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Contents

Original Laboratory Studies

1 The effectiveness of an innovative biorevitalizing mixture carried by the transdermal electodelivery
P. Fargione, F. De Natale, E. Sammarco, N. Chianese, P. Vitiello

A. De Nino, L. Di Donna, L. Maiuolo, F. Mazzotti, A. Napoli, E. Perri, A. Tagarelli, and G. Sindona

General Articles

17 Isoflavones, Phytohormones and Phytosterols
A.C. Dweck

Book Reviews

33 Multifunctional Cosmetics
38 The Antimicrobial Biological Activity of Essential Oils
41 Preservatives for Cosmetics
43 Atlas of Women’s Dermatology. From Infancy to Maturity
44 Color Atlas of Chemical Peels
The effectiveness of an innovative biorevitalizing mixture carried by the transdermal electrodelivery

P. Forgione¹, F. De Natale¹, E. Sammarco¹, N. Chianese², P. Vitiello²
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Key words: Hyaluronic acid; Betaglucan; Vitamic C; Electroporation; Skin aging;

Summary

Today dermatologists have at their own disposal many therapeutical devices able to satisfy the common desire of many of their patients to rejuvenate their skin reducing and slowing down signs of the time passing by.

We report our experience in using a biorevitalizing mixture of hyaluronic acid, vitamin C and betaglucan (trade name HCG2000®) properly carried by electroporation, a non-invasive innovative technique useful in the treatment of face and body skin imperfections.

Riassunto

Sono ormai a disposizione del dermatologo numerosi presidi terapeutici in grado di soddisfare il comune desiderio di molti pazienti di ringiovanire la propria pelle riducendo e rallentando i segni del tempo. Riportiamo la nostra esperienza nell' utilizzo di una miscela di biorivitalizzanti (denominata HCG2000®) costituita da acido ialuronico, vitamina C e betaglucano opportunamente veicolata attraverso l'elettroporazione, tecnica innovativa non invasiva utile nel trattamento degli in estetismi del volto e del corpo.
INTRODUCTION

The solution* used is a calibrated mixture of biostimulant actives of proved effectiveness such as betaglucan, vitamin C arginate, and hyaluronic acid, which stimulates the cellular turnover and the synthesis of collagen, elastin and fibrin.

It is indicated in the treatment via injection of stretch marks, wrinkles and acne scars.

The transdermal electroporation is a methodology that by using peculiar electrical pulsating impulses, cause a temporary expansion of intercellular spaces allowing a fast and selective penetration of drugs and dermocosmetics into the skin basal layer (1,2,3,4).

We report our experience in the use of HCG2000® by electroporation on subjects showing chrono and photo-induced facial skin aging.

MATERIALS AND METHODS

For this study it has been used a cyto stimulant solution* containing:
1) hyaluronic acid
2) betaglucan
3) ascorbic acid dissolved in sterile solution in phials of 2 and 5 ml.

For each treatment we used 1.5 ml of the solution dissolved in a wire gel.

The obtained mixture was ionized in a specific metallic activation room whom which the electronic device called Matiriport® (Burattini Biomedical Engineering, Pozzuoli, NA) is equipped. This allows a fast passage of the used molecules through the pulsed electrical waves of low frequency and high intensity, generated by this special device.

The study was conducted on 30 women aged between 40 and 55, treated by a 16 minutes electroporation sitting once and twice a week.

RESULTS

After 10 treatments a clear reduction in wrinkles depth was observed, many of which just after 5 weeks of treatment. Moreover it was observed a remarkable increase in skin hydration and tone. After the transdermal application of the mixture none of the subjects showed undesirable effects.

The personal opinion of the subjects treated was positive in all cases and extremely positive for the 50% of the cases.

DISCUSSION

The calibrated combination of the three active principles, betaglucan, vitamin C and, hyaluronic acid, that notoriously stimulate and speed up the synthesis of the normal fibrous component of the skin, optimizes the normal production of collagen, elastin and fibrin (5,6,7).

This association, thanks to a patented special procedure, permits the creation of particular ionic bonds activating the different molecules that allow and help their delivery and penetration through the different skin layers.

In this way hyaluronic acid can work with its elective re-hydrating activity maintaining and linking water in skin tissue (8).

The salification of vitamin C makes it stable against the oxidative processes, exalting at the same time its peculiar anti-free radicals activity as well as its stimulating activity in collagen synthesis (9,10).

Finally, betaglucan, strong and well known activator of the skin immune system, works both stimulating the cellular turnover and fighting oxidative stress.

The biorevitalizing mixture we used, is normally injected intradermically or intrapidermically in the desired quantity in the areas to be treated in a rigorous asepsis conditions.

*trade name HCG2000®
Periocular Wrinkles Before

After 10 sittings

Periocular Wrinkles Before

After 10 sittings

Glabellar Wrinkles Before

After 10 sittings
Before & After

Retociliar Winkles

After 8 shakes

Glabellar Winkles

Before

After 8 shakes

The effectiveness of an innovative biodegradable mixture carried by the transdermal electrodelivery
The injection exposes the patient to a possible appearance, in the injection area, of erythema, swelling, pain, itching and microhaematomas. The electroporation uses a reversible electric field, with short and high voltage pulses through which it electropores the double layer lipidic barriers with formation of subcellular pores that increase remarkably the substances delivery speed, even if of high molecular weight. This methodology increases the skin penetration of the active compounds, allowing a higher absorption rate, eliminating possible side effects due to the intradermal or intraepidermal injection and speeding up their therapeutic effects. The obtained results, reported in the figures 1-6, confirm that electroporation is an effective and well tolerated methodology to treat skin ageing and skin photo-ageing by using biorevitalizing molecules.

ACKNOWLEDGEMENTS

The authors would like to thank Mavi Sud S.r.l. for the samples of HCG2000® provided and Mr. Gianfranco Burrattini for the Matripor® project.
The effectiveness of an innovative biorevitalizing mixture carried by the transdermal electrodely delivery

References


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Key words: Hot chili pepper; Olive oil; Nutraceuticals; Oleuropein; Mass spectrometry;

Summary

Hot chili pepper and olive oil are typical foodstuff of the Calabrian agri-food chain. For their acknowledged pharmacological actions they can be considered as functional food or nutraceuticals. The evaluation of both origin and quality of this Mediterranean foods by means of high tech mass spectrometric methodologies is an added value approach that can help local farmers to get important and remunerative international markets.

Riassunto

Le abitudini alimentari dei Calabresi fanno riferimento all’uso quotidiano di peperoncino ed olio d’oliva. Questi alimenti sono ricchi di principi attivi con proprietà terapeutiche ampiamente accertate e pertanto possono esseri definiti come cibi funzionali o nutraceutici.
L’applicazione di metodologie high tech, quali quelle offerte dalla spettrometria di massa, permettono di saggiare la qualità, salubrità e tracciabilità di questi alimenti mediterranei, aggiungendo valore al prodotto finito che può raggiungere mercati internazionali molto più remunerativi di quelli regionali o nazionali.
INTRODUCTION

The image of Calabria, the extreme region of Italian peninsula, is linked to the use of hot chili pepper and virgin olive oil either as typical foods of the Mediterranean diet and as natural preservatives of processed foodstuff such as salami, ham, vegetables, etc. Many products of the total food Calabrian chain are often associated to this traditional marriage (figure 1).

The “accademia del peperoncino” funded in 1994 in Diamante (1), a tourist resort in the province of Cosenza, takes care of the tradition and the history of the Calabrian pepper. At an international level many bodies have been established to deal with the scientific aspect related to the use of chili pepper derivatives either for human nutrition, or as a functional food, with many pharmacological properties, or in cosmetic formulations. The “Pepper Institute” of the New Mexico State University (USA) represents a milestone for the spin off effect that scientific research can exert on the development of a new business in agri-food chain. The therapeutic benefits of chili pepper have been widely described and are associated to the presence of essential elements -such as calcium, iron, magnesium, potassium and phosphorous- to its richness in essential aminoacids- such as tryptophan, lysine and phenylalanine- and to the content of vitamins- A, C, B2 and B6- and, among others, to the presence of a powerful antioxidant such as capsaicin.

The use of chili pepper, very popular in the past in the Mediterranean European and African regions, in eastern countries and in many central and south American states, is now spread throughout all the developed countries and has, incredibly, become a spice commonly used for many typical foods.

Olive oil is a foodstuff widespread in the Mediterranean area, whereas it is replaced by seeds or other vegetable oils in the diet of most of the other developing and developed countries (figure 2). It is known for its typical content of phenols and catecols, the latter are associated to radical scavenger activity which protects the foodstuff during its aging and preserves some of its organoleptic properties.

Fig. 1 Fresh Calabrian chili pepper. The active principle Capsaicin is shown in the insert.

Fig. 2 Typical centennial Olive tree. Some of the Mediterranean cultivars are indicated.
One of the main vectors of phenols in olive drupes and leaves is represented by oleuropein (1, scheme 1), whose presence in fresh olive oils has been only recently demonstrated (123). Among the so-called phenolic component of oils (4) (scheme 1), a parameter normally used to assess the quality of the product, particular importance assume the presence of hydroxytyrosol [(3,4-dihydroxyphenyl)ethanol] (3, scheme 1), tyrosol [(4-hydroxyphenyl)-ethanol] (4, scheme 1), and generally speaking catechols, for their known pharmacological activity. 3 and 4 derive from enzymatic or chemical degradation of secoiridoid glycosides such as 1, which carry the phenol moieties inside the cells. A number of studies report on the biological and pharmacological activity of oleuropein whose presence confers to olive oil an added value due to its known preventive action towards a number of pathologies generated by free-radical action.

