5. RESULTS AND DISCUSSION

5.1 BASIC CHARACTERISATION OF THE RECONSTRUCTED SKIN MODELS

According to the OECD TG 431, the reconstructed human skin models used for the *in vitro* skin corrosion tests should be sufficiently characterised and the skin model have to accomplish several requirements with regard to the structure and barrier.

In the current study, the three most frequently used, commercially available reconstructed human skin models - EpiDerm, EPISKIN and SkinEthic - were evaluated. The tissue architecture was analysed using light and confocal laser scanning microscopy. Epidermal lipid composition was determined by HPTLC and the barrier resistance was evaluated in a time course assay with Triton X-100 in combination with measurements of the Transepidermal Electrical Resistance (TEER).

5.1.1 MORPHOLOGY OF THE RECONSTRUCTED HUMAN SKIN MODELS

Morphology of the reconstructed human epidermal models was evaluated using vertical sections stained with hematoxylin-eosin as described in Materials and Methods. The analysis was focused on overall tissue architecture, evaluation of number of viable cell layers and thickness of the stratum corneum.

Investigation of vertical paraffin sections revealed characteristic epidermal stratification in all three models. All cell layers, typical for human epidermis, were presented including the *stratum basale* (SB), *stratum spinosum* (SS), *stratum granulosum* (SG) and anculeated *stratum corneum* (SC).

Epithelium of EpiDerm cultures consisted of 8-12 viable cell layers (see Figure 23 a). The basal cell layer showed regular organisation, differentiating into the stratum spinosum and 2-3 layers of stratum granulosum, containing keratohyaline granules. The *stratum corneum* was relatively thin (15-30 µm) however organised in manner typical for normal human epidermis *in vivo*. The overall tissue architecture (thin SC and numerous viable cell layers) indicates young epidermis.

The number of viable cell layers in the EPISKIN model varied between 8-12, too. Basal cell layer was regularly organised and differentiating into the well-developed stratum spinosum and stratum granulosum. In lower parts of the epidermis, intercellular spaces were clearly visible. In comparison to EpiDerm and SkinEthic models, the stratum corneum was remarkably thicker (see Figure 23 b).

SkinEthic cultures contained a lower number of viable cell layers (between 7-10) which is typical for skin models in later stage of cultivation (SkinEthic model is cultured for 17 days before supply to customers). The stratum corneum was approximately 20-40 µm thick, and the corneocyte layers were more densely packed in comparison to SC of EPISKIN cultures.

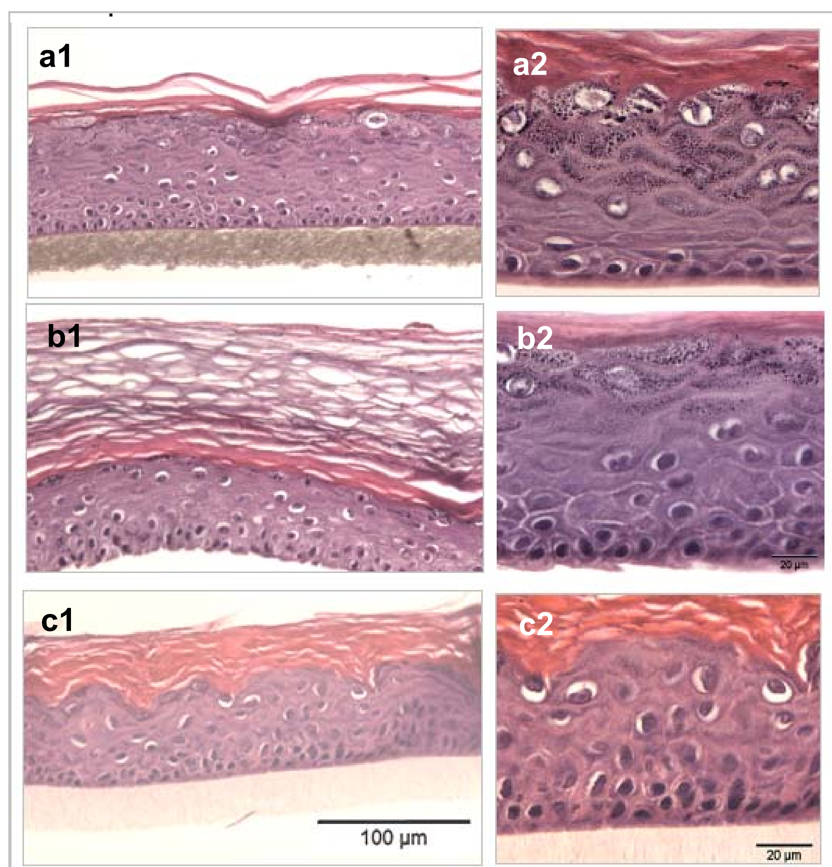


Figure 23. Representative histology images of the three reconstructed human skin models.
a) EpiDerm model (1-magnification 200 x, 2-magnification 600 x)
b) EPISKIN model (1-magnification 200 x, 2-magnification 600 x)
c) SkinEthic model (1-magnification 200 x, 2-magnification 600 x)

Table 19. Basic characteristics of the three reconstructed human skin models.

