a continuous factor. The answer "work of shear" was obtained from the equipment System of Physical and Mechanical Properties Analysis, model TA.XT/Plus (Stable Microsystems, United Kingdom) equipped with the TCC Spreadability rig HDP/SR.

Development of formulations

With the best wax and polymer combination obtained, eight formulations were developed with the vehicle previously mentioned, combining extreme concentrations of waxes and polymers and the presence or not of them (Table II). Building this scale of texture, it was possible to obtain a texture profile of the formulations and to correlate theoretical and practical results.

Table II - Concentrations of wax and polymer .

Ingredient	**Formulation**							
	FA	**FB**	**FC**	**FD**	**FE**	**FF**	**FG**	**FH**
Wax 1	10%	10%	1%	1%	10%	1%	-	-
Polymer 1	0.50%	0.15%	0.15%	0.50%	-	-	0.50%	0.15%

Rheology

The rheological behavior was determined using a Rheometer R/S-CPS Plus (cone/plate and plate/plate) Brookfield, with P50 spindle and temperature probe Pt 100 1/3 DIN, coupled with the Rheo software V2.08 version. The shear rates progressively increased from 0 to 120 s^{-1} for 120s at 25°C, 24h after its preparation. The procedure was repeated in reverse by gradual decrease of shear rate from 120 to 0 s^{-1}, obtaining an ascendant curve and a descendant curve. Apparent viscosity was obtained from rheograms that were mathematically analyzed according to the Power Law model (Equation 1) in which τ is the shear stress (Pa), γ is the shear rate, m is the consistency index ($Pa.s^n$) and n is the flow behavior index (9).

$$\tau = K .(\gamma)^n \qquad (1)$$

Texture analyses

The texture analyses were performed using a TA.XT plus Texture Analyzer (Stable Microsystems, United Kingdom). To evaluate the work of shear parameter, the system was equipped with the TTC Spreadability rig HDP/SR. To evaluate the parameters index of viscosity, consistency and cohesiveness, it was equipped with a Back Extrusion rig A/BE of 35 mm for formulations FA, FB, FD, FE, and FG, and rig A/BE of 45 mm for formulations FC, FF and FH. The formulations were loaded in 125 mL containers with 50 mm diameter. Measurements were made in triplicate. The textural properties of the formulations were calculated via the instrument software. In the spreadability analysis, the work of shear is given from the area under the positive curve. The probe conditions were: return distance 100 mm, return speed 20 mm/sec and contact force 30 g. In Back Extrusion analysis, consistency is given by the area under the positive curve, cohesiveness from the maximum value of the negative curve and Index of viscosity from the area under the negative curve. For this test, the return distance used was 25 mm, the return speed was 20 mm/sec and the contact force was 30g.

Sensory analysis

To evaluate the sensory characteristics of the formulations, a trained panel of ten volunteers, with age between 18 and 30 years, was used and this phase was approved by the ethical committee (CEP/FCFRP nº. 381). The volunteers attended the two training sessions in a sensory analysis cabin. The first training session was based on a protocol where panelists were trained in definitions of sensory analysis to validate their opinions (29). The second session consisted in a training with four wax and polymer standards formulations. After these training sessions, an evaluation of the formulations was made with a simple scale from 1 to 5, which corresponded to very low, low, intermediate,

high and very high, respectively. The volunteers classified the formulations in relation to: spreadability, consistency, cohesiveness and viscosity.

Statistical Analysis

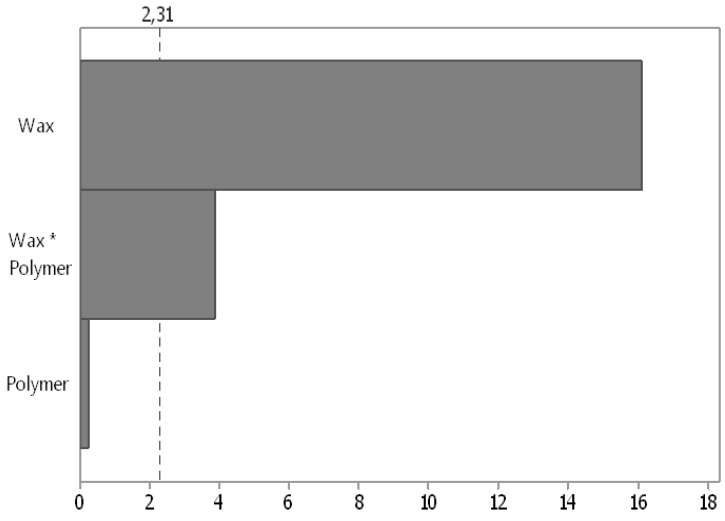
The data obtained from texture analyses were considered normal and correlated in pairs, using an unpaired student t-test. The ranking of sensory analysis was compared by a Kruskal and Wallis one-way analysis of variance test and a Dunn's posttest (α=0.05). The rheological, texture and sensory results were correlated by Spearman's rank correlation test (16,30,31).

Results

Full factorial design of experiments

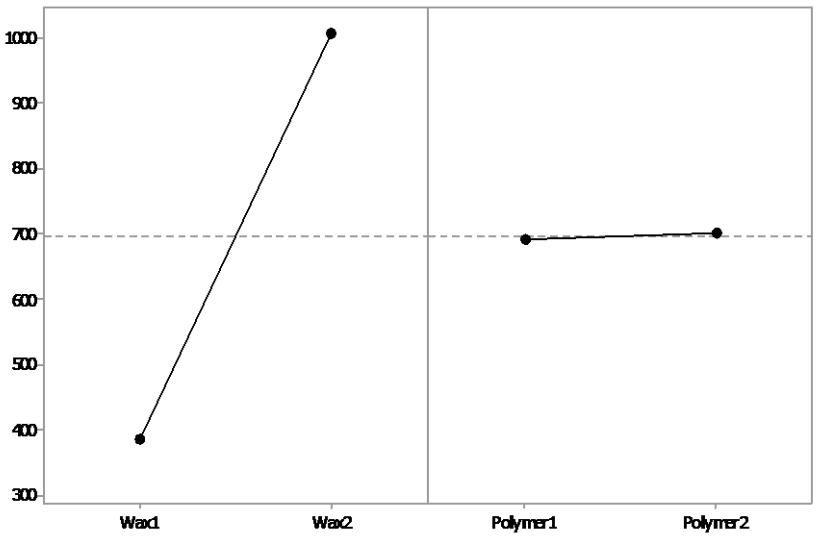
The first test analyzed the following factors: wax (A), polymer (B) and the wax and polymer interaction (AB). After the factorial regression it was observed that only the factors A and AB significantly influenced the response "work of shear" (α=0,05). A Pareto chart was obtained with the absolute effect of the factors (Figure 1).

Fig. 1. Absolute effect of the factors "wax", "polymer" and "interaction between wax and polymer".



Regarding the polymers, both did not significantly influence the work of shear in a different manner. Knowing which factors are significant, it was possible to create factorial plots to assess the main effects. The graphs show the effect of waxes and polymers on the work of shear separately (Figure 2). In graph A, it is observed that Wax 1 contributed to a lower work of shear value, while Wax 2 contributed to higher values. Graph B shows that the two polymers had a similar influence on the work of shear, obtaining values around 700 g/sec. The Polymer 1 provides lower work of shear values.

Fig. 2. Main effects of waxes and polymers on work of shear (g).



The Equation 1, where X_1 is the wax, X_2 is the polymer and X_3 is the interaction between wax and polymer, is the regression equation that demonstrates the influence of the factors on the work of shear of the formulations. This agrees with that observed in Figure 1: the higher influence of wax and the interaction between wax and polymer.

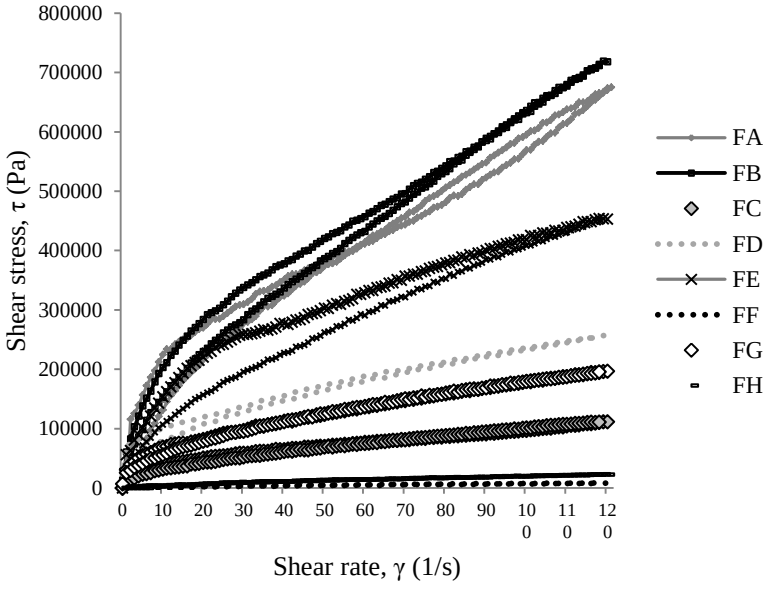
$$\text{Work of shear} = 696{,}3 + 311{,}3\ X_1 + 4{,}1\ X_2 - 74{,}2\ X_3 \quad (1)$$

Thus, the most effective combination of polymer and wax chosen to compose the formulation vehicle was Wax 1 and Polymer 1. The utilized concentrations were 1% and 10% for Wax 1, and 0.15% and 0.5% for Polymer 1 to obtain maximum and minimum standards. From them, interacting or not, it was possible to see clearly the different influence between wax and polymer.

Rheology

Different rheological behavior was observed between the formulations. The formulations with the combination of wax and polymer, FA, FB, FC and FD, and the formulation with maximum concentration of wax, FE, presented higher values of shear stress when compared to the other formulations, and also, formulations FA, FB, FD and FE presented thixotropy (Figure 3). The formulation FG, with maximum concentration of polymer presented low values of work of shear and the formulations FF and FH presented values close to 0.

Fig. 3. Shear stress as a function of shear rate of the formulations.



Regarding the flow index, we observed (Table III) that the interaction between the wax and the polymer at different concentrations resulted in low difference in the flow rate. However, high wax and polymer concentrations separated, resulting in a larger resistance of the material.

Table III – Rheological parameters obtained from Ostwald model.

Formulation	Flow index	Apparent viscosity (Pa.s)	Consistency index ($Pa.s^n$)
FA	0.5	1082.0	63.1
FB	0.5	598.2	53.1
FC	0.4	123.9	13.2
FD	0.4	760.1	36.9
FE	0.4	496.3	56.6
FF	0.7	1.7	0.3
FG	0.5	299.5	20.5
FH	0.7	3.3	0.8

The polymer concentration did not influence the flow index, but the presence of wax did. From formulation FE, with a high concentration of wax and no polymer, to formulation FF, with a low concentration of wax and no polymer, an increase in the flow index was observed. To complement that, the formulations without wax, FG and FH, obtained higher flow index values.

Regarding apparent viscosity, the association between the wax and the polymer in high concentrations and in minimum concentrations resulted in large differences, mainly because of the wax. The minimum concentrations of wax and of polymer, formulations FF and FH, provided values close to zero, indicating that the association between the wax and the polymer results in higher apparent viscosity.

Texture

The texture analyses results (Table IV) showed some tendencies between the parameters and formulations. In all of our analyses, formulations FA and FB, had no significant difference. That result means that with a high concentration of wax, the polymer did not influence textural parameters.

Table IV – Texture parameters of the formulations (mean ± SD).

Formulation	Work of shear (g.sec) Mean ± SD	Index of viscosity (g.sec) Mean ± SD	Consistency (g.sec) Mean ± SD	Cohesiveness (g) Mean ± SD
A	759.3 ± 32.6[a]	2061.4 ± 47.5[a]	2561.0 ± 174.5[a]	211.6 ± 9.2[a]
B	727.7 ± 37.1[a]	1993.2 ± 74.4[a]	2337.3 ± 164.4[a]	198.9 ± 8.4[a]
C	34.2 ± 2.3[c]	491.0 ± 16.8	943.3 ± 6.1	54.1 ± 0.6
D	141.5 ± 5.3	574.6 ± 10.9	983.5 ± 3.6	59.8 ± 0.8
E	424.2 ± 20.9	1993.9 ± 96.2[a]	2268.5 ± 94.9[a]	205.5 ± 23.7[a]
F	13.3 ± 0.4[c]	133.6 ± 15.2[bc]	456.1 ± 13.4[c]	22.1 ± 1.2[c]
G	45.3 ± 3.7	156.1 ± 3.9[c]	448.9 ± 9.3[c]	19.4 ± 0.05
H	17.5 ± 4.6[c]	129.1 ± 3.7[b]	439.5 ± 1.9[c]	21.3 ± 0.1[c]

[a-c] means with the same letter are not significantly different ($P < 0.05$).

When the wax concentration increases to 1%, as in formulations C and D, there is a significant decrease in textural parameters, independent of the polymer concentration. However, in the case of these formulations, a variation in the concentration of polymer from maximum to minimum results in significant changes in textural parameters, showing that the wax has less influence in minimum concentrations.

Regarding work of shear, high concentrations of wax resulted in more difficult to shear the formulations. Changes in wax and polymer concentrations produced significant variations in the work of shear. Formulation FC, with 1% wax and 0.15% polymer, formulation FF, with 1% wax and formulation FH, with 0.15% polymer,

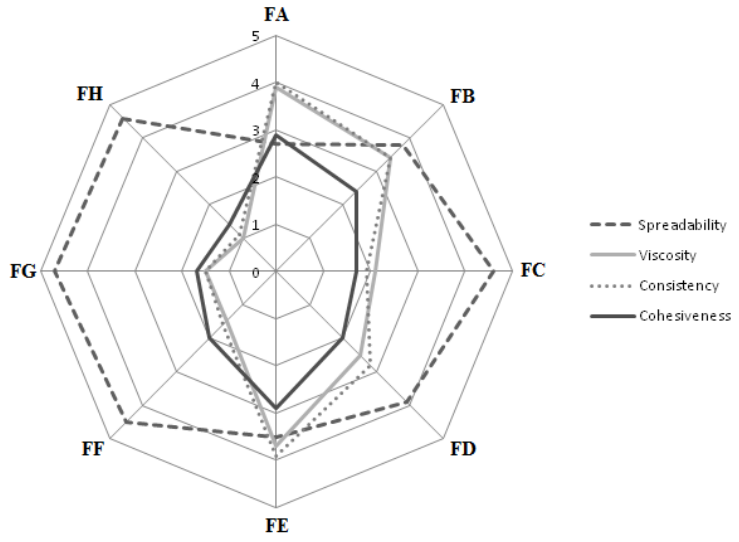
demonstrated no significant difference in this parameter. This means that in minimum concentrations, the influence of wax and polymer, separately or together, is the same for the work of shear. For the other parameters, the wax and polymer interaction produced changes. For the index of viscosity, consistency and cohesiveness, formulations FA, FB and FE were not significantly different. Thus, the removal of the polymer in formulation E did not change these texture characteristics.

Also, an impressive increase is observed from formulation FD to FE, when the wax concentration goes to maximum and the polymer is removed from the formulation. In summary, for the texture parameters, the wax is more influential than the polymer, and an increase in the wax concentration results in an increase in texture parameters.

Sensorial

Our sensorial analysis confirmed the previous steps by radar chart (Figure 4). Notably, the spreadability behavior was different from the others. The spreadability score followed the decrease in the amount of wax and the increase in the amount of polymer. According to statistical analysis and to the trained panel, formulations FC, FF, FG and FH were significantly easier to spread when compared to formulation FA.

Fig. 4. Radar chart of sensory analysis.



In relation to viscosity, consistency and cohesiveness, formulations with a maximum concentration of wax (formulations A, B and E) obtained the highest score for these parameters, and they were significantly more viscous, consistent and cohesive compared to formulations F, G and H.

Spearman's rank correlation

One of the goals of this study was to establish a correlation between rheological, texture and sensory variables to in turn possibly compare theoretical and practical data. Based on Spearman's rank correlation, it was possible to establish correct associations between similar parameters among the analyses (31). The correlation matrix obtained is reported in Table V.

Table V – Correlation matrix of parameters.

		FI	AP	CI	WS	CH	CO	IV	S	CHS	COS	VS
Rheology	Flow Index (FI)	1	-	-	-	-	-	-	-	-	-	-
	Apparent Viscosity (AP)	0.046	1	-	-	-	-	-	-	-	-	-
	Consistency Index (CI)	0.046	0.925	1	-	-	-	-	-	-	-	-
Texture	Work of Shear (WS)	**0.102**	0.944	0.981	1	-	-	-	-	-	-	-
	Cohesiveness (CH)	0.065	0.794	0.869	0.850	1	-	-	-	-	-	-
	Consistency (CO)	0.084	0.850	**0.888**	0.906	0.963	1	-	-	-	-	-
	Index of Viscosity (IV)	-0.010	**0.888**	0.963	0.944	0.944	0.963	1	-	-	-	-
Sensorial	Spreadability (S)	**0.641**	-0.317	-0.392	**-0.373**	-0.533	-0.486	0.011	1	-	-	-
	Cohesiveness(CHS)	0.161	0.733	0.845	0.817	**0.920**	0.911	0.904	-0.598	1	-	-
	Consistency (COS)	0.027	0.831	**0.906**	0.888	0.981	**0.981**	0.981	-0.505	0.939	1	-
	Viscosity (VS)	0.013	**0.864**	0.939	0.920	0.967	0.977	**0.995**	-0.472	0.925	0.995	1

Discussion

The factorial design step demonstrated that the waxes significantly influenced the work of shear of the formulations, agreeing with previous studies (14,24) that demonstrated that the constituents of the oil phase had a significant effect on the spreading of emulsions.

Phosphate derivative surfactants, such as Wax 1, are very stable and have the ability to form stable emulsions (32). Furthermore, the presence of phosphate groups provides better compatibility with skin, influencing the sensory perception of the formulations. Polymer 1 is a hydrophobically modified co-polymer that can act as an emulsifier and viscosity enhancing agent. Its relation with oil ingredients has been studied and the polymer obtained good results in terms of stability and rheological properties (12,13).

The formulations showed non-Newtonian behavior and pseudoplastic character (flow index <1), which is desired for formulations because there is a decrease in viscosity when the shear rate increases (33). A decrease in the flow index when the oil concentration increased may results in a shear thinning behavior associated with creaming (34).

The formulations FB, FD and FE presented hysteresis area, or thixotropy, a natural characteristic of pseudoplastic formulations. This thixotropic behavior, implies that these formulations took more time to rebuild their viscosity after being sheared. This propertie is related to structure recovery properties (2). This may be related to the presence of the wax, which is a consistency agent. Formulations with thixotropy and pseudoplastic flow have a resistance to spreadability that generates a more protective film to the skin (4). Rheology results are consistent with previous studies that observed a high influence of the oil phase, a complex system, on the macroscopic structure of the emulsions, notably spreadability, viscosity and consistency (13,14,16).

The wax and the polymer showed a synergistic effect. On the one hand, the oil concentration may increase the apparent viscosity independent of the emulsifier used and contribute to shear-thinning behavior (16,35). On the other hand, the polymer

chosen has already been shown to act as a good emulsifier and viscosity enhancing agent (12). There seems to be a direct relationship between the polymer concentration and the formulation apparent viscosity (26). Confirming the results obtained in the mentioned studies, the high concentration of wax, formulation FE, and high concentration of polymer, formulation G, produced higher values of apparent viscosity. This influence is clear when the apparent viscosity of the formulations with the minimum concentration of wax, formulation FF, and of polymer, formulation FH, are analyzed. The flow index and apparent viscosity did not have a direct relationship as described in the literature (26).

Concerning consistency, the formulations with a high concentration of wax, formulations FA, FB and FE, demonstrated a high consistency index. Once more, the association between the wax and the polymer seem to bring better results; but in this parameter, wax had a bigger influence. The effect of the wax ratio on the material properties was previously studied (7). It was discovered that the addition of solid wax increases the network strength, and the mechanical properties are governed by the arrangement of the network.

Beri, Norton and Norton (2013) demonstrated that the addition of solid wax provides greater connections, increasing the network strength (7). Binks and Rocher (2009) showed that the stability provided by the wax depends on the concentration (5). In our study, lower concentrations of wax resulted in a greater synergistic effect with polymer in terms of texture parameters.

Regarding index of viscosity, consistency and cohesiveness, formulations FA, FB and FE presented the same behavior. This contradicts a previous study that affirms

that Polymer 1 influences these texture parameters (36). The removal of the polymer did not influence these texture characteristics.

Agreeing with the literature, the sensorial spreading results agreed with the work of shear values (16,25,28). The formulations FA, FB and FE, which have high values of work of shear, presented lower values for that spreading and the formulations FC, FF, FG and FH presented opposite results. The stratum corneum, localized in the epidermis, is the most external layer of the skin. The sensory perception of a given formulation is related to the interaction between the skin surface and physical-chemical properties, reflected in the skin lipid film. The surface lipids are responsible for some parameters, such as the adhesion of solid particles and the skin surface energy, and give the skin surface a more hydrophilic character (37). Therefore, this hydrophilic character can increase the ease of spreadability for a given formulation with minimum or without wax content, as is the case for formulations FC, FD, FF, FG and FH.

Because these sensorial parameters are more related to the physic-mechanical properties of the emulsions (15,16,18,19,21,24,36,38), it is possible to see the major influence of wax in these formulations.

In general, strong correlations were found among the parameters studied. The only negligible correlation occurred between the work of shear and flow index variables. With a correlation coefficient of 0.102, they do not seem to be related. The correlation between the flow Index and sensorial spreadability presented a moderate positive correlation with a coefficient of 0.641. The flow properties can be measured to evaluate the behavior of emulsions subject to shear (4), but they should be compared with

sensorial spreadability (24) because the flow index does not seem to be related with the work of shear parameter.

Savary, Grisel and Picard (2013), demonstrated with another test that spreading measured in a texture analyzer can be linearly related to spreading predicted by the sensory panel (24). In our study, the inverse parameters work of shear and spreadability presented a low negative correlation with a coefficient of -0.373. As previously stated, a formulation with a high work of shear value will be difficult to spread (16,28) on the surface of the skin and also the reverse is true. Thus, this theoretical parameter can be used as a predictor of spreadability.

The following correlations were classified as high positive, with correlation coefficients ranging from 0.864 to 0.888: apparent viscosity/index of viscosity, consistency index/consistency, apparent viscosity/sensorial viscosity. For these correlations, when a parameter increases, their follower increases too.

The strongest correlations were between consistency/sensorial consistency with a coefficient of 0.906; consistency index from rheology/sensorial consistency, with a coefficient of 0.981; cohesiveness/sensorial cohesiveness with a coefficient of 0.920; and index of viscosity / sensorial viscosity, which has the higher correlation coefficient of 0.995. They presented a very high positive correlation, indicating that the texture parameters are highly related to their respective sensorial parameter.

Finally, this work has an important contribution once showed that is possible to correlate physical and mechanical and sensorial parameters with the help of a trained panel, which can help the development of topical formulations predicting the performance into the skin.

Conclusion

Different waxes present a greater effect on the work of shear, a parameter that indicates spreadability. The interaction between the wax and the polymer also influenced this parameter. The best combination of wax and polymer was applied to develop eight formulations. The wax showed more impact compared to the polymer in relation to rheological, texture and sensorial data, except for the work of shear and spreadability, where the polymer acted as a spreadability balancer. The relationships between the rheological, texture and sensory variables were verified. In this way, it is possible to predict sensorial aspects by rheological and texture parameters, making the formulation process easier and more integrated with all stages of the development of new topical formulations.

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3.2. Capítulo 2 – Estudos de estabilidade

3.2.1. Artigo 2:

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Physical Mechanical characterization of cosmetic formulations and correlation between instrumental measurements and sensorial properties

ABSTRACT

OBJECTIVE: The correct choice of raw materials in the development of cosmetic formulations is essential for obtaining stable and pleasant skin care products. Therefore, rheological, texture and sensory analyses are important to understand the behavior and stability of the formulations. In this context, the aim of the present study was to develop cosmetic formulations containing or not (vehicle) UV filters and chicory root extract, to evaluate their stability as well as to characterize their physical and texture properties and correlate them with the sensory attributes.

METHODS: Four formulations containing organic UV filters and chicory extract, each alone or in combination, were developed and evaluated for 180 days with a cone and plate rheometer, a texture analyzer and consumer's sensorial analysis. Thus, the data obtained were correlated in order to observe the different influences.

RESULTS: The developed formulations remained stable after 180 days regarding macroscopic aspects, organoleptic characteristics and pH values. The addition of the UV filters alone and in combination with the active substance resulted in significant increases in rheology, viscosity and consistency. The formulation with the active ingredient showed significant decreases in the texture parameters after 180 days, mainly due to its polysaccharide Inulin. All formulations obtained high scores in sensorial parameters. A strong correlation was mainly found between spreadability and work of shear, and between the texture parameters.

CONCLUSION: The raw materials strongly influenced the physical, texture and sensorial parameters. Finally, the UV filters showed a greater influence on the results of the formulations than the chicory root extract. In conclusion, the association of the mentioned methods allows the correct choice of ingredients and their combinations.

Keywords: Formulation skin care/stability/texture/emulsions**;** Suncare/UV protection; **Polymers/Surfactants;** Rheology; Sensorial.

INTRODUCTION

Gel-cream formulations represent an important strategy in the development of cosmetic products. They are emulsions stabilized with hydrophilic colloid which confers applicability to receiving other cosmetic ingredients such as active substances and sunscreens, resulting in stable and effective topical formulations [1].

Emulsions used as cosmetic formulations have various textures. They may be fluid as in the case of lotions or semi-solid as in the case of creams. These presentations require a complete behavioral study to obtain information about the physical stability and consistency of the product [2]. Therefore, the choice of raw materials is the most important step in cosmetic research and development. Waxes, polymers, surfactants and other ingredients may significantly influence important properties of formulations such as stability, toxicity, physical behavior, efficacy, acceptability, and sensory perception [3].

The choice of the right active ingredient is very important in order to achieve a quality multifunctional cosmetic formulation. The chicory root extract is an ingredient used in traditional Chinese medicine, with diverse natural properties and important functions such as physiological bioactivities and antifatigue and antiaging effects [4]. Inulin is a polysaccharide found in some vegetable species such as chicory roots that appears to be related to physical properties such as the apparent viscosity of the formulations [5].

The correct combination of UV filters results in a high UV protection performance. This protection, however, should not affect the pleasant perception of the formulation. Some UV filters may involve a sensorial loss by causing a greasy and heavy skin feeling [6]. Furthermore, the enhancement of the oily phase originating from the addition of UV filters may have an impact on the stability and physical properties of the preparation [7].

Rheology determines the deformation and flow as a function of the shear strain, shear rate or shear stress applied to the product under analysis. Rheological measurements can be used to rationalize consumer acceptance of the skin feel of a cosmetic product. Parameters such as coverage efficiency, spreadability and stability can be influenced by the flow properties of the formulation [7,8].

In the research and development of cosmetic formulations it is necessary to consider, in addition to rheology, texture analysis which is a group of perceived sensorial physical properties

governed by the internal structure of the material which is determined by molecular interactions of the constituents [9]. Texture analysis is generally used for industrial products mainly in the food, pharmaceutical and cosmetic fields, providing very important analysis parameters such as hardness, firmness and work of shear [3].

Originally, the studies correlating theoretical and sensorial properties were concentrated in the food area [10]. Today, the importance of correlation studies has been discovered as an important tool for producing cosmetic formulations of quality and great acceptance by the consumer. Studies have shown that rheology and texture analysis used in combination are useful and complementary tools for the determination of the role of a particular ingredient in formulations. This techniques can be correlated to skin sensations and performance and, on the basis of their results, it is possible to formulate a cosmetic with excellent sensory properties by using ingredients with pre-defined rheological and textural characteristics [9, 11, 12]

In this context, the aim of the present study was to develop gel-cream cosmetic formulations containing or not UV filters and the active substance, chicory root extract. An additional objective was to evaluate their long-term stability and to characterize and correlate their physical, texture and sensorial properties.

MATERIALS AND METHODS

Materials

Development of formulations

The formulations were developed regarding the specifications of the active substances under study, the sensorial characteristics and their interaction with the raw materials (Table I). Four gel cream formulations were selected to evaluate the influence of the addition of UV filters and of active substances on stability, texture, rheology and sensorial properties. To this end, after development of the vehicle (V), we added organic UV filters (VF), chicory root extract (VA) and a combination of VA and VFA.

Table I – Ingredients of the formulations

I.N.C.I. name/Commercial name	V	VF	VA	VFA

Cetearyl Alcohol and Dicetyl Phosphate and Ceteth-10 Phosphate - Crodafos™CES	5	5	5	5
Acrylates/C10-30 Alkyl Acrylate Crosspolymer - Pemulen™ TR-2 NF Polymer	0.20	0.20	0.20	0.20
Cyclomethicone (and) Dimethicone Crosspolymer - DOW CORNING® 245	5	5	5	5
Cyclopentasiloxane - DOW CORNING® 9040	15	15	15	15
Phenoxyethanol and parabens – Phenova®	0.6	0.6	0.6	0.6
Ethyelenediamine tetraacetic acid – EDTA®	0.05	0.05	0.05	0.05
Butyl Hydroxy Toluene – BHT®	0.01	0.01	0.01	0.01
Propyleneglycol	4	4	4	4
Glycerin	3	3	3	3
C12-15 Alkyl Benzoate - Crodamol™ AB	5	5	5	5
Bis-ethylhexyloxyphenol methoxyphenyl triazine – Tinosorb S®	-	4	-	4
Ethylhexyl Triazone – Uvinul T150®	-	3	-	3
Ethylhexyl Methoxycinnamate - Uvinul A Plus®	-	4	-	4
Ethylhexyl Salicylate - Neo Heliopan® OS	-	4	-	4
Cichorium intybus (Chicory) root extract - Silab®	-	-	1	1

To prepare the formulations the aqueous phase was incorporated into the oil phase under heating at 70 °C. The UV filters were incorporated into the oil phase. The preparations were stirred for 20 minutes and then neutralized with AMP 95 to pH 5.5. The polymer, silicones, preservatives and active ingredients were then added.

METHODS

Accelerated stability test

The formulations developed were submitted to preliminary stability tests. Each formulation was prepared and packaged in glass containers (58 mm in diameter × 180 mm deep) with a lid which were stored at room temperature (25ºC) and challenged by thermal stress at temperatures of 4ºC, 37ºC and 45ºC. Macroscopic aspects, organoleptic characteristics and pH were analyzed for periods of 7, 14, 21, 30, 60, 90

and 180 days. This long period was chosen to determine if an increase in storage time could decrease the physical and mechanical stability of the preparations [13].

Rheological behavior

The formulations were considered stable after the accelerated stability test and were submitted to the determination of rheological behavior. For this test, they were reprepared and conditioned and the analyses were performed on the initial day and 7, 14, 21, 30. 60. 90 and 180 days later. The apparent viscosity was obtained from the rheogram and the curves were adjusted according to the Ostwald model (Equation 1) to obtain the flow index and consistency index [14].

$$\tau = K \cdot (\gamma)^n \qquad (1)$$

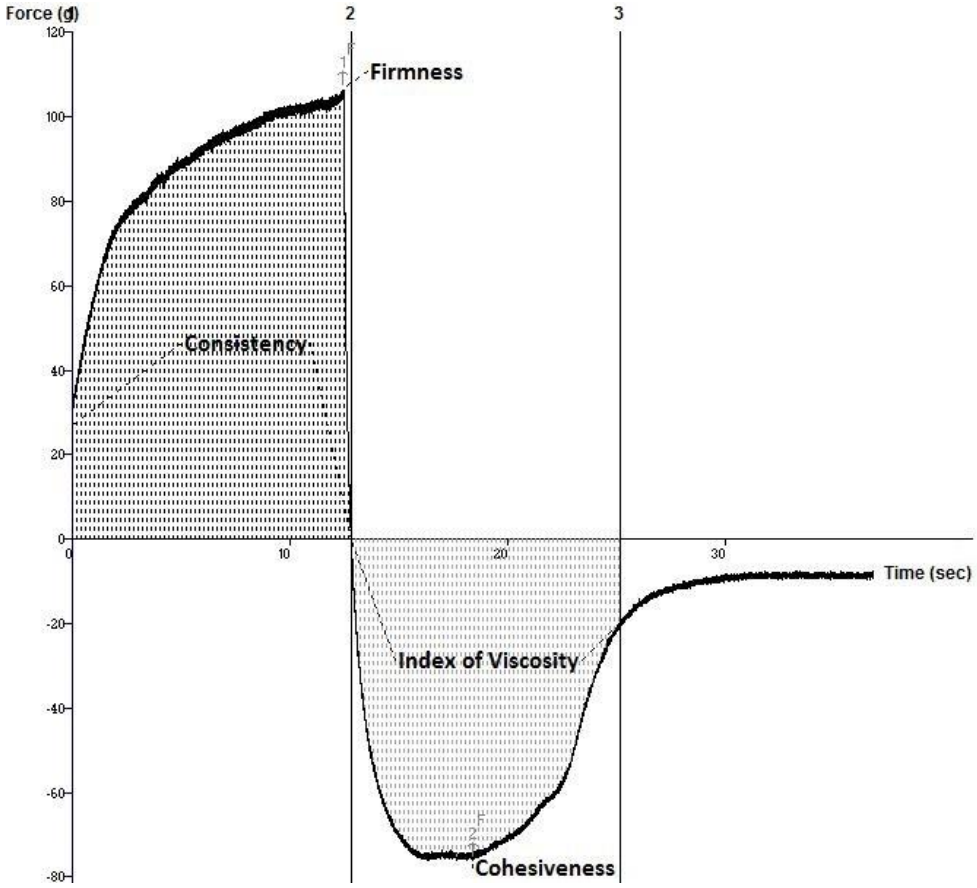
Rheological behavior was determined using a Brookfield DV3T cone and plate rheometer (Brookfield, USA) equipped with a CP-CPA-52Z spindle and coupled to RHEOCALCT® software. In each analysis, 0.5 g of formulation was deposited on the plate. The rotational speed was progressively increased from 0 to 120 rpm with 10 seconds between each speed, forming an upward curve. The downward curve was generated with an inverse decrease of the speed. The hysteresis areas of these curves were studied to evaluate the influence of the UV filters and the active ingredients on the immediate and long-term rheological behavior [13].

