NASUNIN, AN ANTIOXIDANT ANTHOCYANIN FROM EGGPLANT PEELS, AS NATURAL DYE TO AVOID FOOD ALLERGIES AND INTOLERANCES

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Abstract

In recent years, the increasing use of synthetic dyes showed an increase of allergic contact dermatitis with tissues, food, pharmaceutical and cosmetic products. This paper describes a method for extracting quickly, efficiently and economically a natural dye the Nasunin, a purple anthocyanin. To assess the potential sensitizing effect on the skin, have been formulated and applied to the healthy skin of 58 volunteers, patch tests with haptens of the Italian Society of Dermatology, Allergology Professional and Environmental (ISDAPE or SIDAPA, in Italian) standard series, compared with patch tests containing Nasunin extracts. Of all the subjects tested, forty nine were negative to patch tests containg Nasunin, seven showed a positive reaction to irritative and two showed a positive reaction of probable allergic nature. Consequently, these preliminary data show that allergenic effects are negligible and thus the Nasunin could be used as colorant in textile, food, pharmaceutical and cosmetic fields. In particular, the results indicate that the Nasunin, used in concentrations lower than 1% is a natural dye harmless suitable to impart coloration, in addition, to its extraction from eggplant peels allows the recycling of waste products of the food industry.

Keywords: Nasunin, natural dye, eggplant, patch test, allergy, food intolerance

Introduction

The use of color additives is a solution that makes use of the food industry on a daily basis, in order to restore or revive the original color appearance of a food. This aspect is important because it influences the choices of the consumer at the time of purchase. In order to meet this need, the food industry uses natural and artificial substances with properties pigmentation. In recent years, the growing interest in natural products by the consumer, which has become more attentive to the quality of life and environmental protection, has led to a rediscovery of natural dyes. Scientific research, in fact, revealed the extraordinary properties of natural dyes for their use in very diverse and productive activities, mainly in the food industry, in the field of natural cosmetics and in the field of organic textiles and eco-fashion for dyeing natural fibers. For this reason, it is particularly important to study and evaluate such substances, that are not due to potential problems in the consumer, such as food allergies and intolerances. Food allergies and intolerances can both be considered as *individualistic* adverse reactions to food. Pathological forms are individual because they affect a few individuals are in a rather serious; immediate or delayed reactions occur instead with simple effects histamine or, in severe cases, with respiratory and anaphylactic shock. For food allergy refers to the consequence of hypersensitivity of an individual to a particular food caused by a reaction of the immune system to specific proteins of a given food product: the majority of true food allergies are inherited diseases that occur more easily in children of allergic parents. The intolerances are instead due to the inability to metabolize certain individual substances present in food and are not caused by immune reactions, such as sensitivity to lactose and gluten. Interest for allergies and intolerances is nowadays more and more important due to the fact that an increasing number of people are involved, it is estimated that approximately 3-5% of the adult population and 6-8% of children are involved in allergic situations (Sicherer and Sampson, 2006). For this reason it is essential to determine which are the possible triggering agents, so as to be able to remedy the situation (for example comprising through a wide range of possible diagnostic tests, which is the substance to the base of the reaction) by avoiding or minimizing the development of problems in susceptible individuals, which can lead to various symptoms. Possible symptoms that the development of allergic reactions can result, concern various organs or systems: skin (edema, urticaria, atopic dermatitis); gastrointestinal tract (vomiting, nausea, abdominal cramps); problems at the level of the oropharynx, nose, head (headaches), eyes (rhinitis), lungs (bronchial asthma); anaphylactic shock in severe cases.

