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Stratum corneum lipids: the effect of ageing and the seasons

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Abstract **Stratum corneum lipids play a predominant role in maintaining the water barrier of the skin. In order to understand the biological variation in the levels and composition of ceramides, ceramide 1 subtypes, cholesterol and fatty acids, stratum corneum lipids collected from tape strippings from three body sites (face, hand, leg) of female Caucasians of different age groups were analysed. In addition, we studied the influence of seasonal variation on the lipid composition of stratum corneum from the same body sites. The main lipid species were quantified using high-performance thin-layer chromatography and individual fatty acids using gas chromatography. Our findings demonstrated significantly decreased levels of all major lipid species, in particular ceramides, with increasing age. Similarly, the stratum corneum lipid levels of all the body sites examined were dramatically depleted in winter compared with spring and summer. The relative levels of ceramide 1 linoleate were also depleted in winter and in aged skin whereas ceramide 1 oleate levels increased. The other fatty acid levels remained fairly constant with both season and age, apart from lignoceric and heptadecanoic acid which showed a decrease in winter compared with summer. The decrease in the mass levels of intercellular lipids and the altered ratios of fatty acids esterified to ceramide 1, are likely to contribute to the increased susceptibility of aged skin to perturbation of barrier function and xerosis, particularly during the winter months.**

Key words Stratum corneum lipids · Ceramides · Ageing · Seasons · Ceramide 1 linoleate

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Introduction

The unique morphology of the stratum corneum, often simplified to a ‘bricks and mortar’ analogy, is essential for maintaining the water barrier of the skin [4, 10, 18, 19]. The protein-enriched corneocytes are embedded in an intercellular lipid matrix which is composed primarily of ceramides, cholesterol and fatty acids together with smaller amounts of cholesterol sulphate, glucosylceramides and phospholipids. These lipids form multilamellar sheets within the intercellular spaces of the stratum corneum, the organization of which are essential in maintaining the functionality of the skin as an effective barrier to water loss [11, 14]. In addition, they also play an important role in determining the mechanical [29], cohesive [31] and desquamatory [12, 31] properties of the stratum corneum, and therefore have a key role in skin physiology. Depletions in lipid levels as a result of environmental challenges can lead to disturbances in skin function. Changes in stratum corneum lipid levels have been linked with aberrant skin conditions, such as xerosis [3, 31, 34] and decreases in the ceramide levels of the stratum corneum have been proposed as a possible aetiological factor in atopic dermatitis [20].

The incidence of skin xerosis appears greater both with increasing age and during the harsher climatic conditions of the winter season. Although a number of investigators have tried to associate lipid changes with the increased incidence of skin dryness, the evidence remains contradictory. Japanese forearm skin has been reported to show decreases in ceramide levels with ageing [20], whereas no alterations have been found in leg stratum corneum lipids [3]. In addition, decreased stratum corneum lipid levels have been reported in winter xerosis [3, 31], whereas no seasonal changes have been observed in a more recent study on Japanese subjects [43]. Similarly, in terms of water barrier effectiveness, the evidence is also conflicting. Aged skin has been shown to have a barrier more readily penetrated by certain compounds [22], although others have demonstrated that although there is no change in wa-

ter loss with ageing [13, 33], there is an increased susceptibility to damage and a delay in barrier recovery [13].

Ceramide 1 linoleate is the main repository of epidermal linoleic acid [2] and changes in its levels are associated with cutaneous abnormalities: essential fatty acid deficiency (EFAD) [7], atopic dermatitis [44] and acne [42]. In EFAD, the replacement of linoleate with oleate in ceramide 1 is associated with dramatic perturbations in the ultrastructure of the stratum corneum lipids [17]. However, there is only limited information on the biological variation in ceramide 1 fatty acid levels in humans. The present study was designed to gain a greater understanding of the biological variation in stratum corneum lipid levels and their relationship with skin condition. In particular, to examine the influence of ageing and of the seasons on the stratum corneum lipid levels of different body sites in female Caucasians.

Materials and methods

Subjects

A group of 49 healthy female Caucasian volunteers ranging in age from 21 to 60 years took part in the age study. All the subjects had normal skin as judged by visual assessment. They were divided by age into three main age groups (Table 1), the additional subjects over 50 being used to determine the fatty acid species profile. For the seasonal study, 26 healthy female Caucasian volunteers were selected and were sampled during summer (August), spring (April) and winter (January). The sample numbers for each season are shown in Table 1.

