Investigation into the delivery and efficacy of a unique Avena Sativa (Oat) Lipid Extract using Raman spectroscopic, immuno-diagnostic led analysis and skin evaluation.

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Introduction:

The skin's barrier function within the stratum corneum (SC) comprises a complex array of lipids and proteins as a means of protecting the skin and to prevent moisture loss [1,2]. Important factors of the SC lipid behaviour are related to the molecular structures of the lipids, such as the hydrocarbon chain lengths of the ceramides and free fatty acids and the presence of non-saturated free fatty acids [3]. The SC consists of corneocytes embedded in a lipid matrix which forms the only continuous penetration pathway through the SC, and is therefore crucial for the functionality of the barrier [4]. Key lipid classes involved in this process are the ceramides [5,6]. The skins lipid barrier comprises, ceramides, cholesterol and free fatty acids (FFA's). For the formation of the epidermal barrier, FFAs are essential for numerous processes notably the formation of the ceramides of the lipid barrier and to contribute to the structure of the lipid matrix [4]. In addition, cholesterol is required in that it is of fundamental importance for the correct dense lipid organisation of the barrier, and thus for barrier function [7].

Studies have shown that application of a mixture of cholesterol, ceramides, and essential/nonessential free fatty acids (FFAs) in an equimolar ratio enables normal barrier recovery, whereas any 3:1:1:1 ratio of these four ingredients accelerates barrier recovery [8,9]. Xerotic or dry skin is a common occurrence, worsening with age and environmental exposure, and in those skin conditions with a prevalence for dryness [10]. In chronologically aged skin, with decreased epidermal lipid synthesis, and particularly a reduction in cholesterol synthesis, a ratio of 3x-cholesterol to the other lipids was shown to be beneficial also [11]. Omega-hydroxyceramides are required for corneocyte lipid envelope formation and normal epidermal permeability barrier function [12]. Furthermore, it has been shown that lipoxygenases mediate the effect of the essential fatty acid linoleate [13]. This occurs in O-linoleoyl-ω-hydroxyceramide, which, after hydrolysis of the linoleate moiety, is covalently attached to protein via the free ω-hydroxyl of the ceramide, thus, forming the corneocyte lipid envelope, a scaffold between lipid and protein that helps seal the barrier [13].

Emollient products containing various natural oils are essential to any moisturising formulation and may vary depending on the formulators approach. They are now being increasingly recognised for their benefit for a variety of skin diseases and the restoration of cutaneous equilibrium. Unique characteristics of various oils are important when considering their use for topical skin care [14]. Differing ratios of essential fatty acids are major factors of the barrier repair effects of natural oils. Oils with a higher ratio of linoleic acid to oleic acid have stronger barrier repair potential, whereas oils with higher amounts of oleic acid may be detrimental to skin-barrier function, potentially resulting in skin irritation [15,16]. Isolating skin-identical ceramides with the correct stereochemical structures has long been a key desire for the development of effective skin care products.

Restoration of ceramides in depleted skin by phytoceramides from wheat and rice have been shown to provide a benefit in restoring an improvement in the skin's barrier [17,18]. Oats, and their varieties, are globally produced forming an important part of the diet for many people. [19,20]. Oats also possess a variety of beneficial activities especially its anti-inflammatory activity notable in many skin care products in the management of atopic dermatitis. Only in recent years has attention been given to the lipid content of oats, especially its phytoceramide content. Recent studies utilising HPTLC, have shown that glucosylceramides isolated from Ethiopian oat grain (Avena abyssinica) consist of C18- dihydroxy sphingoid bases amide-linked with α- hydroxylated saturated fatty acids (C16-C24) and suggests them to be a potential source of ceramides (CER's) for skin benefits
[21]. Furthermore, it has also been reported that oat lipid extract can activate PPARs and subsequently increase epidermal lipid synthesis and differentiation markers [22]. Oat lipid extracts have been shown to exhibit dual agonism for PPARα and PPARβ/δ and increase direct PPAR target gene induction in primary human keratinocytes. Also, oat oil significantly increases ceramide levels (70%) suggesting a functional translation of PPAR activation by oat oil in keratinocytes and an improved skin barrier function [23].

For proper barrier functioning it is the stereochemical nature, sphingoid base structure, and fatty acid class which are inherent in the skin. Plants contain many so-called phytoceramides but their structure has never been described as skin-identical because of the varied differences in sphingoid bases, which given the rigidity and fluidity between the crystalline states. Furthermore, since cholesterol is not the predominant sterol in plants, unlike the skin, it is argued in the literature that the stereochemical structure of these phytoceramides may not be a replacement for a skin barrier depleted of 'true' ceramides. As such skin-identical lipid mixes are commercially available for skincare use, though these are considered expensive for wider applications, and are derived from bio-fermentation processing.