The component pigments of coloring additives, are small molecules of low molecular weight, generally of non-protein, which in this way should

not be involved in triggering of allergies. These components may possibly stimulate the immune system, only when bound to proteins, act as a complex activities antigena. The additives are natural dyes extracted from natural material, and therefore may contain many other components, including sugars, proteins added/linked to the coloring components, which may lead to an adverse reaction. Reactions to natural color additives, are occasionally reported and are attributed to protein residues, dyes such as annatto in or carmine (Taylor and Dormedy, 1998b). Although the mechanisms involved in the reported cases of adverse reactions caused by the natural color additives, have not been investigated, it is believed that most are due to IgE-mediated reaction, where antibodies react with IgE binding to protein residues that are colored components. By contrast there is no evidence that suggests the involvement of the additives in some types of natural dyes intolerances.

The eggplant (*Solanum melongena L.*) is a vegetable typical of the Mediterranean diet is not only a good and versatile vegetable in the kitchen, but also a wealth of powerful antioxidants, for which it is a valuable ally in the prevention of major diseases, including cardiovascular disease , diabetes, tumors. It contains chlorogenic acid and Nasunin, two molecules that have significant activity against free radicals, that, as now widely confirmed, play a central role in phenomena such as aging, inflammation, cardiovascular disease, cancer. The Nasunin, in particular, is an anthocyanin, a purple pigment, belonging to the family of flavonoids, which together with other anthocyanins imparts to the eggplant its characteristic color. Research shows not only that the Nasunin has strong antioxidant properties, but even a specific action of inhibition of angiogenesis (development of new blood vessels), a process implicated in several diseases, including cancer in the first place. The Nasunin is present in very few foods and eggplants are the natural source that contains significantly more. In particular, this phytonutrient is concentrated in the peel. The eggplant is then rich in soluble fiber (particularly of pectin) and also has interesting levels of potassium. Moreover, this vegetable has a very low glycemic index (GI 15) and is therefore useful in the diet also to prevent and manage disorders such first overweight and diabetes.

On the other hand, it is known that the eggplant can cause food allergies due to the presence of allergens in all the edible parts with preponderance in the peel (Babu and Venkatesh, 2009), but lack detailed studies of the effects on allergies caused by eggplant taken at different concentrations.

Anthocyanins are molecules widely distributed in the plant kingdom, present in glycosylated form (anthocyanins) and in aglycosylated form (anthocyanidins), generally less stable. They are of extreme importance,

because ultimately contribute to the determination of the coloration of various plant products, such as fruit and vegetables, where the color is often determined by a combination of various pigments, ie a combination of multiple molecules belonging to this group, which in their determine the overall chromatic effect. To determine this characteristic of a specific color, anthocyanins are used as a natural color additive, in the color of specific products, such as soft drinks, yogurt, jam, or revive certain types of foods. The goal in this regard would be to be able to increase their content, perhaps in replacement of some dyes but synthetic analogues, since they do not present problems of toxicity to humans, at the same time exploiting the properties that distinguish these molecules (eg important antioxidant activity, prevention of cardiovascular disease).

The extraction of anthocyanins, can take place in aqueous solution sulphited in acidified solution by exploiting their characteristic hydrophilicity, or resorting to the use of ethanol, methanol and carbon dioxide, today there are several techniques for their extraction, which provide for the recovery of anthocyanins found in the peels, in the fresh pomace obtained from the pressing phase (by-product of the wine industry), or even from edible fruits and vegetables, as indicated by a specific European directive. Among the products allowed, for example, include: strawberries, raspberries, blueberries, blackberries, cherries, eggplant, red onions, red cabbage, radish roots. Subsequently, the solution obtained, is subjected to concentration, if the desired product will be in powder form, the concentrated solution will be subjected to dehydration, while if you want to obtain a product in liquid form, after the concentration step, will be subjected to sedimentation in such a way to remove all the impurities (Castaneda-Ovando et al., 2009). Surely it is important to stress, as in the solution obtained from the products mentioned above, there are more molecules, which are naturally present in the starting material, such as organic acids, sugars, nitrogenous components in varying amounts, minerals and of course the anthocyanins, the all in different proportion in function of the starting raw material.

