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A novel toner-emulsion bundle through multi-pathway approach for holistic skin tone management: Addressing brightness, yellowness and redness

WANG Xue[1], WU Mengxi[1], ZHOU Xiaoyin[1], WANG Yang[1], HE Chunyan[1], WANG Dan[1], ZHU Zailing[1], WANG Wenna[1], ZHU Mingjie[1], DU Ana[2], SHENG Le[2], TU Yidong[1], HUANG Nan[1], HSU KungChi[1]

[1] L'Oréal Research and Innovation center, Shanghai, China;

[2] L'Oréal Research and Innovation center, Clark, NJ - 30 Terminal Ave. USA

1. Introduction

Skin tone management, especially skin color, is a common beauty concern for women around most parts of the world. While brightness is universally valued as one of determinant of skin esthetics [1-2], cultural nuances significantly influence the specific aspects of skin tone that are considered ideal. For Chinese women, skin yellowness and redness hold particular importance, intertwined with the traditional concept of *Qi Se*, which closely indicates perceived skin vitality and health[3].This cultural perspective necessitates a multi-faced approach to skin tone management, addressing not only brightness but also the reduction of yellowness and redness.

Skin yellowness can stem from several factors, broadly categorized into internal (systemic) and external causes (i.e. Sun exposure). The accumulation of glycation end products (AGEs), a process where reducing sugars bind to proteins, lipids, or nucleic acids, has a direct relationship with skin yellowness [4]. Furthermore, glycation increases the generation of reactive oxygen species (ROS) [5], creating a vicious cycle that accelerates the inflammation and related pigmentation [6]. Carnosine is a naturally occurring dipeptide, acting by scavenging reactive carbonxyl species, which are precursors to AGEs, theareby preventing the glycation and oxidation [7, 8]. Meanwhile, dandelion extracts have been used for traditional Chinese medicine and native Amerian medicine for long, shown to have anti-oxidant and anti-inflammtory activities.

Hereby, to address these specific concerns for Chinese women, we decoded a multi-faceted approach targeting four pathways, anti-glycation, anti-oxidation, anti-inflammation and depigmentation. By leveraging the potent anti-glycation property of Carnosine, we complemented the active design with dandelion rhizome/root extract and niacinamide, benefiting from their prominent ability of anti-oxidation, anti-inflammation and depigmentation. Finally, a toner-emulsion bundle set with this active composition was developed to deliver comprehensive skin tone improvement in terms of brightness, anti-yellowness and anti-redness.

2. Materials and Methods

A throughout process to evaluate novel formulation efficacy through dimensional pathways from in-vitro to in-vivo and describe its potentialities for brightness, anti-yellowness and anti-redness. The process contains the following three suceessive steps:

Study 1: In-vitro test with 3D full-thickness skin model

Using a 3D full-thickness skin model, we evaluated anti-glycation effect of targeted formula. The assay was conducted with referring methods described by Khmaladze I and Markiewiez E [9-10].

Skin model preparation and Modeling: The pre-warmed culture medium was added to the 6-well plate (2mL/well), and the in vitro artificial full-layer skin model was transferred to the 6-well plate containing the culture medium and cultured overnight in the incubator. The skin models of model group and sample group were transferred to a 6-well plate containing methyl Glyoxal (MGO) in culture medium (500mM, 2mL/well) for saccharification and then cultured overnight.

Sample treatment and evaluation: smear the test samples on model surface evenly and treate the control groups with exposure conditions. The test groups and exposure conditions are shown in Table 1. After exposure, the samples are rinsed thoroughly with PBS until there is no residue, and continue to incubate in a 6-well plate containing fresh artificial full-thickness skin model medium. After post-incubation, the skin model was carefully removed from the plate, the skin model was cut from the chamber. The tissue was homogenized and the supernatant was collected. Later, the content of c (CML) was determined by enzyme-linked immunosorbent assay (ELISA) kit.

Table 1. Grouping and sampling table for Anti-glycation test

Group	Exposure condition	Post-incubation
Negative control group (NC)	PBS	24h
Model control group (M)	MGO	24h
Sample group (emulsion formula)	Emulsion formula + MGO	24h

Study 2: In-vitro test with *Ex vivo* skin tissue

Ex-vivo skin tissue culture medium was used to perform anti-oxidant testing, anti-inflammation testing and depigmentation testing.