Texture Profile

The texture analysis was performed using a TA.XT plus Texture Analyzer (Stable Microsystems, United Kingdom) equipped with two probes: TTC Spreadability rig HDP/SR and Back Extrusion rig A/BE 35 mm at room temperature. The method consists of the insertion of the analytical probe into the sample, with defined speed and depth, leading to a predefined period of recovery between the end of the first compression and the beginning of the second, resulting in a force (g) versus time (t) graph. The analyses were performed on the initial day and after 7, 14, 21, 30. 60. 90 and 180 days.

The first test evaluated the work of shear of the formulations. This measure is obtained from the area under the positive curve. The probe conditions were: return distance 100 mm, return speed 20 mm/sec, and contact force 30 g [15]. The second test evaluated the parameters: index of viscosity, consistency, firmness and cohesiveness. For this, the formulations were loaded in 125 mL containers 50 mm in diameter. In this test, firmness is obtained from the maximum value of the positive curve, consistency from the area under the positive curve, cohesiveness from the maximum value of the negative curve, and index of viscosity from the area under the negative curve [3] (Fig. 1). The return distance used was 25 mm, the return speed was 20 mm/sec and the contact force was 30 g. The data obtained from the negative curve were analyzed as absolute values.

Figure 1 – Typical plot of texture analysis with data graph and its interpretation



Sensory analysis

Fourteen volunteers were selected to evaluate the sensory characteristics of the formulations and this phase was approved by the Ethics Committee of the Institution (CEP/FCFRP nº. 381). They were instructed to apply a standardized amount, 20 mg/cm^2, of the formulations under study. They answered a sensory questionnaire to evaluate spreadability, cohesiveness, consistency, firmness and viscosity of the formulations. The Sensorimeter® SR 100 equipment (Courage-Khazaka, Germany) was used to measure the answers on a 0 to 100 scale according to the sensation obtained with the product.

Statistics

The experimental data obtained were submitted to statistical analysis. The Anderson Darling test was used to evaluate the normality of the populations. When normality was determined, one-way analysis of variance with the Tukey posttest was used (α=0.05). In case of non-normal distribution, the Kruskal-Wallis with Dunn's posttest was applied (α=0.05). The rheological, texture and sensory results were correlated using the Spearman's rank correlation coefficient and Pearson correlation coefficient [16,17]. The use of the two tests was justified in order to check the impact of the data and not to overinterpret the strength of the association of the two tests [18]. All statistical analyses were performed using the Minitab® 17 software (Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

Accelerated stability test

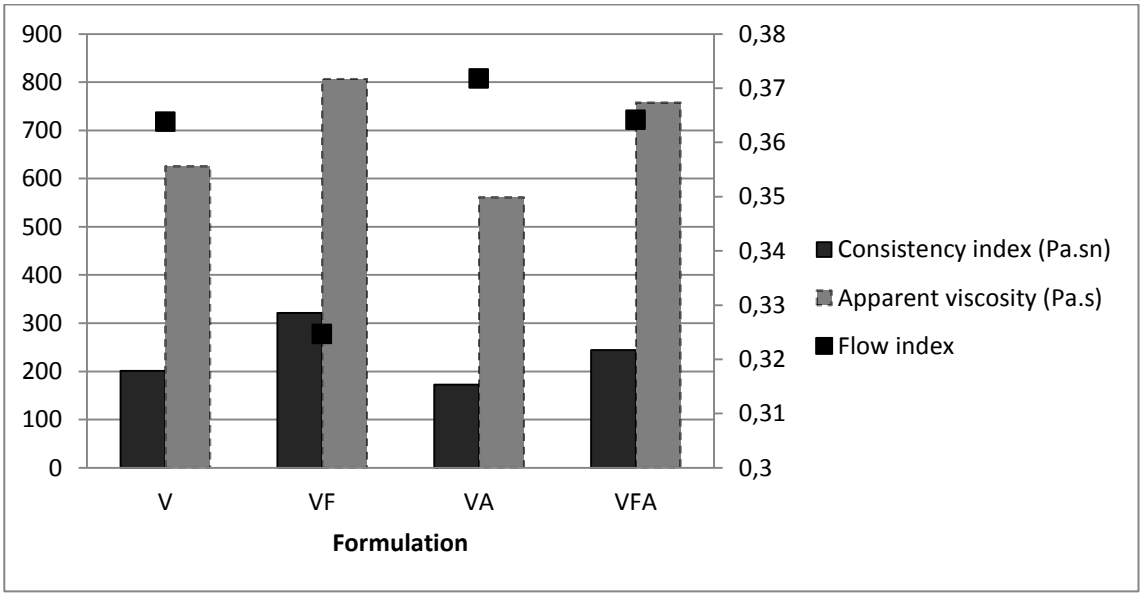
No changes in color, odor, homogeneity or phase separation were observed in the formulations after storage at room temperature or at 4ºC, 37ºC and 45ºC after 180 days. The formulations presented pH values between 4.03 and 5.39, compatible with

skin pH, with no significant variations after this period. Thus, they were considered stable.

Rheology

Regarding the rheological characterization of the formulations, there was no significant difference in flow index, which presented a mean value of 0.36 ± 0.02. Compared to vehicle, the addition of the UV filters alone or in combination with the active ingredients resulted in significant increases in viscosity and consistency (Fig. 2). Furthermore, the addition of the active ingredients did not change significantly any of these parameters (α=0.05). All formulations presented a flow index below 1, indicating pseudoplastic behavior.

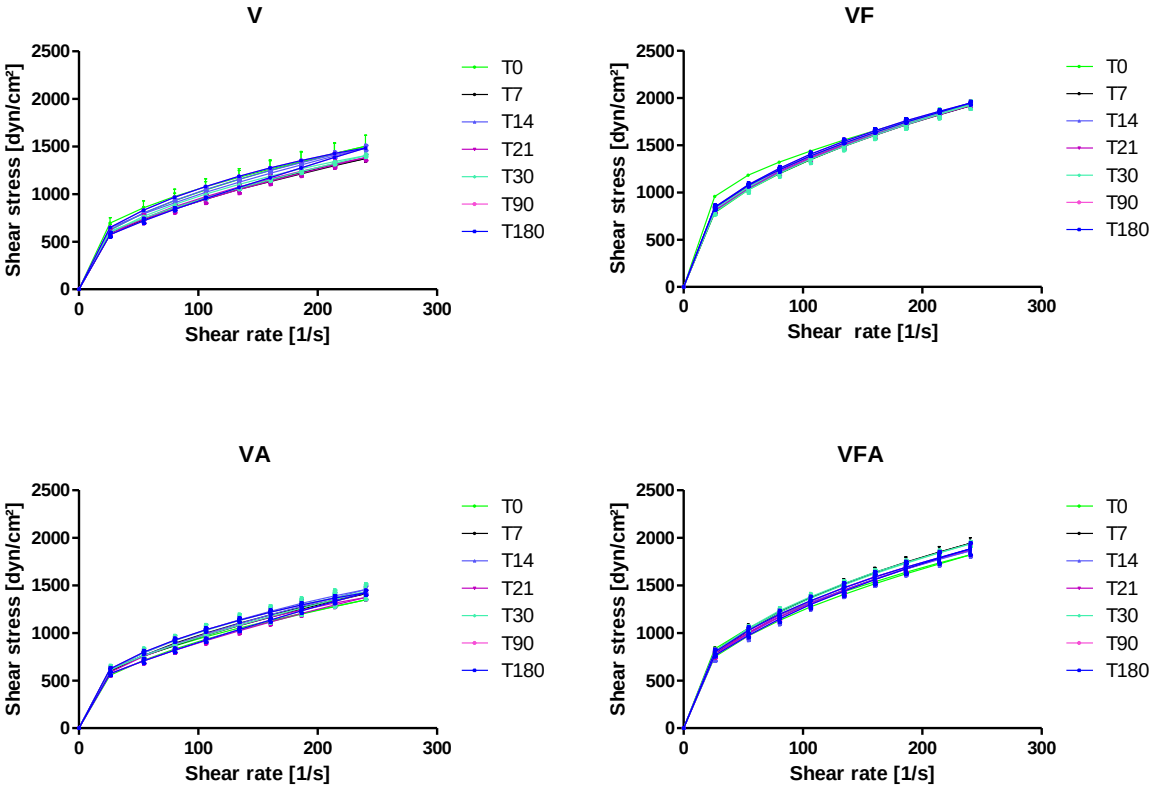
Figure 2 - Flow index, consistency index and apparent viscosity of formulations V, VF, VA and VFA



The rheogram curves indicated that storage did not compromise the structure of the formulations structure since no peaks or alterations were observed in the rheograms after 180 days [13] (Fig. 3). The addition of UV filters resulted in a greater relationship between shear stress and shear rate, detected in a larger area under the curve, which justifies the higher consistency values for the formulations with UV filters

(V and VFA). Usually, if the same experiment is repeated after a long time, the hysteresis tends to decrease. A reduced hysteresis area can be observed , having become smaller over time, probably due to weak flocculation; however, this was not observed in practice [2]. The almost total absence of thixotropy in the formulations demonstrates their rapid structural reorganization after shear [19].

Figure 3 – Rheograms of formulations V (A), VF (B), VA (C) and VFA (D) stored at 25 °C for 0, 7, 14, 21, 30, 60, 90 and 180 days after preparation.



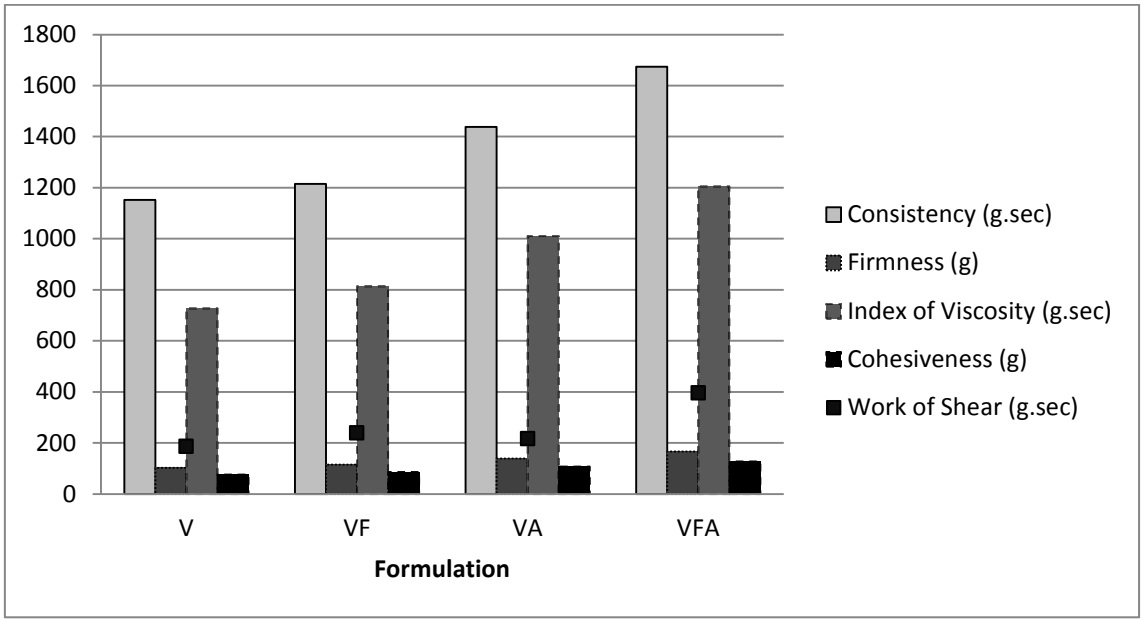
Texture

Interpretation of the physical-mechanical parameters is an important tool that allows the prediction of sensorial characteristics [20]. The texture characterization showed that the VFA formulation presented higher values of cohesiveness, consistency, firmness and viscosity index (Fig. 4). The addition of UV filters with active ingredients such as proteins is interesting for the photostability and efficacy of topical formulations [7]. The increase of fatty substances causes a

structural rearrangement of the formulations, changing their behavior [21,22, 23], so that the oily charge of these formulations influenced their theoretical sensorial properties.

Both the VF and VA formulations presented higher shear values when compared to the V formulation. The VFA formulation presented a significantly higher increase when compared to the vehicle. The values of shear work are highly correlated with the spreadability of the formulation [24]. Thus, the combination of active ingredients and filter caused a loss of spreadability. However, this ingredient acted as a texture agent and therefore its association is indicated when a heavier texture formulation is desired.

Figure 4 –Texture profile characterization of the formulations

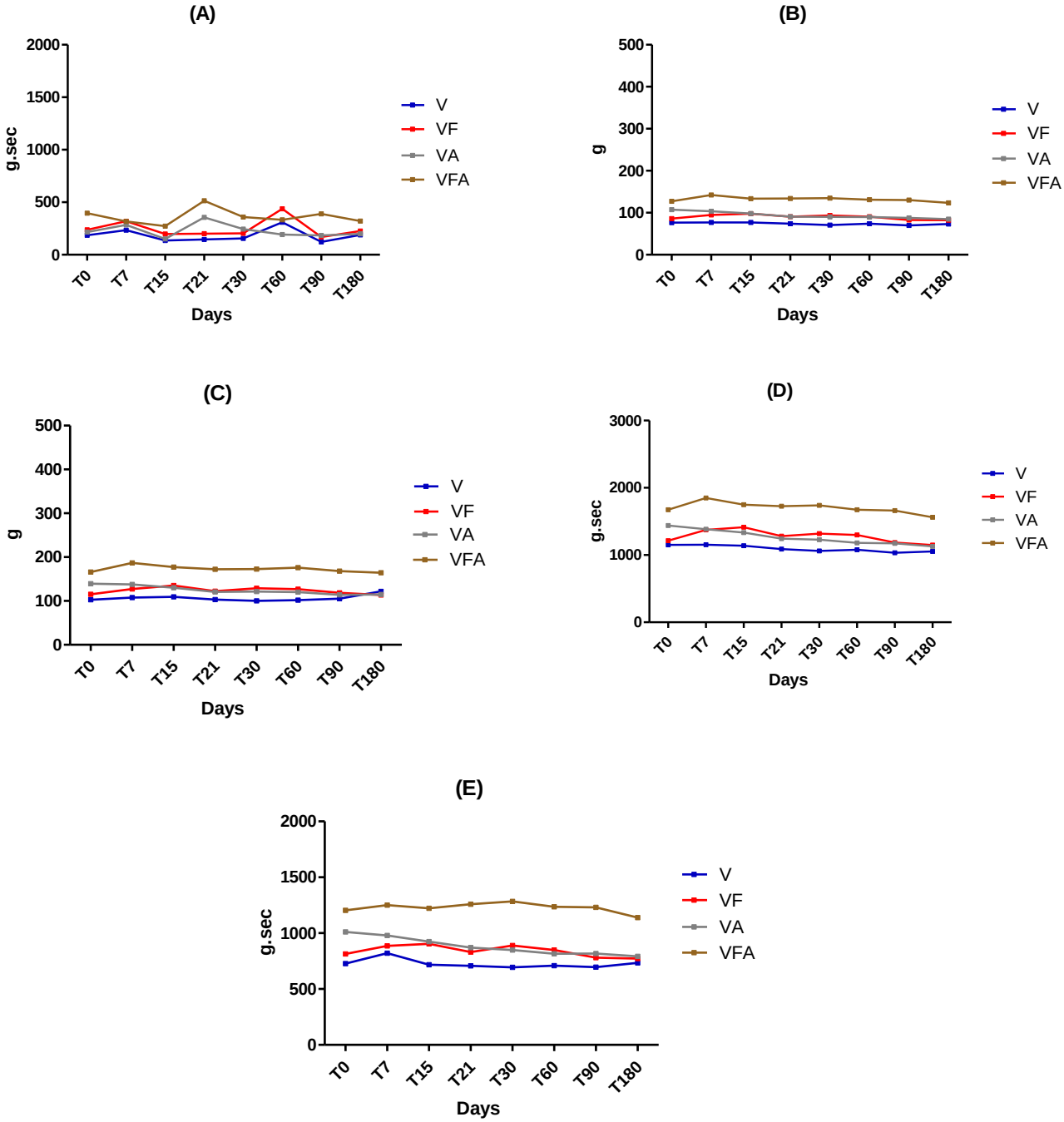


The evaluation of the texture parameters after long-term storage showed that the formulations maintained the same texture behavior after 180 days, except for VA that presented significant decreases in the parameters cohesiveness, firmness and consistency after 180 days (Fig. 5). Regarding the spreadability test, the formulations did not demonstrate significant changes in work of shear after 180 days.

The VA formulation contains the chicory root extract in aqueous solution. The aqueous phase can affect many material properties, including the viscous modulus [21]. The polysaccharide inulin found in chicory extract can affect the physical properties of formulations. As biopolymers they can reduce the interfacial tension acting as surface active agents [5, 25]. In

addition, hydrocolloids located in the aqueous phase can induce the derangement of emulsions by depletion flocculation [26].

Figure 5 – Texture results of formulations V, VF, VA and VFA in terms of Work of Shear (A), Cohesiveness (B), Firmness (C), Consistency (D) and Viscosity (E) stored at 25 °C for 0, 7, 14, 21 and 30, 60, 90 and 180 days after preparation.

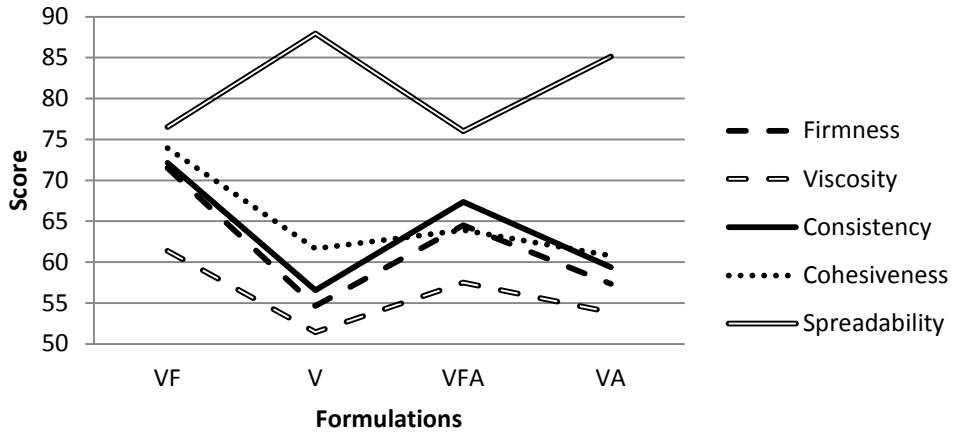


Sensorial properties

The formulations were evaluated by the volunteers concerning physical and mechanical parameters, allowing comparison with the theoretical values obtained. All formulations obtained high scores on the scale (more than 50) in all questions (Fig. 6). Recurrently, the performance of spreadability was inversely correlated with the improvement of texture.

The VF formulation obtained the highest texture values and the lowest spreadability values, similar to the values of the VFA formulation, also reaching higher texture values. Thus, the addition of UV filters increased the sensorial perception of texture with loss of spreadability. This increase was more marked than the one caused by the addition of the active ingredient (VA).

Figure 6 - Sensorial perception of formulations



Statistical correlation

A quick visual analysis of Figures 2, 4 and 6 reveals that the results tended to show a prominent influence of the UV filters on the results, specially regarding rheology and sensorial properties. This tendency was analyzed by the Spearman rank correlation coefficient and by the Pearson correlation coefficient [18]. A combined sensorial and instrumental characterization of formulation properties is an important tool to predict sensorial characteristics from theoretical data [20].

Formulation V, with the lowest work of shear, presented the best spreadability, agreeing with previous studies demonstrating that these two properties are inversely proportional [24]. This relationship was considered to be a perfectly linear negative correlation with a Spearman rho of -1.0 (Table II). The Pearson test revealed strong correlations between the parameters with a Pearson r equal to -0.75.

Table II – Spearman's rank correlation coefficient (above) and Pearson correlation coefficient

		FT	VT	CT	CoT	WST	FS	VS	CS	CoS	SS	VR	CR	FIR
Texture analysis	Firmness (FT)	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
	Viscosity (VT)	1,0 1,0	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
	Consistency (CT)	1,0 1,0	1,0 1,0	- -	- -	- -	- -	- -	-	- -	- -	- -	- -	- -
	Cohesiveness (CoT)	1,0 1,0	1,0 1,0	1,0 1,0	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
	Work of Shear (WST)	0,8 0,9	0,8 0,9	0,8 0,9	0,8 0,9	- -	- -	- -	- -	- -	- -	- -	- -	- -
Sensorial analysis	Firmness (FS)	0,4 0,2	0,4 0,1	0,4 0,1	0,4 0,1	0,8 0,4	- -	-	- -	- -	- -	- -	- -	- -
	Viscosity (VS)	0,4 0,2	0,4 0,2	0,4 0,2	0,4 0,2	0,8 0,4	1,0 1,0	- -	- -	- -	- -	- -	- -	- -
	Consistency (CS)	0,4 0,3	0,4 0,2	0,4 0,3	0,4 0,2	0,8 0,5	1,0 1,0	1,0 1,0	- -	- -	- -	- -	- -	- -
	Cohesiveness (CoS)	0,0 -0,2	0,0 -0,3	0,0 -0,3	0,0 -0,3	0,6 0,1	0,8 0,9	0,8 0,9	0,8 0,9	- -	- -	- -	- -	- -
	Spreadability (SS)	-0,8 -0,5	-0,8 -0,5	-0,8 -0,5	-0,8 -0,5	-1,0 -0,8	-0,8 -0,9	-0,8 -0,9	-0,8 -1,0	-0,6 -0,7	- -	- -	- -	- -
Rheology	Viscosity (VR)	0,0 0,1	0,0 0,1	0,0 0,1	0,0 0,1	0,6 0,5	0,8 0,9	0,8 0,9	0,8 0,9	1,0 0,8	-0,6 -0,9	- -	- -	- -
	Consistency (CR)	0,0 -0,1	0,0 -0,1	0,0 -0,2	0,0 -0,1	0,6 0,3	0,8 0,9	0,8 0,9	0,8 0,9	1,0 1,0	-0,6 -0,8	1,0 0,9	- -	- -
	Flow index (FIR)	0,6 0,4	0,6 0,4	0,6 0,4	0,6 0,4	0,0 0,1	-0,4 -0,8	-0,4 -0,8	-0,4 -0,8	-0,8 -1,0	0,0 0,6	-0,8 -0,8	-0,8 -0,9	- -

A confirmed association was established between the measurements of viscosity and consistency obtained in the sensorial analysis and those obtained for rheology. With a Spearman rho of 0.8 and 0.8 and a Pearson r of 0.876 and 0.916, these measures appear to be strongly correlated.

Both theoretical and practical values showed an increase in consistency, viscosity, cohesiveness and firmness in the formulations with UV filters. This increase is related to the effect of the addition of the oil ingredients to cosmetic formulations [22,23]. Furthermore, a previous study has shown that formulations with high viscosity values are difficult to spread and sticky because viscosity is related to systems' resistance to flow [12].

A perfectly linear positive correlation was found in both correlation tests with a Spearman rho and a Pearson r of 1.0 between the texture parameters firmness, viscosity, consistency and cohesiveness; between the sensorial parameters firmness, viscosity and consistency; and with the rheological parameters viscosity and consistency. This explains the tendency of graph curves to follow the same behavior.

CONCLUSION

The developed formulations remained stable in terms of macroscopic aspects, organoleptic characteristics and pH values after 180 days according to accelerated stability tests and rheograms. All of them presented a flow index below 1, indicating pseudoplastic behavior. The addition of the UV filters alone and in combination with the active ingredients resulted in significant increases in rheology, viscosity and consistency.

The formulation with chicory root extract showed significant decreases of cohesiveness, firmness, consistency and viscosity parameters after 180 days. All formulations achieved high scores regarding the sensorial parameters, indicating a great acceptance by the volunteers.

A strong statistical correlation was detected between spreadability and work of shear, and between the texture parameters using the Spearman's rank correlation coefficient and Pearson correlation coefficient. The raw materials strongly influenced the physical, texture and sensorial parameters. The present results showed that the UV filters influenced the characteristics of the formulations more than the chicory root extract. Finally, the association of the mentioned methods permitted the correct choice of cosmetic ingredients their combinations.

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3.3. Capítulo 3 – Caracterização das formulações

3.3.1. Artigo 3: CALIXTO, L. S. ; CAMPOS, P. M. B. G. M. ; SAVARY, G. ; PICARD, C. . Interactions between UV filters and active substances in emulsion: effect on microstructure, physicochemical and in-vivo properties. International Journal of Pharmaceutics, v. 553, p. 220-228, 2018.

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Interactions between UV filters and active substances in emulsion: effect on microstructure, physicochemical and *in-vivo* properties.

ABSTRACT

The objective of this study was to develop, to characterize and to evaluate the clinical efficacy of topical formulations containing or not active substances and UV-filters, separated and in combination. To this purpose, a stable formulation was developed into which four aqueous active substances and four lipophilic UV-filters were added. Then, they were submitted to microscopic characterization, through optical microscopy and particle size measurement, and macroscopic characterization, through rheology and texture analysis. Finally, a clinical efficacy study was conducted to verify the effect of the formulations on the skin after 1h period application. The formulation containing UV-filters has showed a high polydisperse microstructure and a large amount of liquid crystals. The formulations containing active substances showed higher resistance to deformation, compression and penetration tests. Regarding the spreadability, formulations containing UV filters alone or in combination with active substances showed higher resistance to spread. This behavior was associated with greater clinical efficacy in terms of stratum corneum water content, protection of the skin barrier function and skin surface brightness. It has been demonstrated that the formulation efficacy is more associated with its structure and the way it interacts with the skin surface. Finally, this study showed that the union of these ingredients in the development of multifunctional sunscreens improves the formulations performance.

Keywords: Particle size measurement, Liquid crystal, Rheology, Texture analysis, Clinical efficacy, Topical formulations

1. Introduction

The study of active ingredients has great importance, since topical products are increasingly used by the population. People expect these products treat their skins in a quickly and effectively way. To meet this demand, it is necessary to find substances that have skin enhancement functions and that can be efficiently incorporated. Emulsions are dispersions capable of forming such topical media which are responsible for receiving the different ingredients of the formulation (Ozer, 2007).

Nowadays there is a great concern regarding protection against the damage of UV radiation from sun exposure. It is already known that in a long-term, the exposure to the sun can bring damage like skin burns, premature aging, melasma and skin cancer (Mercurio et al, 2015). Previous studies of our research teams have shown that UV-filters can be safely added to complex formulations without affecting their stability (Calixto et al, 2017; Rowenczyk et al, 2016). Multifunctional formulations combine different ingredients that aim to bring many benefits in a unique product. Anti-aging sunscreens are an example of these formulations, with antioxidant active substances integrated in formulations containing UV-filters. They combine the UV-protection with clinical improvement (Souza and Maia Campos, 2017). These active substances are usually present as aqueous extracts. Spirulina, *Red algae*, *Cichorium intybus* (Chicory) Root and *Medicago sativa* (Alfalfa) are examples of antioxidant substances that bring benefits to the skin as protection against DNA damage, improve skin microrelief, and recovery of photodamaged skin (Maia Campos et al, 2017; Harnedy et al, 2014; Mercurio et al, 2015; Jouandeaud et al, 2006). However, the addition of these substances (UV filters as active substances) may influence the final product characteristics as microstructure, rheology, texture, photostability and in vivo efficacy (Souza and Maia Campos, 2017; Benevenuto et al, 2014; Mercurio et al, 2015). From a fundamental point of view, it is an issue to understand to what extent these characteristics may impact product efficacy compared to the sole expected effect of the actives, usually at very low concentrations in formulation. From an applied point of view changes in formulation characteristics may directly affect sensory, product acceptance and adherence to treatment by consumers.

Rheology and texture analysis are useful and complementary to understand the role of a particular ingredient in a complex formulation (Gilbert, L. et al., 2013a). Formulation

ingredients, as polymers and emollients, can bring considerable changes in physico-chemical parameters (Accili et al., 2004; Gilbert et al., 2013b; Savary et al., 2013). Considerable improvement in mechanical properties can be obtained by the addition of these ingredients, by delivering mechanical strength (Pandey et al, 2017). The formulations can change the mechanical and adhesive properties of skin by interactions with skin surface like increasing the electrical resistance of skin (Tang et al., 2010). To evaluate these effects and interactions, it is necessary to carry out in vitro characterization of formulations combined with different in vivo techniques (Souza and Maia Campos, 2017; Accili et al., 2004).

The biophysical and skin imaging techniques are in vivo, non-invasive techniques that use equipment with different physical and/or physico-chemical principles that allow determining how cosmetic products can act on the skin. Thus, with different equipment, it is possible to correlate physico-chemical parameters related to the structure of the formulations and their interaction with the skin and practical parameters as skin hydration, transepidermal water loss and skin surface (Maia Campos et al, 2017; Try et al, 2010; Melo and Maia Campos, 2016). Thus, the present study aims to study the interactions of active ingredients (active substances and UV filters, combined or not) in formulations, their influence on the intrinsic properties of emulsions and the consequences on skin efficacy after application. To that purpose, we first develop multifunctional cosmetic type emulsions. Then, we determine their physico-chemical properties in order to evaluate the influence of UV filters and active substances on the microstructural organization of the colloids in emulsion and the resulting macroscopic properties in terms of rheology, texture properties and spreadability. In a second step we evaluate their efficacy in order to observe the influence on the performance of products. For this, instrumental techniques were used to correlate theoretical measurements with *in vivo* data.

2. Materials and methods

2.1 Materials

2.1.1 Chemicals

Cyclopentasiloxane and Cyclomethicone (and) Dimethicone Crosspolymer were provided by Dow Corning do Brasil Ltd. (Hortolandia, SP, Brazil). C12-15 Alkyl

Benzoate and Cetearyl Alcohol and Dicetyl Phosphate and Ceteth-10 Phosphate were provided by Croda do Brasil Ltda (Campinas, SP, Brazil). Acrylates/C10-30 alkyl acrylate crosspolymer was provided from Lubrizol do Brasil Aditivos Ltda (Sao Paulo, SP, Brazil); and Butyl hydroxy toluene, glycerin, phenoxyethanol and parabens, ethyelenediamine tetraacetic acid, aminomethyl propanol, propyleneglycol, were provided from Mapric Produtos Farmacêuticos e Cosméticos (Sao Paulo, SP, Brazil).

The UV-filters bis-ethylhexyloxyphenol methoxyphenyl triazine, diethylamino hydroxybenzoyl hexyl benzoate, and ethylhexyl triazone were provided by BASF Personal Care and Nutrition (Monheim, Germany). Ethylhexyl Salicylate was provided by Symrise (Galena, Brazil). Spirulina dry extract was provided by Ouro Fino Agronegócios (Ribeirão Preto, SP, Brazil). *Cichorium intybus* (Chicory) root extract, *Medicago sativa* (Alfalfa) Extract and *Palmaria palmata* extract were provided by Silab (Saint-Viance, France).

