

## Technical Report



Product

**RELISTASE™**

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 **Lipotec**  
We research for you



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## Introduction

A variety of environmental, hormonal, and genetic factors results in skin elasticity loss with age. Mature skin becomes less elastic and less able to resist any deformation leading to many of the visible manifestations of aging such as wrinkles, unfirmness or sagginess. Aged skin presents decreased epidermal turnover, diminished inflammatory response to UV, impaired immune function and a decrease of cutaneous vasculature. Macroscopically, the result is a thicker and atrophic skin [1, 2].

Skin quality deteriorates with age due to the synergistic effects of chronological aging, photoaging, environmental factors, and hormonal deficiency. Chronoaging and extrinsic aging are two processes involved in aging which differ markedly, even though both bear certain similarities. Hormonal aging of skin due to oestrogen loss at menopause is thought to include atrophy; decreased collagen content, water content, and sebaceous secretions and elasticity loss. It has been reported that total collagen declines by an average of 2.1% per post-menopausal year over a period of 15 years [3].

Intrinsically aged skin shows characteristic fine wrinkling and appears smooth [4]. In chronoaging, there is a slower synthesis and turnover of new components by fibroblasts, especially from the age of 40 years, and a greater enzyme action on fibres which imply a loss of skin elasticity and a less supple and more hardened collagen [5]. Consequently, when pressing the skin, it no longer springs back to its initial position but instead sags and forms furrows, culminating in the development of wrinkles.

Solar radiation is the main responsible for extrinsic aging, but some other causes, such as air pollution, are also very important [5]. Excessive exposure to the sun may cause severe photoaging increasing proteolytic activation and showing abnormal extracellular matrix (ECM) turnover. The consequence is an acceleration of collagen and elastic fibres degradation in the dermis resulting in loss of skin's ability to resist stretching [2]. Typically, sun-exposed skin appears papular, coarse, roughened, and deeply wrinkled with marked loss of elasticity and recoil. Later, at the final stage of actinic damage, the skin may become atrophic and the skin blood vessels may look clinically abnormal and prominent [6].

Both chronologic aging and photoaging reduce the elasticity of the skin, which is an essential factor in the formation of wrinkles.

## General description

Elastic fibres are responsible for the normal resilience of the skin. In situations where elastic fibres are fragmented or absent the skin is loose and sagging and lacks the normal recoil [6]. When elastic fibres suffer elongation, their immediate tendency is to return to their initial position with an elastic behaviour (Fig. 1.). This elasticity decreases with time for different reasons such as natural aging or other several factors that accelerate or modify the natural process (extrinsic aging and rapid changes in body weight) [5].



Fig. 1. Relaxed elastic fibres (left) and stretched elastic fibres (right).

Elastic fibres make up an extracellular connective tissue component that is responsible for the elasticity of the skin [6]. Skin elasticity is a mechanical property which is influenced by elastin, a protein in the skin which, together with collagen and glycosaminoglycans, make up the connective tissue. The correlation of all these components must be perfect in order to guarantee the physical properties of the skin. Protein fibres are arranged to form a network submerged in a gel matrix of water and glycosaminoglycans. Fibroblasts are included in this structure and are responsible for synthesising the other components. The protein network gives the tissue its physical properties, such as rigidity and elasticity, elastin being fundamental in this latter parameter [5].

The connective tissue of the skin is composed mostly of collagen which is the most abundant protein in the skin. Collagen makes up 70-80% of the dry weight of the skin and gives the dermis its mechanical and structural integrity [6]. The various collagens and the structures they form all serve the same purpose, to help tissues withstand stretching. Nineteen distinct types of collagens are recognised, all with individual characteristics that serve specific functions in a variety of tissues [7]. One of the most important is type I collagen, which is the most abundant collagen in the human body, representing the 80-85% of the dermal collagen. Type I collagen fibrils have a great tensile strength and elastic resistance [1, 6].