Reconstructed human skin model	Days of cultivation before supply	Model Size (cm^2)	Number of viable cell layers	Approximate thickness of the viable epidermis (µm)	Approximate thickness of the stratum corneum (µm)
EpiDerm	10	0.63	8-12	60-100	15-30
EPISKIN	13	1.1	8-12	60-100	80-100
SkinEthic	17	0.5	7-10	40-60	20-40

All three models demonstrated architecture, which is similar to human epidermis. The variation between tissues within production batch was very low. Similarly, variation between batches was only minor, usually related to the thickness of the viable epidermis. The overall tissue architecture remained constant. Immunohistochemical analysis (investigation of expression and localisation of protein differentiation markers) would better reveal differences between the three reconstructed human skin models, given these do exist.

5.1.2 CONFOCAL LASER SCANNING MICROSCOPY

Confocal laser scanning microscopy (CLSM) allows a functional characterisation of the permeability properties of the skin by direct visualisation of diffusion pathways and cellular distribution of fluorescence markers. Therefore this technique was used to evaluate if the epidermal models EpiDerm, EPISKIN and SkinEthic show the functional characteristics of the human *stratum corneum*.

The cultures were topically stained with sodium fluorescein and the dye was visualised using a dermatological laser scanning microscope (Stratum, OptiScan, Australia), which does not require fixation of the samples in special chambers. The models were investigated directly in the inserts after placement of the laser pen on a tissue surface.

Approximately 5 minutes after application of a fluorescent dye on the tissue surface, the first layers of the stratum corneum became visible. When the dye had reached deeper layers because of the penetration process, these could be visualised too. After 15-20 minutes, the shifted structure of 4 layers of the stratum corneum became visible.

The fluorescence images of the stratum corneum of the native human epidermis demonstrated that the fluorescent dye is mainly located in the lipid layers surrounding the corneocytes (Figure 24, A1-A3). The analysis of the reconstructed human skin models revealed, that they resemble the human *stratum corneum (*Figure 24, B1-D3). However, in some cases, penetration of the fluorescent dye through the corneocytes of the reconstructed epidermis was observed, which indicate altered barrier properties.

Yet, it is of note that the visualisation was difficult since the reconstructed epidermis is much more fragile than the human skin. The *stratum corneum* was frequently damaged by the monitoring procedure using the laser pen. Separation of the SC from the viable epidermis by mechanical pressure during the scanning was frequently observed in EPISKIN cultures. In addition, traces of the medium supplements still present in the reconstructed skin models, affected the quality of visualisation.