---

**Scheme 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$M_r$</th>
<th>R</th>
<th>R'</th>
<th>R''</th>
</tr>
</thead>
<tbody>
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<td>Oleuropein (1)</td>
<td>540</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Lignostroside (2)</td>
<td>524</td>
<td>H</td>
<td></td>
<td></td>
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<tr>
<td>Hydroxytyrosol (3)</td>
<td>154</td>
<td>OH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosol (4)</td>
<td>138</td>
<td>H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialdehyde form (5)</td>
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<td>OH</td>
<td>CH$_3$OOC</td>
<td></td>
</tr>
<tr>
<td>Dialdehyde form (6)</td>
<td>362</td>
<td>H</td>
<td>CH$_3$OOC</td>
<td></td>
</tr>
<tr>
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<td>378</td>
<td>OH</td>
<td>CH$_3$OOC</td>
<td></td>
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<tr>
<td>Geminal diol (8)</td>
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<td>CH$_3$OOC</td>
<td>H</td>
</tr>
<tr>
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<td>CH$_3$</td>
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<td>CH$_3$</td>
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<tr>
<td>Dialdehyde (11)</td>
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<td>H</td>
<td></td>
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<tr>
<td>Dialdehyde (12)</td>
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<td>H</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>Hemiaceatal (13)</td>
<td>352</td>
<td>OH</td>
<td>H</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>Hemiaceatal (14)</td>
<td>336</td>
<td>H</td>
<td>H</td>
<td>CH$_3$</td>
</tr>
</tbody>
</table>
The production of chili pepper and olive oil

Calabria is the region with the highest production of olive oil in Italy and with the highest number of family business in the corresponding food chain. This guarantees, at some extent, the local commercialization of excellent foodstuffs, but prevents the competitiveness of local farms with other national and international producers. The production of virgin olive oil of excellent quality, unfortunately, is not associated to the presence of regional bodies certifying the Calabrian grown olive oils which could promote the international markets penetration of this peculiar foodstuff.

The worldwide need of powdered or crushed chili pepper has promoted the appearance of new agri-food chains going from the production of the fruit till its commercialization after its transformation in food additive.

The demand of chili pepper from the small and medium (SME) Calabrian industries active in the production of sausages and salami and of typical processed foods, such as fishes, vegetables, etc., can not, unfortunately, be met by the local producers of fresh and dried fruits.

Chili pepper and olive oil as Functional Foods

Functional foods can be considered nutritional substances that can affect favorably one or more target functions in the body in a way that are relevant to either improved stage of health and well-being and/or reduction of risk of disease. A functional food must remain food and it must demonstrate its effects in amounts that can normally be expected to be consumed in the diet (5). In this respect virgin olive oil, for its content in catecols, and hot pepper, for the presence of capsaicin and other known active principles, can be regarded as functional foods. The therapeutic agents present in these foodstuffs represent, always, a minor percentage of the total amount of the edible components. Moreover, pesticides and additives, legally or fraudulently added, may be present also at low concentration too. Therefore the characterization of both the quality and the healthiness of olive oil and hot peppers requires the use of modern high tech approaches.

High tech Mass Spectrometric Methods in the Quality and Safety of Calabrian Foods

A French research institute working on the quality and safety of hot chilli pepper has discovered in 2003 and notified to European Union (EU) Commission that an azo-dye known as Sudan I, well known for its carcinogenic properties, was present in powdered or crushed hot chilli pepper originating from India or Pakistan and present in goods commercialized in the EU markets. A directive was issued by the Commission (6) where the presence of this colorant was banned from any foodstuff. More recently a new EU directive has established that Sudan II, III and IV should be totally absent in foods (7).

Unfortunately the low production costs available in undeveloped and developing countries has favored the penetration into the developed countries of uncontrolled foodstuff and raw materials. Powdered chili pepper contaminated with Sudan was spread all over Europe, in Calabria too, by local importers who perform a door-to-door commercialization of the good.
Our group active at the Department of Chemistry of the University of Calabria has set up an extremely sensitive method for the identification and assay of Sudan I, either in foodstuff and in powdered or crushed hot chili pepper. The method can reach at least the low limit of determination of 10 part per billion (10 ppb) (8).

The instrument used to assay the Sudan I in foodstuff is equipped with an atmospheric pressure chemical ionization (APCI) ion source coupled with a triple quadrupole analyzer. The quantitation has been performed by using the deuterated Sudan I as internal standard that provides a better accuracy of the measures and minimizes the errors. Moreover, the use of tandem mass spectrometers, in the applications known as multiple reaction monitoring (MRM), improve the specificity and the sensitivity of the determination.

The MS-MS spectrum of Sudan I (figure 3) shows the abundant ion at m/z 93 due to the formation of the aniline radical cation. Therefore, the transition chosen for the quantitative determination are m/z 249 → m/z 93 and m/z 254 → m/z 98 for the contaminant and the internal standard respectively.

Table I shows the amounts (in ppb) of Sudan I found in some foodstuff produced in Calabria and containing hot chilli pepper and the precision of the methodology (RSD%).

![Fig. 3 APCI MS/MS spectrum of Sudan I.]

<table>
<thead>
<tr>
<th>Typical product</th>
<th>Mean Value (ppm)</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;fresa bread&quot;* I</td>
<td>0.800 ± 0.005</td>
<td>0.62</td>
</tr>
<tr>
<td>Hot chilli pepper</td>
<td>922.313 ± 34.180</td>
<td>3.71</td>
</tr>
<tr>
<td>&quot;nduja&quot;***</td>
<td>93.615 ± 2.030</td>
<td>2.17</td>
</tr>
<tr>
<td>Sausage</td>
<td>0.078 ± 0.005</td>
<td>6.41</td>
</tr>
</tbody>
</table>

* Doubly baked bread, typical from Italian southern regions.
** Spicy sauce from cheek's lard and hot chilli pepper.
This research group is active also in the identification of markers for the traceability of olive oil. This is an important issues since either national or international bodies do not guarantee the consumer on the origin of the foodstuff. In Italy for instance, a labelled Italian virgin olive oil means, by law, that the foodstuff has been bottled in Italy, only! We have recently showed that an important antioxidant molecule, oleuropein, is present in virgin olive oil (3, 4). A survey of olive oils produced thorough Italy has shown that the content of oleuropein varies with the cultivar and the environment where the trees grow.

The quantitation of oleuropein has been accomplished by APCI-MS/MS under MRM condition, using the synthetically labeled d3-oleuropein as internal standard.

Table II shows the variation of the amount of oleuropein in virgin olive oils obtained from different cultivars, grown in different regions under different climate. It can be seen that the amount of oleuropein decreases with the latitude of the harvested drupes; Moreover, It seems that the filtered samples contain more oleuropein than the non-filtered ones. This is probably due to the presence of glycosidases in the water residues present in non-filtered oils leading to the decomposition of the antioxidant.

The identification and assay of minor components in foodstuff of the agri-food chain could provide markers for quality assessment and for the evaluation of its geographical origin. The evaluation of the aroma component of virgin olive oils could represent a source of data for the evaluation of both the specificity of the product and its geographical origin. The major drawback in this field was represented by extraction and concentration procedures and by the availability of instruments with enough sensitivity to detect trace components.

Fig. 4 APCI MS/MS spectra of Oleuropein (A) and labelled oleuropein (B).
Table II