Numerous sensitization reactions, and allergic reactions have been verified real, confirmed and documented in the literature, following ingestion of grapes or their derivatives (Moyer 1990; Parker 1993; Steinman and Potter 1994; Garcia Ortiz et al., 1995; Fernandez-Rivaset al., 1997). On the contrary, it is not reported in the literature, no situation of possible adverse reactions unleashed following ingestion of only anthocyanins. This ultimately suggests that allergic reactions occurred as a result of the ingestion of grapes, are likely due to the protein components, which, though limited in terms of quantity, there are in grape, whereas if you are referring to just the component anthocyanin regardless of the material from which

they were extracted, we have that the probability of causing the occurrence of an adverse reaction is extremely limited and unlikely.

This paper proposes a method for extracting fast, efficient and economical of Nasunin, a natural dye obtained from waste products from the food industry, such as eggplant peels. Subsequently, the Nasusin was used to formulate patch tests that were tested on 58 volunteers, to evaluate the potential sensitizing effect on the skin. The results show that allergenic effects are negligible and therefore the Nasunin could be used as a colorant in various industrial sectors, with a good margin of safety.

Materials, methods and volunteers:

The eggplant were purchased in a local market. All reagents and solvents HPLC grade were purchased from Merck (Darmstadt, Germany). The Sep-Pak C18 Vac cartridges were purchased from Waters S.p.A. (Milan, Italy). Patch tests have been formulated with haptens of the standard series of Italian Society of Dermatology, Allergology, Professional and Environmental (ISDAPE), that includes over 25 haptens of various nature, such as metals (nickel, cobalt, potassium dichromate), dyes (para-phenylene-diamine, blue dispersed, scattered red), substances present in rubber products (thiuram), cosmetics (parabens) or perfume (fragrance mix, balsam of Peru) , preservatives (Kathon) and drugs (neomycin, benzocaine) and with nasunin (solution A) at 1 and 5% using vehicles as water and vaseline. To assess the potential sensitizing effect on the skin, were recruited 58 healthy volunteers (41 females and 17 males), aged between 17 and 71 years, to which were applied the patches.

Extraction procedure:

100 g of fresh peel eggplant cut into small pieces were soaked in 200 mL of 10% acetic acid for 24 hours under constant agitation. After recovery of the solvent, the peels were added 200 mL of glacial acetic acid and left to macerate for an additional 24 h. After filtration, the peels were presented almost colorless, while the extraction liquid was strongly colored in violet. The extracts were pooled and taken to dryness to rotavapor at 40°C. The crude extract was treated first with 200 mL of methanol, brought to dryness and then treated with 200 mL of distilled water until complete dissolution. The solution was filtered by using a Sep-Pak C_{18} cartridge activated with 5 mL of methanol and 5 mL of distilled water. The two eluates were dried, resuspended in a solution of trifluoroacetic acid (TFA) 1% (v/v) and analyzed by high performance liquid chromatography (HPLC).

Chromatographic separation was performed using an HPLC apparatus: Sunicom Oy (Helsinki, Finland) connected with a detector UV/VIS variable wavelength Model 500 equipped with a degasser ERC3TT.

The column used was a Luna ODS (250x4.60 mm, particle size 5 µm) (Phenomenex, CA, USA). The eluents were: A water 0.5% TFA; B methanol 0.5% TFA. The gradient program was as follows: from 75:25 v/v at 30:70 v/v in 25 minutes with a flow rate of 1 mL/min, detector setted at 520 nm. Injection volume of each extract was 10 µL.

Results:

HPLC analysis

The extract of eggplant peels was filtered by using a Sep-Pak C_{18} cartridge, obtaining a first elution in methanol (Solution B colored in brown) and a second in distilled water (Solution A colored in purple). Both eluates were dried and weighed. Were obtained, respectively, 0.6523 grams for the dry residue A and 0.2110 grams for the dry residue B. The two samples were resuspended and analyzed by HPLC (Zhang et al., 2004; Sadilova et al., 2006).