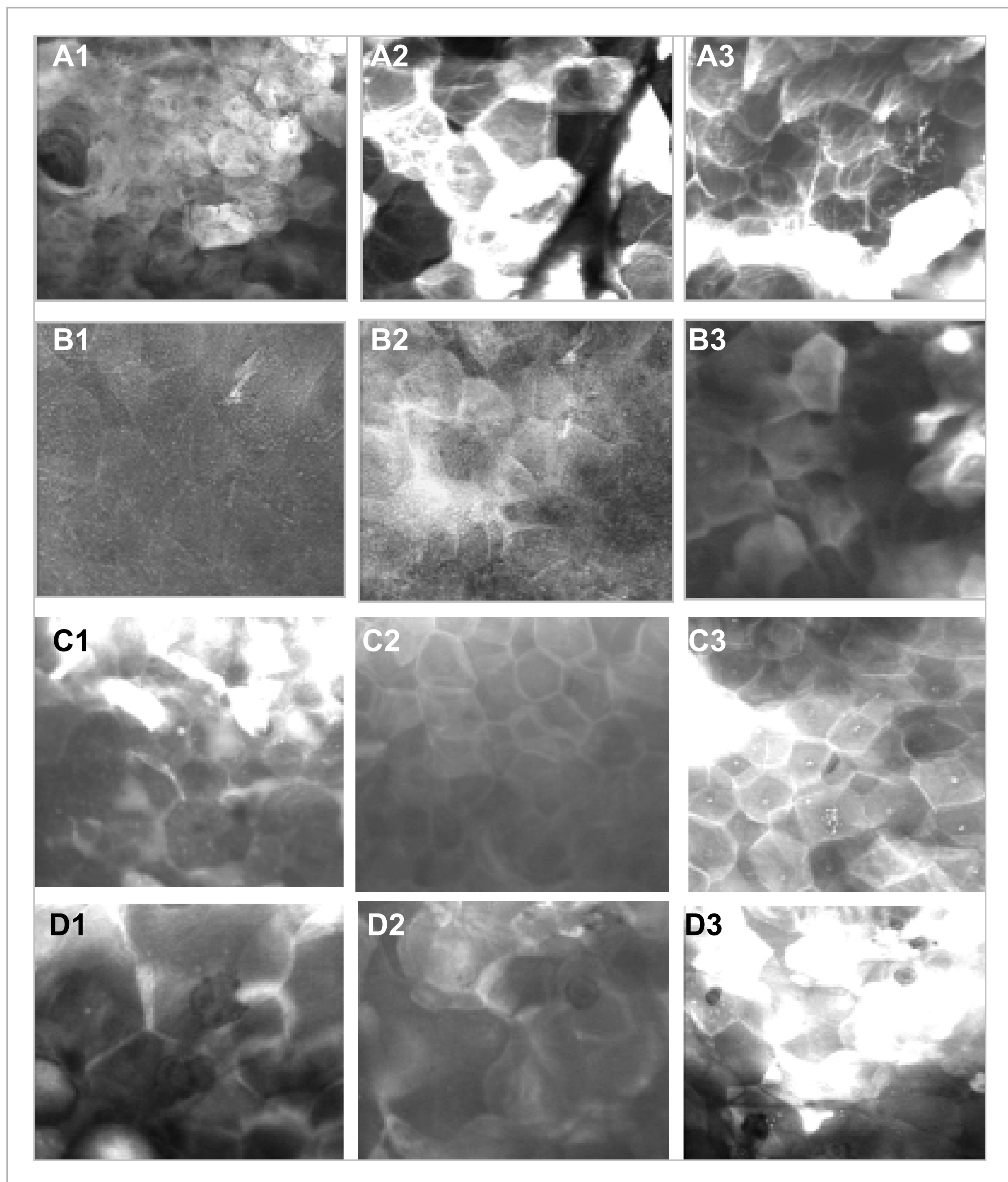


Figure 24. Penetration of fluorescent label across native human epidermis and reconstructed human skin models (representative images). The hexagonal shape of the stratum corneum cells is clearly visible.
A1-A3 Human SC in vivo (forearm, female, age 28 years),
B1-B3 EpiDerm model, C1-C3 EPISKIN model, D1-D3 SkinEthic model

5.1.3 LIPID ANALYSIS

For the normal barrier function of the stratum corneum, both the composition and the organisation of the SC lipids are of crucial importance. Although air-exposed reconstructed human skin models synthesise all lipid classes present in native tissue, some lipids are synthesised and/or metabolised at rates different from those in human skin (Ponec et al., 1998).

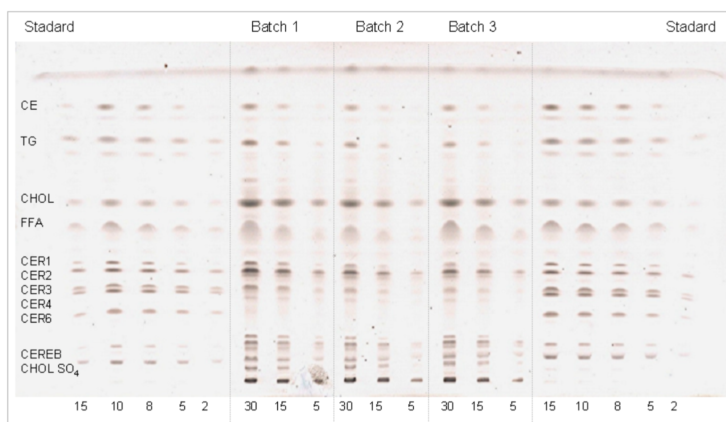
The lipid content and of three different batches of the epidermal models, EpiDerm, EPISKIN, and SkinEthic, was evaluated using high performance thin layer chromatography (HPTLC). Since this technique provides only semi-quantitative information, all quantitative data presented later in the text should be interpreted with caution. In addition, it was not possible to quantify all lipid classes, e.g. phospholipids or diglycerides, as these fractions were not included in standard.

As can be seen from data summarised in Table 20, all three models showed low inter-batch variation in the total content of lipids. Expressed as ratio of the total extracted lipid weight and weight of dehydrated sample, the EpiDerm model contained approximately 12.3 ± 0.5 %, EPISKIN model 10.5 % ± 0.5 % and SkinEthic model 13.2 ± 2.1 % lipids. Related to the tissue surface, EpiDerm model contained 0.417 ± 0.039 mg/cm^2, EPISKIN 0.604 ± 0.047 mg/cm^2 and SkinEthic 0.587 ± 0.043 mg/cm^2 lipids. The lipid extracts from all three reconstructed human skin models contained major lipid classes which can be found in human epidermis. When compared the models amongst each other, some differences were noticed in the content of ceramides, glucosphingolipids (cerebrosides) and free fatty acids (Figure 25 and 26).