2.1.2 Preparation of the emulsions

Four O/W emulsions were prepared, combining or not UV-filters and natural active ingredients. First, a vehicle (formulation V) was developed and its short and long-term stability as well as its sensory perception were previously described by Calixto and Maia Campos, 2017.

This formulation without UV-filters and without active substances was developed to evaluate the effect without the action of these ingredients. After, with the same protocol described below, active substances and UV-filters were incorporated into formulation V. As a result, other three formulations were obtained: formulation VA, with active substances; formulation VF with UV-filters; and formulation VFA, with active substances and UV-filters (Table 1).

Table 1 - List of ingredients used for the oil-in-water emulsions formulations.

Ingredients (INCI name)		Content (%, w/w)			
		V	**VA**	**VF**	**VFA**
Phase A	Glycerin	3	3	3	3
	Ethyelenediamine tetraacetic acid	0.05	0.05	0.05	0.05
	Distilled water	62.14	59.04	47.14	44.04
Phase B	C12-15 Alkyl Benzoate	5	5	5	5
	Cetearyl Alcohol and Dicetyl Phosphate and Ceteth-10 Phosphate	5	5	5	5
	Bis-ethylhexyloxyphenol methoxyphenyl triazine	-	-	4	4
	Ethylhexyl Methoxycinnamate	-	-	4	4

	Ethylhexyl Triazone	-	-	3	3
	Ethylhexyl Salicylate	-	-	4	4
	Butyl Hydroxy Toluene	0.01	0.01	0.01	0.01
Phase C	Acrylates/C10-30 Alkyl Acrylate Crosspolymer	0.2	0.2	0.2	0.2
	Cyclopentasiloxane	15	15	15	15
	Cyclopentasiloxane and Cyclomethicone (and) Dimethicone Crosspolymer	5	5	5	5
	Propyleneglycol	4	4	4	4
	Phenoxyethanol and parabens	0.6	0.6	0.6	0.6
	Cichorium intybus (Chicory) root extract	-	1	-	1
	Spirulina dry extract	-	0.1	-	0.1
	Medicago sativa (Alfalfa) extract	-	1	-	1
	Palmaria palmata extract	-	1	-	1

The phase A was incorporated into the phase B under heating at 70°C. The preparations were stirred for 20 minutes and then neutralized with Aminomethyl Propanol (AMP 95), a mixture of alcohols and amines used as neutralization agent, to pH 5.5. The polymer, silicones and preservatives were then added. In the case of formulations with UV-filters (VF and VFA), they were added to phase B of the vehicle and in the formulations with active substances (VA and VFA) they were added at the end of the process. The formulations were tested in terms of preservation against bacterial development using standard tests performed by an external laboratory.

2.2 Methods

2.2.1 Microstructural characterization

2.2.1.1 Optical microscopy

In order to visualize emulsions microstructure and presence of liquid crystalline phases, a light microscope was utilized: firstly, under the bright field and secondly, under the polarized light (using cross-polarizers). The photomicroscope (DMLP/DC 300, Leica Microsystems, Wetzlar, Germany) was equipped with a camera, a multiplier (x10) and the Leica IM 1000 software (version 1.20 Release 19). Pictures were obtained at a magnification of x50.

2.2.1.2 Particle size measurement

First the refractive index of the oil phase was determined using an electronic refractometer Refracto 30PX / GS (Mettler Toledo, Schwerzenbach, Suisse). The refractive index was determined and as a result, a real part of 1.45 was obtained. The

imaginary part was chosen in accordance with the appearance of the formulations. Thus, the index used to determine the particle size was 1.45-0.00i. Particles sizes measurements were realized by static light scattering using a laser diffraction particle size analyzer SALD-7500 nano (Shimadzu Co., Ltd, Japan) equipped with a violet semiconductor laser (405 nm) and a reverse Fourier optical system (the beam converge before encountering the sample). The measure cell SALD BC 75 consisted in a 7 mL batch and a stirrer and data obtained were analyzed with the WingSALD II-7500 software (version 3.1). The samples were prepared according to protocol reported by Rowenczyk et al. (2016).

2.2.2 Macroscopic properties

2.2.2.1 Rheology

Continuous and oscillatory measurements were realized using a stress controlled rheometer HR1 (TA Instruments, Guyancourt, France). An aluminum cone-plate geometry (angle 1°59'38 ", 40 mm diameter, 47 μm truncation) was used to study the flow properties and viscoelastic behavior. Data analyses were conducted at 25ºC with the software TRIOS®3.0. Samples were loaded with a spatula, and a solvent trap was used to prevent sample drying. The emulsions rested for two minutes after loading without shearing to relax and acclimatize.

To determine the flow behavior increasing shear rates ranging from 0.01 to 1000 s−1 (mode logarithmic) were performed for 150 s with 10 points per decade. The curves representing the evolution of the shear stress and the viscosity as a function of the shear rate were obtained for all emulsions.

Oscillations strain sweep tests were carried out to study the viscoelastic behavior of the emulsions at a fixed frequency of 1Hz and at increasing strain ranging from 0.01 to 100% (mode logarithmic, 30 points / decade). This measure was aimed to determine the viscoelastic parameters G 'and G" modulus, as well as tan δ on the limits of the linear viscoelastic region (LVR). At the point where the module G' loses 10% of its plateau, deformation and stress values ($\gamma_{90\%\ G'}$ and $\sigma_{90\%\ G'}$ respectively) were determined. Both parameters give an indication of the resistance to deformation of formulations and deformability of the droplets network.

All measurements were performed at least twice on two different batches.

2.2.2.2 Texture analyses

All texture analyses were performed using a Texture Analyzer TA.XT Plus (Stable Micro Systems, Cardiff, UK) and the software Texture Exponent 32 (version 5,0,6,0, 2010). The penetration and compression analyses protocol were adapted from Gilbert et al. (2013a). For penetration tests the probe P/0.5R was utilized at a predefined rate of 5 mm/s. The probe P/35 was utilized on compression tests at a predefined rate of 1 mm/s. 1 mL of formulation was used. All measurements were performed at least three times. Curves giving the force (g) as a function of time (s) were recorded. Maximum forces (g) and area under the curve (g.s) were recorded during the compression/penetration phase and during the removal of the probe.

The difficulty of spreading was conducted as reported by Savary et al. (2013). The distance utilized was 12 cm with a speed of 3 mm/s. Four lines of each 50 µL of formulation were deposited using a Microman M250 (Gilson, Villiers-le-Bel, France) in the displacement direction. All measurements were performed at least five times. From the graph Force =f(time) obtained during the experiments was calculated the area under the curve. As spreading is an attribute related to the performance of the formulation during its application to the skin, a bigger area was interpreted as more difficult to spread (Savary et al, 2013). Also, pictures were taken of the spreading traces from a distance of 60 cm in order to evaluate the maximum mobile displacement (cm), as the distance from the beginning to the end of the analysis, and to observe the remaining formulation after spreading.

2.2.3 In vivo effects

2.2.3.1 Experimental Protocol

The clinical study was conducted at the Biometrology analysis room at the University of Le Havre in Le Havre, France (49°30′N 0°08′W) with controlled temperature (20-22ºC) and humidity (45-55%). Fifteen subjects, aged 22 ± 2 years were included in the study after giving their informed consent. The formulations were applied to five regions of 4 x 5cm area located on the forearm (4 formulations). The measurements were determined before application (control – T0) and 60 minutes after the application of 50µL of formulation. Subjects performed acclimatization for 10 minutes before measures for control of physical and emotional factors that could alter the analysis (Berardesca, 1997).

2.2.3.2 Skin brightness

The specular reflecting light from the skin was evaluated by the Glossymeter® GL 200 (Courage-Khazaka, Germany). The device evaluates the brightness of the skin surface by measurements of the reflected light (Try et al, 2010; Melo and Maia Campos, 2016; Li et al, 2017). The measurements were performed by the same operator for 10 seconds, three times for each region randomly.

2.2.3.3 Stratum corneum water content

The stratum corneum water content is a measure related to stratum corneum hydration level. It was determined using the non-invasive skin capacitance equipment Corneometer® CM 825 (Courage-Khazaka, Germany). The device measures the change in the dielectric constant due to skin surface hydration and is expressed in arbitrary units (UA's) where 1 AU corresponds to 0.2 to 0.9 mg of water/gram in stratum corneum (Berardesca, 1997; Souza and Maia Campos, 2017). Three measurements were made for each region randomly by the same operator.

2.2.3.4 Transepidermal water loss

The transepidermal water loss is a property related to skin barrier function. In this test, the equipment Tewameter® TM 300 (Courage-Khazaka, Germany), whose function is to measure water evaporation from the skin surface (Pinnagoda et al, 1990), was utilized. The test is based on the diffusion principle described by Adolf Fick in 1885. The measurements were performed by the same operator for 20 seconds, three times for each region randomly.

2.2.4 Data Analysis

One-way analyses of variance (ANOVA) were applied to test significant differences between the formulations in all parameters ($P<0.05$). The post-test Tukey multiple comparison test was utilized to elucidate the differences. Results were expressed as mean ± standard deviation (SD). These analyses were performed on Minitab® 17 software (Minitab Inc., State College, PA). To highlight significant correlations between the properties, the Pearson's correlation coefficient was calculated ($P = 0.05$) using XLSTAT software (version 2012.1.01, Addinsoft, France).

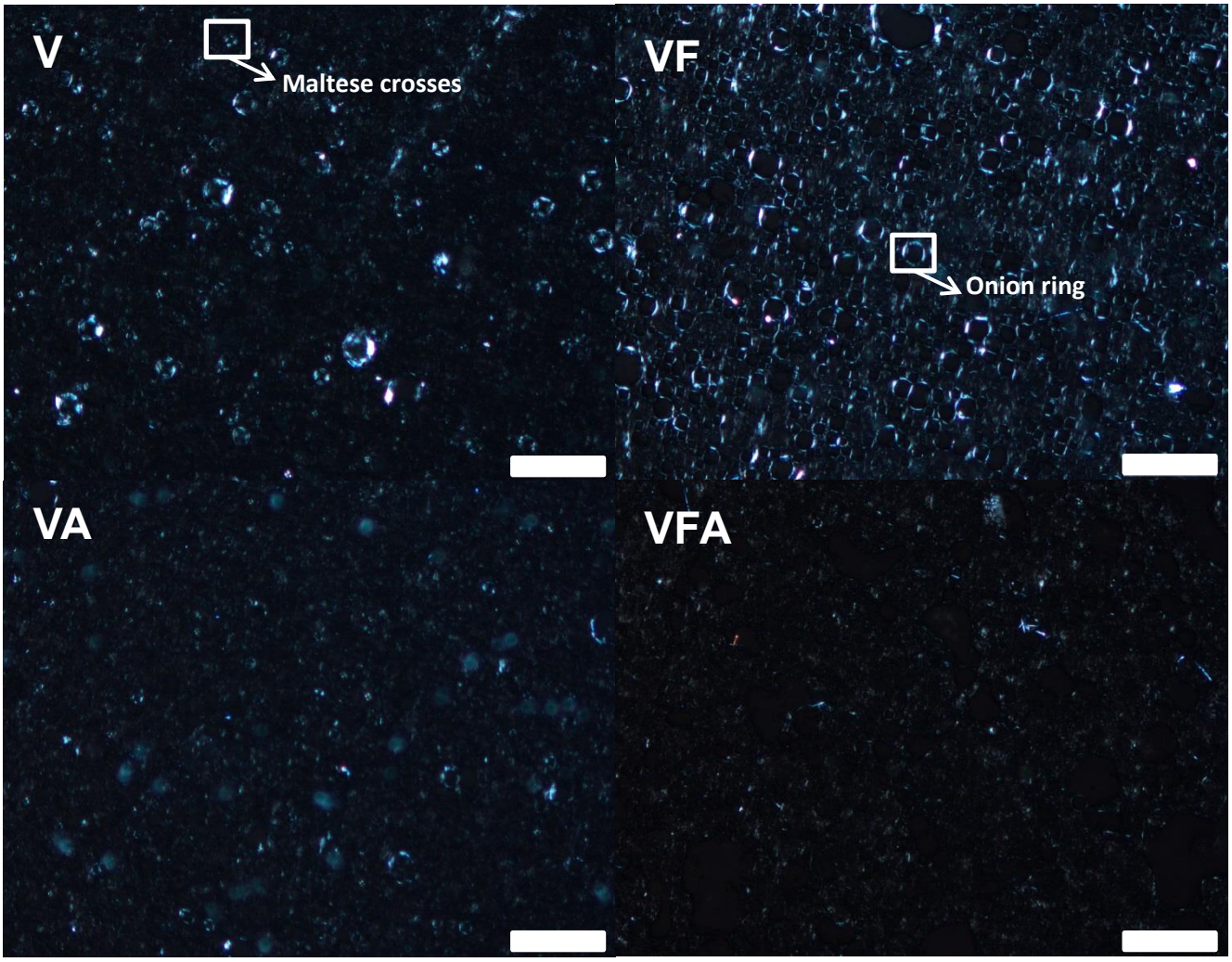
3. Results

3.1 Microstructural characterization

3.1.1 Optical Microscopy

With the images obtained by the optical microscope it was possible to observe the oil phase dispersed in the form of droplets of variable sizes in the continuous aqueous phase. The formulations with UV-filters (VF) presented larger droplets than the formulations without UV-filters (V). With the help of the polarizer it was possible to search for liquid crystal structures: "Maltese cross" and "onion rings" structures were found in formulations V and VA. The VF formulation presented "onion rings" in a larger number and the VFA formulation showed "onion rings" in a smaller number (Figure 1).

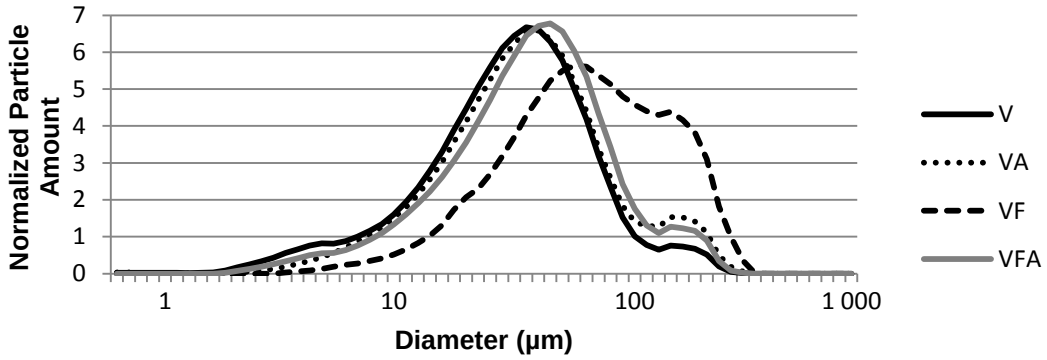
Figure 1 - Micrographs under the polarized light of the formulations V, VA, VF and VFA (The scale bar corresponds to 200 µm).



3.1.2 Particle Size

The data are shown as a volume distribution of droplet sizes (Figure 2) and monitoring of D10, D50 and D90 values. For instance, D10 represents the threshold value in micrometer for which 10% of particles have a smaller size.

Figure 2 - Particle size distributions given in volume particle of formulations.



The size distribution has proved that the formulations were mostly composed of droplets, especially the formulations with filters, in relationship with the observations on light micrographs. The formulations V, VA and VFA presented a bimodal distribution with polydispersity. The formulation V had a lower polydispersity when compared to the other formulations, with a D10 equal to 8.7 µm, a D50 at 29 µm and a D90 at 68.3 µm. The VF curve is slightly bimodal and shifted to higher values of particle size with bigger polydispersity; particle sizes ranged from a D10 of 19.3 µm and a D90 of 157.3 µm, also the formulation obtained the highest D50 of 58,3 µm. Regarding particle size values, the addition of UV-filters increased significantly the particle diameter comparing VF to V ($P < 0.01$). The size distribution of the formulations with active ingredients was very similar to the vehicle. The VA formulation presented D10 value at 11 µm, D50 at 32.4 µm and D90 at 89.9 µm while the VFA formulation showed D10 values at 11.1 µm, D50 at 34.9 µm and D90 at 85.0 µm. Thus, the addition of filters further influenced the particle size distribution than the addition of the active ingredients. Along, there was no important synergistic effect between filters and actives.

3.2 Macroscopic properties

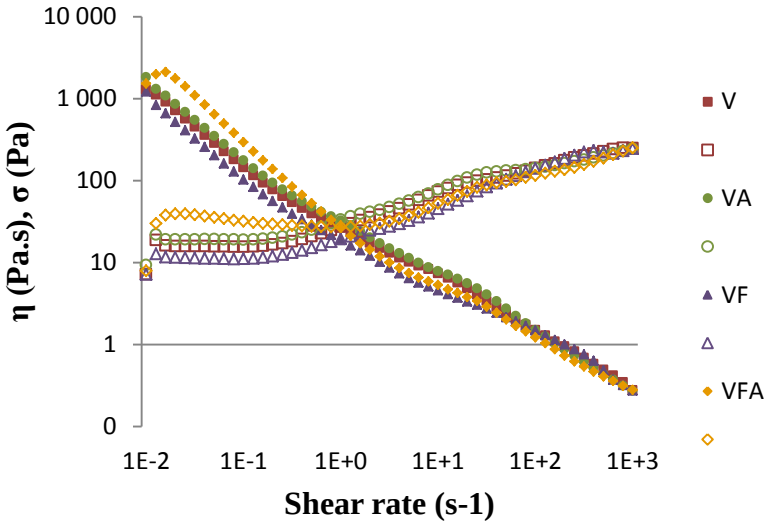
3.2.1 Rheology

The formulations showed a rheofluidifying behavior (Figure 3) that is, a decrease in viscosity with increasing shear rates. The flow tests allowed to characterize the formulations over a wide range of shear. The behavior of the formulations was different

as a function of the shear rate. The addition of filters isolated gave a low viscosity to the formulations on a wide range of shear rates. Combined in combination with the actives they showed a synergistic effect that resulted in higher viscosity values, at least on the first part of the curve (until approximatly1 Pa.s).

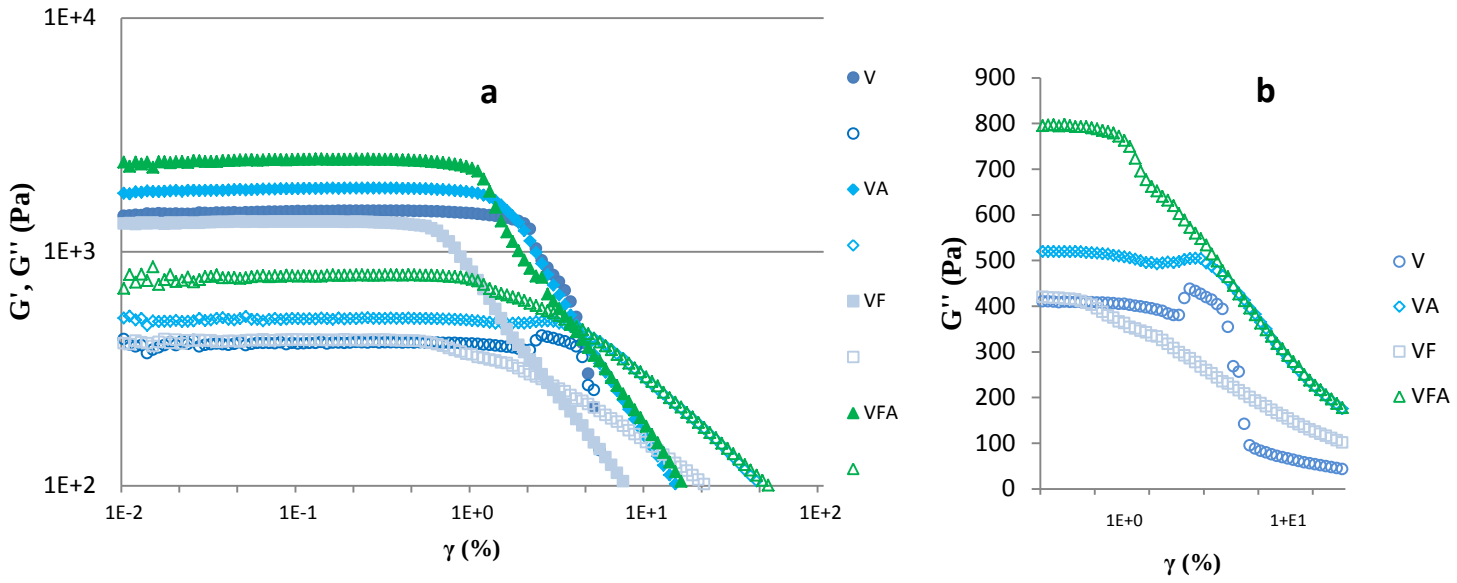
Viscoelastic behavior of the creams was studied using dynamic oscillatory measurements. The formulations exhibit a predominantly elastic behavior with G' higher than G" (Figure 4a). Compared to the vehicle and the vehicle with filters (V and VF), the viscoelastic moduli increase with the addition of the active ingredients (VA and VFA).

Figure 3 – Viscosity and shear stress as a function of shear rates for the different formulations. Solid symbols: viscosity, η; hollow symbols: shear stress, σ.



The evolution values of the linear viscoelastic region (LVR), that is, the behavior of the emulsions at the output of LVR, provide information on the resistance to deformation. It is an index of homogeneity and can be related to the organization of the droplets network. The addition of filters influenced this property more than the addition of actives. With the addition of filters there is a lower resistance to deformation. Formulations V and VA showed a peak of G " (Figure 4b) characteristic of a weak gel type behavior mainly due to the gelling agent. Also, there was a significant increase in tan δ when the formulations were compared with and without filters (Table 2) indicating the enhancement of the viscous behavior. The presence of filters weakened the organization of the droplets and modified its viscoelastic properties.

Figure 4 - Viscoelastic characterization of the formulations. Solid symbols: storage modulus, G'; hollow symbols: loss modulus, G".



From the curve obtained with the penetration and compression tests, the maximum force parameter was extracted (Table 2). The VF formulation, showed significantly low compression values, indicating that the presence of filters disrupts this texture property. After the addition of active ingredients to these formulations, the compression values increase. For this parameter, adding actives has more impact than adding filters. The penetration test demonstrated a great synergistic effect between the presence of filters and actives with higher values for VFA, that was significantly different from all formulations.

Table 2 - Macroscopic characteristics of the formulations.

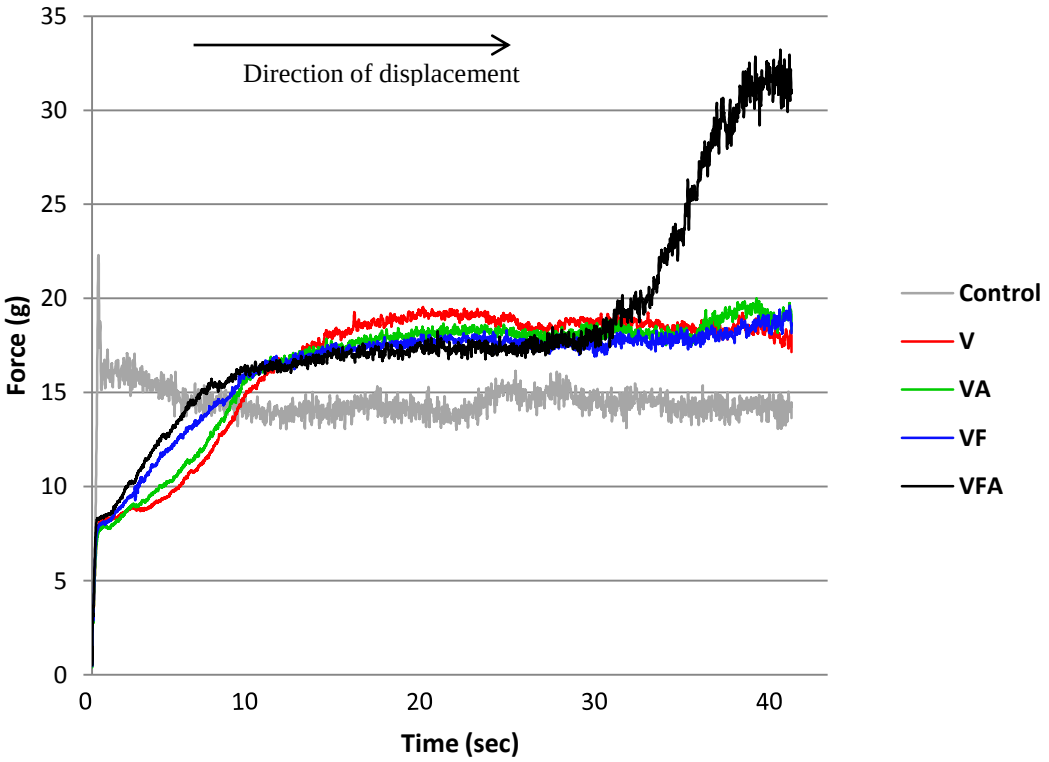
	Viscoelastic parameters						Textural properties		
	G' (Pa)	G'' (Pa)	tan δ	$\sigma_{90\%G'}$ (Pa)	$\gamma_{90\%\,G'}$ (%)	G'=G'' (Pa)	Compression (g)	Penetration (g)	Spreading Ratio
V	$1485.6^{C} \pm 48$	$406.5^{B} \pm 25.5$	$0.27^{B} \pm 0.0$	$1337^{C} \pm 43$	$2^{A} \pm 0.2$	$454.8^{A} \pm 11$	$406.4^{A} \pm 33.3$	$26.5^{B,C} \pm 0.7$	$0,87^{A}$
VA	$1852.9^{B} \pm 23$	$516.3^{B} \pm 10$	$0.28^{B} \pm 0.0$	$1667.6^{B} \pm 20$	$1.4^{A,B} \pm 0.1$	$460.8^{A} \pm 5$	$375.4^{A} \pm 6.7$	$30.4^{B} \pm 1.3$	$0,88^{A}$
VF	$1341.1^{C} \pm 37$	$417.6^{B} \pm 10$	$0.31^{A} \pm 0.0$	$1207^{C} \pm 34$	$0.7^{B} \pm 0.1$	$277.5^{B} \pm 23$	$316.1^{B} \pm 13.5$	$24.4^{C} \pm 3.8$	$0,89^{A}$
VFA	$2421.9^{A} \pm 44$	$771.4^{A} \pm 15$	$0.32^{A} \pm 0.0$	$2179.7^{A} \pm 39$	$1.1^{B} \pm 0.0$	$517.6^{A} \pm 13$	$409.4^{A} \pm 1.4$	$35.3^{A} \pm 2.2$	$0,78^{B}$

Different letters in the same column means significant difference between emulsions for each parameter ($p < 0.05$).

3.2.3 Spreading evaluation

From the graphs resulting from the spreadability analysis it was possible to observe that the initial force required to spread the formulations was low (Figure 5) compared to the one of control without product. This behavior is characteristic of the lubricant role of creams. Then, the area under the curve was calculated and interpreted as the difficulty of spreading. During the analysis, the formulations presented a larger work area (g.sec) than the control with no formulation (578.6 ± 27). The formulation VFA presented a significant higher area value of 739.1 ± 33.4 compared to the other formulations V, VA and VF which presented values of 662.4 ± 27.9, 657.1 ±15.6 and 653.2 ±21.4 respectively .

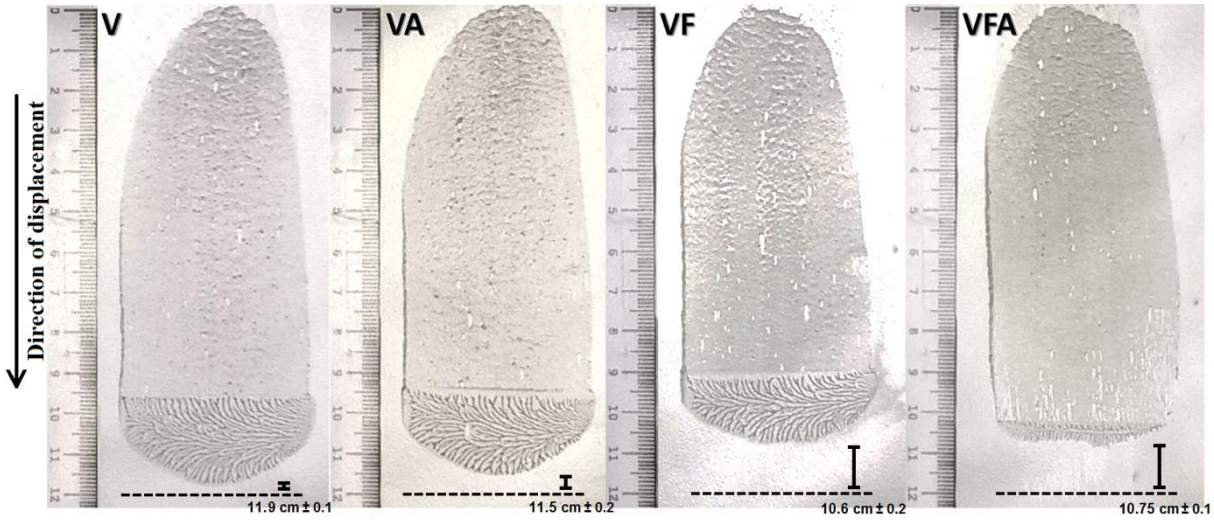
Figure 5 – Friction curves representing the spreading of the formulations and the control-curve with no formulation.



Observing the pictures of the traces obtained (Figure 6), it can be seen that the formulations without filters spread over a longer distance in comparison to the formulations with filter. In addition, it was observed that at the end of the analysis there was still formulation to be spread except for the VFA formulation which ended the analysis with minimal amount of formulation. Moreover, the large final peak of the

curve observed on the previous curves (Figure 5) and the behavior observed in the photos (Figure 6) indicates that, during the analysis, the formulation VFA was more concentrated on the surface of the test, making it necessary a greater force to spread, more than the other formulations. This is confirmed by the value of Spreading ratio, calculated between the control spreading area with no formulation and the spreading area of formulations results (Table 2). The higher the ratio, the easier the spreading. The formulation VFA demonstrated to be significantly more difficult to spread than the others with a ratio of 0.78. There was no significant difference between the rates of the other formulations that also showed similar curves and spreading traces.

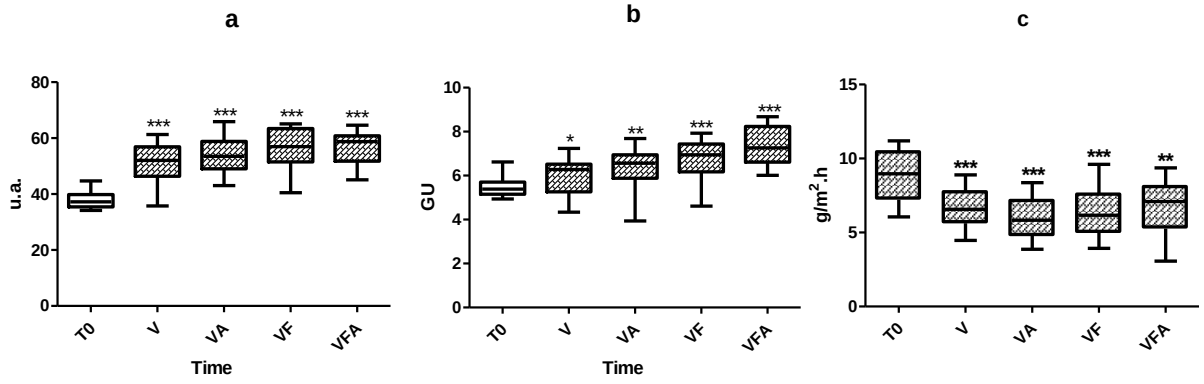
Figure 6 – Example of spreading traces of formulations V, VA, VF and VFA.



In vivo effects

Regarding the effects in vivo, all formulations were able to bring benefits to the skin after 1h. Concerning stratum corneum water content (Figure 7a), the result was the same for the four formulations: a significant impact on hydration levels ($P < 0.001$). However, it can be observed that the presence of filters induced a higher increase of this parameter, especially when filters and active were in combination. All formulations increased the brightness of the skin surface with different significances (Figure 7b). The higher difference was found after applying the formulations with filter, especially the VFA formulation. With the transepidermal water loss results (TEWL) it was possible to ensure that there was no damage to the skin barrier function, since all formulations decreased water loss values after 1h.

Figure 7 - Stratum corneum water content (a) skin surface brightness (b) and transepidermal water loss (c) values before (T0) and 1 hour after application of the formulations. Results are expressed as mean ± standard deviation (n = 15). Statistically significant difference in relation to T0: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.