Skin model preparation and Administration: The obtaine skin tissue was immersed in 75% alcohol, washed for 30s, and then cleaned with sterile PBS buffer. The skin tissues were then cut into tissue blocks, which had a fixed surface area size of $24\pm2mm^2$. After the skin tissues were processed, they were transferred into a 6-well plate with a culture insert. 3.7mL culture solution was added to each well for further culture at 37℃ with 5% CO_2. After 2-day culture in vitro, the skin tissues received irradiation and administration referring the test group in table 2. The *ex-vivo* skin tissue was irradiated by a certain dose of UVA and UVB for 4 consecutive days.

Table 2. Grouping and sampling table for anti-oxidant, anti-inflammation and depigmentation testing

Group	Exposure condition	Stimulation condition
Blank control (BC)	/	/
Model control group (M)	/	30 J/cm2 UVA+50 mJ/cm2 UVB
Sample group (emulsion formula)	Emulsion formula	
Sample group (Bundle formula)	Toner formula + emulsion formula	

Immunofluorescence detection: The skin tissues used for detection was fixed with 4% paraformaldehyde for 24h. The immunofluorescence detection was performed and the images were taken under microscope for observation and analysis to test the content of nuclear transcription factor (NF-κB).

ROS content detection: Firstly, ROS probe working solution was prepared by diluting 10mM probe solution to 60μM probe working solution with tissue medium. After the last administration, the tissue were transferred into a 6-well plate with prepared probe working solution according to the test group. Later, the 6-well plate was placed into CO2 incubator (37℃, 5%CO2) for 1h, and shook the plate every 20min to ensure tissue incubation evenly. After incubation,the residual test substance on the surface of the tissue was washed with sterile PBS, and the inserts were dried with sterile cotton swab. Skin tissue was immediately cut down and placed in 1.5mL centrifuge tube with 4% paraformaldehyde for fixation. After fixation for 24h, frozen embedding and sliced. Cleaned the frozen slices with PBS for 8min. After cleaning, wiped the liquid around the tissue with absorbent paper, dropped a small drop (about 20μL) of fluorescence anti-quenching sealing tablet, and gently covered the cover glass. When the slices were sealed, then took photos(20×) and analyzed with fluorescence microscope.

Melanin distribution test: After fixation of skin tissues with 4% paraformaldehyde for 24h, melanin distribution was assessed via Fontana-Masson staining and observed under a microscope.

Study 3: Clinical study on Chinese women with skin tone & PIH concerns

Subjects

Two groups of Chinese females as subjects were recruited by a local agency in Hangzhou, China, fourty-three for emulsion only and fourty five for bundle use respectively.They were further enrolled by a local experienced dermatologist according to the following main criteria: (1) at least 40 subjects enrolled in this study; (2) Chinese females aged from 20 to 55 years old; (3) all skin types; (4) regular user of cleanser, moisturizing and sunscreen products. (5) present facial sallowness (on a visual scale of ⩾3 severity) [11], discrete hyperpigmentation (on a visual scale of 3-6 severity), skin redness (on a visual scale of 2-6 severity), pore appearance (on a visual scale of 2-6) where the severity scale is based oon SGS standard map (6) no presence or history of a allergic disease to cosmetics or other topical preparations; (7)

no history of photosensitive disease; (8) no obvious skin lesions, scars, hair, etc on the facial skin.

Protocol

The entire study was divided into the treatment phase (baseline to T56D) and regression phase (T56D to T63D). One group of eligible subjects were distributed investigated emulsion while another group were distributed the investigated toner-emulsion bundle for usage. All subjects were asked for continuous usage until D56D twice per day. In real-life clinical, the cleansing products, moisturizing products and sunscreen products were provided. Also, they were asked for visiting the local agency (Hangzhou, Zhejiang, China) for clinical evaluation at T3D, T7D, T28D and T56D after baseline enrollment in the treatment phase. Selfies from each subject were required and asked at T70D and T84D in the regression phase. At each onsite visit in the morning, subjects washed their face and take a 30-minute acclimatization in a well-controlled temperature (21°C ± 1 °C) and humidity (50% ± 5 %) room. Then, a white cap was worn on each subject to avoid the possible influence of hair on attributes perception.