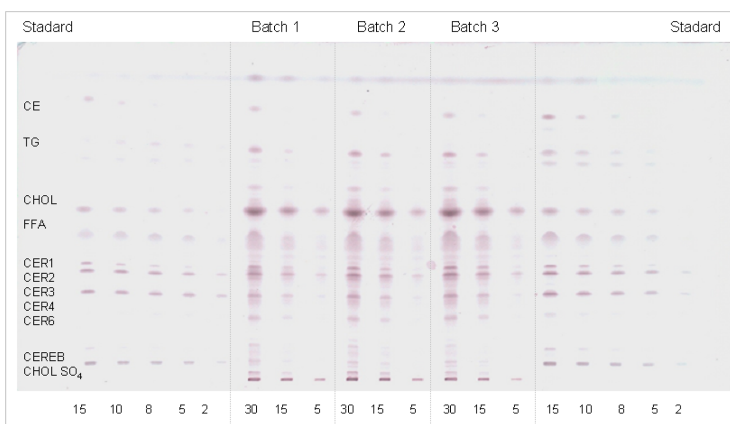
Table 20. Total content of extracted lipids.

	EpiDerm (surface 0.63 cm^2)		**EPISKIN** (surface 1.1 cm^2)		**SkinEthic** (surface 0.5 cm^2)	
	Mean n=3	SD	Mean n=3	SD	Mean n=3	SD
Weight of dried sample (mg)	2.13	0.21	6.4	0.45	2.27	0.4
Total content of extracted lipids (mg)	0.263	0.023	0.664	0.052	0.294	0.022
Total content (mg/cm^2)	0.417	0.037	0.604	0.047	0.587	0.043
Total content (% of sample weight)	12.32	0.51	10.43	0.445	13.2	2.13

EpiDerm model



EPISKIN model



SkinEthic model

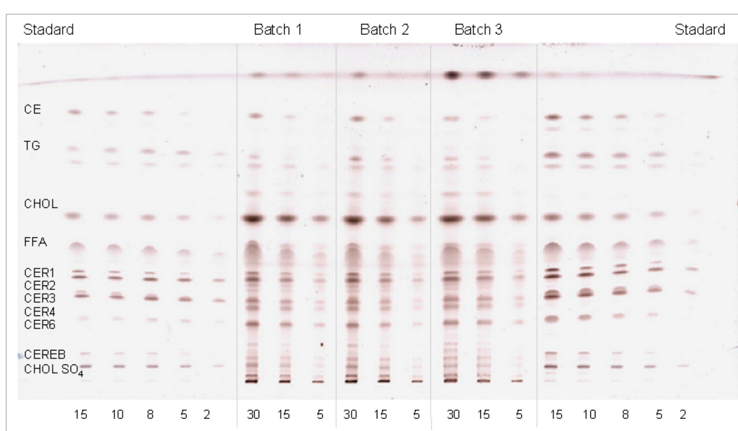


Figure 25. Thin layer chromatographic separation of lipids extracted from the three reconstructed human skin models.
Total epidermal lipids were extracted from three batches and subjected to the separation by HPTLC using the "total lipids development" system (for details see Materials and Methods). A Standard lipid mixture (15 µg, 10 µg, 8 µg, 5 µg, 2 µg) was applied on the right and left side of the TLC plate. Per tissue batch, 30 µg, 15 µg and 5 µg of the lipid extract was applied.
CE - cholesterol esters, TG - triglycerides, CHOL- cholesterol, FFA - free fatty acids, CER - ceramides, CEREB - cerebrosides, CHOL SO_4 - cholesterol sulphate.

Analysis of lipid classes detected by HPTLC

The HPTLC analysis revealed presence of all lipid classes typical for epidermis *in vivo*, including cholesteryl ester (CE), triglicerides (TG), free fatty acids (FFA), ceramides (CER), cerebrosides (CEREB) and cholesteryl sulphate (CHOL SO_4) in all three reconstructed human skin models (see Figure 26). The total content of individual lipid classes detected in the HPTLC assay as well as expression of the result with regard to weight of dehydrated tissue (= dry matter) is summarised in Figure 26 and Table 21.

Ceramides:

Well-developed ceramide profiles were present in all three reconstructed human skin models. The highest amount of ceramides (29% of total lipid content) was present in SkinEthic cultures. EPISKIN models contained about 23.3 % and EpiDerm about 18.5 % of this lipid fraction.

Ceramides 1, 2, 3 and 4 were present in all models in approximately same amounts. Ceramide 2a (fraction of ceramide 2 with higher polarity) and ceramide 5 were also detected in all models but not quantified, as this fractions were not present in standard mixture.

Major differences were observed in the content of ceramide 6, which was low in EpiDerm cultures (0.8 – 2.5 %) and medium in EPISKIN model (5.4 –6.2 %). In the SkinEthic cultures, however, ceramide 6 was one of the dominating ceramides amounting to almost 9.5 – 13 % of the total lipid content.

Cerebrosides:

Glucosphingolipid (GSL) fractions, differing in the polarity, were detected in all reconstructed human skin models. As precursors of ceramides, these lipids have an important function for barrier formation (Boelsma, 1997, Ponec et al., Wertz and Downing, 1991). The overall content of GSL was remarkably higher in the EpiDerm cultures – almost 30 % of the total lipid content detected by HPTLC (see Figures 25 and 26). EPISKIN and SkinEthic cultures revealed lower amount of glucosphingolipids, which corresponded with the higher ceramides level in both models.