Figure 1 (Panel A) shows the HPLC chromatogram of the solution A, in which the two most abundant peaks were identified by comparison with the literature: the peak 1 was identified as delphinidin 3-[4-(cis-p-coumaroyl) -L-ramnosil (1,6) glucopyranoside]-5-glucopyranoside (cis-nasunin), while the peak 2 was delphinidin 3-(4-(trans-p-coumaroyl) -L-ramnosil (1,6) glucopyranoside)-5-glucopyranoside (trans-nasunin) (Takashi et al., 2005) (Figure 2). Figure 1 (Panel B) shows the chromatogram for the sample B, in which have been highlighted peaks compatible, for the conditions used, with the nature of polyphenolic substances, but not identified.

The presence of Nasunin was also confirmed by GC-MS analysis. The spectrum MS showed the peak of molecular mass m/z 919 and other peaks of fragmentation at m/z 757 (loss of hexose group), m/z 465 (loss of the group p-coumaroylhexose), m z 303 (loss of the group of delphinidin).

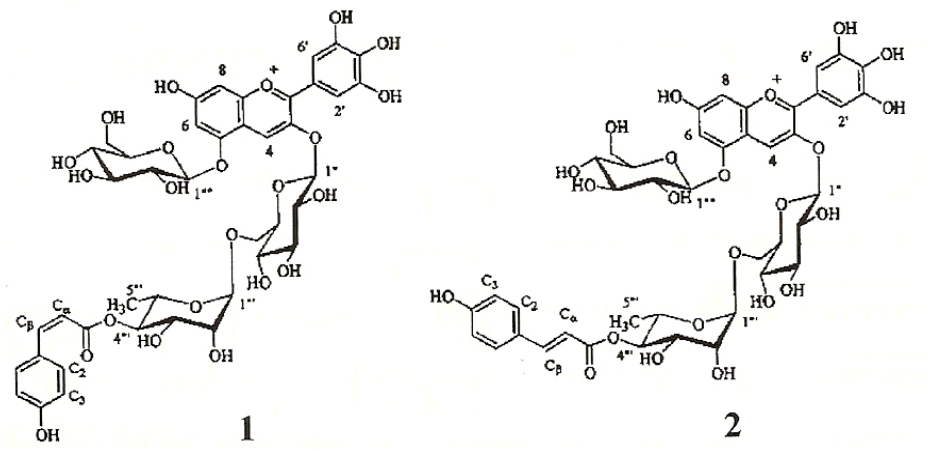

Figure 2. Structural formula of cis-Nasunin (1) and trans-Nasunina (2).