Elastic fibres are insoluble structural elements of connective tissue that have a central core of amorphous, hydrophobic cross-linked elastin surrounded by fibrillar structures with a regular diameter of 10-12 nm, which are called the microfibrillar component of elastic tissue (Fig. 2). Elastin is a well-characterised connective tissue protein and is the major component of the elastic fibres. Although, elastin is found in less amount than collagen in the dermis, it is crucial for skin elasticity. Elastin is initially synthesised as a polypeptide that link to a fibre network

through the formation of desmosines, cross-link compounds not found in other mammalian proteins. During early embryonic development, most of the elastic fibres consist of microfibrils, which form a microfibrillar skeleton upon in which elastin is deposited. In mature, fully developed elastic tissue, well over 90% of the total content consists of elastin [4, 6].

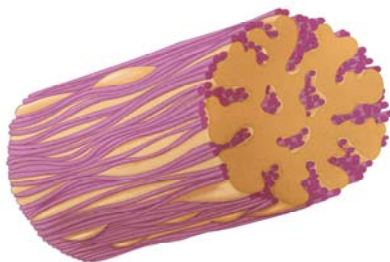


Fig. 2. Elastin core surrounded by fibrillar structures.

Natural aged skin shows general atrophy of the ECM with reduced elastin and disintegration of elastic fibres [4]. In addition, the dermo-epidermal junction becomes flatter, which detracts from its mechanical resistance [6]. There is also a reduction in the amount of peripheral microfibrils. The fibre surface becomes irregular and granular, microfibrils become thicker, and there is a decrease in the amount of glycosaminoglycans and fibroblasts [5]. A major feature of aged skin is also reduced collagen synthesis and increased degradation, resulting in connective tissue damage, and loss of the skin three-dimensional integrity [8]. Reduced synthesis of types I and III collagen is characteristic of chronologically aged skin [9].

Natural aging process and environmental insults contribute to generation of ROS (Reactive Oxygen Species) that stimulate the inflammatory process in the skin activating the transcription factors that regulate the proteolytic degradation of the ECM. In response to UV-induced production of pro-inflammatory cytokines, phagocytic cells such as neutrophils and monocytes infiltrate into skin from capillaries. In addition to keratinocytes, the phagocytic cells themselves secrete cytokines that further enhance recruitment of inflammatory cells. Furthermore, neutrophils release elastases and other proteases that can cause further inflammation, and activation of matrix metalloproteases (MMP) which are known for degrading collagen fibres [2].

Damage to connective tissues is a major complication of the inflammatory response. Elastic fibres are degraded by several types of enzymes, such as neutrophil elastase released during neutrophil infiltration of the epidermis, MMP-12 derived from macrophages, and skin fibroblast elastase produced by fibroblasts [2, 10].

Ultraviolet radiation induces both neutrophil elastase and skin fibroblast elastase. Neutrophil elastase is able to rapidly degrading intact microfibrils and its inhibition has demonstrated to prevent UV-induced wrinkle formation in skin. The secretion and activation of skin fibroblast elastase is thought to be responsible for the degeneration of the three dimensional structure of elastic fibres during the formation of wrinkles [2, 4, 10].

Inflammation, UV irradiation and normal process of aging can activate MMPs leading to increased matrix degradation. MMPs are a group of zinc-dependent endopeptidases capable of degrading ECM components and are involved in the turnover and remodelling of the dermis. In normal skin, the MMPs are expressed in very low levels and are kept in their inactive form bound to endogenous inhibitors [2]. Human macrophage metalloelastase (HME, MMP-12) is the most active MMP against elastin on a molar basis and has broad substrate specificity, being able to degrade also type IV collagen which is the most abundant structural component of basement membranes [11]. On the other hand, MMP-1 initiates cleavage of fibrillar collagen types I and III in the dermis, which is then further degraded by MMP-2 and -9 [2].