Phospholipids:

Phospholipids were also present in all three models (see dark bands located beneath the group of cholesterol sulphate in the HPTLC profiles, Figure 25). However, the quantification was not possible due to the absence of the phospholipid fraction in the

standard. Dark bands, indicating a high content of phospholipids, were observed in EpiDerm TLC profile, while SkinEthic and EPISKIN lipid profiles showed moderate amounts.

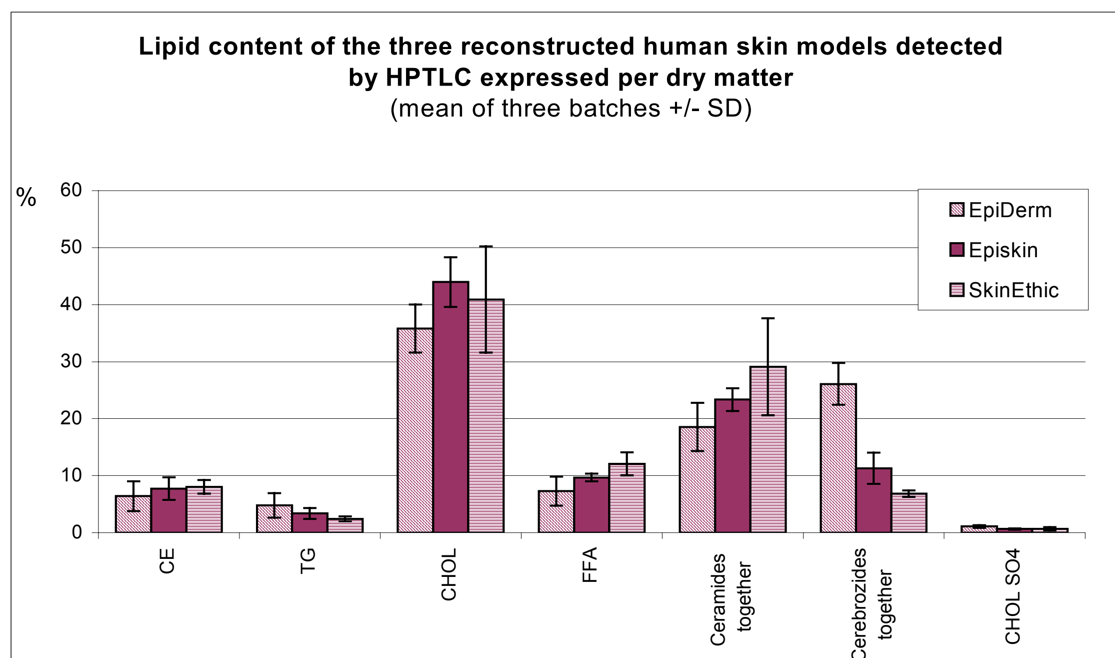


Figure 26. Lipid content of the three reconstructed human epidermal models.
CE - cholesterol esters, TG - triglycerides, CHOL- cholesterol, FFA - free fatty acids, CER - ceramides, CEREB - cerebrosides, CHOL SO_4 - cholesterol sulphate.

Free fatty acids

Group of free fatty acids (FFA) belongs to the one of the main lipid fractions of the *stratum corneum*. In native human epidermis, the content of FFA present about 10 % of the total lipid content (Ponec at al., 1998, Bouwstra et al., 2003). The EpiDerm model contained on average 7.3 %, EPISKIN 9.7% and in SkinEthic cultures 12.1 % of this fraction.

Cholesterol, cholesterol ester and cholesterol sulphate

The amount of cholesterol (CHOL) was high in all three reconstructed human skin models. On average, EpiDerm revealed 38.5 %, EPISKIN 44.0 % and SkinEthic 40.9 % of cholesterol. In addition, small amounts of lanosterol (precursor of cholesterol) were present in all cultures.

Cholesterol ester (CE) and cholesterol sulphate (CHOL SO_4) were detected in all models, in approximately same amounts. Whilst amounts of cholesterol ester were similar to quantities that can be found in native human epidermis (about 8 %), cholesterol sulphate was present in lower amounts (0.6 –1.2 %) compared to *in vivo* epidermis (3 - 4 %)

Di-/Triglycerides

Small amounts of di- and triglycerides were detected in all three epidermal models. However, only triglycerides (TG) were quantified due to the lack of the diglyceride fraction in the standard. The average amount of TG in EpiDerm model was 4.8 %, in EPISKIN 3.4 % and in SkinEthic 2.4 % of the total lipid content. Human epidermis contains approximately 8 - 10% of triglyceride fraction, diglycerides are not present (Bouwstra, 1997).

Table 21. Individual lipid classes extracted from reconstructed human skin models.