Oleuropein content in different extra virgin olive oils.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Italian region</th>
<th>Treatment</th>
<th>ppm value</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carolea</td>
<td>Calabria</td>
<td>Filtered</td>
<td>0.357 ± 0.014</td>
<td>4.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>non-filtered</td>
<td>0.263 ± 0.004</td>
<td>1.38</td>
</tr>
<tr>
<td>Carolea</td>
<td>Abruzzo</td>
<td>Filtered</td>
<td>0.245 ± 0.014</td>
<td>5.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>non-filtered</td>
<td>0.130 ± 0.002</td>
<td>1.47</td>
</tr>
<tr>
<td>Carolea</td>
<td>Apulia</td>
<td>Filtered</td>
<td>0.296 ± 0.002</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>non-filtered</td>
<td>0.225 ± 0.004</td>
<td>1.62</td>
</tr>
<tr>
<td>Coratina</td>
<td>Calabria</td>
<td>filtered</td>
<td>0.175 ± 0.002</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>non-filtered</td>
<td>0.132 ± 0.005</td>
<td>4.06</td>
</tr>
<tr>
<td>Coratina</td>
<td>Abruzzo</td>
<td>filtered</td>
<td>0.116 ± 0.001</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>non-filtered</td>
<td>0.093 ± 0.003</td>
<td>3.48</td>
</tr>
<tr>
<td>Coratina</td>
<td>Apulia</td>
<td>filtered</td>
<td>0.222 ± 0.002</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>non-filtered</td>
<td>0.118 ± 0.001</td>
<td>0.80</td>
</tr>
<tr>
<td>Frantoio</td>
<td>Calabria</td>
<td>filtered</td>
<td>0.344 ± 0.008</td>
<td>2.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>non-filtered</td>
<td>0.214 ± 0.002</td>
<td>0.94</td>
</tr>
<tr>
<td>Frantoio</td>
<td>Abruzzo</td>
<td>filtered</td>
<td>0.203 ± 0.001</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>non-filtered</td>
<td>0.133 ± 0.001</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Gas chromatographic methods have been extensively applied, in connection often with mass spectrometric analysis, to detect, by the headspace method, the composition of the volatile components of foodstuff (9,10). In our approach the solid phase micro-extraction method (SPME) was adopted, since it does not require the use of solvents and can be used for the direct evaluation of analytes either in matrix or by the headspace method. The analysis of the examined virgin oils was carried out with an GC-Ion Trap instrument. The complexity of the mass-chromatograms in terms of number of components might represent a drawback when different samples are to be matched. It was decided therefore to consider chromatograms containing the minimum set of components in relation to their biogenesis (11). Accordingly, hexanal, 1-hexanol, (E)-2-hexenal, (E)-2-hexen-1-ol and (Z)-3-hexenyl acetate were chosen, as markers of linoleic and linolenic acids specific lipoxygenase oxidation, respectively [(path A and B), Scheme 2] (12). The quantitative analysis was therefore oriented towards
the evaluation of the components derived by this metabolic process. The results obtained for all the analyzed samples showed that the number and type of the markers selected for the survey were adequate for the characterization of olive oil as a function of the cultivars, the harvesting time and the growing environmental effect. Different cultivars differ also significantly in the composition of the aroma even if the oil is produced from drupes with identical ripening index. The histograms reported in figures 5 and 6 are indicative of this correlation. Organic oils obtained from drupes of Nocellara del Belice cultivar, grown at Castelvetrano, and Nocellara Etnea cultivar, grown at S. Maria di Licodia (Sicily), with the same ripening index value of 0.18 show enormous difference in the composition of their aroma markers (figure 5). Both products are very rich in 1-hexanol, whereas (E)-2-hexenal, which is very abundant in most of Calabrian and Apulia oils, represents a minor component. Figure 6 shows the chromatogram of volatile compounds of Ogliarola Salentina cultivar, grown at Villa Castelli (Apulia), and Ogliarola Barese cultivar, grown at Conversano (Apulia). A preliminary observation concerns the relative amount of (E)-2-hexenal, always the main component, which points out a characteristic of the Apulian oils, with respect to the others produced in southern Italy, i.e., their typical richness in the aldehyde components. Moreover, the ratios (E)-2-hexen-1-ol/(E)-2-hexenal and 1-hexanol/hexanal in the oil produced from Ogliarola Salentina cultivar probably depend on the activity of alcohol dehydrogenase enzymes which seems to be higher for the saturated than for the unsaturated substrate. The relatively high concentration of (E)-2-hexen-1-ol (0.8 mg/kg), in the case of Ogliarola Barese cultivar, is probably due to an enhanced activity of the enzyme specific for this process.

**New scenarios**

The improvement of the Calabrian olive oil business can be reasonably performed with little efforts. In this field, in fact, it is important to be recognized on a national and international basis for both quality, safety and traceability of the marketed product, goal that can be easily fulfilled by applying the recently high tech methods developed at the University of Calabria.
Similar considerations apply to the production of processed hot chili pepper. In this case particular importance must be devoted to the characterization of the Calabrian foodstuff, in order to demonstrate, unambiguously, the excellence and the safety of this Mediterranean product.

The high tech approach to a better identification of origin and definition of quality of this two peculiar foodstuff of the Mediterranean diet can be performed by means of:

- Evaluation by GC/MS and combined techniques of those markers in the volatile fraction which can be related to cultivars typical of Calabria.
- Evaluation by ESI-MS/MS of the quality and quantity of those micro components present in the fruit playing a pharmacological role.
- Genomics. Correlation, by conventional (PCR_RAPD) and new methodologies (MALDI-TOF/TOF), of plant genomic and cultivar.
- Proteomics. Determination of the protein profiling of specific part of the plants and elaboration of databases useful for a fast screening of the origin and cultivar of a specific fruit.
References


5) Consensus document of the EC project FUFOSE


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Isoflavones, Phytohormones and Phytosterols

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Key words: Isoflavones; Hormones; Sterols; Wrinkle reduction; Anti-inflammatory; Anti-pruritic; Breast and skin firmness; incidence of hot flushes; Menopausal women;

Summary

A review of isoflavones, plant derived hormones and sterols in the care of the skin. The effects have been shown to include protection of the skin, the reduction of wrinkles, reduction in inflammation and a decrease in pruritis. In some commercial raw materials, there has been improvement in breast and skin firmness and reduction in sun burn cell reaction. Internally, these plants are used for reducing the incidence of hot flushes and other discomforts in menopausal women.

Riassunto

Gli isoflavoni, derivati ormonali delle piante, e gli steroi, sono frequentemente utilizzati per la cura della pelle. Di questi composti sono noti gli effetti topici quali sostanze protettive nei confronti degli inquinanti ambientali o quali sostanze attive nel ridurre il numero e la profondità delle rughe o efficaci nel mitigare i processi infiammatori e il prurito.

Dato il loro uso sempre più frequente nei disturbi della menopausa, utilizzati anche per via sistemi­ca, viene descritta e riportata la formulazione chimica, il meccanismo d’azione e i principi attivi contenuti nelle diverse tipologie di estratti vegetali già noti.
Isoflavones have the phenyl group attached to the 3-position, whereas in flavones the phenyl group is attached to the 2-position. The isoflavones are mainly found to occur within the Leguminosae (specifically in the sub-family Papilionoideae), although the literature shows many other species that contain these chemical moieties [Boland and Donnelly]. Isoflavones are also found in other botanical families such as the Compositae, the Iridaceae, the Myristicaceae, and the Rosaceae.

These isoflavones can act as steroidal mimics by filling the stereochemical space that could be occupied by oestrogenic compounds. It is this spacial chemistry that helps explain the effects of many nutritional herbal supplements and topical preparations.

Daidzein is a phyto-estrogen, but is also called a phenolic estrogen, to distinguish it from a steroidal estrogen like 17β-estradiol. The activity of phytoestrogen is much weaker than the steroidal estrogen, varying from 0.005-2% [Brand]. The estrogenic properties are insufficient in strength to replace steroidal estrogens, but they do have significant value when it comes to reducing the effects of ageing and improving the quality of the skin.

Phyto-oestrogens may also be viewed in relation to the phytochemical division of terpenoids, which comprise the largest group of natural plant products. All terpenoids are derived biogenetically from isoprene. The largest group of terpenoids are the triterpenoids, which include, amongst other divisions, the triterpenoid and steroid saponins, and, the phytosterols. The phyto-oestrogens fall into these three categories. In addition, nature has a rich portfolio of phytosterols. It is easy to understand why sterols like stigmasterol (Fig.3) and β-sitosterol (Fig.4) have an effect that is anti-inflammatory and capable of reducing swelling and erythema, when their structure is compared to corticosterone (Fig.5) and hydrocortisone (Fig.6).

The paper concludes with a look at phytohormones and compares them to synthetic hormones and explains their effects against this known background.
The most commonly occurring isoflavones are:
- Biochanin-A: 5,7-dihydroxy-4'-methoxyisoflavone
- Daidzein: 4',7-dihydroxyisoflavone
- (±)-Equol: 4',7-isoflavandiol
- Formononetin: 7-hydroxy-4'-methoxyisoflavone
- Glycitein: 4',7-dihydroxy-6-methoxyisoflavone
- Genistein: 4',5,7-trihydroxyisoflavone
- Genistein-4',7-dimethylether: 5-hydroxy-4',7-dimethoxyisoflavone
- Prunetin: 4',5-dihydroxy-7-methoxyisoflavone

with the associated glucosides
- Genistin: glucosyl-7-genistein
- Glycitin: 4',7-dihydroxy-6-methoxyisoflavone-7-d-glucoside
- Ononin: formononetin-7-O-glucoside
- Sissotrin: biochanin A-7-glucoside

A more detailed list of isoflavones is shown in Appendix I [Boland and Donnelly]. The comparison of effects and functions of plants containing the same isoflavones shows remarkable similarity.

**Daidzein as an example of an isoflavone**

Daidzein is a solid substance that is virtually insoluble in water. Its molecular formula is \( C_{15}H_{10}O_{4} \), and its molecular weight is 254.24 daltons. Daidzein is also known as 7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one and 4', 7-dihydroxyisoflavone. Daidzin, which has greater water solubility than daidzein, is the 7-beta glucoside of daidzein.

Daidzein is an isoflavone. It is also classified as a phytoestrogen since it is a plant-derived non-steroidal compound that has estrogen-like biolo-
Other sources of biochanin A are Baptisia tinctoria (Wild Indigo), Medicago sativa (Alfalfa), Sophora japonica (Japanese Pagoda Tree) and Vigna radiata (Mungbean). These two isoflavones are considerably less estrogenic in their original forms, because the stereochemistry of the methoxy groups means they are not able to bind to the estrogen receptors as efficiently.

However, once these molecules are ingested, bacteria in the colon are able to remove the methyl groups - biochanin A becomes genistein (Fig. 18) and formononetin becomes daidzein (Fig. 1 see above). Daidzein can be further metabolized to equol (Fig. 19).

Other sources of formononetin are Astragalus membranaceus (Astragalus), Cimicifuga racemosa (Black Cohosh), Glycyrrhiza glabra (Licorice root), Medicago sativa (Alfalfa), Pueraria spp. (Kudzu; Pueraria), Sophora japonica (Japanese Pagoda Tree) and Vigna radiata (Mungbean)

Internally, biochanin A and formononetin are then able to be a source of considerable estrogenic activity.