The specific protease inhibitors, tissue inhibitor of metalloproteinases (TIMPs) and skin-derived antileukoproteinase (elafin), regulate the activities of MMPs and elastase [12]. Skin cells produce several classes of TIMPs which are small molecular weight proteins. UV induces MMP transcription by five-fold, however TIMP expression is either not altered or only slightly elevated by UV. Phagocytic cells induce the keratinocytes to synthesise and secrete elafin, an inhibitor of human neutrophil elastase, which eventually limits the damage caused by the inflammatory neutrophils to skin [2].

An increase of elastases activity and a slow elastogenesis end up in elasticity loss which is reflected in a sagging, unfirmed and wrinkled skin. Stimulation of dermal fibroblasts not only induces elastin production but upregulate elastotic enzymes which may rapidly degrade newly produced elastin and existing elastic fibres. Hence, there is a need to protect existing and new elastic fibres from premature enzymatic proteolysis in order to get a firm, elastic and wrinkleless skin [13].

**RELISTASE™** is a new tetrapeptide for skin elasticity and tightness enhancement cosmetic formulations, which was identified by a combinatorial chemistry approach from a library of 331776 peptides. The combinatorial peptide library was screened by monitoring fluorescence of quenched elastin released when digested by elastase to evaluate the inhibitory potency of the peptides on the activity of elastase enzyme.

RELISTASE™ inhibits the excess of elastase activity, helping to improve skin elasticity lost due to the normal process of aging or by extrinsic factors such as excessive solar exposition or air pollution among other causes. By reducing the excess of elastase activity, RELISTASE™ helps to protect elastin and other ECM components which are susceptible to be degraded by these enzymes resulting in a loose, sagging and wrinkled skin. The tetrapeptide also presents collagen boosting properties favouring connective tissue improvement and helping to restore skin three-dimensional integrity by enhancing tensile strength and elastic resistance.

Preventing and improving the body skin elasticity loss together with a collagen booster effect helps to get a more firm, tighter, elastic and younger skin.

## Cosmetic properties

RELISTASE™:

- is a Molecular Cosmetic active peptide that enhances body skin elasticity and tightness especially targeted at mature skins
- helps to protect elastin by inhibiting human neutrophil elastase activity
- improves tightness and overall skin elasticity of the body skin as proved *in vivo*
- favours to restore skin three-dimensional integrity and connective tissue improvement thanks to its excellent collagen booster properties which showed *in vitro*

## Efficacy *in vitro*

### ELASTIN PROTECTION FROM PIG PANCREATIC ELASTASE

The fluorescence released by quenched elastin when digested by pig pancreatic elastase was monitored in order to study RELISTASE™ dose-response inhibition of the porcine elastase.

Samples at 1, 10 and 50  $\mu\text{M}$  were preincubated with the protease reconstituted in Reaction Buffer (0.4 units/mL of pig pancreatic elastase) for 1 hour at room temperature. After preincubation, 25  $\mu\text{L}$  of the substrate (DQ™ Elastin) were added and the samples were incubated in darkness for 2 hours at room temperature. Fluorescence released by the digestion of labelled elastin was measured in an automated multiplate fluorescence reader set for excitation at 485 nm and detection at 530 nm. The results obtained were corrected from the basal fluorescence released with neither elastase nor test items and normalised regarding the release of fluorescence of a control experiment without test items (negative control).