Sample collection

Skin samples were removed from the appropriate body sites by sequential stripping with eight tape strips (Tape 1601, Hadleigh Enterprise, Wickford, Essex, UK). All tapes were stored at –20° C until required. Corneocytes were removed from the tapes by sonication in methanol. The squames were then dried under nitrogen and the lipids were extracted with chloroform/methanol (2:1) for 2 h.

Analysis of stratum corneum lipids

The lipid extracts were evaporated under nitrogen, reconstituted in chloroform (200 μl) and separated on amino-propyl bonded silica gel columns (100 mg Bond Elut NH2, Analytichem International, USA) preconditioned with hexane (2 ml). The samples were loaded onto the columns and cholesterol, ceramide and fatty acid fractions were eluted with 2 ml hexane/ethyl acetate (85:15), chloroform/isopropanol (2:1) and 2% acetic acid in methanol, respectively. All the lipids were then separated using high-performance thin-layer chromatography (HPTLC) on 20 × 10 cm plates (Merck, Darmstadt, Germany). The ceramide species were separated according to the method of Wertz et al. [42] and the fatty acids and cholesterol were separated by eluting up the plate with hexane/ethyl acetate (85:15, then 70:30) to 95 mm. After development, the HPTLC plates were dried, stained (aqueous solution containing 10% copper sulphate, 8% orthophosphoric acid) and then charred at 160° C for 20 min. Finally, the HPTLC plates were cooled and quantitated using a scanning densitometer at 420 nm (Shimadzu CS9000, Shimadzu UK, Phillip Howe Scientific, London, UK). Lipids were identified by their comigration with cholesterol, palmitic acid and *N*-stearoyl DL-dihydrosphingosine standards (Sigma Chemical Company, Poole, UK). Ceramide subclasses were also identified by their rf values obtained from the literature [40, 41]. The mass of each lipid was determined from the appropriate series of standards chromatographed on each plate.

Table 1 Subject groups and sampling strategy

Body site	Subject numbers					
	Age groups (years)			Seasonal sampling		
	21–30	31–40	41–50	Summer	Spring	Winter
Hand	14	14	21	26	5	17
Face	10	6	9	6	0	6
Leg	10	0	10	9	0	9

Ceramide 1 esterified and free fatty acid analysis

Samples of the ceramide fractions from the leg site in the seasonal and age studies were also used to analyse ceramide 1 esterified fatty acids. To isolate ceramide 1 a portion of the ceramide fractions was chromatographed as described above. To locate ceramide 1, CAMAG Test Dye III (Camag Scientific, Wilmington, N.C.), which contains a component comigrating with ceramide 1, was loaded on the end lanes of each plate. Silica gel, containing ceramide 1 was scraped from the plates and extracted in chloroform/methanol (2:1, 2 h). Arachidonic acid (1 μg) was added to the extraction solvent as an internal standard and the extract was filtered through a syringe filter (Gelman Acrodisc CR PTFE 0.45 μm) to remove the silica gel, then dried under nitrogen. The fraction was hydrolysed in 1.2 *N* NaOH in 95% methanol (60° C, 1 h) and then acidified with 0.5 *N* HCl. The methanol was evaporated and the residue redissolved in chloroform. Fatty acids were then purified using solid phase chromatography as described above. Following derivatization of the fatty acids with diazomethane the samples were dried, redissolved in hexane and the fatty acid methyl esters (FAMEs) were analysed by gas chromatography. FAMEs were separated using a silica capillary column crosslinked with polyethylene glycol (SGE Chromatography Products, Milton Keynes, UK. 25QC5/BP20–1.0, 25 m long and 0.53 i.d.). The inlet temperature was set at 250° C and the helium flow rate at 8 ml/min. The temperature programme was set at 150° C for 2 min and programmed for a 4° C rise per min to a final temperature of 220° C for 20 min. The separated fatty acids were quantitated using lauric acid as an internal standard and their levels expressed as relative percentages.