4. Discussion

Our formulations present a traditional O/W emulsion balance between the aqueous phase, where the active substances are contained and the oil phase, where the UV-filters are integrated. The presence of liquid crystal structures indicates the presence of the liquid lamellar phase that seems to be related to the arrangement of fatty alcohols emulsifier and oil molecules at the oil-water interface (Zhang and Liu, 2013). With the addition of filters, there is an increase of oily phase, which increase the presence of oil droplets packaging and of characteristic anisotropic droplets called “onion rings” (Pantelic et al., 2015).

The addition of filters caused an increase in particle size checked by light microscopy and static light scattering. Also, the addition of filters isolated led to a higher number of liquid crystal structures and a higher polydispersity, as noticed on volume particle size distribution. This implied a decrease in rheological and texture parameters. There is no consensus in the literature on the influence of liquid crystals on these parameters, but they appear to be related to the formulations with the lower strength structure and less homogeneous (Lavaselli et al., 2012; Zhang and Liu, 2013; Otto et al., 2013; Mewis and Moldenaers, 1996).

A decrease in the viscosity of emulsions due to the presence of liquid crystals is mainly related to the cetearyl alcohol emulsifiers as Cetearyl Alcohol and Dicetyl Phosphate and Ceteth-10 Phosphate. Although, the lower viscosity may be related to the larger

particle size observed in formulation VF, which is related to increase of the emulsion's internal phase size (Hanno et al., 2015; Otto et al., 2013; Pantelic et al., 2015).

The lower viscosity induced a lower compression and penetration force as a consequence of its behavior. There is a correlation between those tests and rheological parameters, and this explains the same lower results for formulation VF and higher results for formulation VFA (Gilbert et al, 2013a). But, in the case of penetration test there is a more pronounced synergic effect between the actives and the UV-filters and a stronger structure for this formulation was evidenced. An increase in the texture parameters indicates an increase in properties that directly influence the sensory of the formulations as firmness, consistency, cohesiveness (Gilbert et al, 2013a; Gilbert et al, 2013b; Calixto and Maia Campos, 2017).

Regarding rheology results, the formulations V, VA presented a peak of G''. The peak illustrates a "weak gel" behavior (Bais et al., 2005; Rowenczyk et al., 2016). The "weak gel" behavior is provided by the Acrylates/C10-30 Alkyl Acrylate Crosspolymer lead by the reorganization of the droplet network when the percentage of deformation is increased (Ekong et al., 2001), suggesting that the formulations with filters could present a stronger network. Since the formulation V has the largest peak, the addition of active ingredients in the aqueous phase has decreased the "weak gel" character of the formulations and in the same way, the presence of filters annealed this behavior. Nevertheless, this behavior is determined by the presence of some ingredients and related to the microstructure by the organization of droplets. Thus, when analyzing the G'=G" cross point values, it can be observed that they are very similar, except for the VF formulation (Chen and Dickinson, 1998). Also, the VF formulation obtained a low value of γ 90% G', which indicates a less homogeneous system, in relationship with the increase of polydispersity. The formulations VA and VFA obtained higher values of γ 90% G', that is, the addition of actives brought higher resistance to the deformation by the formulation This may be partly due to the impact of active substances to the continuous aqueous phase. Thus, this higher resistance was proven by the smaller traces and higher values of spreading in texture test. Those values are well correlated to sensory spreadability which is a good indicator for products/skin interaction (Gilbert et al., 2013c, Savary et al., 2013). Combining the rheology and texture results and microscopic characterization, it is possible to conclude that the VF formulation has the weakest structure. Combining lipophilic UV filters and aqueous actives to the emulsion

helps to maintain droplets size distribution and network, viscoelastic properties and resistance to deformation.

The formulations showed non-Newtonian behavior and shear-thinning phenomenon, that is, viscosity decreases with shear rate (Gilbert et al, 2013a; Dong et al., 2015). This isothermally and reversibly phenomenon is desired for topical formulations. A pseudoplastic behavior implies a more protective film to the skin generated by a higher resistance to spreadability (Gaspar and Maia Campos, 2003; Gilbert et al, 2013a). Regarding spreadability results, the formulations with filter presented more resistance to spread with smaller traces by the displacement and higher final force peaks on spreadability curves. This leads to a thicker layer of formulation on the skin that increases the effectiveness of the product mainly via occlusion phenomenon (Müller-Goymann, 2004; Savary et al., 2013; Try et al, 2010). The occlusion is correlated with transepidermal water loss (Agner and Serup, 1993; Plessis et al, 2013). Therefore, the *In vivo* results provide important explanations.

All formulations showed enhanced clinical efficacy. The emollients, humectants and silicones present in the vehicle contribute to this result, as they act on the skin barrier, repairing it and optimizing the processes involving water in the lipid barrier (Lynde, 2001). Particle size also appears to be related to increased hydration (Golmohammadzadeh, 2012). The formulations with higher D50, VF and VFA, obtained better clinical efficacy results.

These better results may be due to the large presence of liquid crystals that demonstrated to have good moisturizing properties (Savic, 2006). The liquid crystalline phase of the emulsion can interact with the skin lipids improving its interaction with the stratum corneum (Otto et al, 2009). They can also increase the water content of the stratum corneum by retention of the water molecules in the emulsifier layers of their structure (Zhang and Liu, 2013). The lipids structures can form lamellar structures in the intercellular spaces of the stratum corneum leading to slower water loss resulting in more available water for skin moisturization (Iwai et al, 1998). The decreased TEWL values after application of the product confirm the relationship between hydration and occlusion. This has been described in the literature as an effect of humidity accumulated in the skin/occlusion space because of the saturating of the skin as a result of an

occlusive cover (Aly et al., 1978; Gioia and Celleno, 2002). All formulations significantly improved TEWL, and thus, the skin barrier function (Ersser et al, 2005).

Furthermore, the VFA formulation was the one that obtained the best results in the in vivo tests. This formulation presented higher values of elastic and viscous moduli, higher tan δ and $\sigma_{90\%\ G'}$ values, indicating a viscoelastic behavior more viscous and more resistant to deformation than the other formulations. The in vivo parameters hydration, brightness and TEWL are related to skin surface (Berardesca, 1997; Plessis et al, 2013; Li et al, 2017) so, when the formulation was applied to the skin, it was not instantly absorbed and the cream more viscous remains at the contact interface, resulting in a greater film residue and greater resistance to movement (Tang et al., 2010).

Previous studies showed that the incorporation of UV-filters in combination with antioxidants actives can change the behavior of formulations, without reducing their stabilities and their sensorial characteristics (Calixto and Maia Campos, 2017). Also, the addition of extracts as Spirulina and red algae in formulations can improve the clinical performance of sunscreens (Mercurio et al, 2015; Souza and Maia Campos, 2017).

Our results agree with the literature (Maia Campos et al, 2017; Harnedy et al, 2014; Jouandeaud et al, 2006) and demonstrate that the addition of UV-filters to the formulations with active substances of known effectiveness brought a superior and synergistic result in comparison to the other formulations.

The Pearson's coefficient (r) confirmed the correlation between clinical efficacy and the way the formulations are structured and their interaction with the skin. When the product was more easily disturbed, with a smaller value of γ 90% G' it has shorter traces (r=0.960) and higher values of hydration (r = -0.989) and brightness (r = -0.834). The particle size parameter D50 showed a strong anti-correlation with the compression force (r = -0.921) and the G' and G" cross point (r = -0.905). This characterizes a loss of the viscoelastic properties of the products with higher particle size and the transition between the flow behaviors (Gilbert et al., 2013a). In this study, a synergistic effect was observed between the ingredients in the instrumental properties and *in vivo* performance of the formulations. Though, the presence of UV-filters had more force to perturb the structure of the formulations than the presence of the active ingredients.

5. Conclusion

The development of efficient and stable multifunctional formulations is of major importance because of the strong need to protect against UV radiation and to promote wellbeing through effective topical formulations. The addition of lipophilic filters resulted in more polydisperse formulations with larger particle sizes and weaker structure. They presented many liquid crystal structures especially onion rings. The presence of liquid crystals appears to be associated with the chosen emulsifier and this result proved the incorporation of the UV-filters in the oil phase. It has profoundly influenced the characteristics and efficacy of the formulations.

The addition of UV-filters interfered in the spreadability of the formulations, which presented smaller traces and better efficacy than the formulations without UV-filters. It has been observed that the active ingredients alone have no great influence on the behavior of the formulations. They enhanced weakly the macroscopic properties of emulsions as rheology and texture, as they are in low concentration in emulsion and diluted in the aqueous phase. However, in combination with UV-filters they showed a synergistic effect and were capable to change significantly the rheology, texture and organization of the formulations particles/droplets.

Finally, the combination of UV-filters and active substances resulted in excellent performance *in vivo*. Thus, this study showed that the characterization studies of the formulations are intelligent strategies to predict the final behavior of topical final products as well as its efficacy.

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3.4. Capítulo 4 – Testes de segurança

3.4.1. Artigo 4: CAMPOS, P. M. B. G. M. ; BENEVENUTO, C. G. ; CALIXTO, L. S. ; MELO, M. O. ; PEREIRA, K. C. ; GASPAR, L. R. (in press) Spirulina, Palmaria palmata, Cichorium intybus, and Medicago sativa extracts in cosmetic formulations: an integrated approach of in vitro toxicity and in vivo acceptability studies. Cutaneous and Ocular Toxicology, 2019.

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Application of natural extracts in topical formulations: an integrated approach of *in vitro* toxicity and *in vivo* acceptability studies

Background/Aims: The selection of suitable raw materials in the cosmetic research and development is a key point, not only in order to obtain the expected results but also to avoid undesirable side effects. This study evaluated the *in vitro* toxicity potential of four different plant extracts and their *in vivo* acceptability studies. **Methods:** Spirulina, *Palmaria palmata*, *Cichorium intybus*, and *Medicago sativa* extracts were analysed alone or in combination and added in cosmetic formulations. The *in vitro* toxicity evaluation, Hen's Egg Chorioallantoic Membrane Test (HET-CAM) and 3T3 NRU phototoxicity test were performed to evaluate *in vitro* potential ocular irritation and photo safety, respectively. Twenty subjects were enrolled in the acceptability studies, who were evaluated for the absence of harmful effects of the formulation by visual assessment and by transepidermal water loss, a biophysical technique, for 30 days. **Results:** HET-CAM assay showed that the studied extracts added to a gel-cream formulation had no irritant potential. In addition, the combination of *Palmaria palmata*, alfalfa and chicory extracts did not show phototoxic potential *in vitro*. Acceptability studies showed that the formulation containing the four extracts combined did not provoke any transepidermal water loss (TEWL) alteration, sensory irritation or erythema in the forearms for the period of analysis. **Conclusion:** The studied active ingredients, alone or in combination, present no cytotoxicity potential and when added to a gel-cream formulation had no irritant potential *in vitro*. These results predicting no harmful effects were confirmed in the acceptability tests, which showed no alteration on skin barrier function and no report of irritation perception of sign of erythema, suggesting the potential of these extracts for the development of safe cosmetic products.

Keywords: natural extracts, HET-CAM, phototoxicity, clinical studies, cosmetics

Abbreviations:

3T3 neutral red uptake phototoxicity test (3T3 NRU PT); Analysis of Variance (ANOVA); Bovine Corneal Opacity Permeability (BCOP) Test; Butylated hydroxytoluene (BHT); Dimethyl sulphoxide (DMSO); Disodium ethylenediamine

tetraacetic acid (EDTA); Dubbelco´s modified phosphate buffer saline (DPBS); Hen's Egg Chorioallantoic Membrane Test (HET-CAM); Mean photo effect (MPE); Organization for Economic Co-operation and Development (OECD); Scientific Committee on Consumer Safety (SCCS); Short Time Exposure (STE); Sodium dodecyl sulfate – SDS; irritation score (MSc); Transepidermal water loss (TEWL); Vitamin D receptor (VDR).

1. Introduction

Among the active substances often used in multifunctional cosmetic products, natural extracts and vitamin-like substances have been widely used, mainly on the face region and areas around the eyes, in order to protect the skin and to act on cell renewal and on the recovery of photodamaged skin. This way, the development of skin compatible formulations containing the combination of natural extracts as active ingredients is essential for the cosmetic and dermatological fields in order to obtain effective multifunctional products aiming to keep and restore skin integrity.

In this context, we can highlight the unicellular blue-green alga Spirulina, which presents potential protective effects on the skin and prevent changes related to aging process due to the its rich composition in vitamins, minerals, proteins and polysaccharides [1].

The *Cichorium intybus* root or 'Vitamin-D like', is rich in oligofructosans derived from the chicory root has the potential to restore the functions of the vitamin D receptor (VDR), an intracellular receptor present in most skin cells, by stimulating the molecular network involved in the terminal differentiation of keratinocytes as well as the recuperation of the barrier function [2, 3].

The *Medicago sativa* (alfalfa) extract has been proposed for its 'retinol-like' effect as it is a potential stimulator of cell activity, which decreases during the aging process, it improves epidermal renewal and also regulates keratinocyte differentiation [4] besides its whitening activity. The fraction containing oligosaccharides that are rich in xylose and galactose, obtained from the red algae *Palmaria palmata* has anti-pigmenting activity by inhibiting melanogenesis and limiting the transport of melanosomes and photoinduced pigmentation [5].

Even though popular belief lead to a false perception that natural products does not bring any side effects [6], cosmetic products containing (or not) these type of ingredients for the face and eye regions may accidentally come into contact with the eyes, causing irritation. Some *in vitro* assays are replacing Draize test for the prediction of human ocular irritation, i.e., Bovine Corneal Opacity Permeability (BCOP) Test, Short Time Exposure (STE), and Hen's Egg Chorioallantoic Membrane Test (HET-CAM), among others.

The HET-CAM test is based on the observation of irritant effects (hyperemia, hemorrhage and coagulation) on the chorioallantoic membrane of fertile eggs and has been widely used and has shown satisfactory results for the evaluation of emulsions, in addition to having the advantage of low cost [7, 8]. Besides that, it presents high sensitivity for the identification of absence of irritant potential, i.e. no false negatives, which is important for cosmetics, that are products that cannot cause any side effects. Although this test is not sufficient to replace totally the animal tests for the evaluation ocular irritation potential, it is the only *in vitro* method that evaluates hemorrhage and vascular effects and shows good correlation with findings of *in vivo* tests [8].

According to region and application time, other events must be considered in safety assessment such as photoinduced irritation and toxicity, among others and thus phototoxicity potential evaluation is also important [9].

The European Community and the Organization for Economic Co-operation and Development (OECD) validated the 3T3 NRU phototoxicity test (3T3 NRU PT), which compares the cytotoxicity of a chemical when tested in the 'dark' IC50 (–UV) to the cytotoxicity measured by the activated chemical exposed to a non-cytotoxic dose of ultraviolet A (UVA) light IC50 (+UV) [9]. The 3T3 NRU phototoxicity test identifies phototoxicological hazards with 100 % sensitivity, and thus is accepted as the tier one test that correctly identifies the absence of phototoxic potential. However, as it does not take into consideration the skin bioavailability, positive results in the 3T3 NRU often do not translate into a clinical phototoxicity risk [10, 11].

According to the Scientific Committee on Consumer Safety (SCCS) of European Commission, tests in humans can only be envisaged if no concern is raised in the *in vitro* preclinical toxicological studies performed with the ingredients. Thus, finished cosmetic products are usually tested in small populations to confirm their skin

compatibility and acceptability. Compatibility tests must involve exposure (normal or slightly exaggerated, i.e. patch tests), which closely mimics typical consumer use of the product. Acceptability studies are intended to confirm the fulfilment of the expectations for a cosmetic product in-use [12] as well as to report sensory irritation.

Cosmetic formulations can be clinically evaluated by noninvasive biophysical and skin imaging techniques that allow the analysis of the effects of the formulations under study on real conditions [13]. Many skin parameters can be assessed using these techniques as transepidermal water loss (TEWL), stratum corneum water content skin microrelief, and others. In combination, these tests can provide detailed data about the performance of a cosmetic product on the skin hydration and protection.

On the other hand, TEWL evaluation is especially important to the study of integrity of the skin and the protective effects of cosmetics [15]. In addition, the obtained data informing possible changes in the skin barrier function, which could be a sign of irritation. Patients with skin conditions that affect its barrier function often present an abnormal response to irritating agents, increasing even more the TEWL values [16]. In this context, besides being often used in efficacy clinical studies, the instrumental measurements can also be employed for safety studies [6, 17].

In view of the above considerations, the aim of the present study was to evaluate Spirulina, chicory, alfalfa and *Palmaria palmata* extracts toxicity by using an integrated approach, carried out in two phases; firstly, assessing the potential ocular irritation and the phototoxicity of these extracts alone, in combination and when added in cosmetic formulations by using HET-CAM and 3T3 NRU PT. After that, acceptability studies by visual assessment and by using TEWL measurements to analyze the skin barrier function and evaluate the absence of harmful effects for a period of 30 days.

2. Material and Methods

2.1 Developed formulations

A gel-cream formulation vehicle was developed based on cetearyl alcohol, dicetyl phosphate and ceteth-10 phosphate, acrylates/c10-30 alkyl acrylate crosspolymer, cyclomethicone (and) dimethicone crosspolymer, cyclopentasiloxane, phenoxyethanol and parabens, disodium ethylenediamine tetraacetic acid (EDTA),

butylated hydroxytoluene (BHT), propyleneglycol, glycerin, and c12-15 alkyl benzoate (Table 1).

For the determination of the *in vivo* acceptability study, the active ingredient extracts were added or not (vehicle) to the same gel-cream formulation at the following concentrations: Spirulina (0.1%), chicory root (1%), alfalfa (1%), and *Palmaria palmata* (1%).

[Table 1]

For the *in vitro* analysis (3T3 NRU PT), the active ingredient extracts were tested alone and in three different combinations, following the proportion of the formulations: For ocular irritation potential (HET-CAM) assay), the active ingredients were tested alone, in combinations and added in formulations. The concentrations of the active ingredients were the same as the *in vivo* studies.

2.2 In vitro toxicity potential

2.2.1 Evaluation of ocular irritation potential

Fertile eggs of White Leghorn chickens were used on the tenth day of incubation at 37.8 °C ± 1ºC and 50-60 % relative humidity for the HET-CAM assay [7]. Briefly, the shell membrane was moistened with 0.9 % of sodium chloride (NaCl) (Synth, Brazil) solution and carefully removed and the chorioallantoic membrane (CAM) was exposed and examined for damage or injuries. Sodium dodecyl sulfate (SDS) (Synth, Brazil) 1 % (w/w) solution was used as positive control and a 0.9 % NaCl solution as negative control. The tested extracts and their combinations were applied undiluted (100 %, 300 µL for the liquids or 100 mg for the solid or semi-solid samples). The Spirulina extract was powdered to avoid mechanical injuries to the CAM. The extracts and combinations were also added to the formulation described in item 2.1 to mimic the real conditions of use. After 20 seconds of application, the CAM was rinsed with at least 5 mL of a 0.9 % NaCl solution, the irritant effects (hyperemia, hemorrhage and coagulation) were monitored over a period of 5 minutes (including the initial 20 seconds) and the irritation score (MSc) was calculated according to [7]. The test was performed in quadruplicate. Irritant scores between 0 and 0.9 predicts non-irritant substance, scores between 1 and 4.9 predicts slight irritant, scores from 5 to 8.9 predicts moderate irritants and 9 to 21 predicts severe irritants. Data of the irritation score was statistically analyzed by

ANOVA followed by the post-hoc Tukey test, since the data presented parametric distribution.

2.2.2 Evaluation of phototoxicity

Phototoxicity was evaluated using a 3T3 monolayer fibroblast culture [9, 18, 19]. For the test, 100 µL of a cell suspension ($1x10^5$ cells/mL) of 3T3 fibroblasts was dispensed in two 96-well plates. After 24 h, eight different concentrations of each extract and their combination, diluted in dimethyl sulfoxide (DMSO) and Dulbecco's phosphate-buffered saline (DPBS), were applied in sextuplicate, in the concentration of 100, 68.1, 46.4, 31.6, 21.5, 14.7, 10, and 6.81 µg/mL. For the combinations, each concentration corresponds of a mixture of the extracts, based on the proportion of 0.1:1:1:1 of the 4 extracts: Spirulina *: M. sativa* – alfalfa *: C. intybus* – chicory *: P. palmata, respectively*. For example: for 100 µg/mL, 100 µg/mL (0,01%) of *M. sativa* – alfalfa, *C. intybus* – chicory and *P. palmata* were used, while 10 µg/mL (0,001%) of Spirulina were used. L-histidine and norfloxacin were used as negative and positive controls respectively. After 1 hour of incubation, the +UVA plate was irradiated (total dose = 9 J/cm²) with UVA and the -UVA plate was kept in the dark. The UVA radiation corresponded to a total dose of 9 J/cm^2, which is approximately 22 minutes of exposure to sunlight at mid-day (6.94 mW/cm^2) of a typical September sunny day in the Ribeirao Preto region latitude 21º 10' 39" south and longitude 47º 48' 37" west. In the day after, neutral red medium was added to each well and, after 3 hours of incubation, cells were washed with DPBS and a desorb (ethanol/acetic acid/water) solution was added. Then, +UVA and -UVA plates were analyzed at 540 nm [9, 18]. Data were analyzed with the Phototox Software for the evaluation of phototoxic potential. The substance is predicted to be phototoxic if the mean photo effect (MPE) is higher than 0.15 and is predicted to be 'probably phototoxic' with an MPE > 0.1 and < 0.15 and non-phototoxic with an MPE lower than 0.1 [9].

2.3 Clinical studies

After approval of Ethics Committee in Research (CEP/FCFRP nº381 – CAAE nº 43463115.0.0000.5403), 20 healthy female subjects aged 40 to 60 years were recruited for the evaluation of the clinical effects of the formulations containing Spirulina, chicory root, alfalfa and *Palmaria palmata* extracts, in combination, using biophysical and skin imaging techniques.

All subjects gave written informed consent to participate in the study. The participants had their two forearms demarcated in 4 cm x 4 cm regions. One region received the vehicle formulation, and other the formulation containing all active ingredients in combination.

2.3.1. *In vivo* acceptability evaluation

In order to confirm the absence of harmful effects of the formulation in skin barrier function, transepidermal water loss (TEWL) was measured with an evaporimeter (Tewameter^{TM} TM 210, Courage & Khazaka Electronic GmbH), and recorded as g/m^{2}h after probe equilibration on the skin for 20s. TEWL measurements were performed at baseline (T0) and 30 days after application of the test substances.

A visual assessment of the skin of studied subjects regarding irritant reactions was also performed to confirm the absence of irritation, using a score method as described below:

Reaction	Result
0 – None	Negative (-)
1 – Light Erythema	Uncertain (?)
2 – Distinct Erythema	Positive (+)
3 – Erythema + edema + papules	Positive (++)
4 – Erythema + edema + papules + vesicles	Positive (+++)

Data were analyzed statistically with tests chosen according to the type of sampling distribution detected in preliminary tests of normality distribution and variance homogeneity. Analysis of Variance (ANOVA) was applied to parametric results and the Friedman test was applied to nonparametric results. All statistical tests were performed using the Prism 6 software.

3. Results

3.1 In vitro toxicity potential

3.1.1 Evaluation of ocular irritation potential (HET-CAM)

The values obtained with negative (0.9 % NaCl) and positive controls (1% SDS) were similar to recommended values (Table 1) [8].

M. sativa (alfalfa), Spirulina *and P. palmata* extracts, when applied alone (100%) (MSc: 0, 0, 0.75, respectively) or when added to the gel-cream formulation (undiluted) (MSc: 0) did not induce any reaction in the chorioallantoic membrane

(Tables 2-3). The MSc obtained for *P. palmata* extract (0.75) was considered statistically equivalent to negative control ($p > 0.05$). Only the undiluted chicory root extract (100%) caused a severe irritation (MSc: 11.5) (Figure 1 and Table 2). However, when the same extract was applied at 50 % or when added to the gel-cream formulation, no reaction was observed and thus this extract was also considered non-irritant (MSc: 0) (Tables 2-3 and Figures 1-2).

The combination of the four studied extracts induced severe irritation when applied undiluted (MSc: 10.25) and thus these extracts should be considered as potential ocular irritants (Table 2). However, when added to the gel-cream formulation, this combination was considered safe since it did not produce any reaction in the CAM (MSc: 0) (Table 3 and Figure 2).

Two other combinations were evaluated in the assay; the first one, containing Spirulina, alfalfa and chicory extracts, induced a slight irritation due to hyperemia when applied undiluted (MSc: 3.0) (Table 2), which for instance was considered statistically equivalent to negative control. The other combination containing *P. palmate*, alfalfa and chicory extracts induced no irritation at either dilution studied (Table 2) ($MSc_{100\%}$:0, $MSc_{1\%}$: 0.75) and also when added in the gel-cream formulation (MSc:0). The MSc obtained for this combination of 3 extracts at 1% was considered statistically equivalent to negative control and to this combination at 100% ($p > 0.05$)

Therefore, the gel-cream formulation containing the studied extracts and combinations did not show any irritant potential in the HET-CAM assay and thus could be applied to the face around the eye areas.

[Table 2]

[Table 3]

[Figure 1]

[Figure 2]

3.1.2 Evaluation of phototoxic potential (3T3 NRU PT)

Norfloxacin (positive control) and *L*-histidine (negative control) were within the range recommended by OECD TG 432 [8] (Table 4).

Spirulina and chicory extracts had a phototoxicity potential (MPE_{spir}: 0.941 and 0.900 and MPE_{chic}: 0.217 and 0.259) however alfalfa and *P. palmata* extracts did not exhibit any phototoxicity potential (Table 3, Figure 4). All studied extracts and combinations were non-cytotoxic, with all EC_{50} -UV values higher than the highest tested concentration (100 µg/mL). Thus, they can be considered safe for humans without direct solar exposure (Table 4).

[Figure 3]

Only the combination of *Palmaria palmata,* alfalfa and chicory extracts were not considered phototoxic (Table 4) and therefore has the potential to be applied to cosmetic formulations for the face.

[Table 4]

3.1.3 In vivo acceptability evaluation

Considering that the ingredients under study, alone or in combination, were not considered cytotoxic and that their combination added in the gel-cream formulation were not considered irritant, the TEWL were performed to confirm the absence of harmful effects during a period of one-month application. The results showed that the formulation containing the four extracts combined did not present any TEWL alteration during this period, when comparing T0 and T30 and vehicle X control (Table 5), showing that the skin barrier was preserved and it did not allow the skin water loss (Figure 4). The visual analysis also showed that there was no report of sensory irritation from the subjects or any erythema in the forearms that received the application of the formulation containing the four studied extracts (Table 6).

[Figure 4]

[Table 5]

[Table 6]

4. Discussion

The application of natural extracts in topical formulations is a big highlight in the cosmetic and dermatological research, using these different extracts as active

ingredients with specific activity for the treatment and prevention of skin conditions and diseases.

In this context, the combination of *Palmaria palmata*, alfalfa and chicory extracts was not considered phototoxic for topical use. However, two extracts, Spirulina and chicory, and the combination of the four extracts showed a phototoxic potential. The mechanism that a photosensitizing chemical lead to phototoxicity involves absorption of a photon of UV/visible radiation which lead to electronically excited states that subsequently react via two major pathways: type I reaction that may or may not require oxygen but form superoxide and hydroxyl radicals, or by energy transfer to form excited-state singlet oxygen. These extracts are complex mixtures that present many different compounds that besides their whitening activities, can have antioxidant activity as well [5] and thus can neutralize part of the free radicals formed by the phototosensitizing compounds.

However, additional studies are needed to confirm if this prediction translates into a clinical risk. Thus, they can be considered safe for humans without direct solar exposure. In addition, as these compounds do not necessarily penetrate the skin and cause photoreactions, further experiments considering skin penetration must be performed [9, 10].

The 3T3 NRU-PT is considered a stand-alone test for negative results due to its high sensitivity (100%) for the identification of absence of phototoxic potential [9, 10]. However, if a positive result is obtained, a follow-up test should be performed to obtain data with skin 3D models that contain stratum corneum as a barrier system, taking into account the bioavailability of the compound or extract. Nonetheless, positive results in the 3T3 NRU PT often do not translate to a clinical risk of phototoxicity because they do not take into consideration the cutaneous penetration of a compound [9, 10, 19]. Thus, additional studies must be performed to confirm this prediction.

HET-CAM assay is used worldwide to predict the toxicity of chemicals and is also used to test cosmetic formulations.

Usually, for safety prediction, all chemicals must be labeled with their hazard classes and categories to write the material safety data sheet (MSDS). In the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) chemicals are

classified according to Draize eye test as corrosives, severe irritants, mildly and non irritants. For this classification, the recommended protocol is to apply the chemical undiluted and diluted in the CAM. It is a very sensitive assay for the determination of skin and eye irritation potential, since it has a low number of false negatives.

In the present study, we also included them in a cosmetic formulation in order to test them under actual conditions of use instead of only have their GHS classification.

For the acceptability *in vivo* tests, according to obtained results, the gel-cream formulation containing the studied extracts showed no irritant potential in the HET-CAM assay. In addition, all studied extracts and combinations did not show any cytotoxic potential in *in vitro* cell culture assay (3T3 NRU -UV EC_{50}). These results predicting no harmful effects were confirmed in the *in vivo* acceptability studies, which showed no alteration on skin barrier function and no visual irritation. The combination of *Palmaria palmata,* alfalfa and chicory extracts was not considered phototoxic.

Finally, the integrated approach employing *in vitro* high sensitivity assays, i.e. HET-CAM and 3T3 NRU PT, allowed the evaluation of ingredients alone, in combination, diluted, undiluted and when added in a cosmetic formulation. This strategy also allowed the decision to move forward to *in vivo* acceptability studies, which confirmed the absence of toxicological risks. This way, the ingredients under study can be considered promising extracts for the development of innovative and safe cosmetic formulation for skin care.

5. Conclusion

The studied active ingredients, alone or in combination, present no cytotoxicity potential and when added to a gel-cream formulation had no irritant potential *in vitro.* These results predicting no harmful effects for gel cream formulations were confirmed in the *in vivo* acceptability tests, which showed no alteration on skin barrier function and no report of irritation perception or sign of erythema, suggesting the potential of these extracts for the development of safe cosmetic products.

6. References

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The authors report no conflicts of interest.

Figure 1: HET-CAM (Hen's Egg Chorioallantoic Membrane Test) analysis, showing the results of CAM (Chorioallantoic Membrane) exposure to the extracts under study. (↑ indicates hemorrhage).

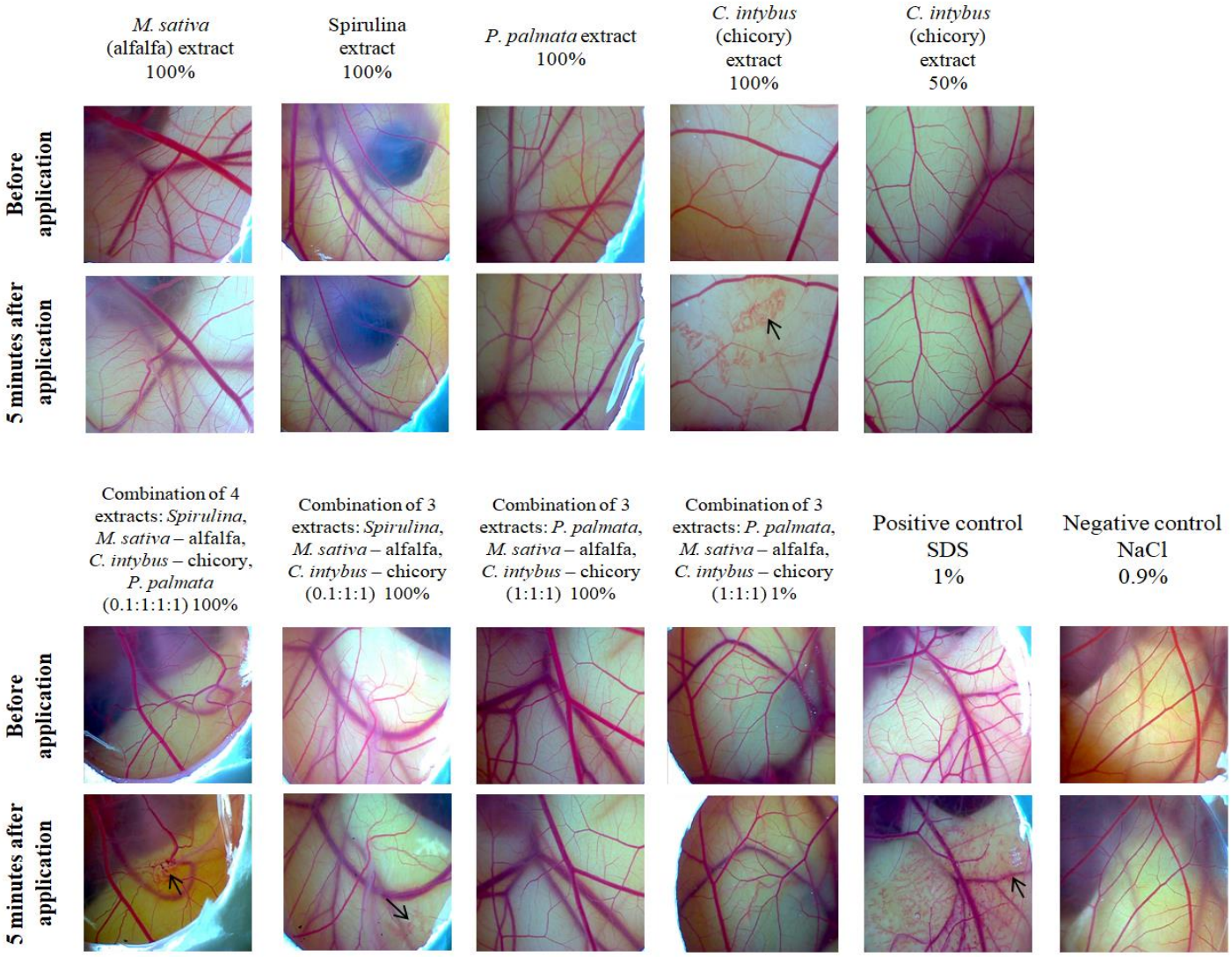

Figure 2: HET-CAM (Hen's Egg Chorioallantoic Membrane Test) analysis, showing the results of the exposure of the CAM (Chorioallantoic Membrane) to the formulations under study.