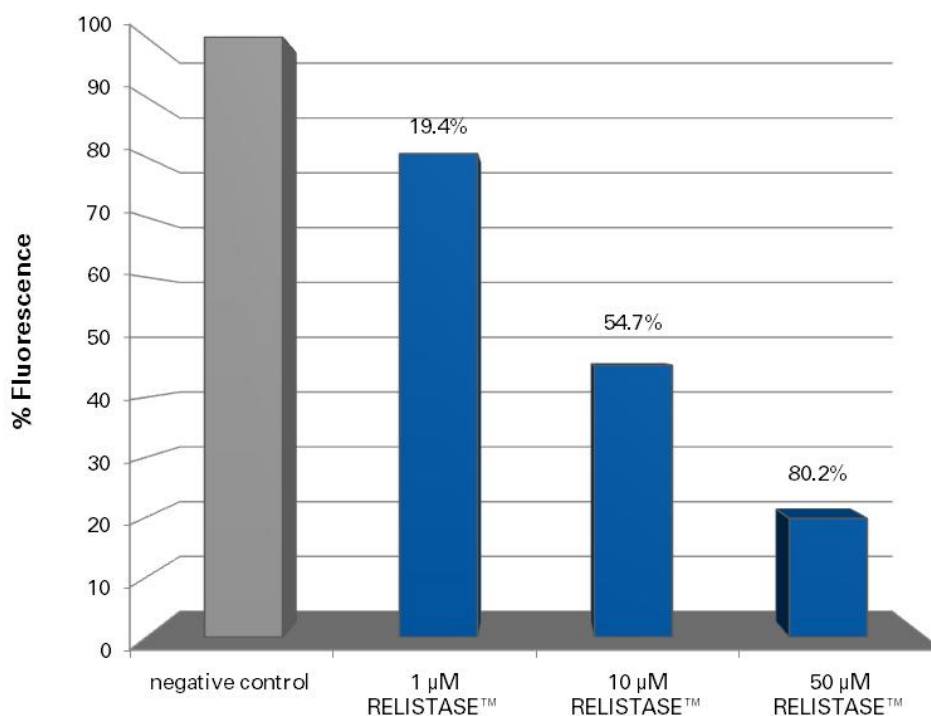


Fig. 3. Inhibition percentage of elastase activity by RELISTASE™ at 1, 10 and 50  $\mu\text{M}$ . Data are shown as MEAN.

Under the experimental conditions, RELISTASE™ proved to be able to protect elastin by inhibiting pig pancreatic elastase activity in a dose-response manner, showing an inhibition of 80.2% at 50  $\mu\text{M}$ .

### INHIBITION OF HUMAN NEUTROPHIL ELASTASE

The fluorescence released by the fluorogenic elastase substrate V (MeOSuc-Ala-Ala-Pro-Val-aminomethylcoumarin) when digested by human neutrophil elastase was monitored in order to study RELISTASE™ dose-response inhibition of the human neutrophil elastase.

Samples at 50, 100 and 500  $\mu\text{M}$  were preincubated with 0.2  $\mu\text{g}/\text{mL}$  of human neutrophil elastase in Reaction Buffer for 1 hour at room temperature. Afterwards, the fluorogenic substrate was added to wells and samples were incubated in darkness for 2 hours at room temperature. The hydrolysis of the fluorogenic elastase substrate V was monitored fluorometrically with a 370 nm excitation filter and a 460 nm emission filter in an automated multiplate fluorescence reader. The results obtained were corrected from the basal fluorescence released with neither elastase nor test items and normalised regarding the release of fluorescence of a control experiment without test items (negative control).