Historically, the flower tea has been used as an antispasmodic, expectorant and mild sedative. It is also recommended for athlete's foot, sores, burns, and ulcers. [Leung & Foster] and has been used in the herbal treatment of cancer, especially of the breast or ovaries [Mills].

Red Clover is also a very popular remedy as the
alternative for hormone replacement therapy and is sold extensively for this purpose.

**Sweet Yellow Melilot (Melilotus officinalis)**

Melilot is soothing, lenitive, astringent, refreshing and anti-irritant and has similar properties to the red clover described above. It is also described as possibly having the additional properties of being anti-inflammatory, anti-oedema, a venous astringent (haemorrhoids) and anaesthetic [Council of Europe].

However, it is perhaps not the isoflavones at force here, but maybe the β-sitosterol or coumarin the roots contain.

Melilotus officinalis L. extract, containing 0.25% coumarin (Fig 20) was studied on acute inflammation induced with oil of turpentine in male rabbits. M. officinalis had anti-inflammatory effects because it reduced the activation of circulating phagocytes and lowered citrulline production.

![Coumarin](image)

*Fig. 20 coumarin.*

These properties were similar to those of hydrocortisone sodium hemisuccinate and coumarin. [Plesca-Manea et al]

**PHYTOSTEROLS AND RELATED COMPOUNDS**

The benefits of these phytosterols may be seen in the common herbal materials indicated for arthritis, such as Frankincense (Boswellia serrata). The boswellic acid present inhibits two inflammatory enzymes, 5-lipoxygenase (which produces leukotrienes) and human leukocyte elastase HLE (which degrades elastase).

![Boswellic acid](image)

*Fig. 21 Boswellic acid.*

Committee on Toxicity of chemicals in food, consumer products and the environment. Working group on phytoestrogens Cellular & molecular mechanisms of phytoestrogen activity The Department of Biochemical Pharmacology, Imperial College School of Medicine prepared a paper for discussion: “Assessment of the estrogenic potency of phyto-compounds”. This reviewed the available information on cellular and molecular mechanisms and phytoestrogen estrogenic potencies.

Out of the 28 points (statements for comment really) the following stood out:

Taking all estrogen receptor binding assays into account the review proposed the following rank order of phytoestrogen potency: estradiol >> coumestrol > 8-prenylnaringenin > equol >= genistein > biochanin A > daidzein > genistein glucuronide* > daidzein glucuronide* > formononetin (the activity of those compounds marked * may be due to the presence of activating enzymes present in the receptor preparation).

Phytoestrogens stimulated *in vitro* cell proliferation at concentrations of 0.1 - 10mM (3 - 4-fold less than estradiol). They did not induce the maximal proliferative effect of estradiol as
higher concentrations inhibited proliferation. The majority of endogenous estrogens (> 90%) were not freely available but bound to plasma proteins. Phytoestrogens bound at 1/100th to 1/10000th the affinity of estradiol. The availability of phytoestrogens in plasma relative to estradiol will be greater. Coumestrol, 8-prenylnaringenin and equol were > 1000-fold less potent than estradiol and the isoflavones > 10 000-fold less potent.

**The Wild Yam (Dioscorea villosa)**

The Wild Yam (Dioscorea villosa) was the source of diosgenin (Fig. 21), a steroidal saponin used as the starting point for the commercial source of pregnanolone (Fig. 22) and progesterone (Fig. 23) used as the first birth control pills. The root of Dioscorea is used for numerous purposes, but the major use is for the suppression of menopausal symptoms like hot flushes [Watson].

![Fig. 22 diosgenin.](image)

There are many other sources of diosgenin
- Trigonella foenum-graecum L. [Fabaceae] Seed 3300-19000ppm
- Solanum nigrum L. [Solanaceae] Fruit 4000-12000ppm
- Daucus carota L. [Apiaceae] Root 5400-6000ppm
- Dioscorea bulbifera L. [Dioscoreaceae] Tuber 4500-4500ppm
- Medicago sativa subsp. sativa [Fabaceae] Seed
- Agave sisalana [Agavaceae] Plant
- Aletris farinosa L. [Liliaceae] Root
- Areca catechu L. [Areaceae] Seed
- Asparagus officinalis L. [Liliaceae] Shoot
- Balanites aegyptiacus (L.) DELILE [Balanitaceae] Fruit
- Chamaelirium luteum (L.) A. GRAY [Liliaceae] Root
- Costus speciosus (J. KONIG) SM. [Costaceae] Rhizome
- Dioscorea composita HEMSL. [Dioscoreaceae] Plant
- Dioscorea sp. [Dioscoreaceae] Root
- Dioscorea villosa L. Plant
- Dioscorea villosa L. [Dioscoreaceae] Tuber
- Jateorhiza palmata MIERS [Menispermaceae] Root
- Lycium chinense MILL. [Solanaceae] Flower
- Melilotus officinalis LAM. [Fabaceae] Seed
- Momordica charantia L. [Cucurbitaceae] Fruit
- Paris polyphylla SM. [Liliaceae] Root
- Smilax china L. [Smilacaceae] Root
- Solanum dulcamara L. [Solanaceae] Plant
- Tribulus terrestris L. [Zygophyllaceae] Shoot

During pregnancy, small frequent doses will help allay nausea [Lust; Grieve]. It is antispasmodic. It is valuable neuralgic affections, spasmodic hiccough and spasmodic asthma [Grieve].

![Fig. 23 pregnanolone.](image)
It is interesting to note that Vitex agnus-castus is a source of natural progesterone. Proprietary preparations containing this material have been available in Germany since the 1950s and many documented studies have investigated the use of these products to treat various gynaecological disorders [Newall]. The fruit of Vitex contains essential oils, iridoid glycosides, and flavonoids. Essential oils include limonene, 1,8 cineole, and sabinene. The primary flavonoids include castican, orientin, and isovitexin. The two iridoidglycosides isolated are agnuside and aucubin. Agnuside serves as a reference material for quality control in the manufacture of Vitex extracts. One other report demonstrated delta-3-ketosteroids in the flowers and leaves of Vitex that probably contained progesterone and 17-hydroxyprogesterone [Brown]. The active constituents have been determined as 17-α-hydroxyprogesterone (leaf), 17-hydroxyprogesterone (leaf), androstenedione (leaf), δ-3-ketosteroids (leaf), epitestosterone (flower), progesterone (leaf), testosterone (flower and leaf) [Phytochemical and Ethnobotanical Databases]. It is highly unlikely that the diosgenin in the plant could ever be synthesised on the topical application to the skin to form a corticosteroid or hormonal derivative. However, it does seem likely that this material (being the precursor to these estrogenic molecules) will to some extent mimic the function of those pharmaceutical active materials and benefit the skin [Dweck, 2002].

However, the production of wild yam was unable to sustain the demand for diosgenin as the starting precursor, for the production of birth control materials, which by this stage was dominated by estrone (Fig. 24 estrone).

**Fenugreek (Trigonella foenum graecum)**

The world turned its attention to Fenugreek (Trigonella foenum graecum) for its source of diosgenin. Fenugreek or Foenugreek seeds are emollient and accelerate the healing of suppurations and inflammations. Externally cooked with water into a porridge and used as hot compresses on boils and abscesses in a similar manner to the usage of linseed [Fluck]. Decotions of whole plant are used as a bath for uterus infections. The seeds are tonic, restorative, aphrodisiac and galactagogue. Their emollient properties are useful for the itch. A cataplasm obtained by boiling the flour of the seeds with vinegar and saltpetre is used for swelling of the spleen [Bonlos]. Extracts of the seeds are incorporated into several cosmetics claimed to have effect on premature hair loss, and as a skin cleanser [Iwu], and it is also reported in Java in hair tonics and to cure baldness [Leung]. Many of the herbal materials found to have an effect on hair growth have a hormonal or hormonal-mimetic basis.

Likewise there are a number of references to fenugreek having galactagogue (increase milk in
nursing mothers) activity [Bunney; Burkill; Mills], which again is indicative of an estrogen-like activity. The plant should be used with caution as Fenugreek is reputed to be oxytocic and in vitro uterine stimulant activity has been documented [Newall et al], so the use of fenugreek during pregnancy and lactation in doses greatly exceeding those normally encountered in foods is not advisable.

**Pomegranate (Punica granatum)**

Pomegranate is one of the many plants that contain substances with hormone-type action. The seeds of Pomegranate, that ancient symbol of fertility, were found to contain an estrone identical with the genuine hormone. Punica granatum seeds are the best source of plant estrone to date [Weiss]. The antioxidant and eicosanoid enzyme inhibition properties of pomegranate (Punica granatum) fermented juice and seed oil flavonoids were studied, which showed strong antioxidant activity (determined by measuring the coupled oxidation of carotene and linoleic acid) close to that of butylated hydroxyanisole (BHA) and green tea, and significantly greater than that of red wine. [Schubert et al]. This is clearly a fruit worthy of further exploration, especially as most of the information to date relates to the use of the bark, seeds and the roots as a taenicide (expelling worms). The rind is used as an astringent [Lust]. The leaf has antibacterial properties and is applied externally to sores [Stuart].

**Other plants that contain estrone**

Dukes data base [Phytochemical and Ethnobotanical Databases] shows the following plants as containing estrone.