Analysis of stratum corneum protein levels

To allow interperson comparisons, the mass of each lipid fraction was normalized to the amount of stratum corneum removed by tape stripping. Stratum corneum removal was estimated by quantification of the detergent-soluble protein of the corneocytes extracted under reducing conditions. The lipid-depleted corneocytes were extracted with 1% sodium dodecyl sulphate, 10 m*M* sodium phosphate buffer and 20 m*M* β-mercaptoethanol at 60° C for 2 h. Aliquots of the protein extracts were then dried, reconstituted in water and quantitated using a modified Pierce BCA protein microassay. Protein concentrations were determined by their absorbance at 540 nm and compared with a range of bovine gamma globulin standards. All the lipid levels were expressed as nanograms lipid per microgram protein.

Results

Age-related changes

The age related changes in the total stratum corneum levels are shown in Fig. 1. All the lipid classes decreased

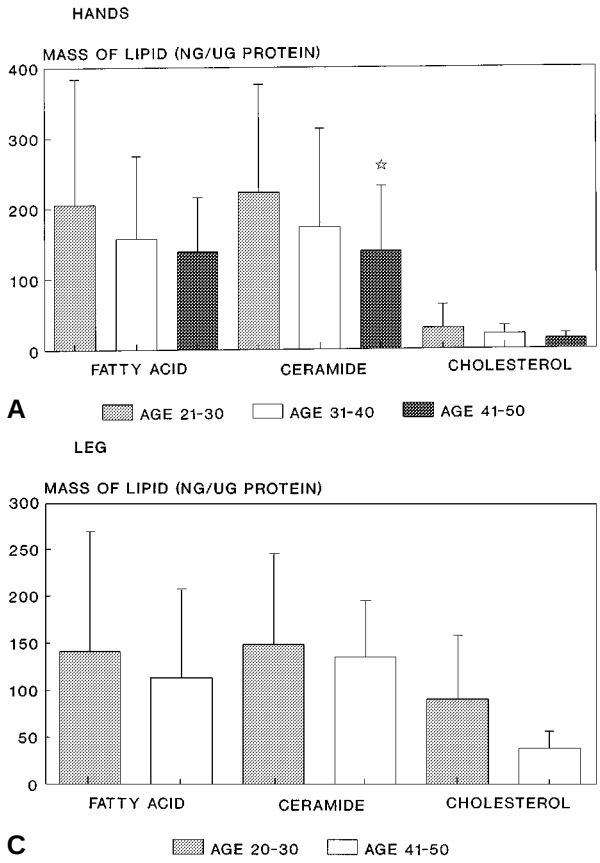

Fig. 1A–C Age changes in the stratum corneum lipids of the hands (**A**), face (**B**) and leg (**C**) shown as means ± standard deviation. *Asterisks* indicate statistically significant reductions in lipid levels compared with the 21–30-year age group calculated using analysis of variance and unpaired Student's *t*-test

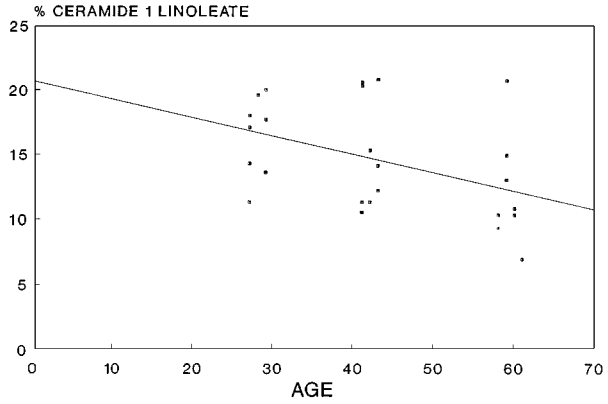

Fig. 2 Age changes in the relative percentage of ceramide 1 linoleate. Linear regression; $P = 0.03$

with increasing age. This was most marked for the levels of all the ceramide species (1–6) in the face and hand, and cholesterol in the face ($P < 0.05$). Although total lipid levels decreased, the percentage ratios of each of the major lipid classes and of the individual ceramide species (1–6) remained constant. Interestingly, body site variations in stratum corneum lipid levels were also observed, the levels being far higher in the face than the hand and leg. In addition, the levels of ceramide 1 linoleate also decreased with increasing age (Fig. 2, Table 2). There were no significant alterations in the other ceramide 1 esterified fatty acids with age (Table 2) or free fatty acid species (Table 3).