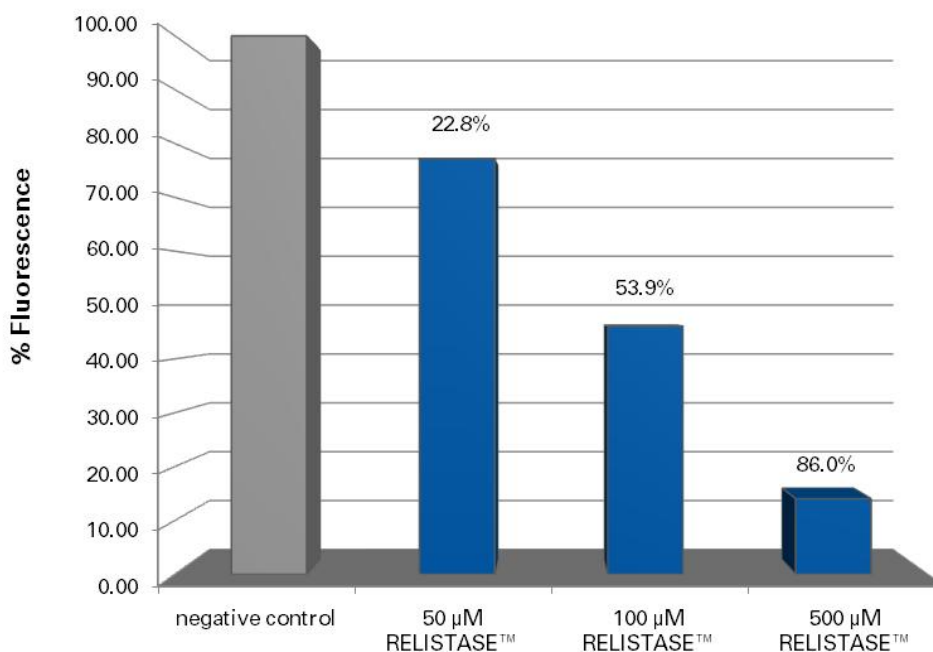


Fig. 4. Inhibition percentage of human neutrophil elastase activity by RELISTASE™ at 50, 100 and 500  $\mu\text{M}$ . Data are shown as MEAN.

RELISTASE™ demonstrated to be able to inhibit human neutrophil elastase in a dose-response manner, showing an elastase inhibition of 86% at 500  $\mu\text{M}$ .

### EVALUATION OF TYPE I COLLAGEN INDUCTION ON HUMAN DERMAL FIBROBLASTS

Collagen induction by RELISTASE™ was evaluated by an Enzyme-linked Immunosorbent Assay (ELISA). Type I collagen from the culture medium was attached to the bottom of a plate well. It was detected with an anti-collagen type I antibody. This antibody was recognised by a labelled secondary antibody. The assay was then quantified by measuring the amount of labelled antibody bound to the matrix, by using a colorimetric substrate. Peroxidase labelled to the secondary antibody converts the colourless substrate (OPD) to a coloured product. This colour was measured and it is proportional to the quantity of type I collagen present in the sample.

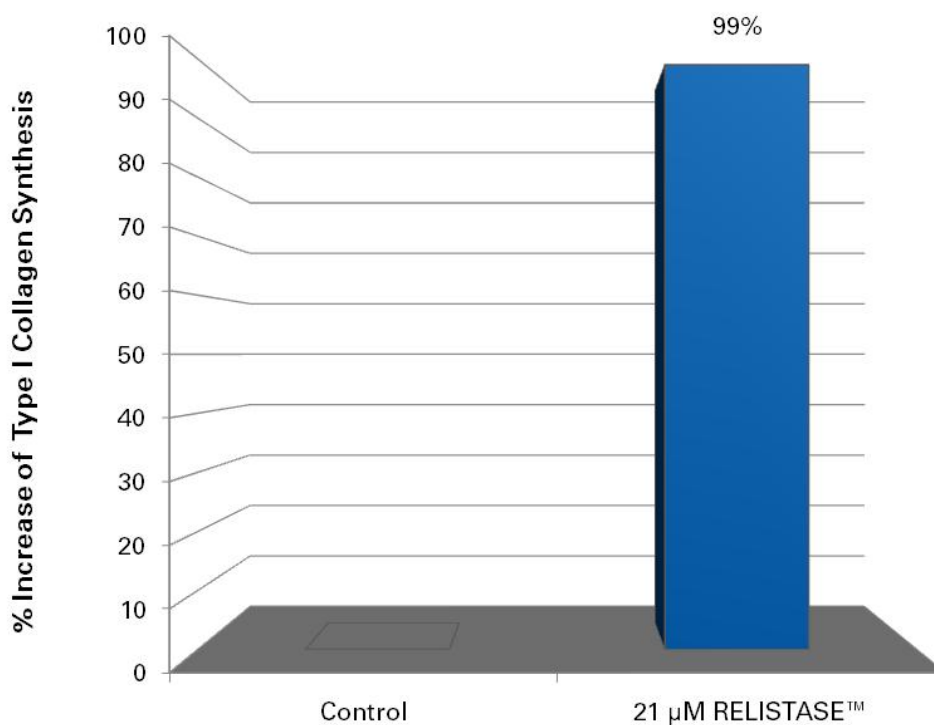


Fig. 5. Increase of type I collagen synthesis induced by 21 µM RELISTASE™ respect to non-treated cells.