Lipid class	% of total lipid content						% of dry matter					
	EpiDerm		EPISKIN		SkinEthic		EpiDerm		EPISKIN		SkinEthic	
	Mean n=3	SD	Mean n=3	SD	Mean n=3	SD	Mean n=3	SD	Mean n=3	SD	Mean n=3	SD
CE	6.4	2.6	7.7	2.0	8.0	1.2	0.52	0.29	0.67	0.18	0.65	0.16
TG	4.8	2.2	3.4	1.0	2.4	0.4	0.39	0.28	0.28	0.05	0.19	0.04
CHOL	35.8	4.2	44.0	4.4	40.9	9.3	2.73	0.69	3.81	0.90	3.22	0.50
FFA	7.3	2.6	9.7	0.7	12.1	2.0	0.56	0.22	0.84	0.17	0.97	0.22
Ceramides together	18.5	4.2	23.3	2.0	29.1	8.5	1.37	0.09	2.22	0.21	2.81	0.64
Cerebrosides together	26.1	3.7	11.3	2.7	6.8	0.6	2.05	0.81	0.95	0.12	0.55	0.08
CHOL SO_4	1.1	0.2	0.6	0.1	0.7	0.3	0.08	0.02	0.05	0.01	0.06	0.03
Sum	100.0	--	100.0	--	100.0	--	7.69	2.04	8.82	1.24	8.44	1.06

First part of the table represents percentile lipid classes as detected in the HPTLC assay, the second part shows the result as ratio of the weight of detected lipids and total weight of the sample (dry matter). In the first type of the calculation the sum of all lipid classes gives always 100%, the second part provides information about how much of the lipid fraction was detected in relation to the total weight of the sample.

SD – standard deviation, CE - cholesterol esters, TG - triglycerides, CHOL- cholesterol, FFA - free fatty acids, CHOL SO_4 - cholesterol sulphate.

5.1.4 EVALUATION OF THE BARRIER FUNCTION OF THE RECONSTRUCTED HUMAN SKIN MODELS

The resistance of barrier of the three reconstructed human skin models was tested in time course assay using Triton X-100 (1% aq.). Duplicate tissues were exposed to 160 µl/cm^2 of Triton for 2, 4, 6, 8 and 10 h. A dose of 160 µl/cm^2 corresponds to volume of 100 µl for EpiDerm (0.63 cm^2), 175 µl for EPISKIN (1.1 cm^2) and 80 µl for SkinEthic model (0.5 cm^2). Two tissues exposed four hours to deionised water served as negative controls. At the end of exposure, tissue viability was determined in the MTT assay and ET 50 value (time at which the tissue viability declines to 50 %) was calculated.

As a second endpoint (related to the tightness of cell layers) the Transepidermal Electrical Resistance (TEER) was used. Initially, the TEER values of non-treated tissues were determined using the Millipore-ERS Volt/ohm-meter. TEER was assessed in each time

point of the ET 50 assay, and TEER 50 (time at which is TEER reduced to 50 %) was calculated.

The mean value of all non-treated EpiDerm tissues measured by Millipore-ERS Volt/ohm-meter was 0.92 ± 0.19 kΩ which corresponds to TEER of 0.51 ± 0.12 kΩ.cm^2. In the time-course assay performed with three different batches of the EpiDerm model, the ET 50 value was 4.98 h in the fist experiment, 5.22 h in the second run and 7.07 h in the third run. In the first run the TEER value decreased below 50 % already after 1.55 hour. The second and third run revealed TEER 50 of 1.62 h and 1.32 h. In both assays the model showed very good reproducibility for both parameters measured.

Surprisingly high variation of resistance was observed between individual tissues of EPISKIN model, ranging from approximately 4.5 to 18 kΩ. The mean value of all non-treated EPISKIN tissues was 10.36 ± 5.02 kΩ, which corresponds to TEER of 11.39 ± 5.51 kΩ.cm^2. Presumptive reason of the high variation is the special insert that is used for the cultivation of the EPISKIN model. The supportive collagen membrane is fitted to the plastic insert with a ring, which must not necessarily close the insert tightly. In the experimental design used for TEER measurement, the leakage of the PBS across this critical area could happen and in consequence affect the measurements. However, using any other experimental design was not possible.

In the fist experiment, the Triton ET 50 value was 9.39 h. In the second and third experiment the ET 50 values were almost identical corresponding to 8.36 and 8.3 hour. The TEER revealed interesting trend during the first four hours. At 4 hour exposure, significant increase of TEER was observed in the first two batches and slight in the third batch of the EPISKIN model (see Figure 27). Only thereafter, the TEER declined rapidly. This phenomenon is most probably linked to the architecture of the stratum corneum (see Figure 23) and its changes during the exposure to Triton.