Table 2 Ceramide 1 ester-linked fatty acid composition (leg). Values are weight % (mean ± SD)

Fatty acid species	Age group (years)		
	26–29 (n = 9)	41–43 (n = 9)	57–60 (n = 10)
16:0	17 ± 8.3	16 ± 6.3	20 ± 7.0
18:0	21 ± 5.4	26 ± 6.3	24 ± 7.1
18:1	30 ± 10	31 ± 8.6	35 ± 8.0
18:2	15 ± 4.3	15 ± 4.3	11 ± 4.0*
24:0	17 ± 16	12 ± 6.6	10 ± 4.6

* $P < 0.05$ compared with 26–29-year age group

Table 3 Relative amounts of free fatty acids in relation to age (leg). Values are weight % (mean ± SD)

Fatty acid species	Age group (years)	
	26–29 (n = 9)	57–60 (n = 10)
14:0	4.7 ± 1.1	3.4 ± 1.1
16:0	23 ± 3.1	22 ± 4.9
16:1	5.2 ± 1.6	2.6 ± 0.7
18:0	16 ± 5.4	23 ± 7.9
18:1	26 ± 8.7	16 ± 8.0
18:2	4.0 ± 1.6	2.6 ± 0.8
20:0	1.8 ± 0.8	2.4 ± 0.9
22:0	2.8 ± 1.0	5.9 ± 2.5
24:0	8.6 ± 3.3	18 ± 6.8

Seasonal changes

The seasonal variations in stratum corneum lipid levels for each of the body sites are represented in Fig. 3. A pronounced seasonal decline in all the lipid levels from summer to spring and winter was observed. As with the age-