RELISTASE™ proved to increase by 99% type I collagen synthesis induction on human dermal fibroblasts cell cultures at 21 µM.

## Efficacy *in vivo*

### CUTANEOUS ELASTICITY AND TIGHTNESS EVALUATION

A panel of volunteers composed of 20 women (mean age 49 years) applied a cream containing 4% RELISTASE™ SOLUTION or a placebo cream on their thighs twice a day for 8 weeks.

Skin elasticity was determined by a Cutometer® SEM 575, Courage & Khazaka which measures the vertical deformation of the skin, when it is sucked into the opening of a measuring probe. Mean values and standard deviations were calculated for T0, T4 and T8 instrumental values. Instrumental data and the variations were statistically compared by means of paired samples t-test. The differences were considered significant when the probability p was  $\leq 0.05$ .

The cream containing 4% RELISTASE™ SOLUTION showed a highly significant improvement on the overall elasticity of 11.7% and 14% after 4 and 8 weeks respectively. No statistically significant variation was detected in the values of the area treated with placebo cream.

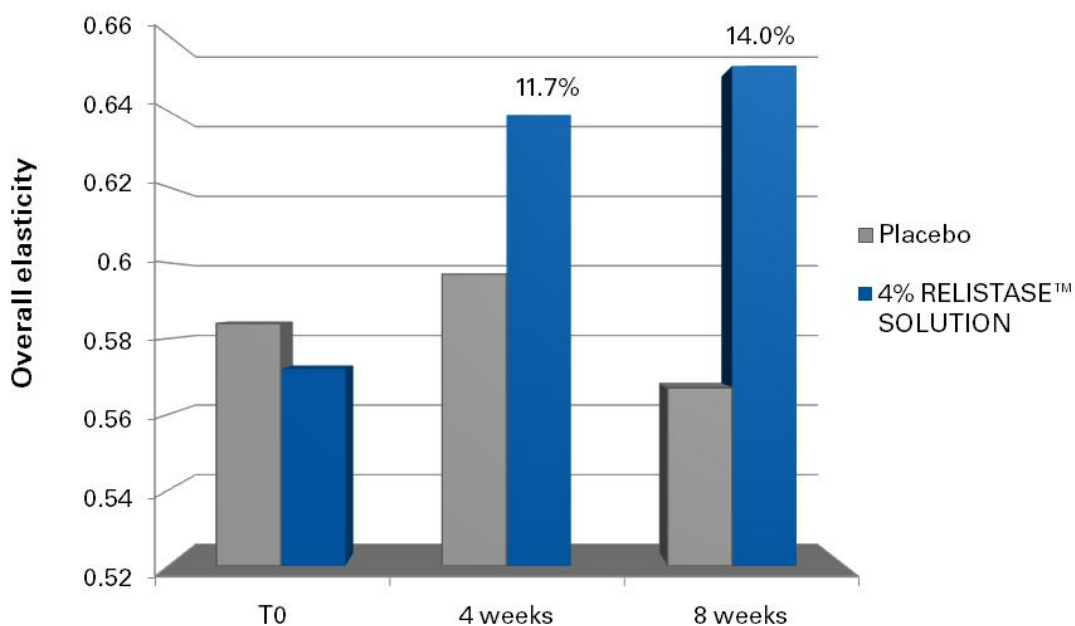


Fig. 6. Overall elasticity of the skin after applying a placebo or a cream containing 4% RELISTASE™ SOLUTION.