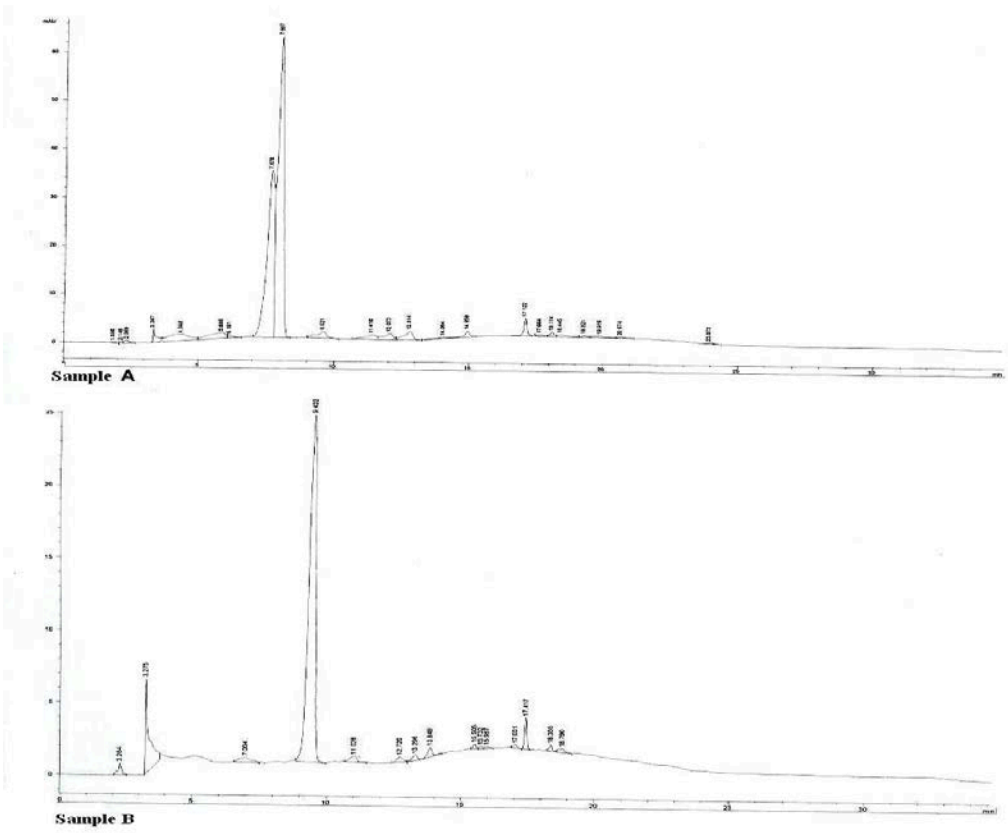

Figure 1. HPLC chromatograms of eggplant peels extracts of A solution (Panel A) and B solution (Panel B).

Dermatological assay

A method for assessing the irritancy of a substance is the patch *in vivo* tests on volunteers, a reliable test that can identify substances responsible for a potential irritant effect on the skin. A type of irritant reaction is strictly limited to the area of application of the substance and regresses in about 2 days. A reaction is regarded as a potentially allergic, if they are present erythema, edema, and vesiculation eczemiforme. The positivity is expressed by a sequence of signs + . For this study, 58 healthy volunteers were recruited, 41 females and 17 males, aged between 17 and 71 years, which have been applied to the disks containing the substances to be tested using strips of adhesive plaster. The patches were applied on the back of the volunteers free from lesions and left in contact with the skin for 48 hours, after which they were removed, and the evaluation was performed. In detail, on all volunteers were tested patches containing haptens of the standard series of Italian Society of Dermatology, Allergology, Professional

and Environmental (ISDAPE), that includes over 25 haptens of various nature (metals, dyes, preservatives and others) and patches with the solution A containing Nasunin. Furthermore, it is appropriate that cortisone therapy by mouth or injecting (from dosages of prednisone 10 mg/day or equivalent) or local cortisone therapy in the skin of the back are suspended at least 15 days before testing. Instead, they can be taken regularly based therapies of anti-histamines. In particular, patch tests were prepared using vehicles such as water and vaseline with concentrations 1 and 5% of Nasunin extract. The solutions in water were placed on supports consisting of cellulose diskettes with a diameter of 1 cm, in amounts comprised between 10 and 40 μL, while for solutions in vaseline diskettes were of 1-4 mM. An irritant reaction and strictly limited to the area of application of substance and subsides in about 2 days (Table 1).

+	Weak erythema
+	Uniform erythema with edema, possibly papules or vesicles
++	Erythema, edema, papules and vesicles evident that can spill over the area of application
+++	Erythema, edema, papules and vesicles very obvious, sometimes confluent in bubbles
IR	Type reaction irritating to different morphology
NT	Not tested

Tab. 1 Quality and quantity assessment of allergic reactions according to ISDAPE

Of all the subjects tested, 49 were negative to patch tests containg Nasunin (Table 2), 7 showed a positive reaction to irritative and 2 showed a positive reaction of probable allergic nature.