Pictures acquisitions and Statistical analysis

Technician captured facial Visia-CR® images under standard 1, standard 2, cross-polarized, parallel-polarized, and ultraviolet modes. For each subject and method, front, 45° left and right as a set was collected.

SPSS 28.0 was used for data statistics and the test data were tested for normal distribution. If the test data are normally distributed, the T-test method is used for statistical analysis. While, if it is non-normal distribution, the rank sum teset method is used for statistical analysis. The rank data is statistically analyzed by the rank sum test. Statistical methods has a significance level $P<0.05$. The significant difference in the values of each test parameter was anaylzed with baseline at each time point.

3. Results and Discussion

Study 1: In-vitro test with 3D full-thickness skin model

To evaluate the efficacy of emulsion formula against glycation, the full-thickness skin model was treated with MGO for saccharification, and then the model was treated with the sample. Carboxymethyl lysine (CML) as a structural form of AGEs, can be used to evaluate the anti-glycation effect of tested substance. The tested CML contect shows that the CML level in the model group (M) was significantly higher thant that in the negative control group (NC), suggesting that the saccharification model was established. Meanwhile, compared with the model group, the CML content of the tested emulsion formula decreased by 137.9%.

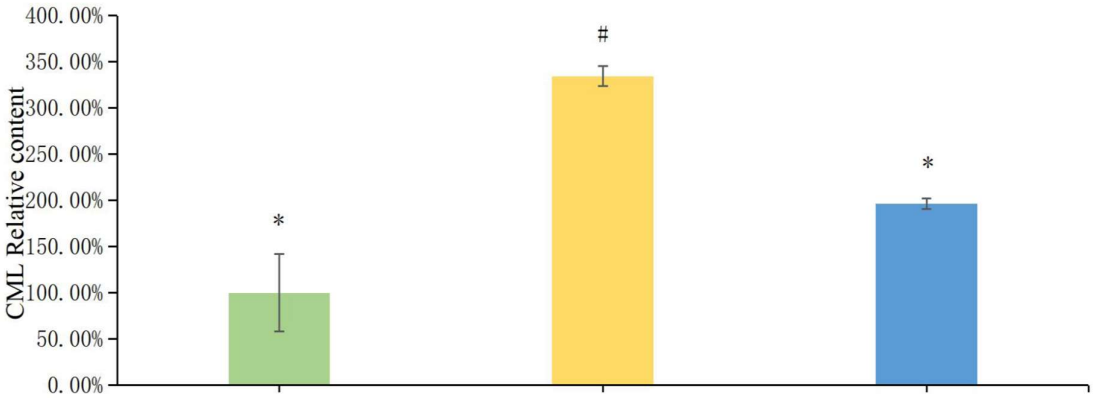

*Figure 1. Effect of test substances on intracellular CML levels. * compared with the model control group, which is treated by methyl glyoxal (MGO), the difference was statistically significant. # compared with negative control group, the difference was statistically significant. ($p<0.05$).*

Study 2: In-vitro test with *Ex vivo* skin tissue

Ex vivo skin tissue was irradiated by a certain dose of UVA and UVB. The skin antioxidant function was evaluated by detecting the change in reactive oxygen species (ROS) content. The skin soothing efficacy was evaluated by detecting the change in nuclear transcription factor (NF-κB) content. The skin whitening efficacy was evaluated by detecting the change in melanin distribution.

From figure 2, the designed emulsion formula contains the novel active composition shows a significant efficacy on anti-oxidation and anti-inflammation, respectively. The ROS fluorescence signals show clear decrease with the treatment of emulsion and bundle consecutively, shown in figure 2.a. Using the integrated optical density to reflect the content of ROS, the treatment of emulsion formula and the bundle group inhibit the production of ROS by 31% ($p<0.01$) and 37% ($p<0.01$), respectively. Additionally, the immunofluorescence analysis demonstrates the UV induced NF-κB can be efficiently suppressed by emulsion formula and bundle formula by 54% and 62% ($p<0.05$), shown in figure 2.b. Melanin distribution was observed via Fontana-Masson staining shown in figure 2.c. Image anyalsis results show that after pairing with the toner formula, the bundle set formula demonstrates overall ability of inhibiting melanin distribution 50% ($p<0.05$), showing significant superiority to toner formula use only (28%).