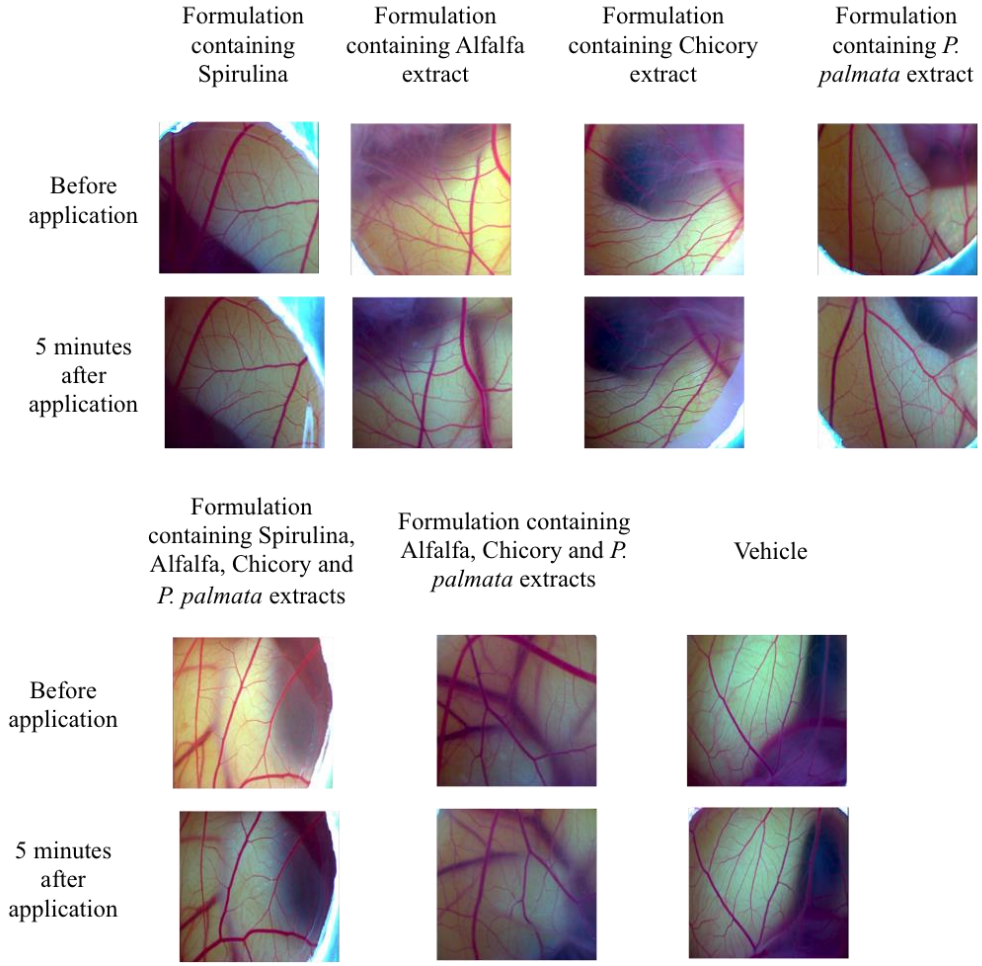

Figure 3. Dose-response curves for the extracts under study obtained with the Phototox 2.0 software. The blue dots with solid line and the yellow dots with dotted lines respectively refer to non-irradiated (-UV) and irradiated (+UV) substances.

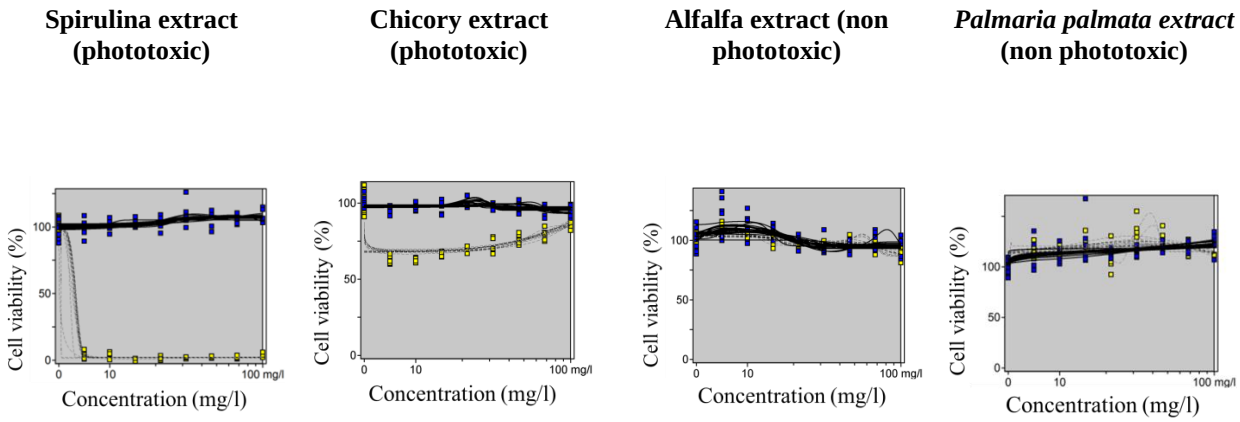

Figure 4: Transepidermal water loss (TEWL) on the forearm region 30 days after application of the formulation containing the combined active ingredients of the four extracts under study and the vehicle region.

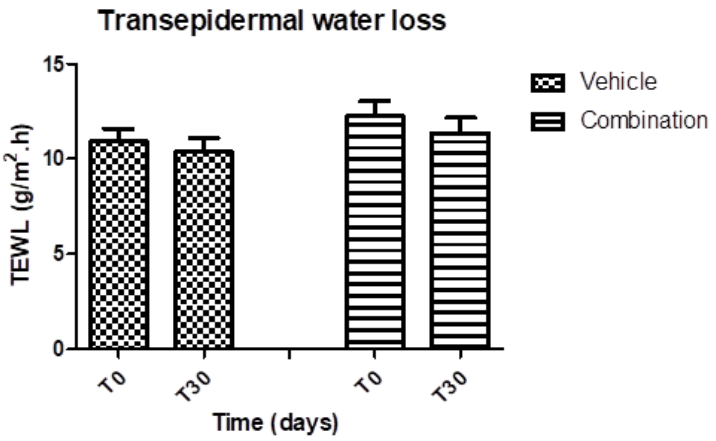

Table 1 – Ingredients of the formulations

Ingredients (I.N.C.I. name)	**Content (%, w/w)**	
	Vehicle	**Combination**
Cetearyl Alcohol and Dicetyl Phosphate and Ceteth-10 Phosphate	5	5
Acrylates/C10-30 Alkyl Acrylate Crosspolymer	0.20	0.20
Cyclomethicone (and) Dimethicone Crosspolymer	5	5
Cyclopentasiloxane	15	15
Phenoxyethanol and parabens	0.6	0.6
Ethyelenediamine tetraacetic acid	0.05	0.05
Butyl Hydroxy Toluene	0.01	0.01
Propyleneglycol	4	4
Glycerin	3	3
C12-15 Alkyl Benzoate	5	5
Cichorium intybus (Chicory) root extract	-	1
Spirulina dry extract	-	0.1
Medicago sativa (Alfalfa) extract	-	1
Palmaria palmata extract	-	1
Distilled water	62.14	59.04

Table 2. Irritant potential of extracts and their combinations in the HET-CAM (Hen's Egg Chorioallantoic Membrane Test) assay.

Extracts/combinations studied	**Mean score ± SD (n=4 eggs)**	**Classification**
M. sativa (alfalfa) extract *100%*	0.0 ± 0.0 ^{B}	non-irritant
C. intybus (chicory) extract *100%*	11.5 ± 1.0 ^{A}	severe irritant
C. intybus (chicory) extract *50%*	0.0 ± 0.0 ^{B}	non-irritant
P. palmata extract *100%*	0.75 ± 1.5 ^{B}	non-irritant
Spirulina extract *100%*	0.0 ± 0.0 ^{B}	non-irritant
Combination of 4 extracts: Spirulina, *M. sativa* - alfalfa, *C. intybus* - chicory, *P. palmata)* (0.1:1:1:1) *100%*	10.25 ± 3.5 ^{A}	severe irritant
Combination of 3 extracts (Spirulina, *M. sativa* -alfalfa, *C. intybus* - chicory) (0.1:1:1) *100%*	3.0 ± 6.0 ^{B}	slight irritant
Combination of 3 extracts (*P. palmata*, *M. sativa* -alfalfa, *C. intybus* - chicory) (1:1:1) *100%*	0.0. ± 0.0 ^{B}	non-irritant
Combination of 3 extracts (*P. palmata*, *M. sativa* -alfalfa, *C. intybus* - chicory*)* (1:1:1) *1%*	0.75 ± 1.5 ^{B}	non-irritant
NaCl 0.9%	0.75 ± 1.5 ^{B}	non-irritant
SDS 1%	12.0 ± 0.0 ^{A}	severe irritant

NaCl: sodium chloride, SDS: sodium dodecyl sulphate.

Different symbols indicate statistically different values ($p < 0.05$): $A \neq B$

Table 3. Irritant potential of extracts added to the gel-cream formulation in the HET-CAM (Hen's Egg Chorioallantoic Membrane Test) assay.

Formulations studied	**Mean score ± SD (n=4 eggs)**	**Classification**
Vehicle formulation (undiluted)	1.25 ± 2.5 ^{A}	slight irritant
Formulation containing *1% M. sativa* (alfalfa) (undiluted)	0.0 ± 0.0 ^{A}	non-irritant
Formulation containing *1% C. intybus* (chicory) (undiluted)	0.0 ± 0.0 ^{A}	non-irritant
Formulation containing *0.1%* Spirulina (undiluted)	0.0 ± 0.0 ^{A}	non-irritant
Formulation containing *0.1% P. palmata* (undiluted)	0.0 ± 0.0 ^{A}	non-irritant
Formulation containing 3 extracts (*P. palmata*, *M. sativa* - alfalfa, *C. intybus* - chicory) (1% each) (undiluted)	0.0 ± 0.0 ^{A}	non-irritant
Formulation containing 4 extracts (Spirulina, *P. palmata, M. sativa* - alfalfa, *C. intybus* - chicory*)* (1% each, except spirulina: 0.1%) (undiluted)	0.0 ± 0.0 ^{A}	non-irritant

Different symbols indicate statistically different values ($p < 0.05$): $A \neq B$, thus all values presented on Table 2 were considered statistically equivalent

Table 4. Phototoxicity of the extracts under study used separately or in combination (n=2).

Extract	**MPE**	**EC50 (-UV) µg/mL**	**EC50 (+UV) µg/mL**	**Probability**
Spirulina	**0.941**	ND	3.515	**Phototoxic**
	0.917	ND	2.712	
M. sativa	0.016	ND	ND	Non-phototoxic
(alfalfa)	-0.067	ND	ND	
C. intybus	**0.217**	ND	ND	**Phototoxic**
(chicory)	**0.251**	ND	ND	
P. palmata	-0.024	ND	ND	Non-phototoxic
	-0.137	ND	ND	
Combination of 4 extracts (Spirulina, *M. sativa* - alfalfa, *C. intybus* - chicory, *P. palmata)* (0.1:1:1:1) (0.001%: 0.01%: 0.01%:0.01%)	**0.252**	ND	65.46	**Phototoxic**
	0.460	ND	30.547	
Combination of 3 extracts (Spirulina, *M. Sativa* - alfalfa, *C. intybus* - chicory (0.1:1:1) (0.001%: 0.01%: 0.01%)	**0.357**	ND	54.362	**Phototoxic**
	0.365	ND	38.656	
Combination of 3 extracts *(P. palmata, M. Sativa* - alfalfa, *C. intybus* - chicory	0.107	ND	ND	Non-phototoxic
	-0.079	ND	ND	
(1:1:1) (0.01%: 0.01%:0.01%)				
L-histidine	-0.064	ND	ND	Non-phototoxic
(negative control)				
Norfloxacin	**0.593**	ND	23.338	**Phototoxic**
(positive control)	**0.447**	ND	28.710	

ND: not determined; half maximal effective concentration (EC50); ultraviolet (UV); mean photo effect (MPE).

Table 5: Visual analysis of irritation

Experimental Time	**Number of Reactive Participants**	**Types of Reaction**	**Measurement of Daily Irritation**	**Percentage of Reactive Participants**
D3	0	None	0	0%
D5	0	None	0	0%
Maximum Measurement of Average Irritation			0	

Table 6 - Comparisons between TEWL values at different study times

Unpaired t-test	**Difference**	**P value**
$T0_{vehicle}$ - $T30_{vehicle}$	n.s.	0.6105
$T0_{combination}$ - $T30_{combination}$	n.s.	0.44
$T0_{vehicle}$ - $T0_{combination}$	n.s.	0.2198
$T30_{vehicle}$ - $T30_{combination}$	n.s.	0.3869

3.5. Capítulo 5 – Efeitos clínicos imediatos

3.5.1. Artigo 5: CALIXTO, L. S.; PICARD, C.; SAVARY, G.; MAIA CAMPOS, P. M. B. G. (in press) Skin characterization and immediate effects of different dermocosmetic treatments in French and Brazilian skin. Journal of Cosmetic Dermatology, 2019.

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Skin characterization and immediate effects of different dermocosmetic treatments in French and Brazilian skin

Abstract

Background All over the world, people face the same skin problems. However, their skin characteristics are different. Thus, it is a challenge to prescribe treatments that will be effective on different skin types. Therefore, it is very important to consider the skin biology when indicating a dermocosmetic treatment.

Objectives To assess skin biophysical parameters in French and Brazilian subjects and to verify the efficacy of four dermocosmetic treatments in these populations.

Methods Five test areas were defined on each volar forearm of the eighteen Brazilian and eighteen French participants using a randomized design. Biophysical measurements in terms of skin hydration, skin barrier function, skin brightness and skin viscoelasticity were performed before and after 60 minutes of treatment.

Results Skin biophysical differences between populations were found. French skin has been shown to be more hydrated regarding epidermal mechanic properties and stratum corneum water content and more radiant when compared with Brazilian skin. However, it showed more signs of cutaneous aging and fatigue effects on skin. The Brazilian skin showed better skin barrier function. In addition, the treatments were effective in both populations.

Conclusions Despite the differences found in French and Brazilian skin, the proposed dermocosmetic treatments showed effective in both populations.

Keywords: dermocosmetic formulation; noninvasive techniques; biophysical and skin-imaging techniques; clinical efficacy.

Introduction

Choosing the right treatment for each type of skin is a challenge faced every day by prescribers and formulators. It is necessary that the treatments be efficient in the diverse types of skin.

Many differences can be found in the skin of people from different continents[1–3]. Physical distance brings changes in environmental and socioeconomic factors, climate and behaviour of populations, which is reflected in their skin. But not going that far, studies show that it is possible to find differences in the skin of people from the same continent[4–7] and even within the population of the same country[8,9].

Countries like Brazil and France are multi-ethnic, that is to say, they present in their population people with different progeny that have different skin[10,11]. That is why studies focusing on the skins of these populations are important to know the structural composition of their skin, as well as their response to different treatments.

Observations across countries are important because they bring extensive knowledge about each region and cross the information to obtain important conclusions for several countries. Differences in skin structure and function can be marked. Skin colours, stratum corneum pH gradient, lipid organization and composition of stratum corneum sheets are characteristics that can be studied, as well as prevalence of skin diseases[5,12,13].

In order to contribute to these observations, the aim of the current study was to assess baseline biophysical parameters of the skin in French and Brazilian subjects and to verify the efficacy of four treatments in these populations.

Finally, this study is an important contribution, since it has shown what is new in the differences between French and Brazilian skin.

Materials and methods

Study Participants

The study was conducted at two research centres: the French participants were evaluated in Le Havre, France (49°30′N 0°08′W) from May to August 2017, and the Brazilian participants were evaluated in Ribeirão Preto, São Paulo, Brazil (21°100S, 47°480W) from May to August 2018. This period was chosen to obtain the same average temperature (17.5ºC ± 2.5) between cities[14,15].

The study protocol conformed to the principles set forth by the Declaration of Helsinki and was approved by the Institutional Ethics Committee (CEP/FCFRP nº. 381). All participants provided written informed consent prior to participation. Thirty-six

participants (eighteen Brazilian and eighteen French) with the same proportion of age (24 years ± 2) were recruited for the study. Participants were excluded in the following situations: pregnancy or lactation; adverse reactions to cutaneous topical treatments; use of drugs that may produce an abnormal skin response; localized or generalized dermatological disorders. The analyses in both Brazil and France were performed in a room with controlled temperature (20-22ºC) and humidity (45-55%).

Study protocol

Four treatments were applied on the skin: a gel-cream vehicle formulation (V); vehicle formulation and anti-aging and whitening agents – Spirulina dry extract, *Cichorium intybus* (Chicory) root extract, *Medicago sativa* (Alfalfa) Extract and *Palmaria palmata* extract (CA), vehicle formulation and UV-filters (CF) and the combination of anti-aging and whitening agents and UV-filters (CM). Formulation details and sensory acceptance have been published previously[16,17]. Thereby, the objective was to verify the response of different populations to treatments with and without sunscreens and active substances.

The measurements were realized before application (T0, basal values) and 60 minutes after application of 50µL of formulation using a randomized design. Five study areas (4 x 5cm) were defined: one control area (C) and four treatments areas (V, CA, CF, CM). Skin on the volar forearm was evaluated using non-invasive biophysical techniques. Subjects performed acclimatization for 20 minutes before measures for control of physical and emotional factors18.

Assessments

A protocol was developed to evaluate the effects of the treatments and to evaluate the characteristics of the different nationalities groups. The parameters skin hydration, skin barrier function, skin brightness and skin elasticity were evaluated using non-invasive biophysical techniques. The evaluations were performed and analysed by the same operator in both countries and the researchers were sure to utilize equipment of the same models and under the same experimental conditions to ensure the condition of comparing the results obtained in France with those obtained in Brazil[3,19].

• *Stratum corneum water content*

The stratum corneum water content was determined using the non-invasive skin capacitance equipment Corneometer® CM 825 (Courage-Khazaka, Germany). The

device measures the change in the dielectric constant due to skin surface hydration and is expressed in arbitrary units (UA's) where 1 AU corresponds to 0.2 to 0.9 mg of water/gram in stratum corneum[18]. Three measurements were randomly made for each region. Changes in skin hydration were evaluated by this analysis.

• *Transepidermal water loss (TEWL)*

In this test, the equipment Tewameter® TM 300 (Courage-Khazaka, Germany), whose function is to measure water evaporation from the skin surface[20], was utilized. The test is based on the diffusion principle described by Adolf Fick in 1885. Three measurements were performed for 20 seconds for each region randomly. The transepidermal water loss is a property related to skin barrier function.

• *Skin viscoelasticity*

To evaluate the mechanical properties of the skin, a Cutometer® SEM (Courage-Khazaka, Germany) was used. This analysis provides information on several skin's properties as viscoelasticity and firmness, as reported in the literature[3,19,21]. In this study, the following parameters were evaluated: maximum amplitude (R0), tiring effects (R3), skin viscoelasticity (R6), skin return to its original state (R8) and tiring effects – fatigue (R9).

• *Skin brightness*

The specular reflecting light from the skin was evaluated by the Glossymeter® GL 200 (Courage-Khazaka, Germany). The device evaluates the brightness of the skin surface by measurements of the reflected light giving information as the radiance of the skin[22]. Three measurements were performed for 10 seconds for each region randomly.

Data Analysis

Statistical analyses were performed using Student's t-test to determine significant differences in skin properties for each region in a first moment between the basal of the different populations, and after, between the results of the four treatments. All analyses were performed using the Minitab® 17 software (Minitab Inc., State College, PA).

Results

Groups Characterization

Before verifying the efficacy of the treatments in the different populations, the groups were characterized and posteriorly, the results obtained for the Brazilian skin were compared with the results obtained for the French skin.

Results for skin viscoelasticity were significantly different between the populations. Based on the literature[19,21,23], five parameters were analysed:

R0 - the final distension of the first curve (Uf);

R3 - the maximum amplitude of the last curve;

R6 - portion of viscoelasticity/ elastic part of the curve (Uv/Ue);

R8 - the final retraction Ua of the first curve;

R9 - the residual deformation at the end of the measuring cycle (R3–R0).

The French group presented higher values in all parameters of this analysis (Table 1). Regarding the gloss on the skin surface, the French group presented higher values than the Brazilian group. The same behaviour was obtained in the analyses of transepidermal water loss, which the values of the Brazilian group were significantly lower than the values of the French group. Both populations showed the same profile in stratum corneum water content.

Table 1 - Parameters of the volar forearms the French and Brazilian participants.

	Parameters	France	Brazil	P-value
R parameters	R0	**0.26 ± 0.04**	0.20 ± 0.07	< 0.0001
	R3	**0.29 ± 0.05**	0.19 ± 0.08	< 0.0001
	R6	**0.36 ± 0.07**	0.32 ± 0.09	< 0.01
	R8	**0.22 ± 0.04**	0.16 ± 0.04	< 0.0001
	R9	**0.02 ± 0.0**	0.01 ± 0.0	< 0.0001
Gloss on the skin surface		**5.5 ± 0.9**	4.8 ± 0.6	< 0.0001
Transepidermal water loss		**8.2 ± 2.4**	7.5 ± 1.6	< 0.0001
Stratum corneum water content		38.4 ± 6.5	37.6 ± 6.5	ns

Data are expressed as mean ± SD. Significant differences between populations are indicated in boldface type ($p < 0.05$) and ns = not significant.

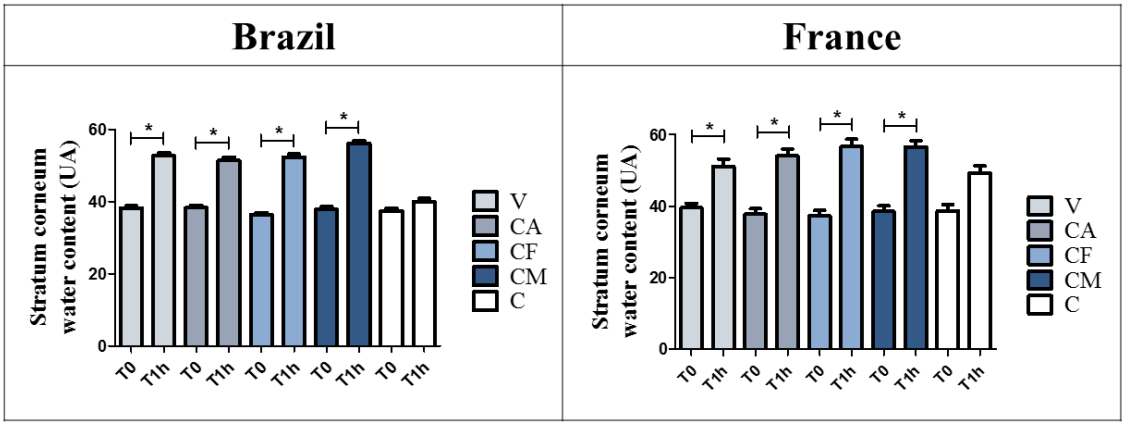
Immediate effects

The treatments proved to be effective after one hour of application in both populations and brought the same benefits to French and Brazilian skin. No alterations in viscoelasticity properties were observed under the study conditions.

All treatments were able to significantly increase stratum corneum water content values after 1h of application (Figure 1), meaning increased skin surface hydration. No differences were observed in the control region after the study time.

Figure 1 - Values of stratum corneum water content before (T0) and after 1h of application (T1h) of the treatments. Results of the Brazilian population on the left and the French population on the right.

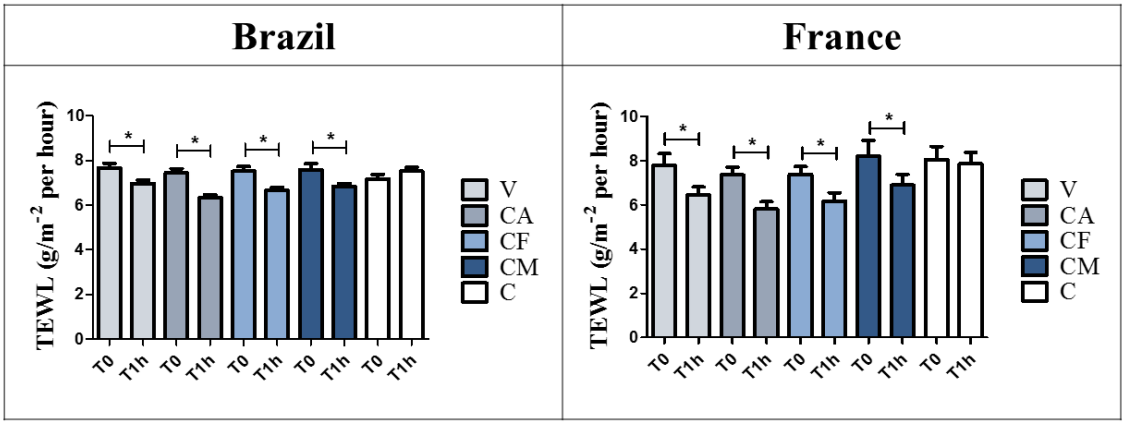
* Statistically significant differences compared to T0 ($p < 0.05$).



Transepidermal water loss (TEWL) is a measure that indicates the functionality of the skin barrier. The lower it is, the greater the balance of its functionality. All treatments reduced TEWL values after 1h (Figure 2). No differences were observed in the control region after the study time.

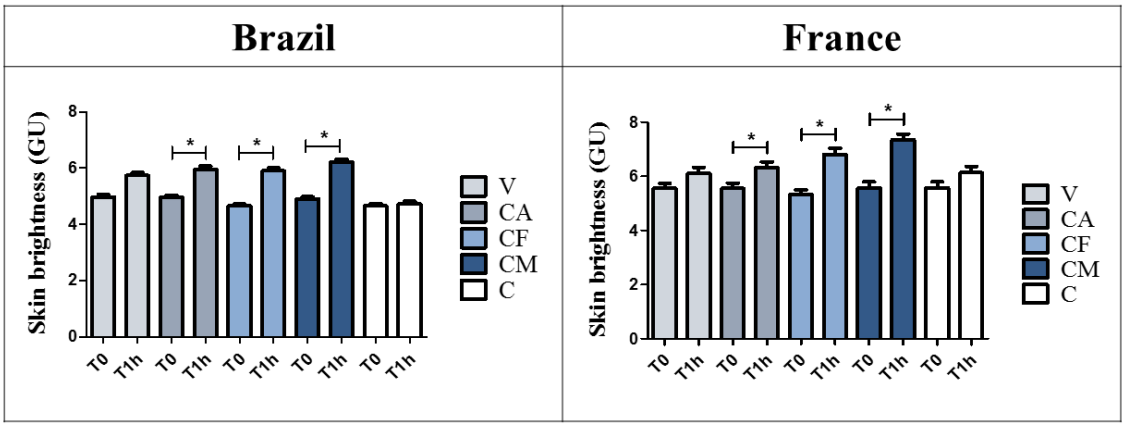
Figure 2 - Transepidermal water loss (TEWL) before (T0) and after 1h of application (T1h) of the treatments. Results of the Brazilian population on the left and the French population on the right.

* Statistically significant differences compared to T0 ($p < 0.05$).



Regarding the skin brightness values, the vehicle formulation was not able to bring benefits to this property after 1h of application. The CA, CF and CM treatments significantly increased skin brightness under the same conditions (Figure 3). No differences were observed in the control region after the study time.

Figure 3 - Values of skin brightness before (T0) and after 1h of application (T1h) of the treatments. Results of the Brazilian population on the left and the French population on the right. * Statistically significant differences compared to T0 ($p < 0.05$).



Discussion

Characterization of populations

Comparative studies among the skins of people from different countries ("across countries" studies) show that many differences can be found in terms of structural composition of the skin, sensations and treatment efficacy[1,5,6,24]. A study comparing Japanese women's skin with Caucasians skin demonstrated differences in terms of barrier function in the stratum corneum, sensory perception and penetration speed[2].

Another across countries studies showed the difference in the prevalence of skin diseases in different countries based on dermatological conditions of different populations[13,25].

Brazil and France are countries with miscegenation, that is, their populations derive from different ethnicities. Previous studies have shown that ethnic specificities can bring structural differences in the skin, but factors such as geographic position, climatic conditions and sun exposure habits have a greater influence than inter-ethnic differences in skin structures[3,26–28].

Therefore, before evaluating the efficacy of the treatments in the skins of the two countries groups, their skin was evaluated to be compared in terms of skin elasticity, skin barrier function, skin brightness and skin hydration using non-invasive biophysical techniques.

From the skin elasticity analysis, we obtained the "R parameters", which give us information about the deformation-relaxation properties of the skin[23]. The French population obtained R0, R3, R6, R8 and R9 values significantly higher than the Brazilian population.

R0 represents the final distension of the first curve and is related to the distensibility of the skin. The R parameters are highly dependent on it[19]. The higher values of R6 indicate a decrease in the viscosity of the interstitial fluid as a consequence of an increase in water content in the dermis which is related to hydration. Consequently, this results in a skin with more cohesive structure and a reduced viscous component.

R3 represents the maximum amplitude of the last cycle and R9 the residual deformation at the end of the measuring cycle[23]. They both represent the effects of skin fatigue. Therefore, French skin showed more signs of fatigue, which is related to skin aging. Parameter R8 is sensitive to epidermal hydration, is highly related to elasticity and decreases with age[19,21]. The differences observed in the R parameters reflect structural differences in the skins of the countries as epidermal hydration and keratinocyte functionality. French skin has been shown to be more hydrated regarding epidermal mechanics and stratum corneum water content; however, it shows more signs of cutaneous aging and fatigue effects on skin.

Regarding the values of gloss on the skin surface, the French population presented significantly higher values than the Brazilian population. This measurement is directly associated the amount of incident light that is reflected from the skin surface and its radiance, which is a complex psychophysical parameter dependent on several factors[22].

Among the factors that influence the skin brightness is colour homogeneity of skin: brighter skin means a more homogeneous skin colour. Considering that Brazil is a country where the sun's incidence is much stronger than France, it is expected that the Brazilian's skin colour is less homogeneous than French skin and therefore it presents a lower brightness[14,15,22,29].

The values of transepidermal water loss bring important information about integrity of the epidermal permeability barrier and its functionality, which may indicate problems as cutaneous sensitivity and skin dryness[20,30,31]. The French population presented significantly higher values of TEWL compared to the Brazilian population. Skin sensitivity is a common concern among the French population[9]. Their loss of water that passes through the epidermis is greater than that of the Brazilian population which can lead to more damaged and sensitive skin because of damage to enzymatic functions and desquamation that result in visible appearance of dry and flaky skin[30,32]. Finally, this result is not associated with skin aging but rather with skin functionality[12].