- Punica granatum L. Pomegranate (Seed) 17ppm, Malus domestica BORKH. Apple (Seed), Zea mays L. Corn (Seed Oil), Humulus lupulus L. Hops (Fruit), Olea europaea L. Olive (Seed), Panax quinquefolius L. American Ginseng (Plant), Phaseolus vulgaris L. Anasazi Bean (Flower), Phoenix dactylifera L. - Date Palm (Seed), Prunus armeniaca L. - Apricot (Seed).

**Date Palm (Phoenix dactylifera)**

Body hormones play a central role in skin appearance and are implicated in skin aging. Studies have shown that the decrease of these hormones plays an important role in skin endogenous aging, reduced skin thickness, and the disturbance of normal collagen turnover which, in turn, results in a decrease in collagen I and III synthesis. Date Palm has seven compounds with regenerative, anti-oxidizing, firming, and soothing properties, extracted from the kernel: phytosterols, phytosteroids, ursolic acid, isoflavons, policosonols, pro-vitamin A and vitamin E.

**Fig. 26 Prasterone or Dehydroepiandrosterone (DHEA).** Some studies suggest that DHEA administration would have a beneficial effect against signs of aging. DHEA is known for its capacity to promote keratinization of the epidermis or to reinforce the barrier function of the skin. The author compared on ex vivo skin, the effects of Date Palm kernel extract with those of DHEA in reference to DHEA as an anti-aging molecule. There was a decrease of wrinkles within only
five weeks of Date Palm kernel extract application and also improved the skin structure in a way superior to that of DHEA [Dal Farra]. The seed and the pollen have both been shown to contain estrone and this may further explain the reasons for this activity [Morton; Duke].

**Plants with a future for topical application**

In view of the benefits seen with those plants containing genistein and daidzein, we looked at other plants that might have potential as topical materials and also looked to see if they contained phytosterols and/or phyto-hormones. The results were promising.

**Calabar Bean (Physostigma venenosum)**

![Stigmasterol](image)


**Suma or Brazilian Ginseng (Pfaffia paniculatata)**

![β-ecdysterone](image)


**Cherimoya (Annona cherimoya)**

![β-sitosterol](image)

Isoflavones, Phytohormones and Phytosterols


Hops (Humulus lupulus)

The hop contains β-sitosterol, estradiol, stigmasterol and estrone. In addition it contains many other materials that are known for their sedative and relaxing attributes.

Regular doses of the herb can help regulate the menstrual cycle [Keville]. It was the girls and women picking hops who first discovered that hops have an effect on genital organs. Before machines were introduced, hop pickers used to spend several weeks at this work, and it had always been known that menstrual periods would come early in young girls while they were doing this work. The reason is that hops contain plant hormones, particularly when very fresh, and these are similar to oestrogens. Considerable amounts have been found, 30,000 to 300,000 i.u. of oestrogen in 100g of hops. This also explains why hops will suppress sexual excitement in men. It has been shown that there are substances called anti-androgens that are able to cancel the effects of the male hormone (androgen) [Weiss]. It was found that hop extract not only recovered the proliferation of hair follicle derived keratinocyte (HFKs) suppressed by androgen but also stimulated the proliferation of HFKs. Furthermore, the effects of hop were evaluated using both animal tests and human volunteers in vivo. It was demonstrated that hop showed a potent acceleration on hair growth. [Okano et al]

Lima Bean or Butter Bean (Phaseolus lunatus)

![Fig. 30 Estradiol.](image)

Sarsaparilla (Smilax ornata)

It is used in concoctions with other plants as a tonic or aphrodisiac [Seaforth]. Sarsaparilla was formerly used in the treatment
of syphilis [Carrington], gonorrhoea [Honychurch], rheumatism and certain skin diseases. Used in soft drinks, the genins are also used in the partial synthesis of cortisone and other steroids [Evans]. As part of a wider treatment for chronic rheumatism it should be considered as it is especially useful for rheumatoid arthritis. It has been shown that Sarsaparilla contains chemicals with properties that aid testosterone activity in the body [Hoffman].

Sarsaparilla contains saponins, sarsaponin and parallin, which yield isomeric sapogenins, sarsapogenin and smilogenin. It also contains sitosterol and stigmasterol in the free form and as glucosides. It is antirheumatic, antiseptic, antipruritic and is indicated for psoriasis, and other cutaneous conditions. Like other steroidal plants it is indicated for chronic rheumatism and rheumatoid arthritis. It is specifically used in cases of psoriasis especially where there is desquamation [British Herbal Pharmacopoeia].
### Appendix

#### Isoflavones

<table>
<thead>
<tr>
<th>Isoflavone</th>
<th>Plant</th>
<th>Part</th>
</tr>
</thead>
<tbody>
<tr>
<td>2′-Hydroxy-5-methoxy-6,7-methylenedioxy-</td>
<td>Calotis armenta</td>
<td>aerial parts</td>
</tr>
<tr>
<td>2′-Hydroxy-5,6-dimethylenedioxy-</td>
<td>Calotis armenta</td>
<td>aerial parts</td>
</tr>
<tr>
<td>6,7,8,3′,4′,5′-Hexamethoxy-</td>
<td>Petalostemon purpureus</td>
<td>bark</td>
</tr>
<tr>
<td>7,8,3′,4′,5′-Pentamethoxy-</td>
<td>Petalostemon purpureus</td>
<td>bark</td>
</tr>
<tr>
<td>Naringin (6,7,4′-trihydroxy-2′-3′,6′-trimethoxy-)</td>
<td>Garcinia nervosa</td>
<td>leaves</td>
</tr>
<tr>
<td>Catechin (6,7,2′-trihydroxy-6,4′,5′-pentamethoxy-)</td>
<td>Garcinia nervosa</td>
<td>leaves</td>
</tr>
<tr>
<td>4-Methylepicondorin (5,4′-dihydroxy-6,7-dimethoxy-)</td>
<td>Iris japonica</td>
<td>aerial parts</td>
</tr>
<tr>
<td>Irisjaponin A (5,7-dihydroxy-6,2′,4′-pentamethoxy-)</td>
<td>Iris japonica</td>
<td>aerial parts</td>
</tr>
<tr>
<td>Irisjaponin B (5,7-dihydroxy-6,2′,4′-pentamethoxy-)</td>
<td>Iris japonica</td>
<td>aerial parts</td>
</tr>
<tr>
<td>Nigricin (4′-hydroxy-5,3′-dimethoxy-6,7-methylenedioxy-)</td>
<td>Iris nigricans</td>
<td>rhizomes</td>
</tr>
<tr>
<td>Isodaiditin (3′-hydroxy-6,7,4′-trimethoxy-)</td>
<td>Iris isodaiditin</td>
<td>rhizomes</td>
</tr>
<tr>
<td>Kashihaeitin (4′-hydroxy-5,3′-dimethoxy-6,7-methylenedioxy-)</td>
<td>Iris kashihaeitin</td>
<td>rhizomes</td>
</tr>
<tr>
<td>2′-Methoxyisoflavanone (2′-hydroxy-3′,4′-dimethoxy-)</td>
<td>Erythrina californica</td>
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</tr>
<tr>
<td>2′,4′-Dihydroxy-7-methoxy-</td>
<td>Erythrina californica</td>
<td>whole plant</td>
</tr>
<tr>
<td>Gerontisclavine A (2′,4′-dihydroxy-5,3′-dimethoxy-)</td>
<td>Cuadrantes cochinchinensis var. gronolobea</td>
<td>root wood</td>
</tr>
<tr>
<td>Aceonoverone (2′-hydroxy-7-methoxy-3′,4′-methylenedioxy-)</td>
<td>Abies alba cieer</td>
<td>roots</td>
</tr>
<tr>
<td>Erythrina senegalensis E</td>
<td>Erythrina senegalensis</td>
<td>stem bark</td>
</tr>
<tr>
<td>Erythrina senegalensis F</td>
<td>Erythrina senegalensis</td>
<td>stem bark</td>
</tr>
<tr>
<td>Erythrina senegalensis G</td>
<td>Erythrina senegalensis</td>
<td>stem bark</td>
</tr>
<tr>
<td>Erythrina senegalensis H</td>
<td>Erythrina senegalensis</td>
<td>stem bark</td>
</tr>
<tr>
<td>Erythrina senegalensis I</td>
<td>Erythrina senegalensis</td>
<td>stem bark</td>
</tr>
<tr>
<td>Erythrina senegalensis K</td>
<td>Erythrina senegalensis</td>
<td>stem bark</td>
</tr>
<tr>
<td>Erythrina senegalensis L</td>
<td>Erythrina senegalensis</td>
<td>stem bark</td>
</tr>
<tr>
<td>Erythrina senagalensis M</td>
<td>Erythrina senegalensis</td>
<td>stem bark</td>
</tr>
<tr>
<td>5-O-Methyl-4′-O-(3-methylbut-2-enyl) alpinumisosiflavone</td>
<td>Mitella thonningi</td>
<td>root bark</td>
</tr>
<tr>
<td>Thonningiisosiflavone</td>
<td>Mitella thonningi</td>
<td>root bark</td>
</tr>
<tr>
<td>Durallone</td>
<td>Mitella dura</td>
<td>seed pods</td>
</tr>
<tr>
<td>6-Demethyldurallone</td>
<td>Mitella dura</td>
<td>seed pods</td>
</tr>
<tr>
<td>Preurallone</td>
<td>Mitella dura</td>
<td>seed pods</td>
</tr>
<tr>
<td>Isothyohynin A 4′-[3-methylbut-2-enyl] ether</td>
<td>Mitella dura</td>
<td>seed pods</td>
</tr>
<tr>
<td>Glyasperin N</td>
<td>Glycyrrhiza aspera</td>
<td>roots</td>
</tr>
<tr>
<td>Kanzoii D</td>
<td>Glycyrrhiza uralinensis</td>
<td>roots</td>
</tr>
<tr>
<td>Kanzoii L</td>
<td>Glycyrrhiza uralinensis</td>
<td>roots</td>
</tr>
<tr>
<td>Kanzoii Y</td>
<td>Glycyrrhiza globra</td>
<td>roots</td>
</tr>
<tr>
<td>Burycaprin A</td>
<td>Glycyrrhiza eurycapra</td>
<td>roots</td>
</tr>
<tr>
<td>Anagynodiosiflavone A</td>
<td>Labiumum amarynagroids</td>
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</tr>
<tr>
<td>Anagynodiosiflavone B</td>
<td>Labiumum amarynagroids</td>
<td>pods</td>
</tr>
<tr>
<td>Apiotin</td>
<td>Labiumum amarynagroids</td>
<td>pods</td>
</tr>
<tr>
<td>Acipotinosiflavone</td>
<td>Labiumum amarynagroids</td>
<td>pods</td>
</tr>
<tr>
<td>Ampoupinosa</td>
<td>Labiumum amarynagroids</td>
<td>roots</td>
</tr>
<tr>
<td>Secundiflor B</td>
<td>Sophora secundiflorata</td>
<td>roots</td>
</tr>
<tr>
<td>Secundiflor C</td>
<td>Sophora secundiflorata</td>
<td>roots</td>
</tr>
<tr>
<td>Isoalbalginin</td>
<td>Lupinus luteus</td>
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</tr>
<tr>
<td>Omoksini</td>
<td>Omokia monogera</td>
<td>root bark</td>
</tr>
<tr>
<td>Eluranaragone</td>
<td>Dennis scandentia</td>
<td>stems</td>
</tr>
<tr>
<td>Eluocaragone</td>
<td>Eriogonum tuberosum</td>
<td>roots</td>
</tr>
<tr>
<td>Ficusin A</td>
<td>Ficus spers</td>
<td>root bark</td>
</tr>
<tr>
<td>Ficusin B</td>
<td>Ficus spers</td>
<td>root bark</td>
</tr>
<tr>
<td>6′,3′,4′,2′-Tetrahydroxy-2′,2′,3′,6′-dimethylpyran [6′,4′,7′,8′,6′-(3′-methylbut-3′-enyl)]</td>
<td>Macha rogsum</td>
<td>fruit</td>
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<tr>
<td>5′,6′-Dihydropseudogonidine</td>
<td>Lonchodaphus subgenuzeneans</td>
<td>roots</td>
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<tr>
<td>Scidendoretin</td>
<td>Erythrina sigmoides</td>
<td>root bark</td>
</tr>
<tr>
<td>Mezvasisosiflavone</td>
<td>Erythrina sigmoides</td>
<td>root bark</td>
</tr>
</tbody>
</table>