In almost all SkinEthic cultures, it was not possible to determine absolute initial value of the electrical resistance using the Millipore-ERS Volt/Ohm-meter as this device enables measurements of resistance only in range 0-20 kΩ. The value shown on the display, however, exceeded 20 kΩ. Thus the initial electrical resistance above 20 was set to "theoretical" 20 kΩ. Only in few cases, it was possible to determine the initial TEER values. The mean-theoretical value (considering all SkinEthic tissues used in study) was 18.53 ± 3.00 kΩ which corresponds to TEER of 9.69 ± 1.12 kΩ.cm^2. The ET 50 value obtained in the first and third experiments was 5.11 h and 5.25 h. In the second run, a dose of 100 µl was erroneously applied instead of 80 µl. Therefore also lower ET 50 value (3.39 h) was obtained. In the first two experiments, the TEER 50 was 1.07 h, third run revealed TEER 50 1.10 h. The very reproducible TEER 50 values for SkinEthic are resulting from rapid

decrease of the electrical resistance to knock-out level (for details see Figure 27c). Similarly as EpiDerm, also SkinEthic RHE revealed good compatibility with both endpoints.

Table 22. Summary of the results obtained in the ET 50 and TEER assay with the three reconstructed human skin models.

	ET 50 (h)					TEER 50 (h)				
	Batch 1	Batch 2	Batch 3	Mean	SD	Batch 1	Batch 2	Batch 3	Mean	SD
EpiDerm	4.98	5.22	7.07	**5.76**	**1.14**	1.55	1.62	1.32	**1.52**	**0.12**
EPISKIN	9.39	8.36	8.3	**8.69**	**0.61**	6.14	6.31	6.31	**6.25**	**0.10**
SkinEthic	5.11	3.39 *	5.25	**4.59**	**1.03**	1.07	1.07	1.10	**1.08**	**0.02**

**in the second run, 100 µl was applied instead of 80 µl*

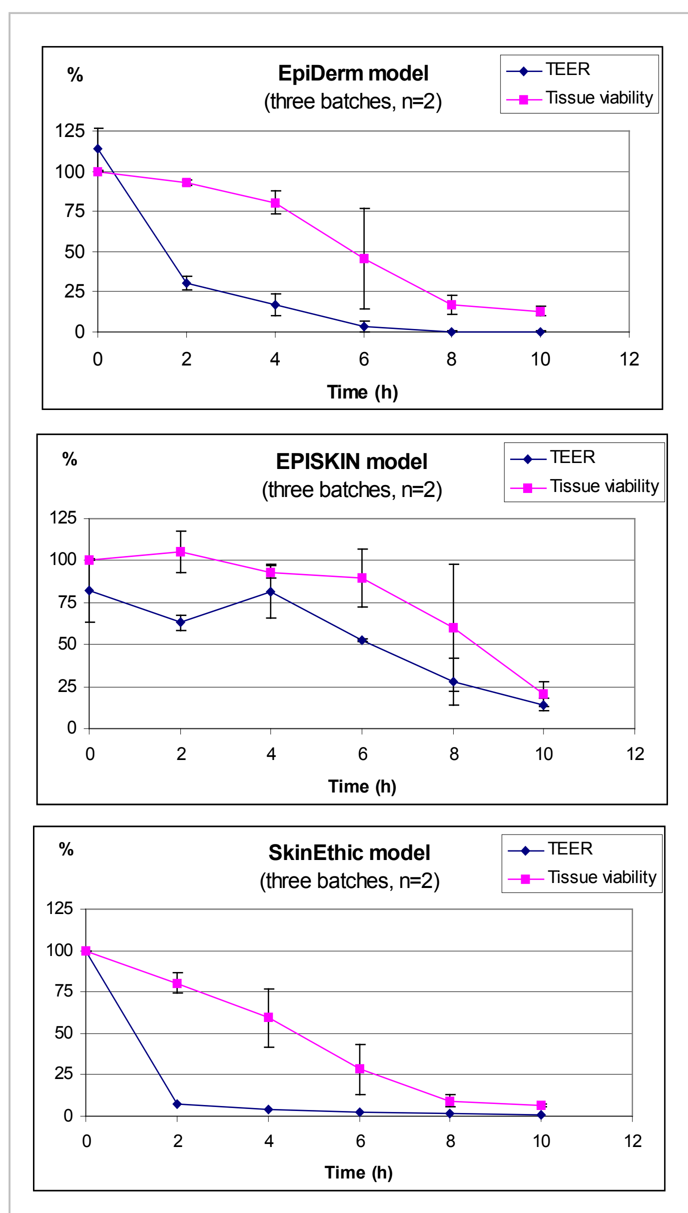


Figure 27. Barrier properties of the three reconstructed human skin models determined in ET 50 and TEER assay after exposure to a dose of 160 µl/cm^2 of Triton X-100 (1%). Means of three independent batches ± SD are presented. The upper (pink) line represents viability decrease and the lower (blue) line, TEER decrease in exposure time-points 2, 4, 6, 8, and 10 h .

5.1.5 DISCUSSION

The aim of the above described study was to evaluate the fundamental characteristics of the three reconstructed human skin models EpiDerm, EPISKIN and SkinEthic according to the requirements of the OECD TG 431.