No differences were found in stratum corneum water content between the two populations. The TEWL analysis represents the diffusion of condensed water through the stratum corneum, while the stratum corneum water content analysis reflects the skin hydration[18,20]. Thus, the hydration level of the two groups was considered equal. Although the results of elasticity have suggested greater hydration in French skin, the results are not compatible because they are techniques with different objectives[19].

Efficacy of treatments

In this study, four treatments were tested to evaluate their efficacy in two different populations: the French and the Brazilian. The correct choice of treatment is very important because it guarantees the success and adherence of the prescription by the patients. In regards to anti-aging treatments it is important to develop formulations with pleasant sensory and proven efficacy[16]. All treatments had the same efficacy results in Brazil and France showing that they have action under different conditions as environment and skin type. No differences were observed in the control region after the study time. Measures of viscoelasticity of the skin before and after application of anti-aging formulas can provide information on the efficacy of the treatment[19]. After one hour of application of the four treatments, no differences were observed in this property in the regions with and without treatment. Because it is a property highly related to skin

aging, it is more difficult to obtain fast results, since the ideal is a continuous treatment[33].

After 1h of application, all treatments were able to significantly increase stratum corneum water content values and reduce TEWL values. These results complement each other and are very important to consider a treatment as effective[34]. The stratum corneum is the outer layer essential skin barrier responsible for regulating processes such as permeation of chemical agents, loss of body fluids, penetration of active substances and, most important, loss of water[18,31,32].

The stratum corneum activity is basically dependent on the function of its two components: the corneocytes and the intercellular lipid bilayer matrix. When the barrier works properly the integrity of the skin is preserved ensuring hydration and preserved skin surface[32,33]. This could be observed one hour after treatment: on the one hand, the increase in water present in the stratum corneum provides us with information about the increased hydration of the skin. On the other hand, the decrease in TEWL ensures that the skin barrier has been conserved and protected[20].

The formulations containing anti-aging and whitening agents and UV-filters separated and in combination (CA, CF and CM treatments) significantly increased skin brightness on both populations under the study conditions. According to previous studies, an increase in this property means greater radiance of the skin, greater homogeneity of the skin colour and its surface[22,35]. Thus, it suggests that the presence of the whitening agents and UV-filters was responsible for this improvement. This information corroborates with the results of decreased TEWL and increased skin hydration, indicating the film-forming effect on the skin. This effect is present on the surface of the skin, where it acts to improve its skin microrelief[18,36,37]. The potential of film-forming effect can maintain the skin barrier function, hydration and integrity and is able to increase the skin radiance[34].

When light is projected on an uneven surface like skin, it deflects at various angles which makes the reflection process diffuse and impairs the skin brightness[21]. By applying a film-forming agent, it will homogenize the surface of the skin and in this way the incident light returns more uniformly which increases the reflected light of the skin and its brightness. Thus, the vehicle formulation, by not having film-forming ingredients, was not able to increase the brightness in the period of one hour.

The active substances can act on the skin through different mechanisms. Spirulina dry extract has high protein concentration, is rich in polysaccharides and pigments, among them the B-complex vitamins and the β-carotene (provitamin A)[38–40]. It can improve the skin barrier function due to its high content of amino acids and polysaccharides that regenerate the skin barrier and reduce TEWL[33,41].

The *Cichorium intybus* (Chicory) root extract is rich in oligofructose and has "Vitamin D-like" activity, since it acts on the increase of the synthesis of vitamin D receptor (VDR), stimulates the expression of genes involved in the cornification processes and desquamation (KLF4, Cytokeratin 1, Involucrin, Cystatin E / M, KLK), and has *in vitro* activity in increasing epidermal thickness and filaggrin synthesis[42–45].

Medicago sativa (Alfalfa) Extract was proposed by its "Retinol-like" effect, presenting potential to stimulate cellular activity that decreases during the aging process, favoring epidermal renewal and regulating the differentiation of keratinocytes. In addition, it has antioxidant action, being able to protect and repair the dermis by stimulating the synthesis of Collagen I and reducing the activity of the metalloproteinases responsible for the destruction of elastin fibers. As a result, the skin is revitalized and the skin barrier function is restored[46–48].

In addition to the benefits described, the whitening action is of extreme importance in order to obtain a multifunctional cosmetic that meets the proposed goals. *In vitro* studies demonstrate the effects of the *Palmaria palmata* extract on the inhibition of tyrosinase enzyme, and the protein complex responsible for the transfer of melanosomes and the inhibition of Stem Cell Factor after induction by ultraviolet radiation[49]. Therefore, the fraction of oligosaccharides acts in different stages involved in the process of skin pigmentation: melanogenesis, transport of melanosomes and pigmentation induced by ultraviolet radiation[50].

In conclusion, important differences in the skin biophysical properties of the French and Brazilian subjects were found. French skin has been shown to be more hydrated and radiant, however, it shows more signs of cutaneous aging and fatigue effects on skin. In addition, Brazilian skin has shown to have a better skin barrier function. Despite these differences, the proposed dermocosmetic treatments were effective in both populations.

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3.6. Capítulo 6 – Efeitos clínicos em longo prazo

3.6.1. Artigo 6: CALIXTO, L. S.; CAMPOS, P. M. B. G. M.; Clinical efficacy of dermocosmetic formulations in the improvement of skin photoaged conditions. Manuscript submitted for publication in Journal of Cosmetic Dermatology.

“Clinical efficacy of dermocosmetic formulations in the improvement of skin photoaged conditions”

ABSTRACT

Skin aging conditions are diverse and present in different structures of the skin. Science looks for active substances from natural sources to improve these conditions, due to their rich composition and potential to apply in dermocosmetic formulations. Among the natural ingredients, the *Palmaria palmata* extract has a high antioxidant potential and its clinical benefits in the improvement of the skin conditions has not yet been studied. This study evaluated the clinical efficacy of dermocosmetic formulations containing active substances obtained of natural sources, Spirulina, *Cichorium intybus* (Chicory) root extract, *Medicago sativa* (Alfalfa) and *Palmaria palmata* extracts in the improvement of skin aging conditions with a complete, non-invasive and multi-instrumental protocol based on novel biophysical and skin imaging techniques as Reflectance Confocal Microscopy - RCM. After the 90-day treatment period study with formulations containing active substances under study, a significant decrease in the skin hyperpigmentation and an increase in the skin brightness and its homogeneity were verified. In addition, these formulations significantly improved the flattening of dermal papillae. Finally, the obtained data showed that the natural origin active substances studied were effective in the skin photoaging alterations control, especially the formulation added with the *Palmaria palmata* extract.

Keywords: Clinical efficacy, Aging skin, Biophysical and Skin Imaging Techniques, Reflectance Confocal Microscopy, *Palmaria palmata* extract

1. INTRODUCTION

There are several natural origin active substances that are studied because of their rich composition and potential application as health enhancing ingredients in food, drug and cosmetic fields. The interest of its use is mainly due to its beneficial effects on the skin as moisturizing and protective effects and improvement in skin appearance [1–3].

When we think of actives of cosmetic interest, we aim substances that are capable of improving skin conditions without disturbing its organization. There is important concern in improving or delaying the signs of cutaneous aging. It is known that they are consequence of oxidative stress, which can be generated by exposition to higher degrees of UVR by sunlight and other environment factors. The oxidative stress can cause changes to skin cells and the keratinization process that are age-associated [4,5].

The changes that come with aging can affect cell cohesion and skin barrier integrity that reflect on changes in skin hydration, sebum content, microrelief parameters, and dermis thickness. Therefore, active substances must act on these properties in a safely and effectively way. Thus, it is necessary to look for cosmetic actives that are stable, safe, with potential effectiveness and that bring multiple benefits to skin aging without causing loss in the sensorial perception of the formulations [6–8].

Studies on human skin have shown that Spirulina dry extract has moisturizing action and that *Cichorium intybus* (Chicory) root extract has protective and restructuring effects [9,10]. Thus, to combine them with other two active substances obtained from natural sources *Medicago sativa* (Alfalfa) and *Palmaria palmata* extracts, which have shown potential to be used in improvement of skin aging conditions [1,11] can result in a multifunctional and more effective dermocosmetic formulation for skin aging treatment.

Maia Campos et al [12] demonstrated the *in vitro* toxicity results and *in vivo* acceptability of these four extracts in combination and suggested their potential for the development of safe cosmetic products. In this context, the synergistic effect of these extracts and the effect of the *Palmaria palmata* extract alone were studied, since no studies in the literature related to its effects on skin aging were found.

To evaluate the effects of cosmetic actives, clinical studies with instruments capable of evaluating performance on the skin at different levels should be carried out. Reflectance

Confocal Microscopy (RCM) is an advanced non-invasive *in vivo* skin imaging technique that can be applied in clinical practice and research. This equipment realizes confocal histology of skin allowing to obtain images of different levels within the skin from structures such as melanocytes, keratinocytes, pilosebaceous units, among others. It also allows the diagnosis of conditions such as solar lentigo, seborrhoeic keratosis, cutaneous hyperchromias and effects of cutaneous aging and sun exposure [4,13–17].

The Reflectance Confocal Microscopy, associated with other imaging and biophysical techniques stratum corneum water content, skin microrelief and viscoelastic characteristics [18,19]. In this context, the aim of this study was to evaluate the influence of active substances obtained of natural sources, Spirulina, *Cichorium intybus* (Chicory) root extract, *Medicago sativa* (Alfalfa) and *Palmaria palmata* extracts in the improvement of skin aging conditions with a complete, non-invasive and multi-instrumental protocol based on biophysical and skin imaging techniques.

Finally, this study is an important contribution because showed the clinical benefits of use of the dermocosmetc formulations with active substances obtained from natural sources evaluated by advanced non-invasive techniques.

2. METHODS

2.1. Studied formulations

All formulations of the study had the same vehicle and the ingredients and suppliers were: Acrylates/C10-30 alkyl acrylate crosspolymer was provided from Lubrizol do Brasil Aditivos Ltda (Sao Paulo, SP, Brazil); and Butyl hydroxy toluene, glycerin, phenoxyethanol and parabens, ethyelenediamine tetraacetic acid, aminomethyl propanol, propyleneglycol, were provided from Mapric Produtos Farmacêuticos e Cosméticos (Sao Paulo, SP, Brazil). Cyclopentasiloxane and Cyclomethicone (and) Dimethicone Crosspolymer were provided by Dow Corning do Brasil Ltd. (Hortolandia, SP, Brazil). C12-15 Alkyl Benzoate and Cetearyl Alcohol and Dicetyl Phosphate and Ceteth-10 Phosphate were provided by Croda do Brasil Ltda (Campinas, SP, Brazil). As we know that characteristics as rheological profile and texture properties are able to influence the performance of formulations [8], we have described previously their

development stages of the formulations, stability studies and characterization of their properties [7,20].

Four extracts to improve conditions associated with skin aging were chosen. Spirulina dry extract was provided by Ouro Fino Agronegócios (Ribeirão Preto, SP, Brazil). *Cichorium intybus* (Chicory) root extract, *Medicago sativa* (Alfalfa) Extract and *Palmaria palmata* extract were provided by Silab (Saint-Viance, France). The *Palmaria palmata* extract was chosen to compose a formulation isolated due its antioxidant activity as protective potential to the skin and the absence of efficacy studies proving their performance in cosmetics [1,2].

2.2. *Clinical* study

Subjects were randomly assigned to three topical formulations with skin compatible composition: the vehicle (Control formulation), the vehicle containing *Palmaria palmata* extract (FRA) in combination or not with the extracts of Spirulina, *Cichorium intybus* and *Medicago sativa* (FCO). A previous study carried out by our research group showed that all these formulations are stable and had their perception approved by a sensory panel [7].

2.2.1. Subjects

After the approval by the Ethics Committee of the Institution (CEP/FCFRP nº. 381), the recruitment of the subjects started based on the following inclusion criteria: women; with age between 40 and 60 years; spots; signs skin aging on the face; Fitzpatrick skin type II-III. Were excluded from the study subjects who presented any of the following exclusion criteria: pregnancy or lactation; history of adverse reactions to the use of cosmetic products; use of drugs that may produce an abnormal skin response; localized or generalized dermatological disorders.

After selection, 45 participants were enrolled in the study and randomly divided into three groups of 15 participants, whom received the three topical creams treatments: Control vehicle, FRA and FCO.

The subjects were instructed to apply the creams on the facial area every night. In the morning, to wash their face and to apply a standard sunscreen that was provided by the researchers. All subjects used the same sunscreen to avoid false results from sun

exposure. The long-term efficacy of the products was evaluated by biophysical and skin imaging techniques at the initial time (before the application of the products) and after 30, 60 and 90 days.

2.2.2. Instruments and parameters

A complete, non-invasive and multi-instrumental protocol was established to verify the general condition of the skin, its functionality, organization, structure and visual appearance. The *in vivo* study was conducted at the biophysical and image analysis laboratory at the University of São Paulo in Ribeirão Preto, Brazil (21°100S, 47°480W) with controlled temperature (20-22ºC) and humidity (45-55%). The study protocol conformed to the principles set forth by the Declaration of Helsinki. All measurements were performed after 20 minutes acclimation and were replicated at least three times by the same operator.

For the determination of the stratum corneum water content (SCWC), Corneometer® CM 825 (Courage & Khazaka, Cologne, Germany) equipment was used. This measure is related to skin hydration parameter [6]. Transepidermal water loss (TEWL) was determined by the Tewameter® TM 210 (Courage & Khazaka, Cologne, Germany) device. This analysis provides information about the barrier function of the skin [10].

Skin microrelief parameters (SELS - Surface Evaluation of Living Skin) were evaluated by analysis of the skin microrelief using a VisioScan® VC98 (Courage & Khazaka, Cologne, Germany) equipment [6,10,21].

In the analysis of the skin microrelief the following SELS parameters were evaluated:

SE_r – is the portion of dark spots representing the roughness;

SE_{sc} – skin desquamation;

SE_{sm} - skin smoothness;

SE_w –number and width of wrinkles.

To measure the thickness and echogenicity of the dermis, 20MHz ultrasound equipment (Dermascan® C, Cortex Technology, Hadsund, Denmark) was used (GIANETI; MAIA CAMPOS, 2014). Skin surface brightness was evaluated using Glossymeter® GL 200

(Courage & Khazaka) [22]. High-resolution full-face photographs for automatic analysis were taken with the equipment Visioface® 1000 D (Courage & Khazaka, Cologne, Germany). Data values were analyzed using the instrument software of each equipment.

Evaluation of the morphological and structural characteristics of the epidermis and papillary dermis was performed with Reflectance Confocal Microscopy using the VivaScope® 1500 microscope (Lucid, New York, U.S.A.) [13–16]. The microscopic images were performed using the Vivastack® imaging system (Lucid, New York, U.S.A.). Images were obtained in three skin sites starting at the stratum corneum surface and every 1.5 μm to the depth of 37.5 μm, every 3 μm to the depth of 112.5 μm and each 4.5 μm to the depth of 132.5 μm [4,14].

2.3. Statistical analysis

One-way ANOVA test was used with Tukey post-test when the data distribution was normal and the Kruskal–Wallis test with Dunn's post-test was applied in case of non-normal distribution. All analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA).

3. RESULTS

3.1. Biophysical techniques

The use of the formulations Control vehicle, FRA and FCO provided a significant increase in the water content of the stratum corneum on the face of volunteers after 30 days of application. The effects were maintained over the period of 60 and 90 days (Table 1). There was no change in transepidermal water loss values after long-term use of the formulations. Regarding skin brightness, the formulation FRA significantly increased this property after 90 days. The other formulations under study did not show significant differences at the end of treatment.

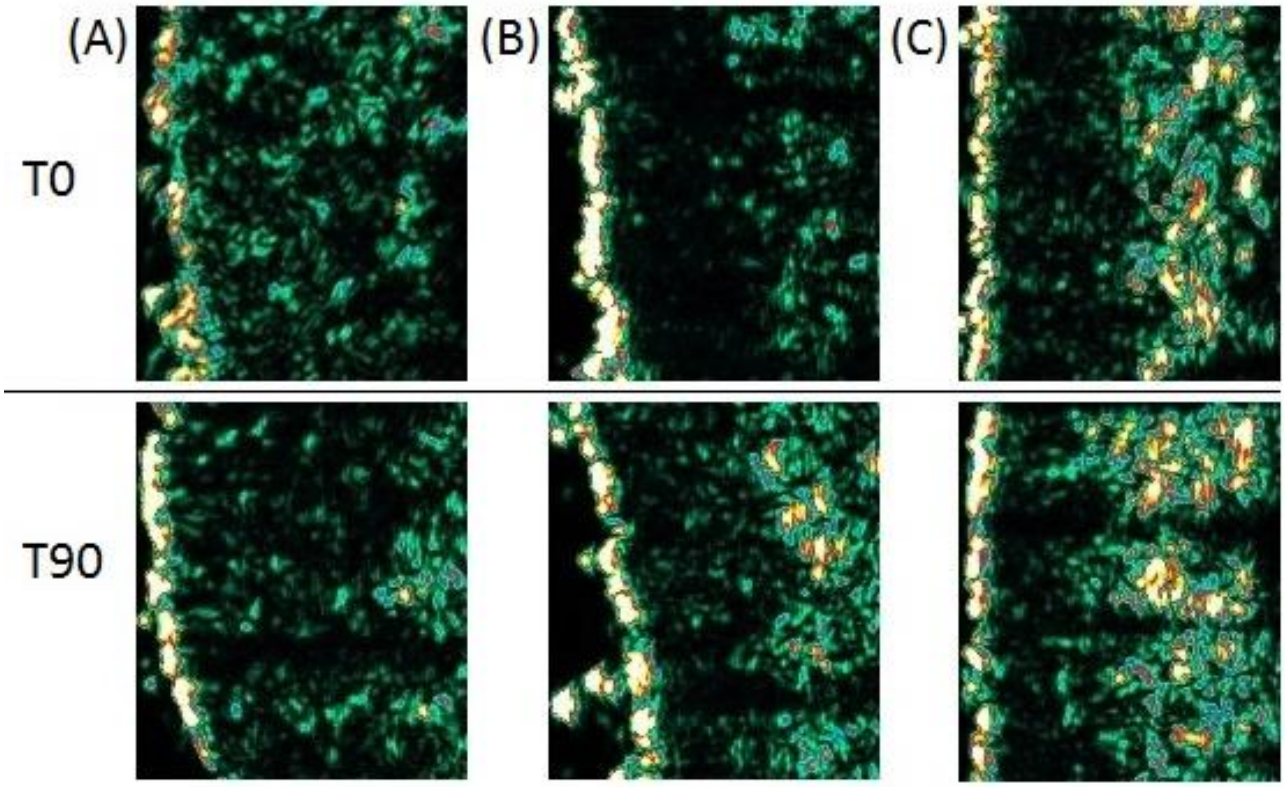
The treatment with the formulation containing the *Palmaria palmata* extract was the only treatment able to significantly decrease skin roughness and desquamation after 60 days of application on face (Table 1).

Table 1 - Skin parameters measured on face at initial time (T0, basal values) and after 30 (T30), 60 (T60) and 90 (T90) days of treatment with Control vehicle, FRA and FCO formulations. Results are expressed as mean ± standard deviation. Significant differences in relation to the initial time are highlighted in bold ($p<0.05$).

Formulation	Parameter	T0	T30	T60	T90
Control vehicle	SCWC	47.5 ± 11.4	**59.7 ± 9.0**	**62.2 ± 8.2**	**70.7 ± 6.3**
	TEWL	10.9 ± 2.4	10.4 ± 2.6	11.8 ± 1.8	12.0 ± 2.3
	Gloss	5.0 ± 1.0	4.5 ± 1.3	4.5 ± 0.8	4.8 ± 1.1
	Dermis thickness	1.9 ± 0.3	1.8 ± 0.3	2.0 ± 0.2	2.1 ± 0.3
	SEr	2.0 ± 1.3	1.3 ± 0.8	1.1 ± 0.5	1.1 ± 0.5
	SEsc	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
	SEsm	27.7 ± 8.7	24.7 ± 3.9	25.6 ± 4.5	26.0 ± 3.3
	SEw	53.8 ± 15.2	50.3 ± 12.8	47.6 ± 6.9	44.2 ± 5.6
FRA	SCWC	41.9 ± 10.9	**53.0 ± 5.7**	**54.4 ± 5.5**	**66.3 ± 10.3**
	TEWL	11.0 ± 3.5	11.1 ± 1.9	12.0 ± 2.1	12.8 ± 2.8
	Gloss	5.2 ± 1.1	4.8 ± 0.9	4.6 ± 0.6	**5.7 ± 1.3**
	Dermis thickness	2.0 ± 0.2	1.9 ± 0.2	2.1 ± 0.2	**2.2 ± 0.3**
	SEr	1.9 ± 0.9	1.7 ± 0.9	**1.2 ± 0.6**	1.5 ± 0.5
	SEsc	0.4 ± 0.2	0.3 ± 0.2	**0.2 ± 0.1**	0.3 ± 0.1
	SEsm	28.5 ± 5.0	24.9 ± 3.3	27.2 ± 2.3	26.9 ± 2.7
	SEw	58.9 ± 12.6	52.2 ± 13.3	47.2 ± 4.0	47.0 ± 6.1
FCO	SCWC	41.4 ± 7.1	**54.1 ± 7.4**	**58.8 ± 11.7**	**69.8 ± 6.7**
	TEWL	12.2 ± 2.9	11,4 ± 2.9	13,0 ± 2.8	12.0 ± 2.1
	Gloss	4.7 ± 0.8	4.8 ± 0.9	4.7 ± 0.7	5.2 ± 1.3
	Dermis thickness	2.0 ± 0.3	2.0 ± 0.2	2.2 ± 0.3	2.1 ± 0.3
	SEr	1.6 ± 1.0	1.3 ± 0.5	1.8 ± 0.7	1.6 ± 0.9
	SEsc	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
	SEsm	26.9 ± 6.2	27.8 ± 5.9	29.1 ± 4.5	26.3 ± 3.4
	SEw	58.1 ± 15.5	49.7 ± 7.6	46.8 ± 4.9	48.6 ± 5.4

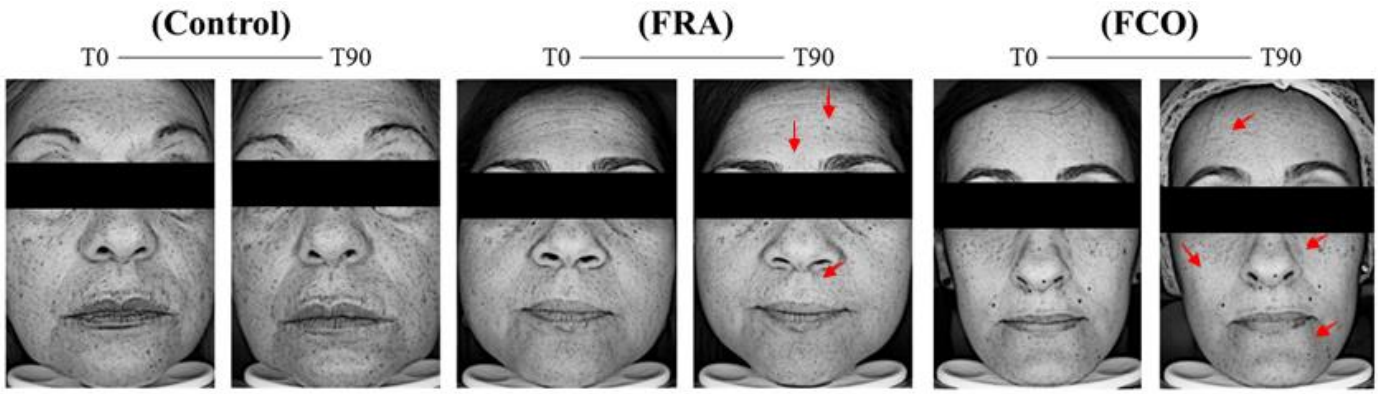
In the long-term study, there was no significant difference in the echogenicity ratio after 30, 60 and 90 days of treatment, but the use of the FRA formulation resulted in a significant increase in the dermis thickness after 90 days (Table 1). In addition, there was a qualitative variation in the number of echogenic pixels after treatment with formulations FRA and FCO. In the representative images (Fig 1) it was possible to observe an improvement in the dermis echogenicity after the 90-day treatment period.

Figure 1 - Representative 20-MHz ultrasound images of variation pattern of the dermis echogenicity. Measures on subjects face before (T0) and after the 90-day treatment period (T90) with the vehicle (A), FRA (B) and FCO (C) formulations. Increases in the echogenicity can be observed after 90 days in (B) and (C).
Skin echogenicity scale: white > red > yellow > green > blue > black.



From the high-resolution full-face photographs it was observed that the formulation FCO reduced skin spots, whitened the skin homogeneously and reduced the spots visible only with UV light (Fig. 2).

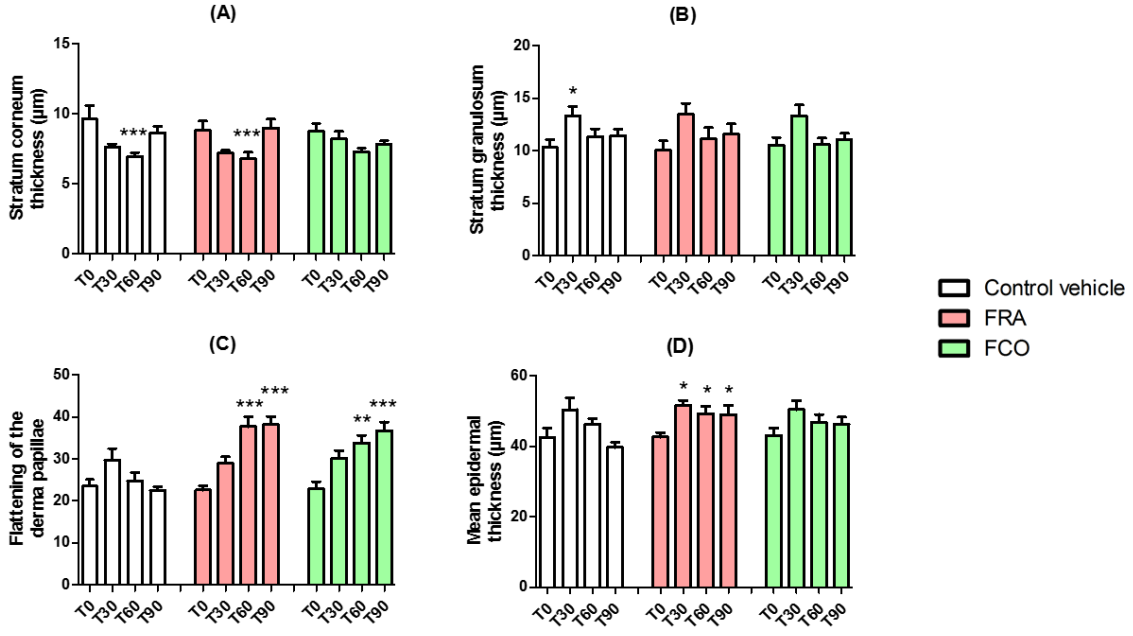
Figure 2 - Macroscopic characteristics of the skin of subjects under UV-like LEDs. The images on the left represent the skin at the initial time (T0) and the images on the right represent the skin after 90 days of treatment (T90). Decreases in pigmentation in spots and increase in the luminosity of the skin can be observed after 90 days use of formulations FRA and FCO compared with the control vehicle action. The arrows indicate the hyperpigmented areas reduction.



3.2 Reflectance Confocal Microscopy

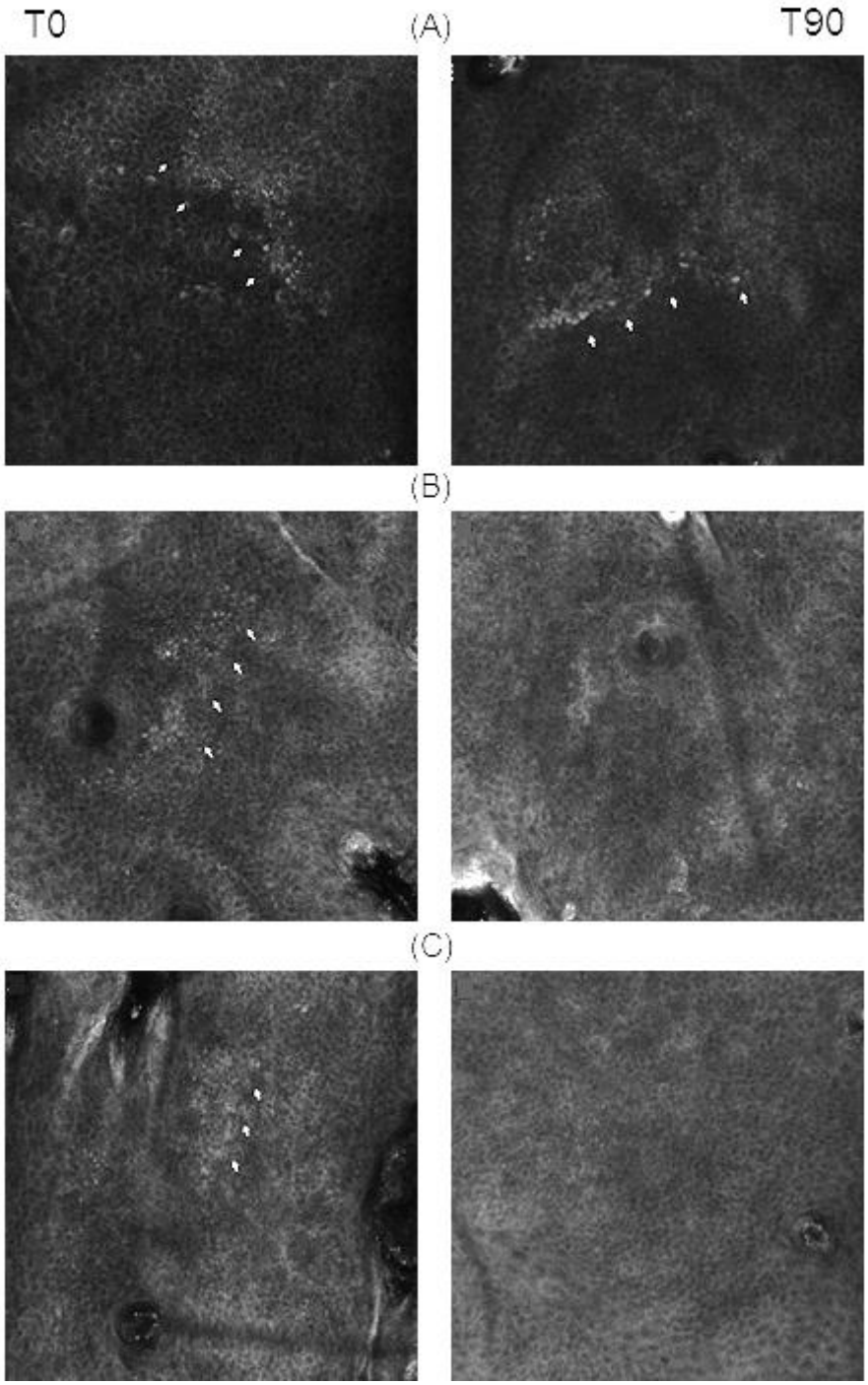
The evaluation of morphological and structural skin characteristics was performed using reflectance confocal microscopy. All formulations leaded to a decrease in the thickness of the stratum corneum after 60 days but only the application of control vehicle and FRA formulation that decrease was significantly different from basal values (Fig. 3A). Regarding stratum granulosum thickness, all formulations showed an increase after 30 days, but only with the control vehicle treatment that increase was significant. (Fig. 3B). After 60 and 90 days of treatment with FRA and FCO, significant differences in flattening of the dermal papillae were observed (Fig. 3C). According to the results of mean thickness of the epidermis, only the formulation FRA showed a significant increase in this property after 30 days which was maintained until the end of the 90 days (Fig. 3D).

Figure 3 - Reflectance Confocal Microscopy evaluated parameters. The graphs show the results on face at initial time (T0, basal values) and after 30 (T30), 60 (T60) and 90 (T90) days of treatment with Control vehicle, FRA and FCO formulations. Statistically significant difference in relation to T0: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.



In the RCM images is possible to observe a whitening effect since there is a hyper reflectance reduction on the skin after 90 days of treatment, which suggests absence of pigmentation (Fig. 4).