#### Glycosides

<table>
<thead>
<tr>
<th>Glycoside</th>
<th>Plant</th>
<th>Part</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prupein-4′-O-apioyl(β-D-glucoside (corriandelin)</td>
<td>Dalbergia coriandelliana</td>
<td>leaves</td>
</tr>
<tr>
<td>Galanthin 5-methyl amin-4′-O-glucoside</td>
<td>Cotonopsis alicsien</td>
<td>leaves</td>
</tr>
<tr>
<td>2′-Hydroxyisoflavanone 5′-6′-dimethylpyran (β-D-glucoside (nodosing)</td>
<td>Cassia nodosa</td>
<td>flowers</td>
</tr>
<tr>
<td>2′-Hydroxyisoflavanone 8′-O-glucoside</td>
<td>Cassia nodosa</td>
<td>flowers</td>
</tr>
<tr>
<td>5,6′,8′- Trimethoxy-3′,4′- methylenedioxy isoflavone 7′-O-(2′-p-coumaryloxy)glucoside</td>
<td>Trichosanthes arguina</td>
<td>seeds</td>
</tr>
</tbody>
</table>
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30) Phytochemical and Ethnobotanical Databases. www.ars-grin.gov/duke/

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Multifunctional Cosmetics

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For the rapid growth of the cosmetic science it is becoming increasingly necessary for all cosmetic chemists and dermatologists involved in cosmetic research and development to have a better understanding of the actual significance of cosmetic products. As a matter of fact, Cosmetic Dermatology is a complex subject and allows for a multiplicity of scientific and philosophical approaches. Cosmetic fashion is one of the most enduring forms of personal expression and our complexions speak frankly of age and health. Throughout history women and men had cosmetics that were intended to beautify them in the same way as modern cosmetics do today, and the growing sophistication of cosmetic science has had a tremendous impact on the growth of the cosmetic industry up to day. Improved technology has vastly increased the range and the combination of materials with which cosmetologists can work to formulate new cosmetics. Now more than ever, consumers can wear cosmetics and, at the same time, improve their skin quality. The better understanding of the skin biology and, the variety of technological and marketing factors permit to formulate more active moisturizers or sunscreens able to improve or to protect this important organ from the environmental assaults. Thus consumers demand also for products that offer more than one primary benefit, performing more than one function.

For these reasons are begun to be created multifunctional personal care products. Consumers in fact, love products that can do more than one thing. And one of the most obvious examples of this kind of multi-functionality is the self-proclaimed two-in-one shampoo that is intended to simultaneously cleanse and condition hair or the deodorant/antiperspirant that control body odour and reduce wetness.

However multifunctional products are becoming increasingly popular for three key reasons: increasing consumer expectations, maturing technology and expanding marketing demands. This is the opinion of the author of the 1st chapter Definition and Principles of Multifunctional cosmetics where all these considerations are reported. Moreover defining multifunctional benefits for a product can help revitalize mature brands. But on a negative note, it is important for the market and scientist alike to remember that multifunctional products may not be well received by certain individuals and demographic segment, who prefer some times conventional ones.
Therefore the successful development of multifunctional products begins with the knowledge of the consumer attitudes and usage patterns, proceeds to the skills essential for the formulation, successively evaluating the product’s prototypes by salon, clinical and consumer test methods.

Of course, the formulator must exercise a greater level of preparation prior to the formulation of a multifunctional product that requires the establishment of an increased number of project goals and criteria for success.

These and other considerations are reported on chapter 2.

Chapter 3 provides a comprehensive look at the different ingredients and formulations that can be used to create multifunctional hair care products.

Indeed, commercial success may be impaired by the very same functional properties that provide the product’s reason for being.

Thus many consumer’s shampoos with traditional UV absorbers are now currently marketed around the world. They protect hair from UV assaults, being known the damages caused by ultraviolet radiations to this fiber also.

Some polymers offer improved curl retention without sacrifice of combing properties; other organo-modified silicones, in shampoo formulations, offer improved foaming characteristics and combing properties; cationic polymers, originally used as film-forming fixatives, have also been shown to result in a lower irritation profile for anionic surfactants and so on.

For all these reasons compatibility and synergism with active ingredients combined with the functional availability of individual raw materials is the key of success.

Therefore the future is a practical one, where personal care products can do more with less.

Consumer needs, in fact, have been the main driving force behind the flurries of innovations and research activities creating the two-in-one conditioning shampoos reported on chapter 4.

The appeal of this important shampoo segment is the convenience the product has to offer. It eliminates the extra steps and the tussles of having to reach for another battle of conditioner in the middle of shampooing and rinsing the hair addressing an important consumer need.

Thus meanwhile the shampoos components remove the hair soil, leaving the hair shiny and lustrous, conditioning compounds restore its body putting back onto the hair an appropriate amount of suitable ingredients.

Moreover, to be really effective the amount of conditioning residues must not be excessive, creating the phenomenon of over conditioning and causing a hair difficult to style. And these results have been obtained by the use of multifunctional polymers derived from silicones, which have revolutionized hair care formulations.

But the concept of multi-functionality is permeating all the modern cosmetic science. Effects, efficacy, and performance that were never considered to be associated are now being combined, i.e., colour and moisture, UV blocking and suppleness and so on. However the functionality of a personal care ingredient is any function that this ingredient may have, for example, acting as a moisturizer or an emollient. This is focused on chapter 5 where the performance and the interaction between individual chemicals and multifunctional cosmetics to increase skin moisturization, skin elasticity, and skin feel, has been controlled by different methodologies.

But, the needs of today’s consumer for multi-functionality are developing faster than the cosmetic industry can identify new active ingredients that can deliver these forms of multi-functionality. However, nail care products offer also an important occasion to produce more than one function in
same product. This is the topic of chapter 6 that explores the opportunity for additional multifunctional nail cosmetics by first reviewing the structure and chemistry of a normal nail and then comparing the needs of this structure with the opportunity presented in each nail care product category as nail strengtheners, decorative nail coatings, polish removers and cuticle treatments.

Cosmeceuticals, combining cosmetic and pharmaceutical functionality is focused on chapter 7. As a matter of fact, Cosmeceuticals have been and has until now used to describe products that yield benefits traditionally thought to be cosmetic in nature, such as moisturization, as well as products that make marketing claims approaching those of drug products, such as reducing (the appearance of) wrinkles.