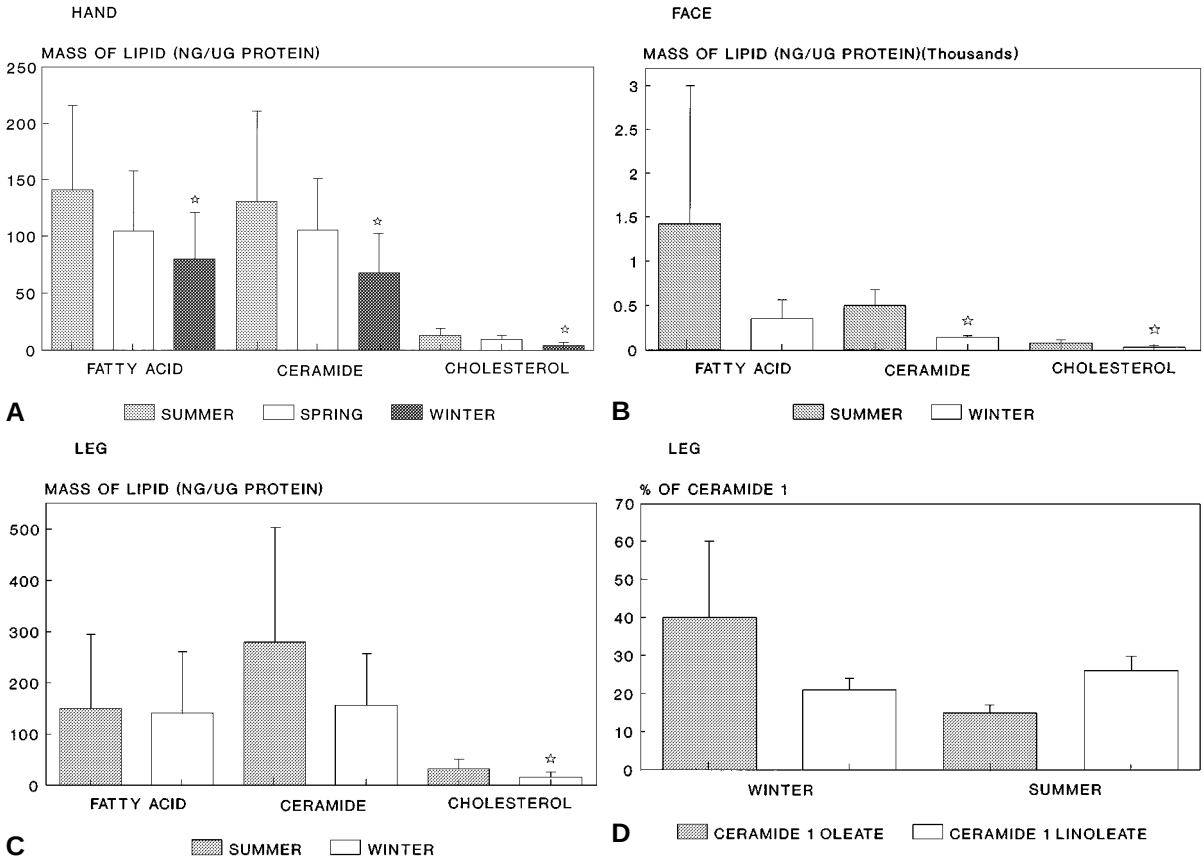

Fig. 3A–D Seasonal changes in the stratum corneum lipid levels of the hands (**A**), face (**B**) and leg (**C**) shown as means ± standard deviation. *Asterisks* indicate statistically significant reductions in the lipid levels in spring and summer compared with winter calculated using analysis of variance and unpaired Student's *t*-test. Seasonal changes are shown in the relative proportions of ceramide 1 esterified fatty acids (**D**)

Table 4 Seasonal variations in the relative percentages of ceramide 1 esterified fatty acids (leg). Values are weight % (± SD)

Fatty acid species	Age group (years)	
	Summer (n = 7)	Winter (n = 10)
15:0	5.1 ± 1.8	1.6 ± 3.5
16:0	6.7 ± 3.9	9.1 ± 4.1
17:0	10 ± 2.1	0.6 ± 2.0*
18:0	26 ± 4.1	22 ± 10
18:1	15 ± 1.7	40 ± 20*
18:2	26 ± 3.7	21 ± 3.2*
24:0	12 ± 4.5	6.8 ± 3.5*

* $P < 0.05$

Table 5 Seasonal variations in the relative percentages of free fatty acids (leg). Values are weight % (± SD)

Fatty acid species	Age group (years)	
	Summer (n = 7)	Winter (n = 10)
14:0	5.3 ± 0.1	0.1 ± 0.3
16:0	18 ± 6.1*	8.0 ± 3.6
16:1	4.0 ± 2.2*	1.1 ± 0.5
18:0	22 ± 7.0	26 ± 5.6
18:1	24 ± 7.2	30 ± 7.0
18:2	5.6 ± 3.4	5.9 ± 3.1
18:3	1.1 ± 1.2	0.7 ± 0.5
20:0	3.0 ± 1.8	2.7 ± 0.7
22:0	4.8 ± 2.3	6.3 ± 1.4
24:0	8.9 ± 5.2*	14 ± 4.6
Others	3.3 ± 1.2*	5.2 ± 2.5

* $P < 0.05$

related changes, all the lipid classes decreased to the same extent, so the relative proportions were maintained. As can be seen from Table 4 there was a 20% decrease in ceramide 1 linoleate levels in winter compared with summer. In addition, the ratio of ceramide 1 linoleate to ceramide 1 oleate showed a dramatic change from the summer months to the winter months (1.74 to 0.51; Fig. 3). Decreases in the percentage levels of esterified saturated fatty acids were also apparent in winter, mainly due to decreases in lignoceric and heptadecanoic acid and the marked elevation in oleic acid. Overall, these changes led to a greater degree of fatty acid unsaturation in winter compared with summer. Alterations in the ratios of the free fatty acids were also observed with the seasons (Table 5). In summer the relative levels of palmitic and

palmitoleic acids were increased and lignoceric acid was decreased.

Discussion

The levels of the intercellular lipids of the stratum corneum, mainly ceramides, fatty acids and cholesterol, are known to be important for water permeability barrier function [4] and normal skin functioning [31]. Although recent studies have suggested that there are significant ethnic differences in stratum corneum lipid profiles [37], our study confirms that Caucasian skin shows a similar age-related decline in stratum corneum ceramide levels to that previously reported for Japanese subjects [20]. The age-related decline was not restricted to ceramides. We also found significant reductions in facial cholesterol levels and numerical decreases in fatty acid levels. Indeed, the ratios of the different lipids remained constant. These findings mirror those previously observed in murine stratum corneum [13]. Overall, the total lipid levels were decreased by approximately 30%, which may reflect the slower keratinocyte metabolism of the aged [15], leading to decreased biosynthetic capacity.