Figure 4 - Dermo-epidermal junction (JDE) RCM images. Accumulation of melanin distributed irregularly among keratinocytes can be seen before the treatment (T0) – more hyper reflexive keratinocytes with the control vehicle (A), FRA (B) and FCO (C) formulations. Reduction of skin hyperpigmentation after the 90-day treatment period with the formulations containing natural origin active substances, FRA and FCO was observed. The arrows indicate the hyperpigmented areas reduction. (Scale: 500 x 500 μm)



4. DISCUSSION

The significant increase in the stratum corneum water content infers in a constant and cumulative increase in the hydration of the skin with the continuous use of the formulations [19]. Transepidermal water loss (TEWL) analyses alongside with stratum corneum water content (SCWC) is important for assessing the skin barrier function. TEWL values reflect the impairment of epidermal functions during treatment and SCWC values reflect the skin hydration [23]. Since alterations in these values can mean disturbances in permeation, healing and barrier dysfunction, it is important to monitor these properties at all stages of the study [18]. All treatments under study were able to significantly increase SCWC values without changing TEWL values. Therefore, the skin has increased its levels of hydration without disrupting its organization.

A skin hydration effect by increases of stratum corneum water content after the 90 days of study was verified and when the active substances were added in the vehicle formulation this effect was not limited to the upper cell layers but is also present in the deeper layers since a dermis thickness and skin echogenicity were observed, which suggests an increase of water content.

In this case, the association of the active substances (FCO) harmed the effect on the brightness exerted by the *Palmaria palmata* extract that when isolated, was able to clean and tone the skin, causing a radiance effect on the face [22].

Assessing the characteristics of the dermis, the thickness and the relation between number of hypoechoic pixels with total number of pixels was calculated, and the smaller this relation, the more echogenic the skin and the greater is its density. For this, low-echogenicity pixels were calculated in relation to the total number of pixels in the upper dermis, lower dermis and total dermis [24].

There is no consensus in the literature regarding the relationship between dermis thickness and skin aging [25], although it is known that the increase in the thickness of the dermis is related to the connection with water molecules. Thus, the significant increase in dermis thickness after 90 days with the use of the formulation with *Palmaria palmata* extract suggests an increase of skin hydration [26].

Acoustic impedance is based on sound propagation, thus, ultrasound analysis is related to the resistance or difficulty that the material opposes the passage of sound. The

echogenicity of the skin may vary with increasing or decreasing water in the skin, which is represented by echogenicity pixels on high frequency ultrasonography [24,27]. The skin echogenicity is related to collagen and elastin fibers, photoaging and hydration [28]. The increase in skin echogenicity suggests protection against skin damage and increased hydration[29,30].

In the analysis of the skin microrelief, among all treatments, the best result for this analysis was obtained with the formulation containing the *Palmaria palmata* extract (FRA), which reduced the roughness and desquamation of the skin after 60 days. This result reflects in the skin homogeneity and it is important because is related to dryness and desquamation of the stratum corneum demonstrating once again the moisturizing effect of the formulation [19].

The high-resolution full face photographs were used to detect visible spots and those visible only with UV light. The equipment provides a skin image illuminated by white LEDs and by UV-like LEDs, respectively [6]. According to the qualitative analysis, increase in the luminosity of the skin could be observed after the use of the formulations FRA and FCO, this result is also related to the radiance of the skin as well as the brightness analysis. The combination of the active substances has shown to have a whitening effect on both visible and UV-light visible spots. These spots are mainly related to sun exposure and can lead to hyperpigmentation problems [16,17].

Increasing the thickness of the viable epidermis by increasing the thickness of the granular layer indicates an improvement of skin general conditions and also suggests that the skin is more hydrated [4,31].

The flattening of the dermal papillae is related to skin aging and this increase suggests that the dermal papillae are deeper and better defined, indicating an improvement in the structural and supporting conditions of the skin [4,14]. Previous results in a *in vivo* pre-clinical study have shown that increase in thickness of viable epidermis suggests intra and extracellular hydration [19], also this parameter is related to the maintenance of skin euthermia and stimulation of cell renewal [4,31]. These results agree with the desquamation result observed in skin microrelief analysis. Thus, both formulations containing active substances were able to act in different layers of the skin, improving their conditions with more pronounced effects than vehicle.

From the analysis of the morphological pattern of the dermo-epidermal junction (JDE) it was possible to observe that the vehicle formulation did not act in the reduction of the pigmentation of the dermo-epidermal junction [13]. On the other hand, the use of the formulations with the active substances (FRA and FCO) resulted in a decrease of this pattern of pigmentation (Fig. 4) [16]. Microscopic analysis correlates with the visual analysis obtained by high-resolution full face photographs at Fig. 2. Skin pigmentation pattern characterization on JDE reflects the visible and UV-light visible spots on the face [16,17].

There are studies demonstrating the *in vitro* antioxidant activity of the extracts that are the active substances of the formulations under study and its potential for application in skin care [1,9,11,32,33]. However, it is the first time that the association of Spirulina dry extract, *Cichorium intybus* (Chicory) root extract, *Medicago sativa* (Alfalfa) Extract and *Palmaria palmata* extract is studied for cosmetic application purposes and also the effectiveness of the *Palmaria palmata* extract alone as a cosmetic active is demonstrated.

There is still difficulty in maintaining the improvement in properties such as roughness and desquamation since the skin is subjected to the effects of skin aging by internal and external environmental factors as pollution and solar radiation. Skin care should be daily and prolonged. Though, it was possible to verify that the topical formulations containing natural origin active substances were able to hydrate the skin on its surface and depth and with superior performance to the control formulation. This improvement also had an effect on the improvement of conditions of skin aging and hyperpigmentation control.

In conclusion, an improvement of skin microrelief and homogeneity and an increase of granular layer and viable epidermis thickness were observed after the 90-day treatment period with the formulations containing the active substances when compared to the vehicle. In addition, these formulations significantly improved the flattening of dermal papillae and reduced the skin hyperpigmentation, showing their efficacy in the skin photoaging alterations control, especially the formulation added with the *Palmaria palmata* extract.

Finally, in this study a complete, non-invasive and multi-instrumental protocol based on biophysical and skin imaging techniques was developed and allowed to evaluate the

clinical efficacy of the natural origin active substances in the improvement of skin aging conditions compared to a control vehicle formulation.

Acknowledgments

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Disclosure of interest

The authors report no conflicts of interest.

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3.7. Capítulo 7 – Análise sensorial

3.7.1. Artigo 7: CALIXTO, L. S.; MAIA CAMPOS, P. M. B. G.; PICARD, C.; SAVARY, G.; Brazilian and French sensory perception of cosmetic formulations: a cross-cultural study. Journal of Sensory Studies, Submitted for publication 2019.

Brazilian and French sensory perception of cosmetic formulations: a cross-cultural study

Abstract

Brazil and France are two major beauty markets worldwide. Despite this, there is not much cross-information on sensory analysis of cosmetic products between both population of these countries. The objective of this study was to compare the sensory perception of cosmetic formulations between Brazilian and French assessors and establish cross-culturally preferences. For this, a total panel of 100 consumers of cosmetics evaluated four different products. The same protocol was followed in both countries. The panelists were able to perceive differences in the products and the method proved to be repeatable in both countries. The presence of UV-filters in the formulation was noticed and displeased both populations. Brazilians, although dissatisfied with the sensory aspect of the sunscreen, are willing to use it for its UV protection.

Practical Applications

This work delivers important information on the sensory perception of cosmetics by people from different countries. Our protocol had consistent results and proved to be repeatable in populations of countries with different geographic position, climate, culture and society. Our data provide insight into consumer perception and demonstrates that characteristics such as hydration, oily residue and spreading are perceived in the same way by different people, but that the hedonic responses are influenced by several factors. Thereby, this study brings important knowledge to develop products that will be appreciated worldwide.

Keywords

Cross-cultural; Sensory analysis; Cosmetic formulations; Consumers' perception; Brazilian assessors; French assessors.

INTRODUCTION

The research and development of cosmetic formulations is a complex process involving different aspects. Among them, the sensory analysis stands out as a tool that predicts the acceptance of a product by a population studying the way the consumer interacts with this product (Pensé-Lhéritier, 2015; Varela & Ares, 2012).

In order to develop products that will be appreciated by the market, it is necessary to have great concern with the sensory aspect. It is necessary to combine different ingredients, in order to associate the well-being sensation with proven efficacy claim, to obtain innovative products with excellence (Brummer & Godersky, 1999; Calixto, Maia Campos, Savary, & Picard, 2018; Eudier, Hirel, Grisel, Picard, & Savary, 2018; Souza & Campos, 2017). To ensure that a product reaches the requirements of different populations, it is necessary to make substantial sensory evaluations with different communities of interest.

Cross-cultural and cross-national studies are helpful to understand consumer behavior and sensory perception in groups from different countries. From them, we can obtain market data, purchase behavior and consumer attitudes (Dant, Perrigot, & Cliquet, 2008; Hersleth et al., 2013; Kim, Jombart, Valentin, & Kim, 2013; Monteiro et al., 2017; Moussaoui & Varela, 2010; Nagashima, 1970).

Souiden & Diagne (2009) studied the impact of personal, socio-cultural and marketing variables on the attitude of Canadian and French males toward the consumption of men's cosmetics. The groups from different nationalities had different motivations to buy product based on their self-image, lifestyle, among others. Advertising and attractiveness had a strong positive impact on both populations. These analyzes provide more complete results regarding the acceptance of cosmetics by different societies.

Brazil and France are among the largest global beauty markets. For cultural, geographic and socioeconomic reasons, these countries are strong consumers and producers of cosmetics (Ficheux et al., 2016; Ficheux, Wesolek, Chevillotte, & Roudot, 2015; Infante, Calixto, & Maia Campos, 2016; Lopaciuk & Loboda, 2013). There are studies comparing the efficacy of products between these two populations (Calixto, Picard, Savary, & Maia Campos, 2019; Calixto, Picard, Savary, & Maia Campos,, 2018; Mercurio, Jdid, Morizot, Masson, & Maia Campos, 2016), but there is a lack of information about the comparison of sensory perception between French and Brazilians.

In this context, the objective of this study was to compare the sensory perceptions after the application of cosmetic formulations between Brazilian and French consumer assessors and establish cross-culturally preferences between them. Sensory evaluations focused on skin-care formulations containing UV filters, anti-aging active substances and these in combination.

MATERIALS AND METHODS

Samples

Four gel-cream cosmetic formulations were evaluated: a vehicle (S1), which some ingredients were added as UV-filters (S2), a combination of natural extracts with anti-aging properties – Alfafa, Cichorium root, Spirulina and Red Algae extracts (S3) and UV-filters in combinations with these extracts (S4) (Table 1). Details of the vehicle formulation have been published previously (Calixto & Maia Campos, 2017; Calixto, Infante, & Maia Campos, 2018; Calixto, Maia Campos, Savary, & Picard, 2018). We aimed to verify how the two groups of assessors would perceive the sensory effects of the addition of these ingredients.

Table 1 – Description of samples

S1	Gel-cream vehicle
S2	Gel-cream vehicle + UV-filters
S3	Gel-cream vehicle + extracts in combination
S4	Gel-cream vehicle + UV-filters + extracts in combination

Consumer panel

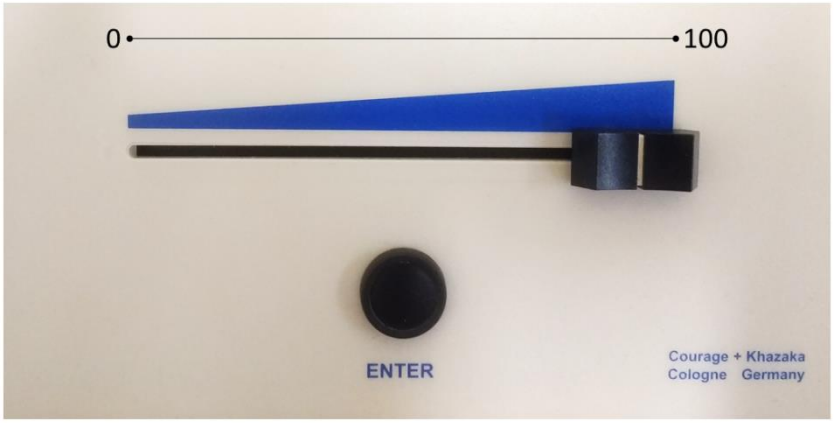
According to the objective to compare the perceptions between the Brazilian and the French assessors, one hundred untrained regular consumers of cosmetics (fifty Brazilian and fifty French) aged from 21 to 61 years were selected to the study. The study was conducted conformed to the principles of the Declaration of Helsinki and was approved by the Institutional Ethics Committee (CEP/FCFRP nº. 381). The evaluations were performed in the native language of each country, that is, in Portuguese in Brazil and in French in France. The sessions were conducted under the guidance of the same researcher in both countries and we used the same equipment under the same experimental conditions to ensure the requirement of comparing the results obtained.

Experimental conditions

The evaluations were conducted at two research centers: the French consumers participated at the Biometrology analysis room at the University Le Havre Normandy in Le Havre, France (49°30′N 0°08′W) from May to August 2017, and the Brazilian consumers participated at the Biophysical and image analysis laboratory at the University of São Paulo in Ribeirão Preto, Brazil (21°100S, 47°480W) from May to August 2018. This period was chosen to obtain the same average temperature between cities of 17.5 ± 2.5 (Climate-data, 2018b, 2018a).

The formulations were evaluated by the assessors with a specific instrument named Sensorimeter® SR 100 (Courage-Khazaka, Germany). This equipment translates subjective opinions in objective measurements. A scale from 0 to 100 was chosen to evaluate the consumer's sensation, in which the higher the score, the higher was the impact of the characteristic (Figure 1). The analyzes in both Brazil and France were performed in a room with controlled relative humidity and temperature. The consumers received a standardized amount of formulation of 50µL to spread on a 4 x 5cm area in a pre-delimited region on the forearm.

Figure 1 - Evaluation scale named Sensorimeter® SR 100



Descriptors

We performed a descriptive test using intensity scales with consumers (Parente, Ares, & Manzoni, 2010; Varela & Ares, 2012). In order to perform a cross-cultural sensory analysis, we had to choose a methodology that would fit both populations. Free-Choice Profiling (FCP) is a method used with consumer assessors when wanted to target consumers' perceptions about products and their preferences. Participants need to be able to use an evaluation scale and be consumers of the product under study (Hersleth et al., 2013; Varela & Ares, 2012). Usually, consumers are free to develop the sensory lexicon themselves. As we wanted a standardized analysis between the groups of both countries, we decided to establish the vocabulary to be used to assist the learning process (Murray, Delahunty, & Baxter, 2001).

Ten descriptors were defined to evaluate the formulations regarding appearance, sensations before, during and after application. "Integrity of shape", "Cohesiveness" (Stringiness), "Spreading" (Difficulty of spreading) and "Stickiness" were adapted from literature (Gilbert, Picard, Savary, & Grisel, 2012). "Viscosity" and "Consistency" were present to verify the perception of these characteristics which are highly noticed in gel-cream formulations due to the presence of waxes (Calixto, Infante, & Maia Campos, 2018). "Oily residue" and "White residue" were descriptors that aimed to verify if the addition of UV-filters influenced the oily character of the formulation, as well as, they are characteristics of formulations with high proportions of oil phase. Finally, the descriptors "Absorption" and "Hydration" have been chosen to verify if consumers could perceive the absorption of the formulation immediately and its consequent hydration of the skin (skin-feeling properties). The assessors received the descriptors definitions (Table 2) and they were allowed to ask questions during the analysis.

Table 2 - List of descriptors and their definitions

Appearance
1. Integrity of shape (Verify if the formulation holds the given shape in the package) 0 - Flattens 100 – Retains shape
Before application
2. Viscosity (Verify the malleability of the formulation when you touch) 0 - Soft 100 - Hard
3. Consistency (Verify if the formulation has solid characteristics) 0 - Soft 100 - Hard
4. Cohesiveness (Proportion of product deforming/stretching rather than breaking when fingers separate) 0 - Absence of filaments 100 - Very large filaments
During application
5. Spreading (easy to push / spread the product on the skin) 0 - Extremely hard 100 - Extremely easy
6. Stickiness (Force required to take off the fingers of the skin) 0 - Slightly 100 - Very
7. Absorption (Check the absorption intensity) 0 - Null 100 - Very important
After application
8. Oily residue (Amount of oil perceived after spreading) 0 - Null 100 - Very important
9. White residue (Amount of product left on the skin) 0 - Null 100 - Very important
10. Hydration (Hydration sensation soon after application of the product) 0 - Null 100 - Very important

Statistical analysis

The analyses were accomplished with XLSTAT software V. 2018 (Addinsoft, Paris). Analysis of variance (ANOVA) with Tukey post-hoc test was used to determine significant differences on the scores provided by the two populations. Differences were considered significant at $p < 0.05$. Pearson's correlation and principal component analysis (PCA) were carried out to compare the results obtained in each country and to understand in what way the characteristics of the formulations impacted the sensory perception by the consumers.

RESULTS AND DISCUSSION

Sensory perception of formulations

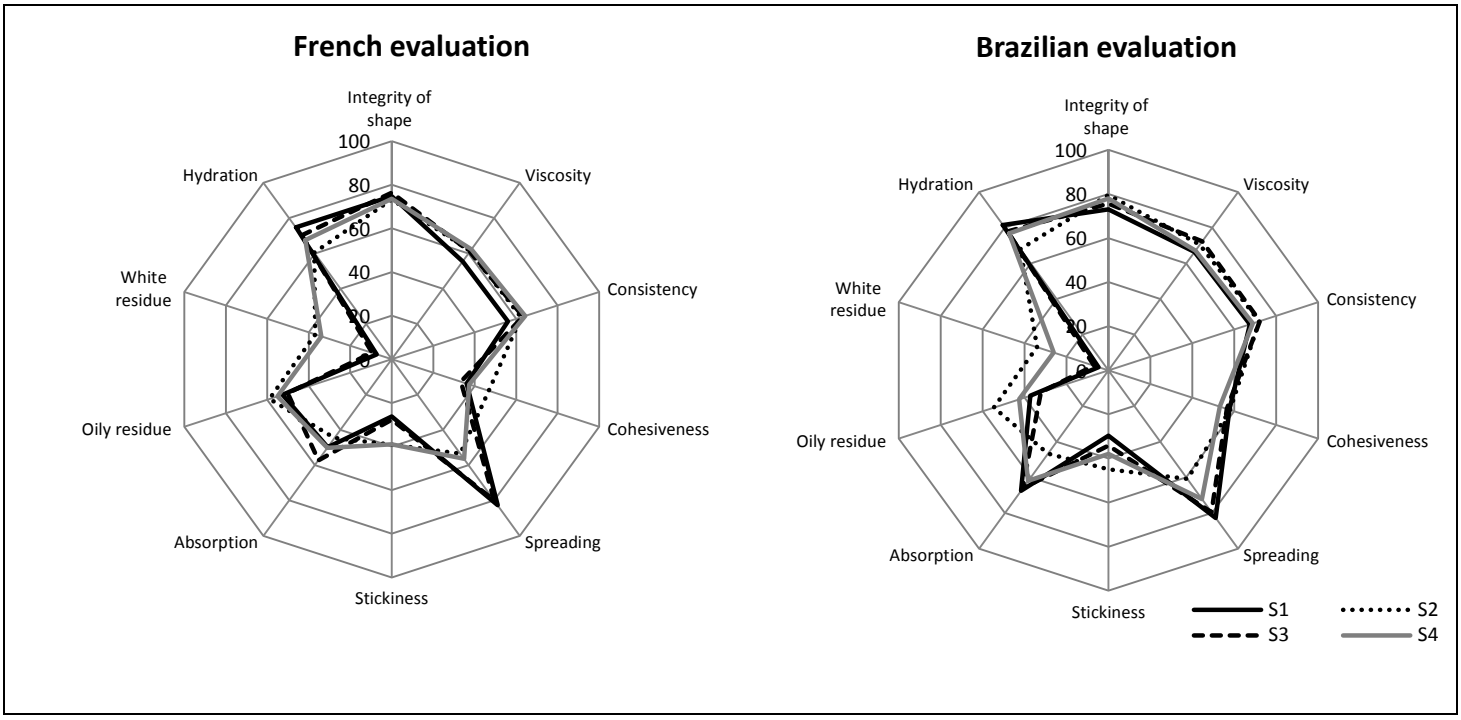
We chose a descriptive test as it involves discrimination and description of the sensory attributes of a product in order to gather information to understand the similarities and differences between the formulations (Varela & Ares, 2012). This analysis is essential in different stages of cosmetic development. It provides information on product specificities that may help in the innovative process within the laboratory, but also indicates how that formulation will be accepted by the market (Pensé-Lhéritier, 2015). We aimed to get the sensory evaluations of different populations on the same formulations. All assessors were able to perform the test and had no difficulties in handling the equipment. The results in Figure 2 show the consumers' evaluations in terms of descriptors for the four gels-creams.

In the Brazilian evaluation, the assessors attributed similar scores to the four samples with respect to the parameters: "Integrity of shape", "Viscosity", "Consistency" and "Cohesiveness" (Figure 2). In the parameters "Spreading", "Absorption" and "Oily residue" the formulations were well distinguished and significant differences ($p<0.05$)

were perceived with the addition of UV-filters (formulation S2) for these parameters. Regarding "Stickiness", the formulation S1 was considered significantly less sticky than other formulations ($p<0.05$).

Formulations that did not contain UV-filters, S1 and S3, had a “White residue” result close to zero and the formulation S2 presented significantly higher values for this property. The formulation S1 presented higher “Hydration” values than S2 ($p<0.05$).

Figure 2 - Mean intensity scores of the sensory perception of Brazilian and French consumers



In the French evaluation, all formulations presented a similar score for the "Integrity of shape", “Cohesiveness”, "Stickiness" and “Oily residue” characteristics (Figure 2). Statistical analysis ($p<0.05$) showed that the presence of UV-filters in the formulation S2 disturbed the characteristics "Spreading" and "White residue" compared to the other formulations. Formulation S1 was evaluated as less consistent / viscous (“Viscosity” and “Consistency”) than the others ($p<0.05$). This result agrees with data obtained from

instrumental measurements for the same formulations (Calixto & Maia Campos, 2017; Calixto et al., 2018).

The addition of extracts in S3 significantly improved the properties "Cohesiveness" and "Absorption". Regarding "Hydration", the formulation S1 presented a significantly higher hydration than the S2 formulation.

Both juries gave globally very similar textural profiles with some differences. According to the scores, the Brazilian assessors classified the formulations without UV-filters, S1 and S3, in a similar way and the multifunctional formulation S4 following the same trend. The S2 formulation differed from the others and disrupted most of the properties. The French assessors attributed similar scores to S1 and S3 as well as to the Brazilian assessors, however, it better distinguished the formulations with UV-filters, approaching the S2 and S4 scores. Thus, this population showed more sensitivity to the presence of this ingredient in the formulations.

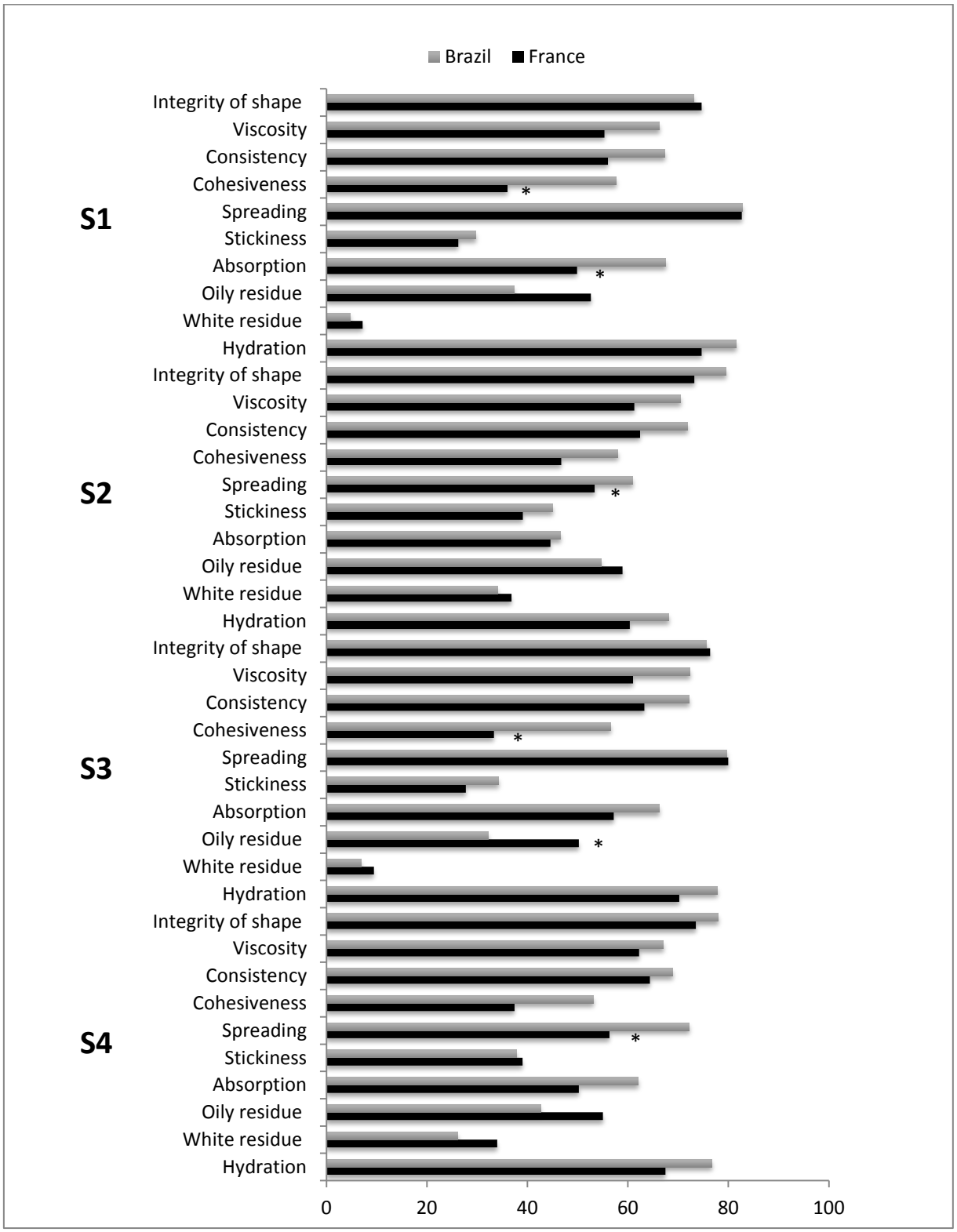
As the Brazilian assessors, the French group found it difficult to differentiate the descriptors “Viscosity” and “Consistency”. This is common when attributes are very similar (Nogueira, Cabeço-silva, Schacher, Adolphe, & Broega, 2011; Pensé-Lhéritier, 2015). For the French, even in combination with other ingredients, the presence of UV-filters stands out.

As reported in the literature, untrained consumers proved to be a valid instrument in the differentiation of cosmetic formulations with different ingredients (Moussaoui & Varela, 2010; Murray et al., 2001; Oliver, Cicerale, Pang, & Keast, 2018; Varela & Ares, 2012). The consumers from both countries were able to understand the sensory lexicon, to differentiate the formulations and to verify the impact of UV-filters and extracts on formulations.

Correlation of results of both juries

The intensity of the scores varied between the groups of the two countries (Figure 3). This suggests heterogeneity in consumer scores between the two countries and demonstrates that they do not have the same references and familiarity with the products (Hersleth et al., 2013; Kim et al., 2013; Parente et al., 2010). Brazilians attributed higher value scores, especially for properties related to the product as “Viscosity”, “Consistency” and “Cohesiveness”. Their perception of “Absorption” was higher than the French and this was reflected in a greater perception of “Hydration” as well. The French found it more difficult to spread formulations containing UV-filters (they attributed higher values of “Spreading”) compared to Brazilian and attributed higher scores to "Oily residue" than those given by the Brazilians.

Figure 3 - Sensory profile of formulations by Brazilian and French consumers
* mean significantly different at $p<0.05$.

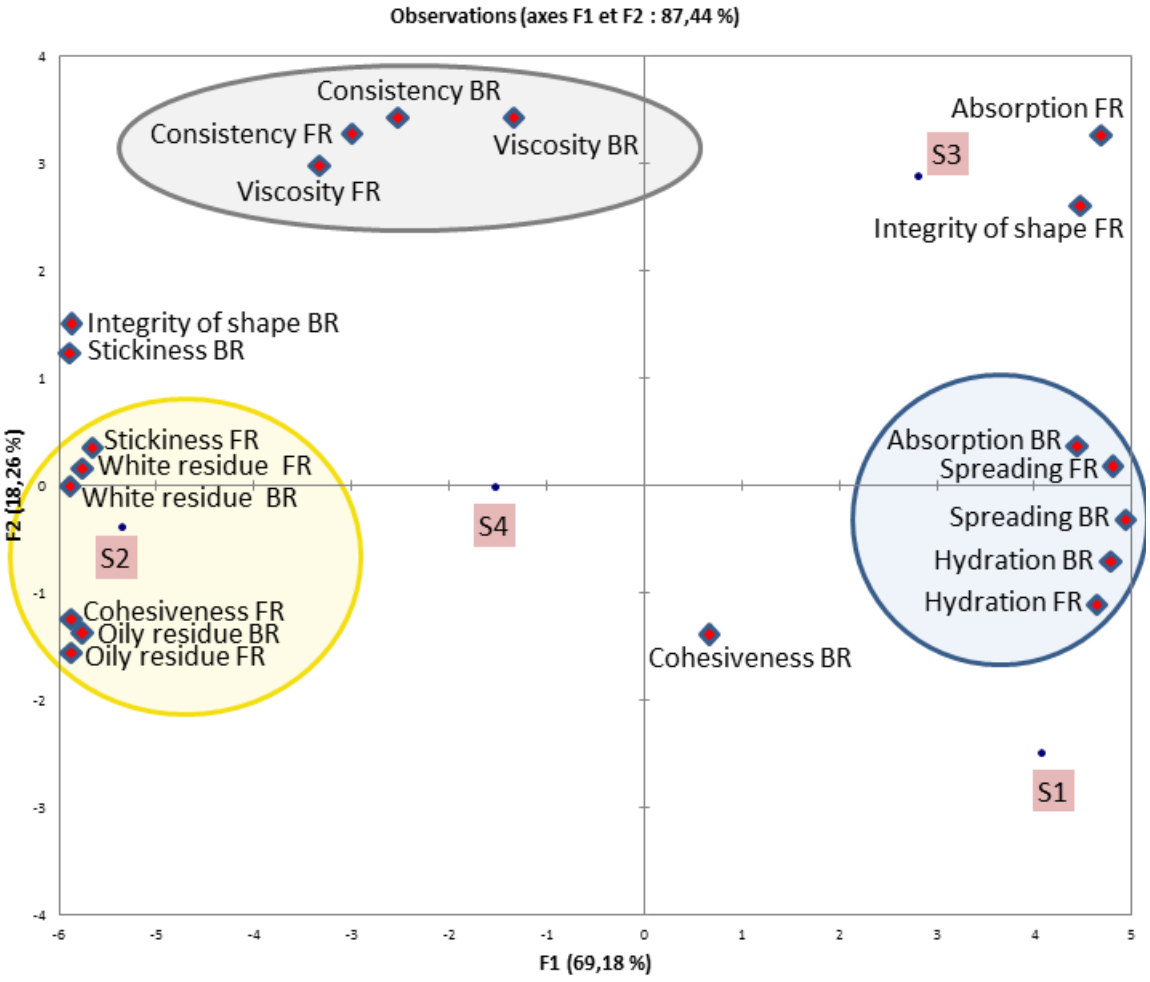


A principal component analysis (PCA) was conducted on the 10 descriptors for each sample analyzed and the results are shown in Figure 4. The properties related to the sensation felt when interacting with the formulations (“Spreading”, “Absorption” and “Hydration”) are positioned in the quadrants on the right side. The formulations without filter, S1 and S3 remained close to these properties. The properties related to the residue left by the formulation on the skin (“Stickiness”, "Oily residue" and "White residue") are found in the quadrants on the left side and close to them is located the formulation S2. The properties “Viscosity” and “Consistency”, which were considered very similar by the assessors, are located close to the central axis, as well as the formulation S4.

The addition of extracts in vehicle formulation S1 did not have an important impact when comparing its results with those of formulation S3 except for the firmness (“Viscosity” and “Consistency” attributes). These two formulations were more associated with wellness properties, with better spreadability, less residue, greater sensation of absorption and better perception of hydration after application.