Several studies have described the isolation and identification of oat lipids and individual oat 'ceramides'. However, as described herein, this is the first reported preliminary investigation of oat lipids utilising confocal Raman spectroscopy for the identification of oat ceramides from Avena sativa. In this investigation, we analysed a specific oat lipid extract, naturally extracted from Avena sativa, using ethanol, comprising 'skin identical' ratios of sterol, fatty acids and phytoceramides. Confocal Raman spectroscopy is a widely accepted sensitive approach for the study of the skin's barrier in a space-resolved manner [23,26]. In order to gain a further understanding of the lipid nature and its effects on skin barrier lipids, we utilised a multi-method approach led by Raman spectroscopy, transmission electron microscopy (Lipbarvis®), and immunostaining, to ascertain the structure, and beneficial efficacy of Oat Lipid Extract, comprising a complex of ceramides, polyunsaturated fatty acids, sterols, phospholipids, triacylglycerols, tocopherols, tocotrienols and other polar lipids.

Materials & Methods:

Oat Lipid Extract is naturally derived from a previously unconsidered by-product of the fractionation of oat oil from the oat kernel. The by product is produced as a viscous residue during the extraction of oil from oats by fractionation with a polar solvent. Neutral and polar lipids may be separated from oats by extracting using a polar solvent such as ethanol, however high levels of residual solvents and sugars and low proportion of desirable skincare components make this an unusable ingredient in personal care. Oat Lipid Extract has been developed as an oat oil fraction which has been further refined, to contain high polar lipid content and other desirable components, but which does not contain significant water / solvent residue.

Lipid profiling
Profiles of lipid classes and total amounts of Oat Lipid Extract were carried out using GLC and HPTLC.
Lipid class analyses - Oil lipid class compositions were determined by single- dimension double-development high-performance thin-layer chromatography (HPTLC).

Oxidative stability of OAT LIPID EXTRACT
Analysis of the oxidative stability of the Oat Lipid Extract [29] was performed using the RapidOxy method (Anton Paar, Germany)

Confocal Raman Spectroscopic lipid analysis
Raman spectra were obtained using a confocal Raman spectroscopy Xplora (Horiba, Jobin Yvon) performed on 10 μm thick frozen skin sections in the presence of the test product using a 532 nm laser.

Skin Samples
The potential of the Oat Lipid Extract to increase the ceramide content of human epidermis was measured using confocal Raman spectroscopy and immunostaining of human skin using an ex-vivo Perfex™ abdominoplasty model [31].
Immunostaining: After fixation for 24 hours in buffered formalin, skin samples were dehydrated and impregnated in paraffin using a Leica PEARL dehydration automat and then embedded using a Leica EG 1160 embedding station.
Polar lipids were stained on frozen sections using ReZolve-L1™ reagent (Rezolve scientific).
The effects of the Oat Lipid Extract compounds were evaluated using RT-qPCR technology in reconstructed human epidermis (RHE).

Lipid Lamellar Formation - Electron Microscopy Analysis (Lipbarvis® TEM)
The effects of Oat Lipid Extract were further evaluated via Lipbarvis® transmission electron microscopy (Dähnhardt GmbH).
Oat Lipid Extract Treatment and Suction Blisters: According to Good Clinical Practice
(GCP) the test product Oat Lipid Extract was applied to the forearms of six healthy female caucasian consenting volunteers willing to comply with the inclusion and exclusion criteria of the protocol. Transmission Electron Microscopy (Lipbarvis® TEM): One small part of each suction blister samples (ø 3 mm) was fixed overnight in Karnovsky's medium at 4°C, washed twice with 0.2M sodium cacodylate buffer for 10min each, and postfixed with 1% RuO4 in 0,13M sodium cacodylate buffer at 4°C for 90 minutes. Specimens were then washed twice with aqua bidest, dehydrated in an ethanol series and embedded in epoxy resin. Polymerisation was carried out overnight at 60°C. Briefly, perpendicular sections of the suction blister samples were prepared with an ultramicrotome (Ultracut S Leica Microsystems, Wetzlar, Germany) using a diamond knife (35°, Diatomee, Switzerland). Sections were mounted onto copper grids. The subsequent counterstaining was carried out with uranyl acetate and lead citrate. For TEM examination a TEM CM 10 (FEI, Eindhoven, Netherlands) with an acceleration voltage of 80 kV was used. Images were captured with a CCD camera (IDS, Obersulm, Germany) connected directly to the TEM.