Japan was progressive in attempting to make such a new product classification, referred as quasi-drugs.

The new classification includes substances causing a mild action on the body and demonstrated to be safe. And to defend this concept of Cosmeceutical, also in 1983 the International Society of Cosmetic Dermatology and this Journal of Applied Cosmetology were founded.

Today it is well known by new available techniques as, for example, petrolatum (which is not a drug) is able to alter the structure and function of skin.

Therefore the challenge of a cosmeceutical formulation is to select the right active ingredients, to formulate the right vehicle to control in vitro and in vivo its efficacy and the absence of side effects, creating a product that yield the expected therapeutic benefits. This product may be defined as a clinically correct cosmetic.

A convergence between consumer demand and increasing technological sophistication has led to oral care products that can do a number of things all at once. This is the topic of chapter 8.

Today, multi-functional oral care products promise at least two, if not three or four, different ways to bolster the oral health and beautify the face: toothpastes that clean also battle gum disease and turn pearly whites wither; mouthwash fights plaque as well as halitosis, and so on.

These innovations in oral care products are a direct response to the busy modern consumer, who wants products that can do a number of things all at once. And the innovations are created by Cosmetics Chemists well versed in the physiochemical properties of the raw materials, surface and colloid/chemistry, and rheology, who formulate their oral care products in collaboration with stomatologists and dermatologists.

The purpose of chapter 9 has to provide an understanding of the state of the art of sun-protection to facilitate development of innovative multi-functional products incorporating sun protective ingredients.

As a matter of fact, sunscreens have been known to reduce the efficacy of moisturizers, moisturizing ingredients could disrupt the protective film of a sunscreen product, meanwhile antioxidants exert a protective effect on sunscreen ingredients, enhancing their photostability.

The solar spectrum tends, in fact, to degrade photo labile sunscreens more effectively than the spectrum of atypical solar simulator. Thus the sunscreen efficacy and of course its SPF, diminishes during UV exposure.

For all these reasons the right assessment of both UVB and UVA protection is a formidable problem that has to be studied deeply measuring the efficacy of the sunscreen product both in vitro and in vivo with the collaboration of expert dermatologists.

It appears that multi-functionality is here to stay! As these trends have continued, consumer products
companies have sought to differentiate their products lines.
The addition of *antibacterial agents* to a variety of cosmetic products is focused on chapter 10 that provides basic knowledge and practical suggestion to formulate this special class of cosmetics. The amount of antibacterial agent to use depends, in fact, not only on the type of antibacterial agent, but also on the proposal product application.

Therefore testing for the presence and quantities of the antimicrobial agent is necessary when the multifunctional product containing the antibacterial agent is undergoing micro-efficacy testing. Thus to make on *antibacterial hand wash* claim, it is necessary to perform *in vitro* and *in vivo* tests that include MIC (Minimum Inhibitory Concentration) time-kill, general use hand wash methods, and residual efficacy tests (e.g. Agar Patch).

However the efficacy and the claim of the product are under the manufacturer’s responsibility. Therefore the perceived and/or real performance characteristics for multifunctional skin care products requires a clinical trial design that can objectively quantify multiple clinical end points that are supportive of each specific marketing claim.

And *Claims Support Strategies for Multifunctional Products* are reported and discussed on chapter 11.

Thus the evaluation of a product that can *lightness darkness around the eyes* requires, for example, a clinical study design based on (1) identification of a significant number of selected test subjects (2) use of clinical end point defined by scientific literature (3) control of subject by dermatologists that provide precision and reliability (4) discussion of results demonstrating statistical differences before and after the cosmetic treatment.

The image analysis scanning of the silicon replica may provide demonstrated quantitative changes in perioral or periocular fine line wrinkles helping to differentiate performance of one product versus another. Therefore it is clear that the increasing use of functionally active raw ingredients in multifunctional personal care products will continually require innovative claim testing approaches to accurately quantify claims in a compelling, defensible fashion to meet the needs of the market place as well as the end users ultimate needs.

In summary the global arena of multifunctional personal care products will continue to evolve to meet the consumer needs.

However the key will always remain that the active ingredients used or/and the final formulated products must be not only effective but also safe.

**Chapter 13** covers another important topic: the role of packaging.

Packaging is, in fact, an important tool in designing and supporting multifunctional products in terms of both functionality and appearance. This is because of a question commonly asked by product development staff is: what packaging material should I use? Several are the vital factor to weigh for arriving at the best packaging decision as (a) barrier requirements determined by the product ingredients; (b) graphic and aesthetic requirements; (c) dispensing requirements connected with the market appeal; (d) process requirements; (e) safety requirements; (f) economic requirements, and finally (g) the environmental impact.

Replying to this check list may help to choose among the multitude of plastics, glass metal, and paper alternative packaging being offered in today’s market.

In conclusion, packaging plays a key role in supporting the multifunctional nature of a product, because of the message it conveys to the consumer on the shelf and for improving its stability and
facility of dispensing. Chapter 13 ends the book reporting some ideas based on customer focus versus product focus, which today represents the new concept of consumer research. A growing demand for customization and personalization is driving, in fact, the development of new kinds of multifunctional cosmetic products.

Consumer search is now more important than ever. Thus changing marketing tactics have also played a role in the escalation of product functionality. On one hand multifunctional products (MEPs) can serve to expand the target market by appealing to a broader base of people. On the other hand (MPPs) can narrow a market by appealing only to people who prefer the combination of functions offered.

Therefore, consumer research needs to dig more deeply to better understand consumer need and performance. It means that power is shifting to the customer and so the marketer’s focus must follow.

If marketers can raise the level of creativity in ideation and concept development to at least the same levels of quality, it will be a multifunctional slam dunk.

Thank to all the interesting topics and discussions reported, this book has to be considered a fundamental reference not only for the Cosmetic Chemists and Dermatologists expert in Cosmetic Dermatology, but also for all the people involved in the cosmetic marketing area and for all those scientists and students of the chemical and medical community interested to understand and develop innovative and multifunctional cosmetic products.

P. Morganti
Editor-in-Chief
The Antimicrobial Biological Activity of Essential Oils

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Many of today's proposed uses of plant materials in toiletry and cosmetic products originate from the traditional medicinal/herbal uses of these plants, their extracts and essential oils. Thus, it is possible to establish a traditional use for selected plant materials as cosmetic or diet supplement aids.

No doubt, fresh plant material should be preferred and carefully dried plant material is more often used to obtain its active compounds' content.

In addition to simple extraction methods, as the preparation of infusions, there are more selective methods, such as the distillation of volatile oils and water and the pressing of plant material for fixed oils, some volatile oils and various saps and juices.

However the use of fresh plant material adds interest to a product and can help to elevate and distinguish it from a mass of apparently similar products.

But it is important to remember that plant extracts, for example, fall into two clearly defined categories: total plant extracts and partial plant extracts.

Total plant extracts are prepared by taking either the whole plant or one or more of the organs of the plant and extracting them by suitable maceration or percolation processes to yield a total extract from that plant in the chosen solvent.

Partial plant extracts are prepared by selectively separating from either the whole plant or one or more of the organs of the plant, a particular range of constituents by either chemical or physical methods. This may be oil, juice or sap.

The extraction and biological use of fixed oils, volatile oils, absolutes and concretes is the main topic of this interesting book.

Plant volatile oils have been recognized since antiquity to have biological activities. Chief among these are their antibacterial, antifungal and antioxidant properties. Thus have born aromatherapy and aromachology known both in medical and chemical community.

Volatile oils have been shown, in fact, to act as hepatic protective agents in aging mammals. Such studies highlighted the beneficial impact of dietary supplementation with certain volatile oils upon polyunsaturated fatty acids (PUFA), in particular the long chain C20 and C22 acids.

In addition to protecting liver PUFA's the volatile oils also demonstrated a positive effect upon docosahexaenoic acid (DHA) levels in aging rodent retinas.

On the other hand, many plant essential oils have demonstrated effective antioxidant capacities, pro-
tecting PUFA during vital periods of the lifespan.

As a matter of fact, lipid peroxidation, widely recognized as a primary toxicological event is caused by the generation of free radicals from a variety of sources including organic hydroperoxides, redox cycling compounds and iron-containing compounds.

The secondary events, including changes in membrane structure, permeability and fluidity, lysosomal destabilization and stimulation of apoptosis, are cellular changes resulting from initial damage. Tertiary effects, such as hydrophilic degeneration, and necrosis, are the observable and final stages. Thus, the consequences of lipid peroxidation are profound to the organism concerned resulting in loss of membrane functions and integrity and ultimately to cellular necrosis and death. Therefore the use of essential oils, as antioxidant compounds, are to be preferred because of their antioxidant effectiveness.

Moreover their solubility in phospholipids bilayer of biological membrane appear to be of importance being directly related to their ability to penetrate the cell walls of a bacterium or fungus, influencing its primary enzymatic energy metabolism.

Thus today, the search for various naturally occurring compounds with antimicrobial activity has become quite intense due to the growing concern about the polluting effect of some synthetic fungicides and development of resistance among the pathogens against such fungicides.

And many essential oils have been found to have different degrees of fungicide toxicity against different pathogens.

Furthermore, biologically active natural compounds are of interest to the pharmaceutical industry for the control of human diseases of microbial origin and for the prevention of lipid peroxidative damage, which is implicated in several pathological disorders, such as ischemia-reperfusion injury, coronary atherosclerosis, and carcinogenesis as well as in ageing processes.