On the other hand, the formulation with UV-filters S2 is placed near the properties considered undesirable to sensory perception, presenting high values of white residue, stickiness and oily residue (Eudier et al., 2018; Souza & Campos, 2017; Wagemaker et al., 2013). The formulation combining UV-filters and extracts, S4, was centrally positioned between these properties, demonstrating that the presence of aqueous extracts attenuated the negative characteristics of the formulation S2. Finally, the consumers’ perception of both countries agrees with the results obtained with the instrumental analyzes; the synergic effect between the presence of UV-filters and extracts favors the spreadability, improves the texture and benefits the *in vivo* efficacy of these formulations (Calixto et al., 2018)..

Figure 4 - Principal component analysis correlating the results of the analyzes in the two countries for each formulation. The first principal component (F1) and the second principal component (F2) were used. BR = Brazil ; FR = France



Pearson's correlation coefficient was calculated in order to establish the relationship between the results obtained by the two populations (Gilbert et al., 2012). The attributes "Spreading", "Stickiness", "Oily residue", "White residue" and "Hydration" of the Brazilian and French assesors were considered highly correlated (Pearson coefficients between 0.871 and 0.995).

"Viscosity", "Consistency", "Absorption" presented intermediate correlation (Pearson coefficients between 0.506 and 0.777). The attributes with the weakest correlations were

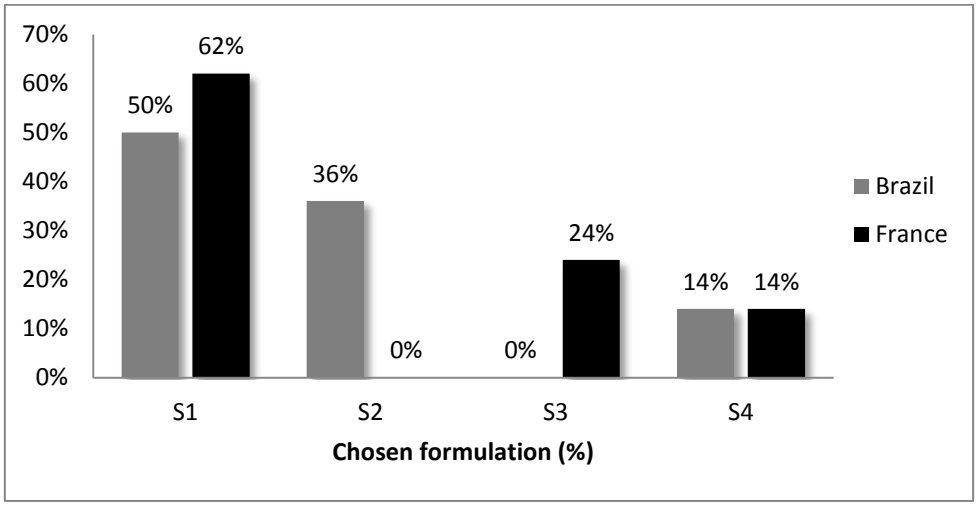
"Cohesiveness" and "Integrity of shape" with coefficients of 0.320 and -0.629 respectively.

Consumers have found it easier to evaluate properties related to the sensation provided by the formulation and the residue left over the skin after application. Properties related to sensation are also called skin-feeling, they are divided into primary (at the beginning of the application) and secondary (at the end of the application) (Brummer; Godersky, 1999). There were differences of opinion between the two groups for the appearance attributes that differentiate the characteristics of the formulations, especially "Integrity of shape", that presented a negative correlation.

Differences in assessors' preference

The important differences in sensory perception caused by the addition of UV-filters were highlighted in the result of the "preferred formulation" (Figure 5). When asked about which formulation they chose as their preferred, no French assessor chose the formulation S2 whereas for the Brazilian assessors, this formulation was the second most chosen. In both populations the vehicle formulation S1 was the most chosen, demonstrating that the addition of ingredients in vehicle formulation was disapproved by both groups.

Figure 5 - Choice of formulations between Brazilian and French populations (n=100)



Cross-cultural studies are important to verify differences in perception and preference of products in populations of different countries. They bring us information about consumer attitudes toward products, motivations, sense of generalization and purchase behavior (Dant et al., 2008; Hersleth et al., 2013; Monteiro et al., 2017; Nagashima, 1970; Souiden & Diagne, 2009). Culture and familiarity with products are factors that affect sensory properties in assessors from different countries (Hersleth et al., 2013; Kim et al., 2013).

The Brazilian population is very concerned about the deleterious effects of exposure to solar radiation and does not consider sensory perception as a priority factor when buying a cosmetic (Infante, Calixto, & Campos, 2016). Still, they consider their skin very sensitive and the sun is one of the reasons according to them (Taieb, Auges, Georgescu, Perez Cullell, & Miséry, 2014).

Sun protection is not the number one priority for the French. Among those who consume solar products, they apply them for less than 2 months per year (Ficheux et al., 2015). Their purchase priority is basic hygiene products such as soap, deodorant and shampoo and fragrances. Also, the consumption of UV-filters is made by the female population and the most frequent application area is the body, in contrast to the

Brazilians who prioritize the face area (Ficheux et al., 2016; Weber & Capitant de Villebonne, 2002).

UV-filters' sensory can plays a fundamental role in their *in vivo* efficacy. If the consumer does not like the sensory of the product he applies less product to the skin, and it is known that the smaller the amount of sunscreens applied, the less the SPF protection (Couteau, Diarra, & Coiffard, 2016; Souza & Campos, 2017).

Notably, in the case of this study, because they are countries with different climates, Brazilian assessors, although they feel that the sunscreen formulation has a heavier appearance and leaves much residue on the skin, they feel protected and were willing to choose it.

Also, studies show that compared with Brazilian skin, French skin was more hydrated (Mercurio, Jdid, Morizot, Masson, & Maia Campos, 2016 ; Calixto, Picard, Savary, & Maia Campos, 2019). Therefore, the lower sensation of dry skin makes the French more restricted in the choice of cream that they will use preferring a soft cream, since they do not feel the need of strong protection.

CONCLUSIONS

Our results combine the sensory perception of groups from different countries that evaluated cosmetic formulations and verified that properties related to sensation are perceived in the same way with a good accordance between both textural descriptive profiles. These results are unprecedented in the literature and provide as perspectives information that can help in the development of cosmetics to be appreciated by diverse populations.

The protocol of analysis was repeatable in both populations and important knowledge was brought about the preference of each population. Brazilians and French were able to

judge differences in the appearance and the skin-feelings caused by the products. The presence of UV-filters on sunscreens displeased both groups. However, for the group most exposed to UV radiation, the protection generated by these ingredients is more important than the sensory disturbance. Thus, hedonic responses are more difficult to control in cross-country studies.

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4. DISCUSSÃO

Na etapa de Pesquisa & desenvolvimento das formulações, as mesmas foram elaboradas com diferentes concentrações de ceras e polímeros a partir do planejamento fatorial completo de experimentos, o qual demonstrou que as ceras objeto de estudo influenciaram significativamente o trabalho de cisalhamento das formulações, principalmente pelo aumento da concentração de fase oleosa na formulação (GORE; PICARD; SAVARY, 2018; TERESCENCO et al., 2018).

Além disso, na avaliação das propriedades mecânicas das formulações em estudo, a combinação de cera e polímero escolhidos para compor a formulação veículo foi aquela que apresentou menores valores de trabalho de cisalhamento, que é uma propriedade preditora da percepção sensorial da espalhabilidade. Estudos relatados na literatura mostraram que quanto maior o trabalho para cisalhar, menor o parâmetro espalhabilidade na percepção sensorial (SAVARY; GRISEL; PICARD, 2013; CALIXTO; MAIA CAMPOS, 2017). Dessa forma, a aplicação do sistema de análise das propriedades físico-mecânicas (texturômetro) como preditor de propriedades sensoriais de cosméticos é de grande valia no desenvolvimento de formulações cosméticas, uma vez que o mesmo possibilita um screening das formulações para a analise sensorial.

O estudo de concentração de ingredientes demonstrou que a presença de cera e de polímero juntos na formulação veículo apresentou um efeito sinérgico sobre as propriedades reológicas, de textura e sensoriais das formulações (CALIXTO; INFANTE; MAIA CAMPOS, 2018). Essas propriedades são de extrema importância e influenciam a estabilidade, o comportamento e a percepção do produto pelo consumidor. Por isso, ao se pensar em desenvolver uma formulação multifuncional que combine diversos benefícios, é importante considerar o impacto da escolha dos ingredientes sobre a performance da formulação.

As substâncias ativas de origens naturais *Palmaria palmata*, Spirulina, Alfafa e Raiz de chicória e os filtros solares escolhidos foram adicionados à formulação veículo com o objetivo de obter um cosmético com proteção UV (FPS 30) e efeito *anti-aging*.

Considerando que, além dos benefícios de proteção e eficácia clínica, a adição de ingredientes ativos e filtros solares pode influenciar o comportamento das formulações cosméticas, os ingredientes em estudo foram adicionados

separadamente e em combinação à formulação veículo para avaliar a influência dos mesmos na estabilidade, propriedades reológicas, de textura e sensoriais da formulação (GASPAR; CAMPOS, 2007; MAIA CAMPOS et al., 2012; WAGEMAKER et al., 2015).

De acordo com o estudo de estabilidade, todas as formulações estudadas se mantiveram estáveis tanto em relação às características organolépticas quanto ao comportamento reológico após 180 dias de avaliação. As propriedades de textura também foram acompanhadas e a formulação que continha apenas o extrato de raiz de chicória apresentou ganho de viscosidade, consistência e firmeza após esse período, o que sugere que o polissacarídeo Inulina presente nesse extrato, em longo prazo pode interferir nas características de textura da formulação (TONELI et al., 2008; EL-SAYED et al., 2015; CALIXTO; MAIA CAMPOS, 2017).

Em comparação com o veículo, a adição de filtros solares UV isoladamente ou em combinação com os ingredientes ativos resultou em aumentos significativos nos valores de viscosidade mínima aparente e índice de consistência no estudo reológico. A partir dos reogramas obtidos, foi possível observar que as formulações apresentaram um comportamento não-Newtoniano do tipo pseudoplástico, uma vez que houve diminuição da viscosidade com o aumento da taxa de cisalhamento. Esse comportamento é desejado em formulações cosméticas, pois aumenta a facilidade de fluir das mesmas e consequentemente, sua espalhabilidade. Além disso, é possível observar que a formulação veículo apresentou maior tixotropia do que as outras, que apresentaram menor área de histerese. Essa propriedade tem influência no aspecto de espalhabilidade e no FPS, no caso das formulações com filtro solar (GASPAR; MAIA CAMPOS, 2003; GIANETI et al., 2012).

A partir do teste oscilatório, foi possível analisar o comportamento viscoelástico das formulações por meio de um gráfico relacionando o módulo elástico (G’) e o módulo viscoso (G’’) com a deformação. Todas elas exibiram um comportamento predominantemente elástico com G' superior a G". Analisando a região viscoelástica linear (SAOS), observou-se que a adição de ingredientes teve influência sobre o valor dos módulos viscoelásticos em relação à formulação veículo. Saindo da região SAOS, foi possível obter informações sobre a resistência à deformação e a homogeinidade da organização das partículas. A formulação veículo apresentou

grande resistência à deformação. Algumas formulações apresentaram um pico de G" na região LAOS, onde G’ e G’’ não são definidas igualmente. Esse pico é característico de um comportamento de tipo de gel fraco principalmente devido ao agente gelificante (ROWENCZYK et al., 2016).

A influência da adição de ingredientes sobre o comportamento reológico das formulações se deve principalmente à mudanças microestruturais como aumento do tamanho de partícula, mudança da forma das gotículas e presença de cristais líquidos (TAHERIAN; FUSTIER; RAMASWAMY, 2006; BERI; NORTON; NORTON, 2013; ZHANG; LIU, 2013; WAGEMAKER et al., 2015; TERESCENCO et al., 2018). Tais mudanças além de alterarem a percepção sensorial de propriedades relacionadas à textura, como viscosidade, coesividade e pegajosidade, acabam perturbando também a percepção de espalhabilidade, hidratação e resíduo oleoso (GILBERT et al., 2013b; RIGON et al., 2013; CALIXTO; MAIA CAMPOS, 2017; CALIXTO et al., 2018).

Ainda em relação à avaliação das propriedades físico mecânicas das formulações, o presente trabalho tinha como objetivo explorar as inúmeras possibilidades da aplicação do texturômetro, um instrumento que possui uma base universal, capaz de realizar avaliações de força, tempo e distância e nos permite acoplar diversos probes próprios ao equipamento, para melhor compreender como a adição de ingredientes alteraria as características das formulações.

Uma das referidas possibilidades é a análise da espalhabilidade. Em modo tensão, o aparelho é capaz de deslocar uma placa, em velocidade constante sobre uma superfície de polipropileno e calcular a força necessária para espalhar uma quantidade de produto sobre essa superfície, com base em medidas de fricção. Esse protocolo foi desenvolvido para simular uma aplicação real de produto e os seus resultados demonstraram correlação com dados obtidos em análise sensorial com participantes (SAVARY; GRISEL; PICARD, 2013; CALIXTO et al., 2018).

Entre outros protocolos de análise de textura, destacam-se os testes em modo compressão, onde diferentes acessórios podem comprimir as formulações e fornecer informações à respeito da dureza, adesividade, consistência e força de compressão das formulações (GILBERT et al., 2013c, 2013b; CALIXTO; MAIA CAMPOS, 2017; FONSECA-SANTOS et al., 2017). Um outro protocolo muito utilizado é o

teste de penetração, onde é possível obter dados relacionados à espalhabilidade, trabalho de cisalhamento e força de penetração das formulações em estudo (GILBERT et al., 2013a; CALIXTO; INFANTE; MAIA CAMPOS, 2018; DUBUISSON et al., 2018).

Nossos resultados mostraram que a formulação com filtros e ativos em combinação possuía uma microestrutura diferente das demais e isso resultou em mudanças macroestruturais refletidas em valores mais altos de compressão, penetração, fricção, coesividade, consistência, firmeza e índice de viscosidade (CALIXTO; MAIA CAMPOS, 2017; CALIXTO et al., 2018).

Ao alterar as características física e físico-mecânicas das formulações, se altera também a maneira com a qual elas interagem com a pele e sua ação imediata. A capacidade de formação de filme, oclusão e característica residual do cosmético possui influência na eficácia em curto prazo do produto (ZHAI; MAIBACH, 2002; MÜLLER-GOYMANN, 2004; TRY; NICOD; HUMBERT, 2010; SAVARY et al., 2019). Após uma hora de aplicação da formulação contendo ativos e filtros, observou-se aumento da hidratação, por meio do aumento da quantidade de água no estrato córneo, aumento do brilho da pele e proteção da barreira cutânea (CALIXTO et al., 2018).

Após a definição das formulações, as mesmas foram submetidas a estudos de eficácia clínica, os quais foram realizados após a devida aprovação do Comitê de ética da Faculdade de Ciências Farmacêuticas de Ribeirão Preto/SP (CEP/FCFRP nº. 381). A avaliação da eficácia clínica em longo prazo por 90 dias foi realizada em três das doze formulações do estudo em três grupos de participantes. O primeiro grupo recebeu a formulação veículo sem as substâncias ativas antienvelhecimento; o segundo grupo recebeu a formulação contendo a associação das quatro substâncias ativas objeto de estudo; o terceiro grupo recebeu a formulação contendo o ativo extrato de alga vermelha, o qual foi estudado sozinho devido às evidências do seu potencial de aplicação em formulações dermocosméticas (YUAN; CARRINGTON; WALSH, 2005; CAMPOS et al., 2019).

O aumento significativo do conteúdo aquoso do estrato córneo infere-se num aumento constante e cumulativo da hidratação da pele com o uso contínuo das formulações (MAIA CAMPOS et al., 2012). A análise de perda de água

transepidérmica (TEWL) juntamente com o conteúdo aquoso do estrato córneo (SCWC) é importante para avaliar a função de barreira da pele. Os valores de TEWL refletem o comprometimento das funções epidérmicas durante o tratamento e os valores de SCWC refletem a hidratação da pele (YE; LI; MAN, 2018). Todos os tratamentos em estudo foram capazes de aumentar significativamente os valores de SCWC sem alterar os valores de TEWL. Portanto, a pele aumentou seus níveis de hidratação sem perturbar sua organização.

A impedância acústica baseia-se na propagação do som, portanto, a análise ultrassonográfica está relacionada à resistência ou dificuldade que o material se opõe à passagem do som. A ecogenicidade da pele pode variar com o aumento ou diminuição da água na pele, que é representada por pixels de ecogenicidade na ultrassonografia de alta frequência (GNIADECKA; JAMEC, 1998; JASAITIENE et al., 2011). A ecogenicidade da pele está relacionada às fibras de colágeno e elastina, fotoenvelhecimento e hidratação (SOUZA; CAMPOS, 2017). O aumento da ecogenicidade da pele sugere proteção contra danos à pele e aumento da hidratação (CRISAN et al., 2015; TULINA et al., 2018).

Não há consenso na literatura sobre a relação entre a espessura da derme e o envelhecimento cutâneo (POURADIER et al., 2013), embora se saiba que o aumento da espessura da derme está relacionado à sua conexão com as moléculas de água. O aumento significativo dessa propriedade após 90 dias de uso da formulação acrescida do extrato de *Palmaria palmata* sugere aumento da hidratação da pele (MLOSEK et al., 2013), uma vez que, outros parâmetros avaliados mostarram o efeito hidratante da formulação. Além disso, outros estudos mostraram que o efeito hidratante de formulações não se limita às camadas superiores da pele, mas também às camadas mais profundas (CAMPOS P.M.B.G.M et al., 1999).

Na análise do microrrelevo da pele, dentre todos os tratamentos, o melhor resultado foi obtido com a formulação contendo o extrato de *Palmaria palmata*, que reduziu a rugosidade e a descamação da pele após 60 dias. Este resultado reflete na homogeneidade da pele e é importante porque está relacionado à secura e descamação do estrato córneo demonstrando mais uma vez o efeito hidratante da formulação (MAIA CAMPOS et al., 2012).

A análise com o microscópio confocal de reflectância à laser demonstrou o aumento da espessura da epiderme viável pelo aumento da espessura da camada granulosa, o que indica melhora das condições gerais da pele por um possível estímulo à renovação celular (MAIA CAMPOS; MELO; MERCURIO, 2016; MERCURIO et al., 2016). O achatamento das papilas dérmicas está relacionado ao envelhecimento da pele e o aumento da profundidade e melhor definição das papilas observados após o tratamento indica uma melhora nas condições estruturais e de suporte da pele (LAGARRIGUE et al., 2012; MERCURIO et al., 2016).

A partir da análise do padrão morfológico da junção dermo-epidérmica (JDE) foi possível observar que a formulação veículo não atuou na redução da pigmentação da junção dermo-epidérmica enquanto o uso das formulações contendo as substâncias ativas resultou em uma diminuição desse padrão de pigmentação (MARTINI; MERCURIO; MAIA CAMPOS, 2017; BRAHIMI; GUITERA, 2018).

Sendo assim, as formulações contendo substâncias ativas de origem natural, quando usadas topicamente durante 90 dias, foram eficazes na hidratação da superfície e profundidade da pele e com efeitos mais pronunciados em relação à formulação veículo. Uma melhoria também foi observada nas condições gerais da pele envelhecida e controle da hiperpigmentação.

Os estudos multicêntricos em diferentes populações se demonstraram pertinentes na avaliação da eficácia e da percepção sensorial já que as formulações avaliadas neste estudo apresentaram os mesmos efeitos sobre a pele brasileira e sobre a pele francesa (HERSLETH et al., 2013; MONTEIRO et al., 2017). Além disso, graças ao protocolo desenvolvido para a análise sensorial, os participantes dos dois países conseguiram diferenciar essas formulações e verificar a influência de cada ingrediente.

É importante ressaltar que os ganho de aspecto oleoso pela adição de filtros na formulação foi percebido pelas duas populações e à ele foi atribuido um aspecto negativo. Porém, a população brasileira se mostra disposta a escolher esse produto apostando na promessa de um efeito protetor enquanto o público francês apresenta comportamento contrário.

Sabe-se que existe uma grande conscientização da população brasileira à respeito dos malefícios oriundos da exposição solar sem proteção e também uma grande preocupação dessa população com a beleza e o bem estar (INFANTE; CALIXTO; CAMPOS, 2016). A França possui tradição no desenvolvimento de cosméticos e eles são amplamente utilizados pela população, porém, o protetor solar não se apresenta na lista dos produtos mais utilizados (FICHEUX et al., 2015, 2016; GOMEZ-BERRADA et al., 2018).

Em síntese, o presente trabalho apresenta como contribuição a aplicação da análise de textura como ferramenta no desenvolvimento de formulações cosméticas norteando o desenvolvimento de cosméticos multifuncionais inovadores, com eficácia comprovada, sensorial aprovado e ampla aceitabilidade pelo consumidor.

5. CONCLUSÃO

Esse estudo traz importantes resultados acerca da importância da correta escolha dos ingredientes de uma formulação cosmética. A adição de substâncias ativas e filtros solares na formulação veículo teve como objetivo unir tratamento e proteção UV em um mesmo produto multifuncional.

O acréscimo desses ingredientes, especialmente os filtros solares trouxe grandes mudanças nas propriedades micro e macroestruturais das formulações. Essas mudanças podem ter como consequência mudanças no sensorial e na eficácia das formulações.

A análise de textura foi a técnica utilizada para caracterizar as propriedades fisico-mecânicas das formulações. Ela trouxe muitas informações à respeito da performance desses sistemas e foi capaz de predizer comportamentos relacionados ao aspecto sensorial.

Na avaliação da eficácia clínica em curto prazo em população brasileira e francesa, as formulações aumentaram a hidratação e brilho da pele, bem como reduziram a TEWL, atuando na proteção da função barreira da pele das duas populações.

Segundo a análise sensorial, a presença de filtros solares teve um efeito negativo na percepção de consumidores brasileiros e franceses, os quais concordaram que as formulações com esse ingrediente apresentam maior resíduo branco, resíduo oleoso e pior espalhabilidade.

Após tratamento de três meses com a formulação multifuncional, foi observado aumento na hidratação, melhora no brilho e nas condições relacionadas ao fotoenvelhecimento, principalmente quando o extrato de alga vermelha estava presente na formulação.

Por fim, as formulações desenvolvidas foram caracterizadas por diversos testes físicos e estruturais, com destaque para a análise de textura, e os dados obtidos auxiliaram na compreensão do comportamento das mesmas quando em contato com a pele. Além disso, elas apresentaram sensorial agradável e aceito por diferentes populações e eficácia comprovada por técnicas de biofísica e imagem da pele.

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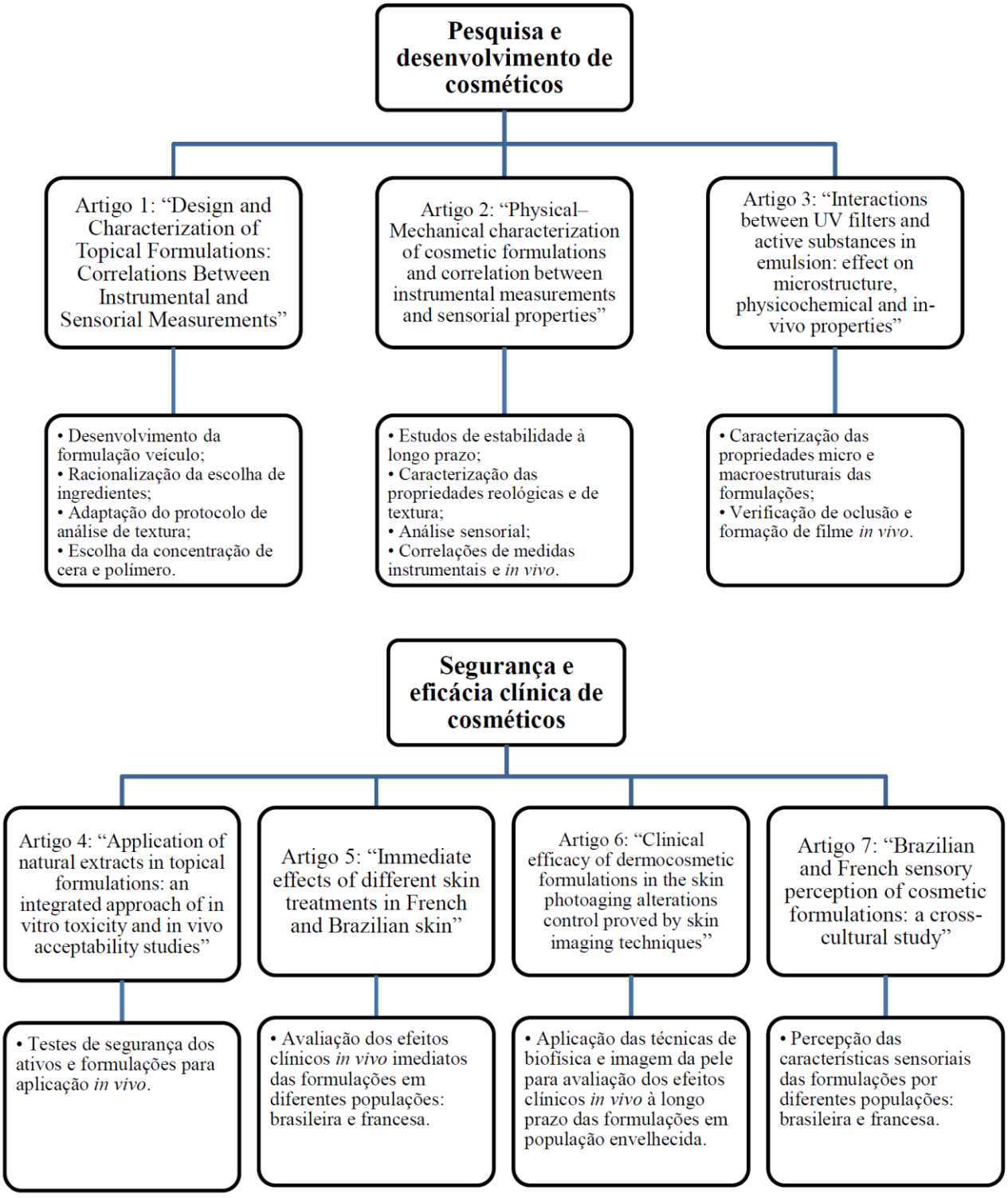
ANEXOS

APÊNDICE 1 – Divisão dos trabalhos em artigos



DICE 2 - Parecer Consubstanciado do Comitê de Ética

FACULDADE DE CIÊNCIAS FARMACÊUTICAS DE RIBEIRÃO PRETO - FCFRP/

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: DESENVOLVIMENTO DE FORMULAÇÕES DERMOCOSMÉTICAS MULTIFUNCIONAIS:AVALIAÇÃO DAS PROPRIEDADES FÍSICAS E DA EFICÁCIA

Pesquisador: Patrícia Maria Berardo Gonçalves Maia Campos

Área Temática:

Versão: 2

CAAE: 43463115.0.0000.5403

Instituição Proponente: Faculdade de Ciências Farmacêuticas de Ribeirão Preto - USP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.343.184

Apresentação do Projeto:

O projeto de pesquisa está relacionado com a área de Pesquisa & Desenvolvimento de Cosméticos. O texto relata a atuação dos dermocosméticos com destaque para os produtos multifuncionais. Inclui resumo, descrição do estado da arte, objetivos, métodos e forma de análise dos resultados, além de referências bibliográficas e cronograma de execução.

Objetivo da Pesquisa:

De acordo com a pesquisadora, o objetivo do trabalho "é desenvolver formulações dermocosméticas multifuncionais, com base nas características estruturais e biofísicas da pele, e avaliar as propriedades físico -mecânicas dessas formulações, bem como estudar a eficácia clínica por meio de técnicas avançadas de biofísica e análise de imagem da pele".

Avaliação dos Riscos e Benefícios:

No caso de eventuais efeitos adversos, haverá acompanhamento pela pesquisadora e pelo médico responsável pela pesquisa (declaração em anexo). No caso de reações adversas, a voluntária será imediatamente excluída da pesquisa e serão tomadas todas as providências necessárias para a sua recuperação. Os benefícios previstos são "Melhora na elasticidade e hidratação da pele da voluntária, além de proteção solar dos fotoprotetores da formulação".

Endereço: Avenida do café s/nº
Bairro: Monte Alegre **CEP:** 14.040-903
UF: SP **Município:** RIBEIRAO PRETO
Telefone: (16)3315-4213 **Fax:** (16)3315-4892 **E-mail:** cep@fcfrp.usp.br

ATENDIDO. A informação está na página 7 do projeto.

4) Na Folha de Rosto o número de telefone para contato da pesquisadora e da Direção da FCFRP/USP está incorreto.
ATENDIDO. Ver novo documento de informações gerais.

5) Qual ou quais as referências que servem como base para apoiar a afirmação que está no TCLE "Serão avaliados os efeitos de uma formulação cosmética contendo microalga Spirulina, obtida por processo biotecnológico, extrato da raiz de chicória, extrato de alfafa e extrato de algo vermelha, a qual é compatível e segura para uso na pele"?
ATENDIDO. O termo biotecnológico foi substituído no novo TCLE. As informações sobre o uso dos ativos em cosméticos estão em anexo.

6) O termo processo biotecnológico no TCLE é de fácil entendimento pelas voluntárias? Não ficou claro como será o processo de recrutamento e seleção das voluntárias
ATENDIDO. O termo foi substituído no novo TCLE.

7) Qual é o critério para a classificação de peles humanas em II, III e IV? O que representa está classificação, pele normal ou pele com dermatoses?
ATENDIDO, a classificação foi incluída na página 9 do projeto de pesquisa.

8) Atenção para o item 5 do TCLE "A disponibilidade de tratamento médico e a indenização que legalmente teria direito, por parte da Instituição à Saúde, em caso de danos que justifiquem diretamente causados pela pesquisa". O texto está confuso e não ficou claro qual a Instituição responsável pelo pagamento da indenização. Seria a FCFRP/USP?
ATENDIDO. O termo foi modificado no TCLE.

Conclusões ou Pendências e Lista de Inadequações:
Não pertinente.

Considerações Finais a critério do CEP:
Aprovado pelo CEP/FCFRP em sua 148ª reunião ordinária. Em atendimento às Resoluções vigentes, deverá ser encaminhado ao CEP, através da Plataforma Brasil, o relatório final da pesquisa conforme modelo de Relatório aprovado pelo Comitê, bem como comunicada qualquer alteração, intercorrência ou interrupção da mesma. Informamos que, de acordo com a Resolução 466/12,

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item IV.5, letra d, o TCLE deve "ser elaborado em duas vias, rubricadas em todas as suas páginas e assinadas, ao seu término, pelo convidado a participar da pesquisa, ou por seu representante legal, assim como pelo pesquisador responsável, ou pela (s) pessoa (s) por ele delegada (s), devendo as páginas de assinaturas estar na mesma folha". Sugerimos que o TCLE seja apresentado ao sujeito da pesquisa em documento impresso frente e verso.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_489720.pdf	16/11/2015 14:15:11		Aceito
Outros	Whitonyl_ExtratodeAlgaVermelha.pdf	16/11/2015 14:13:50	Patrícia Maria Berardo Gonçalves Maia Campos	Aceito
Outros	Vitanol_ExtratodeAlfafa.pdf	16/11/2015 14:13:12	Patrícia Maria Berardo Gonçalves Maia Campos	Aceito
Outros	Vederine_ExtratoderaizChicoria.pdf	16/11/2015 14:12:12	Patrícia Maria Berardo Gonçalves Maia Campos	Aceito
Outros	SpirulinaSafetyIFSCC2015.pdf	16/11/2015 14:10:42	Patrícia Maria Berardo Gonçalves Maia Campos	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_versao2.pdf	16/11/2015 14:09:36	Patrícia Maria Berardo Gonçalves Maia Campos	Aceito
Projeto Detalhado / Brochura Investigador	PROJETOMaiaCampos_versao2.pdf	16/11/2015 14:09:14	Patrícia Maria Berardo Gonçalves Maia Campos	Aceito
Outros	CartadeReenvioCEP.pdf	16/11/2015 14:07:37	Patrícia Maria Berardo Gonçalves Maia Campos	Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_489720.pdf	30/03/2015 16:52:44		Aceito
Folha de Rosto	MV Projetao.jpg	27/03/2015 17:29:16		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

RIBEIRAO PRETO, 30 de Novembro de 2015

Assinado por:
Cleni Mara Marzocchi Machado
(Coordenador)

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UF: SP **Município:** RIBEIRAO PRETO
Telefone: (16)3315-4213 **Fax:** (16)3315-4892 **E-mail:** cep@fcfrp.usp.br