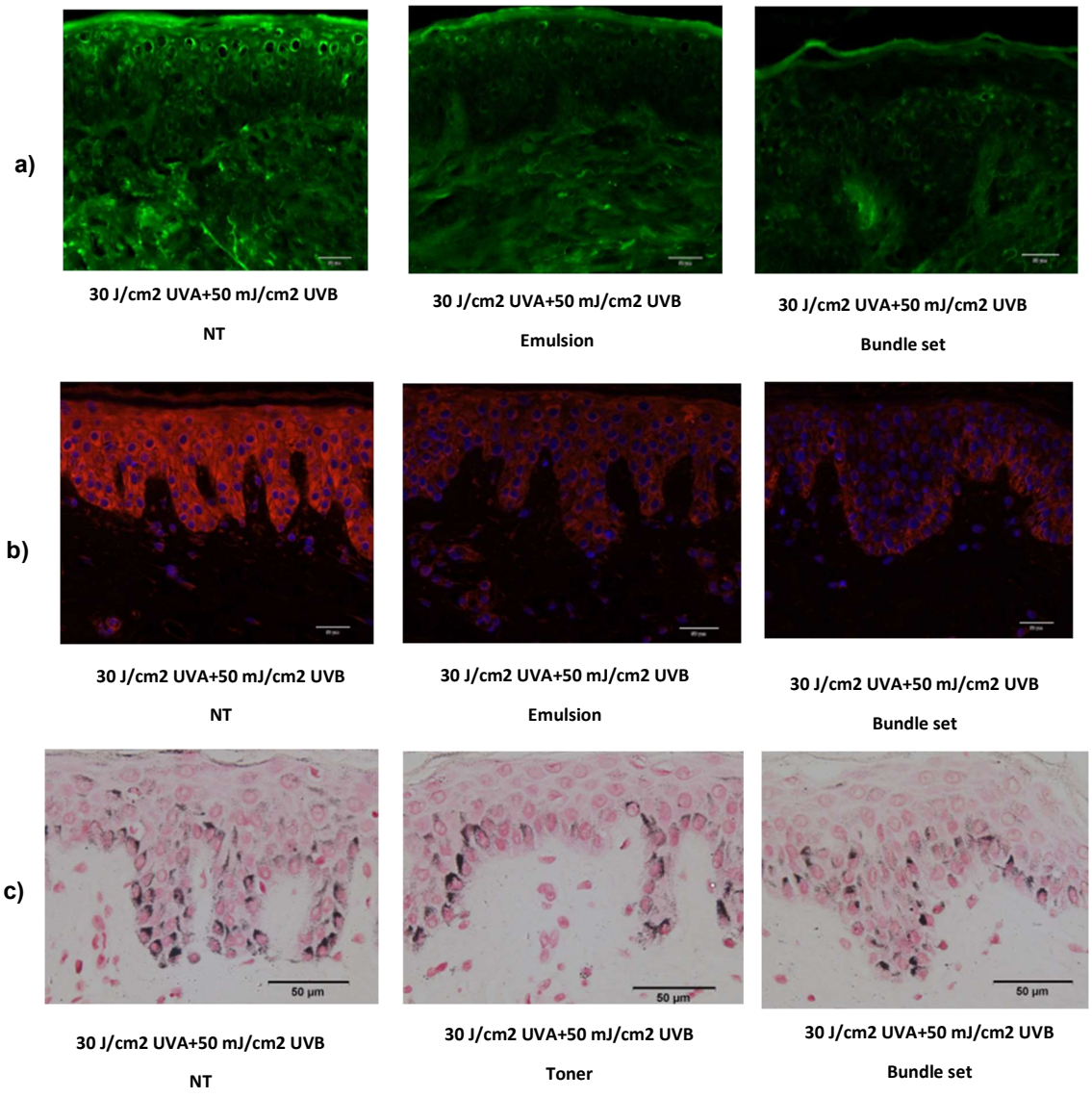

Figure 2. a) Relative oxygen species (ROS) and b) Nuclear transcription factor (NF-kB)was detected under fluorescence microscope after UV exposure and relative sample treatment. c) Melanin particle distribution on Ex-vivo skin tissue was observed by Fontana-Masson staining after UV exposure and relative sample treatment .