Statistics
Analysis of the study objectives was performed using Microsoft Excel and Statistica (StatSoft).

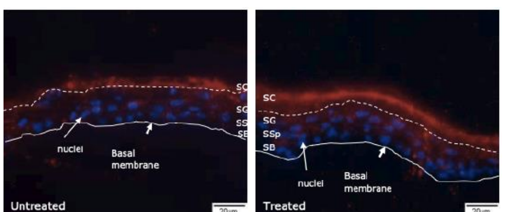
Results & Discussion:

Approximate Composition*	Percentage
Ceramides	1.36
Hydroxyceramides	
Glycosyl Inositol Phosphoryl Ceramides (Proceramide)	1.32
Glucosylceramide	1.32
Total Ceramides	4.00

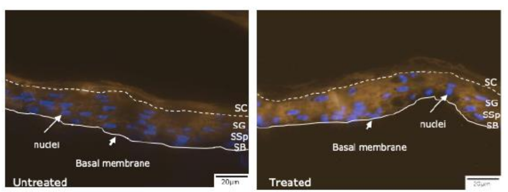
Ceramide Class*	Oat Lipid Extract Skin Identical (%)	Oat Lipid Extract Total incl. Isomers (%)
Non-hydroxy-sphingosine [NS]	0.07	0.52
Non-hydroxy-phytosphingosine [NP]	0.13	0.13
Omegahydroxy-6-hydroxy-sphingosine [EOH]	0.47	0.47
Alphahydroxy-sphingosine [AS]	0.05	0.19
Alphahydroxy-phytosphingosine [AP]	0.05	0.05
Total	**0.78**	**1.36**

Table II: Ceramide classes present in Oat Lipid Extract

In addition a large proportion of phospholipids were also present (15mg/100g) with phosphatidylcholine being the most predominant species. The largest proportion of lipids present were identified as neutral lipids (60mg/100g) comprising triacylglycerols (37mg/100g), free fatty acids (11mg/100g), sterols (10mg/100g) and diacylglycerol (2mg/100g). Not surprisingly the sterols were identified mainly as β– sitosterol and avenasterol. Fatty acid profiling from neutral and polar fractions revealed relatively high amounts of both mono- and polyunsaturated fatty acids (12.97mg/100g and 10.87mg/100g respectively), as compared to saturated fatty acid content (5.45mg/100g).



The immunofluorescence labelling of hyaluronic acid (red) and nuclei staining (blue) in semithin sections of the suction blister samples. In both samples (treated and untreated) the red fluorescence labelling is clearly detectable in all layers (stratum corneum (SC), stratum granulosum (SG) stratum spinosum (SSp) and stratum basale (SB)) of the epidermis. The basal membrane and the interface between SG and SC are marked by lines. The intensity of fluorescence in the treated samples is significantly higher than in the untreated samples.



Ceramides fluorescent staining. Untreated: test site without product treatment, Treated: Oat Lipid Extract treatment twice daily for 8 weeks. Observations show immunofluorescence labelling of the ceramides (yellow) and nuclei staining (blue) in semithin sections of the suction blister samples. In both samples (treated and untreated) the yellow fluorescence is distributed over the whole epidermis. In the lower layers of the epidermis (SSp & SG) mostly glycosylceramides are labelled whereas in the SC only ceramides are labelled. The basal membrane and the interface between SG and SC are marked by lines. The intensity of fluorescence in the treated samples is significant higher as in the untreated samples.

Conclusions:

Oat ceramides in the form of Oat Lipid Extract can be effectively delivered into the stratum corneum and are proven to be a good source of skin supplementing lipids. Our profiling has shown that Oat Lipid Extract is unique in its composition, containing phytoceramides, and a significant proportion of the ceramide classes required by the skin. This unique ratio is indicative of skin barrier improvement, which we have demonstrated via immunostaining, Raman analysis, Lipbarvis® TEM and immunostaining, as well as showing an up-regulation hyaluronan synthase through a gene array study.

Further studies are required to provide evidence of the liquid crystalline changes that occur, and the molecular arrangement in the stratum corneum remains to be investigated. This preliminary Raman study has given good insight into the possibility of Oat Lipid Extract mimicking the structure and function of the skin's barrier.

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