The area treated with the cream containing 4% RELISTASE™ SOLUTION showed a highly significant decrease in maximal deformation values of -5.5% and -15.6% after 4 and 8 weeks of treatment. However, no statistically significant variation was detected in the values of the area treated with placebo cream.

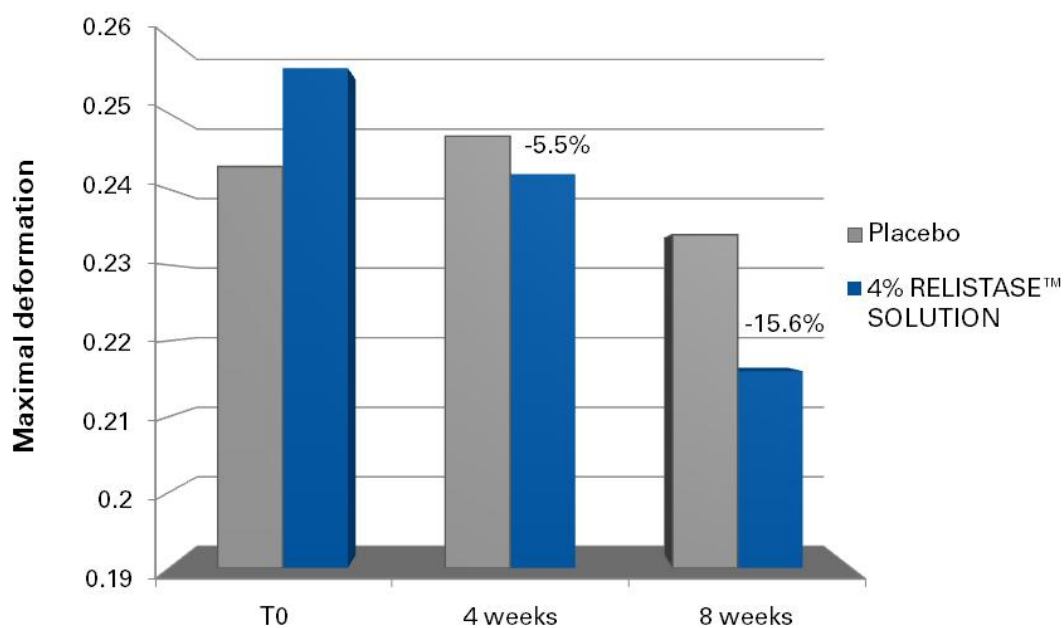


Fig. 7. Maximal deformation of the skin after applying a placebo or a cream containing 4% RELISTASE™ SOLUTION.

RELISTASE™ showed a highly significant improvement maximal deformation and overall elasticity of 15.6% and 14% respectively under the assayed conditions.

At the light of the results, it can be concluded that RELISTASE™ is a good cosmetic active ingredient for helping to improve elasticity and tightness of the skin.

## Safety

The toxicological profile of RELISTASE™ for cosmetic purposes was assessed *in vitro* and *in vivo*. A full toxicological report and a summary of all the safety tests performed are available on request.

### **IN VITRO TESTS**

#### **Cytotoxicity test on human epidermal keratinocytes**

The results showed no significant signs of cytotoxicity at the concentrations assayed.

#### **Cytotoxicity test on 3T3 fibroblasts**

The results showed no significant signs of cytotoxicity at the concentrations assayed.

#### **Ocular Irritation (HET-CAM test)**

According to the results of the HET-CAM test, RELISTASE™ is classified as slight ocular irritation.

#### **NRU Phototoxicity test**

The results showed no signs of phototoxicity at the concentrations assayed.

#### **Bacterial reverse mutation test (Ames test)**

The product produced no mutagenic neither promutagenic activity in any of the five bacterial strains used.

### **IN VIVO TESTS**

#### **Skin sensitisation and cutaneous compatibility test**

A HRIPT (Human Repeated Insult Patch Test) was performed on 101 volunteers aged 19 to 70. RELISTASE™ at 0.05%, did not provoke any primary or cumulative irritation reaction, nor any cutaneous sensitisation.