Table 3 shows the data of skin tone attributes at the end of treatment phase T56D and the end of regression phase T63D for two different groups. From the statistical analysis of clinical assessment results, the treatment of the emulsion significantly improved the skin tone in 5 dimensions, at the same time, reduced the redness at T56D.

Furthermore, the 8-week application of the said bundle set shows significant stronger efficacy on skin fairness, skin radiance, brightness and skin redness compared with emulsion use only. The bundle set improves skin fairness, brightness, and radiance by 20%, 19% and 26% ($p<0.05$), respectively. Moreover, the skin sallowness, the attribute that evaluates the skin yellowness, was also improved significantly by 20%, Also worthy of noticing, all these efficacies maintain until day 63, still showing an improvement rate of 20%, 19%, 25% and

20% ($p<0.05$). Besides, from the instrumental test results, the set also improves skin redness by decreasing redness area ratio and a* value by 50% ($p<0.05$) and 19% ($p<0.05$), respectively.Figure 3 shows the average improvement case on real skin.

*Table 3 data table of skin tone attributes at T56D and T63D timepoint. All values are statistically significantly improved (P < 0.001) compared with T0 (baseline). Smaller scale indicates better efficacy. * shows the significant improvement compared with emulsion use only*

Attribute		Baseline (BL)	D56	%Change vs. BL	D63	%Change vs. BL
Skin Fairness	Emulsion	3.83±0.80	3.14±0.86	-17.93% (-0.69)	3.14±0.86	-17.93% (-0.69)
	Bundle	4.29±0.91	3.42±0.88	-20.21% (-0.87)*	3.42±0.88	-20.21% (-0.87)*
Skin Radiance	Emulsion	4.41±0.67	3.50±0.60	-20.58% (-0.91)	3.60±0.59	-18.21% (-0.80)
	Bundle	5.01±0.65	3.70±0.61	-26.16% (-1.31)*	3.74±0.61	-25.28% (-1.27)*
Skin Sallowness	Emulsion	3.87±0.81	3.10±0.85	-19.82% (-0.77)	3.14±0.84	-18.92% (-0.73)
	Bundle	4.27±0.95	3.39±0.88	-20.57% (-0.88)	3.39±0.88	-20.57% (-0.88)
Skin Dullness	Emulsion	4.01±0.90	3.41±0.84	-15.07% (-0.60)	3.41±0.84	-15.07% (-0.60)
	Bundle	4.33±0.90	3.56±0.83	-17.95% (-0.78)	3.56±0.83	-17.95% (-0.78)
Skin Brightness	Emulsion	3.95±0.74	3.31±0.74	-16.18% (-0.64)	3.31±0.74	-16.18% (-0.64)
	Bundle	4.54±0.74	3.66±0.75	-19.56% (-0.89)*	3.66±0.75	-19.56% (-0.89)*
a* Value	Emulsion	22.41±6.84	20.05±5.90	-10.52% (-2.36)	20.52±5.96	-8.46% (-1.90)
	Bundle	20.41±5.97	16.52±4.29	-19.07% (-3.89) *	17.96±4.47	-11.97% (-2.44)
Redness area proportion	Emulsion	56.82%±14.99%	35.73%±18.35%	-37.12% (-21.09%)	40.59%±18.15%	-28.56% (-16.23%)
	Bundle	59.03%±11.61%	28.93%±13.01%	-51.00% (-30.01%)*	39.73%±15.15%	-32.70% (-19.30%)