For the problem of food and cosmetic preservation and to ameliorate the quality of life the use of phytochemicals and essential oils are widely increasing.

Many natural compounds extracted from plants have demonstrated biological activities and among these volatile oils from aromatic and medicinally plants are particularly interesting.

Thus plants and more importantly, their extracts or essential oils are incorporated into toiletry and cosmetic products for a variety of reasons.

However, in order to enhance the product performance the plant material need to be present in known quantities and thus it is essential to know the plant equivalent of the chosen extract. It is only by having an accurate plant equivalent that manufacturers of toiletry and cosmetic products can decide on a reasonable incorporation rate for an extract or essential oil into their products.

The public are becoming, in fact, more aware and responsible with respect to environmental issues and have realized the need to protect the remaining and precious natural resources.

Along with this environmental awareness is a growing consciousness of what we are putting in and on our bodies. Therefore we are living a real green revolution.

This new awareness has resulted in the opening up of a new niche in the market-place for products which are both environment friendly and safe to use.

Hence the advent of ozone friendly aerosols, re-cycle containers, products not tested on animals, products free from chemical flavours, colours and preservatives, and therefore natural cosmetics.

But the definition and regulation of natural product is still somewhat confused in the cosmetic industry.

However essential oils are complex mixtures of compounds and due to this complexity, are subject
to considerable variation.
In plants grown in the soil, the quality of essential oil produced is affected by the variety of plant grown, the type of soil and weather conditions during growth and the method of extraction used.
Different kind of essential oils may be also obtained by different extraction methods and solvent. Thus, a water based solvent will not extract non-polar materials whereas a solvent such as isopropyl myristate would extract oil-soluble components.
Another aspect of the essential oils’ extraction is the production of aromatic waters as by-products of the distillation of volatile oils.
However in order to enhance the antibacterial/antioxidant performance of the essential oils the plant needs to be used in known quantities and thus it is essential to know also the plant equivalent of the chosen extract or distilled.
Plant, in fact, and more importantly their essential oils are incorporated into toiletry and cosmetic products for a variety of reasons.
However their addition has to enhance the product’s performance and should not be a mere marketing ploy.
The antibacterial, antifungal, antioxidant-properties of many used essential oils are reported and described in this fascinating book, necessary for chemists or medical doctors involved in aromatherapy and/or aromachology.
The antibacterial activity of essential oil melaleuca alternifolia or wild oregano is discussed together with, for example, the fungicidal activity of different naturally occurring essential oils from many aromatic plants.
Their chemical composition, bacteriological activity and clinical studies are often reported for the majority of essential oils discussed by more than 100 chapters of the book.
In my opinion this unusual book is surely useful for all the students or the marketing managers who want to know and understand in a better way the fascinating world of aromatherapy and aromachology living around the natural and complementary medicine.

P. Morganti
Editor-in-Chief
Cosmetics as food provide a rich source of nitrogen and, therefore are easy to be contaminated. However, no real comparison can be made between them in terms of risk and deterioration due to microorganisms, because the usage period is much longer for cosmetics.

To prevent cosmetics’ deterioration it is therefore necessary to add preservatives to suppress the proliferation of bacteria, fungi and yeasts which may contaminate them.

Preservatives used in cosmetics and their quality standard have been drawn up by EU Cosmetic Directive and the Food and Drug Administration (FDA) in USA, indicating upper limits on safety also. For each antimicrobial agent it is necessary to check such items as the solubility, safety, effective pH range, prohibitions on use, smell, color and their actual effect in a cosmetic product when considering their inclusion in cosmetics.

The ideal preservative or preservative system has to have a broad-spectrum activity, be effective at low concentrations, water-soluble and oil-insoluble, stable at all temperatures and pH, colorless and odorless, compatible with all ingredients used, easy to handle and to analyze and at low cost. What is important to underline is the necessity to use them at the lower dose possible because they all have potential for being toxic, or irritating, or sensitizing, being biologically active products. However European Cosmetics Directive requires that cosmetics do not cause harm and remain safe under normal and reasonable conditions of use. Therefore they must not be contaminated with microorganisms which may be pathogenic and should not cause skin sensitization.

The second edition of this interesting book is organized in 10 chapters and Reference tables reporting all the preservatives reviewed by the Cosmetic Ingredient Review (CIR) or reported in the positive lists of different countries: Brasil, Canada, EU, Japan and Mexico.

After all the general information reported on chapter 1, as introductory news chapter 2 lists all the Common Preservatives commonly used in the Cosmetic field, the better and practical way of using them, the stability, the analysis the legal rules applied worldwide. Thus the reader may easily understand in a exhaustive way what is necessary to know about parabens, isothiazolinone, phenolic and halogenated compounds, quaternia, alcohols and acid preservatives, that represent the more commonly used chemicals for cosmetics.

Other Preservatives not widely used are all listed in chapter 3.

The ideal preservative does not exist and for this reason combination of preservatives are used. At this purpose, many manufacturers offer Combination and Solutions of preservatives mixed together. And this is the topic of chapter 4, where combination of compounds with a broader spectrum activity (antibacterial and antifungal) with their percentages of use are reported.
This is another part of the book useful for the different kind of cosmetic products. But it is also interesting for dermatologists who may better understand the necessity to use different combinations of preservatives to fasten the cosmetic stability of the product for protecting the customer 'health'. "Preservative as Active Ingredients" are reported on chapter 5.

There are, in fact, several product categories that utilize chemicals to kill microorganisms and make a claim of this action. Making such a claim in the US and sometimes in EU also, changes the legal status of a product from a cosmetic to a drug. Way of using Pyrithione Zinc, Selenium sulfide, Ketoconazole, Salicylic Acid, and other antibacterial and antifungal chemicals are described in this chapter.

Chapter 6 is entirely dedicated to Natural Preservatives. In reviewing the property of the ideal preservative with what actually occurs in nature, it is easy to conclude that an ideal preservative will never be found naturally!

This is the conclusion of the author of the book, who however, reports example of natural preservative blends present on the market.

Chapter 7 is focused on Non-Traditional Preservatives. For all these chemicals based on higher molecular glycols and enzyme systems, the author considers them as preservatives that are not on the approved list of preservatives in the EU or Japan.

Thus companies market these products for some other function, which leads marketers to proclaim 'preservative-free' on their label.

Among the multifunctional phospholipids, same structural modified molecules such as cocamidopropyl PG-Dimonium Chloride Phosphate and Sodium coco PG-Dimonium Chloride Phosphate have been shown to exhibit a broad range of functional characteristics including gentle cleansing and foaming properties, anti-irritation effect when combined with anionic surfactants, skin conditioning, and broad spectrum antimicrobial and preservative enhancement.

All microorganisms need sufficient water and nutrients to grow, hence water activity is the topic of chapter 8 where all the ingredients capable to lower its activity are reported together with the instrumentation necessary to determine this activity. Antioxidants and Chelating Agents are respectively described on chapters 9 and 10.

Antioxidants are added to cosmetic formulation to prevent degradation of ingredients by oxidation meanwhile chelating agents enhance both the activity of preservatives and antioxidants.

By the description of all the chemicals used at this purpose, ends this totally comprehensive agile and easy to consult book full of news and useful advice to better formulate a stable cosmetic product.

Useful for all the students of the chemical community that wish to understand the problem of cosmetic preservation, the fascinating topic of this book may be of interest for expert cosmetic chemists and dermatologists.

P. Morganti
Editor-in-Chief
Dermatology is a morphologically oriented specialty and non-dermatologists have often difficulty interpreting what they see. As a matter of fact the diagnosis of skin disease must be approached in an orderly and logical manner and an eye scan over wide areas is inefficient.

It is most productive to mentally divide the skin surface into several sections and carefully study each section. Thus many dermatologists now advocate a complete skin examination for all their patients. Because of an awareness that some patients are uncomfortable undressing completely when they have specific request such a treatment of a plantar wart, other dermatologists advocate a case-by-case approach.

However the physician should learn the surface characteristics of all the common entities of a disease process and gain experience by examining known entities.

For all these reasons this color Atlas of Women’s Dermatology may be of interests for many young dermatologists, providing many interesting pictures of skin diseases afflicting girls and women. Thus each diagnosis reported on the book, has a unique code, each code representing a single diagnostic concept with only one meaning.

The relationship between diagnosis seen within this text and other dermatological diagnosis can be easily reviewed and the physician has the possibility to learn and understand the common entities and gain experience by examining them. Most skin diseases begin, in fact, with a basic lesion that is referred to as a primary lesion.

Identification of the primary lesion is the key to accurate interpretation and description of cutaneous disease. Its presence provides the initial orientation and allows the formulation of a differential diagnosis.

Therefore the value of the diagnostic codes reported on this book lies not only within the unique number associated with a diagnosis, but within a greater network of meaning.

The organization of the different chapters in a topographic format, the reported diagnosis for each disease and the clear definition of all the photos give to this book an interesting clinical significant helping to better interpret what is meaning directly observed. Its reading may be therefore surely useful for all physicians and dermatologists as a helpful tool in their daily practice.
In copertina / Front cover

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Neuro cells 2A treated with H$_2$O$_2$ 30µM and BETAFFE PLUS 0.5 µg/ml (immunofluorescence with phalloidin, x100). On kind permission of Prof. Graziella Biagini, Istituto di Morfologia Umana Normale, Università Politecnica delle Marche, Ancona- Italy

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