## Technical data

### PRESENTATION

Solution containing 0.01% of active ingredient.

<b>Trade name</b>	RELISTASE™ SOLUTION
<b>Product code</b>	PD170

### INGREDIENTS

INCI name	CAS No	EINECS No
GLYCERIN	56-81-5	200-289-5
ACETYLRGINYLTRYPTOPHYL DIPHENYLGLYCINE	-	-

### SPECIFICATIONS

Appearance:	Transparent solution
Colour:	Colourless to yellow
Specific gravity:	1.20 - 1.30 g/mL
Refractive index:	1.447 - 1.487

### DOSAGE

A dosage of 4% of RELISTASE™ SOLUTION is recommended in final cosmetic formulations.

### PROCESSING

RELISTASE™ SOLUTION can be incorporated at the final stage of the manufacturing process, provided always that the temperature is between 35°C – 40°C.

The pH of the final cosmetic formulation should range from 5.0 to 7.0.

### INCOMPATIBILITIES

Strong oxidants.

### SOLUBILITY

RELISTASE™ SOLUTION is soluble in water.

### STORAGE AND SHELF LIFE

RELISTASE™ SOLUTION should be stored in a dark, clean and cool place. If stored in these conditions shelf life is at least 18 months.

## FORMULATION

RELISTASE™ SOLUTION can be added to all types of skin care formulations where an elastic and tight enhancer effect is desired.

## Firming Cream for Mature Skin

### PROPERTIES

pH = 5.5 – 7.0

Viscosity = 80000 – 120000

cps (6/5.0)

### PROCEDURE

Mix A phase in an appropriate vessel. Add A1 phase while stirring and then heat up to 70°C. Weight B phase in another vessel and dissolve while heating up to 80°C. Pour the first mixture onto B phase. Let cool to 50°C and add C and D phase while stirring. Adjust pH with E phase.

PHASE	INGREDIENTS	% BY WEIGHT
A	WATER (AQUA)	q.s.p 100
	BUTYLENE GLYCOL	3
	PENTYLENE GLYCOL	2.5
	GLYCERIN	5
	PHENOXYETHANOL, METHYLPARABEN, ETHYLPARABEN, BUTYLPARABEN, PROPYLPARABEN, ISOBUTYLPARABEN	0.8
	IMIDAZOLIDINYL UREA	0.2
	DISODIUM EDTA	0.3
A1	SODIUM POLYACRYLATE, WATER (AQUA)	0.3
B	PROPYLENE GLYCOL DICAPRYLATE/DICAPRATE	3
	SHEA BUTTER (BUTYROSPERMUM PARKII)	4
	MALTOOLIGOSYL GLUCOSIDE, HYDROGENATED STARCH HYDROLYSATE, WATER (AQUA)	5
	MINERAL OIL (PARAFFINUM LIQUIDUM)	2
	TRIETHYLHEXANOIN	2
	GLYCERYL STEARATE, BEHENYL ALCOHOL, GLYCERYL STEARATE CITRATE, DISODIUM ETHYLENE DICOCAMIDE	3
	PEG-15 DISULFATE	
	CETEARETH-25	2
	STEARYL ALCOHOL	2
	CETEARYL ALCOHOL	2
GLYCERYL STEARATE, PEG-100 STEARATE	2	
C	DIMETHICONE	2
	RELISTASE™ SOLUTION (code PD170)	4
	SERILESINE® (code PD060)	5
	DECORINYL® (code PD090)	5
D	FRAGRANCE (PARFUM)	0.2
E	SODIUM HYDROXIDE (20% in aqueous solution)	q.s.

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*Note: Graphs and photographs of efficacy tests are available for customer use provided that the final product contains the same concentration of active as the formulations in our tests. Customers must request written permission for use of the graphic material and/or ingredient tradenames to Lipotec. Customers are responsible for compliance with local and international advertising regulations.*

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