It is important to note that, among the 49 volunteers who did not show any kind of reaction to the extract, 17 were positive for haptens of the standard series ISDAPE. Of the 2 volunteers having shown a positive reaction to the extracts of eggplant (allergic contact dermatitis, ACD), 1 (+++) was found to be negative for haptens ISDAPE, while the other (++) were positive to haptens Balsam of Peru, p- phenylenediamine and cobalt chloride (Table 2). Of the 7 subjects having shown an irritative reaction (IR) to eggplant extracts, 5 were negative for haptens ISDAPE and 2 were positive, one at formaldehyde and the other to p-phenylenediamine (Table 2). It should be emphasized that all the positive subjects were treated with the concentration to 5% (a concentration considered remarkable for a dye), while no subject was positive at a concentration of 1%.

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N. Patients	Nasunin	Haptens*
49	Negatives	
2	ACD	1 negative 1 positive: balsam of Peru paraphenylenediamine cobalt chloride
7	IR	5 negatives 1 positive (formaldehyde) 1 positive (paraphenylenediamine)

Tab. 2. Results of patch tests with haptens of ISDAPE standard series and with Nasunin performed on 58 volunteers.*Haptens of Italian Society of Dermatology, Allergology Professional and Environmental (ISDAPE) standard series

Discussion

The Nasunin is the most important anthocyanin present in the eggplant peels at a concentration of 700 mg/100 g and is responsible for the purple color of this vegetable. It was isolated for the first time by Kuroda and Wada in 1933 (Kuroda and Wada, 1933; Kuroda and Wada, 1935) and its structure was determined by Sakamura et al., 1963.

It is an anthocyanin acylated rather stable and is presented as a mixture of cis-trans isomers of delphinidin. Its structure was indeed identified as: delphinidin 3 - (4 - (p - coumaroyl) – L – ramnosil - (1,6) glucopyranoside)-5-gluco-pyranoside. It had a high antioxidant activity (Kahkonen and Heinonen, 2003; Yasuko et al., 2000) and a powerful scavenger of hydroxyl radicals and superoxide radicals (Tateyama and Igarashi, 2006).

Spectrophotometric studies have shown that the Nasunin forms a complex with Fe^{3+} attributing its antioxidant action to the ability to inhibit the formation of hydroxyl free radicals for chelation with the iron. This was very important because superoxide radicals generated *in vivo*, were generally converted to hydrogen peroxide and as other free radicals could damage lipids, proteins and DNA (Halliwell et al., 1995).

The Nasunin has anti-angiogenic activity, in fact at a concentration higher than 10 mM is able to suppress the growth of microvessels (Matsubara et al., 2005) and is also involved in the prevention of other

diseases such as atherosclerosis and diabetic retinopathy (Mercier et al., 1965).

Several studies have also demonstrated that the Nasunin may prevent the toxic effects of Paraquat, a pesticide that can cause damage (oxidative stress) in several organs such as liver, lungs, kidneys and heart (Kimura et al., 1999). For its ability to suppress both the increase in the peroxidation of liver lipids, both the reduction of the activity of catalase, induced by Paraquat own, the Nasunin, preventing the increase of atherogenic factors, may be useful in preventing the development of atherogenesis due to this pesticide.

The patch test on extracts containing Nasunin showed only a moderate irritant at high concentrations (5%). Therefore it may be used in concentrations sufficient to impart coloration (below 1%), concentration that induced no irritative process on the skin *in vivo*.

Conclusion

In the present work were described preliminary results for future studies in use of Nasunin as natural dye. They represented the necessary basic information to a better characterization of this substance and to a better understanding of health risk. In conclusion, the development of a method for obtaining this anthocyanin extracted from eggplant peels, in a quick and efficient way, had two advantages, in fact allowed to obtain a dye both for the textile field, that for other sectors, such as cosmetic, pharmaceutical, food, thus avoiding the problems associated with the use of synthetic dyes, in addition the use of eggplant peels to extract the Nasunin, could promote the recycling of waste materials by reducing the environmental impact.

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