Histological examination of vertical paraffin sections showed a completely stratified epithelium in all reconstructed human skin models. Despite of slight differences in the tissue architecture (mainly related to the thickness of the SC), all three models closely resembled human epidermis. Very low intra-batch and acceptable inter-batch variation was observed, which is of crucial importance for the quality of data obtained by *in vitro* testing.

The organisation of the outermost layers of the stratum corneum was further investigated using confocal laser scanning microscopy. Due to the difficulties with the visualisation, resulting mainly from limited mechanical stability of the reconstructed human skin models, the pictures have only medium quality. The dermatological confocal laser microscope Stratum, Optiscan seems not to be suitable for the analysis of the very fragile *in vitro* epidermal samples. Nevertheless, the basic information about the SC organisation of the three models was obtained. As can be seen from the representative images presented in Figure 24 B, C, D, the keratinocytes of the SC of RHS models show typical hexagonal structure and penetration pathways close to native human epidermis (Figure 24 A).

Lipid analyses revealed the presence of all major epidermal lipid classes in all three models. Only small differences were noted in the lipid composition of independent tissue batches, however, differences between the three models become obvious. Differences were found in the content of ceramides and glucosphingolipids. Especially, the content of polar ceramide 6 was very low in EpiDerm model, moderate in EPISKIN and highest in SkinEthic cultures. Glucosphingolipids (precursors of ceramides) were abundantly present in EpiDerm cultures, whilst EPISKIN and SkinEthic showed only medium content. This difference is most probably related to the different cultivation stage of the three models, varying from 10 to 17 days (see Table 19). In all cultures, small amounts of diglycerides and lanosterol were present, which do not occur in native human epidermis (Vicanova, 1997).

The time-course assay with Triton X-100 and TEER measurements revealed some obvious differences between the three models, too. Both SkinEthic and EpiDerm models, revealed similar result in the ET 50 assay with Triton X-100 (in both cases ET 50 approximately 5 h). It has been observed, that these two models react similarly also to another surfactant - 5% sodium dodecyl sulphate (SDS), while EPISKIN model shows less sensitivity to the same chemical. This has been now confirmed also for Triton X-100. In the present study, the EPISKIN ET 50 values were considerably higher in comparison to EpiDerm and SkinEthic.

Information, which could explain these differences, can be obtained from TEER experiment and from the morphology of the reconstructed human skin models. The TEER values on non-treated tissues were very high in the SkinEthic (>20 kΩ). In addition, the surface of the SkinEthic model is highly hydrophobic (probably due to the high ceramide content). However, once the lipid layer is disturbed, the penetration of the chemicals proceeds rapidly and the TEER quickly decreases (see Figure 27). In contrast, the TEER values of the EpiDerm model were relatively low, since the model is highly hydrated. High content of water increases the ion exchange and thus decreases the overall TEER value. The hydration of the EpiDerm model is most probably caused by the special shipping conditions set up by MatTek. Although the hydration may enhance penetration of hydrophilic substances (Salminen and Roberts, 2000), the resistance values following to Triton exposure did not decrease as rapidly as with SkinEthic. This may be due to the higher number of viable cells and a slightly different lipid composition of the EpiDerm model. The most interesting behaviour showed the EPISKIN model. The initial Triton penetration across the SC appears to resemble the penetration into the EpiDerm and SkinEthic models. It takes, however, longer until the chemical reaches the compact layer of the SC laying beneath the thicker SC layer (see Figure 23 b). For unknown reasons, the TEER increase approximately at 4 hours after exposure and only then declines significantly. A possible explanation for the TEER increase at 4 hours could be swelling of the SC, which temporarily disturbs ion exchange.

Another interesting effect was observed in tissues exposed for four hours to deionised water. SkinEthic cultures showed no measurable reaction - the TEER values constantly remained above 20 kΩ. In EpiDerm cultures a slight increase of TEER following four-hour exposure to water was observed. Although, not so obvious as in Triton experiment with EPISKIN cultures, both effects may have the same cause. In EPISKIN model, the TEER of negative controls slightly decreased after exposure to deionised water. This effect is in concordance with a slight reduction of viability after exposure of EPISKIN models to deionised water.

It seems, that measurement of the TEER might be also able to detect impaired barrier of the reconstructed human skin models. One experiment performed with a non-qualified tissue batch, provided by one of the manufacturers for research purposes, revealed significantly different values in comparison to qualified tissues (data not shown in this thesis). However, to evaluate this hypothesis systematic investigations with “qualified” and “non-qualified” tissues should be performed. This is, however, a challenging issue as manufacturers release only occasionally such a type of products.

In summary, all three reconstructed human skin models tested in the present study reflected many of the characteristics of normal human epidermis and therefore provide a morphologically relevant *in vitro* means to perform skin irritation, skin corrosion and other "skin-related" studies. In conclusion, all skin models provided promising means for studying the effects of topically applied chemicals.