In this study, we showed that the levels of stratum corneum lipids in a group of Caucasian females were subject to dramatic seasonal variation. All the lipid species analysed were depleted in winter compared with summer, mirroring the ageing influence on stratum corneum lipid levels. The seasonal variation in lipid levels observed in this study is consistent with the decrease in stratum corneum lipid levels in winter xerosis [31] and the winter decline in skin surface lipids [1] observed by others. Our findings are also consistent with the seasonal decreases in epidermal glucosylceramide levels reported by Niemenen et al. [25]. However, these studies are in marked contrast to a recent mixed-sex study performed by Yoshikawa et al. on Japanese subjects, in which no seasonal changes in lipid levels were apparent [43]. Although the control mechanisms that regulate lipid biosynthesis are not fully understood, the differences between the results reported here and those of Yoshikawa et al. may reflect gender-related differences. Female sex hormones have been shown to influence skin thickness [5, 36] and keratinocyte proliferation [38] and are implicated in sphingolipid lipogenesis [8]. In addition, the seasonal decrease in stratum corneum lipid levels we observed may reflect circannual fluctuations in hormone levels. Alternatively, decreases in skin temperature [1] may influence the overall biosynthetic capacity of the epidermis, leading to decreased lipid biosynthesis in winter.

In addition to the age and seasonal decline in the mass levels of the different ceramide species, the relative proportion of ceramide 1 linoleate was decreased in winter compared with summer and in aged leg stratum corneum. The crucial role of essential fatty acids in stratum corneum function has been appreciated for decades [7]. In EFAD the replacement of linoleate with oleate in ceramide 1 is associated with dramatic perturbations to the ultrastructure of the stratum corneum lipids and desquamatory abnormalities [17]. EFA insufficiency is also associated with other less-serious conditions. For example, ceramide 1 linoleate levels are reduced in atopic dermatitis [44], acne [42] and dry skin [6]. Recent studies in our own laboratory have shown that ceramide 1 linoleate is important for maintaining stratum corneum flexibility [28] and bilayer fluidity [26] as previously observed for glycerol [30]. We envisage that the depletion of ceramide 1 linoleate both with increasing age and in winter may contribute to the formation of an intrinsically weaker stratum corneum with an increased susceptibility to dysfunctions of desquamation, e.g. xerosis. In addition to the changes in ceramide 1 linoleate, there was a marked elevation in esterified oleic acid and decline in lignoceric and heptadecanoic acid percentage levels in winter. This will lead to a greater degree of fatty acid unsaturation, disrupting lipid packing and contributing towards an impaired barrier during the winter season. Seasonal changes were also observed in the relative amounts of the free fatty acids: increased levels of both palmitic and palmitoleic acids were observed in summer. The changes in the lipid levels between the seasons are believed to be due to intrinsic differences in the epidermal lipids. However, it is possible that during summer, sebum-derived free fatty acids may contribute slightly to the free fatty acid pool, although this was established as negligible using squalene as a marker.

Despite a marked age- and seasonal-related decline in lipid levels, transepidermal water loss does not appear to increase with either age [13] or season [1]. The preservation of barrier function may in part be due to the stratum corneum maintaining the relative proportions of the lipids present and hence the organisation of the lipid bilayers [23]. It should also be emphasized that the effectiveness of the stratum corneum water barrier is dependent not only on its lipid content and composition, but also on its overall morphology [9, 27]. Indeed, the increase in corneocyte size that accompanies both the ageing process [24] and exposure to winter conditions [16] may compensate for the lipid depletion. Although water loss from the skin does not appear to alter with age or season, the reduction in lipid levels observed may explain the increased susceptibility of the barrier to damage in the elderly [13] and the increased incidence of xerosis in winter [3, 31, 34]. The reduction in lipid levels may in turn reduce the water content of the stratum corneum. This may influence the activity of the stratum corneum proteases thought to be involved in desquamation [21, 31, 32, 39] and will interfere with the generation of natural moisturizing factors [35] leaving the stratum corneum more prone to xerosis.

In summary, the present study demonstrated a seasonal and age-related reduction in stratum corneum lipid levels probably reflecting decreased epidermal lipid biosynthesis. As the expression of skin xerosis is related to the levels and types of stratum corneum lipids, these changes are likely to contribute to the increased susceptibility of aged skin, and skin in winter to xerosis.

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