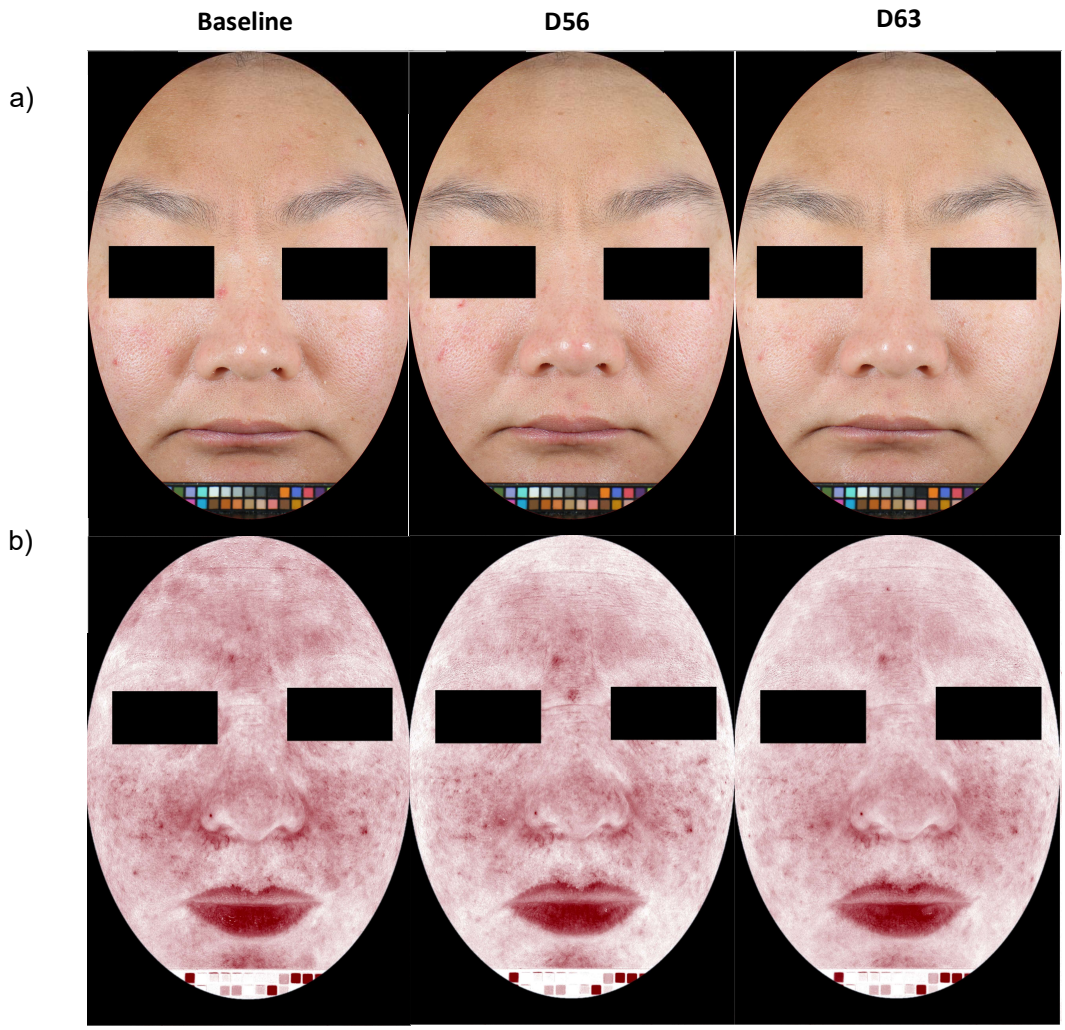

Figure 3. a) VISIA-CR image, standard 1 mode, b)VISIA-CR image, red channel

4. Conclusion

In conclusion, by leveraging the anti-glycation, anti- oxidation, anti-inflammation, and depigmentation effect of the novel composition, we developed a toner-emulsion bundle set for skin tone management through multi pathway integrated mechanism. The emulsion formula shows strong anti-glycation, anti-oxidation and anti-inflammation ability in in-vitro tests. Paring with toner use then exhibits better depigmentation effect than current toner formula use only. In in-vivo tests, the bundle use of toner and emulsion from both clinical assessment and instrumental assessment provides a holistic understanding of delivered skin tone improvement. It proves that the bundle set can effectively improve not only skin brightness, but also skin yellowness in a long-term manner. Furthermore, it can also improve skin redness under a continuous use. The study shows that by leveraging the four potential mechanisms that are interactively linked with skin tone, the aim of holistic improvement on skin tone in real clinical environment can be achieved.

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Conflict of Interest Statement

NONE

Reference

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[11] Clinical method for evaluation of anti-glycation cosmetic products appendix A.1